Polyunsaturated fatty acids and the fetal origins hypothesis : a study on cardiovascular risk indicators in children

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Thesis |

**Polyunsaturated fatty acids
and the fetal origins hypothesis**

A study on cardiovascular risk indicators in children

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Polyunsaturated fatty acids and the fetal origins hypothesis –

A study on cardiovascular risk indicators in children

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Polyunsaturated fatty acids and the fetal origins hypothesis

A study on cardiovascular risk indicators in children

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
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*De band, die 't harte bindt
Der moeder aan het kind,
Gebaard met wee en smarte,
Aan hare borst met melk gevoed,
Zo lang gedragen onder 't harte,
Verbint het bloed.*

Joost van den Vondel, 1637

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Chapter 1 | General introduction

Cardiovascular diseases in the Netherlands

Cardiovascular diseases, including ischemic heart disease and cerebrovascular diseases, are responsible for about 30% of all deaths worldwide.¹ Together they have become the leading causes of death, surpassing infectious diseases and cancer. In the United States of America 724,859 people died as a consequence of heart diseases in 1998.² Cerebrovascular diseases accounted for 158,448 deaths. In the Netherlands approximately 37% of all deaths are caused by cardiovascular diseases, accounting for the death of 50,545 people in 1997.³ Within Europe, cardiovascular disease mortality rates differ substantially between countries (Figure 1.1). In general, the age-adjusted mortality rates from ischemic heart disease are lower in Mediterranean countries than observed in Northern European countries.

Figure 1.1: Age-standardized death rates for ischemic heart disease and cerebrovascular diseases in a selection of European countries. Death rates are given per 100,000 of the World Health Organization standard population.⁴

Although the age-adjusted mortality rates for cardiovascular diseases are declining in the Netherlands,^{3,5} cardiovascular diseases still remain among the largest of all medical problems. The number of Dutch patients admitted to a hospital because of cardiovascular diseases increased by 53% between 1972 and 1997.³ This increase was mainly caused by the increased admittance of older subjects (aged 65 years or over). Due to the improvement of medical care, patients with cardiovascular diseases live longer. But since these patients have an increased risk for recurrent atherosclerotic events, heart failure and other complications, the need for medical treatment increases. The progress of medical science has also led to higher age-limits for advanced treatments. As a consequence, the number of interventions and the costs per intervention increases. In 1994, the total costs related to cardiovascular diseases in the Netherlands has been valued at € 2.8 billion and was only exceeded by the costs for psychological disorders.⁶ In the period between 1988 and 1994, the costs for cardiovascular diseases had increased by approximately seven percent each year. Even higher increases of the costs are expected for the future.⁶ These figures illustrate the large economic and medical impact of cardiovascular diseases in the Netherlands and emphasize the need to continue the search for preventive measures.

On the childhood origins of cardiovascular diseases

Cardiovascular risk indicators

During the past century, scientific research has contributed substantially to our understanding on the etiology of cardiovascular diseases and the underlying processes of inflammation, atherosclerosis and thrombosis.^{7,8} Prospective population surveys, such as the Framingham Heart Study,⁹ the Northwick Park Heart Study¹⁰ or the Seven Countries Study,¹¹ disclosed the most important characteristics related to an increased risk for the development of cardiovascular diseases. Among the principal risk indicators identified are a high serum cholesterol concentration,¹²⁻¹⁵ hypertension,¹⁶⁻¹⁹ and smoking.²⁰⁻²² Additional risk variables are diabetes,²³⁻²⁵ obesity,^{26,27} elevated plasma triacylglycerol levels,²⁸⁻³² a sedentary life style³³⁻³⁵ and a positive family history of early atherosclerotic events.³⁶⁻³⁸ A substantial number of other indicators have been identified. A survey conducted by Hopkins and Williams resulted in a list of 246 potential coronary risk variables.³⁹ More recently, biochemical markers for inflammation such as C-reactive protein (CRP) were added to this long list.^{40,41}

It is believed that combined – acting simultaneously or sequentially – such environmental and genetic factors may contribute in a synergistic way to the development of cardiovascular diseases. Many of these characteristics are already present in adolescents and children and it has been suggested that subjects at risk for the development of cardiovascular diseases can be identified at a young age.^{42,43}

Clustering of cardiovascular risk indicators

Cardiovascular risk indicators are not independent entities. They tend to cluster in certain individuals. In adults, the combination of hyperinsulinemia, hypercholesterolemia, hypertension and coronary artery disease – first described by Reaven⁴⁴ – is well recognized and has become known as “Syndrome X”, “the metabolic syndrome” or “the insulin resistance syndrome”.⁴⁵⁻⁴⁷ Obesity is often considered a component of this syndrome,⁴⁸⁻⁵⁰ but the clustering of cardiovascular risk indicators is observed in the non-obese as well. A similar clustering of cardiovascular risk variables is also observed in children and adolescents.⁵¹⁻⁵³ It is believed that the development of insulin resistance plays a central role and contributes to the clustering of the other cardiovascular risk indicators.^{54,55}

Cardiovascular risk indicators and the childhood origin of atherosclerosis

One of the most important processes related to the development of cardiovascular diseases is atherosclerosis. Apart from relatively rare cases suffering from genetic disorders like familial hypercholesterolaemia, clinically relevant or symptomatic atherosclerotic lesions are generally not observed at a pediatric age. However, evidence from autopsy studies suggests that the subclinical development of atherosclerosis start during childhood already.^{56,57} In young adults and children, early stages of the atherosclerotic process (*i.e.* fatty streaks, intermediate lesions, fibrous plaques or even more advanced lesions) can be found in the aorta and coronary arteries.⁵⁸⁻⁶¹ In a study based on more than 500 autopsies, approximately one third of the deceased children under 9 years of age had simple intimal fatty streaks in the left coronary artery.⁶² At puberty, more than 50% had larger lesions containing foam cells, extracellular lipid, and lipid in smooth muscle cells. By the age of 20 to 30 years, one third of young adults had developed raised lesions with fibromuscular caps and extracellular lipid cores. The size and number of lesions like these increases with age and are associated with the ante-mortem presence of several cardiovascular risk indicators.⁶³⁻⁷⁰ Combinations of obesity, high blood pressure, elevated serum concentrations of triacylglycerol, total cholesterol and low-density lipoprotein cholesterol (LDL-cholesterol), or low serum concentrations of high-density lipoprotein cholesterol (HDL-cholesterol), during childhood are all related to the extent of the atherosclerotic lesions present.^{64,67,69} These findings demonstrate an association between cardiovascular risk indicators and the development of atherosclerosis in the young, and they seem to justify the evaluation of such risk variables in children.

Tracking of cardiovascular risk indicators from childhood into adulthood

The development of the multi-factorial hypothesis for the origins of cardiovascular diseases and the recognition that atherosclerosis starts at a young age already, prompted researchers to study cardiovascular risk indicators in children during the 1960s and 1970s.⁷¹ Since that time, numerous studies have contributed to the currently available

information on risk variables in children and on the trends of such variables over time.^{51,72-77} An important observation was that children tend to maintain their ranking order with respect to several cardiovascular risk indicators from childhood into adulthood; a phenomenon referred to as "tracking". Tracking has been demonstrated for serum lipid and lipoprotein concentrations,^{78,79} obesity,⁸⁰⁻⁸² blood pressure,^{83,84} fitness (aerobic power),⁸⁵ and plasma insulin concentration.^{86,87} The occurrence of tracking implies that the evaluation of cardiovascular risk indicators in children can indeed be used to identify groups of individuals at potential risk for developing cardiovascular diseases later in life. In this light, the recently reported increases in the prevalence of obesity and non-insulin-dependent diabetes mellitus among children and adolescents certainly justify some concerns for the future.⁸⁸⁻⁹¹

On the fetal origins of cardiovascular diseases

Aside from the classical risk variables discussed above, a new and intriguing risk indicator has been identified during recent years. It suggests that adult cardiovascular diseases may not only arise from the prolonged effects of genetic and environmental factors acting since childhood but could also in part originate from experiences during the short period of life before birth. A small size at birth (or during infancy) has namely been associated to an increased risk for the development of adult heart disease, Type II (non-insulin-dependent) diabetes mellitus and related risk factors such as hypertension and dyslipidemia.⁹²⁻⁹⁶ Different research groups have found similar relations in both men and women from various populations.⁹⁷⁻¹⁰⁴ These findings started a whole new field of research on the origins of cardiovascular diseases.

The fetal origins hypothesis

The associations found between infant size at birth and adult diseases led to the formulation of the so-called "fetal origins hypothesis" or the "Barker hypothesis", named after the man who revived the concept that experiences before birth contribute to the development of adult diseases.¹⁰⁵ According to this hypothesis, the propensity to develop coronary heart disease, Type II diabetes mellitus, hypertension and other disorders are consequences of intra-uterine adaptations to fetal undernourishment.¹⁰⁶ These adaptations are presumed to persist during adult life and considered detrimental in the postnatal abundance of food.¹⁰⁷ The risk for adult diseases may thus be "programmed" or "imprinted" by the environment *in utero*.^{108,109} Studies on subjects born around the time of the Dutch famine in the winter of 1944-1945 seem to support this concept. Prenatal exposure to the famine was associated with a higher risk for obesity, glucose intolerance and a more atherogenic lipid profile in adult life.¹¹⁰⁻¹¹³ The prevalence of coronary heart disease was higher in people prenatally exposed to the Dutch famine (during early gestation only) compared to those born before or after the famine.¹¹⁴ A similar study on subjects born during the Leningrad siege between 1941

and 1944 did not reveal such associations.¹¹⁵ The comparability of the conditions before, during and after the Leningrad siege and the Dutch famine are, however, debated.¹¹³

Evidence for the fetal origins hypothesis in children

According to Whincup, studies on the fetal origins of cardiovascular diseases in children are important since they may complement the epidemiological studies in adults in several ways.¹¹⁶ First, potential confounding factors such as adult life style (e.g. smoking, alcohol use or certain dietary habits) are hardly operant during childhood. Secondly, the effects of the fetal environment may interact with postnatal factors such as growth, especially the development of obesity;^{98,99} studies in children allow us to evaluate these interactive effects. Additionally, most studies in adults are based on pre-war birth cohorts; studies in children (born under current perinatal circumstances) further allow us to examine the temporary relevance of the fetal origins hypothesis.¹¹⁶

In children, birth weight or other measures of size at birth have indeed been related to a diversity of cardiovascular risk indicators. Negative relations between size at birth and systolic blood pressure have been reported in many birth cohorts,¹¹⁷⁻¹²² including a cohort of Dutch children.¹²³ This association appears to be dependent on current body weight, body mass index or on other parameters of postnatal growth. It further seems that the relation between small size at birth and childhood blood pressure becomes stronger with increasing age but may be attenuated during puberty.^{117,119,124,125} Indices of glycaemic control (such as fasting insulin or pro-insulin concentration, glycated hemoglobin level or glucose intolerance) in children have also been associated to a small size at birth.^{120,126,127} Moreover, serum concentrations of cholesterol and triacylglycerol were negatively related to size at birth in some of the studied cohorts.^{120,126,128} A lower weight at birth has also been related to (central) adiposity in children,^{126,129-131} but this relation with body fatness has been refuted in other studies.¹³²⁻¹³⁴ The reported associations between small size at birth and cardiovascular risk indicators were not found in all populations studied.^{135,136} In some studies, the associations found did not seem to indicate a relation with impaired fetal nutrition (e.g. as reflected by a low ponderal index).^{121,135}

Postnatal "catch-up" growth, the return to the genetically determined growth trajectory after birth, might be a more sensitive indicator of intra-uterine growth restraint caused by environmental factors than a small size at birth itself. Postnatal "catch-up" growth has been associated to an increased body weight, body mass index, percent body fat, and waist circumference in British five-year-olds.¹³⁷ These variables predict adult obesity and are related to a variety of cardiovascular risk indicators in children.^{81,138} Moreover, postnatal "catch-up" growth has indeed been associated to adult coronary heart disease mortality.^{104,139} The combination of a small size at birth and subsequent development of obesity during childhood thus seems to be of particular

importance for the development of an unfavorable cardiovascular risk profile.^{116,126,139,140}

Suggested mechanisms to explain the fetal origins of adult diseases

A diversity of mechanisms have been proposed to explain how intra-uterine adaptations to fetal undernourishment could eventually lead to the development of adult diseases.¹⁰⁶ Fetal growth is characterized by periods of rapid cell division and different tissues grow during various so-called "critical periods".¹⁴¹ The availability of oxygen and nutrients during such periods of cell division may influence the rate of growth and permanently determine the number of cells in particular organs.¹⁰⁶ In animal experiments, periods of undernourishment have been related to reduced numbers of cells in various tissues.^{142,143} Furthermore, such alterations in the diet (e.g. low protein intake) of pregnant animals have been demonstrated to cause persisting changes in the metabolism of their offspring.^{144,145} In humans, a reduction of the number of pancreatic β -cells or renal nephrons at birth have been suggested to explain the increased risk for the development of Type II diabetes mellitus or hypertension later in life.^{146,147}

Alternatively, nutritional factors may permanently alter gene expression in certain tissues or could affect the selection of cell lines that will ultimately form a particular organ (clonal selection).¹⁰⁸ The effects of nutrients are not necessarily direct and on a cellular level; it is very well possible that they could be mediated through various hormone systems (e.g. insulin, glucocorticoids, gut hormones *etc.*).^{108,148-150} Several epigenetic mechanisms have also been proposed, including the possibility of genomic imprinting in response to environmental factors such as nutrition during development.^{151,152} Others suggest that the associations found between infant size at birth and adult diseases, especially Type II diabetes mellitus, can readily be explained by common genetic variations rather than by fetal nutrition.¹⁵³⁻¹⁵⁵ Insulin plays a key role in fetal growth, and a disturbed insulin metabolism caused by such genetic factors could be responsible for both a low infant weight at birth and the propensity to develop Type II diabetes mellitus later in life.

In short, a large diversity of mechanisms have been proposed to explain the relations between a small size at birth and adult diseases but scarce evidence for each is available. At present, it seems unlikely that only one factor (one gene, an enzyme, a hormone, one specific nutrient) or mechanism can explain all of the observed phenomena.¹⁵⁶

Essential fatty acids and the early origins of cardiovascular diseases

The suggestion that nutrition plays a central role in the fetal origins of adult diseases is now supported by three sets of evidence.¹⁵⁷ First, support comes from the epidemiological findings of the Dutch Hunger Winter studies (discussed above).¹¹⁰⁻¹¹³ Secondly, manipulation of the maternal diet in pregnant animals permanently alters the

physiology of their offspring in a way that is consistent with the phenomena observed in human epidemiological studies.^{144,158-161} And finally, the third line of evidence comes from the current knowledge of the regulation of fetal growth, in which the fetal supply of nutrients play a key role.^{157,162}

So far, only a very small number of human studies have been conducted to identify the nutrients involved. Those who did mostly focussed on the effects of the balance of macro-nutrients (*e.g.* the protein/carbohydrate balance) of the maternal diet during pregnancy.¹⁶³⁻¹⁶⁵ Hardly any work has been done to explore the importance of specific nutrients and their balance on fetal growth and later outcome.¹⁶⁶ For instance, based on animal experiments the availability of specific amino acids, such as taurine, may have critical roles.¹⁶⁷ Other specific nutrients that have been implicated, but have not been studied in this context so far, are essential fatty acids and their derivatives.¹⁶⁸⁻¹⁷⁰ Beneficial effects of dietary essential fatty acids have been shown in relation to adult cardiovascular diseases and related disorders like dyslipidemia, hypertension, thrombosis, and insulin resistance.¹⁷¹⁻¹⁸³ Whether the fetal availability of these substances may influence later cardiovascular risk is unknown. But recent findings from animal experiments certainly seem to support the idea that the availability of specific fatty acids early in life may be of importance.¹⁸⁴

Essential fatty acids and their derivatives

At the start of the previous century, Burr and Burr discovered that a fat-free diet causes retarded growth, scaly dermatitis, trans-epidermal water loss, renal disorders, reproductive failure and even death in rats.^{185,186} Subsequent supplementation with small amounts of vegetable oils markedly improved the deficiency symptoms.^{185,186} Their findings clearly demonstrated that certain fatty acids are essential constituents of the diet in animals. Several decades later, two essential fatty acids (*i.e.* linoleic acid and α -linolenic acid) were identified and proven to be indispensable for humans as well.¹⁸⁷⁻¹⁹¹ Since man lack the required enzymes for the synthesis of linoleic acid and α -linolenic acid, these fatty acids have to be obtained through the diet. Once consumed, linoleic acid and α -linolenic acid can be converted into a diversity of products with important biological functions (Figure 1.2). Because there is no interconversion between the products derived from linoleic acid and those derived from α -linolenic acid, the derivatives are either designated to the so-called n-3 (or ω -3) family (derived from α -linolenic acid) or to the n-6 (or ω -6) family (derived from linoleic acid) of fatty acids.

The n-3 and n-6 fatty acids are found especially in (membrane) phospholipids. They are not just a source of energy, but are also necessary for maintaining particular bodily functions. Linoleic acid is important for maintaining the integrity of the epidermal water barrier^{192,193} and is also the precursor for dihomo- γ -linolenic acid and arachidonic acid, which are both substrates for the production of eicosanoids from the 1- and 2-series, respectively.^{194,195} Eicosapentaenoic acid is a derivative of α -linolenic

acid, and is the precursor for the 3-series eicosanoids. These eicosanoids are active mediators of many physiological and pathological processes like, for instance, immunological responses, blood pressure regulation, reproduction, body temperature regulation, and blood coagulation.^{196,197} Additionally, arachidonic acid and docosahexaenoic acid are important structural cellular components in the brain, and are believed to fulfill an array of membrane associated functions. These may include alterations in cellular ion transport, hormone receptor number or affinity, signal transduction and membrane fluidity.¹⁹⁸⁻²⁰¹ Moreover, essential fatty acids and their products may regulate gene expression, directly or via regulatory proteins such as the so-called peroxisome proliferator-activated receptors (PPARs).²⁰²⁻²⁰⁴

When the availability of essential fatty acids or their products is lower than required, the human body starts to synthesize fatty acids of similar molecular structure. One example is eicosatrienoic acid (Mead acid; 20:3n-9), which is a derivative of oleic acid (18:1n-9).²⁰⁵ Eicosatrienoic acid is therefore regarded as a marker for an essential fatty acid shortage. Another example is docosapentaenoic acid (Osbond acid; 22:5n-6), which is considered a marker for a limited availability of especially docosahexaenoic acid.²⁰⁶ Relatively high amounts of these fatty acids have been found in fetal blood and tissue.^{207,208} This finding has led to the suggestion that current fetal essential fatty acid availability might not be adequate, or at least, seems marginal.^{208,209}

Fetal essential fatty acid availability

Because the fetus can not synthesize linoleic acid and α -linolenic acid, these fatty acids have to be obtained from the maternal circulation. The source of the derived products is less well known. They may be synthesized by the placenta or by the fetus itself. However, since the fetal and placental capacity to do so seems rather low,²¹⁰⁻²¹³ it is more likely that these products are also derived from the maternal circulation by placental transfer. The close relationship found between maternal dietary fatty acid intake and blood fatty acid concentrations on one hand and fetal blood fatty acid concentrations on the other hand, support this notion.²¹⁴⁻²¹⁶ Fatty acid transport across the placenta might be selective.²¹⁷⁻²¹⁹

When comparing fetal blood concentrations of fatty acids with maternal blood concentrations (expressed as % by wt of total fatty acids), higher concentrations of long-chain poly-unsaturated fatty acids and lower concentrations of the essential fatty acids linoleic acid and α -linolenic acid are found in fetal blood.^{214,220,221} The concentrations (% by wt of total fatty acids) of long-chain polyunsaturated fatty acids of the n-3 and n-6 family also increases from maternal blood, placenta, fetal blood, fetal liver and finally fetal brain.²²² These observations suggest a preferred transfer of the long-chain polyunsaturated derivatives of linoleic acid and α -linolenic acid to fetal tissues. The term "biomagnification" has been introduced to describe this phenomenon.²²²

Based on blood and tissue concentrations, the quantitative accretion of n-3 and n-6 long-chain polyunsaturated fatty acids by the fetus increases progressively during gestation.²²³⁻²²⁶ The last trimester of pregnancy is therefore regarded as an important period for fetal fatty acid supply. Preterm infants are devoid of this important phase in life and are born with lower essential fatty acid concentrations in their blood and tissues than infants carried to term.²²⁷

Infants born with a lower weight at birth also have lower blood and tissue essential fatty acid concentrations.²²⁸ Considering that growth retardation is one of the prominent features of essential fatty acid deficiency,^{185-187,190} the availability of essential fatty acids might be a determinant of fetal growth. In premature infants, birth weight was positively related to the proportions of arachidonic acid, dihomo- γ -linolenic acid and docosahexaenoic acid measured in blood or tissue lipid fractions.²²⁹⁻²³³ Moreover, higher blood and tissue concentrations of essential fatty acid deficiency markers (like Mead acid) have been found in low-birth-weight neonates.²²⁸ Such findings suggest an important role for essential fatty acids in fetal growth.²³⁴ A few studies have indicated that the maternal intake of specific fatty acids (particularly n-3 fatty acids) might influence the length of gestation and thereby infant weight at birth.^{235,236} More recent work has, however, largely failed to confirm that birth weight might be influenced in such a way.²³⁷

The combination of the suggested inadequacy of fetal essential fatty acid availability under current dietary conditions, the potential importance of essential fatty acids for fetal growth, and the wide array of regulatory functions of essential fatty acids and their products, renders them as attractive candidate nutrients involved in the fetal origins of cardiovascular diseases.

Fetal versus maternal nutrition

The availability of nutrients to the fetus is ultimately dependent upon the diet of its mother, but factors like maternal metabolism, uterine and umbilical blood flows, and placental transfer and metabolism play a very important role as well.^{157,162} The way in which the placenta is able to deliver the required substances will eventually determine the nutritional status of the developing fetus. For this reason, measuring maternal nutrient intake or nutritional status (e.g. using biochemical indicators) does not necessarily provide an accurate estimate of fetal nutrient availability. This aspect may in part explain why the influence of maternal nutrition on infant size at birth seems absent or small in many human studies.²³⁸⁻²⁴¹ Fetal nutrition should thus be distinguished from maternal nutrition.¹⁵⁷ The fetal availability of specific nutrients like fatty acids might thus be studied at best by using fetal biochemical indicators such as measurements of neonatal blood or tissue fatty acid concentrations.

Figure 1.2: Main pathways for the conversion of the essential fatty acids linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) into their longer-chain polyunsaturated derivatives. The nomenclature used for fatty acids is as follows: the number of carbon atoms, followed by a colon and the number of double bonds; the fatty acid family is indicated by designation of the first double bond from the methyl terminus of the carbon chain.

Common genetic variations

The risk for the development of cardiovascular diseases is in part determined by hereditary factors.^{242,243} Numerous rare and common genetic variations have now been identified and studied in relation to cardiovascular diseases, non-insulin-dependent diabetes mellitus, obesity, dyslipidemia, hypertension or serum concentrations of fibrinolytic or coagulation factors. Well-known are the mutations in the LDL-receptor gene, leading to familial hypercholesterolaemia. These mutations are found in approximately 1 out of 400-500 individuals. Relatively rare mutations like these, however, will contribute little to the total variation of serum lipid and lipoprotein concentrations observed within a population. Other mutations, found at a much higher rate in the general population are probably of more importance. A few well-known common variants (so-called "polymorphisms") are discussed here in more detail.

Hydrophobic molecules such as cholesterol, triacylglycerol and other lipids are transported in the aqueous surroundings of the blood by circulating lipoprotein particles. These lipoprotein particles contain so-called apolipoproteins, the protein moieties of these particles. Common variations in the genes coding for these apolipoproteins may affect serum concentrations of lipoproteins (like LDL or HDL) and thereby influence the risk for cardiovascular diseases.^{244,245} Apolipoprotein E (apo E), for instance, is present in both apolipoprotein B-containing particles (such as LDL) as well as apolipoprotein A1-containing lipoproteins (*i.e.* HDL). It has been estimated that genetic variation at the apo E locus may account for up to 10-15 % of the observed inter-individual variation in serum total cholesterol and LDL-cholesterol concentrations.²⁴⁶⁻²⁴⁸

The most studied polymorphism of apo E is the combination of variations in amino acids at residues 112 and 158. The $\epsilon 2$ allele (substitution of arginine at position 158 by cysteine) is associated with lower concentrations of serum total cholesterol and LDL-cholesterol, whereas the $\epsilon 4$ allele (substitution of cysteine at position 112 by arginine) relates to higher levels of total cholesterol and LDL-cholesterol.^{249,250} These effects of apo E genotype are observed at a paediatric age already,²⁵¹ as well as during infancy.²⁵² Variations at the apo E locus have also been shown to both influence the clustering of cardiovascular risk indicators in children²⁵³ and the tracking of cholesterol and lipoprotein levels from childhood into adulthood.²⁵⁴⁻²⁵⁷ Moreover, genetic variation at the apo E locus in children and young adults is related to the parental history of myocardial infarction.^{253,258} Similarly, genetic variations in other apolipoproteins (*e.g.* apo B, apo A-I or apo CIII) may also affect serum concentrations of lipids and lipoproteins in children and thereby contribute to the risk of heart disease in adult life.²⁵⁹

Apart from variations in genes coding for apolipoproteins, variations in other genes like those coding for enzymes involved in lipid and lipoprotein metabolism are important as well. One example is the enzyme called cholesteryl ester transfer protein (CETP).²⁶⁰ CETP plays a key role in reverse cholesterol transport; the transport of cell-

derived cholesterol back to the liver mediated by HDL particles. It mediates the transfer of cholesteryl esters from HDL to apo B containing lipoproteins like LDL and very-low-density lipoprotein (VLDL), in exchange for triacylglycerol.²⁶¹ An increased CETP activity is negatively related to serum levels of HDL cholesterol and positively related to VLDL and LDL cholesterol concentrations.²⁶¹ In adults, plasma CETP concentrations, CETP activity and HDL cholesterol levels have been associated with common variations in the CETP gene, like the *Taq* IB polymorphism.²⁶²⁻²⁶⁴ The presence of this polymorphism (*i.e.* B1 allele) has also been related to the progression of coronary atherosclerosis.²⁶⁵

Common genetic variations like these may not only be important predictors of circulating cardiovascular risk factor levels in children (and thus as co-variables), they could also influence the individual response of a subject to an environmental stimulus. Like for instance the response to dietary interventions, medical treatments, alcohol consumption or smoking.²⁶⁵⁻²⁷² Recently, the effects of the $\epsilon 2$ and $\epsilon 4$ alleles at the apo E locus on serum LDL-cholesterol concentration were found to be more pronounced in adults with a lower body weight during infancy compared to adults with a higher body weight during infancy.²⁷³ The hypothesized effects of fetal essential fatty acid availability on the cardiovascular risk profile later in life may depend upon the presence of such common genetic variations.

Outline of this thesis

Objectives of the study

The primary objective of the study described in this thesis was to investigate whether the availability of essential fatty acids and their long-chain polyunsaturated derivatives during fetal life, as is reflected by the fatty acid composition of umbilical cord plasma phospholipids, relates to the cardiovascular risk profile of children. Secondly, to study associations and possible interactions with a selection of common gene variants. For this purpose cardiovascular risk indicators and gene polymorphisms were studied in a cohort of Dutch seven-year-olds.

Study population

Subjects were recruited from a birth-cohort of children. Between 1989 and 1995, pregnant women from the Maastricht region were asked to participate in a longitudinal observational study investigating the changes in essential fatty acid status during pregnancy and their relation to pregnancy outcome.^{215,216,274,275} Three antenatal clinics participated: the University Hospital in Maastricht, Hospital "De Wever" in Heerlen, and the School for Midwifery in Kerkrade. These centers are all located in the province of Limburg in the southern part of the Netherlands. Selection criteria for entering the study were a gestational age of less than 16 weeks at entry and no apparent sign of any cardiovascular, neurological, renal, or metabolic disorder at the time of recruitment. In

order to determine the changes in essential fatty acid status during pregnancy, maternal blood samples were collected during the first, second and third trimester of pregnancy, and shortly after delivery. Directly after parturition, an additional blood sample was obtained from the umbilical vein to estimate fetal essential fatty acid availability. Local hospital staff members recorded individual maternal and infant characteristics on standardized data sheets. Additional information was retrieved from questionnaires and medical records. In a sub-sample of these women, the habitual maternal dietary fatty acid intake was assessed.^{215,276} This unique cohort of women and children provides an excellent opportunity to study potential relations between fetal essential fatty acid availability and cardiovascular risk later in life. Therefore, a follow-up study was performed between 1997 and 2000. All singleton babies born alive before 1994 – and of whom an umbilical blood sample was available for the determination of the essential fatty acid status at birth – were candidates to be included. In total, 750 children were eligible for follow-up.

Participation during the follow-up study

With the aid of the Dutch population registry 97% of the eligible population could be traced (Figure 1.3). Three of the children had died and 34 lived abroad. The parents of the remaining 691 children received an invitation for the follow-up evaluation. Despite repeated invitations, 133 parents did not respond. Eventually we were able to contact the parents of 558 children. Of these, 231 refused to participate. The most important reasons for not participating were: lack of time or interest (39%), medical problems of the child (12%), medical problems of another family member (3%), burden of the protocol (12%), travel distance or lack of transportation (10%), the lack of a financial reward (1%), reason unknown (11%) or for a diversity of other reasons (13%). In total 297 children and their parents finally showed up at the appointments made for the follow-up on cardiovascular risk profile. The birth characteristics of these 297 children were not significantly different from the 453 children who did not attend our clinic (Table 1.1), suggesting that the investigated children comprised a representative sample of the entire cohort.

Measurements during the follow-up study

The children were investigated before puberty, at approximately seven years of age. Fasting blood samples were collected for measurement of blood lipids and lipoproteins, fibrinolytic and coagulation factors, indices of glycaemic control and to determine the essential fatty acid status at seven years of age. Leukocytes were isolated to extract DNA for analysis of gene polymorphisms. Anthropometric data was collected to study relations with body size and body composition. Additionally, resting blood pressure was measured and maximal treadmill exercise performance was assessed.

Figure 1.3: Participation during the follow-up study on cardiovascular risk profile. Values are absolute numbers or proportions of the total eligible population.

A questionnaire and a structured interview with one or both parents was used to collect additional information like socio-economic status, infant nutrition, and family history for cardiovascular diseases and related disorders.

Table 1.1: Comparison of birth characteristics between participants and non-participants.

Characteristic	Participants (n = 297)	Non-participants (n = 453)
Sex ratio (M : F)	164 : 133	254 : 199
Length of gestation (wk)	39.9 (39.7; 40.1)	39.9 (39.7; 40.0)
Birth weight (g)	3301 (3243; 3360)	3254 (3207; 3301)
Weight standard deviation score ¹	-0.13 (-0.23; -0.02)	-0.20 (-0.29; -0.12)
Crown-heel length at birth (cm)	49.9 (49.6; 50.2)	49.9 (49.6; 50.1)
Occipital-frontal circumference (cm)	34.4 (34.2; 34.6)	34.3 (34.1; 34.5)
Ponderal index ² (g/cm ³)	2.7 (2.6; 2.7)	2.6 (2.6; 2.7)
Born SGA ¹	37 (12)	60 (13)
Born LGA ¹	19 (6)	26 (6)
Mode of delivery		
vaginal	228 (77)	333 (74)
extraction (vacuum/forceps)	49 (16)	68 (15)
caesarian section	19 (6)	49 (11)
Birth order		
1	211 (71)	339 (75)
2	65 (22)	95 (21)
3 or more	20 (7)	17 (4)

Values are mean (95 % CI) or number (%). Totals may vary due to missing data.

¹According to Dutch references (appropriate for length of gestation, infant sex and birth order).²⁷⁷ Standard deviation score (or z-score) is the distance between observed birth weight and the average birth weight of the reference population expressed in standard deviation units.²⁷⁸ SGA, small for gestational age (birth weight $\leq$ 10th percentile). LGA, large for gestational age (birth weight $\geq$ 90th percentile).

²Ponderal index was calculated as birth weight (g) divided by [length (cm)]³ $\times$ 100.

No statistically significant differences were found between participants and non-participants.

Presentation of the results in the present thesis

The distribution of essential fatty acid concentrations in maternal and umbilical plasma phospholipids depends upon various maternal and infant characteristics. In *Chapter 2* some of these characteristics are discussed and the distribution of the concentrations of individual n-3 and n-6 polyunsaturated fatty acids in maternal and umbilical cord blood samples are presented. The relations between the concentrations of polyunsaturated fatty acids in umbilical cord plasma phospholipids and infant size at birth are described in *Chapter 3*. Further reported are the associations between umbilical cord plasma phospholipid n-3 and n-6 polyunsaturated fatty acid concentrations (measured at birth) and factors related to the cardiovascular risk profile of the children (measured at follow-up). These are fasting glucose, insulin, proinsulin, and leptin concentrations (*Chapter 4*), body size and body composition (*Chapter 4*), and blood lipids and lipoproteins (*Chapter 5*). The influences of common gene variants on plasma lipid and lipoprotein concentrations are reported in *Chapter 6*. An overall discussion of the results, their implications, and potential sources of erroneous conclusions is given in *Chapter 7*. Several appendices were added to provide additional information.

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Chapter 2 | The n-3 and n-6 polyunsaturated fatty acid composition of plasma phospholipids in pregnant women and their infants. Relationship with maternal linoleic acid intake

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Abstract

The availability of long-chain polyunsaturated fatty acids during infancy has been related to neonatal growth and development. Fatty acid concentration at birth is an important predictor of postnatal level. The primary aim of this study was to provide a description of the distribution of n-3 and n-6 polyunsaturated fatty acids in the plasma phospholipid fraction of pregnant women remaining on a Western-style diet and their neonates. The plasma phospholipid polyunsaturated fatty acid composition was determined by gas-liquid chromatography in 889 mother-infant pairs. Blood samples were taken during the first, second and third trimester of pregnancy, at delivery, and from the umbilical vein at birth. Mean ($\pm$ SD) fatty acid concentrations are reported in mg/L and as percentage of total fatty acids (% wt/wt). In addition, the 10th, 25th, 50th, 75th and 90th percentiles are given. The distribution of docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6) concentrations in umbilical plasma phospholipids is also reported as a function of gestational age and maternal linoleic acid intake during pregnancy. This data can be used as a reference for future studies and may aid in identifying term infants with a relatively low long-chain polyene status at birth.

Introduction

The biological significance of n-3 and n-6 polyunsaturated fatty acids (PUFAs) for optimal neonatal growth and development has received considerable attention.¹⁻⁴ The involved long-chain polyenes (LCPs), such as arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3), can be derived from the parent essential fatty acids linoleic acid (18:2n-6) and α -linolenic acid (18:3n-3), respectively (see Figure 1.2, Chapter 1).¹ The derivatives dihomo- γ -linolenic acid (20:3n-6), arachidonic acid and eicosapentaenoic acid (20:5n-3) are direct precursors of prostaglandins and other eicosanoids. Arachidonic acid and docosahexaenoic acid are important structural and functional components of cellular membranes. Concentrations of these PUFAs in plasma lipid fractions are often used as a marker for dietary exposure or to indicate a subject's essential fatty acid status. The availability of detailed reference values for plasma PUFA concentrations in pregnant women and their neonates might be useful for comparative investigations and for metabolic and nutritional studies.

Although fatty acid concentrations in maternal and umbilical plasma lipid fractions have been described before,⁵⁻¹¹ most of the available information is based on relatively small numbers of subjects. To our knowledge, none of the previously published papers reported percentile values for absolute (mg/L) and relative (% wt/wt) PUFA concentrations. During a longitudinal observational study, we determined the fatty acid composition of plasma phospholipids in a large population of pregnant women and their neonates. The main purpose of the present paper was to describe the distribution of the n-3 and n-6 PUFA concentrations. In addition, we studied the relationship between the fatty acid profiles and the maternal linoleic acid intake in a sub-sample of the population.

Subjects and Methods

Study population

At their first visit to the antenatal clinic, pregnant women were asked to participate in a longitudinal observational study of the changes in essential fatty acid status during pregnancy and their relation to the outcome of pregnancy.^{6,12,13} Three antenatal clinics participated: the University Hospital in Maastricht, Hospital "De Wever" in Heerlen and the School for Midwifery in Kerkrade. These centers are all located in the province of Limburg in the southern part of the Netherlands. The entry criteria for the study were gestational age of less than 16 weeks at and no signs of cardiovascular, neurological, renal, or metabolic disorders. Plasma samples were available in a total of 1212 mother-infant pairs. After excluding cases on the basis of multiple pregnancy ($n = 24$), premature delivery (pregnancy duration < 37 completed weeks, $n = 89$), post-term delivery (pregnancy duration ≥ 43 completed weeks, $n = 4$), infant death ($n = 5$), inappropriate birth weight (≤ 2.3 th percentile or ≥ 97.7 percentile, according to Dutch

reference data,¹⁴ $n = 36$), unknown gestational age or birth weight ($n = 37$), maternal diabetes ($n = 40$) or maternal hypertension ($n = 162$), a total study population of 889 women and neonates was left for analysis. Approval for this study was obtained from the Ethics Committee of the University Hospital Maastricht and all participating women gave their written informed consent.

Local hospital staff members recorded individual maternal and infant characteristics on a standardized data sheet. Additional information was obtained by questionnaires or retrieved from medical records. Pregnancy duration (in weeks) was calculated from the recorded date of delivery and the self-reported first day of the last menstrual period, and fractions were expressed in decimals. If the last menstrual period was unknown, gestational age was based on the early ultrasound measurements. The maternal and infant characteristics are summarized in Table 2.1.

Dietary intake of fatty acids

To determine the habitual dietary fatty acid intake of the pregnant women, a cross-check dietary history was carried out in a sub-sample of the study population during the second trimester, at approximately 22 weeks of gestation. A trained dietitian questioned 288 selected women about their usual food consumption patterns over the preceding 2 months. The food consumption data were encoded according to the system of the Netherlands Nutrition Databank and converted into nutrient intake data using the Netherlands food table (version NL3a). This version of the Netherlands food table does not allow the calculation of individual $n-3$ and $n-6$ fatty acid intake other than that of linoleic acid (18:2 $n-6$) and total PUFA (for more details see Al *et al.*¹³ and Badart-Smook *et al.*¹⁵).

Blood collection and determination of fatty acid composition

Maternal venous blood samples were collected in EDTA-treated tubes during the first, second, and third trimester, and after delivery. Directly after parturition, an additional blood sample was obtained from the umbilical vein. Plasma was separated by centrifugation and stored under nitrogen at -80°C until analysis. The fatty acid composition of maternal and umbilical plasma phospholipids was determined by capillary gas-liquid chromatography, as described before.⁶ Briefly, after the addition of 1, 2-dinonadecanoyl phosphatidyl-choline (internal standard) total lipid extracts from 100 μL plasma were prepared using modified Folch extraction.^{16,17} Phospholipids were isolated by solid phase separation from total lipid extracts on aminopropyl-silica columns ($\pm 90\%$ recovery).¹⁸ To check for carry-over of other lipid fractions during this procedure, heptadecaenoic acid (17:1) was added to the samples. After saponification of the isolated phospholipids, fatty acids were converted to the corresponding methyl esters (FAMES).¹⁹ The FAMES were analyzed by capillary gas-liquid chromatography using a 50m CP-Sil 5 CB non-polar capillary column (Chrompack, Middelburg, the Netherlands).

Table 2.1: Characteristics of the study population ($n = 889$).

	Mean $\pm$ SD or frequency (%)
Maternal characteristics	
Age (y)	29.3 $\pm$ 4.4
Height (cm)	166.3 $\pm$ 6.4
Weight at study entry (kg)	65.7 $\pm$ 11.3
Weight increase during pregnancy (kg)	11.6 $\pm$ 4.1
Parity at entry (%)	
nullipara	73.2
primipara	21.7
multipara	5.1
Dietary fatty acid intake (g/day) ¹	
total fat	97.7 $\pm$ 33.5
PUFA	18.4 $\pm$ 8.3
MUFA	37.1 $\pm$ 13.1
SFA	40.7 $\pm$ 13.7
linoleic acid (18:2n-6)	15.6 $\pm$ 7.5
Infant characteristics	
Sex (% female)	45.2
Weight (g)	
boys	3397 $\pm$ 420
girls	3277 $\pm$ 420
Weight $\leq 10^{\text{th}}$ percentile (%) ²	11.5
Weight $\geq 90^{\text{th}}$ percentile (%) ²	5.6

PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

¹Measured by dietary history midway gestation in a sub-sample of 288 women.

²According to Dutch reference data (appropriate for gestational age, infant sex and birth order).¹⁴ Note that infants with a birth weight $\leq 2.3^{\text{rd}}$ percentile or $\geq 97.7^{\text{th}}$ percentile were excluded.

Fatty acid concentrations are reported in mg/L plasma and as a percentage of total fatty acids (% wt/wt). Quantification was based on the amount of internal standard (19:0) recovered, assuming that equal mass amounts of long-chain polyunsaturated fatty acid methyl esters give rise to equal gas chromatography (GC) peak areas.²⁰

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). In addition, the 10th, 25th, 50th (median), 75th, and the 90th percentiles are reported. Spearman rank correlation tests were applied to study the effect of the gestational age at birth and maternal PUFA intake. Differences between means were evaluated by either paired or unpaired two-tailed Student's *t* tests or by non-parametric Wilcoxon signed rank tests. Due to incomplete data, not all analyses were based on the same number of subjects. A two-tailed *P* value < 0.05 was considered statistically significant. All statistical analyses were performed using the StatView version 4.5 statistical software (Abacus Concepts Inc., Berkeley, CA, USA).

Results

The mean ($\pm$ SD) and percentiles of PUFA concentration in maternal plasma phospholipids at delivery are given in Table 2.2 (the data on the distribution of PUFA concentrations observed during pregnancy are given in *Appendices A, B, and C*). The women tended to maintain their ranking order with respect to their plasma phospholipid fatty acid concentrations during pregnancy (Spearman rank correlation coefficients for the associations between values at the first trimester of pregnancy and those determined at delivery were 0.52 for linoleic acid, 0.63 for arachidonic acid, and 0.56 for docosahexaenoic acid; $P < 0.0001$ for all). The PUFA composition of umbilical plasma phospholipids is reported for boys and girls separately (Tables 2.3 & 2.4). The concentrations of the other identified fatty acids are reported in Table 2.5.

The well-known differences in PUFA concentrations between mothers and neonates were observed. The total concentration of phospholipid-associated fatty acids was 45.39 mg/L (95% CI: 27.71 - 63.07) higher in girls than in boys. A similar difference of approximately 7% was observed for the individual concentrations (mg/L) of the predominant fatty acids (Tables 2.3 & 2.4). However, the proportions (% wt/wt) of individual fatty acids did not differ significantly between boys and girls. These findings indicate that it was the size of the phospholipid pool rather than its composition that differed. The PUFA concentrations in maternal plasma were important predictors of the PUFA concentrations in neonatal plasma (Spearman $r = 0.39-0.50$; $P < 0.0001$ for all PUFAs with a mean concentration $> 1\%$ wt/wt).

The PUFA composition of umbilical cord plasma phospholipids was related to the gestational age at birth (maternal PUFA concentrations at delivery were not related to the length of gestation). The concentration (% wt/wt and mg/L) of 22:6n-3 was positively related to gestational age at birth ($P < 0.0001$ for both). In contrast, concentrations of arachidonic acid (20:4n-6), dihomo- γ -linolenic acid (20:3n-6) and docosapentaenoic acid (22:5n-6) hardly differed between infants born at different gestational ages. Moreover, concentrations (mg/L and % wt/wt) of docosapentaenoic acid (22:5-3) and docosatretraenoic acid (22:4n-6) were positively associated with the length of gestation, whereas concentrations of eicosatrienoic acid (20:3n-9) were negatively related to gestational age at birth ($P < 0.01$ for all).

Legend to Table 2.2 (next page): Mean $\pm$ SD gestational age at delivery was 40.2 ± 1.2 wk. ND, not detectable; $\Sigma n-6$ and $\Sigma n-3$, sum of all n-6 and n-3 fatty acids; $\Sigma n-6$ LCP, sum of n-6 long-chain polyenes (20:3n-6, 20:4n-6, 22:4n-6, and 22:5n-6); $\Sigma n-3$ LCP, sum of n-3 long-chain polyenes (20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3); $\Sigma n-7 + n-9$, sum of all n-7 and n-9 fatty acids; ΣSFA , $\Sigma MUFA$, $\Sigma PUFA$, sum of all saturated, monounsaturated, and polyunsaturated fatty acids, respectively. The total amount and all individual fatty acid concentrations (mg/L and % wt/wt) were significantly different between mothers and neonates ($P < 0.0001$, by paired Student's t test or Wilcoxon signed rank test in 724 mother-infant pairs).

Table 2.2: The polyunsaturated fatty acid composition of maternal plasma phospholipids at delivery ($n = 760$).¹

Fatty acid	mg/L							% wt/wt						
	Mean	SD	Percentile					Mean	SD	Percentile				
			10	25	50	75	90			10	25	50	75	90
18:2n-6	361.54	76.87	262.74	310.93	359.67	410.82	460.89	20.69	2.48	17.94	19.29	20.53	22.15	23.92
18:3n-6	0.62	0.42	0.00	0.36	0.56	0.84	1.12	0.03	0.02	0.00	0.02	0.03	0.05	0.06
20:2n-6	7.77	2.05	5.23	6.46	7.63	9.09	10.31	0.44	0.08	0.35	0.39	0.44	0.49	0.54
20:3n-6	61.21	17.36	40.70	49.47	59.59	70.55	83.82	3.48	0.63	2.76	3.07	3.41	3.82	4.32
20:4n-6	149.31	37.04	105.73	122.93	146.59	171.80	196.32	8.57	1.55	6.66	7.60	8.43	9.44	10.35
22:2n-6	0.33	0.20	0.00	0.23	0.34	0.45	0.57	0.02	0.01	0.00	0.01	0.02	0.03	0.03
22:4n-6	6.57	1.88	4.41	5.32	6.37	7.56	9.07	0.38	0.08	0.28	0.33	0.37	0.42	0.48
22:5n-6	8.98	3.55	4.86	6.59	8.50	10.83	13.85	0.51	0.17	0.31	0.40	0.49	0.61	0.73
24:2n-6	3.02	1.17	1.73	2.23	2.86	3.61	4.53	0.18	0.07	0.10	0.13	0.17	0.21	0.26
Σ n-6	599.57	115.56	451.45	545.61	601.88	674.56	744.59	34.31	2.13	32.00	33.12	34.38	35.64	36.67
Σ n-6 LCP	226.06	52.06	162.26	191.22	223.07	258.58	296.03	12.94	1.76	10.89	11.85	12.88	13.94	15.00
18:3n-3	3.99	4.69	1.65	2.45	3.69	4.98	6.41	0.23	0.48	0.11	0.15	0.21	0.28	0.34
20:3n-3	0.36	0.29	0.00	0.18	0.39	0.53	0.65	0.02	0.02	0.00	0.00	0.02	0.03	0.04
20:4n-3	2.40	1.17	1.13	1.60	2.18	3.02	3.97	0.14	0.06	0.07	0.10	0.12	0.17	0.21
20:5n-3	6.51	5.78	2.83	3.79	5.24	7.11	10.12	0.36	0.51	0.18	0.23	0.30	0.40	0.53
22:5n-3	9.54	2.83	6.44	7.57	9.25	11.16	13.10	0.54	0.12	0.40	0.46	0.53	0.61	0.69
22:6n-3	66.79	18.65	44.58	54.18	65.16	77.66	91.11	3.83	0.78	2.91	3.29	3.76	4.26	4.90
Σ n-3	89.34	25.13	60.42	71.85	87.35	102.75	120.81	5.12	1.26	3.99	4.42	4.99	5.65	6.32
Σ n-3 LCP	84.98	23.77	57.54	68.29	82.84	98.23	115.63	4.87	1.03	3.76	4.17	4.77	5.37	6.08
20:3n-9	4.27	2.44	1.66	2.57	3.77	5.51	7.60	0.24	0.13	0.11	0.16	0.22	0.31	0.41
Σ n-7 + n-9	227.71	50.40	166.52	196.08	225.34	258.71	293.25	13.02	1.49	11.28	12.07	12.99	14.04	14.82
Σ PUFA	693.53	130.91	526.35	611.44	696.97	779.59	863.54	39.70	1.54	37.77	38.73	39.72	40.73	41.64
Σ MUFA	225.02	49.45	164.79	193.47	223.15	254.74	289.50	12.87	1.45	11.17	11.95	12.85	13.83	14.59
Σ SFA	798.83	146.44	614.51	701.75	795.24	891.35	989.63	45.78	1.29	44.34	45.04	45.78	46.46	47.13
Total fatty acids	1746.3	321.6	1338.5	1540.3	1754.9	1953.1	2167.6	100.00						

¹For legend see previous page.

Table 2.3: The polyunsaturated fatty acid composition of umbilical cord plasma phospholipids in boys ($n = 430$).¹

	mg/L							% wt/wt						
	Mean	SD	Percentile					Mean	SD	Percentile				
			10	25	50	75	90			10	25	50	75	90
Fatty acid														
18:2n-6	43.46 ^a	13.43	29.83	35.16	41.86	49.80	57.72	7.59	1.37	6.09	6.74	7.46	8.40	9.17
18:3n-6	0.30	0.22	0.00	0.17	0.31	0.43	0.57	0.05	0.04	0.00	0.02	0.06	0.08	0.10
20:2n-6	2.08 ^d	0.54	1.44	1.72	2.03	2.38	2.78	0.37	0.07	0.29	0.32	0.36	0.40	0.45
20:3n-6	28.83 ^d	6.99	20.92	24.07	28.19	32.48	37.88	5.08	0.86	4.00	4.48	5.03	5.56	6.28
20:4n-6	94.84 ^d	19.51	72.36	81.03	93.46	105.16	118.80	16.70	1.69	14.69	15.69	16.69	17.78	18.72
22:2n-6	0.23	0.15	0.00	0.14	0.24	0.32	0.41	0.04	0.03	0.00	0.02	0.04	0.06	0.07
22:4n-6	4.53 ^b	1.14	3.17	3.73	4.43	5.12	6.03	0.80	0.14	0.62	0.70	0.79	0.88	0.98
22:5n-6	4.77	1.80	2.68	3.38	4.62	5.81	7.24	0.84	0.27	0.49	0.63	0.81	1.01	1.20
24:2n-6	3.40 ^b	1.12	2.13	2.63	3.29	4.07	4.78	0.60	0.16	0.40	0.50	0.59	0.70	0.81
Σ n-6	182.41 ^d	36.00	141.65	157.81	180.87	201.77	225.00	32.06	1.69	29.93	31.03	32.13	33.25	34.07
Σ n-6 LCP	132.96 ^d	25.23	103.25	115.93	131.43	147.89	164.07	23.41	1.58	21.53	22.56	23.50	24.41	25.33
18:3n-3	ND	—	—	—	—	—	—	ND	—	—	—	—	—	—
20:3n-3	ND	—	—	—	—	—	—	ND	—	—	—	—	—	—
20:4n-3	0.47	0.28	0.00	0.32	0.49	0.66	0.83	0.08	0.05	0.00	0.06	0.09	0.11	0.14
20:5n-3	1.23 ^a	0.69	0.58	0.85	1.12	1.46	2.01	0.22	0.11	0.11	0.15	0.20	0.26	0.35
22:5n-3	2.61 ^a	1.14	1.38	1.79	2.42	3.16	4.06	0.46	0.17	0.26	0.33	0.43	0.56	0.69
22:6n-3	34.82 ^b	10.14	23.27	28.06	33.53	40.10	48.23	6.12	1.30	4.47	5.16	6.06	7.01	7.83
Σ n-3	39.39 ^b	11.50	26.64	31.67	38.08	45.18	54.98	6.93	1.47	5.04	5.77	6.88	8.04	8.93
Σ n-3 LCP	39.13 ^b	11.45	26.56	31.42	37.89	45.04	54.65	6.88	1.47	5.02	5.73	6.78	7.98	8.91
20:3n-9	2.62	1.73	0.93	1.47	2.22	3.39	4.75	0.46	0.30	0.17	0.26	0.41	0.61	0.85
Σ n-7 + n-9	70.63 ^c	19.02	50.79	58.60	68.12	80.52	90.86	12.36	1.88	10.37	11.19	12.17	13.14	14.60
Σ PUFA	224.64 ^d	43.65	176.01	194.89	220.84	250.04	274.26	39.49	1.75	37.34	38.37	39.49	40.63	41.72
Σ MUFA	68.09 ^c	18.15	48.89	56.89	65.46	76.84	87.08	11.92	1.70	10.12	10.82	11.76	12.67	13.75
Σ SFA	270.56 ^d	52.37	211.07	234.51	267.79	302.46	331.72	47.53	1.58	45.79	46.73	47.60	48.45	49.26
Total fatty acids	569.43 ^d	110.02	447.16	489.48	562.20	631.83	701.28	100.00						

¹For legend see Table 2.2. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, ^d $P < 0.0001$ for difference between boys and girls (two-tailed Student t tests).

Table 2.4: The polyunsaturated fatty acid composition of umbilical plasma phospholipids in girls ($n = 350$).¹

	mg/L							% wt/wt						
	Mean	SD	Percentile					Mean	SD	Percentile				
			10	25	50	75	90			10	25	50	75	90
Fatty acid														
18:2n-6	46.29	22.81	31.89	37.53	43.87	50.90	61.29	7.44	1.39	6.11	6.63	7.34	8.27	8.86
18:3n-6	0.31	0.23	0.00	0.16	0.31	0.44	0.59	0.05	0.04	0.00	0.03	0.05	0.07	0.09
20:2n-6	2.29	0.74	1.52	1.82	2.19	2.61	3.15	0.37	0.07	0.29	0.32	0.37	0.41	0.45
20:3n-6	31.67	7.85	22.53	26.24	31.26	35.85	42.35	5.18	0.85	4.17	4.60	5.08	5.71	6.24
20:4n-6	103.20	22.89	77.05	87.55	101.78	114.45	134.44	16.84	1.61	14.92	15.80	16.69	17.90	18.87
22:2n-6	0.24	0.16	0.00	0.13	0.25	0.33	0.44	0.04	0.03	0.00	0.02	0.04	0.05	0.07
22:4n-6	4.78	1.29	3.31	3.87	4.63	5.41	6.55	0.78	0.14	0.61	0.68	0.77	0.87	0.96
22:5n-6	4.99	1.93	2.75	3.64	4.82	6.06	7.28	0.81	0.26	0.51	0.64	0.78	0.94	1.16
24:2n-6	3.68	1.26	2.23	2.80	3.55	4.41	5.25	0.60	0.16	0.40	0.49	0.60	0.71	0.78
Σ n-6	197.42	47.89	151.55	168.74	194.70	218.88	246.46	32.10	1.69	29.87	30.94	32.13	33.25	34.13
Σ n-6 LCP	144.63	30.04	111.10	124.05	142.63	161.99	182.37	23.61	1.54	21.74	22.77	23.63	24.58	25.46
18:3n-3	ND	—	—	—	—	—	—	ND	—	—	—	—	—	—
20:3n-3	ND	—	—	—	—	—	—	ND	—	—	—	—	—	—
20:4n-3	0.51	0.34	0.00	0.34	0.52	0.71	0.88	0.08	0.05	0.00	0.06	0.09	0.11	0.14
20:5n-3	1.31	0.70	0.61	0.86	1.20	1.68	2.16	0.22	0.11	0.10	0.15	0.20	0.28	0.34
22:5n-3	2.80	1.21	1.46	1.96	2.59	3.47	4.17	0.46	0.17	0.26	0.33	0.44	0.57	0.69
22:6n-3	37.17	11.01	25.30	29.25	35.35	41.86	53.14	6.08	1.36	4.35	5.03	5.98	7.04	8.04
Σ n-3	42.03	12.49	28.42	33.23	39.69	47.86	59.64	6.88	1.53	4.91	5.71	6.81	7.94	9.08
Σ n-3 LCP	41.79	12.38	28.34	33.02	39.49	47.51	59.64	6.84	1.53	4.90	5.68	6.75	7.92	9.03
20:3n-9	2.66	1.64	1.01	1.56	2.28	3.30	5.13	0.44	0.25	0.17	0.26	0.38	0.56	0.77
Σ n-7 + n-9	75.35	22.38	53.42	62.97	72.89	84.90	97.29	12.33	1.91	10.40	11.12	12.06	13.17	14.47
Σ PUFA	242.40	55.59	187.48	207.54	238.75	270.35	302.03	39.47	1.72	37.31	38.46	39.49	40.67	41.50
Σ MUFA	73.35	22.30	51.78	61.13	70.56	81.82	93.66	11.88	1.74	10.13	10.82	11.66	12.62	13.66
Σ SFA	292.65	66.38	222.53	250.61	286.86	322.77	363.90	47.62	1.24	46.23	46.81	47.72	48.41	49.07
Total fatty acids	614.82	141.50	469.69	525.02	603.68	683.44	760.93	100.00						

¹For legend see Table 2.2.

Table 2.5: Concentrations of saturated, n-7 and n-9 unsaturated, and *trans* fatty acids in maternal and umbilical cord plasma phospholipids.

Fatty acid	Maternal plasma		Umbilical plasma	
	mg/L	% wt/wt	mg/L	% wt/wt
14:0	5.20 ± 1.88	0.29 ± 0.08	1.41 ± 0.59	0.24 ± 0.08
15:0	2.97 ± 0.99	0.17 ± 0.04	0.67 ± 0.31	0.11 ± 0.05
16:0	543.16 ± 102.12	30.96 ± 1.21	163.93 ± 34.17	27.69 ± 1.42
17:0	4.84 ± 1.77	0.28 ± 0.09	1.56 ± 0.70	0.27 ± 0.11
18:0	168.48 ± 33.53	9.61 ± 0.85	84.58 ± 17.22	14.31 ± 1.04
20:0	9.14 ± 1.99	0.52 ± 0.09	5.09 ± 1.43	0.86 ± 0.16
22:0	26.04 ± 6.18	1.49 ± 0.29	8.93 ± 2.84	1.51 ± 0.37
23:0	9.79 ± 2.38	0.56 ± 0.10	1.23 ± 0.51	0.21 ± 0.07
24:0	16.46 ± 4.20	0.94 ± 0.19	8.95 ± 2.49	1.51 ± 0.29
16:1n-7	8.24 ± 4.25	0.46 ± 0.21	3.05 ± 1.52	0.51 ± 0.20
18:1n-7	25.46 ± 8.85	1.46 ± 0.44	14.13 ± 3.92	2.38 ± 0.39
20:1n-7	ND	ND	ND	ND
18:1n-9	154.22 ± 36.21	8.78 ± 1.20	39.58 ± 12.79	6.66 ± 1.32
18:2n-9	ND	ND	ND	ND
20:1n-9	2.49 ± 0.69	0.14 ± 0.03	0.31 ± 0.18	0.05 ± 0.03
22:1n-9	ND	ND	ND	ND
22:3n-9	ND	ND	ND	ND
24:1n-9	28.48 ± 7.40	1.63 ± 0.35	13.14 ± 3.67	2.21 ± 0.38
Σ Trans	5.42 ± 4.16	0.31 ± 0.23	0.40 ± 0.51	0.07 ± 0.08

Mean ± SD. ND, not detectable or detected in trace amounts only.

The ratio of the sum of all essential fatty acids and their derivatives to the sum of all non-essential fatty acids of the n-7 and n-9 families (Σ n-3 + n-6 / Σ n-7 + n-9) and the ratio of all n-3 fatty acids to all n-6 fatty acids (Σ n-3 / Σ n-6) were positively related to the length of gestation ($P < 0.0001$ for both). The distribution of the relative concentrations of arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) in umbilical plasma phospholipids are plotted as a function of gestational age (Figure 2.1).

The maternal concentration (% wt/wt) of docosahexaenoic acid at delivery was dependent on maternal parity at study entry. Mean ± SD 22:6n-3 concentration was 3.8 ± 0.8, 3.7 ± 0.8, and 3.6 ± 0.9 % wt/wt ($P < 0.05$ by ANOVA) for nullipara, primipara, and multipara, respectively. Umbilical cord plasma concentrations of 22:6n-3 did not relate to maternal parity at study entry. Also, we did not observe a significant relationship between parity and maternal plasma and umbilical cord plasma concentrations of arachidonic acid (20:4n-6).

The average dietary fatty acid intake, according to the dietary history in a sub-sample of 288 women midway in gestation, is given in Table 2.1. The average intake of linoleic acid (18:2n-6) in this population of pregnant women was approximately 85% of total PUFA intake. The relationship between maternal 18:2n-6 intake and the PUFA concentrations in maternal and umbilical plasma phospholipids are shown in Table 2.6. Higher intake of 18:2n-6 was associated with higher concentrations (% wt/wt) of

18:2n-6 and lower concentrations (% wt/wt) of 22:6n-3 in maternal and umbilical plasma phospholipids. The proportion of infants with a 22:6n-3 concentration (% wt/wt) below the 10th percentile (appropriate for gestational age) was 3%, 10%, 11%, and 22% for mothers in the 1st, 2nd, 3rd, and 4th quartile of linoleic acid intake, respectively. A higher intake of 18:2n-6 did not relate to higher 20:4n-6 concentrations.

A higher intake of PUFAs other than linoleic acid (mostly α -linolenic acid) was related to higher concentrations of α -linolenic acid (18:3n-3) in maternal plasma and to higher concentrations of its derivative eicosapentaenoic acid (20:5n-3) in umbilical cord plasma (not shown). The higher intake of PUFAs other than linoleic acid did not relate to higher 22:6n-3 concentrations in maternal or umbilical cord blood. There was a positive relation between the intake of linoleic acid and the intake of other PUFAs ($r = 0.50$, $P < 0.0001$).

Discussion

The PUFA composition of maternal plasma phospholipids changes during pregnancy. This was described by Al *et al.*⁶ The PUFA concentration in maternal plasma lipids is known to be a strong predictor of the PUFA concentration in umbilical plasma lipids.^{5,6} Both are influenced by the maternal dietary intake of fatty acids (Table 2.6).^{13,21-24} Umbilical fatty acid concentrations measured after delivery, as in the present study, are essentially similar to concentrations found in fetal plasma when measured at a comparable gestational age.²⁵ Therefore, they seem to be a reasonable reflection of the intrauterine fatty acid supply. During intrauterine life, the placenta plays a crucial role in the delivery of essential fatty acids and their derivatives to the fetus.^{26,27} A selective transfer of LCPs seems to occur. As a result, the proportion (but not the absolute concentration) of LCPs is higher and the proportion of their parent essential fatty acids is lower in fetal and umbilical samples, compared with the corresponding proportion in maternal plasma (Tables 2.2-2.4).^{5,25} Similar findings were obtained when studying the fatty acid composition of plasma cholesterol esters, triglycerides and total lipids.⁸⁻¹¹ During postnatal life, the essential fatty acids and their derivatives are obtained from the infant's diet (initially from the breast milk or artificial formula). As a result, the fatty acid composition of plasma phospholipids will change and ultimately reach a pattern similar to that found in adults.^{9,26}

As stated above, the PUFA concentrations in umbilical plasma are related to the PUFA levels in maternal plasma.^{5,6} In addition, the PUFA composition of umbilical plasma phospholipids is also dependent on the duration of pregnancy (the present paper).^{25,28,29} Most pronounced is the increased absolute and relative concentration of docosahexaenoic acid (22:6n-3) (Figure 2.1).²⁸ This has also been described in fetal plasma phospholipids during pregnancy²⁵ and in neonatal plasma cholesteryl esters¹⁰ and triglycerides.¹¹

Figure 2.1: Distribution of the umbilical cord plasma phospholipid concentration of arachidonic acid (20:4n-6) and docosa-hexaenoic acid (22:6n-3) as a function of gestational age at birth. The number of infants in each gestational age group is given underneath the figure. The lines represent the 10th, 25th, 50th, 75th and 90th percentiles (P). The triangles represent the 25th, 50th and 75th percentiles for the preterm infants who were excluded only because of premature birth (average gestational age at birth: 35 ± 1.5 wk, *n* = 39). *P* < 0.0001 for the association between docosahexaenoic acid concentration and gestational age at birth (Spearman rank correlation test). The arachidonic acid concentration was not significantly related to the length of gestation. For more plots see *Appendix G*.

A preference for transport of 22:6n-3 to the fetal circulation by the placenta might explain these observations.³⁰ Although only limited information is available, an increase in fetal 22:6n-3 biosynthesis does not seem likely because stable fetal liver delta-5 and delta-6 desaturase activities have been found throughout the third trimester.³¹

Table 2.6: Average n-3 and n-6 polyunsaturated fatty acid concentrations in maternal and umbilical plasma phospholipids at delivery according to the maternal linoleic acid (18:2n-6) intake at mid-gestation (g/day).

Fatty acid (mg/L)	Quartiles of linoleic acid intake ¹			
	I	II	III	IV
Maternal plasma				
18:2n-6 ^a	342.89	376.37	382.73	383.94
20:3n-6	61.23	64.75	62.25	59.49
20:4n-6	141.70	155.78	142.72	146.02
18:3n-3	3.69	3.93	4.15	3.40
20:5n-3 ^a	6.33	5.99	6.02	5.25
22:5n-3 ^a	9.77	10.38	9.74	8.84
22:6n-3 ^a	70.16	71.25	65.21	64.39
Umbilical plasma				
18:2n-6	45.75	41.87	43.31	48.19
20:3n-6	31.38	29.45	30.48	30.03
20:4n-6	100.27	95.60	92.14	95.29
20:5n-3 ^b	1.52	1.32	1.19	1.26
22:5n-3 ^b	2.96	2.66	2.34	2.58
22:6n-3 ^c	40.27	35.55	33.34	33.48
Fatty acid (% wt/wt)	Quartiles of linoleic acid intake ¹			
	I	II	III	IV
Maternal plasma				
18:2n-6 ^d	20.04	20.51	21.58	21.89
20:3n-6 ^a	3.57	3.50	3.46	3.35
20:4n-6	8.29	8.52	8.02	8.37
18:3n-3	0.21	0.21	0.23	0.21
20:5n-3 ^a	0.37	0.32	0.33	0.31
22:5n-3 ^b	0.57	0.56	0.54	0.51
22:6n-3 ^b	4.11	3.88	3.66	3.69
Umbilical plasma				
18:2n-6 ^c	7.34	7.28	7.73	8.11
20:3n-6	5.07	5.12	5.43	5.14
20:4n-6	16.28	16.60	16.48	16.27
20:5n-3 ^a	0.25	0.23	0.22	0.22
22:5n-3	0.48	0.47	0.42	0.44
22:6n-3 ^b	6.51	6.21	5.93	5.73

¹The average ($\pm$ SE) linoleic acid intake was 7.57 ± 0.22 , 12.34 ± 0.14 , 16.79 ± 0.17 , and 25.71 ± 0.72 g/day, for the quartiles I, II, III and IV respectively.

^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, ^d $P < 0.0001$ for relation with linoleic acid intake (Spearman rank correlation test between the continuous variables, $n = 224$ for maternal plasma and $n = 233$ for umbilical plasma).

Uauy *et al.*³² even reported decreased LCP synthesis rates in infants born after a longer duration of pregnancy. The PUFA composition of umbilical plasma phospholipids is also related to the infant size at birth.²⁹ A higher weight at birth is associated with lower

proportions of both arachidonic acid (20:4n-6) and docosaheptaenoic acid (22:5n-3) in umbilical plasma phospholipids of term neonates (see Chapter 3).²⁹ Moreover, the concentrations of eicosatrienoic acid (20:3n-9) are higher and the ratio $\Sigma n-3 + n-6 / \Sigma n-7 + n-9$ and the ratio 22:6n-3/22:5n-6 are lower in heavier infants,²⁹ indicating a lower biochemical essential fatty acid status.³³⁻³⁵ These observations suggest that (under the present dietary conditions) the maternal-to-fetal delivery of LCPs may not fully keep up with fetal accretion rate in larger infants. Previously, contrasting findings had been described in smaller samples of premature infants and low-birth-weight neonates.^{26,36-38}

Apart from the aspects discussed above, several other factors should be taken into consideration when the information reported in this paper is used. First, the comparability of fatty acid concentrations between laboratories is limited by differences in the analytical procedures. Especially the time of addition of the internal standard is important since this influences the absolute concentration (mg/L).³⁹ Secondly, differences in dietary habits between populations are likely to be the reason for differences in the distribution of fatty acid concentrations. Therefore, the information presented here may be applicable only to populations with comparable dietary habits. Considering the high average intake of linoleic acid in our population (a characteristic feature of the Western-style diet) and the putative beneficial effects of a higher (n-3) LCP intake,^{40,41} it is questionable whether the percentiles given in this paper should be regarded as a pursuable standard. As shown in Table 2.6, higher intake of linoleic acid is related to lower maternal and umbilical 22:6n-3 concentrations.

Since the LCP status at birth is an important predictor of the postnatal LCP status,³⁸ an appropriate prenatal supply of LCPs (as determined by *e.g.* maternal dietary habits, maternal circulating LCP concentrations and the length of gestation) is important.⁴² The studies on premature infants (who have a low LCP status at birth) show that LCP-enriched formulas may have beneficial effects on the postnatal development.⁴³ The findings in term neonates have been less consistent.⁴⁴ Among the variables that may explain this inconsistency could be the LCP status at birth.⁴² Probably, term neonates with a relatively low LCP status at birth will benefit most from LCP-enriched formulas. The information reported in this paper may aid in identifying such infants.

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Chapter 3 | Essential fatty acid composition of plasma phospholipids and birth weight: a study in term neonates

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Abstract

Background Essential fatty acids (EFAs) in umbilical cord blood samples are associated with attained birth weight in premature infants and low-birth-weight neonates.

Objective The objective was to investigate relations between the EFA composition of cord and maternal plasma phospholipids and birth weight in term neonates.

Design This was a cross-sectional study in 627 singletons born at term. The plasma phospholipid EFA composition of the mothers was determined by liquid-gas chromatography at study entry (≤ 16 weeks gestation), at delivery, and in cord plasma at birth. Birth weights were normalized to SD scores.

Results In cord plasma, the dihomo- γ -linolenic acid concentration was positively related to weight SD scores. Both arachidonic acid (AA) and docosahexaenoic acid (DHA) were negatively related to weight SD scores. EFA-status indicators showed similar negative associations, whereas eicosatrienoic acid concentrations were positively related to neonatal size. In maternal plasma, proportions of n-3 long-chain polyenes (LCPs) and n-6 LCPs decreased during pregnancy. Larger decreases in AA, DHA, n-3 LCP and n-6 LCP fractions were observed in mothers of heavier babies. Higher concentrations of LCPs in maternal plasma were, however, not related to a larger infant size at birth.

Conclusions A lower biochemical EFA status in umbilical cord plasma and a larger decrease in maternal plasma LCP concentrations are associated with a higher weight-for-gestational-age at birth in term neonates. Our findings do not support a growth-stimulating effect of AA or DHA; however, they do suggest that maternal-to-fetal transfer of EFAs might be a limiting factor in determining neonatal EFA status.

Introduction

Growth retardation is one of the prominent features of essential fatty acid (EFA) deficiency in both animals^{1,2} and humans.^{3,4} Linoleic acid (18:2n-6) and the longer-chain polyunsaturated fatty acids (PUFAs) of the n-6 family are believed to be important for optimal growth. Lower concentrations of EFAs were found in red blood cell membranes, plasma phospholipids, and in the walls of the umbilical artery of low-birth-weight neonates.⁵ In premature infants, birth weight was positively associated with the proportions of arachidonic acid (AA; 20:4n-6) and dihomo- γ -linolenic acid (20:3n-6) in plasma triacylglycerols and choline phosphoglycerides.^{6,7} In some studies, positive associations between birth weight and docosahexaenoic acid (DHA; 22:6n-3) were described as well.⁷⁻⁹ On the Basis of these findings, a smaller size at birth seems to be related to a lower EFA status.

Besides individual EFA concentrations in plasma, erythrocytes, and tissue phospholipids, there are other indicators of biochemical EFA status. When the availability of EFAs does not meet functional requirements, the human body produces more fatty acids of comparable chain length and degree of unsaturation, such as eicosatrienoic acid (20:3n-9). Therefore, higher concentrations of eicosatrienoic acid indicate a lower EFA status. Similarly, docosapentaenoic acid (22:5n-6) is produced when DHA availability is marginal, and the ratio between DHA and docosapentaenoic acid can be used as an indicator of DHA status.¹⁰ A more general marker of EFA status is the ratio between the sum of all n-3 and n-6 fatty acids and the sum of all non-EFAs from the n-7 and n-9 families.¹¹ Crawford *et al.*⁵ reported "grossly abnormal" concentrations of two of these indexes in the umbilical artery walls of low-birth-weight neonates (birth weight < 2500 g) which suggests again that a small size at birth is associated with a lower EFA status.

Conclusions drawn from observations in premature infants or low-birth-weight neonates might, however, not be applicable to the more common situation of term birth. Few comparable studies in healthy term neonates have been conducted. In the present study we determined both EFA concentrations and EFA-status indexes in umbilical cord plasma phospholipid samples obtained from term neonates. These indexes of biochemical EFA status were used to investigate relations with infant birth weight. In addition, we studied relations between neonatal weight and observed changes in maternal plasma EFA composition during pregnancy.

Subjects and Methods

Study population

As part of other investigations, pregnant women were asked to participate in longitudinal observational studies investigating of changes in EFA status during pregnancy and the relation of these changes to pregnancy outcome.¹²⁻¹⁵ Three antenatal

clinics located in the province of Limburg in the southern part of the Netherlands participated: the University Hospital in Maastricht, Hospital "De Wever" in Heerlen, and the School for Midwifery in Kerkrade. Selection criteria for inclusion in these studies were a gestational age of ≤ 16 wk at entry, a diastolic blood pressure < 90 mmHg, and no signs of cardiovascular, neurologic, renal, or metabolic disorders at the time of recruitment. In the present analysis, the available data for 752 singletons born between January 1990 and January 1994 during these observational studies were used. After exclusion of infants with unknown gestational age or birth weight ($n = 6$), who were born prematurely (gestational age < 37 wk, $n = 43$), or who died ($n = 2$) and of mothers with diabetes ($n = 14$) or pregnancy-induced hypertension ($n = 71$), a total study population of 627 infants was left for analysis. Approval for these studies was obtained from the Ethics Committee of the University Hospital Maastricht, and all participating women gave their written, informed consent.

Gestational age and birth weight

Local hospital staff members recorded individual maternal and infant characteristics on a standardized data sheet. Additional information was obtained from medical records or by using questionnaires. Gestational age at birth (in wk) was calculated from the recorded date of delivery and the self-reported first day of the last menstrual period; fractions were expressed in decimals. If the last menstrual period was unknown, gestational age was based on early ultrasound measurements. Infants were categorized into 5 weight-for-gestational-age categories. Infants with a birth weight $\leq 10^{\text{th}}$ percentile were classified as small for gestational age (SGA) and those with a birth weight $\geq 90^{\text{th}}$ percentile as large for gestational age (LGA). Because most infants were classified as appropriate for gestational age (AGA), this category was divided into 3 subcategories: 1) a birth weight $> 10^{\text{th}}$ percentile but $\leq 25^{\text{th}}$ percentile, 2) a birth weight $> 25^{\text{th}}$ but $< 75^{\text{th}}$ percentile, and 3) a birth weight $\geq 75^{\text{th}}$ but $< 90^{\text{th}}$ percentile. This classification was based on the percentiles given by the Dutch reference standard (appropriate for length of gestation, infant sex, and birth order).¹⁶ In addition, recorded birth weights were converted into SD scores:¹⁷

$$\text{SD score} = (\text{birth weight}_{\text{observed}} - \text{mean birth weight}_{\text{reference}}) / \text{SD}_{\text{reference}} \quad (1)$$

In this way, a continuous measure for birth weight-for-gestational-age was created. A SD score of -2 corresponds to the 2.3^{rd} percentile and a SD score of 2 corresponds to the 97.7^{th} percentile of birth weight-for-gestational-age, respectively.

Blood collection and determination of fatty acid composition

Maternal venous blood samples were collected in EDTA-treated evacuated tubes at study entry (≤ 16 wk, mean ($\pm$ SD) gestational age at entry was 11 ± 3 wk) and after delivery.

Table 3.1: Fatty acid composition of umbilical cord plasma phospholipids of term neonates.¹

	Boys (n = 347)	Girls (n = 280)	All (n = 627)	Range
Total fatty acids (mg/L)	571.5 ± 6.07 ^a	613.9 ± 7.40	590.4 ± 4.79	251.5 – 1218.5
Fatty acids (% wt/wt)				
18:2n-6	7.54 ± 0.07	7.41 ± 0.07	7.48 ± 0.05	4.30 – 11.98
20:3n-6	5.06 ± 0.05	5.18 ± 0.05	5.11 ± 0.03	2.55 – 8.04
20:4n-6	16.74 ± 0.08	16.89 ± 0.09	16.81 ± 0.06	11.04 – 21.30
22:4n-6	0.81 ± 0.01	0.79 ± 0.01	0.80 ± 0.01	0.47 – 1.38
22:5n-6	0.86 ± 0.02	0.83 ± 0.02	0.85 ± 0.01	0.25 – 1.93
Σn-6	32.08 ± 0.09	32.17 ± 0.10	32.12 ± 0.07	24.11 – 36.51
Σn-6 LCPs	23.46 ± 0.08	23.68 ± 0.08	23.56 ± 0.06	18.38 – 27.79
18:3n-3	ND	ND	ND	ND
20:5n-3	0.23 ± 0.01	0.23 ± 0.01	0.23 ± 0.00	0.00 – 1.07
22:5n-3	0.47 ± 0.01	0.47 ± 0.01	0.47 ± 0.01	0.14 – 1.21
22:6n-3	6.20 ± 0.07	6.21 ± 0.08	6.21 ± 0.05	3.12 – 10.48
Σn-3	7.04 ± 0.08	7.04 ± 0.09	7.04 ± 0.06	3.57 – 11.69
Σn-3 LCPs	6.98 ± 0.08	6.70 ± 0.09	6.99 ± 0.06	3.54 – 11.69
20:3n-9	0.49 ± 0.02	0.44 ± 0.02	0.47 ± 0.01	0.00 – 2.32
Σn-7+n-9	12.44 ± 0.10	12.28 ± 0.12	12.37 ± 0.08	6.09 – 24.45
ΣSFAs	47.60 ± 0.08	47.69 ± 0.08	47.64 ± 0.06	39.33 – 57.27
ΣMUFAs	11.89 ± 0.09	11.77 ± 0.10	11.83 ± 0.07	5.75 – 23.53
ΣPUFAs	39.52 ± 0.09	39.59 ± 0.10	39.55 ± 0.07	31.47 – 43.73

¹Values are mean ± SEM. Σn-6 and Σn-3, the sum of all n-6 and n-3 fatty acids, respectively; Σn-6 LCPs, the sum of all n-6 long-chain polyenes (20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6); Σn-3 LCPs, the sum of all n-3 long-chain polyenes (20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3); Σn-7+n-9, the sum of all n-7 and n-9 fatty acids; ΣSFAs, ΣMUFAs and ΣPUFAs, the sum of all saturated, monounsaturated, and polyunsaturated fatty acids, respectively; ND, not detected.

^aSignificantly different from girls, $P < 0.0001$ (unpaired Student's *t* test).

Directly after parturition, a blood sample was obtained from the umbilical vein. Plasma was separated from blood cells by centrifugation (2000 × *g*, 4 °C, 15 min) and stored under nitrogen at –80 °C until analyzed.¹⁸ The fatty acid composition of maternal and umbilical cord plasma phospholipids was determined as described previously.¹² In short, after the addition of 1,2-dinonadecanoyl phosphatidylcholine (internal standard), total lipid extracts from 100 µL plasma were prepared by using a modified¹⁹ version of Folch *et al.*'s²⁰ extraction method. Phospholipids were isolated by solid-phase extraction of total lipid extracts on aminopropyl-silica columns.²¹ To check for carry-over of other lipid fractions during this procedure, heptadecaenoic acid (17:1) was added to the samples. After saponification of the isolated phospholipids, fatty acids were converted to the corresponding fatty acid methyl esters.²² The fatty acid methyl esters were analyzed by capillary gas-liquid chromatography with use of a 50-m CP-Sil 5 CB non-polar capillary column (Chrompack, Middelburg, The Netherlands). Plasma total phospholipid fatty acids were expressed in absolute concentrations (mg/L) and the individual fractions of fatty acids and fatty acid groups as relative values (% by wt of total fatty acids).

Table 3.2: Characteristics of the study population according to weight-for-gestational-age percentile category at birth.^{1,2}

	SGA, $\leq 10^{\text{th}}$ (<i>n</i> = 81)	AGA			LGA, $\geq 90^{\text{th}}$ (<i>n</i> = 41)
		$> 10^{\text{th}}$ to $\leq 25^{\text{th}}$ (<i>n</i> = 95)	$> 25^{\text{th}}$ to $< 75^{\text{th}}$ (<i>n</i> = 339)	$\geq 75^{\text{th}}$ to $< 90^{\text{th}}$ (<i>n</i> = 71)	
Maternal characteristics					
Age (y)	28.9 $\pm$ 4.1	28.9 $\pm$ 4.6	29.5 $\pm$ 4.2	29.3 $\pm$ 4.2	29.4 $\pm$ 3.9
Height (cm)	163.7 $\pm$ 6.9	164.4 $\pm$ 5.9	166.6 $\pm$ 6.6	168.5 $\pm$ 6.0	170.8 $\pm$ 6.0
Weight at study entry (kg)	61.7 $\pm$ 11.4	61.3 $\pm$ 9.6	65.2 $\pm$ 11.0	71.4 $\pm$ 15.8	71.3 $\pm$ 12.0
Weight increase during pregnancy (kg)	9.7 $\pm$ 3.8	10.7 $\pm$ 3.7	11.8 $\pm$ 3.8	12.4 $\pm$ 4.4	12.5 $\pm$ 3.9
Parity (%)					
0	67	71	74	75	68
1	30	23	21	21	27
2	1	5	4	4	5
3 or more	2	1	1	0	0
Smoking (%)	44	28	27	18	17
Mode of delivery (%)					
Vaginal	77	79	77	79	68
Extraction (vacuum/forceps)	11	12	17	13	17
Caesarian section	12	9	6	8	15
Infant characteristics					
Sex (M : F)	36 : 45	49 : 46	204 : 135	38 : 33	20 : 21
Gestational age (wk)	40.1 $\pm$ 1.3	40.0 $\pm$ 1.0	40.1 $\pm$ 1.2	40.6 $\pm$ 1.2	40.4 $\pm$ 1.3
Birth weight (g)	2661 $\pm$ 255	3006 $\pm$ 178	3380 $\pm$ 247	3856 $\pm$ 222	4166 $\pm$ 227
Weight SD score	-1.62 $\pm$ 0.39	-0.87 $\pm$ 0.17	-0.05 $\pm$ 0.36	0.90 $\pm$ 0.19	1.63 $\pm$ 0.32
Crown-heel length (cm)	47.6 $\pm$ 2.0	48.8 $\pm$ 1.7	50.3 $\pm$ 1.7	51.8 $\pm$ 1.6	52.5 $\pm$ 1.4
Occipital-frontal circumference (cm)	33.2 $\pm$ 1.3	34.0 $\pm$ 1.2	34.4 $\pm$ 1.3	35.5 $\pm$ 1.2	36.1 $\pm$ 1.2
Apgar score after 5 min	9.3 $\pm$ 1.2	9.7 $\pm$ 0.8	9.6 $\pm$ 0.8	9.7 $\pm$ 0.6	9.6 $\pm$ 0.6
Apgar score ≤ 7 (%)	9	2	2	1	3

¹SGA, small for gestational age; AGA, appropriate for gestational age; LGA, large for gestational age. The percentile categories are based on Dutch reference standards (appropriate for length of gestation, infant sex and birth order).¹⁶

²Mean $\pm$ SD; *n* = 627.

Table 3.3: Fatty acid composition of umbilical cord plasma phospholipids in term infants according to gestational age at birth.¹

	37 wk (n = 26)	38 wk (n = 69)	39 wk (n = 162)	40 wk (n = 189)	41 wk (n = 143)	42 wk (n = 38)	<i>P</i> for trend ²	
							crude (n = 627)	adjusted (n = 614)
Total fatty acids (mg/L)	594.6 ± 22.5	590.8 ± 16.2	580.2 ± 10.0	582.8 ± 7.79	613.6 ± 10.1	581.9 ± 18.7	0.3335	0.3233
Fatty acids (% wt/wt)								
18:2n-6	8.35 ± 0.21	7.79 ± 0.13	7.53 ± 0.09	7.38 ± 0.08	7.39 ± 0.10	6.96 ± 0.21	< 0.0001	< 0.0001
20:3n-6	5.04 ± 0.18	5.33 ± 0.12	5.24 ± 0.06	5.03 ± 0.06	5.00 ± 0.07	5.01 ± 0.14	0.0105	0.0039
20:4n-6	17.04 ± 0.30	16.61 ± 0.21	16.72 ± 0.12	16.93 ± 0.11	16.69 ± 0.13	17.21 ± 0.25	0.4008	0.3204
22:4n-6	0.72 ± 0.02	0.73 ± 0.01	0.80 ± 0.01	0.81 ± 0.01	0.82 ± 0.01	0.84 ± 0.02	< 0.0001	< 0.0001
22:5n-6	0.78 ± 0.05	0.82 ± 0.03	0.86 ± 0.02	0.85 ± 0.02	0.85 ± 0.02	0.87 ± 0.05	0.3751	0.2357
Σn-6	33.09 ± 0.24	32.38 ± 0.18	32.26 ± 0.12	32.06 ± 0.13	31.83 ± 0.14	31.88 ± 0.27	< 0.0001	0.0003
Σn-6 LCPs	23.57 ± 0.28	23.49 ± 0.18	23.62 ± 0.11	23.62 ± 0.11	23.35 ± 0.12	23.92 ± 0.21	0.9713	0.9291
18:3n-3	ND	ND	ND	ND	ND	ND		
20:5n-3	0.19 ± 0.02	0.22 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.24 ± 0.01	0.24 ± 0.02	0.0303	0.0423
22:5n-3	0.34 ± 0.03	0.38 ± 0.01	0.45 ± 0.01	0.48 ± 0.01	0.52 ± 0.01	0.57 ± 0.03	< 0.0001	< 0.0001
22:6n-3	5.12 ± 0.27	5.48 ± 0.13	6.07 ± 0.09	6.25 ± 0.09	6.61 ± 0.12	7.08 ± 0.19	< 0.0001	< 0.0001
Σn-3	5.74 ± 0.29	6.19 ± 0.14	6.90 ± 0.11	7.08 ± 0.11	7.51 ± 0.14	8.06 ± 0.22	< 0.0001	< 0.0001
Σn-3 LCPs	5.72 ± 0.29	6.15 ± 0.14	6.86 ± 0.11	7.04 ± 0.11	7.46 ± 0.14	7.98 ± 0.22	< 0.0001	< 0.0001
20:3n-9	0.44 ± 0.06	0.53 ± 0.03	0.47 ± 0.02	0.47 ± 0.02	0.43 ± 0.02	0.41 ± 0.03	0.0299	0.0060
Σn-7+n-9	12.90 ± 0.36	13.22 ± 0.22	12.33 ± 0.11	12.27 ± 0.13	12.22 ± 0.21	11.68 ± 0.20	< 0.0001	< 0.0001
ΣSFAs	47.55 ± 0.18	47.31 ± 0.17	47.72 ± 0.09	47.78 ± 0.11	47.57 ± 0.14	47.44 ± 0.20	0.4809	0.6155
ΣMUFAs	12.37 ± 0.30	12.61 ± 0.20	11.78 ± 0.09	11.73 ± 0.12	11.73 ± 0.20	11.21 ± 0.17	< 0.0001	< 0.0001
ΣPUFAs	39.18 ± 0.34	39.02 ± 0.19	39.54 ± 0.12	39.54 ± 0.13	39.70 ± 0.16	40.28 ± 0.24	0.0003	0.0006

¹Values are unadjusted means ± SEM. For legend see also Table 3.1; ND, not detected.

²*P* values for linear trends with the continuous variable gestational age are given crude and adjusted for potential confounding factors (maternal age, weight at entry, weight increase during pregnancy, smoking, parity, mode of delivery, 5-min Apgar score, infant sex and weight SD score).

Table 3.4: Fatty acid composition of umbilical cord plasma phospholipids in term infants according to weight-for-gestational-age percentile category at birth.¹

	SGA, $\leq 10^{\text{th}}$ (<i>n</i> = 81)	AGA				<i>P</i> for trend ²	
		$>10^{\text{th}}$ to $\leq 25^{\text{th}}$ (<i>n</i> = 95)	$>25^{\text{th}}$ to $<75^{\text{th}}$ (<i>n</i> = 339)	$\geq 75^{\text{th}}$ to $<90^{\text{th}}$ (<i>n</i> = 71)	LGA, $\geq 90^{\text{th}}$ (<i>n</i> = 41)	crude (<i>n</i> = 627)	adjusted (<i>n</i> = 614)
Total fatty acids (mg/L)	573.9 $\pm$ 12.6	576.7 $\pm$ 13.3	588.7 $\pm$ 6.48	621.2 $\pm$ 12.5	615.9 $\pm$ 20.2	0.0006	< 0.0001
Fatty acids (% wt/wt)							
18:2n-6	7.60 $\pm$ 0.13	7.51 $\pm$ 0.12	7.48 $\pm$ 0.06	7.35 $\pm$ 0.14	7.42 $\pm$ 0.19	0.0426	0.2131
20:3n-6	4.73 $\pm$ 0.10	5.03 $\pm$ 0.09	5.18 $\pm$ 0.05	5.18 $\pm$ 0.09	5.35 $\pm$ 0.11	< 0.0001	< 0.0001
20:4n-6	17.61 $\pm$ 0.18	17.01 $\pm$ 0.14	16.68 $\pm$ 0.09	16.60 $\pm$ 0.18	16.23 $\pm$ 0.22	< 0.0001	< 0.0001
22:4n-6	0.80 $\pm$ 0.02	0.82 $\pm$ 0.02	0.79 $\pm$ 0.01	0.78 $\pm$ 0.02	0.79 $\pm$ 0.02	0.1925	0.0574
22:5n-6	0.78 $\pm$ 0.03	0.85 $\pm$ 0.03	0.85 $\pm$ 0.02	0.86 $\pm$ 0.03	0.95 $\pm$ 0.05	0.0006	0.0001
Σ n-6	32.57 $\pm$ 0.17	32.32 $\pm$ 0.17	32.06 $\pm$ 0.09	31.80 $\pm$ 0.19	31.81 $\pm$ 0.23	0.0007	0.0021
Σ n-6 LCPs	23.92 $\pm$ 0.16	23.71 $\pm$ 0.14	23.49 $\pm$ 0.08	23.42 $\pm$ 0.18	23.31 $\pm$ 0.21	0.0371	0.0166
18:3n-3	ND	ND	ND	ND	ND		
20:5n-3	0.23 $\pm$ 0.01	0.22 $\pm$ 0.01	0.23 $\pm$ 0.01	0.24 $\pm$ 0.02	0.22 $\pm$ 0.01	0.9161	0.5722
22:5n-3	0.51 $\pm$ 0.02	0.47 $\pm$ 0.02	0.46 $\pm$ 0.01	0.48 $\pm$ 0.02	0.45 $\pm$ 0.02	0.0373	0.0003
22:6n-3	6.56 $\pm$ 0.16	6.28 $\pm$ 0.13	6.13 $\pm$ 0.07	6.32 $\pm$ 0.17	5.74 $\pm$ 0.19	0.0072	< 0.0001
Σ n-3	7.42 $\pm$ 0.17	7.11 $\pm$ 0.14	6.96 $\pm$ 0.08	7.17 $\pm$ 0.19	6.52 $\pm$ 0.21	0.0108	< 0.0001
Σ n-3 LCPs	7.38 $\pm$ 0.17	7.06 $\pm$ 0.14	6.91 $\pm$ 0.08	7.13 $\pm$ 0.19	6.49 $\pm$ 0.21	0.0112	< 0.0001
20:3n-9	0.35 $\pm$ 0.03	0.43 $\pm$ 0.03	0.48 $\pm$ 0.02	0.49 $\pm$ 0.03	0.61 $\pm$ 0.05	< 0.0001	< 0.0001
Σ n-7+n-9	11.67 $\pm$ 0.20	12.00 $\pm$ 0.16	12.49 $\pm$ 0.11	12.59 $\pm$ 0.21	13.16 $\pm$ 0.28	< 0.0001	< 0.0001
Σ SFAs	47.42 $\pm$ 0.15	47.73 $\pm$ 0.17	47.66 $\pm$ 0.08	47.57 $\pm$ 0.15	47.77 $\pm$ 0.14	0.3284	0.4106
Σ MUFAs	11.26 $\pm$ 0.19	11.50 $\pm$ 0.15	11.94 $\pm$ 0.10	12.02 $\pm$ 0.18	12.49 $\pm$ 0.23	< 0.0001	< 0.0001
Σ PUFAs	40.27 $\pm$ 0.16	39.79 $\pm$ 0.18	39.43 $\pm$ 0.09	39.38 $\pm$ 0.22	38.86 $\pm$ 0.23	< 0.0001	< 0.0001

¹Values are unadjusted means $\pm$ SEM. For legend see also Table 3.1 and Table 3.2. ND, not detected.²*P* values for linear trends with the continuous variable weight SD score are given crude and adjusted for potential confounding factors (maternal age, weight at entry, weight increase during pregnancy, smoking, parity, mode of delivery, 5-min Apgar score, infant sex and pregnancy duration).

In total, 39 fatty acids were identified but, for clarity, only the concentrations of 9 individual fatty acids and 8 fatty acid groups are reported (Table 3.1). In addition to the reported concentrations, two indexes of EFA status were calculated: the DHA-status index (ratio of DHA to docosapentaenoic acid (22:5n-6); a higher ratio indicates a higher DHA status)¹⁰ and the EFA-status index (ratio of $\sum n-3+n-6$ to $\sum n-7+n-9$; a higher ratio indicates a higher EFA status).¹¹

Statistical analysis

Values are reported as mean $\pm$ SEMs, unless specified otherwise. Differences between means were evaluated by either paired or unpaired two-tailed Student's *t* tests. Relations between variables were analyzed with simple and multiple regression models. To test for linear trends, the continuous variable weight SD score was used instead of the weight-for-gestational-age categories. Maternal age, maternal weight at study entry, weight increase during pregnancy, smoking habits, parity (ordinal), mode of delivery (dummies for extraction and caesarian section with vaginal delivery as a reference) and the 5-min Apgar score were included as potential confounding factors. When the total study sample was analyzed, infant sex was introduced as an additional factor. Because of a skewed distribution of some variables (20:5n-3, 20:3n-9 and DHA-status index) log or square-root transformed data were used in the analyses. Because of incomplete data records, not all analyses were based on the same number of subjects. A two-tailed *P* value < 0.05 was considered statistically significant. All statistical analyses were performed by using STATVIEW (version 4.5; Abacus Concepts Inc., Berkeley, CA).

Results

Characteristics of the women and their neonates are listed in Table 3.2. Birth weight ranged from 1875 to 4350 g in girls and from 2050 to 4620 g in boys. In total, 81 (13 %) infants were born SGA according to Dutch references.¹⁶ Forty-one (7 %) neonates were born LGA. On average, birth weight deviated from the reference mean by -0.16 SDs (range in weight SD score: -3.23 to 2.57). Weight SD scores did not differ significantly between boys and girls (mean $\pm$ SD: -0.14 ± 0.87 and -0.18 ± 0.92 , respectively) by unpaired Student's *t* test.

Infant sex and the EFA composition of plasma phospholipids

The total amount (mg/L) of fatty acids in umbilical cord plasma phospholipids of female neonates was larger than that observed in males (Table 3.1). This difference, however, was not due to a higher concentration of one particular fatty acid or fatty acid group. The proportions of each individual fatty acid and that of specific fatty acid groups did not differ between boys and girls. In addition, the total amount of fatty acids and the relative fatty acid composition of maternal plasma phospholipids did not differ between boys and girls, nor was there any sex-related difference in the observed

changes in relative fatty acid concentrations in maternal plasma phospholipids during pregnancy. For these reasons, further comparisons were done with boys and girls combined. Infant sex was used as an additional factor in the multiple regression models, but no major effect of sex on fatty acid fractions or relations were found.

The fatty acid α -linolenic acid (18:3n-3) was present in such low concentrations in umbilical plasma phospholipids that it could not be detected in most of the samples. In only 34% of the infants α -linolenic acid was detected in measurable amounts. The median α -linolenic acid concentration in these subjects was 0.12 % by wt of total fatty acids [inter quartile range (IQR): 0.07 % by wt of total fatty acids]. In the total group of infants, median α -linolenic acid concentration was 0.00 % by wt of total fatty acids (IQR: 0.08 % by wt of total fatty acids). The number of infants in whom α -linolenic acid could be measured did not differ significantly between boys and girls, gestational age groups, or weight-for-gestational-age groups (chi-square tests).

Gestational age and the fatty acid composition of umbilical plasma phospholipids

Infants born after a shorter duration of pregnancy had relatively higher linoleic acid and higher Σ n-6 fatty acid concentrations in their umbilical cord plasma phospholipids (Table 3.3). In contrast, 20:4n-6 and Σ n-6 long chain polyene (LCP) concentrations were not significantly related to gestational age at birth. Most pronounced, however, were the differences in the n-3 fatty acid fractions. Neonates born at a later gestational age had higher umbilical cord plasma concentrations of eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (22:5n-3), DHA, Σ n-3 and Σ n-3 LCPs, whereas the proportions of eicosatrienoic acid (20:3n-9) and Σ n-7+n-9 fatty acids were lower in these infants. The total fatty acid content was not related to gestational age at birth.

Birth weight and the fatty acid composition of umbilical cord plasma phospholipids

The relative EFA composition of umbilical cord plasma phospholipids was associated with weight-for-gestational-age at birth (Table 3.4). Fractions of AA, Σ n-6, Σ n-6 LCPs and Σ PUFAs were lower in infants born LGA. Both dihomog- γ -linolenic acid (20:3n-6) and docosapentaenoic acid (22:5n-6) concentrations were, however, higher in heavier neonates. Furthermore, docosapentaenoic acid (22:5n-3), DHA, Σ n-3 and Σ n-3 LCP concentrations were higher in the smaller infants, whereas the proportion of EPA was not related to weight-for-gestational-age at birth. In addition to these observations, 20:3n-9, Σ n-7+n-9 fatty acids and the sum of monounsaturated fatty acids (Σ MUFAs) concentrations were evidently higher in umbilical cord plasma samples of heavier infants. The total amount of plasma phospholipid fatty acids was also higher in heavier infants.

Gestational age, birth weight and indicators of EFA status

The EFA-status index was higher in the umbilical cord plasma of neonates born at a later time point but was lower in those born relatively large for gestational age (Figure

3.1, A & C). Similarly, the DHA-status index was higher in plasma of infants born after a longer duration of gestation and lower in those with a higher weight-for-gestational-age at birth (Figure 3.1, B & D). In Table 3.5, the association between weight-for-gestational-age at birth and the EFA-status index is compared with the relations of key prognostic indicators of fetal growth and infant size at birth.

EFA composition of maternal plasma phospholipids

Of all 627 selected neonates, information on the EFA composition of 582 maternal plasma samples taken at study entry (≤ 16 wk gestation) and of 568 maternal plasma samples taken at delivery were available. In a total of 546 cases (87%), the fatty acid compositions of both samples were known.

The well-known differences in relative fatty acid composition between maternal and umbilical cord plasma were observed (Tables 3.2 & 3.6). The concentration of α -linolenic acid was significantly higher in plasma phospholipids of mothers of infants in whom α -linolenic acid concentrations were measurable than in those of mothers of infants with undetectable amounts of α -linolenic acid (0.27 ± 0.10 % compared with 0.19 ± 0.10 % by wt of total fatty acids; $P < 0.0001$).

During pregnancy, the total fatty acid concentration increased and its composition changed significantly (Table 3.6). At delivery, relatively more saturated fatty acids and MUFAs were found, whereas the fraction of PUFAs was lower. The EFA-status index and the DHA-status index also decreased during pregnancy (Table 3.6). The observed changes in the mother's plasma EFA compositions were related to the size of their infants (Figures 3.2 & 3.3). The biggest decrease in plasma concentrations of AA, DHA, $\sum n-6$ LCP and $\sum n-3$ LCPs were observed in mothers of heavier infants, whereas the largest reduction in the fraction of linoleic acid was found in the mothers of relatively smaller neonates.

No cross-sectional association was found between maternal fatty acid concentrations and infant size at birth at study entry or at delivery. There also was no relation between maternal plasma fatty acid concentrations and the total duration of gestation. The fatty acid concentrations in maternal plasma were strong predictors of umbilical cord plasma fatty acid composition. However, the reported relations between umbilical cord plasma fatty acid composition and normalized birth weight were independent of the observed maternal concentrations.

Discussion

To our knowledge, this was the first study to show relations between umbilical cord plasma phospholipid EFA composition and size at birth in term neonates. Both the pattern of individual fatty acid fractions and the EFA-status indexes seemed to indicate a higher biochemical EFA status in umbilical cord plasma of smaller infants than in the plasma of larger ones.

Figure 3.1: Mean ($\pm$ SEM) essential fatty acid (EFA)-status index (ratio of $\Sigma n-3+n-6$ to $\Sigma n-7+n-9$) and docosahexaenoic acid (DHA)-status index (ratio of 22:6n-3 to 22:5n-6) in umbilical cord plasma phospholipids of infants according to gestational age at birth (A & B) and infant birth weight, *i.e.*, weight-for-gestational-age category (C & D). The categories are small for gestational age (SGA; birth weight $\leq 10^{\text{th}}$ percentile), appropriate for gestational age (AGA; divided into 3 subcategories: 1) birth weight $> 10^{\text{th}}$ but $\leq 25^{\text{th}}$ percentile, 2) $> 25^{\text{th}}$ but $< 75^{\text{th}}$ percentile, and 3) $\geq 75^{\text{th}}$ but $< 90^{\text{th}}$ percentile), and large for gestational age (LGA; birth weight $\geq 90^{\text{th}}$ percentile). All percentiles were based on Dutch reference standards (appropriate for length of gestation, infant sex, and birth order).¹⁶ The P values shown are for linear trends with the continuous variable gestational age or weight SD score.

Table 3.5: Association of weight-for-gestational-age at birth with the essential fatty acid-status index measured in umbilical cord plasma phospholipids and prognostic indicators of fetal growth.¹

	β	SE	t
Univariate analysis			
EFA-status index	-0.343 ^a	0.060	-5.8
Maternal weight at study entry (kg)	0.019 ^a	0.003	6.5
Weight increase during pregnancy (kg)	0.049 ^a	0.009	5.6
Maternal height (cm)	0.041 ^a	0.005	7.7
Maternal smoking (yes = 1; no = 0)	-0.353 ^a	0.079	-4.5
Maternal age (y)	0.010	0.008	1.2
Multivariate analysis			
EFA-status index	-0.369 ^a	0.055	-6.7
Maternal weight at study entry (kg)	0.018 ^a	0.003	6.3
Weight increase during pregnancy (kg)	0.051 ^a	0.008	6.3
Maternal height (cm)	0.024 ^a	0.005	4.7
Maternal smoking (yes = 1; no = 0)	-0.266 ^b	0.072	-3.7
Maternal age (y)	0.004	0.008	0.5

Essential fatty acid (EFA)-status index, ratio of $\Sigma n-3 + n-6$ to $\Sigma n-7 + n-9$.

¹Results of simple and multiple regression analyses with weight SD score (appropriate for length of gestation, infant sex, and birth order)¹⁶ as the dependent variable and the EFA-status index and prognostic indicators of fetal growth as independent variables.

^a $P < 0.0001$; ^b $P < 0.001$.

EFA as determinants of fetal growth

The concept that EFAs such as AA or DHA may serve as potential fetal growth factors was not supported by our results. Proportions of AA and DHA in umbilical cord plasma phospholipids were negatively related to neonatal size at birth (Table 3.4). In addition, no relation was found between the concentrations of these fatty acids in maternal plasma and infant birth weight. These findings are in contrast with previous observations in premature infants and low-birth-weight babies.^{5-7,23} In most of these studies, lower proportions of AA, DHA, or both were found in smaller neonates. An explanation for this inconsistency between studies could be that additional (pathologic) factors associated with premature birth or severe growth retardation affected both EFA concentrations and intrauterine growth in these populations. Another factor that might play a role is gestational age at birth. Both birth weight and the biochemical EFA status of newborns are related to the duration of pregnancy (see also *Chapter 2*).^{24,25} No adjustment for differences in gestational age at birth might therefore have confounded some of the previously reported associations. When birth weights were interpreted in relation to gestation duration in comparisons of SGA with AGA or LGA infants, no differences in plasma or vessel wall AA concentrations were found by several investigators.^{7,8,26,27} In one study, the reported relative DHA concentration was even significantly higher in SGA babies.⁷ However, most of these findings were based on observations in relatively small numbers of infants.

Table 3.6: Fatty acid composition of maternal plasma phospholipids.¹

	Study entry (<i>n</i> = 582)	Delivery (<i>n</i> = 568)	Change ^{2, a} (<i>n</i> = 546)
Total fatty acids (mg/L)	1329.5 ± 10.5	1759.4 ± 13.0	433.0 ± 14.6 (404.2; 461.7)
Fatty acids (% wt/wt)			
18:2n-6	21.48 ± 0.11	20.72 ± 0.10	-0.76 ± 0.10 (-0.96; -0.57)
20:3n-6	3.08 ± 0.03	3.46 ± 0.03	0.37 ± 0.03 (0.32; 0.42)
20:4n-6	9.61 ± 0.06	8.55 ± 0.06	-1.07 ± 0.05 (-1.17; -0.97)
22:4n-6	0.39 ± 0.00	0.38 ± 0.00	-0.02 ± 0.00 (-0.02; -0.01)
22:5n-6	0.35 ± 0.01	0.53 ± 0.01	0.18 ± 0.01 (0.17; 0.19)
Σn-6	35.55 ± 0.08	34.33 ± 0.08	-1.24 ± 0.08 (-1.40; -1.08)
Σn-6 LCPs	13.44 ± 0.07	12.92 ± 0.07	-0.53 ± 0.06 (-0.64; -0.42)
18:3n-3	0.21 ± 0.01	0.22 ± 0.00	0.01 ± 0.01 (-0.01; 0.02)
20:5n-3	0.55 ± 0.02	0.35 ± 0.01	-0.20 ± 0.02 (-0.23; -0.17)
22:5n-3	0.75 ± 0.01	0.55 ± 0.01	-0.20 ± 0.01 (-0.22; -0.19)
22:6n-3	4.04 ± 0.04	3.87 ± 0.03	-0.18 ± 0.03 (-0.24; -0.11)
Σn-3	5.69 ± 0.05	4.93 ± 0.04	-0.77 ± 0.04 (-0.85; -0.68)
Σn-3 LCPs	5.48 ± 0.05	4.91 ± 0.04	-0.58 ± 0.04 (-0.66; -0.49)
20:3n-9	0.19 ± 0.01	0.25 ± 0.01	0.06 ± 0.00 (0.05; 0.06)
Σn-7+n-9	11.95 ± 0.05	13.08 ± 0.06	1.13 ± 0.06 (1.02; 1.25)
ΣSFAs	44.36 ± 0.05	45.60 ± 0.04	1.24 ± 0.05 (1.14; 1.33)
ΣMUFAs	11.72 ± 0.05	12.80 ± 0.06	1.08 ± 0.06 (0.97; 1.19)
ΣPUFAs	41.44 ± 0.06	39.63 ± 0.06	-1.83 ± 0.07 (-1.96; -1.67)
DHA-status index	12.85 ± 0.25	8.21 ± 0.24	-4.71 ± 0.21 (-5.11; -4.30)
EFA-status index	3.50 ± 0.02	3.05 ± 0.02	-0.45 ± 0.02 (-0.50; -0.41)

¹Values are mean ± SEM (95% CI). For legend see also Table 3.1. DHA-status index, ratio of 22:6n-3 to 22:5n-6; EFA-status index, ratio of Σn-3 + n-6 to Σn-7 + n-9.

²Difference between fatty acid concentrations at delivery and at study entry (≤ 16 wk).

^aAll differences were significant at *P* < 0.0001 (paired Student's *t* test), except for α-linolenic acid (18:3n-3).

AA and DHA were also shown to be related to postnatal growth. During the first year after birth, negative effects of fish-oil-supplemented formula (rich in DHA and EPA) on infant growth were described in premature infants.²⁸ A reduction in the AA status was regarded as a causative factor in the observed growth restriction.²⁹ However, some intervention studies in term neonates found no such effect of DHA supplementation (with or without AA) on postnatal growth.³⁰⁻³³ To our knowledge, no studies of the effects of formulas supplemented with AA alone on infant growth have been conducted. A potential effect of AA and DHA on neonatal growth, therefore, is still controversial.

In contrast with AA and DHA concentrations, concentrations of dihomo-γ-linolenic acid (20:3n-6) were positively related to normalized birth weight (Table 3.4). Lower concentrations of dihomo-γ-linolenic acid in the blood or vessel walls of smaller than of larger neonates were reported previously and were also found in premature infants.^{6-8,26,27,34} The positive association between dihomo-γ-linolenic acid concentration and size at birth seems to be more consistent than that reported for AA or

DHA. Therefore, dihomog- γ -linolenic acid may be more important for intrauterine growth than AA. No study has yet evaluated the effect of dihomog- γ -linolenic acid supplementation on intrauterine or postnatal growth.

Maternal-to-fetal EFA supply

The n-3 and n-6 long-chain PUFAs (especially AA and DHA) are important structural and functional components of cell membranes. Therefore, a larger infant probably accretes more of these substances than does a smaller one. Because the fetal capacity to convert linoleic acid and α -linolenic acid into LCPs is limited,³⁵⁻³⁸ most of these LCPs are obtained from the maternal circulation via the placenta. The observation that umbilical cord plasma EFA concentrations are positively associated with both maternal plasma EFA concentrations and maternal dietary EFA intake¹⁴ supports this notion.

In a subgroup of the participating women, previously published information on the dietary intake of fatty acids^{14,15} was available (based on food frequency questionnaires and dietary history). The most important finding was a relatively high intake of linoleic acid ($\pm 6\%$ of total energy intake and $\pm 85\%$ of total PUFA intake). Such a high linoleic acid intake might explain the low α -linolenic acid concentrations found in umbilical cord plasma. Indeed, the dietary ratio of linoleic acid to other PUFAs (mainly α -linolenic acid) was significantly lower in mothers of infants in whom α -linolenic acid could be detected than in mothers of infants with undetectable amounts of α -linolenic acid ($P < 0.05$). Moreover, higher maternal plasma α -linolenic acid concentrations were found in mothers of infants with detectable α -linolenic acid concentrations. The maternal intake of fatty acids, however, did not differ between the weight-for-gestational-age groups (data not shown). The current finding that decreases in maternal plasma n-3 LCP and n-6 LCP fractions are more pronounced in women who gave birth to larger infants (Figure 3.2 & 3.3) implies that the EFA transfer from the mother to the fetus is related to fetal growth. It is possible that larger infants are born when the maternal-to-fetal transfer of LCPs is more efficient. However, an increased LCP transfer could also be an adaptation to an increased fetal LCP accretion. Because umbilical cord plasma EFA concentrations are positively related to maternal plasma EFA concentrations, whereas birth weights of infants are not, the latter explanation seems more likely.

The observed lower relative concentrations of AA and DHA, higher concentrations of plasma 20:3n-9 (Table 3.4), and lower EFA-status and DHA-status indexes (Figure 3.1) in the plasma of heavier neonates suggest that the maternal-to-fetal supply of EFAs is limited. It seems that even an increased maternal-to-fetal LCP flux cannot prevent a lower biochemical EFA status in plasma of heavier neonates. We showed previously that the biochemical EFA status determined on the basis of EFA concentrations in the cord plasma and vessel walls of twins and triplets is lower than observed in singletons.^{39,40}

Figure 3.2: Mean ($\pm$ SEM) changes in linoleic acid (18:2n-6), arachidonic acid (AA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3) concentration in maternal plasma phospholipids during pregnancy for the total study group and according to infant birth weight, *i.e.*, weight-for-gestational-age category. The categories are small for gestational age (SGA; birth weight $\leq 10^{\text{th}}$ percentile), appropriate for gestational age (AGA; divided into 3 subcategories: 1) birth weight $> 10^{\text{th}}$ but $\leq 25^{\text{th}}$ percentile, 2) $> 25^{\text{th}}$ but $< 75^{\text{th}}$ percentile, and 3) $\geq 75^{\text{th}}$ but $< 90^{\text{th}}$ percentile), and large for gestational age (LGA; birth weight $\geq 90^{\text{th}}$ percentile). All percentiles were based on Dutch reference standards (appropriate for length of gestation, infant sex, and birth order).¹⁶ The *P* values shown are for linear trends with the continuous variable weight SD score.

Figure 3.3: Mean ($\pm$ SEM) changes in n-6 long-chain polyenes ($\Sigma n-6$ LCPs) and n-3 long-chain polyenes ($\Sigma n-3$ LCPs) in maternal plasma phospholipids during pregnancy for the total study group and according to birth weight, *i.e.* weight-for-gestational-age category. The *P* values shown are for linear trends with the continuous variable weight SD score. For legend see also Figure 3.2.

These observations seem to support the concept that a larger total fetal tissue mass is related to an increased EFA accretion and the idea that the supply of EFA is limited. However, these hypotheses are based on observed associations and further evidence is needed to validate them. Studies using more advanced techniques, such as stable isotopes, are needed to evaluate complex dynamic processes like LCP accretion and placental transport efficiency.

Changes in linoleic acid concentration

The pregnancy-associated decrease in linoleic acid concentration was more pronounced in mothers of smaller infants than in mothers of larger infants (Figure 3.2). The reason for this is not clear. Relative linoleic acid concentrations tend to remain stable until the end of pregnancy and the decrease shown in Figure 3.2 occurs mainly around the time of delivery.¹² In contrast, the observed decreases in the fractions of AA and DHA start before 16 and 22 wk of gestation, respectively.¹² Thus, it seems unlikely that these opposite patterns of observed decreases are directly related, *e.g.* because of competitive or selective placental transfer of fatty acids.

Nutritional sufficiency of EFA status in plasma of term neonates

Although low EFA concentrations did not seem to be associated with limited growth in the present study, the specific neonatal demand for EFAs may not have been met.^{41,42} We are presently conducting a long-term follow-up study on the children born during our studies to investigate potential functional consequences of early EFA status in later life. Without such information, statements about the nutritional sufficiency of the EFA status of term infants, on the basis of EFA concentrations found in umbilical cord plasma, remain speculative.

In summary, under the present dietary conditions, EFAs such as AA and DHA do not seem to be important determinants of fetal growth in term neonates. The biochemical EFA status measured in umbilical cord plasma of term neonates is even negatively associated with size at birth. This lower EFA status in plasma of heavier infants occurred despite a larger decrease in LCP fractions in maternal plasma. These findings suggest that the maternal-to-fetal EFA transfer capacity is a limiting factor in determining neonatal EFA status. However, the implications of these findings for mother and children are not known and remain to be investigated.

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Chapter 4 |

Components of the insulin resistance syndrome in seven-year-old children: relations with birth weight and the polyunsaturated fatty acid content of umbilical cord plasma phospholipids

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Abstract

Aims/hypothesis According to the fetal origins hypothesis, there is a relation between fetal nutrition and adult glucose intolerance. In adults, insulin resistance has been associated with dietary polyunsaturated fatty acids. We examined whether the availability of polyunsaturated fatty acids during fetal life, as indicated by the fatty acid composition of cord blood samples, relates to childhood body composition and glycaemic control.

Methods Fatty acid concentrations in umbilical cord blood were determined by gas-liquid chromatography in a birth-cohort of infants. When the children were seven years old, fasting glucose, insulin, proinsulin, and leptin levels were measured in 259 of these children, and relations with cord plasma fatty acid concentrations were studied.

Results Cord plasma phospholipid γ -linolenic acid and dihomo- γ -linolenic acid concentrations were negatively related to insulin concentration and calculated insulin resistance (homeostasis model assessment) at seven years of age. The γ -linolenic acid concentration was also negatively related to body fatness and proinsulin and leptin concentrations at seven years of age. No association was found with other polyunsaturated fatty acid concentrations at birth. Adjusted for age, sex, current weight, and gestational age, a lower birth weight related to higher values of the insulin resistance variables. The highest insulin concentrations were found in children with a low birth weight and a low γ -linolenic acid concentration at birth. The relations between γ -linolenic acid concentration at birth and fasting insulin and calculated insulin resistance remained statistically significant when adjusted for birth weight.

Conclusion/interpretation These findings indicate that fetal availability or metabolism of γ -linolenic acid could be involved in the early origins of insulin resistance.

Introduction

Insulin resistance is an important component of the metabolic syndrome that encompasses a cluster of risk factors for cardiovascular diseases including Type II (non-insulin-dependent) diabetes mellitus, dyslipidaemia, obesity, and hypertension.^{1,2} A small size at birth has been associated with an increased risk for the development of insulin resistance and Type II diabetes mellitus later in life.³⁻⁸ According to the fetal origins hypothesis, which was based on these epidemiological findings, the propensity to develop insulin resistance might be a consequence of structural and functional adaptations to a limited availability of nutrients during fetal life.⁹

Birth weight is, however, only a crude indicator of fetal nutrition and other environmental or genetic factors could explain the associations between size at birth and adult Type II diabetes mellitus. Still, there is some evidence that fetal nutrition could indeed be involved,¹⁰ like the epidemiological findings of the Dutch Hunger Winter study¹¹ and results from experiments in pregnant animals.¹²⁻¹⁴ The availability or balance of particular nutrients rather than the availability of macronutrients could be important.¹⁰ Thus far, only a limited number of human studies have been conducted on the nutritional factors that could play a role. One such group of nutrients that could be important in the fetal origins of adult diseases are essential fatty acids and their polyunsaturated derivatives.^{15,16} The importance of the fatty acid composition of our diet for the development of insulin resistance has been recognized for some years now.¹⁷⁻¹⁹ Several studies have shown that the fatty acid composition of serum lipid fractions and muscle membrane phospholipids – which at least partly reflect dietary fat quality – are closely related to insulin action.²⁰⁻²³ For instance, the development of Type II diabetes has been related to lower concentrations of linoleic acid (18:2n-6) and higher concentrations of palmitoleic acid (16:1n-7), γ -linolenic acid (18:3n-6), and dihomo- γ -linolenic acid (20:3n-6) in serum cholesterol esters of 50-year-old men.²⁴ Previous comparisons between breast-fed and bottle-fed subjects seem to indicate that nutritional factors early in life, like the availability of polyunsaturated fatty acids (PUFAs), could indeed play a role in the development of adult Type II diabetes mellitus.^{25,26}

Whether the fetal availability of PUFAs relates to the development of insulin resistance later in life is not known. The primary objective of our study was therefore to explore whether the availability of PUFAs in utero relates to fasting glucose, insulin, proinsulin and leptin concentrations later in life. For this purpose we conducted a follow-up study on a cohort of children in whom umbilical cord plasma phospholipid PUFA concentrations had been determined at birth. We used these umbilical cord plasma phospholipid PUFA concentrations as biomarkers of intrauterine PUFA availability.

Table 4.1: Characteristics of the study population by sex.¹

	Boys (<i>n</i> = 142)	Girls (<i>n</i> = 117)
Length of gestation (wk)	39.8 (39.1; 41.0)	39.9 (39.1; 41.0)
Birth weight (g)	3377 (504)	3203 (499) ^b
Age (y)	7.3 (0.3)	7.3 (0.3)
Weight (kg)	25.5 (4.4)	24.8 (4.0)
Height (cm)	127.8 (5.0)	126.1 (5.9) ^a
BMI (kg/m ²)	15.4 (14.3; 16.4)	15.4 (14.3; 16.5)
Sum four skinfolds (mm)	24.1 (19.9; 26.4)	28.4 (23.4; 33.0) ^c
Body fat (%)	16.2 (13.8; 18.0)	20.9 (18.3; 23.7) ^c
Fat mass (kg)	4.1 (3.2; 4.8)	5.1 (4.0; 6.4) ^c
Fat-free mass (kg)	21.1 (2.7)	19.4 (2.3) ^c
Leptin (µg/L)	2.7 (1.9; 3.6)	3.8 (2.6; 4.9) ^c
Glucose (mmol/L)	4.7 (0.4)	4.6 (0.3) ^a
Insulin (pmol/L)	32.9 (25.0; 44.0)	36.2 (27.8; 48.0)
Proinsulin (pmol/L)	6.4 (5.4; 7.5)	6.9 (5.6; 8.5) ^a
Proinsulin to insulin ratio	0.20 (0.14; 0.25)	0.19 (0.15; 0.25)
Insulin resistance (HOMA)	1.0 (0.7; 1.3)	1.0 (0.8; 1.4)
Beta-cell function (HOMA)	78.1 (60.6; 99.6)	90.9 (71.2; 123.9) ^b

BMI, body mass index; HOMA, homeostasis model assessment.

¹Values are mean (SD) except for gestational age, BMI, sum four skinfolds, percent body fat, fat mass, leptin, insulin, pro-insulin, and the HOMA indexes for insulin resistance and β -cell function, where values are geometric mean (inter-quartile range).

^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$ for differences between sexes.

Subjects and Methods

Study cohort

Subjects were recruited from a Dutch birth-cohort of children. As infants, these children and their mothers participated in a previous study of essential fatty acids during pregnancy and pregnancy outcome. No interventions were provided. To explore the potential long-term effects of fetal essential fatty acid availability, a follow-up study was carried out between 1997 and 2000. All singleton babies born alive before 1994, and for whom an umbilical cord blood sample was available for the determination of the essential fatty acid status at birth, were included. In total, 750 children were eligible for follow-up (for more details on this cohort see Rump *et al.*).²⁷ With the aid of the Dutch population registry, 97% of the eligible population could be traced. Three children had died and 34 lived abroad. The parents of the remaining 691 children received a written invitation for the follow-up evaluation. We were able to contact the parents of 558 children. Of these, 297 participated, 231 refused, and 30 retracted their initial consent. Birth characteristics (*i.e.* birth weight, crown-heel length, occipital-frontal circumference, ponderal index, gestational age, birth order) of the children who participated did not differ from the children who did not attend our clinic. Approval for this study was obtained from the Ethics Committee of the University Hospital

Maastricht and the parents of all participating children gave their written informed consent.

Data collection at follow-up

All children were investigated before puberty, at about seven years of age (range: 6.7 – 8.1 years). The same physician (PR) examined all children and performed all venipunctures. A questionnaire and a structured interview with one or both parents were used to collect additional information. Included were information on maternal smoking during pregnancy, infant nutrition, parental education, and family history of Type II diabetes. A family history of Type II diabetes was considered when a parent or grandparent of the child had Type II diabetes. Anthropometric measurements were done by one observer (PR) as previously described.²⁸ The percentage of body fat, fat mass, and fat-free mass were estimated from these skinfold measurements using equations appropriate for children.²⁹ The use of other equations³⁰ did not influence the main findings of this study.

Blood sampling and laboratory analyses

Not all parents and children agreed to a venipuncture, and no more than two puncture attempts were made. As a consequence, blood samples were obtained in 264 of the 297 children. The blood samples were collected in EDTA-treated evacuated tubes after an overnight fast. Plasma was separated from blood cells by centrifugation and stored in small portions at -80°C until analysis. The fatty acid composition of umbilical cord plasma phospholipids was determined by capillary gas-liquid chromatography, as described before.³¹ Glucose was determined enzymatically (Glucose HK-method, Hoffmann-La Roche, Basel, Switzerland) and specific insulin and total proinsulin were measured as described by Ruige *et al.*³² Plasma leptin concentration was measured using a human leptin radioimmunoassay (Linco Research, St. Charles, MO, USA). Estimates of pancreatic beta-cell function and relative insulin resistance were calculated from the fasting insulin and glucose concentrations using the homeostasis model assessment (HOMA) equations.³³

Statistical analysis

All analyses were restricted to those children for whom a blood sample was obtained at follow-up. Five cases were excluded for missing values, leaving 259 children for analysis. Data are presented as mean $\pm$ standard deviation (SD), unless specified otherwise. Differences between groups were evaluated by unpaired two-tailed Student's *t* tests or χ^2 (chi square) tests. Linear regression models were used to explore relations between plasma phospholipid fatty acid concentrations, birth weight and the outcome variables. During these analysis continuous variables were used instead of categories.

Table 4.2: Unadjusted mean anthropometric measurements and biochemical parameters in seven-year-old children according to tertiles of γ -linolenic acid concentration in the plasma phospholipid fraction of umbilical cord blood at birth.

	Tertiles of γ -linolenic acid (% wt/wt)			<i>P</i> for trend ¹	
	< 0.04 (<i>n</i> = 85)	0.04-0.07 (<i>n</i> = 87)	≥ 0.07 (<i>n</i> = 87)	unadjusted	adjusted ²
Weight (kg)	25.6	24.9	25.1	NS	—
Height (cm)	127.4	126.7	127.0	NS	NS
BMI (kg/m ²)	15.7	15.4	15.5	NS	NS
Sum four skinfolds (mm)	28.7	26.7	26.6	0.009	NS
Body fat (%)	19.8	18.0	18.4	0.003	NS
Fat mass (kg)	5.2	4.6	4.7	0.010	NS
Fat-free mass (kg)	20.4	20.3	20.4	NS	NS
Leptin (μg/L)	4.2	3.8	3.3	0.003	0.047
Glucose (mmol/L)	4.7	4.7	4.6	NS	NS
Insulin (pmol/L)	40.4	37.3	34.7	0.004	0.011
Proinsulin (pmol/L)	7.2	6.9	6.6	0.025	NS
Proinsulin to insulin ratio	0.20	0.21	0.22	NS	NS
Insulin resistance (HOMA)	1.2	1.1	1.0	0.004	0.011
Beta-cell function (HOMA)	94.8	91.1	85.2	0.033	NS

BMI, body mass index; HOMA, homeostasis model assessment; NS, not significant.

¹*P* values are derived from simple (unadjusted) and multiple (adjusted) linear regression using continuous variables.

²Adjusted for sex, age, current weight and γ -linolenic acid concentration at follow-up.

Because of a skewed distribution of some variables, log-transformed data were used when appropriate. Multiple regression models were applied to control for potential confounding factors. A two-tailed *P* value of less than 0.05 was considered to be statistically significant. All statistical analyses were done using STATVIEW (version 4.5, Abacus Concepts Inc., Berkeley, CA, USA).

Results

Sex, current weight and body composition

None of the children were diabetic (fasting glucose levels < 7.0 mmol/L). The characteristics of the participating boys and girls are reported in Table 4.1. Most of the outcome variables were higher in children with a higher current weight, length, fat mass, or fat-free mass (not shown).

Relations with plasma phospholipid fatty acid composition

The most consistent relations were found between the outcome variables and the γ -linolenic acid (18:3n-6; a derivative of the essential fatty acid linoleic acid) content of the umbilical cord plasma phospholipid fraction. A higher concentration of γ -linolenic

acid was associated with lower fasting insulin, proinsulin, HOMA insulin resistance, and HOMA beta-cell function (Table 4.2). In addition, indices of body fatness (fasting leptin, sum four skinfolds, percent body fat, and fat mass) were also negatively related to the γ -linolenic acid levels at birth, whereas, weight, height, BMI, and fat-free mass did not relate to umbilical cord γ -linolenic acid concentrations (Table 4.2).

In contrast, plasma phospholipid γ -linolenic acid concentration at seven years of age was positively related to the parameters of glycaemic control (fasting glucose, insulin, proinsulin, HOMA insulin resistance, and HOMA beta-cell function) and fasting leptin concentration ($P < 0.05$ for all, unadjusted and adjusted for age, sex and current weight; data not shown). Plasma phospholipid γ -linolenic acid concentration at seven years of age did not relate to the anthropometric indices of body fatness (sum skinfolds, percent body fat, or fat mass). The associations found between γ -linolenic acid concentration at birth and fasting insulin and leptin concentrations, and HOMA insulin resistance values remained statistically significant when adjusted for age, sex, current weight and the plasma phospholipid γ -linolenic acid content at seven years of age (Table 4.2). The associations found between γ -linolenic acid concentration at birth and indices of body fatness (sum four skinfolds, percent body fat, and fat mass) were statistically significant ($P < 0.05$ for all) when adjusted for sex, age and the plasma phospholipid γ -linolenic acid content at seven years of age. However, when also corrected for current weight, these relations were no longer significant (Table 4.2). No significant association or interaction was found between γ -linolenic acid concentrations at birth and γ -linolenic acid concentrations at seven years.

In addition to γ -linolenic acid, umbilical cord plasma phospholipid dihomogamma-linolenic acid (20:3n-6; the elongation product of γ -linolenic acid) concentrations were also negatively related with fasting insulin concentration and HOMA insulin resistance at age seven ($P < 0.05$ for both, after adjustment for sex, age and current weight). Plasma phospholipid dihomogamma-linolenic acid at seven years of age did not relate to the outcome variables. The concentration of linoleic acid (18:2n-6), arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3), docosahexaenoic acid (22:6n-3), the sum of all n-6 fatty acids, the sum of all n-6 long-chain polyenes (20:3n-6, 20:4n-6, 22:4n-6, and 22:5n-6), the sum of all n-3 fatty acids, the sum of all n-3 long-chain polyenes (20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3) and the ratio of n-3 to n-6 fatty acids, in umbilical cord plasma phospholipids were not related any of the outcome variables. Adjustments for sex, age and current weight or the fatty acid concentrations measured at seven years of age did not change these results.

Relations with birth weight

Children of lower birth weight had a lower fat-free mass but not a lower fat mass at age seven. After adjustment for sex, age, current weight, and length of gestation, birth weight was negatively related to BMI, sum four skinfolds, percentage of body fat, fat

mass, leptin, insulin, HOMA insulin resistance and HOMA beta-cell function (for more information see *Chapter 7*).

Low birth weight and low γ -linolenic acid concentration at birth

The highest fasting insulin concentration and HOMA insulin resistance values were seen in children of low birth weight who had a low γ -linolenic acid concentration at birth (Table 4.3). These children also had the lowest proinsulin to insulin ratio (Table 4.3). Additionally, children in the lowest tertile of birth weight and the lowest tertile of γ -linoleic acid concentration at birth had the highest mean fasting leptin concentration and percentage of body fat (not shown). We found no statistically significant association between birth weight and the γ -linolenic acid concentration in umbilical cord plasma phospholipids. The associations between γ -linoleic acid concentration at birth and fasting insulin concentration and HOMA insulin resistance at seven years of age remained statistically significant ($P < 0.01$, for both) when adjusted for birth weight by multiple regression analysis. The product terms (birth weight times γ -linoleic acid) for interactions were not statistically significant.

Family history of Type II diabetes mellitus

Because genetic factors play a role in the origins of insulin resistance and Type II diabetes, we investigated relations with a positive family history of diabetes mellitus. Eight children were born to mothers who reported diabetes or impaired glucose tolerance during pregnancy; 78 children had a parent or grandparent with Type II diabetes. As shown in Table 4.4, a low γ -linolenic acid concentration at birth was related to a positive family history of Type II diabetes. Of the eight children born to mothers with diabetes or impaired glucose tolerance during pregnancy, seven had a below median γ -linolenic acid concentration at birth ($P = 0.03$, χ^2 test). Of the children with a positive family history for Type II diabetes, 51 (65%) had a below median γ -linolenic acid concentration at birth ($P = 0.005$, χ^2 test).

The γ -linolenic acid concentration at follow-up was not related to a maternal or family history of diabetes. Excluding children with a positive family history of diabetes from the analysis did not change the main findings of our study. Fasting plasma insulin, proinsulin and leptin concentrations, HOMA insulin resistance, and percent body fat at age seven remained negatively associated with the γ -linoleic acid content of umbilical cord plasma ($P < 0.05$, for all).

Multiple regression analysis

To determine how the different variables were related to fasting insulin concentrations and HOMA insulin resistance, backward step-wise multiple regression analyses were performed (Table 4.5).

Table 4.3: Fasting insulin concentration, proinsulin to insulin ratios and insulin resistance (HOMA) values in seven-year-old children according to tertiles of umbilical cord plasma phospholipid γ -linolenic acid concentration and birth weight.

Tertiles of birth weight (g)	Tertiles of γ -linolenic acid (% wt/wt)			<i>P</i> for trend ¹
	< 0.04	0.04-0.07	$\geq$ 0.07	
Insulin (pmol/L)				
< 3100	44.4 (35)	34.3 (24)	27.8 (29)	0.001
3100-3500	31.2 (26)	32.4 (35)	34.7 (27)	NS
$\geq$ 3500	34.7 (24)	37.6 (28)	32.8 (31)	NS
<i>P</i> for trend ¹	0.020	NS	NS	...
Proinsulin to insulin ratio				
< 3100	0.16	0.18	0.23	0.011
3100-3500	0.21	0.20	0.19	NS
$\geq$ 3500	0.20	0.19	0.19	NS
<i>P</i> for trend ¹	0.025	NS	NS	...
Insulin resistance (HOMA)				
< 3100	1.3	1.0	0.8	0.001
3100-3500	0.9	0.9	1.0	NS
$\geq$ 3500	1.0	1.1	0.9	NS
<i>P</i> for trend ¹	0.013	NS	NS	...

Values are unadjusted geometric means (number of subjects). HOMA, homeostasis model assessment; NS, not significant

¹*P* values are from multiple linear regression using continuous variables (adjusted for sex, age, current weight, length of gestation, and γ -linolenic acid concentration at seven years).

Removed from both models ($P > 0.1$) were age and the product terms birth weight times γ -linolenic acid concentration at birth, birth weight times current weight, and family history of diabetes times γ -linolenic acid concentration at birth. There was a significant interaction between birth weight and a family history of Type II diabetes. A lower birth weight was associated higher fasting insulin concentrations and HOMA insulin resistance values only in children with a positive family history for Type II diabetes mellitus ($P < 0.001$ for both). In children with a negative family history for Type II diabetes mellitus, birth weight was not related to fasting insulin concentration nor to HOMA insulin resistance ($P = 0.6$ for both). Further adjustments for maternal smoking, infant feeding mode, maternal education, paternal education, or birth order had no major influence on the outcome.

Discussion

In this study, body composition and insulin resistance variables at seven years of age

related to birth weight and PUFA concentrations in umbilical cord blood. This data suggests that a low intrauterine availability of γ -linolenic acid could predispose individuals to an increased body fatness and insulin resistance later in life. This would be in accordance with the general concept of the fetal origin hypothesis that a limited availability of nutrients early in life could have detrimental effects for the subsequent development of insulin resistance and Type II diabetes.⁹

Similar to previous studies conducted in adults and children,^{3-8,34} we found that (after correction for sex, age, current weight and length of gestation) a small size at birth related to higher levels of the insulin resistance variables and an increased body fatness later in life. In our cohort of seven-year-olds, a low birth weight not only related to a higher HOMA index for insulin resistance, but also to higher values of HOMA beta-cell function and lower proinsulin-to-insulin ratios. These findings support an association between birth weight and insulin resistance, but do not indicate a relation with an impaired beta-cell function.

In this study, fasting insulin concentrations and the HOMA index for insulin resistance were highest in children who had a low birth weight in combination with a low umbilical cord plasma phospholipid γ -linolenic acid concentration. After adjustment for birth weight, cord blood γ -linoleic acid concentration remained associated with HOMA insulin resistance and fasting insulin levels. We found no significant relation between birth weight and the γ -linolenic acid content of umbilical cord plasma phospholipids. Therefore, the association between cord blood γ -linolenic acid concentrations and childhood insulin resistance does not seem to be mediated through a reduction in fetal growth. According to a previous study,¹¹ poor nutrition during late gestation could lead to permanent changes in insulin-glucose metabolism, even when the effects on intrauterine growth are small.

It is not clear how γ -linolenic acid influences insulin metabolism. Suggested mechanisms explaining the effects of dietary fatty acids on insulin metabolism involve changes in number and affinity of insulin receptors, alterations in membrane fatty acid composition, changes in the translocation of glucose transporters, changes in intracellular glucose storage, or alterations in the expression of lipogenic and glycolytic genes.^{17,19,35} Such mechanisms could involve the so-called proliferator-activated-receptors (PPARs). Gamma-linolenic acid has been shown to have a relatively high affinity for PPAR α and PPAR δ , these transcription factors playing central roles in glucose and lipid homeostasis.^{36,37}

The observed association between a low γ -linolenic acid concentration in umbilical cord plasma phospholipids and the insulin resistance variables at age seven could reflect a genetic predisposition rather than a limited γ -linoleic acid availability dependent on maternal dietary intake. Investigators have shown that the muscle membrane fatty acid composition of children relates to maternal indices of insulin resistance.³⁸

Table 4.4: Frequency of maternal diabetes mellitus or impaired glucose tolerance during pregnancy and of family history for Type II diabetes mellitus according to tertiles of umbilical cord plasma phospholipid γ -linolenic acid concentrations at birth.

Tertile of γ -Linolenic acid (% wt/wt)		Maternal diabetes or IGT during pregnancy	Family history of Type II diabetes	Combined
		<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
< 0.04	(<i>n</i> = 85)	4 (5)	32 (38)	33 (39)
0.04-0.07	(<i>n</i> = 87)	3 (3)	28 (32)	30 (34)
≥ 0.07	(<i>n</i> = 87)	1 (1)	18 (21)	19 (22)
<i>P</i> (χ^2 square test)		NS	0.04	0.04

IGT, impaired glucose tolerance.

Table 4.5: Multiple regression analyses of fasting insulin concentration and insulin resistance (HOMA) in seven-year-old children with sex, current weight, birth weight, family history of diabetes and plasma phospholipid γ -linolenic acid concentrations.

	Coefficient ¹	(95 % CI)	<i>P</i>
Log insulin (pmol/L)			
Sex (0 = boy; 1 = girl)	3.2	(-0.5; 5.8)	0.019
Weight (kg)	1.0	(0.7; 1.3)	<0.001
Birth weight (kg)	0.9	(-2.3; 4.3)	0.602
Family history of diabetes (0 = no; 1 = yes) ²	30.1	(13.0; 47.3)	<0.001
γ -Linolenic acid at birth (% wt/wt)	-4.1	(-7.8; -0.4)	0.028
γ -Linolenic acid at seven years (% wt/wt)	6.6	(4.3; 8.9)	<0.001
Birth weight $\times$ family history	-8.8	(-13.9; -3.6)	<0.001
Log insulin resistance (HOMA)			
Sex (0 = boy; 1 = girl)	4.2	(-0.3; 8.7)	0.070
Weight (kg)	1.6	(1.0; 2.1)	<0.001
Birth weight (kg)	1.7	(-4.1; 7.5)	0.566
Family history of diabetes (0 = no; 1 = yes) ²	49.6	(20.3; 78.9)	0.001
γ -Linolenic acid at birth (% wt/wt)	-7.1	(-13.4; -0.8)	0.028
γ -Linolenic acid at seven years (% wt/wt)	11.1	(7.2; 15.1)	<0.001
Birth weight $\times$ family history	-14.3	(-23.0; -5.5)	0.002

HOMA, homeostasis model assessment.

¹Coefficients are percentage changes in the outcome variable per unit change in predictor variables (Note that one unit change in γ -linolenic acid concentration equals 0.1 % wt/wt).

²Includes maternal diabetes or impaired glucose tolerance during pregnancy.

They postulate that a genetic predisposition for the incorporation, elongation, and desaturation of specific fatty acids is inherited by the child and contributes to the risk for future development of insulin resistance.³⁸ In this study, a positive family history of diabetes was related to a lower γ -linolenic acid content of umbilical cord plasma phospholipids. However, the associations found between γ -linolenic acid concentration at birth and the insulin resistance variables at age seven were independent of family

history. It thus seems that the associations found do not simply reflect a genetic predisposition.

The observed associations between γ -linolenic acid concentrations at birth and at seven years of age on one hand and the insulin resistance variables on the other hand could be an epiphenomenon. Insulin plays a crucial role in fatty acid metabolism and is known to enhance the activity of the enzyme delta-6-desaturase.³⁹ Delta-6-desaturase stimulates the conversion of linoleic acid (18:2n-6) into γ -linolenic acid. A higher plasma concentration of insulin could thus explain a higher γ -linolenic acid content of plasma lipid fractions. Conversely, low levels of circulating insulin could relate to lower γ -linolenic acid concentrations. Findings in Type I (insulin-dependent) diabetes mellitus patients⁴⁰ and animal models of reduced beta-cell function⁴¹ indicate that insulin deficiency relates to a lower delta-6-desaturase activity, which can be restored by insulin treatment. The associations found between plasma phospholipid γ -linolenic acid concentration and the insulin resistance variables could thus reflect such a 'mechanism' of reverse causality. Furthermore, less than 40% of the children participated during the follow-up study. Although the birth characteristics (including γ -linolenic acid concentration at birth) of the children who participated did not differ from the children who did not attend our clinic, residual bias due to selection cannot be excluded.

In conclusion, the results of the present explorative study indicate that a limited availability of γ -linolenic acid early in human life could be one of the factors involved in the fetal origins of insulin resistance.

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Chapter 5 | Fasting triacylglycerol concentrations in seven-year-old children are related to the polyunsaturated fatty acid composition of umbilical cord plasma phospholipids at birth

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Abstract

Objective According to the fetal origins hypothesis, fetal nutrition is related to adult glucose intolerance and coronary heart disease. Glucose intolerance and coronary heart disease have been associated to dietary polyunsaturated fatty acids. We explored whether the availability of polyunsaturated fatty acids (PUFAs) during fetal life, as indicated by the fatty acid composition of cord blood samples, relates to childhood lipid and lipoprotein concentrations.

Design The phospholipid PUFA composition of umbilical cord blood was determined by gas-liquid chromatography in a birth-cohort of infants. At seven years of age, fasting concentrations of plasma lipids and lipoproteins were measured in 264 of these children and relations with cord plasma fatty acid concentrations were studied.

Results Umbilical cord plasma phospholipid γ -linolenic acid (18:3n-6) and dihomo- γ -linolenic acid (20:3n-6) concentrations were negatively related to fasting triacylglycerol concentrations measured at seven years of age. The highest triacylglycerol concentrations were seen in children with low 18:3n-6 and 20:3n-6 concentrations at birth and high 18:3n-6 and 20:3n-6 concentrations at seven years of age. Adjustment for potential confounding factors did not attenuate the associations. The fatty acid composition of umbilical cord plasma phospholipids was not related to total cholesterol, HDL cholesterol, LDL cholesterol, apolipoprotein A1, apolipoprotein B, and lipoprotein(a) concentrations in this cohort of seven-year-old children.

Conclusion These findings could indicate that the fetal availability or metabolism of γ -linolenic acid influences the cardiovascular risk profile in children.

Introduction

A small size at birth has been associated with an increased risk for the development of adult coronary heart disease, and components of the metabolic syndrome, e.g. Type II diabetes, hypertension and dyslipidemia.^{1,2} Different research groups have found similar relations in both men and women from various populations.³⁻⁵ These associations led to the formulation of the fetal origins hypothesis.⁶ According to this hypothesis the propensity to develop coronary heart disease is a consequence of structural and functional adaptations to a limited availability of nutrients during fetal life. That fetal nutrition may indeed be involved is supported by the findings of the Dutch Hunger Winter Study and experiments in animals.⁷⁻¹⁰ Thus far, a limited number of human studies have been conducted in an attempt to identify the nutritional factors that could play a role. These studies mainly focussed on the effects of the balance between macro-nutrients of the maternal diet during pregnancy.^{11,12} Hardly any work has been done to explore the importance of specific nutrients and their balance on fetal growth and later outcome.

Recently, we reported on the negative association between concentrations of γ -linolenic acid (18:3n-6) and dihomo- γ -linolenic acid (20:3n-6) in umbilical cord plasma phospholipids and components of the insulin resistance syndrome in Dutch 7-year-old children.¹³ Children who had a lower γ -linolenic acid concentration at birth had a higher fasting concentration of insulin, proinsulin, and leptin and a higher percentage of body fat at age seven.¹³ These findings suggest that the fetal availability of γ -linolenic acid could be involved in the fetal origins of insulin resistance. The objective of the present study was to explore whether the umbilical cord plasma phospholipid concentration of γ -linolenic acid and dihomo- γ -linolenic acid (20:3n-6) also relate to another component of the metabolic syndrome in 7-year-olds, *i.e.* their plasma lipid and lipoprotein concentrations.

Subjects and Methods

Study population

Subjects were recruited from a Dutch birth-cohort of children. As infants, these children and their mothers participated in a previous study of essential fatty acids during pregnancy and pregnancy outcome. No interventions were provided. To explore the potential long-term effects of fetal essential fatty acid availability, a follow-up study was performed between 1997 and 2000. All singleton babies born alive before 1994 – and of whom an umbilical cord blood sample was available for the determination of the essential fatty acid status at birth – were candidates to be included. In total, 750 children were eligible for follow-up (for more details on this cohort see Rump *et al.*).¹⁴ Using the Dutch population registry, 97% of the eligible population could be traced. Three children had died and 34 lived abroad. The parents of the remaining 691 children

received a written invitation for the follow-up evaluation. A reminder was sent within fourteen days. When no response was obtained, phone-calls were made in an attempt to contact the remaining parents. Eventually we were able to contact the parents of 558 children. Of these, 297 participated, 231 refused, and 30 retracted their initial consent. Birth characteristics (*i.e.* birth weight, crown-heel length, occipital-frontal circumference, ponderal index, gestational age, birth order, and the γ -linolenic acid composition of umbilical cord plasma phospholipids) of the children who participated did not differ from the children who did not attend our clinic. Approval for this study was obtained from the Ethics Committee of the University Hospital Maastricht and the parents of all participating children gave their written informed consent.

Data collection at follow-up

All children were investigated at approximately seven years of age (range: 6.7 – 8.1 years). The same physician (PR) examined all children and performed all venipunctures. A questionnaire and a structured interview with one or both parents were used to collect additional information. Included were information on maternal smoking during pregnancy, infant nutrition, parental education, and family history of cardiovascular diseases. Children who never received human milk were considered formula-fed and the remaining children regarded as breast-fed. Three categories of parental education were distinguished (low: primary school, lower vocational or lower general education; middle: intermediate general or intermediate vocational education; and high: higher general and higher vocational education or university). A family history of cardiovascular disease was considered when a first or second-degree family member of the child (parent, grandparent or sibling of parent) had suffered from myocardial infarction ≤ 55 years. Anthropometric measurements were performed as described in detail by Gerver and de Bruin.¹⁵

Blood sampling and laboratory analyses

Not all parents and children agreed to a venipuncture, and no more than two puncture attempts were made. As a consequence, blood samples were obtained in 264 of the 297 children. The blood samples were collected in EDTA-treated evacuated tubes after an overnight fast. Plasma was separated from blood cells by centrifugation and stored in small portions at -80°C until analysis. The fatty acid composition of umbilical cord plasma phospholipids was determined by capillary gas-liquid chromatography, as described by Al *et al.*¹⁶

All lipid and lipoprotein concentrations were measured in duplicate. Total cholesterol, triacylglycerol, and (after precipitation of apolipoprotein B containing lipoproteins) HDL cholesterol were determined enzymatically.¹⁷ The intra-assay and inter-assay variation coefficients were 1.4% and 3.0% (total cholesterol), 2.5% and 4.3% (triacylglycerol), 3.6% and 7.8% (HDL cholesterol), respectively. LDL cholesterol was calculated using the equation of Friedewald *et al.*¹⁸

Table 5.1: Neonatal and childhood characteristics of the study population by sex.¹

	Boys (n = 145)	Girls (n = 119)
Neonatal characteristics		
Length of gestation (wk)	40.0 (39.1; 41.0)	40.1 (39.1; 41.0)
Birth weight (g)	3375 (500)	3209 (497) ^b
Infant nutrition		
Formula-fed	77 (53)	64 (54)
Breast-fed	68 (47)	55 (46)
Childhood characteristics		
Age (y)	7.3 (0.3)	7.3 (0.3)
Weight (kg)	25.5 (4.4)	25.0 (4.2)
Height (cm)	127.7 (5.0)	126.3 (6.1) ^a
BMI (kg/m ²)	15.5 (1.9)	15.6 (1.7)
Sum four skinfolds (mm)	23.1 (19.9; 26.6)	26.9 (23.5; 33.1) ^c
Total cholesterol (mmol/L)	4.04 (0.57)	4.10 (0.71)
Triacylglycerol (mmol/L)	0.48 (0.34; 0.63)	0.51 (0.38; 0.72)
HDL cholesterol (mmol/L)	1.37 (0.31)	1.34 (0.31)
LDL cholesterol (mmol/L)	2.43 (0.53)	2.50 (0.64)
LDL to HDL cholesterol ratio	1.89 (0.65)	1.97 (0.73)
Apolipoprotein A1 (mg/L)	1365 (22)	1350 (23)
Apolipoprotein B (mg/L)	715 (15)	748 (17)
Lipoprotein(a) (mg/L)	79 (17; 224)	74 (37; 290)

¹Values are mean (SD) or number (%) except for gestational age, sum four skinfolds, triacylglycerol and lipoprotein(a), where values are median (inter-quartile range).

^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ for differences between sexes.

Apolipoprotein A1 and apolipoprotein B were analyzed by an immunoturbidimetric reaction (UNI-KIT Apo A1 and UNI-KIT Apo B, Hoffmann-La Roche, Basel, Switzerland). The coefficients of variation within and between assays were 2.4% and 5.1% for Apo A1, and 3.0% and 5.8% for Apo B. An enzyme-linked immunosorbent assay (ELISA) was applied to measure plasma lipoprotein(a) concentration (TintElize, Biopool, Umeå, Sweden). Variation coefficients were 7.7% and 3.4%.

Statistical analysis

All analyses were restricted to the 264 children of whom a blood sample was obtained at follow-up. Data are presented as mean $\pm$ standard deviation (SD), unless specified otherwise. Differences between groups were evaluated by unpaired two-tailed Student's *t* tests or χ^2 tests. Linear regression models were used to explore relations between plasma phospholipid fatty acid concentrations and lipid and lipoprotein concentrations at follow-up. Continuous variables were used instead of categories. Because of a skewed distribution of some variables (lipoprotein(a), triacylglycerol), log transformed data were used during the analyses of these variables. Multiple regression models were applied to control for potential confounding factors. A two-tailed *P* value < 0.05 was

considered statistically significant. All statistical analyses were performed using STATVIEW (version 4.5, Abacus Concepts Inc., Berkeley, CA, USA).

Results

The characteristics of the study population are shown in Table 5.1. For both umbilical cord blood samples and for blood samples obtained at follow-up, the fatty acid composition of plasma phospholipids is reported in Table 5.2. The fatty acid composition of plasma phospholipids did not differ between boys and girls. See also *Appendix D* and *E*.

Table 5.2: Fatty acids in plasma phospholipids at birth and at 7 years of age ($n = 264$).

	At birth		At 7 years	
	Mean	SD	Mean	SD
Fatty acid (% wt/wt)				
18:2n-6	7.67	1.29	23.19	2.27
18:3n-6	0.05	0.04	0.08	0.06
20:3n-6	5.18	0.86	2.97	0.54
20:4n-6	16.61	1.60	9.20	1.23
Σ n-6	32.16	1.70	36.78	1.81
Σ n-6 LCP	23.41	1.52	12.92	1.34
18:3n-3	ND	—	0.19	0.07
20:5n-3	0.23	0.11	0.51	0.22
22:6n-3	6.11	1.39	2.82	0.69
Σ n-3	6.94	1.57	4.55	0.86
Σ n-3 LCP	6.89	1.57	4.23	0.87
Σ SFA	47.62	1.43	45.44	1.02
Σ MUFA	11.88	1.65	13.12	1.38
Σ PUFA	39.50	1.76	41.44	1.55
Σ n-3 / Σ n-6	0.22	0.05	0.13	0.03
Total fatty acids (mg/L)	592	118	1060	148

ND, not detected. Σ n-6 LCP, sum of n-6 long-chain polyenes (20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6). Σ n-3 LCP, sum of n-3 long-chain polyenes (20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). α -Linolenic acid was not detectable in cord blood samples in more than 50% of the population.

Lipids, lipoproteins, and (dihomo)- γ -linolenic acid concentrations

Triacylglycerol concentration at seven years of age was negatively associated with both γ -linolenic acid and dihomom- γ -linolenic acid concentrations measured in the phospholipid fraction of umbilical cord plasma (Table 5.3). These associations remained statistically significant when adjusted for sex, age at follow-up, and current weight. In contrast, phospholipid γ -linolenic acid and dihomom- γ -linolenic acid concentrations measured at seven years of age were positively related to childhood triacylglycerol concentrations.

The geometric mean concentration of triacylglycerol in seven-year-old children according to tertiles of γ -linolenic acid and dihomo- γ -linolenic acid, measured at birth and at seven years of age, are depicted in Figures 5.1 A and B. Triacylglycerol concentration was highest in children with the lowest γ -linolenic acid and dihomo- γ -linolenic acid concentrations at birth and the highest γ -linolenic acid and dihomo- γ -linolenic acid concentrations at seven years of age.

Table 5.3: Plasma triacylglycerol concentrations in seven-year-old children according to tertiles of phospholipid γ -linolenic acid and dihomo- γ -linolenic acid concentrations in umbilical cord plasma at birth.

Fatty acid (% wt/wt)	Triacylglycerol (mmol/L) ¹	P for trend ²	
		Crude	Adjusted ³
γ -Linolenic acid			
Low (< 0.04)	0.54 $\pm$ 0.02		
Middle (0.04 – 0.07)	0.51 $\pm$ 0.03	0.016	0.045
High ($\geq$ 0.07)	0.44 $\pm$ 0.02		
Dihomo- γ -linolenic acid			
Low (< 4.83)	0.54 $\pm$ 0.03		
Middle (4.83 – 5.46)	0.51 $\pm$ 0.02	0.017	0.019
High ($\geq$ 5.46)	0.44 $\pm$ 0.02		

¹Values are geometric mean $\pm$ SE.

²P values are derived from simple and multiple linear regression using continuous variables.

³Adjusted for sex, age at follow-up, and current weight.

The difference in log-triacylglycerol concentration between children who changed from the lowest tertile of γ -linolenic acid at birth to the highest tertile at age seven and children who changed from the highest tertile of γ -linolenic acid at birth to the lowest tertile at age seven, was 34% (95% CI: 21% – 47%). For dihomo- γ -linolenic acid, the observed difference in log-triacylglycerol concentration between these categories was 28% (95% CI: 8% – 48%). A difference of 30% in log-triacylglycerol concentration corresponds to a difference in triacylglycerol concentration of $\pm$ 0.30 mmol/L. The proportion of children that had increased or decreased their rank order of γ -linolenic acid or dihomo- γ -linolenic acid concentration between birth and seven years of age is shown in Table 5.4.

After correction for potential confounding factors by multiple regression analysis the association between triacylglycerol concentration at seven years of age and γ -linolenic acid or dihomo- γ -linolenic acid measured at birth remained statistically significant (Table 5.5). Children with a higher γ -linolenic acid concentration in the plasma phospholipid fraction tended to have a higher dihomo- γ -linolenic acid concentration as well ($r = 0.20$, $P = 0.001$ at birth and $r = 0.33$, $P < 0.001$ at seven years; Spearman rank correlation tests).

A

B

Figure 5.1: Fasting plasma triacylglycerol concentrations in seven-year-old children according to tertiles of plasma phospholipid γ -linolenic acid (A) or dihomo- γ -linolenic acid (B) concentrations in umbilical cord blood at birth and in blood obtained at seven years of age ($n = 264$). Values are geometric means ($P = 0.006$ for effect of γ -linolenic acid concentrations at birth, $P < 0.001$ for effect of γ -linolenic acid concentrations at follow-up, $P < 0.001$ for effect of dihomo- γ -linolenic acid concentrations at birth, and $P = 0.002$ for effect of dihomo- γ -linolenic acid concentrations at follow-up results from multiple regression models with log-triacylglycerol concentration as dependent variable and γ -linolenic acid or dihomo- γ -linolenic acid concentrations at birth and at seven years of age as independent variables).

Triacylglycerol concentration at seven years of age was also positively related to docosahexaenoic acid concentration at birth ($P = 0.02$). This association was no longer statistically significant when length of gestation was entered as a co-variable. At seven years of age, docosahexaenoic acid concentrations did not relate to triacylglycerol levels. No relation was found between triacylglycerol levels at follow-up and umbilical cord plasma phospholipid concentrations of the other fatty acids and fatty acid groups listed in Table 5.2. Total cholesterol, HDL cholesterol, LDL cholesterol, apolipoprotein A1, apolipoprotein B, and lipoprotein(a) concentrations, measured at seven years of age, did not relate to the phospholipid fatty acid composition of umbilical cord plasma at birth. Adjustment for the fatty acid concentrations determined at seven years of age, or for sex, age at follow-up, and current weight, did not alter these results.

Lipids, lipoproteins, and birth weight.

No statistically significant association was found between birth weight and plasma triacylglycerol or the other lipid and lipoprotein concentrations measured at seven years of age, nor when adjusted for length of gestation or for sex, age at follow-up, and current weight.

Table 5.4: The proportion of children increasing, decreasing or maintaining their rank order of plasma phospholipid γ -linolenic acid and dihomo- γ -linolenic acid concentrations over a period of seven years.

Tertile at birth	Tertile at seven years of age		
	Low	Middle	High
γ -Linoleic acid			
Low	37 %	26 %	37 %
Middle	36 %	36 %	28 %
High	27 %	39 %	34 %
Dihomo- γ -linolenic acid			
Low	45 %	30 %	25 %
Middle	34 %	36 %	30 %
High	20 %	34 %	46 %

No differences between breast-fed children and formula-fed children

The phospholipid fatty acid concentrations of umbilical cord blood and of blood obtained at follow-up did not differ significantly between breast-fed children and formula-fed children. Total cholesterol, triacylglycerol, HDL cholesterol, LDL cholesterol, apolipoprotein A1, apolipoprotein B, and lipoprotein(a) concentrations did not differ between breast-fed children and formula-fed children either, and no statistically significant interactions were observed.

Table 5.5: Parameters for regression of γ -linolenic acid (% wt/wt) and dihomo- γ -linolenic acid (% wt/wt) concentrations in umbilical cord plasma phospholipids on log-triacylglycerol (mmol/L) at seven years of age, unadjusted and adjusted for potential confounding factors.

Additional variables in model	β^1	95% CI	P value
γ-Linolenic acid (18:3n-6)			
Unadjusted	-12.3	(-22.3; -2.4)	0.016
18:3n-6 at 7 years	-13.2	(-22.4; -3.9)	0.006
18:3n-6 at 7 years and family history ²	-12.7	(-21.9; -3.4)	0.007
18:3n-6 at 7 years and anthropometric variables ³	-11.3	(-20.7; -2.0)	0.018
18:3n-6 at 7 years of and socio-economic factors ⁴	-13.3	(-22.9; -3.6)	0.007
18:3n-6 at 7 years and neonatal characteristics ⁵	-11.7	(-21.2; -2.2)	0.016
All listed variables	-10.1	(-20.0; -0.2)	0.046
Dihomo-γ-linolenic acid (20:3n-6)			
Unadjusted	-5.0	(-9.1; -0.91)	0.017
20:3n-6 at 7 years	-7.4	(-11.7; -3.1)	<0.001
20:3n-6 at 7 years and family history ²	-7.2	(-11.3; -2.9)	0.001
20:3n-6 at 7 years and anthropometric variables ³	-7.9	(-12.2; -3.6)	<0.001
20:3n-6 at 7 years and socio-economic factors ⁴	-7.2	(-11.7; -2.7)	0.002
20:3n-6 at 7 years and neonatal characteristics ⁵	-7.0	(-11.4; -2.5)	0.002
All listed variables	-7.6	(-12.3; -3.0)	0.001

¹Results of multiple regression analyses with plasma phospholipid 18:3n-6 (% wt/wt) or 20:3n-6 (% wt/wt) at birth as independent variables and log-triacylglycerol (mmol/L) as dependent variable. The regression coefficients are percentage changes in the outcome variable per unit change in 18:3n-6 or 20:3n-6 concentration at birth (Note that for 18:3n-6 one unit equals 0.1 % wt/wt and that for 20:3n-6 one unit equals 1 % wt/wt).

²Family history of cardiovascular disease (dichotomous; yes vs no).

³Anthropometric variables: height, weight, and sum four skinfolds

⁴Socio-economic factors: maternal and paternal education (entered as dummy variables) and number of children in household.

⁵Neonatal characteristics: birth weight, length of gestation, maternal smoking during pregnancy (dichotomous; yes vs no) and infant nutrition (dichotomous; breast-fed vs formula-fed).

Discussion

In the present study, childhood triacylglycerol concentration appeared related to concentrations of γ -linolenic acid and its elongation product dihomo- γ -linolenic acid measured seven years earlier in umbilical cord blood. Both γ -linolenic acid and dihomo- γ -linolenic acid can be derived from the parent essential fatty acid linoleic acid (18:2n-6), the most abundant PUFA in a typical Western-style diet. A high linoleic acid intake does, however, not necessarily guarantee a higher availability of these metabolites.¹⁹ In contrast, an increased intake of γ -linolenic acid results in an increased concentration of γ -linolenic acid and dihomo- γ -linolenic acid in blood lipid fractions.¹⁹ Dietary γ -linolenic acid may thus be a more important source of γ -linolenic acid and dihomo- γ -linolenic acid than linoleic acid. The presented data seem to suggest that a low intrauterine availability of γ -linolenic acid predispose individuals to higher

triacylglycerol concentrations in childhood. An elevated plasma triacylglycerol concentration is increasingly recognized as an important component of the metabolic syndrome associated with the risk for atherosclerosis and the development of cardiovascular diseases.²⁰⁻²²

We have previously shown that a lower umbilical plasma concentration of γ -linolenic acid also relates to higher fasting insulin and proinsulin concentrations in 7-year-olds.¹³ Circulating triacylglycerol concentration is closely related to insulin sensitivity.²³ The association found between triacylglycerol and umbilical cord blood γ -linolenic acid concentration could be explained by an alteration in insulin metabolism. How γ -linolenic acid may influence insulin metabolism, however, is not clear. Maybe peroxisome proliferator-activated receptors (PPARs) are involved.²⁴ Interestingly, gamma-linolenic acid has been shown to have a relatively high affinity for PPAR α and PPAR δ , these transcription factors are involved in glucose and lipid homeostasis.^{25,26}

The positive associations found between triacylglycerol concentration and phospholipid γ -linolenic acid and dihomo- γ -linolenic acid concentrations measured at seven years of age, are consistent with previous findings in adults and children.^{27,28} Why fasting triacylglycerol concentration is negatively related to the γ -linolenic acid and dihomo- γ -linolenic acid content of umbilical cord plasma and positively related to the γ -linolenic acid and dihomo- γ -linolenic acid content of plasma phospholipids at age seven is not known. Whether this reflects different effects of γ -linolenic acid depending on the moment of exposure, an adaptation to a low γ -linolenic acid availability in fetal life with the inability to handle a higher availability during childhood, or some other mechanism needs further exploration.

Although birth weight is positively related with umbilical cord plasma phospholipid dihomo- γ -linolenic acid concentration in this cohort,¹⁴ no relation was found between birth weight and childhood plasma triacylglycerol and other lipid and lipoprotein concentrations. We also found no significant relation between birth weight and the γ -linolenic acid content of umbilical cord plasma phospholipids. It thus seems that γ -linolenic acid availability early in life relates to triacylglycerol concentration later in life independent of attained weight at birth. According to Ravelli *et al.*,⁸ a limited availability of nutrients during late gestation may lead to permanent changes in insulin metabolism, even when the effects on birth weight are small.

The phospholipid fatty acid composition of umbilical cord blood collected after birth is similar to that of fetal blood collected in utero at a comparable gestational age,²⁹ but it does not necessarily reflect the PUFA availability during earlier stages of pregnancy. This may be of relevance since exposure to the Dutch famine during early gestation, rather than during late gestation, has been associated to adult plasma cholesterol and lipoprotein concentrations.³⁰ This could explain why we did not find a relation with cholesterol and lipoprotein levels.

The use of umbilical cord plasma phospholipid fatty acid composition as a biomarker for fetal PUFA availability has limitations. The PUFA content of cord

plasma phospholipids depends upon a complex of different factors, which include maternal PUFA intake, maternal PUFA reserves and placental PUFA delivery, in combination with endogenous maternal, placental and fetal fatty acid metabolism. We can not differentiate between these factors. Moreover, we can not exclude the possibility of reverse causality. Alterations in insulin metabolism by other unknown factors could influence both plasma triacylglycerol concentration and, through effects on delta-6-desaturase, the γ -linolenic acid and dihomo- γ -linolenic acid content of plasma phospholipids. Another limitation of the present study is the fact that less than 40% of the eligible children participated. Although the birth characteristics, including γ -linolenic acid and dihomo- γ -linolenic acid concentration in umbilical plasma, did not differ between those who participated and those who did not attend our clinic, residual confounding due to selection can not be fully excluded. Controlled intervention studies are therefore needed to establish whether the availability of γ -linolenic acid is indeed the primary factor involved and whether the quality of fat intake during pregnancy in women could have an influence on the cardiovascular risk profile in her offspring.

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Chapter 6 | Interaction between a common variant of the cholesteryl ester transfer protein gene and the apolipoprotein E polymorphism: effects on plasma lipids and lipoproteins in a cohort of seven-year-old children

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Abstract

Background/Aim Common variations in genes, like apolipoprotein E (apo E) and cholesteryl ester transfer protein (CETP), are important determinants of plasma lipid and lipoprotein levels. Both, apo E and CETP are contributing to the reverse transport of cholesterol back to the liver. Therefore, the effects of variations at the CETP locus may very well interact with apo E genotype.

Methods & Results As part of an ongoing study, the combined effects of apo E genotype and heterogeneity at the CETP gene locus on plasma lipid and lipoproteins were studied in a birth-cohort-based sample of 257 Dutch prepubescent boys and girls (aged 6.7 – 8.1 years). Children with an apo E2E3 genotype (carrying the $\epsilon 2$ allele; $\text{arg}_{158} \rightarrow \text{cys}$) had lower concentrations of LDL cholesterol and apolipoprotein B (apo B) than those with an apo E4E3 genotype (carrying the $\epsilon 4$ allele; $\text{cys}_{112} \rightarrow \text{arg}$) or apo E3E3 genotype (homozygous for the parent $\epsilon 3$ allele). These associations were statistically significant in children who were homozygous ($P = 0.004$ for LDL; $P = 0.002$ for apo B) or heterozygous ($P < 0.0001$; for LDL and apo B) for the absence of the *Taq* IB polymorphism at the CETP gene locus (B2 allele), but not in those homozygous for the presence of this variant (B1B1). The highest plasma HDL cholesterol concentrations were observed in children with the CETP B2B2 genotype. The difference in HDL cholesterol level between CETP genotype groups was statistically significant only in E2E3 carriers ($P = 0.01$). The LDL/HDL ratio was significantly lower in E2E3 carriers, but not when combined with a CETP B1B1 genotype.

Conclusion Our findings indicate that apo E genotype and heterogeneity at the CETP gene locus have an additive and interactive influence on plasma lipid and lipoprotein levels in children.

Introduction

High serum concentrations of low-density lipoprotein (LDL) cholesterol and low concentrations of high-density lipoprotein (HDL) cholesterol are associated with an increased risk for the development of coronary heart disease in adults.¹ Childhood levels of lipids and lipoproteins persist over time and tend to predict adult values.^{2,3} More importantly, childhood levels of LDL and HDL cholesterol are related to the presence and severity of asymptomatic atherosclerosis of the aorta and coronary arteries.^{4,5} Common variations at the apolipoprotein E (apo E) gene locus are important determinants of plasma lipid and lipoprotein levels in adults and children.⁶⁻⁸ Variation at the apo E locus may also influence the serum lipid and lipoprotein changes from childhood into adulthood.⁹ Moreover, apo E genotype seems to predispose individuals to develop coronary heart disease later in life.¹⁰

Three common apo E isoforms are recognized: the $\epsilon 2$ allele (arg₁₅₈ $\rightarrow$ cys), the $\epsilon 4$ allele (cys₁₁₂ $\rightarrow$ arg), and the parent $\epsilon 3$ allele. The $\epsilon 2$ allele is associated with lower total cholesterol, LDL cholesterol and apolipoprotein B (apo B) concentrations, whereas the $\epsilon 4$ allele relates to higher levels.⁶⁻⁸ Higher concentrations of HDL cholesterol have been found in subjects carrying the $\epsilon 2$ allele.¹¹ These findings have been attributed to a reduced affinity of apo E2 for the apo E/remnant-receptor, leading to a reduced clearance of cholesterol from apo E-containing lipoproteins (*e.g.* HDL, chylomicrons, very-low-density lipoprotein) by the liver.¹² A compensatory up-regulation of the apo B/E LDL-receptor, resulting in an enhanced hepatic uptake of LDL, may explain the lower LDL cholesterol and apo B concentrations seen in $\epsilon 2$ carriers.¹² Apo E is also considered a contributor to reverse cholesterol transport; the transport of cell-derived cholesterol back to the liver mediated by apo E-containing HDL particles.^{13,14} Genetic variation at the apo E locus may influence this process.^{15,16}

Variation at the apo E gene locus has also been associated with the metabolic response to dietary cholesterol, including changes in the plasma concentrations of an enzyme called cholesteryl ester transfer protein (CETP).¹⁷ CETP plays a key role in reverse cholesterol transport. It mediates the transfer of cholesteryl esters from HDL to apo B containing lipoproteins like LDL and very-low-density lipoprotein (VLDL), in exchange for triacylglycerol.¹⁸⁻²⁰ In adults, plasma CETP concentrations, CETP activity and HDL cholesterol levels have been associated with common variations in the CETP gene, like the so-called *Taq* IB polymorphism.²¹⁻²³ The presence of this polymorphism (*i.e.* B1 allele) has also been related to the progression of coronary atherosclerosis.²⁴

So far, limited information is available on the relations between the *Taq* IB CETP polymorphism and plasma lipid and lipoprotein concentrations in children.²⁵ The study of genotype-phenotype relationships in children is important since it allows the observation of associations in the absence of atherosclerotic diseases and adult life style factors that may obscure or alter such relations, like smoking and alcohol consumption.²⁰ Considering the role of apo E in the hepatic clearance of cholesterol

from apo E containing lipoproteins like HDL and as a contributor to reverse cholesterol transport, the effects of variations at the CETP locus may very well interact with apo E genotype. Therefore, we investigated the association between CETP genotype and fasting plasma lipid and lipoprotein concentrations in children and whether these associations were modulated by variations at the apo E gene locus.

Subjects and Methods

Study population

Subjects were recruited from a Dutch birth-cohort of children. As infants, these children and their mothers participated in a previous observational study of plasma essential fatty acid concentrations during pregnancy and their relation to pregnancy outcome. No interventions were provided during this study. To explore the potential long-term effects of fetal essential fatty acid availability on the cardiovascular risk profile of children, a follow-up study was performed between 1997 and 2000.

In total, 750 children were eligible for follow-up (for more details on this cohort see Rump *et al.*).²⁶ With the aid of the Dutch population registry 97% of the eligible population could be traced. Three of the children had died and 34 lived abroad. The remaining 691 children and their parents were invited for the follow-up evaluation. Of these, 297 participated, 231 refused, 133 did not respond, and 30 did not show up at appointments made or retracted their initial consent. Birth characteristics (*i.e.* birth weight, birth length, head circumference, ponderal index, length of gestation and birth order) of the children who participated did not differ from the children who did not attend our clinic (data not shown).

Genomic DNA was available in 257 (141 boys and 116 girls) of the participating children. The present analyses focus on the combined effects of apo E and CETP genotype on plasma lipid and lipoprotein concentrations in children and was therefore limited to these 257 subjects. Approval for this study was obtained from the Ethics Committee of the University Hospital in Maastricht and the parents of all participating children gave their written, informed consent.

Data collection at follow-up

All children were investigated at approximately seven years of age. Mean age (range) at follow-up was 7.3 (6.7 - 8.1) years. The same physician (PR) examined all children and performed all venipunctures. Anthropometric measurements were performed as described in detail by Gerver and de Bruin.²⁷ Blood samples were collected in EDTA-treated evacuated tubes after an overnight fast. Plasma was separated from blood cells by centrifugation and stored in small portions at -80 °C until analysis.

Total cholesterol, triacylglycerol, and (after precipitation of apo B containing lipoproteins) HDL cholesterol were determined enzymatically, as described before.²⁸ The intra-assay and inter-assay variation coefficients were 1.4% and 3.0% (total

cholesterol), 2.5% and 4.3% (triacylglycerols), 3.6% and 7.8% (HDL cholesterol), respectively. LDL cholesterol was calculated using the equation of Friedewald *et al.*²⁹ Apolipoprotein A1 (apo A1) and apolipoprotein B (apo B) were analyzed by an immunoturbidimetric reaction (UNI-KIT Apo A1 and UNI-KIT Apo B, Hoffmann-La Roche, Basel, Switzerland). The coefficients of variation within and between assays were 2.4% and 5.1% for apo A1 and 3.0% and 5.8% for apo B. An enzyme-linked immunosorbent assay (ELISA) was applied to measure plasma Lp(a) concentration (TintElize, Biopool, Umeå, Sweden). Variation coefficients were 7.7% and 3.4%.

DNA isolation and apo E and CETP genotyping

Genomic DNA was extracted from isolated leukocytes using standard procedures.²⁸ The *Taq* IB polymorphism of the CETP gene and apo E genotypes were determined by PCR and subsequent restriction-fragment-length polymorphism analysis as described by Kuivenhoven *et al.*²² and Plat and Mensink,²⁸ respectively. After repeated attempts, the apo E genotype could not be determined in 6 children and the CETP genotype could not be determined in 16 children.

Statistical analysis

Data are presented as mean $\pm$ SE. Associations of apo E and CETP genotypes with plasma lipid and lipoprotein concentrations and the demographic characteristics weight, height and BMI were evaluated by one-way analysis of variance (ANOVA). E2E2 and E2E3 genotypes (E2 group) and the E4E4 and E4E3 genotypes (E4 group) were compared to the E3E3 genotype. The distributions of boys and girls among apo E and CETP genotype groups was investigated with chi-square tests. The interaction between apo E genotype and CETP genotype was studied using stratified analyses, and when significant ($P < 0.05$ by ANOVA) effects were found, subsequent post-hoc pair-wise comparisons between the individual genotype groups were performed (using a Bonferroni correction for multiple testing; $\alpha = 0.0165$). Because of their low frequency and unequal distribution among CETP genotype groups, subjects with the rare apo E genotypes E2E2 ($n = 2$) and E4E4 ($n = 3$) were excluded during the analysis of interaction with CETP genotype. Children with the E2E4 genotype ($n = 10$) were excluded during all analyses because they carry an allele that is common to both the E2 group and the E4 group. Because of a skewed distribution of Lp(a) and triacylglycerol concentrations, log-transformed values were used. For these two variables the reported means are geometric means. The assumption of a Hardy-Weinberg equilibrium for the distribution of apo E and CETP genotypes was tested using gene counting and chi-square analysis.

Results

Frequency of apo E and CETP polymorphisms

The allele frequencies of the apo E polymorphism were 0.10, 0.76 and 0.14 for the $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$ alleles respectively. The CETP gene B1 and B2 alleles were found at frequencies of 0.55 and 0.45. For both the apo E and the CETP polymorphism, the observed genotype frequencies were in Hardy-Weinberg equilibrium. The frequencies of the CETP genotypes B1B1, B1B2 and B2B2 did not differ between the E2E3, E3E3 and E4E3 genotype groups ($P = 0.44$ by chi-square analysis).

Plasma lipids, lipoproteins and Apo E or CETP genotype.

Plasma lipid and lipoprotein concentrations did not differ between boys and girls. There were no statistically significant differences between the apo E and CETP genotype groups with respect to the distribution of sex, height, weight and BMI (not shown). There was a clear association of apo E and CETP genotypes with plasma lipid and lipoprotein concentrations (Table 6.1). The lowest levels of total cholesterol, LDL cholesterol, LDL/HDL cholesterol ratio, apo B and the highest concentration of HDL cholesterol were seen in the apo E2 genotype group. Concentrations of apo A1 did not differ significantly between the apo E genotype groups, but there was a tendency of a higher apo A1 level in E2 carriers. Variation at the CETP locus was associated to plasma total cholesterol, LDL cholesterol, HDL cholesterol, apo B and apo A1 concentrations (Table 6.1). The highest LDL cholesterol and apo B concentrations and the highest LDL/HDL cholesterol ratio were found in children with the B1B1 genotype. The highest HDL cholesterol and apo A1 concentrations were found in B2B2 carriers. In this population, triacylglycerol and Lp(a) concentrations did not differ among the apo E and CETP genotype groups.

Combined effects of Apo E and CETP genotypes

The associations between the *Taq IB* polymorphism in the CETP gene and plasma lipid and lipoprotein concentrations were most pronounced in subjects with an apo E E2E3 genotype. Children with a CETP B1B1 genotype had a statistically significant higher apo B concentration than children with a B1B2 or B2B2 genotype, but only when combined with the apo E2E3 genotype (Figure 6.1). Apo B concentrations did not differ between CETP genotype groups in children with the apo E E3E3 and E4E3 genotypes (Figure 6.1). Conversely, a statistically significant lower apo B concentration in E2E3 carriers compared to E3E3 and E4E3 carriers was seen only when combined with a B1B2 or a B2B2 CETP genotype (Figure 6.1). The differences in apo B concentration were not statistically significant between the apo E genotype groups in children with a CETP B1B1 genotype.

Table 6.1: Total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, LDL/HDL cholesterol ratio, apolipoprotein A1 (apo A1), apolipoprotein B (apo B), triacylglycerol and lipoprotein (a) (Lp(a)) concentrations in a cohort of seven-year-old children according to genetic variation at either the apolipoprotein E (apo E) or the cholesteryl ester transfer protein (CETP) loci.¹

	Apo E genotype ²			P
	E2 group (n = 38)	E3E3 (n = 146)	E4 group (n = 57)	
Total cholesterol (mmol/L)	3.73 ± 0.08 ^{ab}	4.12 ± 0.05 ^a	4.24 ± 0.08 ^b	0.0002
HDL cholesterol (mmol/L)	1.46 ± 0.05 ^a	1.32 ± 0.03 ^a	1.38 ± 0.04	0.03
LDL cholesterol (mmol/L)	2.01 ± 0.08 ^{ab}	2.53 ± 0.05 ^a	2.62 ± 0.07 ^b	<0.0001
LDL/HDL cholesterol ratio	1.47 ± 0.09 ^{ab}	2.04 ± 0.06 ^a	1.98 ± 0.08 ^b	<0.0001
Apo A1 (mg/L)	1434 ± 35	1346 ± 18	1342 ± 28	0.07
Apo B (mg/L)	625 ± 28 ^{ab}	755 ± 13 ^a	760 ± 16 ^b	<0.0001
Triacylglycerols (mmol/L)	0.51 ± 0.04	0.50 ± 0.02	0.48 ± 0.03	0.79
Lp(a) (mg/L)	72 ± 16	80 ± 9	68 ± 15	0.75

	CETP genotype			P
	B1B1 (n = 76)	B1B2 (n = 113)	B2B2 (n = 52)	
Total cholesterol (mmol/L)	4.15 ± 0.07	3.93 ± 0.06	4.17 ± 0.09	0.02
HDL cholesterol (mmol/L)	1.28 ± 0.03 ^a	1.36 ± 0.03	1.45 ± 0.05 ^a	0.009
LDL cholesterol (mmol/L)	2.62 ± 0.07 ^a	2.33 ± 0.05 ^a	2.43 ± 0.08	0.002
LDL/HDL cholesterol ratio	2.15 ± 0.08 ^{ab}	1.81 ± 0.06 ^a	1.82 ± 0.10 ^b	0.001
Apo A1 (mg/L)	1343 ± 24	1325 ± 20 ^a	1439 ± 36 ^a	0.009
Apo B (mg/L)	782 ± 19 ^a	689 ± 14 ^a	739 ± 20	0.0003
Triacylglycerols (mmol/L)	0.51 ± 0.02	0.47 ± 0.02	0.55 ± 0.04	0.12
Lp(a) (mg/L)	75 ± 11	65 ± 10	71 ± 15	0.79

¹Values are means ± SE, except for triacylglycerol and Lp(a) concentrations (geometric means ± SE).

²E2 group, E2E2 and E2E3 carriers; E4 group, E4E3 and E4E4 carriers; subjects with the E2E4 genotype (n = 10) were excluded.

^{ab}P < 0.0165 for pair-wise differences between genotype groups indicated by the same number.

Plasma HDL cholesterol and LDL cholesterol levels differed significantly between the CETP genotype groups only in children with an apo E2E3 genotype (Table 6.2). The P value for the difference in LDL cholesterol between E2E3/B1B1 and E2E3/B2B2 carriers was 0.025, and thus just above our pre-defined criterion of statistical significance ($\alpha = 0.0165$). Conversely, LDL cholesterol concentrations were significantly lower in E2E3 carriers compared to both E3E3 and E4E3 carriers in children with a CETP B1B2 or B2B2 genotype (Table 6.2). Additionally, the higher concentration of HDL cholesterol observed in apo E2 carriers was most pronounced in subjects carrying a CETP B2 allele. However, the differences in plasma HDL cholesterol levels between apo E genotype groups was statistically significant in B2B2 carriers only (Table 6.2). The ratio of LDL cholesterol to HDL cholesterol was

significantly lower in E2E3 carriers except when combined with a CETP B1B1 genotype (Table 6.2). The P value for the difference in LDL/HDL ratio between E2E3/B2B2 and E4E3/B2B2 carriers was 0.022, slightly higher than our pre-defined criterion of statistical significance ($\alpha = 0.0165$). In children with an apo E2E3 genotype, apo A1 concentrations for B1B1, B1B2, and B2B2 carriers were 1407 ± 63 , 1361 ± 45 , and 1556 ± 76 mg/L, respectively (P value = 0.08, by ANOVA). Apo A1 concentrations for B1B1, B1B2, and B2B2 carriers were 1310 ± 30 , 1343 ± 27 , and 1405 ± 55 mg/L for E3E3 carriers and 1328 ± 69 , 1298 ± 38 , and 1416 ± 64 mg/L for E4E3 carriers, respectively (ANOVA P value > 0.2 for both). Concentrations of triacylglycerol and Lp(a) did not differ significantly between the stratified CETP and apo E genotype groups (not shown).

Figure 6.1: Mean ($\pm$ SE) plasma apolipoprotein B concentration in seven-year-old children according to combinations of genetic variation at the cholesteryl ester transfer protein (CETP) and apolipoprotein E (apo E) loci. Subjects with the rare apo E genotypes E2E2 ($n = 2$), E4E4 ($n = 3$), and E2E4 ($n = 10$) were excluded. The difference in apolipoprotein B concentration between CETP genotype groups was statistically significant in apo E2E3 carriers only ($P < 0.0001$; ANOVA). The difference in apolipoprotein B concentration between apo E genotype groups was statistically significant in children with a CETP B1B2 or B2B2 genotype ($P < 0.0001$ and $P = 0.002$, respectively; ANOVA). ^a $P < 0.0165$ for difference with B1B2/E3E3, B1B2/E4E3, and B1B1/E2E3. ^b $P < 0.0165$ for difference with B2B2/E3E3, B2B2/E4E3, and B1B1/E2E3.

Discussion

Our findings indicate an additive and interactive effect of heterogeneity at the CETP gene locus and the apo E genotype on plasma lipid and lipoprotein concentrations in children. The apo E and CETP allele frequencies found in the present study were similar to previous reported frequencies in Dutch adult populations.^{24,30} According to Hubacek *et al.*,²⁵ common polymorphisms in the CETP gene do not determine plasma lipid levels during childhood. This conclusion was based on their finding that CETP *Taq* IB allele frequencies did not differ between Czechoslovakian children from opposite ends of the cholesterol distribution curve.²⁵ In the present cohort, total cholesterol concentrations in B1 homozygotes were indeed similar to those in B2 homozygotes. However, the observed associations between the CETP *Taq* IB polymorphism and plasma HDL cholesterol and apo A1 levels in our cohort of seven-year-olds, which are consistent with previous findings in adult populations,²¹⁻²³ indicate that this CETP gene variation does relate to plasma lipid and lipoprotein levels in children. The relations found might be explained by the increased concentration and activity of circulating CETP associated with this polymorphism.^{22,23} It should be emphasized, however, that the *Taq* IB polymorphism itself is not located in a coding region of the CETP gene.²² This polymorphism could be a non-functional marker in close linkage disequilibrium with a mutation elsewhere in the CETP gene or in nearby genes. For instance, the recently described -629 A/C polymorphism located in the promoter region of the CETP gene, which is tightly linked with the *Taq* IB polymorphism.³¹ Alternatively, the *Taq* IB polymorphism may have an effect on transcriptional regulation of the CETP gene.

The observation that the association between CETP genotype and plasma lipids and lipoproteins is most pronounced in apo ϵ 2 carriers is new. Plasma CETP activity is positively related to apo E concentrations³² and apo E concentrations are higher in subjects with an E2E3 genotype.¹² Further, higher plasma concentrations of CETP and lower levels of HDL cholesterol are found in response to a high-cholesterol diet in adults carrying an ϵ 2 allele.¹⁷ Maybe due to such an increased CETP responsiveness, the impact of heterogeneity at the CETP gene locus is larger in ϵ 2 carriers. The relations between the apo ϵ 2 allele and plasma LDL and HDL cholesterol concentrations has been attributed to the reduced affinity of apo E2 to the apo E/remnant-receptor and a compensatory up-regulation of the apo B/E LDL-receptor, resulting in an increased clearance of the apo B-containing LDL particles.¹² A higher CETP activity may lead to higher concentrations of LDL cholesterol and apo B.³³ This could explain the higher LDL cholesterol and apo B concentrations in ϵ 2 carriers with a B1B1 genotype when compared to children with a B1B2 or B2B2 genotype. This association between the CETP *Taq* IB polymorphism and plasma levels of apo B and LDL cholesterol is generally not observed in adult populations. The reason for this

difference between children and adults is not clear, maybe the absence of adult life style factors like alcohol consumption or smoking play a role.²⁰

In transgenic mice expressing a defective apo E (like apo E2), co-expression of CETP leads to an increase of cholesteryl esters in apo B containing lipoproteins.³⁴ Additionally, an increase in the concentration of plasma apo B and (VLDL + LDL) cholesterol concentrations has been observed in mice over-expressing CETP.³⁵ This increase in apo B concentration could be a consequence of a down-regulation of the apo B/E LDL-receptor.^{18,36} The lower apo B and LDL cholesterol concentrations in $\epsilon 2$ carriers, generally explained by an up-regulation of this particular receptor, might thus in theory be compensated by an increased CETP activity such as associated with the B1B1 genotype.

Table 6.2: High-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and the LDL/HDL cholesterol ratio in a cohort of seven-year-old children according to combinations of genetic variation at the apolipoprotein E and the cholesteryl ester transfer protein (CETP) loci.¹

Apo E genotype ²	CETP genotype			<i>P</i>
	B1B1 (69)	B1B2 (102)	B2B2 (51)	
HDL cholesterol (mmol/L)				
E2E3 (36)	1.28 ± 0.07 (12) ^a	1.43 ± 0.07 (14)	1.68 ± 0.13 (10) ^{a,c}	0.01
E3E3 (135)	1.25 ± 0.04 (45)	1.35 ± 0.04 (64)	1.33 ± 0.07 (26) ^c	0.20
E4E3 (51)	1.25 ± 0.07 (12)	1.36 ± 0.05 (24)	1.48 ± 0.09 (15)	0.11
<i>P</i>	0.90	0.60	0.04	...
LDL cholesterol (mmol/L)				
E2E3	2.40 ± 0.12 ^a	1.81 ± 0.11 ^{a,c,d}	1.92 ± 0.08 ^{c,d}	0.001
E3E3	2.60 ± 0.08	2.43 ± 0.06 ^c	2.56 ± 0.12 ^c	0.20
E4E3	2.84 ± 0.17	2.48 ± 0.10 ^d	2.56 ± 0.11 ^d	0.15
<i>P</i>	0.14	<0.0001	0.004	...
LDL/HDL cholesterol ratio				
E2E3	1.95 ± 0.16 ^{a,b}	1.32 ± 0.11 ^{a,c,d}	1.21 ± 0.11 ^{b,c}	0.0007
E3E3	2.20 ± 0.11	1.92 ± 0.08 ^c	2.07 ± 0.15 ^c	0.11
E4E3	2.34 ± 0.19	1.90 ± 0.11 ^d	1.80 ± 0.13	0.03
<i>P</i>	0.36	0.003	0.002	...

¹Values are means ± SE (number of cases).

²Subjects with the apo E genotypes E2E2 (*n* = 2), E4E4 (*n* = 3) and E2E4 (*n* = 10) were excluded.

^{a,b}*P* < 0.0165 for pair-wise differences between CETP genotype groups indicated by the same number (horizontally).

^{c,d}*P* < 0.0165 for pair-wise differences between apo E genotype groups indicated by the same letter (vertically).

In mice, overexpression of CETP also resulted in a higher (VLDL + LDL) cholesterol/HDL cholesterol ratio, which was related to an increase in the size of atherosclerotic lesions in the aorta.³⁷ In the present study, the highest mean LDL/HDL cholesterol ratios were seen in children carrying the CETP B1B1 genotype.

Because of the small sample size, we were unable to study the effects of CETP genotype in children homozygous for $\epsilon 2$. This is of interest since children homozygous for $\epsilon 2$ may develop type III hyperlipoproteinemia (dysbetalipoproteinemia).³⁸ Our observations in children heterozygous for the $\epsilon 2$ allele suggest that a genetically based increase in CETP activity (such as associated with a B1B1 genotype) may indeed be an additional factor in the development of this phenotype, as proposed by Fazio and colleagues based on their experiments in mice.³⁴ Whether an interaction between apo E and CETP genotype, as seen in the present study, is also observed in adults, remains to be explored. The observed interaction between CETP and apo E genotype may also have implications for the prevention of cardiovascular diseases. Interventions designed to reduce the activity of CETP (e.g. newly developed CETP inhibitors)^{39,40} may provide interesting approaches in lowering LDL cholesterol and increasing HDL cholesterol, especially in subjects with a genetic predisposition to a high CETP activity. Our results seem to indicate that the effectiveness of such interventions may depend upon apo E genotype.

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Chapter 7 | General discussion

Introduction

A low weight at birth is associated with an increased risk for the development of adult heart disease, Type II diabetes mellitus and related risk factors such as hypertension and dyslipidemia. This relationship between infant weight at birth and adult diseases led to the formulation of the fetal origins hypothesis.¹ According to this hypothesis, the propensity to develop coronary heart disease, Type II diabetes mellitus, hypertension and other disorders are consequences of intra-uterine adaptations to fetal undernourishment.² The risk for adult diseases may thus be “programmed” or “imprinted” by a limited and/or unbalanced availability of nutrients *in utero*.^{3,4} Only a small number of human studies have been conducted to identify the nutrients involved. One group of nutrients that has been implicated, but has not been studied in this context so far, are essential fatty acids and their longer-chain, more-unsaturated derivatives, abbreviated as LCP's (long-chain polyenes).⁵⁻⁷ Beneficial effects of dietary essential fatty acids have been described in relation to adult cardiovascular diseases and related disorders. Whether the availability of these substances during fetal life may influence later cardiovascular risk is not known. The primary objective of the study described in the present thesis was therefore to explore whether the availability of essential fatty acids and their longer-chain more-unsaturated derivatives during fetal life relates to the cardiovascular risk profile of children. For this purpose we studied cardiovascular risk indicators in a cohort of Dutch children with known essential fatty acid status at birth.

Main findings of the study

Essential fatty acids and birth weight

The first step in exploring the potential role of essential fatty acids in the fetal origins of cardiovascular diseases was to study relations between birth weight and the fatty acid concentrations in umbilical cord blood samples. Previous studies had demonstrated that infants born with a lower weight also have a lower essential fatty acid status at birth.⁸⁻¹³ These findings suggest that the availability of essential fatty acids could be a determinant of fetal growth. Especially arachidonic acid (20:4n-6) is considered an important fatty acid for growth.^{9,10,13,14} However, these studies included mostly premature infants and low-birth-weight neonates. Therefore, confirmation of these findings for infants born at term was needed. In the study described in *Chapter 3* we investigated the relation between birth weight and the essential fatty acid concentrations measured in umbilical cord plasma phospholipids of the neonates from our cohort. Surprisingly, the proportion of arachidonic acid and docosahexaenoic acid (22:6n-3) in umbilical cord plasma phospholipids was negatively related to neonatal weight at birth. This finding is in total contrast with previous observations in premature infants and low-birth-weight neonates. Other long-chain polyene concentrations (docosatetraenoic acid (22:4n-6), docosapentaenoic acid (22:5n-3), Σ n-6 LCPs, and Σ n-3 LCPs) were

also negatively related to infant size at birth (*Chapter 3*). Additionally, markers for the general essential fatty acid status (like Mead acid, the ratio of $\Sigma n-3 + n-6$ to $\Sigma n-7 + n-9$ fatty acids, and the ratio 22:6n-3 to 22:5n-6) indicated a lower essential fatty acid status in larger neonates rather than in smaller neonates. Therefore, these findings suggest that, in term neonates, a high weight at birth is related to a lower biochemical essential fatty acid status in general. An explanation for the inconsistency with studies in premature infants and low-birth-weight neonates could be that additional (pathologic) factors associated with premature birth or severe growth retardation affected both essential fatty acid status and intrauterine growth in these populations. Another explanation could be a lack of an appropriate adjustment for gestational age at birth (see *Chapter 3*).

In contrast to arachidonic acid and docosahexaenoic acid, concentrations of dihomo- γ -linolenic acid in umbilical cord blood were positively related to size at birth. The positive association between dihomo- γ -linolenic acid concentration and size at birth seems more consistent than those reported for arachidonic acid and docosahexaenoic acid. Higher concentrations of dihomo- γ -linolenic acid in blood lipid fractions or vessel walls of larger neonates have been reported previously and have been found in premature infants as well.^{9-11,15-17} Maybe dihomo- γ -linolenic acid is important for intrauterine growth. No study has yet evaluated the effect of dihomo- γ -linolenic acid supplementation (or supplementation of its precursor γ -linolenic acid) on intrauterine or postnatal growth. In fact, the suggestion that arachidonic acid and docosahexaenoic acid are determinants of neonatal growth have not been supported by intervention studies either. The most cited intervention study is the study performed by Carlson *et al.*¹⁴ In this trial a negative effect of fish oil supplemented formula (rich in docosahexaenoic acid and eicosapentaenoic acid) on infant growth had been found.¹⁴ The importance of arachidonic acid was suggested based on the observed reduction in blood arachidonic acid concentrations accompanying this intervention.¹⁸ However, an alternative explanation could be that the supplemented n-3 fatty acids themselves inhibited growth. Recent intervention studies in term neonates did not find any effect of docosahexaenoic acid supplementation (with or without arachidonic acid) on postnatal growth.¹⁹⁻²² Studies using formulas with the addition of arachidonic acid alone are lacking. Firm conclusions on cause and effect cannot be drawn from observed associations; and since conclusive evidence from controlled intervention studies is also lacking it is difficult to uphold the suggestion that essential fatty acids like arachidonic acid have a stimulatory effect on fetal growth, even in premature infants.

The relative concentrations of n-3 and n-6 long-chain polyenes in maternal plasma phospholipids decreases during the second half of pregnancy.²³ In the present study, this decrease was found to be more pronounced in mothers of larger infants (*Chapter 3*). The second half of pregnancy is considered an important period for fetal essential fatty acid accretion. Between 37 and 42 weeks of gestational age, the essential fatty acid status in cord blood samples still increases. Especially the proportion of the

n-3 long-chain polyene docosahexaenoic acid increases in umbilical cord blood during this period (Chapter 2 & 3). A heavier infant probably accretes more long-chain polyenes than a smaller neonate does. The larger decrease in plasma long-chain polyene concentrations of mothers of heavier babies might thus be explained by an increased mother-to-fetal transport, driven by an increased fetal accretion of these substances. If this is true, the lower LCP concentrations found in heavier neonates could suggest that this increase in the maternal-to-fetal LCP flux is not sufficient to maintain cord plasma long-chain polyene levels and maybe fetal essential fatty acid status. On the other hand, the lower long-chain polyene concentrations in fetal blood of heavier neonates may result from the fact that these substances are divided over larger body stores.

Essential fatty acids and cardiovascular risk indicators in children

Consistent relations between umbilical cord blood fatty acid concentrations and cardiovascular risk variables measured at age seven were only found for γ -linolenic acid and its direct elongation product dihomo- γ -linolenic acid (Chapter 4 & Chapter 5). A lower concentration of γ -linolenic acid in umbilical cord blood related to higher plasma insulin, proinsulin, leptin, and triacylglycerol concentrations and to a higher percentage of body fat at age seven. A lower dihomo- γ -linolenic acid concentration at birth also related to higher plasma insulin and triacylglycerol concentrations in seven-year-olds. These variables are all components of the so-called insulin resistance syndrome. Our findings thus seem to indicate a relation between the availability of γ -linolenic acid and/or dihomo- γ -linolenic acid during fetal life and the development of insulin resistance during childhood. This suggestion is supported by the fact that concentrations for both fatty acids in umbilical cord blood were negatively related with the homeostasis model assessment (HOMA) index for insulin resistance. Based on the absence of consistent relationships, the availability of other more abundant essential fatty acids (*i.e.* linoleic acid, arachidonic acid and docosahexaenoic acid) does not seem to be an important factor in the development of an adverse cardiovascular risk profile during childhood.

Birth weight and cardiovascular risk indicators in children

Similar to previous studies conducted in adults and children,²⁴⁻³⁰ we found that (after correction for sex, age, current weight and length of gestation) a small size at birth related to higher levels of the insulin resistance variables and an increased body fatness later in life (Table 7.1 and Figure 7.1). In our study, fasting insulin concentration and the HOMA index for insulin resistance were highest in children who had a low birth weight in combination with a low umbilical cord plasma phospholipid γ -linolenic acid concentration (Chapter 4). After adjustment for birth weight, cord blood γ -linoleic acid concentration remained associated with HOMA insulin resistance and fasting insulin levels. We found no significant relation between birth weight and the γ -linolenic acid content of umbilical cord plasma phospholipids. These findings indicate that the

relations between γ -linolenic acid and the risk variables are not be mediated through an effect on fetal growth. It seems that the effects of birth weight and that of the fetal availability of γ -linolenic acid are additive rather than interactive. According to the findings of the Dutch Hunger Winter study,³¹ the availability of nutrients during late gestation could still be important even when the effects on intrauterine growth are small.

Gene polymorphisms

Common genetic variations can be important predictors of circulating cardiovascular risk factor levels in children and may influence the response of a subject to an environmental stimulus. The potential effects of fetal essential fatty acid availability on the cardiovascular risk profile of children could therefore be dependent upon the presence of such gene variants. In the present study, two common gene polymorphisms were investigated (*Chapter 6*). Their selection was based on a relatively high population frequency of the rare allele and on the fact that relations between their presence and plasma lipid concentrations had been reported several times before. These polymorphisms were the apolipoprotein E polymorphism ($\epsilon 2$ allele: arg₁₅₈ $\rightarrow$ cys; $\epsilon 4$ allele: cys₁₁₂ $\rightarrow$ arg; and the parent $\epsilon 3$ allele) and the *Taq* IB polymorphism in the cholesteryl transfer protein gene (CETP) gene (B1 allele: presence of *Taq* IB site in intron 1; B2 allele: absence of *Taq* IB site). The apo E $\epsilon 2$ allele is associated with lower total cholesterol, LDL cholesterol and apolipoprotein B (apo B) concentrations, whereas the $\epsilon 4$ allele relates to higher levels.³²⁻³⁴ Higher plasma CETP concentrations, an increased CETP activity and lower HDL cholesterol levels are associated with the CETP *Taq* IB B1 allele.³⁵⁻³⁷

The selected polymorphisms in the apo E and CETP genes were indeed important predictors of circulating lipid and lipoprotein concentrations in children (*Chapter 6*). Additionally, we could demonstrate a genotype-genotype interaction between these common gene variants, especially in relation to plasma apolipoprotein B concentration (*Chapter 6*). Our primary aim, however, was to investigate the influence of these common genetic variations on the potential association between essential fatty acid concentrations in umbilical cord blood on one hand and the plasma lipid and lipoprotein concentrations measured at age seven on the other hand. No statistically significant relations were found between essential fatty acid concentrations at birth and the lipid and lipoprotein concentrations determined at age seven, except for triacylglycerol (*Chapter 5*). Triacylglycerol concentrations were, however, not related to the studied variations in the apo E and CETP gene (*Chapter 6*). Therefore, the two selected polymorphisms were not informative in relation to our hypothesis (*i.e.* that potential effects of fetal essential fatty acid availability on the cardiovascular risk profile of children could be modulated by the presence of common gene variants).

Table 7.1: Unadjusted mean anthropometric measurements and biochemical parameters in 7-year-old children according to birth weight.

	Birth weight (g)					P for trend ¹	
	< 2500 (n = 16)	2500-2999 (n = 49)	3000-3499 (n = 109)	3500-3999 (n = 63)	≥ 4000 (n = 22)	unadjusted	adjusted ²
Weight (kg)	24.7	24.1	24.5	26.3	28.4	<0.001	—
Height (cm)	125.9	125.3	126.0	128.7	132.0	<0.001	<0.001
BMI (kg/m ²)	15.5	15.3	15.4	15.9	16.2	0.012	0.003 ³
Sum four skinfolds (mm)	28.8	27.8	26.5	28.5	26.1	NS	<0.001 ³
Body fat (%)	20.0	19.1	18.4	19.0	17.9	NS	<0.001 ³
Fat mass (kg)	5.1	4.8	4.6	5.2	5.2	NS	<0.001 ³
Fat-free mass (kg)	19.6	19.3	19.9	21.1	23.2	<0.001	<0.001
Leptin (μg/L)	5.1	4.0	3.5	3.8	3.1	NS	<0.001 ³
Glucose (mmol/L)	4.8	4.6	4.7	4.7	4.7	NS	NS
Insulin (pmol/L)	48.3	37.7	35.5	37.5	38.2	NS	0.006 ³
Proinsulin (pmol/L)	7.4	6.7	6.7	7.2	6.6	NS	NS
Proinsulin to insulin ratio	0.16	0.20	0.22	0.21	0.22	NS	0.017
Insulin resistance (HOMA)	1.4	1.1	1.0	1.1	1.1	NS	0.009 ³
Beta-cell function (HOMA)	108.1	96.9	86.4	86.1	94.4	0.031	0.010 ³
Total cholesterol (mmol/L)	4.10	4.04	4.10	4.09	3.88	NS	NS
LDL-cholesterol (mmol/L)	2.47	2.49	2.47	2.50	2.23	NS	NS
HDL-cholesterol (mmol/L)	1.39	1.29	1.39	1.32	1.42	NS	NS
Apolipoprotein A1 (mg/L)	1376	1331	1376	1331	1399	NS	NS
Apolipoprotein B (mg/L)	750	749	733	740	650	NS	NS
Triacylglycerol (mmol/L)	0.54	0.58	0.54	0.58	0.49	NS	NS
Lipoprotein(a) (mg/L) ⁴	67	92	73	73	39	NS	NS

BMI, body mass index; HOMA, homeostasis model assessment; NS, not significant.

¹P values are derived from simple (unadjusted) and multiple (adjusted) linear regression using continuous variables.

²Adjusted for length of gestation, sex, age at follow-up, and current body mass.

³For negative association with birth weight.

⁴Values are geometric means.

Figure 7.1: Percent body fat and plasma leptin concentration in 7-year-old children according to tertiles of birth weight and current weight ($n = 259$). Children with a lower weight at birth had a higher body fatness and plasma leptin concentration, especially in children with a high current weight. $P < 0,001$ for the associations of body fatness and plasma leptin with birth weight and current weight (adjusted for length of gestation, age, and sex by multiple regression analysis).

Infant nutrition

It is generally accepted that breast-feeding has important beneficial effects on the wellbeing of infants and children. In relation to later cardiovascular risk, however, the effect of breastfeeding is less clear. Both favorable and adverse influences on cardiovascular risk have been attributed to breastfeeding. Ravelli *et al.*³⁸ report higher mean LDL-cholesterol and lower mean HDL-cholesterol concentrations, a lower LDL/HDL ratio, and higher mean glucose concentrations after an oral glucose tolerance test in bottle-fed individuals when compared to exclusively breast-fed adults. The incidence of Type II diabetes mellitus is lower among Pima Indians who were breast-fed during infancy.^{39,40} Additionally, the prevalence of obesity is lower in breast-fed children.^{41,42} A clear dose-dependent effect of breastfeeding on the prevalence of childhood obesity was observed during some studies,^{41,43} but not during others.⁴⁴ On the other hand, Leeson and colleagues⁴⁵ reported that breastfeeding is associated with a lower arterial distensibility in early adult life. In addition, the lowest prevalence of obesity in 6-year-old children was found in association with the shortest period of breastfeeding in another study.⁴⁶ In the same study, the risk of overweight or obesity was greatest for those children who either never received breast-milk or those breast-fed for the longest period of time.⁴⁶

The in *Chapter 4 & 5* reported cardiovascular risk indicator levels did not differ between breast-fed children and formula-fed children (not shown). In addition, the phospholipid γ -linolenic acid and dihomo- γ -linolenic acid concentrations, of umbilical cord blood and of blood obtained at follow-up, did not differ between breast-fed children and formula-fed children. Interestingly, infant feeding mode did seem to have an influence on the associations found between triacylglycerol and the phospholipid concentrations of these fatty acids (Figure 7.2). The negative relation with γ -linolenic acid and dihomo- γ -linolenic acid measured at birth was more pronounced in children who were breast-fed during infancy. Whereas, the positive relation with γ -linolenic acid and dihomo- γ -linolenic acid measured at seven years of age was more pronounced in children who were formula-fed during infancy. However, the interaction terms of the multiple regression models did not reach statistical significance.

In contrast to breast milk, most of the commercially available formulas in the Netherlands do not contain γ -linolenic acid or dihomo- γ -linolenic acid.⁴⁷ Breast-fed children, therefore, have a higher γ -linolenic acid and dihomo- γ -linolenic acid availability during infancy than formula-fed children. It is tempting to conclude that this could explain observed differences between breast-fed children and formula-fed children. However, the involvement of other factors that differ between breast-fed children and formula-fed children can not be excluded. The mother's decision to breast-feed her child may depend on factors that could also have an influence on the cardiovascular risk profile of her offspring. For instance, maternal obesity has been shown to interfere with the initiation and duration of breastfeeding.^{48,49} Mothers of heavier babies may switch to bottle-feeding earlier than mothers of smaller infants

because of a different energy intake and suckling behavior of her child.^{48,50} The observed differences between breast-fed children and formula-fed children may also be accounted for by associated differences in social factors. In the present study, the information on infant nutrition was obtained seven years after birth. The results might have been biased by this fact (*i.e.* recall bias).

Limitations of the study

Study design

The study described in this thesis is in essence a non-concurrent prospective study (also called a historical cohort study or a retrospective cohort study).⁵¹ Historical data (essential fatty acid concentrations measured in umbilical cord blood shortly after birth) were related to prospectively measured outcome variables (cardiovascular risk indicators measured at age seven). The absence of a random allocation of the studied subjects is critical in the interpretation of the results obtained through a cohort study.^{51,52} The associations found may indicate causal relationships but the strength of evidence is less than when obtained by a well-designed randomized trial. Children with different levels of exposure for a particular fatty acid may also differ with respect to other (unknown) factors. The guarantee that children with different fatty acid concentrations at birth are otherwise comparable is namely lost by the absence of a random allocation.

Although the associations found did not attenuate after correction for several potential confounding factors, residual confounding can not be excluded. Thus, inherent to the design of our study we are left with the uncertainty that confounding factors could have caused the observed associations. Non-participation and non-response can introduce major biases that can complicate the interpretation of findings in cohort studies.⁵¹ If children are selectively lost to follow-up, the associations found may be the result of non-participation of a particular group of children. As shown in *Chapter 1* (Figure 1.3), only about 40% of the eligible children participated. The birth characteristics of these children, however, were not significantly different from the children who did not attend our clinic (Table 1.1). Moreover, the concentrations of γ -linolenic acid and dihomo- γ -linolenic acid in umbilical cord blood did not differ between participants and non-participants. Mean $\pm$ SD concentrations for participants and non-participants were: $0,05 \pm 0,04$ % wt/wt and $0,05 \pm 0,04$ % wt/wt for γ -linolenic acid and $5,15 \pm 0,88$ % wt/wt and $5,06 \pm 0,87$ % wt/wt for dihomo- γ -linolenic acid, respectively. These findings all seem to indicate that the investigated children comprised a representative sample of the entire cohort. Nevertheless, outcome variables could not be compared between participants and non-participants, and a residual bias due to a selective loss to follow-up can not be fully excluded.

We included all children for whom fatty acid concentrations at birth were known. This permitted the study of several candidate fatty acids in relation to the

outcome variables, which was an important advantage considering the explorative nature of our study. The risk of this approach is that due to multiple testing of associations some relations will appear statistically significant purely due to chance. The importance of such change findings may subsequently be overestimated.

Figure 7.2: Relations between fasting plasma triacylglycerol concentrations in 7-year-old children and plasma phospholipid γ -linolenic acid and dihomo- γ -linolenic acid concentrations in umbilical cord blood or in blood obtained at seven years of age according to infant feeding mode. Values are geometric means $\pm$ SE. P values are from linear regression models with log-triacylglycerol level as dependent variable and either γ -linolenic acid or dihomo- γ -linolenic acid concentration as the independent variable.

Table 7.2: Multiple regression analyses of log-transformed insulin concentration (pmol/L) in seven-year-old children with plasma phospholipid γ -linolenic acid concentrations, adjusted for the arachidonic acid to linoleic acid ratio.

	Coefficient ¹ (95% CI)	P
Model 1		
Arachidonic acid to linoleic acid ratio	-6.6 (-24.2; 11.0)	0.460
Model 2		
γ -Linolenic acid at birth (% wt/wt)	-5.7 (-9.6; -1.9)	0.004
γ -Linolenic acid at seven years (% wt/wt)	5.7 (3.2; 8.1)	<0.001
Arachidonic acid to linoleic acid ratio	-9.9 (-26.7; 6.9)	0.247

¹Coefficients are percentage changes in log-insulin concentration per unit change in predictor variables (Note that one unit change in γ -linolenic acid concentration equals 0.1 % wt/wt).

The fact that γ -linolenic acid concentration at birth related to several outcome variables (triacylglycerol, insulin, proinsulin, leptin, and body fatness), which are all components of the same so-called insulin resistance syndrome, and the fact that adjustments for potential confounding factors did not attenuate these associations, both seem to indicate that the associations found are more than just change findings.

The observed associations between γ -linolenic acid concentrations at birth and at seven years of age on one hand and the insulin resistance variables on the other hand could result from a 'mechanism' of reverse causality. Insulin has a positive influence on the activity of the enzyme delta-6-desaturase.⁵³ Delta-6-desaturase stimulates the conversion of linoleic acid (18:2n-6) into γ -linolenic acid (Figure 1.2). A higher plasma concentration of insulin could thus explain a higher γ -linolenic acid content of plasma lipid fractions. Conversely, low levels of circulating insulin could relate to lower γ -linolenic acid concentrations. Other factors with an influence on insulin metabolism (including genetic factors) could differ between infants with different γ -linolenic acid concentrations at birth and explain our observations. Our data may thus also fit the "fetal insulin hypothesis", which has been proposed as an alternative explanation for the associations between birth weight and adult cardiovascular diseases.⁵⁴ Interestingly, we observed an interaction between birth weight and a positive family history of Type II diabetes in relation to childhood insulin concentrations. Birth weight was negatively related to insulin concentrations only in children with a positive family history of diabetes (*Chapter 4*). Recently, common gene variants, that are associated with adult impaired glucose tolerance and Type II diabetes mellitus, have also been related to a low weight or ponderal index at birth.^{55,56} It could be of interest to study such polymorphisms in relation to γ -linolenic acid concentrations at birth.

In an attempt to test the hypothesis of reverse causality, the relation between γ -linolenic acid and insulin concentration was studied correcting for the arachidonic acid to linoleic acid ratio at age seven. Since delta-6-desaturase is considered to be the rate-limiting enzyme in the conversion of linoleic acid into arachidonic acid, this ratio indirectly reflects delta-6-desaturase activity. No statistically significant relation

between this ratio and insulin concentration was found. Further, the observed associations between insulin concentration and the γ -linolenic acid content of umbilical cord blood and blood obtained at seven years of age were not attenuated when corrected for the arachidonic acid to linoleic acid ratio (Table 7.2). The arachidonic acid to linoleic acid ratio determined at birth was not related to the insulin concentrations at age seven either ($P = 0.21$). These observations do not support the idea of reverse causality. A small fraction of umbilical cord plasma is still available for some of the children in our cohort. Additional clues might be found by studying insulin concentrations in these samples. If a positive relation between umbilical plasma insulin levels and γ -linolenic acid concentration is not found, such a mechanism of reverse causality seems less likely.

Use of plasma phospholipid fatty acid concentrations

In the studies described in *Chapters 3, 4 and 5*, we used essential fatty acid concentrations (*i.e.* expressed as % by wt of total fatty acids) measured in the umbilical cord plasma phospholipid fraction as a measure for the availability of these fatty acids during fetal life. Fatty acid concentrations in maternal plasma lipids measured during pregnancy are known to be strong predictors of fatty acid concentrations in umbilical plasma (*Chapter 2*).^{23,57} Both, maternal and umbilical fatty acid concentrations, are influenced by the maternal dietary intake of fatty acids (Table 2.6).⁵⁸⁻⁶² Additionally, umbilical fatty acid concentrations measured after delivery, as in the present study, are essentially similar to concentrations found in fetal plasma when measured at a comparable gestational age.⁶³ For these reasons, we assume that umbilical cord blood fatty acid concentrations are a reasonable reflection of the intrauterine fatty acid supply. The turnover of fatty acids in plasma phospholipids is, however, relatively fast. Therefore, fatty acid concentrations in umbilical plasma phospholipids probably only reflect the availability of essential fatty acids during late gestation. This may be of relevance since exposure to the Dutch famine during early gestation, rather than during late gestation, has been associated to adult plasma cholesterol and lipoprotein concentrations.⁶⁴ It could explain why we did not find a relation between umbilical plasma fatty acid concentrations and childhood cholesterol and lipoprotein levels. Other results might have been obtained when erythrocyte membrane or umbilical vascular wall tissue fatty acid concentrations were used as a proxy for essential fatty acid availability during fetal life. Since these data are available for a subset of our study population, such a study could easily be performed in the near future.

Fatty acid concentrations can be expressed as an absolute concentration (in mg/L) or as a relative concentration (% by wt of total fatty acids), as in *Chapter 2*. The use of each expression has its own particular limitations. Absolute concentrations are more dependent on differences in analytical procedures such as the time-point at which the internal standard is added to the samples.⁶⁵ When compared to relative concentrations, the comparability of absolute concentrations between laboratories is

smaller, and the measurement errors are, usually, larger. Another important limitation of absolute concentrations is their dependence on the total lipid pool size. The absolute concentration of individual fatty acids will be higher in a subject with a higher total amount of phospholipids in his blood. For this reason, absolute concentrations of individual fatty acids can not be used for the study of associations with cardiovascular risk indicators that could relate to the total phospholipid pool size (like plasma lipid concentrations).

As shown in Figure 7.3, the total amount of phospholipid associated fatty acids (which reflects the phospholipid pool size) is strongly related to plasma cholesterol concentration. For these reasons, relative concentrations were used during the studies described in *Chapters 3, 4 and 5*. The use of relative concentrations, however, has important limitations as well.⁶⁶ The individual fatty acid concentrations measured are not obtained from independent observations, this is especially true for relative concentrations of the more abundant fatty acids. An increase in one proportion relates with a decrease in some other proportion, because the sum of all fatty acids is 100%. As a result, associations between individual fatty acid concentrations can be found. This problem of interdependency between fatty acid concentrations is not unique to relative fatty acid concentrations; a similar problem would arise when absolute concentrations are used. Since the amount of phospholipid associated fatty acids might be higher in one person compared to another because of a larger phospholipid pool, positive relations between absolute fatty acid concentrations can just be found as a result of the differences in phospholipid pool sizes. These methodological problems, inherent to studies in which fatty acid concentrations are related to one another, are hard to overcome. The artificial effects created are superimposed on the true biological relations that could be present. Therefore, a careful interpretation is needed when studying relations between individual fatty acid concentrations measured in the same matrix.

Use of cardiovascular risk indicators as outcome variables

Major cardiovascular events are rare in children. Therefore, the risk for the development of cardiovascular diseases had to be studied indirectly, using cardiovascular risk indicators. Evidence from autopsy studies indicate that the development of atherosclerosis starts during childhood, and that childhood cardiovascular risk indicator levels are related to the extent of the atherosclerotic lesions present. Moreover, children tend to maintain their ranking order from childhood into adulthood with respect to several cardiovascular risk indicators; a phenomenon referred to as "tracking" (for references see *Chapter 1*). These findings imply that the evaluation of cardiovascular risk indicators in children is a useful tool in identifying groups of individuals who are at a potential risk for developing cardiovascular diseases later in life. An important question that remains is whether the differences in risk indicator levels found in children are clinically relevant. Individual risk indicator levels

measured during childhood may not relate to true cardiovascular events in adulthood. Whether fatty streaks seen in arteries of children are related to adult cardiovascular disease is not known. At this point, childhood cardiovascular risk indicators (like childhood cholesterol) have been associated with adult risk indicators (like adult cholesterol), but not with true endpoints like coronary heart disease morbidity or mortality.⁶⁸ Moreover, it is not known whether interventions started early in life, and designed to reduce cardiovascular risk indicator levels in children, are safe and equally effective in the prevention of cardiovascular events than interventions started in adulthood.⁶⁸

Figure 7.3: Relation between plasma total cholesterol concentration and the total fatty acid content of plasma phospholipids in 7-year-old children ($P < 0,0001$; $r = 0,75$; $n = 259$).

Many children with higher risk indicator levels will have normal levels in adulthood even in the absence of prescribed individual interventions.⁶⁷ Risk indicator level screening could thus unjustly label children as patients with a "disease", causing unjustified anxiety and stigmatization. Therefore, the measurement of cardiovascular risk indicators in children should not be used as a universal screening tool to identify individuals with an increased risk and to select them for individual interventions, like

drug therapy.^{68,69} This does not mean that lowering risk indicator levels in children could not have beneficial effects. Based on the indirect evidence available, selective screening and the subsequent treatment of children who are at the highest risk (e.g. children from families with hypercholesterolemia, children with diabetes, or obese children) is recommendable.^{69,70} For other children, a population-wide approach (e.g. improving dietary composition, increasing physical activity, discouraging cigarette smoking, and avoiding excessive weight gain) is considered a more appropriate strategy to lower the risk for cardiovascular diseases later in life.^{69,70} An advantage of the latter approach is that even a relatively small reduction of population-average risk indicator levels in childhood could substantially decrease the incidence of coronary heart disease, when carried into adulthood.⁶⁹ In this light, small differences in risk indicator levels found between groups of children could be clinically relevant indeed.

Critique on studies on the fetal origins hypothesis

The fetal origins hypothesis is important because of the potential impact on public health it might have. One of the biggest problems, however, is that it seems impossible to provide conclusive evidence. To test the hypothesis, investigators used retrospective studies, based on existing data sets and natural occurring events, like the Dutch Hunger Winter. As a consequence, the associations found could be biased and may not indicate causal relationships. Therefore, it is not astonishing, that the most important points of critique on studies on the fetal origins hypothesis are the possibility of selection bias and confounding, for instance by socioeconomic status.⁷¹⁻⁷³ Other major concerns are the inconsistencies reported within and across the studies and the considerable broadening of the hypothesis.⁷⁴ The fetal origins hypothesis extended from cardiovascular diseases and related disorders to stroke, atopy, cancer, osteoporosis, depression, and aging. A more precise expression of the hypothesis tested, and adherence to that expression has therefore been suggested.⁷⁴

When studying the relation between potential cause and subsequent events, which are separated by decades, losses to follow-up are almost inevitable. Indications for the presence of a selection bias can be found, e.g. by comparing participants with non-participants, but a selection bias can never be completely excluded. The use of a high quality data set, and an almost complete follow-up,⁷⁶ may overcome this problem of selection but does not exclude the possibility of confounding. And although recent studies indicate that the association between size at birth and adult cardiovascular risk is not simply explained by differences in socio-economic background,^{76,77} confounding by other unknown factors can not be excluded. However, to conduct randomized trials – which may provide the stronger evidence needed – one would first have to know which intervention might have the desirable effect.

The study described in this thesis was explorative in nature, therefore, the hypothesis was not precisely expressed; a selection bias might have occurred, and

confounding can not be fully excluded. Thus, many of the above mentioned points of critique are applicable to the study described in this thesis as well. But this does not mean we should simply disparage the results obtained. The shortcomings mentioned not necessarily lead to wrong conclusions.⁷⁸ A bias, when present, may be small or irrelevant. Furthermore, the findings of the present study allow many points of critique to be addressed during future studies. A more precise hypothesis can now be generated and tested. Knowing a candidate nutritional factor that could be involved, intervention studies using a random allocation of studied subjects can be conducted, which will avoid many methodological problems and reduce the possibility of confounding. Therefore, despite the reservations discussed above, there seems to be sufficient evidence to justify further scrutiny.

Interpretations and recommendations for future studies

From the study described in the present thesis it can be concluded that concentrations of essential fatty acids, measured in umbilical cord blood, are related to several cardiovascular risk indicators determined in children at seven years of age. Based on the findings described, it seems that a lower availability of γ -linolenic acid (and/or its direct elongation product dihomo- γ -linolenic acid) during fetal life is related to a less favorable risk profile. Increasing the maternal intake of γ -linolenic acid during pregnancy is expected to enhance the fetal availability of both γ -linolenic acid and dihomo- γ -linolenic acid. Our findings therefore suggest that γ -linolenic acid supplementation in pregnant women may have beneficial effects on the risk profile of her offspring. In addition, some observations indicate that an increased γ -linolenic acid availability during infancy may have an additive effect (see above). The positive associations found at age seven, on the other hand, seem to suggest that a lower intake of this fatty acid is recommendable later in life. Because of the many limitations mentioned before, our study does not provide sufficient evidence to fully support these hypotheses. As stated above, other mechanisms could explain our findings as well. At this point it would be premature to actually recommend a higher intake of this fatty acid to pregnant women or infants. However, the relations described do indicate that γ -linolenic acid is an interesting nutrient in light of the fetal origins hypothesis, and thus for further scrutiny.

Ideally, the potential effect and safety of an increased availability of γ -linolenic acid during fetal life is studied using randomized supplementation trials during pregnancy. As a first step, the efficacy of γ -linolenic acid supplementation could be tested by short-term animal experiments. Another option could be to recruit children from previous randomized trials. Intervention studies have been performed to analyze the effects of γ -linolenic acid supplementation on the development of atopic diseases in children. The measurement of cardiovascular risk indicators, especially components of the insulin resistance syndrome, in those children allows comparisons between

randomized groups within a short time window. New studies are also needed to investigate the effects of γ -linolenic acid supplementation in pregnant women and infant formula. For a truly prospective test, these studies would need a long follow-up period. Because of the relatively small effects found, the tested population should also be large enough to accurately estimate population-wide benefits. If studies like these confirm that a higher availability of γ -linolenic acid during fetal life and maybe infancy, relates to a more favorable risk profile later in life, population-wide supplementation of γ -linolenic acid during pregnancy and infancy could be a relatively simple new tool in the prevention of cardiovascular diseases.

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Abbreviations | Fatty acid nomenclature and list of units and abbreviations

18:2n-6	linoleic acid
18:3n-3	α -linolenic acid
18:3n-6	γ -linolenic acid
20:3n-6	dihomo- γ -linolenic acid
20:3n-9	eicosatrienoic acid or Mead acid
20:4n-6	arachidonic acid
20:5n-3	eicosapentaenoic acid or timnodonic acid
22:4n-6	docosatetraenoic acid or adrenic acid
22:5n-3	docosapentaenoic acid or clupanodonic acid
22:5n-6	docosapentaenoic acid or Osbond acid
22:6n-3	docosahexaenoic acid or cervonic acid

AA	arachidonic acid
AGA	appropriate for gestational age
ANOVA	analysis of variance
Apo	apolipoprotein
β	regression coefficient
BMI	body mass index
$^{\circ}\text{C}$	degree Celsius
CETP	cholesteryl ester transfer protein
CI	confidence interval
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EFA	essential fatty acid
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
F	female
FAME	fatty acid methyl ester
FFM	fat-free mass
FM	fat mass
g	gram
GA	gestational age
GC	gas chromatography

Abbreviations

h	hour
HDL	high-density lipoprotein
HOMA	homeostasis model assessment
IQR	inter quartile range
L	liter
LCP	long-chain polyene
LC-PUFA	long-chain polyunsaturated fatty acid
LDL	low-density lipoprotein
LGA	large for gestational age
Lp(a)	lipoprotein(a)
M	male
m	meter
min	minute
mol	mole
MUFA	monounsaturated fatty acid
<i>n</i>	number of subjects
ND	not detected
NIDDM	non-insulin-dependent diabetes mellitus
NS	not statistically significant
<i>P</i>	probability value
PCR	polymerase chain reaction
PL	phospholipid
PUFA	polyunsaturated fatty acid
<i>r</i>	correlation coefficient
RIA	radio-immunoassay
SD	standard deviation
SE	standard error
SFA	saturated fatty acid
SGA	small for gestational age
TG	triacylglycerol
VLDL	very low-density lipoprotein
wk	week
wt	weight
y	year

Summary |

**Polyunsaturated fatty acids
and the fetal origins hypothesis**

Cardiovascular diseases are the most important cause of death in the Dutch population. Although the mortality rates for cardiovascular diseases are declining, they remain a substantial public health problem. Previous research has shown that multiple factors play a role in the origins of cardiovascular diseases. For instance, it has become clear that hypertension, diabetes, hypercholesterolemia, and obesity increase the risk for cardiovascular diseases. Besides genetic factors, environmental factors like physical activity, smoking and nutrition play a role in the etiology. Usually, the symptoms of cardiovascular diseases occur for the first time during adulthood. In (young) children, however, early stages of the atherosclerotic process can be found during autopsy studies. Additionally, it is known that many children tend to maintain their ranking order with respect to several cardiovascular risk indicators from childhood into adulthood. Such observations support the idea that a combination of factors, acting since childhood, contributes to the origins of cardiovascular diseases.

In the past twenty years or so, research findings suggest that factors active during the short period of life before birth may increase the risk for adult cardiovascular diseases. Epidemiological studies in different populations have shown that a low birth weight is associated with an increased coronary heart disease mortality. Subjects with a low birth weight are also more likely to suffer from diabetes, hypertension, or dyslipidemia. Associations like these have led to the formulation of the fetal origins hypothesis. According to this hypothesis, the risk for chronic diseases in adult life is in part determined by the availability of nutrients during the fetal period. It is believed that this is a consequence of permanent physiological and structural adaptations of the body. The mechanisms involved are hardly understood, but results from animal experiments and observations in subjects exposed to the Dutch famine during the winter of 1944 – 1945 have supported the suggestion that fetal nutrient availability is important. Only a very small number of human studies have been conducted to identify the specific nutritional factors involved. The primary aim of the study described in the present thesis was to explore the potential involvement of prenatal essential fatty acid availability.

Essential fatty acids belong to the long-chain polyunsaturated fatty acids. Strictly speaking, only linoleic acid and alpha-linolenic acid are essential fatty acids. Because the human body lacks the required enzymes needed for the synthesis of linoleic acid and alpha-linolenic acid, these fatty acids have to be obtained through the diet. This means that a fetus has to receive these nutrients from the mother by placental transfer. Once obtained, linoleic acid and alpha-linolenic acid can be converted into other long-chain polyunsaturated fatty acids, like gamma-linolenic acid, dihomo-gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid, or docosahexaenoic acid. Because the fetal capacity for these conversions seems rather low, these products are also considered as essential fatty acids. Essential fatty acids have many biological functions. They are not just a source of energy, but are also active mediators of physiological processes and regulators of gene expression. They may conduct such functions directly

or indirectly. Observations from previous studies suggest that fetal essential fatty acid availability is limited. Furthermore, essential fatty acids seem to have an influence on the intrauterine growth of the fetus. This renders them as attractive candidate nutrients in relation to the fetal origins hypothesis.

To investigate the relation between fetal essential fatty acid availability and later cardiovascular disease risk, a cohort of children was studied at seven years of age. The mothers of these children had previously participated in a study on maternal and fetal essential fatty acid status and pregnancy outcome. From the children of this cohort the essential fatty acid composition of umbilical cord plasma phospholipids was known. The fatty acid composition of cord plasma phospholipids can be used as an indicator of fetal essential fatty acid availability. In total, 297 of the eligible 750 children participated in the follow-up study on cardiovascular risk indicators. More information on the rationale for the study, design of the study, and participation is given in general introduction of this thesis (*Chapter 1*).

In *Chapter 2*, the means and distributions are reported for the fatty acid concentrations measured in the phospholipid fraction of cord plasma and maternal plasma at the time of birth. As described previously, the absolute fatty acid concentrations (expressed in mg/L) were significantly higher in maternal plasma than in umbilical cord plasma. In addition we observed that boys had lower absolute fatty acid concentrations than girls had. Both observations are probably the result of a difference in the size of the phospholipid pool. The relative fatty acid concentrations (expressed in % of total fatty acids) did not differ between boys and girls. The well known differences in relative fatty acid concentrations were observed between mothers and children, namely a higher content of linoleic acid and alpha-linolenic acid and lower concentrations of the derived products dihomo-gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid in mothers. The relative concentration of docosahexaenoic acid in umbilical cord plasma was related to the duration of pregnancy and maternal dietary linoleic acid intake. Mothers with a higher intake of linoleic acid during pregnancy more frequently had children with a lower docosahexaenoic acid concentration appropriate for gestational age than mothers with a lower linoleic acid intake.

In order to investigate the relation between the fetal essential fatty acid availability and later cardiovascular disease risk, we first studied the association between birth weight and relative essential fatty acid concentrations in umbilical cord plasma (*Chapter 3*). Results from previous studies in premature infants and low-birth-weight neonates (weight < 2500 gram) indicated that the overall biochemical essential fatty acid status of a child is positively related to the weight attained at birth. In our study however, heavier infants had a lower biochemical essential fatty acid status than lighter infants. Dihomo-gamma-linolenic acid was the only essential fatty acid positively related to birth weight (dihomo-gamma-linolenic acid is also positively

related to size at birth in premature infants and low-birth-weight neonates). In addition, we observed a decline in maternal concentrations of n-6 and n-3 long-chain polyunsaturated fatty acids (*e.g.* arachidonic acid and docosahexaenoic acid) during pregnancy. This decrease was most pronounced in mothers of heavier infants. Observations like these seem to suggest that more fatty acids are transported from the mother to the fetus via the placenta when the fetus is relatively large. Because the biochemical essential fatty acid status is lower in heavier infants compared to lighter infants, the capacity for the maternal-to-fetal delivery of essential fatty acids seems limited. Based on the results of this study, it seems less likely that the general biochemical essential fatty acid status at birth is negatively related to the cardiovascular risk profile at seven years of age. For an adverse risk profile is related to a low weight at birth. However, the involvement of the availability of individual fatty acids (like dihomo-gamma-linolenic acid) or a relation with essential fatty acid status independent of birth weight could still be important.

Chapter 4 and *Chapter 5* describe the relations between essential fatty acid concentrations in umbilical cord plasma and several cardiovascular risk indicators determined at age seven. A relation was found with the concentrations of two essential fatty acids. A higher concentration of gamma-linolenic acid and dihomo-gamma-linolenic acid in umbilical cord plasma was associated with lower fasting concentrations of triacylglycerol and insulin at seven years of age. A higher concentration of gamma-linolenic acid was also related to lower levels of proinsulin and leptin, and to a lower percentage of body fat. The concentration of gamma-linolenic acid and dihomo-gamma-linolenic acid in umbilical cord plasma were both negatively related to the homeostasis model assessment (HOMA) index for insulin resistance. Together these findings suggest that the fetal availability of gamma-linolenic acid and/or dihomo-gamma-linolenic acid could be of importance in the development of Syndrome X (also called the metabolic syndrome or the insulin resistance syndrome). An implication of this might be that a higher maternal intake of these fatty acids during pregnancy could lower the risk of insulin resistance in her offspring.

We did not find associations between umbilical cord plasma concentrations of gamma-linolenic acid and dihomo-gamma-linolenic acid and plasma concentrations of total cholesterol, LDL cholesterol, HDL cholesterol, apolipoprotein A, apolipoprotein B, and lipoprotein(a) at seven years of age. Umbilical cord plasma concentrations of linoleic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid were not related to the measured risk indicators for cardiovascular diseases (*Chapters 4 & 5*). Markers for the general essential fatty acid status (*e.g.* Mead acid or the essential fatty acid status index) did not relate to the cardiovascular risk indicators either. It seems therefore that the availability of individual fatty acids (in particular gamma-linolenic acid and dihomo-gamma-linolenic acid) is more important than the essential fatty acid status at birth in general.

A relation between the fetal availability of essential fatty acids and the later risk for cardiovascular diseases could be influenced by genetic differences between subjects. For this reason, two common gene variations were studied, *i.e.* the *Taq* IB polymorphism in the cholesteryl ester transfer protein (CETP) gene and the apolipoprotein E polymorphism. Both polymorphisms, however, proved not to be informative in relation to this hypothesis. We did observe the expected associations with plasma concentrations of lipids and lipoproteins. In addition, an interaction between these genetic variants was seen (see *Chapter 6*).

The limitations of the study are discussed in detail in the general discussion section of this thesis (*Chapter 7*). Discussed are the use of plasma phospholipid fatty acid concentrations as a proxy for essential fatty acid availability, the potential consequences of the low participation rate, the possibility of confounding, and alternative hypothesis to explain some of our findings. In light of these limitations a serious recommendation can not be given regarding the maternal intake of gamma-linolenic acid and dihomo-gamma-linolenic acid during pregnancy. Preferably, the effect of an increased maternal intake of these fatty acids during pregnancy on the cardiovascular risk profile of the next generation is tested during future, more controlled, randomized trials. In this way, it could also become clear whether the associations found reflect a causative relation indeed.

**Samenvatting | Meervoudig onverzadigde vetzuren
en de *fetal origins* hypothese**

Hart- en vaatziekten vormen de belangrijkste oorzaak van overlijden in de Nederlandse bevolking. Hoewel de sterfte aan coronaire hartziekten daalt, blijven deze aandoeningen een aanzienlijk volksgezondheidsprobleem. Uit eerder onderzoek is gebleken dat vele factoren een rol spelen bij het ontstaan van hart- en vaatziekten. Zo is duidelijk geworden dat o.a. hypertensie, diabetes, hypercholesterolemie en obesitas het risico op hart- en vaatziekten verhogen. Naast erfelijke factoren spelen ook omgevingsfactoren zoals lichamelijke activiteit, roken en voeding een rol bij de etiologie. De klachten en symptomen van hart- en vaatziekten komen meestal pas op volwassen leeftijd tot uiting. Echter op de (vroege) kinderleeftijd kunnen bij obductie reeds de eerste tekenen van atherosclerose worden waargenomen. Daarnaast is bekend dat veel kinderen hun rangorde ten aanzien van verschillende risico-indicatoren voor hart- en vaatziekten handhaven tot op volwassen leeftijd. Dergelijke bevindingen ondersteunen het idee dat een combinatie van factoren al vanaf de kinderleeftijd bijdraagt aan het ontstaan van hart- en vaatziekten.

Gedurende de laatste twee decennia zijn er aanwijzingen gevonden dat ook factoren actief in de periode voor de geboorte het risico voor hart- en vaatziekten op volwassen leeftijd kunnen verhogen. Epidemiologische studies in verschillende populaties hebben laten zien dat mensen met een laag geboortegewicht op latere leeftijd een grotere kans hebben om te overlijden aan de gevolgen van coronaire hartziekten. Ook hebben zij vaker last van diabetes, hypertensie en hypercholesterolemie. De gevonden associaties hebben geleid tot de formulering van de "fetal origins" hypothese. Volgens deze hypothese bepaalt de beschikbaarheid van voeding tijdens de foetale periode mede het risico op chronische aandoeningen in het latere leven. Dit zou het gevolg zijn van permanente structurele en fysiologische aanpassingen in het lichaam. Welke mechanismen hierbij een rol spelen is nog nauwelijks bekend, maar dat de beschikbaarheid van voedingsstoffen tijdens de foetale periode inderdaad belangrijk kan zijn, werd ondersteund met behulp van dierexperimentele studies en observaties bij mensen die voor hun geboorte waren blootgesteld aan de hongerwinter in 1944 – 1945. Welke specifieke voedingsfactoren hierbij een rol spelen is tot nu toe nog nauwelijks bestudeerd. Het onderzoek beschreven in dit proefschrift had als hoofddoel het verkennen van een mogelijke betrokkenheid van de prenatale beschikbaarheid van essentiële vetzuren.

Essentiële vetzuren behoren tot de lange-keten meervoudig onverzadigde vetzuren. In strikte zin zijn alleen linolzuur en alfa-linoleenzuur essentiële vetzuren. Door het ontbreken van de benodigde enzymen kunnen linolzuur en alfa-linoleenzuur niet door het lichaam worden gevormd, daarom moeten zij via de voeding worden opgenomen. Dit betekent dat een foetus deze voedingsstoffen van de moeder moet krijgen via transport door de placenta. Eenmaal in het lichaam kunnen linolzuur en alfa-linoleenzuur worden omgezet in andere lange-keten meervoudig onverzadigde vetzuren, zoals gamma-linoleenzuur, dihomogamma-linoleenzuur, arachidonzuur, eicosapentaen- en docosahexaen- zuur. Omdat de foetale capaciteit voor deze

omzettingen beperkt lijkt te zijn, worden deze vetzuren vaak ook tot de essentiële vetzuren gerekend. Essentiële vetzuren vervullen een groot aantal biologische functies. Zij vormen niet alleen een bron van energie, maar reguleren direct of indirect ook fysiologische processen en beïnvloeden de expressie van genen. Eerder onderzoek doet vermoeden dat de foetale beschikbaarheid van essentiële vetzuren beperkt is. Ook zouden essentiële vetzuren een invloed hebben op de intra-uteriene groei van de foetus. Dit maakt dat zij interessante kandidaat-voedingsstoffen zijn in relatie tot de fetal origins hypothese.

Om het verband tussen de foetale beschikbaarheid van essentiële vetzuren en het latere risico op hart- en vaatziekten te bestuderen, werd een cohort kinderen op 7-jarige leeftijd onderzocht. De moeders van deze kinderen hadden eerder deelgenomen aan een onderzoek naar de maternale en neonatale vetzuurstatus en de zwangerschapsuitkomst. Van de kinderen in dit cohort was bij de geboorte de essentiële vetzuursamenstelling van fosfolipiden in navelstrengplasma bekend. Deze vetzuursamenstelling van fosfolipiden kan worden gebruikt als een indicator voor de foetale beschikbaarheid van essentiële vetzuren. Van de oorspronkelijk 750 kinderen in het cohort namen er uiteindelijk 297 deel aan het vervolgonderzoek naar risico-indicatoren voor hart- en vaatziekten. Meer informatie over de achtergronden van deze studie, de studieopzet en de participatie is gegeven in de algemene inleiding van dit proefschrift (*Hoofdstuk 1*).

In *Hoofdstuk 2* worden gemiddelden en spreiding van de gemeten vetzuurconcentraties in fosfolipiden van navelstrengplasma en moederlijk plasma ten tijde van de bevalling gerapporteerd. Zoals eerder al beschreven is, waren de absolute concentraties (uitgedrukt in mg/L) van de vetzuren hoger bij de moeders dan bij hun kinderen. Verder zagen we dat jongens iets lagere absolute vetzuurconcentraties hadden dan meisjes. Beide observaties worden waarschijnlijk verklaard door een verschil in de grootte van de fosfolipidenpool waarin gemeten is. De relatieve vetzuurwaarden (uitgedrukt in % van de totale hoeveelheid vetzuren) verschilden niet tussen jongens en meisjes. Tussen moeders en kinderen werden de bekende verschillen in relatieve vetzuurconcentraties waargenomen, namelijk een hogere concentratie van linolzuur en alfa-linoleenzuur en lagere concentraties van de afgeleide vetzuren dihomogamma-linoleenzuur, arachidonzuur, eicosapentaeenzuur, en docosahexaeenzuur bij moeders. De relatieve concentratie van docosahexaeenzuur in navelstreng plasma was afhankelijk van de duur van de zwangerschap alsmede de moederlijke inname van linolzuur. Moeders met een hogere inname van linolzuur in de zwangerschap hadden vaker een kind met een lagere docosahexaeenzuurconcentratie in navelstrengplasma voor de duur van de zwangerschap dan moeders met een lagere linolzuurinname.

Om het verband tussen de foetale beschikbaarheid van essentiële vetzuren en het latere risico op hart- en vaatziekten te bestuderen, werd in eerste instantie gekeken naar de relatie tussen de relatieve essentiële vetzuurconcentraties in navelstrengplasma en het geboortegewicht (*Hoofdstuk 3*). Resultaten van eerdere studies bij prematuur

geboren kinderen en kinderen met een laag geboortegewicht (< 2500 gram) deden vermoeden dat in het algemeen de biochemische essentiële vetzuurstatus positief gerelateerd is met het geboortegewicht van een kind. Wij vonden in ons cohort van à term geboren kinderen echter dat zwaardere kinderen bij geboorte een lagere biochemische essentiële vetzuurstatus hadden dan lichtere kinderen. Het enige essentiële vetzuur dat een positieve associatie had met geboortegewicht was dihomogamma-linoleenzuur (dihomo-gamma-linoleenzuur is ook bij prematuur geboren kinderen positief gerelateerd aan het geboortegewicht). Verder zagen we dat de concentraties van n-6 en n-3 lange-keten meervoudig onverzadigde vetzuren (zoals arachidonzuur en docosahexaeenzuur) in moederlijk plasma daalden gedurende de zwangerschap. Deze daling was sterker bij moeders van zwaardere baby's. Deze observaties suggereren dat er meer essentiële vetzuren van moeder naar kind worden getransporteerd via de placenta als het kind relatief zwaarder is. Omdat de biochemische vetzuurstatus van zwaardere kinderen lager is dan die van lichtere kinderen, lijkt de capaciteit voor deze aanvoer beperkt te zijn. De resultaten van deze studie maken het minder waarschijnlijk dat de biochemische essentiële vetzuurstatus in het algemeen negatief gerelateerd zou zijn aan het risicoprofiel voor hart- en vaatziekten op 7-jarige leeftijd. Een ongunstig risicoprofiel is immers gerelateerd aan een laag geboortegewicht. Echter de betrokkenheid van de beschikbaarheid van individuele vetzuren (zoals dihomogamma-linoleenzuur) of een relatie met essentiële vetzuren onafhankelijk van geboortegewicht behoren nog steeds tot de mogelijkheden.

Hoofdstuk 4 en Hoofdstuk 5 beschrijven de relaties tussen essentiële vetzuurconcentraties in navelstrengplasma en verschillende risico-indicatoren voor hart- en vaatziekten gemeten op 7-jarige leeftijd. Een associatie met de concentraties van twee essentiële vetzuren werd gevonden. Een hoger gehalte aan gamma-linoleenzuur en dihomogamma-linoleenzuur in navelstrengplasma relateerde aan een lager nuchter gemeten plasma concentratie van triacylglycerol en insuline op 7-jarige leeftijd. Een hogere concentratie van gamma-linoleenzuur was ook gerelateerd aan een lagere concentratie pro-insuline en leptine, en een lager lichaamsvetpercentage. De concentraties van gamma-linoleenzuur en dihomogamma-linoleenzuur in navelstrengplasma waren beiden negatief gerelateerd aan de "homeostasis model assesment" (HOMA) index voor insulineresistentie. Samen suggereren deze bevindingen dat de foetale beschikbaarheid van gamma-linoleenzuur en/of dihomogamma-linoleenzuur van belang zou zijn voor de latere ontwikkeling van "Syndrome X" (ook wel "the metabolic syndrome" of "the insulin resistance syndrome" genoemd). Een implicatie hiervan kan zijn dat een hogere inname van deze vetzuren bij zwangere vrouwen het latere risico op insulineresistentie bij hun kinderen zou kunnen verlagen.

Er werden geen relaties gevonden tussen de navelstrengconcentraties van gamma-linoleenzuur en dihomogamma-linoleenzuur en de plasma concentraties van totaal cholesterol, LDL-cholesterol, HDL-cholesterol, apolipoproteïne A, apolipoproteïne B en lipoproteïne(a) op 7-jarige leeftijd. De navelstreng-

plasmaconcentraties van linolzuur, arachidonzuur, eicosapentaeenzuur en docosahexaeenzuur waren niet gerelateerd aan de gemeten risico-indicatoren voor hart- en vaatziekten op 7-jarige leeftijd (*Hoofdstukken 4 & 5*). Ook markers voor een algemeen lagere biochemische essentiële vetzuurstatus bij geboorte (zoals Meadzuur en de essentiële vetzuurstatusindex) waren niet gerelateerd aan risico-indicatoren gemeten op 7-jarige leeftijd. Het lijkt daarom dat de beschikbaarheid van individuele vetzuren (in het bijzonder gamma-linoleenzuur en dihomogamma-linoleenzuur) belangrijker is dan de algemene biochemische essentiële vetzuurstatus bij geboorte.

Een verband tussen de foetale beschikbaarheid van essentiële vetzuren en het latere risico op hart- en vaatziekten zou afhankelijk kunnen zijn van genetische verschillen tussen personen. Om deze reden werden ook twee veelvoorkomende genetische variaties bestudeerd, namelijk het *Taq* IB polymorfisme in het gen dat codeert voor het cholesterylester transfer proteïne (CETP) en het polymorfisme in het apolipoproteïne E gen. Beiden bleken echter niet informatief voor de bovenstaande vraagstelling. Wel vonden we de te verwachten associaties met plasmaconcentraties van lipiden en lipoproteïnes op 7-jarige leeftijd en zagen we een interactie tussen deze genetische varianten (zie *Hoofdstuk 6*).

In *Hoofdstuk 7* worden de verschillende beperkingen van het onderzoek in detail besproken. Er wordt ondermeer ingegaan op het gebruik van vetzuurconcentraties in plasma fosfolipiden als maat voor de beschikbaarheid van essentiële vetzuren, de potentiële gevolgen van de beperkte deelname aan het onderzoek, een mogelijke vertekening van de resultaten en alternatieve verklaringen voor onze bevindingen. Met het oog op deze beperkingen, is een serieuze aanbeveling over de maternale inname van gamma-linoleenzuur en dihomogamma-linoleenzuur gedurende de zwangerschap nog niet te geven. Bij voorkeur zouden de effecten van een verhoogde inname van deze vetzuren tijdens de zwangerschap op het risicoprofiel voor hart- en vaatziekten in de volgende generatie in toekomstige, meer gecontroleerde, studies moeten worden onderzocht. Op deze manier zou ook meer duidelijkheid verschaft kunnen worden ten aanzien van de vraag of de gevonden associaties inderdaad een causaal verband weergeven.

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Curriculum Vitae |

Patrick Rump was born in Sliedrecht on September 25th in 1967. After completing high school (HAVO) at St. Canisius in Almelo in 1985, and college (VWO) at Pius X College in Almelo in 1987, he studied Medicine and Biology at the University of Nijmegen. As a student he performed studies on in situ fibre-optic oxygen measurement, on ion transport mechanisms in cell volume regulation, and on cellular magnesium homeostasis, at the Departments of Physiology and Cell Physiology of the Faculty of Medicine, and at the Department of Animal Physiology of the Faculty of Science, respectively. In addition, he worked on a research project involving the PRimed In Situ (PRINS) labelling technique for metaphase chromosomes at the Max-Planck Institute for Molecular Genetics in Berlin. He has a Master of Science degree in Biology from the University of Nijmegen (received in 1995) and finished his medical training at St. Radboud University Hospital in Nijmegen in 1996. During the following years he worked as a PhD-fellow for the Department of Human Biology at the Nutrition and Toxicology Research Institute Maastricht (NUTRIM) of the University of Maastricht. The results of the research performed during this period are for the greater part described in the present thesis. Patrick Rump is currently working as a resident in Clinical Genetics at the Department of Clinical Genetics of the Academic Hospital in Groningen.

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Appendices | Maternal polyunsaturated fatty acid concentrations during the first, second, and third trimester of pregnancy | A, B & C

Polyunsaturated fatty acid concentrations in seven-year-old boys and girls | D & E

Anthropometric characteristics and cardiovascular risk indicator levels in seven-year-old boys and girls | F

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Appendix A: The polyunsaturated fatty acid composition of maternal plasma phospholipids during the first trimester of pregnancy ($n = 837$).¹

	mg/L							% wt/wt						
	Mean	SD	Percentile					Mean	SD	Percentile				
			10	25	50	75	90			10	25	50	75	90
Fatty acid														
18:2n-6	283.22	58.17	214.20	241.92	277.90	317.01	357.58	21.39	2.65	18.00	19.67	21.27	23.16	24.90
18:3n-6	0.51	0.42	0.00	0.27	0.43	0.67	1.02	0.04	0.03	0.00	0.02	0.03	0.05	0.07
20:2n-6	6.17	1.79	3.92	4.88	6.00	7.33	8.52	0.46	0.09	0.35	0.40	0.45	0.51	0.58
20:3n-6	41.91	12.78	26.08	32.74	40.96	49.91	57.89	3.13	0.66	2.33	2.67	3.07	3.54	4.02
20:4n-6	127.42	29.65	92.13	106.50	123.94	145.63	166.48	9.60	1.46	7.66	8.65	9.59	10.53	11.38
22:2n-6	0.18	0.19	0.00	0.00	0.18	0.27	0.34	0.01	0.01	0.00	0.00	0.01	0.02	0.03
22:4n-6	5.17	1.53	3.47	4.09	4.91	6.08	7.27	0.39	0.08	0.29	0.33	0.39	0.44	0.50
22:5n-6	4.55	2.02	2.23	3.00	4.21	5.71	7.27	0.34	0.12	0.19	0.25	0.33	0.41	0.50
24:2n-6	2.28	0.83	1.34	1.68	2.16	2.71	3.46	0.17	0.06	0.11	0.13	0.17	0.21	0.25
Σ n-6	471.65	86.37	364.08	411.44	464.30	525.52	581.11	35.56	1.99	32.94	34.19	35.65	36.87	38.04
Σ n-6 LCP	179.05	40.30	129.85	149.07	175.05	206.27	232.56	13.46	1.63	11.28	12.36	13.52	14.54	15.47
18:3n-3	2.74	1.65	1.11	1.70	2.55	3.45	4.56	0.21	0.12	0.09	0.14	0.20	0.26	0.33
20:3n-3	0.45	0.37	0.00	0.28	0.45	0.61	0.76	0.03	0.03	0.00	0.02	0.03	0.04	0.06
20:4n-3	1.88	1.02	0.87	1.27	1.68	2.34	3.10	0.14	0.07	0.08	0.10	0.13	0.17	0.22
20:5n-3	7.34	5.26	3.31	4.44	6.04	8.43	12.71	0.55	0.38	0.28	0.35	0.46	0.62	0.87
22:5n-3	9.86	2.72	6.80	7.89	9.46	11.41	13.50	0.75	0.17	0.53	0.62	0.74	0.85	0.96
22:6n-3	53.33	16.34	34.01	42.06	51.03	62.82	73.94	3.99	0.88	2.97	3.34	3.89	4.52	5.18
Σ n-3	75.60	22.28	50.91	59.54	72.47	86.75	103.19	5.67	1.19	4.41	4.88	5.46	6.25	7.08
Σ n-3 LCP	72.41	21.81	48.16	57.21	69.16	83.59	98.88	5.43	1.19	4.17	4.65	5.24	5.99	6.90
20:3n-9	2.57	1.33	1.19	1.62	2.35	3.29	4.34	0.19	0.10	0.10	0.13	0.18	0.24	0.32
Σ n-7 + n-9	157.95	32.58	118.34	134.66	154.95	178.14	198.14	11.90	1.23	10.33	11.03	11.86	12.72	13.49
Σ PUFA	550.19	100.10	427.29	478.92	546.28	609.86	676.27	41.45	1.52	39.55	40.41	41.50	42.49	43.31
Σ MUFA	157.23	32.23	117.39	133.81	154.86	177.80	197.80	11.85	1.20	10.30	11.01	11.83	12.66	13.39
Σ SFA	592.03	107.59	459.90	514.34	584.24	656.79	730.49	44.59	1.32	42.96	43.82	44.64	45.46	46.17
Total fatty acids	1327.5	236.0	1033.4	1164.9	1313.7	1470.4	1621.7	100.00						

¹Mean ± SD gestational age was 11.1 ± 2.8 wk. For legend see Table 2.2 (Chapter 2).

Appendix B: The polyunsaturated fatty acid composition of maternal plasma phospholipids during the second trimester of pregnancy ($n = 780$).¹

	mg/L							% wt/wt						
	Mean	SD	Percentile					Mean	SD	Percentile				
			10	25	50	75	90			10	25	50	75	90
Fatty acid														
18:2n-6	348.71	60.66	278.57	307.41	342.45	385.34	436.87	21.39	2.49	18.31	19.73	21.26	23.04	24.50
18:3n-6	0.63	0.46	0.00	0.34	0.55	0.84	1.21	0.04	0.03	0.00	0.02	0.03	0.05	0.07
20:2n-6	8.71	1.89	6.57	7.41	8.58	9.70	11.18	0.53	0.08	0.44	0.47	0.52	0.58	0.64
20:3n-6	55.90	14.32	39.40	46.07	54.66	64.56	75.00	3.40	0.60	2.68	2.99	3.37	3.76	4.15
20:4n-6	140.55	29.49	104.48	119.29	138.12	159.01	179.92	8.61	1.31	6.93	7.76	8.59	9.50	10.23
22:2n-6	0.26	0.22	0.00	0.14	0.27	0.36	0.46	0.02	0.01	0.00	0.00	0.02	0.02	0.03
22:4n-6	6.46	1.84	4.51	5.25	6.16	7.43	8.83	0.39	0.09	0.29	0.33	0.39	0.44	0.51
22:5n-6	7.24	2.63	4.09	5.27	7.00	8.78	10.42	0.44	0.14	0.26	0.34	0.44	0.54	0.62
24:2n-6	2.80	1.00	1.63	2.13	2.69	3.32	4.06	0.17	0.06	0.10	0.13	0.17	0.21	0.25
Σ n-6	571.52	86.00	469.55	508.71	567.73	626.67	680.81	35.01	2.00	32.58	33.73	35.02	36.35	37.46
Σ n-6 LCP	210.14	41.01	160.50	181.89	205.85	236.99	264.22	12.84	1.51	10.82	11.86	12.92	13.84	14.69
18:3n-3	4.01	1.92	1.85	2.82	3.79	5.10	6.38	0.25	0.11	0.12	0.17	0.24	0.31	0.38
20:3n-3	0.55	0.30	0.00	0.39	0.57	0.74	0.88	0.03	0.02	0.00	0.02	0.04	0.05	0.05
20:4n-3	2.52	1.22	1.27	1.72	2.29	3.03	4.07	0.15	0.07	0.09	0.11	0.14	0.18	0.23
20:5n-3	7.01	5.61	3.34	4.39	5.91	7.91	10.99	0.43	0.34	0.23	0.28	0.37	0.47	0.62
22:5n-3	10.23	2.87	7.10	8.21	9.79	11.59	14.10	0.62	0.13	0.47	0.53	0.61	0.70	0.80
22:6n-3	66.64	16.67	47.11	55.19	64.48	76.14	89.61	4.09	0.86	3.09	3.50	4.01	4.56	5.21
Σ n-3	90.94	23.00	65.23	75.38	88.09	102.96	119.14	5.57	1.15	4.35	4.84	5.43	6.08	6.92
Σ n-3 LCP	86.39	22.64	61.52	71.22	83.78	97.69	114.53	5.29	1.15	4.11	4.56	5.14	5.80	6.67
20:3n-9	3.45	1.77	1.51	2.18	3.13	4.36	5.79	0.21	0.10	0.10	0.14	0.19	0.26	0.35
Σ n-7 + n-9	203.37	37.91	156.49	178.90	202.28	226.38	251.13	12.43	1.34	10.69	11.53	12.43	13.31	14.14
Σ PUFA	666.28	96.58	548.56	596.28	663.66	732.77	788.09	40.81	1.59	38.75	39.77	40.87	41.90	42.75
Σ MUFA	201.89	37.36	155.75	177.66	200.21	225.00	248.96	12.34	1.30	10.65	11.47	12.34	13.22	14.03
Σ SFA	733.58	110.82	596.43	662.74	727.37	805.77	878.96	44.87	1.19	43.35	44.12	44.87	45.61	46.40
Total fatty acids	1634.0	236.4	1336.9	1472.8	1632.1	1789.0	1931.1	100.00						

¹Mean ± SD gestational age was 22.5 ± 1.5 weeks. For legend see Table 2.2 (Chapter 2).

Appendix C: The polyunsaturated fatty acid composition of maternal plasma phospholipids during the third trimester of pregnancy ($n = 808$).¹

	mg/L							% wt/wt						
	Mean	SD	Percentile					Mean	SD	Percentile				
			10	25	50	75	90			10	25	50	75	90
Fatty acid														
18:2n-6	383.52	63.75	306.69	341.66	377.75	424.16	465.58	21.79	2.30	18.91	20.25	21.81	23.24	24.73
18:3n-6	0.64	0.45	0.10	0.36	0.55	0.85	1.26	0.04	0.02	0.00	0.02	0.03	0.05	0.07
20:2n-6	8.64	1.79	6.47	7.39	8.49	9.67	10.97	0.49	0.08	0.40	0.44	0.49	0.53	0.59
20:3n-6	60.11	14.76	42.04	49.71	58.63	69.07	79.24	3.40	0.61	2.66	2.97	3.37	3.76	4.23
20:4n-6	144.05	29.19	108.04	122.99	142.26	161.67	183.01	8.17	1.22	6.61	7.37	8.15	8.94	9.69
22:2n-6	0.30	0.19	0.00	0.20	0.31	0.42	0.51	0.02	0.01	0.00	0.01	0.02	0.02	0.03
22:4n-6	6.54	1.67	4.64	5.35	6.33	7.49	8.80	0.37	0.08	0.28	0.32	0.36	0.42	0.47
22:5n-6	8.17	2.89	4.76	6.10	7.78	10.07	12.05	0.46	0.15	0.28	0.35	0.45	0.56	0.66
24:2n-6	2.98	1.11	1.70	2.23	2.85	3.62	4.40	0.17	0.06	0.10	0.13	0.16	0.21	0.25
Σ n-6	615.27	89.03	505.18	553.61	614.66	668.26	728.84	34.92	1.90	32.58	33.74	34.97	36.19	37.34
Σ n-6 LCP	218.87	40.17	170.41	191.32	214.75	244.43	274.45	12.41	1.41	10.64	11.48	12.41	13.31	14.25
18:3n-3	4.43	2.02	2.14	3.01	4.19	5.48	6.99	0.25	0.11	0.12	0.18	0.24	0.31	0.38
20:3n-3	0.48	0.31	0.00	0.34	0.50	0.65	0.81	0.03	0.02	0.00	0.02	0.03	0.04	0.04
20:4n-3	2.52	1.15	1.33	1.75	2.31	3.07	3.94	0.14	0.06	0.08	0.10	0.13	0.17	0.21
20:5n-3	6.36	4.23	3.08	4.04	5.39	7.40	10.20	0.36	0.22	0.19	0.23	0.31	0.41	0.57
22:5n-3	9.87	2.60	6.92	7.97	9.54	11.25	13.42	0.56	0.11	0.43	0.48	0.54	0.63	0.71
22:6n-3	69.31	16.39	49.89	57.40	67.56	78.99	90.97	3.93	0.76	3.05	3.40	3.87	4.43	4.96
Σ n-3	92.88	21.85	68.46	77.16	89.88	105.39	120.43	5.26	0.96	4.18	4.59	5.12	5.84	6.49
Σ n-3 LCP	88.06	21.29	64.19	73.13	85.46	99.99	114.62	4.99	0.95	3.92	4.33	4.86	5.56	6.19
20:3n-9	3.84	2.02	1.75	2.52	3.48	4.78	6.43	0.22	0.11	0.10	0.14	0.20	0.27	0.36
Σ n-7 + n-9	223.34	40.07	177.26	195.09	220.26	245.89	272.63	12.66	1.35	10.97	11.76	12.64	13.49	14.39
Σ PUFA	712.36	100.29	591.53	643.55	711.65	773.54	839.31	40.43	1.54	38.59	39.47	40.43	41.48	42.36
Σ MUFA	221.32	39.46	175.62	194.21	218.49	243.37	271.25	12.55	1.32	10.90	11.65	12.53	13.36	14.26
Σ SFA	796.86	115.01	658.73	717.95	792.65	861.99	941.15	45.19	1.48	43.86	44.55	45.27	45.91	46.55
Total fatty acids	1762.7	243.5	1465.3	1593.4	1755.4	1903.6	2073.9	100.00						

¹Mean ± SD gestational age was 32.4 ± 1.1 weeks. For legend see Table 2.2 (Chapter 2).

Appendix D: The polyunsaturated fatty acid composition of plasma phospholipids in 7-year-old boys ($n = 145$).¹

	mg/L							% wt/wt						
	Mean	SD	Percentile					Mean	SD	Percentile				
			10	25	50	75	90			10	25	50	75	90
Fatty acid														
18:2n-6	242.49	42.22	192.78	212.83	238.98	267.97	301.35	22.98	2.25	20.01	21.42	22.90	24.25	25.96
18:3n-6	0.93	0.63	0.00	0.54	0.90	1.20	1.59	0.09	0.06	0.00	0.05	0.08	0.11	0.15
20:2n-6	3.33	0.83	2.44	2.83	3.25	3.78	4.35	0.32	0.06	0.24	0.28	0.31	0.36	0.39
20:3n-6	31.74	7.17	23.48	26.77	31.22	35.59	41.04	3.01	0.53	2.35	2.66	2.96	3.29	3.69
20:4n-6	96.81	18.07	74.86	85.76	95.29	108.83	119.78	9.20	1.27	7.36	8.29	9.19	10.12	10.76
22:2n-6	ND	—	—	—	—	—	—	ND	—	—	—	—	—	—
22:4n-6	4.80	0.96	3.69	4.13	4.73	5.17	6.01	0.46	0.07	0.37	0.41	0.45	0.50	0.54
22:5n-6	3.42	0.99	2.28	2.72	3.29	4.03	4.64	0.33	0.08	0.22	0.27	0.32	0.37	0.42
24:2n-6	2.04	0.56	1.52	1.76	2.00	2.25	2.76	0.20	0.06	0.14	0.16	0.19	0.22	0.26
Σ n-6	386.21	56.74	317.20	346.72	382.65	425.63	457.08	36.64	1.82	34.21	35.42	36.65	37.86	38.84
Σ n-6 LCP	136.76	22.67	111.93	120.98	133.89	151.67	165.47	12.99	1.33	11.11	12.24	12.97	13.86	14.68
18:3n-3	2.09	0.84	1.20	1.48	1.95	2.57	3.26	0.20	0.07	0.13	0.14	0.19	0.24	0.28
18:4n-3	1.44	0.62	0.80	1.04	1.38	1.76	2.24	0.14	0.05	0.08	0.10	0.13	0.16	0.19
20:4n-3	ND	—	—	—	—	—	—	ND	—	—	—	—	—	—
20:5n-3	5.53	3.20	2.93	3.68	4.94	6.30	8.57	0.52	0.25	0.31	0.36	0.46	0.60	0.76
22:5n-3	9.72	2.07	6.98	8.32	9.68	10.77	11.91	0.92	0.15	0.75	0.82	0.92	1.00	1.11
22:6n-3	29.74	8.67	20.63	23.63	28.09	35.26	39.50	2.82	0.73	2.10	2.33	2.65	3.22	3.65
Σ n-3	48.51	12.44	35.31	39.95	47.05	55.56	63.62	4.59	0.92	3.64	3.96	4.39	5.03	5.62
Σ n-3 LCP	44.98	12.13	31.71	36.45	42.85	51.80	59.35	4.26	0.93	3.29	3.65	4.05	4.66	5.32
20:3n-9	1.13	0.62	0.00	0.79	1.07	1.39	1.89	0.11	0.06	0.00	0.08	0.10	0.14	0.17
Σ n-7 + n-9	140.72	26.02	109.98	121.94	137.88	159.12	176.16	13.33	1.45	11.48	12.44	13.36	14.15	15.11
Σ PUFA	435.85	63.33	357.39	389.43	431.36	477.83	519.81	41.33	1.55	39.38	40.29	41.53	42.39	43.20
Σ MUFA	139.59	25.70	108.95	120.96	137.02	158.15	175.09	13.22	1.42	11.40	12.34	13.21	14.03	15.04
Σ SFA	479.45	69.67	398.89	429.41	477.51	524.26	570.82	45.45	1.00	44.16	44.90	45.50	46.02	46.49
Total fatty acids	1054.9	150.9	866.8	943.0	1052.7	1155.0	1263.3	100.00						

¹Mean ± SD age was 7.26 ± 0.25 years. For legend see Table 2.2 (Chapter 2).

Appendix E: The polyunsaturated fatty acid composition of plasma phospholipids in 7-year-old girls ($n = 118$).¹

	mg/L							% wt/wt						
	Mean	SD	Percentile					Mean	SD	Percentile				
			10	25	50	75	90			10	25	50	75	90
Fatty acid														
18:2n-6	249.92	39.96	200.49	223.56	255.46	271.68	300.52	23.44	2.28	20.11	22.20	23.78	25.00	25.79
18:3n-6	0.80	0.57	0.00	0.52	0.78	1.09	1.39	0.07	0.05	0.00	0.05	0.07	0.10	0.13
20:2n-6	3.37	0.85	2.53	2.69	3.42	3.89	4.28	0.32	0.07	0.25	0.29	0.32	0.36	0.40
20:3n-6	31.09	7.31	22.95	26.50	30.09	35.02	41.82	2.91	0.54	2.24	2.46	2.92	3.28	3.63
20:4n-6	98.05	18.71	77.32	85.67	93.77	112.38	125.65	9.19	1.19	7.65	8.30	9.23	9.93	10.55
22:2n-6	ND	—	—	—	—	—	—	ND	—	—	—	—	—	—
22:4n-6	4.70	1.12	3.59	3.88	4.53	5.33	6.12	0.44	0.08	0.35	0.39	0.44	0.50	0.54
22:5n-6	3.23	1.02	2.16	2.55	3.03	3.79	4.53	0.30	0.08	0.22	0.25	0.30	0.35	0.41
24:2n-6	2.29	1.22	1.54	1.81	2.14	2.55	2.83	0.22	0.11	0.14	0.18	0.20	0.24	0.28
Σ n-6	394.24	55.74	328.66	354.22	393.52	422.91	465.06	36.96	1.78	34.72	35.53	37.25	38.36	39.22
Σ n-6 LCP	137.08	24.26	108.29	120.83	133.92	153.10	169.73	12.84	1.35	10.94	11.98	12.90	13.72	14.59
18:3n-3	2.01	0.80	1.17	1.48	1.89	2.42	3.03	0.19	0.07	0.12	0.14	0.17	0.22	0.28
18:4n-3	1.43	0.52	0.81	1.10	1.40	1.76	2.09	0.13	0.05	0.08	0.10	0.13	0.17	0.18
20:4n-3	ND	—	—	—	—	—	—	ND	—	—	—	—	—	—
20:5n-3	5.26	2.09	3.05	3.90	4.77	6.18	8.37	0.49	0.18	0.30	0.37	0.46	0.58	0.71
22:5n-3	9.43	1.94	7.29	8.18	9.11	10.36	12.04	0.88	0.12	0.74	0.79	0.88	0.95	1.03
22:6n-3	30.02	8.08	21.38	24.23	28.59	33.93	40.35	2.81	0.65	2.05	2.35	2.75	3.19	3.54
Σ n-3	48.15	10.74	36.56	41.01	46.26	54.24	62.06	4.51	0.77	3.69	4.00	4.44	4.93	5.37
Σ n-3 LCP	44.71	10.48	33.48	37.79	43.43	50.49	58.45	4.19	0.78	3.39	3.66	4.11	4.60	5.06
20:3n-9	1.06	0.70	0.00	0.75	1.00	1.37	1.86	0.10	0.06	0.00	0.08	0.10	0.13	0.16
Σ n-7 + n-9	139.87	25.33	111.36	123.25	138.85	153.59	174.19	13.09	1.34	11.68	12.20	12.89	13.72	15.31
Σ PUFA	443.45	61.66	368.42	401.92	441.79	476.59	518.29	41.57	1.54	39.49	40.67	41.68	42.81	43.50
Σ MUFA	138.81	24.93	110.59	122.18	137.76	151.77	172.72	12.99	1.32	11.61	12.08	12.80	13.65	15.20
Σ SFA	484.85	66.31	404.52	440.58	479.09	525.30	562.96	45.44	1.06	44.21	44.79	45.37	46.10	46.98
Total fatty acids	1067.1	145.1	889.3	997.1	1060.9	1151.6	1256.6	100.00						

¹Mean ± SD age was 7.27 ± 0.26 years. For legend see Table 2.2 (Chapter 2).

Appendix F: Distribution of anthropometric characteristics and cardiovascular risk indicators in 7-year-old boys and girls.

	Boys (<i>n</i> = 145) ¹							Girls (<i>n</i> = 119) ¹						
	Mean	SD	Percentile					Mean	SD	Percentile				
			10	25	50	75	90			10	25	50	75	90
Weight (kg)	25.4	4.3	20.9	22.6	24.5	27.9	30.8	25.0	4.2	20.4	22.0	24.4	27.5	30.7
Height (cm)	127.5	5.1	121.0	124.2	127.8	131.0	134.3	126.2	6.0	119.0	122.6	125.5	129.8	134.2
BMI (kg/cm ²)	15.6	1.9	13.8	14.4	15.1	16.4	17.7	15.6	1.7	13.8	14.4	15.2	16.6	18.2
SBP (mmHg)	101	7	93	97	101	105	109	100	7	92	69	99	105	110
DBP (mmHg)	61	6	54	57	62	65	68	62	5	55	58	62	65	67
Cholesterol (mmol/L)	4.04	0.57	3.3	3.6	4.0	4.5	4.7	4.10	0.71	3.35	3.65	4.09	4.49	4.94
LDL-C	2.43	0.53	1.76	2.08	2.44	2.80	3.11	2.50	0.64	1.73	2.05	2.49	2.92	3.23
HDL-C	1.37	0.31	0.99	1.15	1.35	1.60	1.77	1.34	0.31	0.96	1.15	1.30	1.54	1.76
Triacylglycerol (mg/L)	0.53	0.30	0.27	0.34	0.48	0.63	0.86	0.58	0.27	0.30	0.38	0.51	0.72	0.96
Apo A1 (mg/L)	1364	219	1070	1204	1355	1540	1660	1350	228	1110	1201	1350	1488	1613
Apo B (mg/L)	715	152	530	605	725	815	885	748	169	535	631	745	834	980
Lp(a) (mg/L)	150	175	7	17	79	224	450	178	212	14	37	74	290	536
Glucose (mmol/L)	4.7	0.4	4.3	4.5	4.7	4.9	5.1	4.6	0.3	4.3	4.5	4.6	4.8	5.0
Insulin (pmol/L)	35	15	19	25	35	44	53	40	18	21	28	36	48	61
Proinsulin (pmol/L)	6.6	1.6	4.6	5.4	6.4	7.5	8.9	7.2	2.0	4.6	5.6	7.2	8.6	9.4
Leptin (µg/L)	3.1	2.5	1.6	1.9	2.4	3.6	4.9	4.6	3.3	2.1	2.6	3.6	4.9	8.9

SBP, systolic blood pressure; DBP, diastolic blood pressure.

¹For some of the variables the total number of observations may be lower due to missing data.

Appendix G: The figures on the following two pages depict the distribution of the umbilical cord plasma phospholipid concentration (in % wt/wt and in mg/L) of linoleic acid (A; 18:2n-6), dihomog- γ -linolenic acid (B: 20:3n-6), arachidonic acid (C, 20:4n-6), docosatetraenoic acid (D; 22:4n-6), eicosapentaenoic acid (E; 20:5n-3), docosapentaenoic acid (F; 22:5n-3), docosa-hexaenoic acid (G, 22:6n-3) and eicosatrienoic acid (H; 20:3n-9) as a function of gestational age at birth. The figures on page 173 depict the distribution of the umbilical cord plasma phospholipid $\Sigma n-3 + \Sigma n-6 / \Sigma n-7 + \Sigma n-9$, 22:6n3/22:5n6, and $\Sigma n-3 / \Sigma n-6$ ratio's in relation to gestational age at birth. The number of infants in each gestational age group is given underneath the figure. The lines represent the 10th, 25th, 50th, 75th and 90th percentiles (P). The triangles represent the 25th, 50th and 75th percentiles for the preterm infants (average gestational age at birth: 35 $\pm$ 1.5 wk, $n = 39$). For more information see Chapter 2.

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Notes |

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