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# n-3 Fatty acids in red blood cells from pregnant and non-pregnant women in Iceland. The relationship to n-3 fatty acid intake, lifestyle and pregnancy outcome

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PhD THESIS
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# Ómega-3 fitusýrur í rauðfrumum þungaðra og óþungaðra kvenna á Íslandi. Tengsl við neyslu, lífshætti og útkomu meðgöngu

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# ÁGRIP Á ÍSLENSKU

Fæðingarþyngd íslenskra barna er óvenju há borið saman við tölur frá flestum öðrum Evrópuþjóðum og útkoma meðgöngu góð. Langt fram á 20. öldina einkenndist fæði Íslendinga af neyslu sjávarfangs og lýsis, sem er ríkt af fjölómettuðu ómega-3 fitusýrunum eikósapentaensýru (EPA) og dókósahexaensýru (DHA). EPA er forveri prostaglandína og annarra eikósanóíða sem auka blóðflæði til fylgju. DHA gegnir mikilvægu hlutverki fyrir þroska fósturs og fylgjan flytur DHA sértækt úr blóði móðurinnar til fóstursins. Mikið er af DHA í frumuhimnum heila og taugakerfisins, þar sem hún eykur fljótanleika himnanna og er talin taka þátt í boðefnaflutningi. Fyrstu vikur meðgöngunnar skipta ekki síður máli fyrir þroska fósturs en seinni hluti meðgöngu. Það er því mikilvægt að konur á barneignaraldri hafi forða af ómega-3 fitusýrum þegar til getnaðar kemur.

Fitusýrusamsetning rauðfrumna segir til um stöðu fjölómettaðra fitusýra hjá einstaklingi, en mikilvægt er að rauðfrumusýni séu geymd við rétt skilyrði, þar sem járnið í rauðfrumunum getur hvatað peroxun fjölómettuðu fitusýranna. Markmið þessarar rannsóknar var að kanna fitusýrusamsetningu rauðfrumna frá þunguðum og óþunguðum konum á barneignaraldri, og bera hana saman við neyslu, lífshætti og útkomu meðgöngu. Annað markmið var að kanna stöðugleika fjölómettaðra fitusýra í rauðfrumum við mismunandi geymsluskilyrði.

Fylgni milli neyslu ómega-3 fitusýra, lífsháttа og útkomu meðgöngu var könnuð meðal 549 þungaðra kvenna tvisvar á meðgöngu. Rauðfrumusýni voru tekin úr 176 þessara kvenna, fitusýrusamsetning könnuð og borin saman við neyslu, lífshætti og útkomu meðgöngu. Rauðfrumusýni voru einnig tekin úr 45 óþunguðum konum á barneignaraldri, fitusýrusamsetning könnuð og borin saman við neyslu og lífshætti. Að lokum voru rauðfrumusýni tekin úr 13 óþunguðum konum á aldrinum 25 til 55 ára og skipt í sjö hluta sem geymdir voru mislengi, ýmist með eða án viðbætts andoxunarefnis.

Jákvæð fylgni var milli neyslu ómega-3 fitusýra og hluts þeirra í rauðfrumum bæði meðal þungaðra og óþungaðra kvenna. Fjölþátta aðhvarfsgreining á neyslu, lífsháttum og útkomu meðgöngu hjá öllum þunguðu konunum leiddi í ljós að neysla lýsis í byrjun meðgöngu tengdist aukinni þyngd nýbura þegar leiðrétt hafði verið fyrir meðgöngulengd og lífsháttum. Reykingar og áfengisneysla tengdust aftur á móti minni fæðingarþyngd. Aukinn hlutur ómega-3 fitusýra í rauðfrumum í byrjun

meðgöngu tengdist léttari fylgju þegar leiðrétt hafði verið fyrir fæðingarþyngdinni. Hlutur DHA var hærri í rauðfrumum því lengra sem konurnar voru komnar á leið þegar þær hófu þátttöku í rannsókninni, óháð neyslu DHA. Reykingar tengdust lægri hlut DHA í rauðfrumum á fyrri hluta meðgöngu, en neysla á léttum bjór auknum hlut DHA í rauðfrumum á seinni hluta meðgöngu. Líkamsrækt og notkun getnaðarvarnarpillu tengdust auknum hlut DHA í rauðfrumum óþungaðra kvenna.

Fjölómettaðar fitusýrur í rauðfrumusýnum voru stöðugar í fjórar vikur við −20°C án þess að andoxunarefni hefði verið bætt í sýnin, en eftir það fór hlutur fjölómettaðra fitusýra lækkandi. Andoxunarefnið bútýl hýdroxýtólúen (BHT) varðveitti fitusýrusamsetningu rauðfrumna í 17 vikur við -20°C. Rauðfrumusýnin frá þunguðu konunum voru geymd við -20°C án viðbætts andoxunarefnis í allt að 20 vikur. Þegar niðurstöður fitusýrugreiningar gáfu til kynna að fjölómettaðar fitusýrur í hluta sýnanna hefðu peroxast, var leiðréttingaraðferð, sem algengt er að nota á orkuinntöku, þróuð til að leiðrétta fyrir peroxuninni. Enginn marktækur munur var á fitusýrusamsetningu þeirra rauðfrumusýna sem ekki voru talin peroxuð, og fitusýrusamsetningu allra rauðfrumusýnanna eftir leiðréttingu.

Niðurstöður rannsóknarinnar gefa til kynna að lýsisneysla í byrjun meðgöngu og góð staða ómega-3 fitusýra í rauðfrumum tengist heilbrigðri aukningu í fæðingarþyngd og léttari fylgju. Há fæðingarþyngd og lág fylgjuþyngd hafa verið tengd minni hættu á háþrýstingi og hjarta- og æðasjúkdómum á fullorðinsárum, og ómega-3 fitusýrur gætu verið einn af þeim þáttum sem forrita langtímaheilsu einstaklingsins. Það er því mikilvægt að konur hugi að neyslu sjávarfangs eða lýsis í byrjun meðgöngunnar. Þó hlutur DHA aukist í rauðfrumum eftir því sem líður á fyrri hluta meðgöngunnar óháð neyslu DHA, geta lífshættir haft sín áhrif, bæði á fitusýrusamsetningu frumuhimna og á útkomu meðgöngunnar. Reykingar auka peroxun í líkamanum og tengjast bæði lægri hlut DHA í rauðfrumum og minni fæðingarþyngd. Þó neysla á léttum bjór auki hugsanlega nýmyndun DHA í líkamanum, ættu þungaðar konur að forðast neyslu hans, því áfengisneysla, jafnvel í litlu magni, tengdist lægri fæðingarþyngd afkvæmis. Óþungaðar konur sem voru á getnaðarvarnarpillunni höfðu hærri hlut DHA í rauðfrumum en þær sem ekki voru á pillunni, og ýmislegt bendir til að östrógenið í pillunum hvetji nýmyndun DHA í líkamanum. Við þjálfun er hugsanlegt að nýmyndun og/eða innsetning DHA í himnur rauðfrumna aukist, og er þá líklega vörn líkamans gegn rauðfrumurofi sem er fylgifiskur þjálfunar.

Þær aðstæður geta skapast að ekki séu tök á að geyma rauðfrumusýni við kjörhitastig. Í því tilfelli er ekki óhætt að geyma sýnin nema fjórar vikur við -20°C áður en fitusýrugreining fer fram. Með því að bæta andoxunarefninu BHT út í sýnin fyrir frystingu má þó auka geymsluþolið verulega, eða í minnst 17 vikur. Vísindamenn sem út frá niðurstöðum fitusýrugagna telja hluta rauðfrumusýna hafa peroxast, geta notað heildarstyrk fitusýra í sýnunum til að leiðrétta fyrir peroxuninni. Það er sérlega gagnlegt ef ekki er unnt að nota eingöngu þann hluta rauðfrumusýnanna sem ekki er talinn peroxaður, án þess að niðurstöðurnar líði fyrir valskekkju.

Ómega-3 fitusýrur eru lífsnauðsynlegar fitusýrur sem við verðum að fá úr fæðunni. Neysla ómega-3 fitusýra er mjög mikilvæg barnshafandi konum og konum á barneignaraldri, þar sem staða þeirra í byrjun meðgöngu virðist tengjast útkomu meðgöngunnar. Góð útkoma meðgöngu hefur verið tengd betri heilsu afkvæmisins síðar á ævinni.

Lykilorð: Ómega-3 fitusýrur, rauðfrumur, meðganga, fæðingarþyngd, fylgjuþyngd, lípíðperoxun, konur á barneignaraldri

# ABSTRACT

Birthweight in Iceland is higher than in most other European countries and pregnancy outcome is good. The traditional diet of Icelanders was high in seafood and cod liver oil, which are rich in the n-3 long-chain polyunsaturated fatty acids (LCPUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA is a precursor of prostaglandins and other eicosanoids that increase the blood supply to the placenta. DHA plays an important role in fetal development and the placenta transports DHA specifically and in concentrated amounts from the maternal to the fetal blood. DHA is incorporated into cell membranes, especially those of the central nervous system and the retina, where it increases the fluidity of the membranes and takes part in various cell functions. The first weeks of pregnancy are at least as important for fetal development as the later half of pregnancy. It is therefore important that women of reproductive age have a reservoir of n-3 LCPUFA when they become pregnant.

Fatty acid (FA) composition of red blood cells (RBC) is often used to monitor the PUFA status of the individual. Iron in the RBC may induce peroxidation of PUFA, making storage conditions of the RBC samples crucial. The objective of this study was to investigate the FA composition of RBC from pregnant and non-pregnant women of reproductive age, and to compare it with dietary intake, other lifestyle factors and outcome of pregnancy. Another objective was to investigate the stability of PUFA in RBC during storage.

The relationship between dietary intake of n-3 LCPUFA, other lifestyle factors and outcome of pregnancy was investigated early and late in pregnancy among 549 women. RBC samples were obtained from 176 of these women, FA composition determined and the relationship to dietary intake, other lifestyle factors and outcome of pregnancy investigated. RBC samples were also obtained from 45 non-pregnant women of reproductive age, FA composition determined and the relationship with dietary intake and other lifestyle factors investigated. Finally, RBC samples were obtained from 13 non-pregnant women aged 25 to 55 years and divided into seven portions, which were stored for different lengths of time, with or without added antioxidant.

Dietary intake of n-3 LCPUFA correlated positively with n-3 LCPUFA in RBC among both pregnant and non-pregnant women. In the whole cohort of

pregnant women, there was a positive correlation between liquid cod liver oil (L−CLO) intake early in pregnancy and birthweight adjusted for duration of gestation and other confounding factors. On the other hand, smoking and alcohol consumption were inversely correlated with birthweight. The proportion of n-3 LCPUFA in RBC early in pregnancy was inversely correlated with placental weight, adjusted for birthweight. The proportion of DHA in RBC was positively correlated with gestational length at entry, adjusted for dietary intake of DHA. Smoking was inversely correlated with the proportion of DHA in RBC early in pregnancy, while light beer consumption was positively correlated with the proportion of DHA in RBC late in pregnancy. Among the non-pregnant women, oral contraceptive use and physical activity were positively correlated with DHA in RBC.

The PUFA composition of RBC was stable during four weeks storage at −20°C without addition of antioxidant, but further storage led to a decrease in the proportion of PUFA. Addition of the antioxidant butyl hydroxytoluene (BHT) preserved the FA composition of the RBC for 17 weeks at -20°C. The RBC samples from the pregnant women were stored at -20°C without addition of antioxidant for up to 20 weeks. When the FA data analysis indicated that the RBC samples were partly lipid peroxidized, an adjustment method designed for nutritional data analysis was modified to analyze the RBC FA data. There was no difference in FA composition between unadjusted data from RBC samples that were considered not peroxidized and adjusted data from all the RBC samples.

The results of this study indicate that L-CLO intake early in pregnancy and a relatively high n-3 LCPUFA status, as assessed by RBC level, are associated with a healthy increase in the weight of the neonate and a decrease in the weight of the placenta. High birthweight in combination with a low placental weight have been related to a lower risk of hypertension and cardiovascular disease in adult life. n-3 LCPUFA could be one of the factors programming later health of the growing individual, and therefore regular intake of L-CLO early in pregnancy could be important for the health of the offspring. Even though the DHA level in RBC increases in the first trimester of pregnancy, independent of DHA intake, lifestyle may affect both the FA composition of cell membranes and the outcome of pregnancy. Smoking induces lipid peroxidation and was associated with a lower proportion of DHA in RBC and also with lower birthweight. Even though light beer consumption might stimulate biosynthesis of DHA, alcohol consumption cannot be

recommended at any stage of pregnancy, as its use, even at low levels, was related to lower birthweight of the neonate. Non-pregnant women who used oral contraceptives had higher levels of DHA in RBC than those who did not, and there are indications that oestrogen in the pills elevates the biosynthesis of DHA. Increased biosynthesis and/or incorporation of DHA in RBC membranes of physically active people might serve to increase the fluidity of the membranes and counteract exercise-induced hemolysis.

Some centres for health services do not have access to a freezer colder than -20°C. In such cases it is not safe to store RBC samples for more than four weeks before lipid extraction and FA analysis. With addition of the antioxidant BHT, they are stable for a considerably longer period, at least 17 weeks. Researchers analyzing FA data from RBC samples that are considered partly lipid peroxidized can use total FA concentration in the RBC to adjust the data for the effect of lipid peroxidation. This is especially useful when it is impossible to include only the part of the samples considered not to be peroxidized in the data analysis without causing selection bias.

n-3 PUFA are essential FA that we have to consume in our food. Including n-3 LCPUFA in the diet of women in the periconceptional period is especially important, since n-3 LCPUFA status in the first trimester is associated with the outcome of pregnancy. Optimal outcome of pregnancy has been related to improved health of the offspring later in life.

Key words: n-3 Long-chain polyunsaturated fatty acids, red blood cells, pregnancy, birthweight, placental weight, lipid peroxidation, women in reproductive age

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Many people offered their help and support during the years this work was undertaken, and I owe them all a debt of gratitude. I particularly want to thank my supervisor, Dr. Philos. Guðrún V. Skúladóttir, research professor in the Department of Physiology, Faculty of Medicine, University of Iceland, who supported me through the entire project. Without her this thesis would not have been written, as she took the initiative for all three studies and coordinated the work of the institutes involved. She was always prepared to discuss the obstacles and problems I encountered on this journey, and to take part in conversations on the field of n-3 PUFA. Furthermore, she was always willing to read and re-read my manuscripts and give invaluable guidance about scientific writing. I also thank other members of the PhD committee, Dr. Laufey Steingrímsdóttir, former division director of research at the Public Health Institute of Iceland, and currently professor at the Agricultural University of Iceland; Dr. Arnar Hauksson, chief physician of the Center of Prenatal Care, and associate professor in the Faculty of Medicine, University of Iceland; Dr. Ingibjörg Harðardóttir, professor in the Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Iceland; and Dr. Þórarinn Sveinsson, associate professor in the Department of Physiology, Faculty of Medicine, University of Iceland. They all inspired me throughout my work with their knowledge, advice and encouragement.

I would also like to thank my co-authors of the papers on which this thesis is based. First, I would like to mention Anna Sigríður Ólafsdóttir, PhD, nutritionist, assistant professor, Iceland University of Education, who was my co-author for Paper I. Learning from her experience in writing a scientific paper was invaluable to me. I also want to thank Hólmfríður Þorgeirsdóttir, M.Sc., nutritionist, the Public Health Institute of Iceland, who calculated and cleaned the data from the food frequency questionnaire; and Dr. Geir Gunnlaugsson, director of the Centre for Child Health Services and professor of public health, School of Health and Education, Reykjavik University, who co-operated with us on the study of non-pregnant women of reproductive age.

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# CLARIFICATION OF CONTRIBUTION

**Paper I** – This paper describes the relationship between liquid cod liver oil intake early in pregnancy and birthweight. I analyzed part of the data and participated in writing the manuscript.

**Paper II** – This paper describes the relationship between smoking and the proportion of n-3 LCPUFA in RBC early in pregnancy. Furthermore, it describes the relationship between the proportion of n-3 LCPUFA in RBC early in pregnancy and placental weight. I performed all the FA analysis of RBC and analyzed all of the data. I wrote the manuscript in collaboration with Guðrún V. Skúladóttir.

**Paper III** – This paper describes the relationship between physical activity, oral contraceptive use and the proportion of DHA in RBC from women of reproductive age. I performed all the FA analysis of RBC and analyzed all of the data. I wrote the manuscript in collaboration with Guðrún V. Skúladóttir.

**Paper IV** – This paper describes the stability of PUFA in RBC during storage. I participated in planning the research, performed all the FA analysis of RBC and analyzed all of the data. I wrote the manuscript in collaboration with Guðrún V. Skúladóttir.

**Paper V** – This paper describes an adjustment method used for data analysis of FA composition in RBC samples considered partly lipid peroxidized. I modified the adjustment method and analyzed all of the data. I wrote the manuscript in collaboration with Guðrún V. Skúladóttir.

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**II** Anna R. Magnusardottir, Laufey Steingrimsdottir, Holmfridur Thorgeirsdottir, Arnar Hauksson, Gudrun V. Skuladottir. Red blood cell n-3 polyunsaturated fatty acids in first trimester of pregnancy are inversely associated with placental weight. Acta Obstet Gynecol Scand 2009;88:91-97.

**III** Anna R. Magnusardottir, Laufey Steingrimsdottir, Holmfridur Thorgeirsdottir, Geir Gunnlaugsson, Gudrun V. Skuladottir. Docosahexaenoic acid in red blood cells of women of reproductive age is positively associated with oral contraceptive use and physical activity. Prostaglandins Leukot Essent Fatty Acids 2009;80:27-32.

**IV** Anna R. Magnusardottir, Gudrun V. Skuladottir. Effects of storage time and added antioxidant on fatty acid composition of red blood cells at -20°C. Lipids 2006;41:401-404.

**V** Anna R. Magnusardottir, Laufey Steingrimsdottir, Holmfridur Thorgeirsdottir, Arnar Hauksson, Gudrun V. Skuladottir. A method for adjustment of peroxidized fatty acid data: the relationship between n-3 polyunsaturated fatty acids in diet and red blood cells from women in early pregnancy. Unsubmitted manuscript.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **AA** | **Arachidonic acid (20:4n-6)** |
| **A-A** | **Adjusted-all** |
| **ALA** | **α-Linolenic acid (18:3n-3)** |
| **ATP** | **Adenosine triphosphate** |
| **$BF_3$** | **Borontrifluoride** |
| **BHT** | **Butylated hydroxytoluene** |
| **BMI** | **Body mass index (body weight / height$^2$)** |
| **CI** | **Confidence interval** |
| **CLO** | **Cod liver oil** |
| **CV** | **Coefficient of variation** |
| **DHA** | **Docosahexaenoic acid (22:6n-3)** |
| **DNA** | **Deoxyribonucleic acid** |
| **DPA** | **Docosapentaenoic acid (22:5n-3)** |
| **EDTA** | **Ethylene diamine tetra-acetate** |
| **EPA** | **Eicosapentaenoic acid (20:5n-3)** |
| **FA** | **Fatty acid** |
| **FABPpm** | **Fatty acid binding protein in plasma membrane** |
| **FAME** | **Fatty acid methyl esters** |
| **FATP** | **Fatty acid transfer protein** |
| **FFQ** | **Food frequency questionnaire** |
| **GC** | **Gas chromatography** |
| **LA** | **Linoleic acid (18:2n-6)** |
| **L-CLO** | **Liquid cod liver oil** |
| **LCPUFA** | **Long-chain polyunsaturated fatty acids** |
| **MUFA** | **Monounsaturated fatty acids** |
| **NEFA** | **Non-esterified fatty acid** |
| **PC** | **Phosphatidylcholine** |
| **PE** | **Phosphatidylethanolamine** |
| **p-FABPpm** | **Placental fatty acid binding protein in plasma membrane** |
| **$PGE_2$** | **Prostaglandin $E_2$** |
| **$PGE_3$** | **Prostaglandin $E_3$** |
| **PL** | **Phospholipids** |
| **PUFA** | **Polyunsaturated fatty acids** |
| **RBC** | **Red blood cells** |
| **RNA** | **Ribonucleic acid** |
| **ROS** | **Reactive oxygen species** |
| **SD** | **Standard deviation** |
| **SFA** | **Saturated fatty acids** |
| **TG** | **Triglyceride** |
| **$TXA_2$** | **Thromboxane $A_2$** |
| **U-A** | **Unadjusted-all** |
| **U-NP** | **Unadjusted-not peroxidized** |

# INTRODUCTION

This thesis is based on a study of n-3 long-chain polyunsaturated fatty acids (LCPUFA) in the diet and red blood cells (RBC) of pregnant and non-pregnant women in Iceland. The thesis will begin by describing the biochemistry and physiological role of n-3 LCPUFA. The focus will then turn to the role of the placenta in transporting n-3 LCPUFA from maternal to fetal blood, and the specific role of n-3 LCPUFA for growth and development. The scientific background for research on n-3 LCPUFA for pregnant and non-pregnant women will be provided. The RBC will be introduced and lipid peroxidation and vulnerability of RBC PUFA to peroxidation will be described. The methods used will be given in the Methods section of the thesis, and the Results section will go on to describe both published and unpublished results of the study. Although most of the results can also be found in five scientific papers listed in the Appendix, some are only described in the Results section of the thesis. In the Discussion section of the thesis, the results of the study will be put into the context of other studies and conclusions drawn.

Traditional fish and cod liver oil (CLO) consumption in Iceland makes it an interesting field for observational studies of n-3 LCPUFA intake and blood status. Pregnant and non-pregnant women of reproductive age are populations of special interest, since n-3 LCPUFA are essential for fetal growth and development.

## 1 Polyunsaturated fatty acids (PUFA)

Every cell membrane is composed of a lipid bilayer. Phospholipids (PL) are the fundamental building blocks of each layer. PL are amphipathic molecules, consisting of two hydrophobic fatty acid (FA) chains and one phosphate-containing hydrophilic group. The phosphate group with its attached water-soluble molecule extends to the surface of the membrane, either the inner or the outer surface. The water insoluble FA form the core of the bilayer membrane.

A fatty acid is a chain of carbon atoms with hydrogens attached. At the acid end of the carbon chain there is a carboxyl (acid) group and at the methyl end (omega or n end) there is a methyl group (Fig. 1). Saturated fatty acids (SFA) have only single bonds between the carbon atoms in the chain. Monounsaturated fatty acids (MUFA) have one double bond, and FA with two or more double bonds are

named polyunsaturated fatty acids (PUFA). PUFA with the first double bond at carbon atom number six, or three counted from the omega end, are termed n-6 PUFA and n-3 PUFA, respectively (1). Linoleic acid (LA, 18:2n-6) with 18 carbon atoms and two double bonds and arachidonic acid (AA, 20:4n-6) with 20 carbon atoms and four double bonds are n-6 PUFA, while α-linolenic acid (ALA, 18:3n-3) with 18 carbons and three double bonds, eicosapentaenoic acid (EPA, 20:5n-3) with 20 carbon atoms and five double bonds and docosahexaenoic acid (DHA, 22:6n-3) with 22 carbon atoms and six double bonds are n-3 PUFA (Fig. 1). LCPUFA contain twenty or more carbons and two or more double bonds in the chain. AA is an n-6 LCPUFA while EPA and DHA are n-3 LCPUFA.

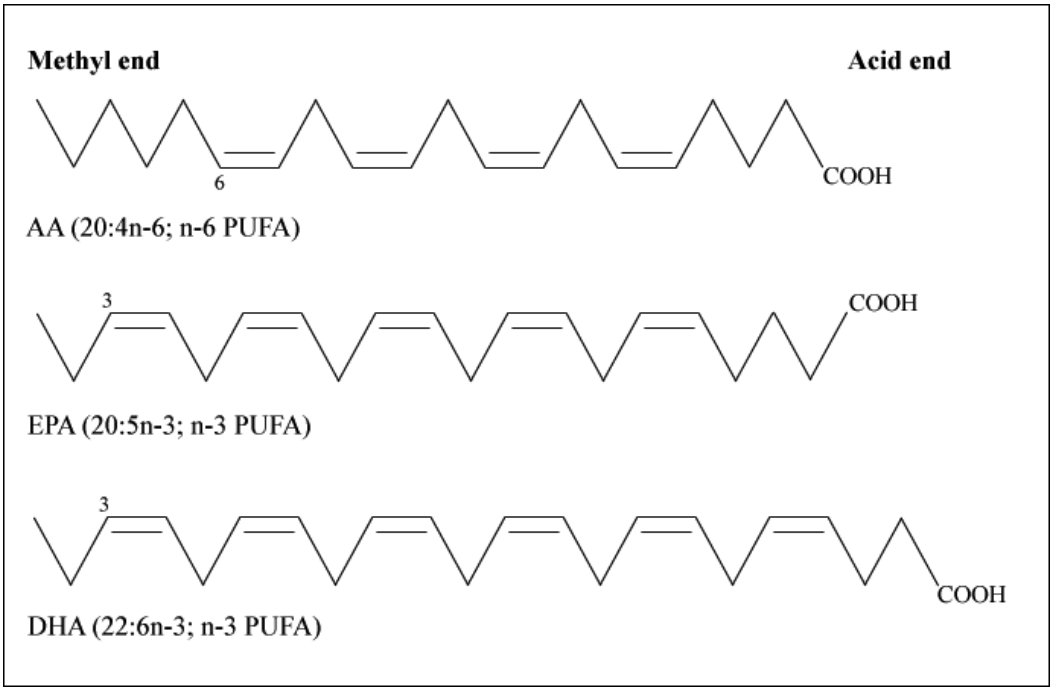



**Fig. 1 Structure of arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).**

Vertebrates cannot insert double bonds more proximal to the methyl end of FA chains than at the seventh carbon atom, which means that humans and other vertebrates cannot synthesize ALA or LA (1). However, the liver, brain, heart and lung of mammals have an enzyme system that desaturates ALA and LA and elongates them to make their longer-chain derivatives (2, 3). The chains are elongated from the carboxyl end with two carbon units at a time, without altering the methyl end. This makes n-3 and n-6 PUFA non-interconvertible. EPA and DHA are

made from ALA, but AA from LA (reviewed in (4)) (Fig. 2). It is the same enzyme system that elongates and desaturates nonessential n-9 FA, n-6 and n-3 PUFA. The pathway takes place in the endoplasmic reticulum, until the final chain-shortening step, which most probably takes place in peroxisomes (4) (Fig. 2).

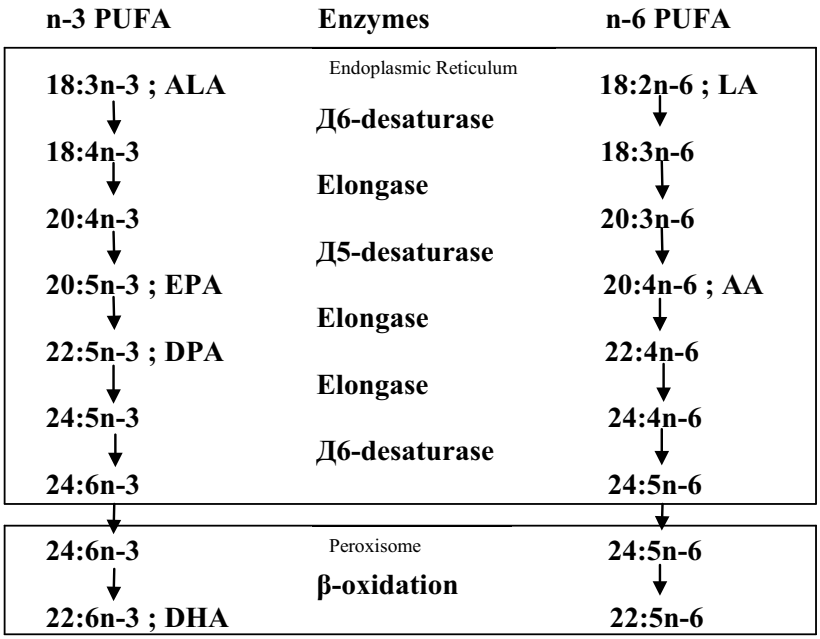



**Fig. 2 Elongation and desaturation of the essential fatty acids linoleic acid (LA) and α-linolenic acid (ALA).**

## 1.1 Food sources and biological functions

LA is found in many plant oils, but ALA is mainly found in green leafy vegetables, soybeans, flaxseed and canola oil (5). The elongated and desaturated enzyme products are also found in food. AA is found in meat and eggs, and EPA and DHA are found in seafood. When terrestrial animals are fed fish oil, herring or fish meal, as has been common in Iceland, n-3 LCPUFA are found in the meat and eggs (6, 7). Fish intake in Iceland was high, 73 ± 53 g/day on average in 1990 (8) but decreased substantially during the following decade, being 40 ± 76 g/day on average in 2002 (9). Liquid cod liver oil (L-CLO) intake in Iceland is still substantial, 17% of women and 22% of men took L-CLO daily in 2002 (9).

FA are the major components of cell membranes. SFA and MUFA are also used as an energy source, as are LA and ALA (10, 11). However, n-3 and n-6 LCPUFA have various physiological roles, such as being the precursors of

eicosanoids (prostaglandins (PG), thromboxanes (TX) and leukotrienes) and docosanoids (resolvins and protectins) (Fig. 3). Eicosanoids are important autocrine/paracrine hormones, mediating immune response, blood pressure regulation and blood coagulation, among other functions (reviewed in (12, 13)). Derivatives of AA, i.e. $PGE_2$ and $TXA_2$ have been associated with thrombosis, vasospasm, arrhythmia and chronic inflammatory processes (reviewed in (14)). EPA in endothelial cells gives rise to $PGE_3$, the effect of which is vasodilation and antithrombosis (14). DHA is found in high amounts in cell membranes of brain, retina and sperm (15, 16). Neuroprotectin D1 is a DHA derived docosanoid, which is a potent inhibitor of oxidative stress-induced apoptosis in the brain (reviewed in (17)), and DHA derived protectins and resolvins mediate the termination of acute inflammation (reviewed in (18)). Moreover, n-3 and n-6 LCPUFA are important for lipid raft formation, signal transduction, neurotransmission, endocytosis/exocytosis, ion channel activity, DNA polymerase inhibition, and regulation of gene expression (reviewed in (19)).

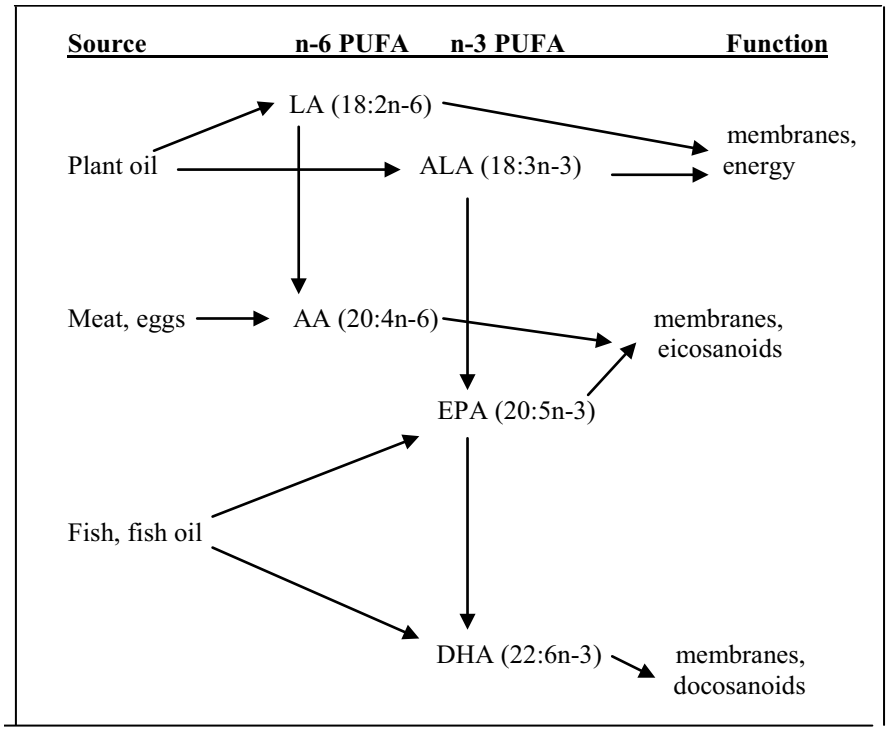



**Fig. 3 Food sources of n-3 and n-6 PUFA and their main functions (reviewed in (19, 20)).**

## 1.2 Essentiality

Since mammals cannot synthesize ALA and LA they have to get these FA from food. Much research in the last decades has focused on the essentiality of n-3 PUFA. It is the longer-chain derivatives, EPA and DHA, with their important physiological roles, that are functionally essential, rather than ALA itself.

The fetal brain is particularly vulnerable to n-3 PUFA deficiency. After 19 days of n-3 PUFA deficient maternal diet, DHA content of rat fetus brain was significantly lower than that of brain from rat fetuses whose maternal diet contained ALA (21). Maternal supplementation for only 5 days with DHA restored the DHA concentration in brain and liver (21). Brain growth continues in the postnatal period. The proportion of DHA in RBC, cerebellum and forebrain of rat pups reflected the amount of DHA in the formula (22). Brain DHA was strongly correlated with DHA in RBC (22). Preformed DHA was much more effective than ALA for elevating the DHA content of brain and retina of the guinea pig (23). A rich source of ALA does not prevent DHA deficiency in the non-human primate if the diet is deprived of DHA. Rhesus monkey neonates of mothers fed n-3 PUFA deficient diet throughout pregnancy had 70-90% lower content of n-3 PUFA in brain and retina than control monkey neonates (24). A diet rich in ALA restored the n-3 PUFA content of cerebral cortex at 15 weeks, but at three years of age, DHA content of retina was still lower than in control monkeys (24).

It was shown as early as 1929 that feeding rats a diet deprived of all FA had pathological consequences (25). The animals seemed to be cured by supplementation with LA (26). Since then it has been shown that rhesus monkey infants deprived of n-3 PUFA pre- and postnatally have impaired visual function (27). Symptoms ascribed to n-3 PUFA deficiency: scaly skin and substantial skin atrophy, were seen in four human patients on long-term gastric tube feeding (28). Within four weeks of essential FA supplementation, the symptoms disappeared and n-3 PUFA in plasma and RBC increased dramatically, while n-6 PUFA were unchanged or decreased slightly. Symptoms related to vision or the nervous system were not found because of the advanced age of the patients and underlying disease. The minimal daily requirement of EPA and DHA in the complete absence of ALA was assumed to be 0.1-0.2% of total energy intake (28).

The conversion of ALA to n-3 LCPUFA seems to be limited in the human body. Non-pregnant young women given $^{13}C$ labeled ALA were able to convert it to n-3 LCPUFA, although mainly to EPA (11). After 21 days, 21% of the administered dose had been converted to EPA, but only 6% to docosapentaenoic acid (DPA, 22:5n-3) and 9% to DHA (11). In the first 24 hours, 22% of the administered dose of $^{13}C$ labeled ALA was oxidized (11). In young men, more ALA seemed to be oxidized, and conversion of DPA to DHA was either very low or absent (10). Whether the conversion of ALA to longer-chain derivatives in the human body meets the demand for EPA and DHA remains elusive. When food contains much more LA than ALA, as modern Western food does, most of the LCPUFA products are n-6 LCPUFA, as high levels of LA block the desaturation of ALA (29, 30). High intake of LA can also inhibit the incorporation of EPA from supplements into membranes (31). Recommendations for lipid intake during pregnancy and lactation have recently been proposed by the Perinatal Lipid Intake Working Group, developed by the European Commission research projects Perinatal Lipid Metabolism and Early Nutrition Programming together with representatives of other societies in the field (32). According to the consensus statement, pregnant and lactating women should aim to achieve an average dietary intake of at least 200 mg/d of DHA, and women of childbearing age should aim to consume one to two portions of sea fish per week, including oily fish (32).

Considering the functional importance of n-3 LCPUFA in the vulnerable state of pregnancy and the changing dietary intake of people in Iceland, it was of relevance to investigate dietary intake of n-3 LCPUFA among pregnant women in Iceland and how it correlates with biochemical n-3 LCPUFA status and outcome of pregnancy.

## 1.3 The n-6/n-3 PUFA ratio

The ratio of n-6/n-3 PUFA in the human diet has changed from being roughly 1-2:1 in preagricultural times (33) to 10-20:1 in the modern Western diet (34). This change is attributed both to increased intake of n-6 PUFA and to decreased intake of n-3 PUFA, particularly n-3 LCPUFA (33, 34). FA of both series, n-6 and n-3 PUFA, are incorporated into all cell membranes in the body. An imbalance in dietary intake of n-6 and n-3 PUFA changes the ratio of n-6/n-3 PUFA in membranes

(35). A high ratio of n-6/n-3 PUFA has been associated with increased platelet aggregation (36), cardiovascular disease (37, 38), inflammatory processes (39, 40), and proliferation of cancer cells (41). Decreasing the ratio of n-6/n-3 PUFA in the diet of women of reproductive age could be important in the prevention of major (42) and perinatal depression (43, 44), osteoporosis (45) and breast cancer (46).

## 2 Placenta

Trophoblast cells on the surface of a fertilized ovum invade the endometrial stroma in the uterus, a process called implantation (47). After implantation the trophoblast proliferates rapidly to form the placenta and the umbilical cord (Fig. 4). The chorionic plate on the fetal side of the placenta sends out numerous chorionic villi. Embryonic capillaries grow into the umbilical cord and into the villi. Septa from the decidual plate on the maternal side of the placenta separate the fetal cotyledons, each forming an intervillous space (47). The intervillous space is supplied with blood from the mother (48) (Fig. 4). The microvilli effectively enhance the total area available for exchange between the mother and the fetus. The fetal and maternal blood streams are separated by two membranes, the microvillous facing the maternal blood, and the basal facing the fetal blood. By the 10$^{th}$ week after fertilization, placental development is complete, but the placenta continues to grow, increasing the maternal blood flow and the total surface area of the villi (47).

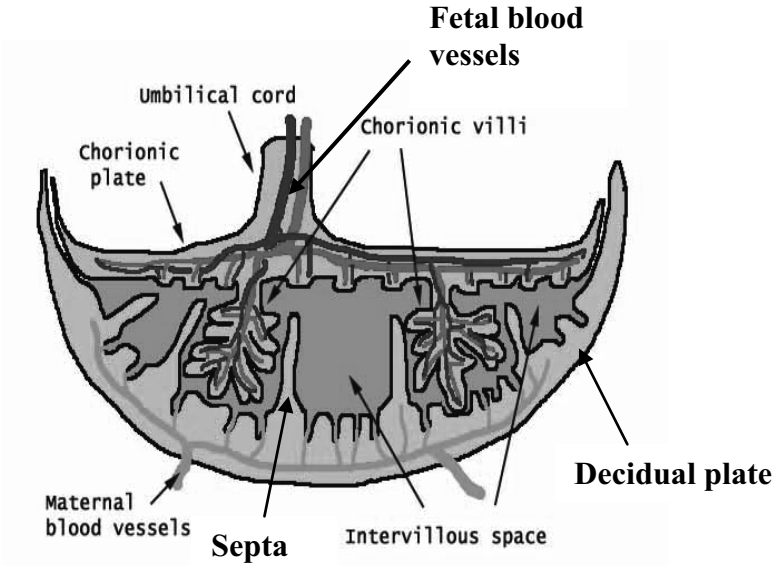



**Fig. 4 The placenta. Image copyright 2007 by David G. King. Reprinted with permission.**

Cell division and fetal growth depend on nutrients and oxygen. The major function of the placenta is to provide the fetus with nutrients and oxygen from maternal blood and to eliminate carbon dioxide and other excretory products from fetal blood by transferring them to the maternal blood. The placenta is also a highly metabolically active organ, synthesizing, for example, eicosanoids from AA (49), even though FA synthesis in placental tissue is low (50).

The placenta grows throughout gestation, concomitant with or ahead of fetal growth (51), leading to a positive correlation between placental weight and birthweight (52). Brief periods of fetal undernutrition during the critical period of organ development in the first trimester of pregnancy may permanently reduce the number of cells in the organ, and decrease fetal growth (53). This has been called fetal programming (54), and according to the Barker hypothesis it might be translated into pathology later in the life of the individual (55). Low birthweight has been associated with disturbed lipid metabolism, abnormal response of insulin to glucose, and disturbed immune function (56-59). Furthermore, low birthweight in combination with a high placental weight (a high placental weight to birthweight ratio) has been associated with chronic hypertension and cardiovascular disease in adulthood (56, 57, 59, 60), while low placental weight has been associated with hypertension in combination with type 2 diabetes (60). Controlled trials on animals have confirmed that maternal nutrition is critical in early and mid-pregnancy, with undernutrition leading to increased risk of pathology at a later stage of the life of the animal (61, 62).

## 2.1 Fatty acid transport

All FA can readily cross lipid bilayers like those separating the maternal and fetal blood streams in the placenta. FA transfer can occur by simple diffusion but is further facilitated by fatty acid transfer proteins (FATP) and fatty acid binding proteins (FABP), such as the plasma membrane fatty acid binding protein (FABPpm) (reviewed in (63, 64)). These proteins have been found on both the maternal and the fetal side of the placenta (reviewed in (65)). In addition, there is an FABP found exclusively on the maternal facing membranes of the human placenta (p-FABPpm) that preferentially binds DHA and AA, compared with LA and the MUFA oleic acid (18:1n-9) (66-69). The proportions of DHA and AA are significantly higher on the

fetal than the maternal side, regardless whether you compare plasma PL (70, 71), triglycerides (TG) (72, 73), cholesterol esters (73), non-esterified fatty acids (NEFA) (74), or RBC total lipids (71, 75). NEFA are the major source of FA for transport over the placenta (49, 76), and the concentration of NEFA increases in maternal plasma in the third trimester of pregnancy (77). Lipoprotein receptors (78-80) and lipoprotein lipases (81, 82) on the microvillous membrane of the placenta act to derive NEFA from circulating TG. RBC lipids may also act as a reservoir of LCPUFA for placental transfer (75, 83). Most studies indicate that little if any synthesis of LCPUFA takes place within the placenta (2, 66, 76, 84), while synthesis of AA was found in one study (85). DHA is selectively transferred from the mother to the fetus through the placenta, but AA is in part used within the placenta for synthesis of eicosanoids (49, 66, 69, 86). Based on autopsy of premature infants that died within 3 days of birth, it was estimated that the whole body accretion of n-3 LCPUFA was 50 mg/kg body weight/day in the third trimester (87). Arachidonic acid accretion mainly occurs postnatally (88, 89). The maternal diet is the most important determinant of LCPUFA availability to the growing fetus (90-92), with a change in FA composition of plasma and RBC lipids profoundly affecting the delivery of n-3 and n-6 LCPUFA to the fetus (69, 93).

The bulk of DHA and AA transported over the placenta to the fetus is stored in fetal adipose tissue, to be mobilized postnatally, probably to support postnatal brain growth and retinal development (94). In spite of both adipose stores (95) and liver synthesis (96, 97), the concentration of DHA in the human infant brain is dependent on intake of preformed DHA (16, 98). If the neonates diet containes no preformed DHA, the DHA stores are used up in the first two months after birth (95). Intake of preformed AA does not seem to affect the brain concentration (16), probably because the synthesis of AA is more effective than the synthesis of DHA.

## 3 Pregnancy outcome

In the Faeroe Islands, fish intake is higher, duration of gestation longer, and birth weight higher than in Denmark (99, 100). The hypothesis that intake of marine fat might increase birth weight by prolonging gestation, was based on the physiological role of prostaglandins in parturition (reviewed in (101)). With increased dietary intake of marine food, the balance between n-6 LCPUFA derived prostaglandins and

those derived from n-3 LCPUFA would change, from the biologically more active to the biologically less active, and also from parturition-stimulating to parturition-inhibiting prostaglandins (100).

## 3.1 Duration of gestation

Longer duration of gestation (100) and a lower risk of preterm birth (102) has been found in observational studies among women with moderate to high intake of marine food during pregnancy, compared with women with low intake of marine food. Duration of gestation was positively associated with the ratio of n-3 LCPUFA to AA in maternal RBC (103, 104), and with DHA status of term (70, 105) and preterm (106, 107) neonates. In some studies feeding animals with n-3 LCPUFA, duration of gestation was longer than in control groups. Pregnant rats given fish oil had longer duration of gestation than control rats (108). Induced preterm delivery in pregnant sheep was delayed or reverted with intravenous fish oil emulsion (109). Some human controlled intervention trials where n-3 LCPUFA supplements were given in later half of pregnancy have shown longer duration of gestation (110, 111), and others have shown a reduced risk of preterm delivery (112), at least in high-risk singleton pregnancies (113, 114). Two studies beginning supplementation between the 15$^{th}$ and 20$^{th}$ week of pregnancy found no difference between the intervention and control groups, but both found DHA in cord plasma PL to be positively related to duration of gestation independent of supplement group (115, 116). The amount of n-3 LCPUFA in the supplements was highly variable between the studies, ranging from 0.1 g/d (112) to 2.7 g/d (110, 113, 114). An effect was seen when the lowest (111, 112) and highest (110, 113, 114) amounts were given, but no clear effect when the amount was 0.2 (116) or 1.2 (115) g/d. The background intake of fish was also highly variable, with high background intake probably blunting the effect of supplementation in some of the studies. Seeing an increase in maternal status of n-3 LCPUFA does not guarantee an increase in the fetal or neonatal status. Supplementation with 0.2 g/d n-3 LCPUFA from the 15$^{th}$ week of pregnancy increased the proportion of DHA in maternal RBC by 20% compared with control, but failed to affect the DHA status of the neonate (117). n-3 LCPUFA supplementation from 20 weeks of pregnancy to delivery providing 3.4 g/d DHA +

EPA, increased both maternal and neonatal DHA status, compared with control, and the maternal DHA status remained elevated at six weeks post-partum (118).

## 3.2 Neonatal birth dimensions

Higher birthweight, independent of duration of gestation, has been found in observational studies among women with moderate to high, compared with low intake of marine food during pregnancy (99, 119-121). Positive correlation has also been found between preterm infant birthweight and n-3 and n-6 LCPUFA status in plasma choline phosphoglycerides (106) and in umbilical vessel walls (107, 122). A recent study found term infant birthweight and head circumference to be positively correlated with the DHA status in maternal plasma PL, especially early in pregnancy (123). The same study found birthweight and crown-heel length to be inversely correlated with AA and dihomo-γ-linolenic acid (DGLA, 20:3n-6) status in maternal plasma PL late in pregnancy and at delivery (123). In contrast, one study found the decrease in proportions of AA and DHA in maternal plasma PL during pregnancy, and the proportions of AA and DHA in umbilical plasma PL to be inversely correlated with birthweight of the neonate (124). Controlled intervention trials where fish oil was given to pregnant rats (108), and studies on pregnant women from week 15 (116), week 18 (115), week 20 (112, 113), week 30 (110), or in the third trimester (111) have failed to show a difference in birthweight adjusted for duration of gestation, between intervention and control groups. It has been speculated that increased birthweight is either caused by fish chemicals other than n-3 LCPUFA, or that n-3 LCPUFA assert their effect before 15 weeks of pregnancy (102).

Since n-3 LCPUFA supplement intake is substantial in Iceland, it was relevant to investigate the relationship between n-3 LCPUFA intake both early and late in pregnancy and pregnancy outcome among women in Iceland.

# 4 Other lifestyle factors

## 4.1 Tobacco and alcohol

Tobacco use and harmful and hazardous alcohol use are among the major preventable risk factors for non-communicable diseases (125). The prevalence of smoking has decreased in Iceland in the last two decades, while total alcohol consumption has increased, with a shift from intake of strong spirits to intake of wine and beer (126). Smoking and alcohol consumption during pregnancy present health risks for the unborn child. It has repeatedly been shown that maternal smoking is related to fetal growth retardation (52, 127-130), and some studies have found a relationship between maternal smoking and spontaneous abortion (131) and preterm delivery (132). Heavy (133-136) and moderate (133, 137) maternal alcohol consumption has been associated with fetal growth retardation. Heavy drinking during pregnancy has also been associated with other adverse pregnancy outcomes, such as multiple congenital anomalies and the fetal alcohol syndrome (138, 139). PUFA are highly labile and susceptible to oxidative damage (140), and cigarette smoking has been shown to increase lipid peroxidation (141). Agostoni and colleages (142) recently reported that maternal smoking is associated with a reduction in LCPUFA pools in infants, and this may have structural and functional consequences. If this is the case, it is plausible that one of the reasons for a negative impact of active and passive maternal smoking on birth outcome (143) and postnatal survival and health (144) may be an insufficiency of LCPUFA during the critical period of fetal and neonatal growth and development.

## 4.2 Oral contraceptives

Studies have shown that when $^{13}C$ labeled ALA is given to human subjects, endogenous biosynthesis of DHA is higher in women (11) than men (10). Furthermore, when women and men consumed the same rigidly controlled diet for three weeks, containing ALA but no n-3 LCPUFA, the women had a higher level of DHA in plasma cholesteryl esters than the men at the end of the study (145). The stimulating effect was believed to be mediated by oestrogen, since synthesis of DHA tended to be higher in women using oral contraceptives compared to those not using

oral contraceptives (11, 145). Oestrogen therapy resulted in a significant increase in the proportion of DHA in plasma cholesteryl esters of male to female transsexuals, while testosterone therapy resulted in a parallel decrease in plasma estradiol and DHA of female to male transsexuals (145).

### 4.3 Physical activity

Studies have shown that heavy endurance exercise increases both oxidative stress (146, 147) and hemolysis (148), leading to lipid peroxidation and RBC membrane rupture. Exercise-induced oxidative stress and hemolysis is partly counteracted by increased antioxidant activity (146) and increased fluidity of the RBC membranes (149, 150). The double bonds of PUFA are vulnerable to lipid peroxidation (140), but DHA is known to increase membrane fluidity (151-153).

In observational studies, it is important to monitor potential confounding factors. Investigating lifestyle factors like tobacco and alcohol use and physical activity of both pregnant and non-pregnant women, and oral contraceptive use by the non-pregnant women was therefore relevant to our study.

## 5 Red blood cells (RBC)

Mature RBC are non-nucleated biconcave discs that carry oxygen from the lungs to every cell in the body and carbon dioxide from every cell to the lungs where it is exhaled (154). RBC are produced in the bone marrow, with the help of many substances, such as iron, vitamin $B_{12}$, folate, manganese, cobalt, vitamin C and vitamin E (154). Anemia can therefore have several different causes, but iron-deficiency anemia is the most common, especially among pregnant women (155). After a number of cell divisions the pronormoblast loses its nucleus, ribosomal RNA and protein synthetic apparatus and becomes a mature RBC. Mature RBC generate energy through the anaerobic, glycolytic pathway, as they have no mitochondrions. Before protein synthesis stops, the cytoplasm is filled with hemoglobin, an oxygen-carrying protein that contains iron atoms (154). RBC circulate in the blood for 120 days. By then the RBC membrane has lost its elasticity and is easily ruptured. The ruptured cells are taken up by macrophages in the spleen, liver and bone marrow and

degraded (154). During pregnancy, extra oxygen and nutrients are delivered from the maternal to fetal blood. This is met by a 50% increase in plasma volume and an increased rate of production of RBC. The increase in RBC production is proportionately less than the increase in plasma volume, leading to a 12 to 20% decrease in hemoglobin concentration during normal pregnancy, a condition called hemodilution (156). Nevertheless, the total hemoglobin amount is greater during pregnancy than in the non-pregnant state (155).

Mature RBC cannot synthesize lipids de novo. All of the cell lipids are in the membrane, and if some of them are lost, they have to be compensated for by interchange with the plasma. A change in diet changes FA composition of RBC membranes, but the change takes from 10 days to four or even six weeks (157, 158). The PUFA composition of RBC membrane lipids is a good indicator of dietary PUFA intake over the preceding three months (157, 159-161). Using RBC PUFA as a biomarker of PUFA status for the women in the study was therefore considered the best choice. FA composition of RBC has been used as a biomarker of disease risk (37, 162), and it has been suggested that maternal RBC may act as a source of n-6 and n-3 LCPUFA for placental transfer to the fetus (75, 83). Investigating the relationship between the RBC status of n-3 LCPUFA, lifestyle and outcome of pregnancy was therefore relevant.

## 6 Lipid peroxidation

Reactive oxygen species (ROS) are constantly produced from molecular oxygen in living cells (163). The ROS, superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide ($H_2O_2$), cannot by themselves initiate lipid peroxidation in the concentration produced *in vivo*. However, a trace of transition metals such as iron can catalyze the formation of the highly reactive hydroxyl radical ($OH^{\cdot}$) by the Fenton and the Haber-Weiss reactions (Fig. 5) (154, 164). The hydroxyl radical will immediately react with any organic compound, and in order to initiate peroxidation of membrane lipids, it has to be produced near the membrane (154).

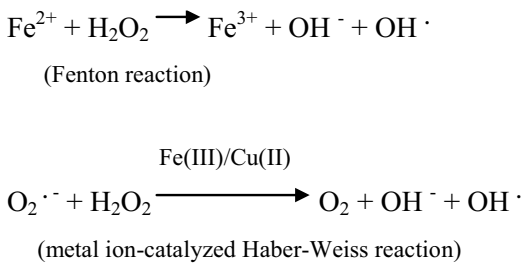



**Fig. 5 The Fenton and the Haber-Weiss reactions**

The lipid peroxidation process can be divided into three steps: initiation, propagation and termination (Fig. 6). Hydroxyl radical, other ROS and several iron-oxygen complexes initiate lipid peroxidation by abstracting an H · from a $–CH_2$- group in an FA chain, forming a carbon-centered lipid radical (R ·) and starting a chain of reactions (reaction 1, Fig. 6).

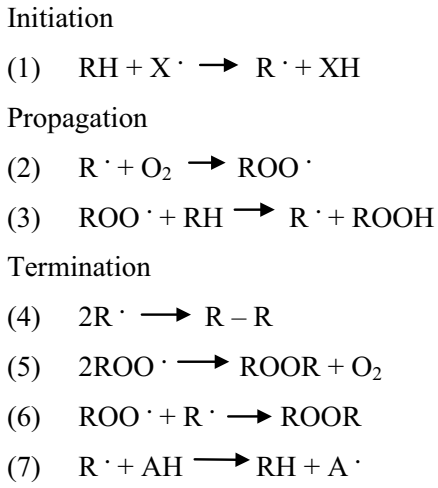



**Fig. 6 The initiation, propagation and termination of lipid peroxidation. RH, unsaturated FA; X ·, free radical; R ·, carbon-centered lipid radical; ROO ·, peroxyl radical; ROOH, lipid hydroperoxide, AH, antioxidant; A ·, antioxidant radical.**

The carbon radical thus formed is usually stabilized by a molecular rearrangement to a conjugated diene (Fig. 7). The diene rapidly reacts with molecular oxygen to form a peroxyl radical (ROO ·, reaction 2, Fig. 6). The peroxyl radical attacks the adjacent FA chain in the lipid molecule, abstracting another hydrogen atom to produce a lipid hydroperoxide (ROOH) and a new carbon radical, ready to react with another oxygen molecule, leading to a chain of reactions (reaction 3, Fig. 6). The hydrogen atom in the methylene group placed between two

vinylgroups (Fig. 7) in a PUFA is the most vulnerable to attack from the peroxyl radical.

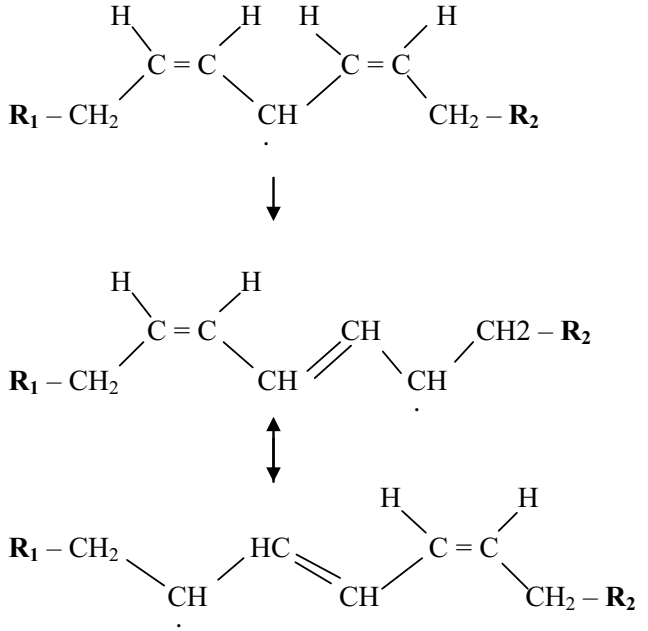



**Fig. 7 Carbon radical and formation of a conjugated diene.**

Lipid peroxides are comparatively stabile, but are however rapidly degraded in the presence of transition metals and transition metal complexes like free heme or hemoglobin. There are two types of reactions that can terminate the peroxidation process. Either two lipid radicals combine to form a molecule with an even number of electrons (reactions 4-6, Fig. 6), or the lipid radical receives an electron from another molecule, an antioxidant (AH). All cells that live in the presence of oxygen must have an antioxidant defence system to trap ROS before they harm the membrane or other organelles or molecules in the cell. When an antioxidant gives away an electron a new, more stabile radical (A ·) is formed that will not propagate the chain reaction (reaction 7, Fig. 6). Vitamin E is the predominant lipophilic antioxidant and probably the most important inhibitor of lipid peroxidation. It tends to concentrate in the interior of membranes and competes with the PUFA for the peroxyl radicals (165). α-Tocopherol, the most active form of vitamin E, gives hydrogen atoms four times more rapidly to peroxyl radicals than the acylchain (154). The tocopheryl radical that is formed during α-tocopherols action as a chain-breaking

antioxidant is recycled to α-tocopherol by the hydrophilic antioxidant vitamin C (ascorbic acid) (154, 166). Thus the redox status of the cell is maintained.

## 6.1 RBC PUFA stability

The RBC membrane is rich in PUFA, and a mature RBC is filled with hemoglobin (154). RBC contain various enzymes and antioxidants that act either as free radical scavengers or inhibitors of free radical propagation reactions (154). These scavengers and inhibitors prevent oxidation very efficiently in a healthy individual (154), but when RBC are removed from the body and frozen to at least -20°C, enzyme activity decreases substantially (167). However, enzymes are seldom totally inactivated by freezing (168). Due to high susceptibility of PUFA to peroxidation initiated by free radicals, storage condition of RBC samples is critical in studies on FA composition of RBC lipids. At centres for health service, isolated RBC samples may be frozen slowly to –20°C or –30°C. Slow freezing produces a few, large ice crystals and clusters of ice crystals that can rupture membranes, while rapid freezing produces more numerous small ice crystals, leaving the membrane intact (167). Rupture of RBC membranes releases otherwise sequestered iron, making it available for catalyzing free radical reactions (154).

When temperature falls from the freezing point of water to -20°C, ice formation takes place. Density, thermal conductivity, specific heat, enthalpy and latent heat change (169), but if cells are stored at or below -20°C, these variables should be nearly constant, even though a proportion of the water in the cells is unfrozen at this temperature (169). Below -30°C ice formation is very slow, but there is a layer of unfrozen water molecules around every protein and next to the membrane, even at very low temperatures (170). This allows diffusion of substrates and enzymes, and it has been demonstrated that chemical reactions do take place in frozen cells (167). Crystals are usually made of pure water, which means that solutes are expelled from the area of the crystal. As more water freezes, the concentration of solutes in the liquid phase increases, which lowers the freezing point. Even though reaction rate decreases when temperature falls below initial freezing point, increasing solute concentration in the liquid phase opposes this and increases the reaction rate (167). The concentration of oxygen seems to matter for the stability of RBC PUFA, as venous RBC stored at -20°C without addition of antioxidant were stable for four

weeks, while capillary RBC were not (171). Duration of storage matters, as a lower proportion of LCPUFA was found in venous RBC stored for six months at -20°C without addition of antioxidant than at baseline (172). The physiological or pathological state of the individual can also affect the stability of PUFA in RBC. PUFA in RBC from schizophrenic (173) and autistic (174) individuals have less stability during storage at -20°C than PUFA in RBC from healthy controls. The reason for less stability is not known, but antioxidant status might possibly be affected by these pathological states. The stability of PUFA in RBC has been shown to depend on the status of antioxidants naturally present in the RBC (175). Artificial antioxidants can also be added to RBC samples before storage, and butylated hydroxytoluene (BHT) has been shown to preserve the PUFA composition of RBC samples from healthy individuals for one year at -50°C (171) and for two years at −80°C (172).

Results from studies examining the vulnerability of individual PUFA to peroxidation are inconsistent. In RBC samples containing ethylene diamine tetra-acetate (EDTA) as anticoagulant, n-3 and n-6 LCPUFA were more stable than less unsaturated PUFA during two-day transport at room temperature (176). Some studies have shown that n-3 PUFA are more prone to *in vitro* oxidation than n-6 PUFA (152, 177), while others have found AA and the other n-6 PUFA to be particularly vulnerable to autoxidation during frozen storage (171). RBC are best stored with added antioxidant at -70°C or -80°C, but there are many unanswered questions concerning stability of RBC PUFA during storage at a higher temperature, with or without addition of antioxidant. It was therefore relevant to investigate the stability of RBC at -20°C, with and without addition of the antioxidant BHT.

## 6.2 Residual adjustment

In free-living human populations, variation in total energy intake, and consequently in intake of many nutrients, is related to variation in physical activity, body size and metabolic efficiency. The variation represented in dietary studies is further increased by the well-known problem of underreporting of food intake. Dietary intake of many nutrients is strongly positively correlated with total caloric intake, even though the correlation is often non-linear. Bearing this in mind, Walter Willett and coworkers (178, 179) designed an adjustment method where nutrient intake was adjusted for

total energy intake. Energy adjustment controls for the effect of inter-individual differences in physical activity, body size or metabolic efficiency. Energy adjustment also makes it possible to include all the participants in the data analysis, instead of excluding the low energy reporters. Low energy reporters are unevenly distributed across employment grades, which causes selection bias if they are excluded (180, 181). The basis for the residual adjustment method is the strong, positive correlation between total energy intake and intake of most nutrients. However, nutrients that affect the central nervous system, which is uncorrelated with body size, and nutrients whose metabolism is unaffected by physical activity, are usually not correlated with energy intake. Both may be the case for n-3 LCPUFA. If so, the best approach might be to use absolute intake, instead of adjusting for the caloric intake (178). To deal with our problem with partly peroxidized PUFA in RBC samples stored inappropriately, the adjustment method of Walter Willett was modified for use on the FA data, in order to adjust for the effect of peroxidation.

# AIMS OF THE STUDY

Even though the importance of n-3 LCPUFA for women of reproductive age and their contribution to fetal growth have been studied extensively over the last few decades, we have still a long way to go. During the first few months of pregnancy placental development takes place, and the fetal organs are developing with a high rate of cell division. Later in pregnancy the fetus undergoes a growth spurt with a high rate of placental transfer of nutrients. Low birthweight, high placental weight and increased placental weight to birthweight ratio have been associated with increased risk of hypertension and cardiovascular disease in adult human life. Higher birthweight has been found in observational studies among women with moderate to high compared to low intake of marine food during pregnancy. This association has, however, not been confirmed in controlled intervention studies where pregnant women have been supplemented with n-3 LCPUFA from the 15$^{th}$ week of pregnancy or later.

The overall aim of the study was to add to knowledge of n-3 LCPUFA in diet and RBC of pregnant and non-pregnant women in Iceland, and how it relates to lifestyle and pregnancy outcome. The specific aims of the thesis were:

1. To determine whether there is a relationship between n-3 LCPUFA in RBC during healthy pregnancy and dietary n-3 LCPUFA, other lifestyle factors and pregnancy outcome.

2. To determine whether there is a relationship between n-3 LCPUFA in RBC and dietary n-3 LCPUFA and other lifestyle factors among healthy non-pregnant women of reproductive age.

3. To investigate the stability of PUFA in RBC from healthy, non-pregnant women during storage at -20°C, with and without addition of the antioxidant BHT.

4. To modify an adjustment method designed for nutritional data analysis for use in analyzing FA data from partly lipid peroxidized RBC.

# MATERIALS AND METHODS

## 7 Study design

The study consisted of three main parts: 1) A study of dietary intake, RBC FA composition, lifestyle and pregnancy outcome of healthy and low-risk pregnant women early and late in pregnancy (Cohort 1). 2) A study of dietary intake, RBC FA composition and lifestyle of healthy non-pregnant women of reproductive age (Cohort 2). 3) A study of the stability of RBC PUFA from healthy, non-pregnant women during storage (Cohort 3).

### 7.1 Pregnant women (Cohort 1)

The participants were 549 healthy and low-risk pregnant women who were enrolled during a routine first visit, in the 11$^{th}$ to 15$^{th}$ week of pregnancy, to the Center of Prenatal Care in Reykjavik, Iceland, during the period 1999 to 2001. The women answered a semi-quantitative food frequency questionnaire (FFQ) and a questionnaire on other lifestyle factors in the 11$^{th}$ to 15$^{th}$ week and again in the 34$^{th}$ to 37$^{th}$ week of pregnancy. Information on pregnancy outcome was collected from maternity records. Results from this study have been published previously (127, 182, 183). The first 176 women enrolled gave a blood sample at the same two time points that they answered the questionnaires. RBC were isolated and FA composition analyzed (Fig. 8). These 176 women were all singleton pregnant. Paper I includes data from all 549 pregnant women. Paper II and Paper V include data from the first 176 women enrolled in the larger study. The National Bioethics Committee of Iceland approved the study and the Icelandic Data Protection Commission was notified of the processing of personal data. The women gave informed consent for their participation in the study.

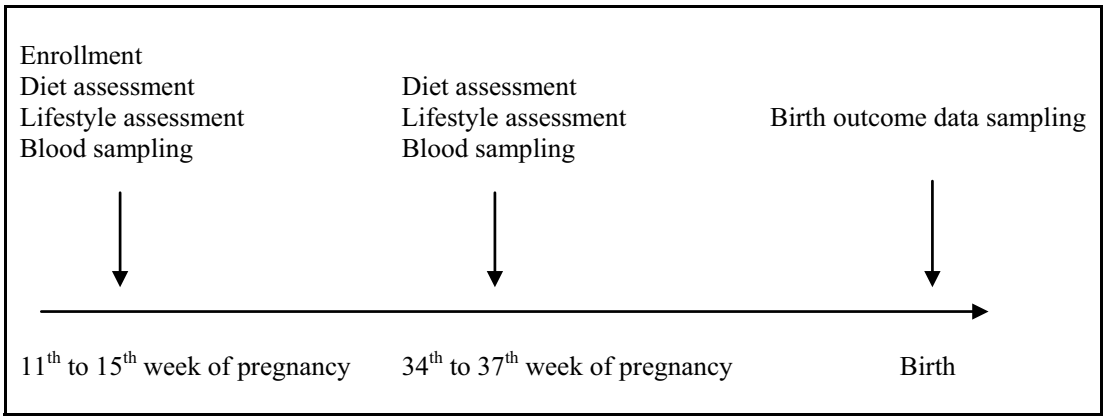



**Fig. 8 Design of the study on the pregnant women early and late in pregnancy.**

## 7.2 Non-pregnant women (Cohort 2)

The participants were 45 healthy, non-pregnant women of reproductive age. They were enrolled during a visit to the Centre for Child Health Services in Reykjavik, Iceland, for vaccination of their 18-month-old children during the years 2001 and 2002. None of the women in Cohort 2 participated in Cohort 1. The women answered the same FFQ as the pregnant women and a similar lifestyle questionnaire without pregnancy-related questions. Blood samples were collected from the women, RBC isolated and FA composition analyzed. Paper III includes data from Cohort 2. The National Bioethics Committee of Iceland approved the study, and the Icelandic Data Protection Commission was notified of the processing of personal data. The women gave informed consent for their participation in the study.

## 7.3 Stability of RBC PUFA during storage (Cohort 3)

The participants were 13 healthy, non-pregnant women, aged 25 to 55 years, who volunteered to participate in the study. Blood samples were collected and RBC isolated. The RBC sample from each participant was divided into seven portions: one baseline sample with, and another without addition of the antioxidant BHT, samples without BHT stored at -20°C for 2, 4, 9 or 17 weeks, and one sample with BHT stored at -20°C for 17 weeks (Fig. 9). Paper IV includes data from Cohort 3.

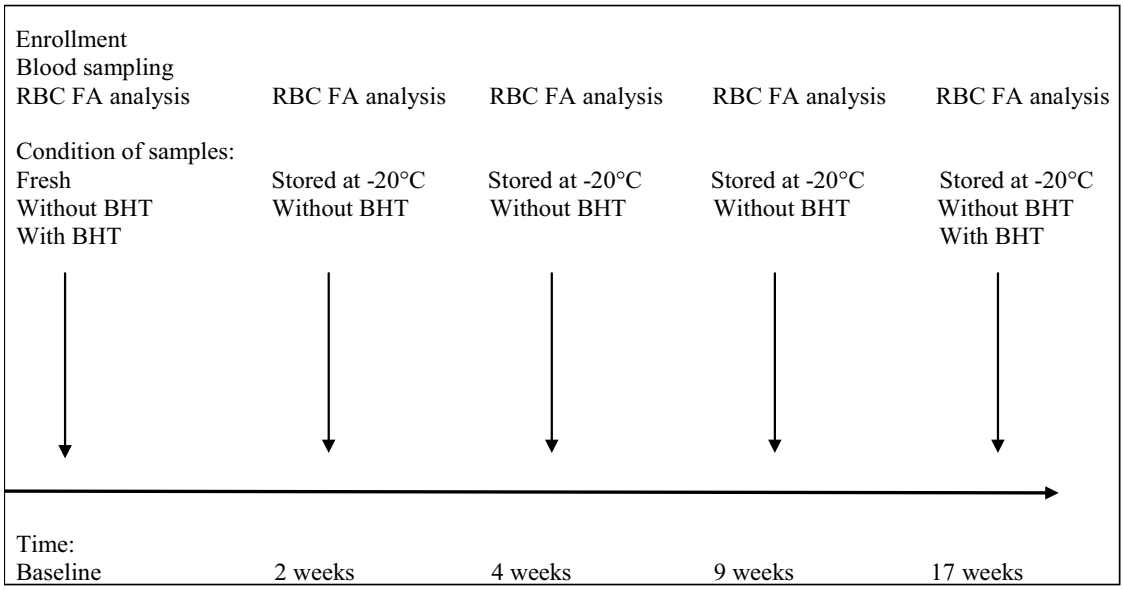



**Fig. 9 Design of the study on the stability of RBC PUFA during storage.**

# 8 Criteria for exclusion and missing data

One hundred and thirteen women were excluded from Cohort 1 and two from Cohort 2 due to withdrawal or health problems that came up or were discovered after enrolment (Table 1). All of the women in Cohort 3 were healthy. In some figures and tables, there were fewer data points because of missing data or because of technical accidents making a part of the FA data from RBC unusable.

## 8.1 Missing data and technical accidents

A few women did not fill in one or both questionnaires or did not give a blood sample at either time point (usually the second). Some women did not answer all questions in the lifestyle questionnaires. Furthermore, data on head circumference and/or placental weight were lacking in several maternal records (Table 1).

No antioxidant was added to the RBC samples from the pregnant women in Cohort 1 before being frozen at -20°C. All of the RBC samples were analyzed within 20 weeks. Fifty-six of the RBC samples from 11$^{th}$ to 15$^{th}$ week of pregnancy and 80 RBC samples from 34$^{th}$ to 37$^{th}$ week of pregnancy were considered lipid peroxidized. This data was either adjusted for total FA concentration (Paper V) or excluded (Paper II, Paper V, and other results from Cohort 1 included only in this

thesis). An accident in dilution of the internal standard made it impossible to calculate total FA concentration for 38 RBC samples from 11$^{th}$ to 15$^{th}$ week of pregnancy and for 14 samples from 34$^{th}$ to 37$^{th}$ week of pregnancy. Therefore, adjustment for total FA concentration was not possible for these RBC samples, and they were excluded from Paper V. The RBC sample from one woman in Cohort 3 was lost (Paper IV). Unless otherwise stated (Paper V), there was no significant difference detected between the women who were included and those who were excluded from the analyses.

**Table 1 Criteria for exclusion of participants and missing data.**

| | Paper I (Cohort 1) | Paper II (Cohort 1) | Paper III (Cohort 2) | Paper IV (Cohort 3) | Paper V (Cohort 1) | Thesis[a] (Cohort 1) |
|---|---|---|---|---|---|---|
| Number enrolled | 549 | 176[b] | 45 | 13 | 176[b] | 176[b] |
| Twins or triplets | 5 | | | | | |
| Hypertension/Preeclampsia/Intrahepatic cholestasis | 62 | 14 | | | 14 | 14 |
| Diabetes mellitus (gestational or chronic) | 4 | 3 | | | 3 | 3 |
| Preterm birth (≤ 259 d) | 17 | 4 | | | 4 | 4 |
| Intruterine growth retardation (> 259 d and < 2500 g) | | 1 | | | 1 | 1 |
| Miscarriage or stillbirth | 17 | 6 | | | 6 | 6 |
| Anaemia | | 4 | | | 4 | 4 |
| Oligohydramnion/polyhydramnion | | 2 | | | 2 | 2 |
| Moved abroad before giving birth | 8 | 1 | | | 1 | 1 |
| Variables missing for analysis | | 19[c] | 3 | | 4 | 4 |
| Technical accidents | | 45[d] | | 1 | 36[e] | 54[d] |
| Essential hypertension | | | 2 | | | |
| Total number of participants excluded | 113 | 99 | 5 | 1 | 75 | 93 |
| Participants participating (number, (%)) | 436 (79%) | 77 (44%) | 40 (89%) | 12 (92%) | 101 (57%) | 83 (47%) |

[a]Other figures and tables in the thesis.

[b]Papers II and V, and other figures and tables in the thesis, include data from the first 176 women enrolled in Cohort 1.

[c]Most of the women had missing data on placental weight.

[d]Those RBC samples were considered peroxidized.

[e]Data on total FA concentration in RBC were missing.

# 9 Analysis of red blood cell fatty acids

## 9.1 The blood samples

Non-fasting venous blood samples were collected in Vacuette® tubes containing EDTA. RBC were isolated immediately by centrifuging whole blood at 3000 rpm for 10 min at 4°C. They were then washed three times with isotonic saline solution. The RBC samples from Cohorts 1 and 2 were divided into two portions before they were frozen at -20°C and stored. No antioxidant was added to the RBC samples from Cohort 1 before they were frozen at -20°C. The antioxidant BHT dissolved in methanol (500 mg/L) was added to the RBC samples from Cohort 2 at a final concentration of 10-20 mg/L before they were frozen at -20°C. The duplicates were lipid-extracted and their FA composition analyzed on the same or adjacent days within 20 weeks from collection of the blood sample. The RBC samples from Cohort 3 were divided into seven portions, and BHT was added to defined portions at a final concentration of 42 mg/L. Baseline portions with or without BHT were lipid-extracted within three hours of collection, and FA composition analyzed. The other portions were frozen at -20°C and stored until FA analysis after a defined time interval.

## 9.2 RBC total lipid extraction

Total RBC lipids were extracted with isopropanol/chloroform (2:1 v/v, Merck, Darmstadt, Germany) as described by Bligh & Dyer (184), except that isopropanol was used instead of methanol. The antioxidant BHT was added to the extraction medium at a final concentration of 50 mg/L. The procedure of lipid extraction and FA analysis of RBC is shown in Fig. 10. Thawed RBC samples were transferred to teflon-lined, screw-capped glass test tubes and hemolyzed with distilled water. Isopropanol was added to the RBC samples (10:1 v/v) and agitated for 45 min at room temperature. Chloroform was added to the mixture (isopropanol: chloroform 2:1 v/v). Phosphatidylcholine, diheptadecanoyl (PC 17:0) (Sigma Chemical Co., St. Louis, MO) was used as an internal standard to monitor recovery. After agitating for 45 min at room temperature, the samples were centrifuged at 1700 rpm for 30 min at

20°C. The sediment contained heme and other proteins, and the supernatant contained total lipids and solvents (monophasic). The supernatant was transferred to another teflon-lined, screw-capped test tube, chloroform was added (isopropanol: chloroform 1:1 v/v), and distilled water (isopropanol: chloroform: distilled water 1:1:0.8 v/v/v). After vortexing and centrifuging at 1700 rpm for 15 min at 20°C, the solution became diphasic with the lower phase containing the lipids. The upper phase was removed and discarded and the lower phase transferred to a new teflon-lined screw-capped test tube. The extraction medium was evaporated under a stream of nitrogen at room temperature, leaving the lipids as an oily layer on the test tube walls.

## 9.3 Fatty acid methylation

The RBC total lipids were transmethylated for 45 min at 110°C using 14% boron trifluoride/methanol (Sigma Chemical Co., St. Louis, MO). Heneicosanoic acid (C 21:0) methyl ester (Sigma Chemical Co., St. Louis, MO) was used as an external standard. Before methylation, the samples were flushed with nitrogen to replace oxygen. After transmethylation, the test tubes were chilled at room temperature. The fatty acid methyl esters (FAME) were extracted three times with hexane. The combined portions were evaporated under a stream of nitrogen at room temperature, leaving the FAME as an oily layer on the test tube walls. The lipids were dissolved in isooctane and transferred to gas chromatography (GC) vials, closed tight and stored at –20°C until FA analysis.

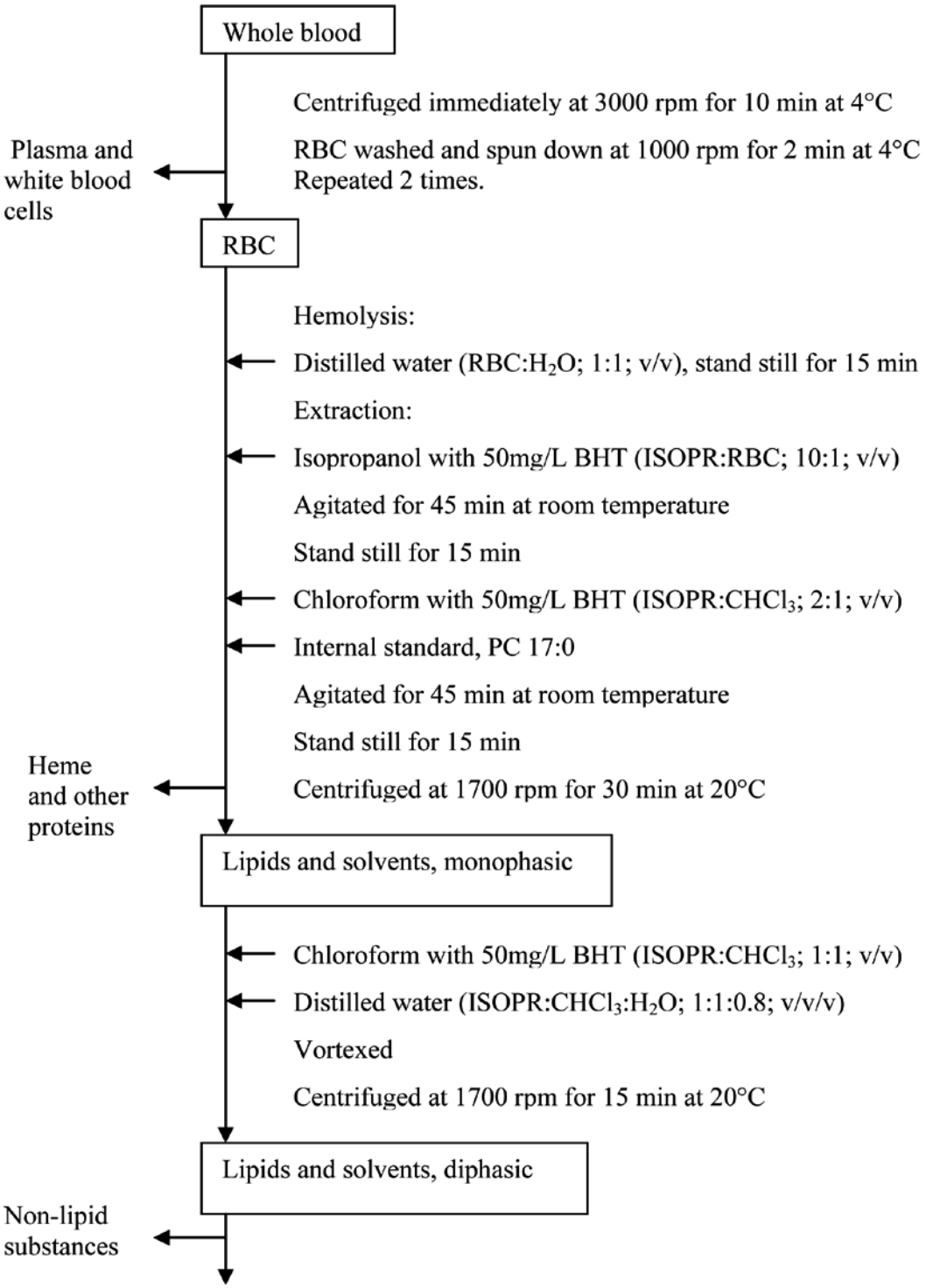
## 9.4 Fatty acid analysis

Samples from Cohort 1 were analyzed by high resolution capillary GC (HP Series II 5890 A, Hewlett Packard co., Palo Alto, CA, USA) equipped with a flame ionization detector and a GC-Capillary polyethylene glycol column from Chrompack (CP-WAX 52CB 0.32 mm inner diameter (i.d.) x 0.2 μm film thickness x 25 m). The injector and detector temperatures were maintained at 235°C and 250°C, respectively. The column temperature was programmed to have an initial temperature of 90°C for 2 min, then rising by 30°C/min to 165°C and at 3°C/min to 225°C and then held isothermal for 6 min. The carrier gas was hydrogen at 31.8 kPa.

Samples from Cohorts 2 and 3 were analyzed by GC (Agilent 6890 N, Agilent, Palo Alto, CA, USA) equipped with a flame ionization detector and a GC-Capillary column (5% phenyl, 95% dimethyl-polysiloxane) from Chrompack (CP-SIL 8CB 0.25 mm i.d. x 0.12 µm film thickness x 25 m). The injector and detector temperatures were maintained at 280°C and 300°C, respectively. The oven temperature was programmed to have an initial temperature of 150°C for 4 min, then rising at 4°C/min to 230°C and at 20°C/min to 280°C and then held isothermal for 4 min. The carrier gas was hydrogen at 110 kPa.

The FAME peaks were identified and calibrated against those of commercial standards (Sigma; Nu-Chek-Prep, Elysian, MN). The RBC values are presented as % of total FA with chain length from $C_{14}$ to $C_{24}$ and as concentration in mg/L RBC. The intraassay coefficient of variation (CV, measurement error based on 50 measurements) between duplicates run on the same column was 5.2% for DHA and 3.9% for AA. The CV between duplicates run on different columns, one on the CP-SIL and the other on the CP-WAX column, was 5.3% for DHA and 1.3% for AA. Since the CV between the two columns was not higher than the CV between duplicates run on the same column, it can all be ascribed to variation from one analysis to the next and not to different columns. Instrumental control and data handling was done by HP 3365 Chemstation, Version A.02.12. (Hewlett Packard co.).

When calculating the total FA concentration (mg/L) of RBC, it was assumed that equal amount of FAME in mg gave rise to equal peak areas on the GC graph. As the amount of internal standard (PC 17:0) added to each RBC sample was known, the amount of FA in RBC could be calculated. When calculating recovery of the RBC FA, it was assumed that the recovery of the external standard (C 21:0 methyl ester) was 100%, and that the recovery of the FA in RBC was similar to that of the internal standard. The recovery was 83.0±5.8% (mean±SD).


Whole blood
Centrifuged immediately at 3000 rpm for 10 min at 4°C
RBC washed and spun down at 1000 rpm for 2 min at 4°C
Repeated 2 times.
Plasma and white blood cells
RBC
Hemolysis:
Distilled water (RBC:$H_2O$; 1:1; v/v), stand still for 15 min
Extraction:
Isopropanol with 50mg/L BHT (ISOPR:RBC; 10:1; v/v)
Agitated for 45 min at room temperature
Stand still for 15 min
Chloroform with 50mg/L BHT (ISOPR:$CHCl_3$; 2:1; v/v)
Internal standard, PC 17:0
Agitated for 45 min at room temperature
Stand still for 15 min
Centrifuged at 1700 rpm for 30 min at 20°C
Heme and other proteins
Lipids and solvents, monophasic
Chloroform with 50mg/L BHT (ISOPR:$CHCl_3$; 1:1; v/v)
Distilled water (ISOPR:$CHCl_3$:$H_2O$; 1:1:0.8; v/v/v)
Vortexed
Centrifuged at 1700 rpm for 15 min at 20°C
Lipids and solvents, diphasic
Non-lipid substances

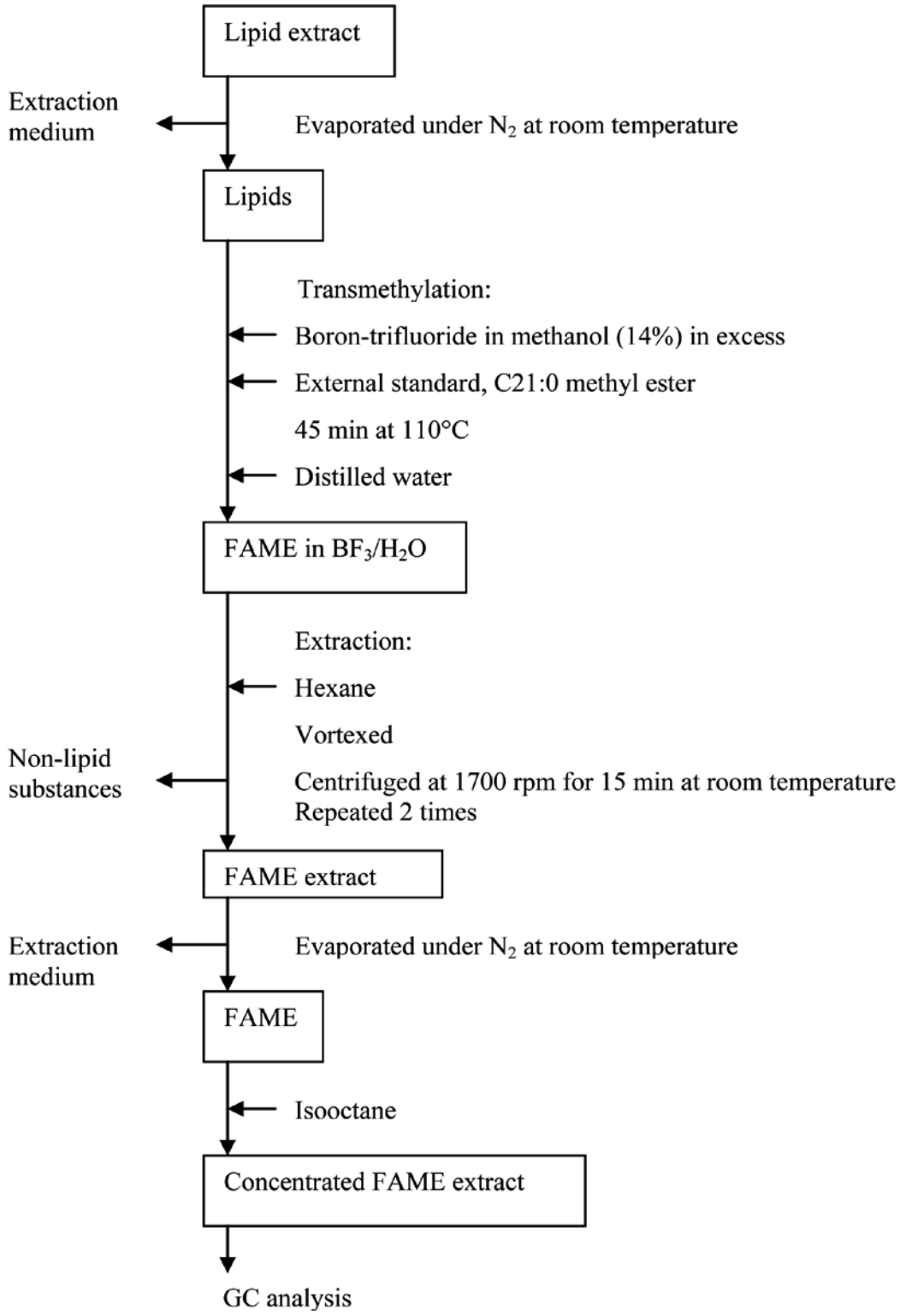



**Fig. 10 The procedure of lipid extraction and FA analysis of RBC. ISOPR, isopropanol; $CHCl_3$, chloroform.**

## 9.5 Modified adjustment method

No antioxidant was added to the RBC samples from Cohort 1 before they were frozen at -20°C. The distributions of the proportions of AA and DHA in RBC from both time points of pregnancy were bimodal, and a strong correlation was found between total FA concentration of RBC and all RBC FA proportions. In contrast, the antioxidant BHT was added to the RBC samples from Cohort 2 before they were frozen at -20°C. The distributions of the proportions of both AA and DHA in their RBC were normal, and no correlation between total FA concentration and FA proportions. The RBC samples from Cohort 1 that were in the lower mode of the bimodal distribution of the proportion of AA (≤9.5% of total FA early in pregnancy, <9% of total FA late in pregnancy) were considered lipid peroxidized.

An adjustment method designed by Willett *et al.* (178, 179) was modified for use on the FA data, to adjust for the effect of lipid peroxidation. The residual adjustment method of Willett *et al*. adjusted nutrient intake for total energy intake. By the modified method, the RBC FA data were adjusted for total FA concentration by calculating the equation for the regression line between each RBC FA proportion and total FA concentration ($y = a + b*x$, where y is the predicted FA proportion for a given total FA concentration, x; a and b are constants). The residuals between the regression line and each FA proportion value were found by subtracting the predicted FA proportions from the obtained proportions, $y' - y = y' - a - b*x$ (where y' is the obtained value and y is the predicted value). Residuals are by definition either negative or positive, and their mean is zero. A constant was added to the residuals to convey the sense of actual FA proportion values. The constant used by Willett and coworkers was the nutrient intake referring to the mean caloric intake of the cohort, assuming that the mean caloric intake of the cohort was the same as the average, true caloric intake of the population. In the FA data analysis of the RBC from Cohort 1, the constant added to the residuals of the regression line was the FA proportion referring to the mean total FA concentration of the higher mode of the bimodal distribution of the proportion of AA, or 1047.08 mg/L early in pregnancy and 1074,26 mg/L late in pregnancy. The constant was calculated from the equation for the regression line, $y_k = a + b*x_m$ (where $y_k$ is the constant and $x_m$ is 1047.08 mg/L or 1074,26 mg/L for early or late pregnancy, respectively). The sum of the residual and

the constant gave the residually adjusted FA proportion value, which was thus standardized to the mean total FA concentration of RBC samples not considered lipid peroxidized.

## 10 Food frequency questionnaire (FFQ)

Dietary intake was estimated using the semi-quantitative FFQ developed by Steingrímsdóttir at the Icelandic Nutrition Council (Appendix I). It was designed to assess the entire diet over the preceding three months. It contained questions about the average frequency of consumption of 130 food items, mixed dishes, beverages and supplements. The participants estimated portion sizes from pictures of three different portions of common food items, and from general household measures. The FFQ were scanned and food and nutritive intake calculated using The Icelandic Nutrition Database (ISGEM) and a nutrient and food calculating program (ICEFOOD). Regarding marine foods, the intake of lean fish, oily fish and fish oil was calculated, as well as the intake of each type of FA. The FFQ has been validated against a reference method and several nutrient biomarkers (185).

## 11 Lifestyle questionnaires

Information on lifestyle, health and socio-economic factors, as well as food choices, was gathered from the pregnant women with two questionnaires in the $11^{th}$ to $15^{th}$ week of pregnancy and in the $34^{th}$ to $37^{th}$ week of pregnancy (Appendix II a and b). When the women returned the questionnaire, the midwives weighed the women, and measured their height and blood pressure. Gestational length at entry was calculated from last menstrual period, reestimated at $20^{th}$ week of pregnancy with an ultrasoundscan and corrected if not in accordance with the date of last menstrual period.

The women in Cohort 2 (18 months post-partum) completed an adjusted version of the lifestyle questionnaires, where pregnancy related questions were left out (Appendix III).

## 12 Pregnancy outcome

Only healthy pregnant women without antenatal and intrapartum complications were included in the study, and all their neonates were born healthy after term pregnancy. The data on pregnancy outcome were collected from maternity records at the Department of Obstetrics and Gynecology, National University Hospital in Reykjavík, Iceland with permission (Appendix IV). The pregnancy outcome variables used in the study were duration of gestation, birthweight, crown-heel length and head circumference of the neonate, and placental weight. Z-scores or standard deviation scores were not calculated, since a reference for birthweight in Iceland was not available. Duration of gestation was based on routine fetal biometry at a 20$^{th}$ week ultrasound examination. The placenta was weighed untrimmed, after washing to remove all clots, and after inspection of the cotyledons and membranes for any missing tissue.

## 13 Statistical analysis

The data were presented as means ± SD, or as percentages (i.e. percent of participants using n-3 LCPUFA supplements). FA values in RBC were presented as percent of total FA and as FA concentrations. Normality of distribution was ascertained with histograms and the Kolmogorov-Smirnov test. Log transformation of skewed distributions did not considerably change correlation coefficients or the significance of statistical tests. Thus, for easier interpretation, all variables were used untransformed. Log contrast models have been recommended instead of habitual regression models for analysis of proportions (186). The reason for this is a methodological problem related to correlational analysis of proportions within the same matrix (187). In the study presented in this thesis, log contrast models and habitual regression models gave similar associations. We chose to use the habitual regression models, since they were easier to interpret.

The two subgroups of women who did not smoke (had never smoked or had quit smoking more than six months prior to study entry) and those who did smoke (who quit smoking less than six months prior to study entry and women who smoked at study entry) (Paper II), women using and not using L-CLO (Paper I), women using and not using n-3 LCPUFA supplements (Paper II) and women in the lower and

higher mode of distribution of the proportion of AA in RBC (Paper V), were compared using independent *t*-test (continuous and normally distributed variables), Mann-Whitney *U*-test (continuous but non-normally distributed variables), Chi-square test (categorical variables with lowest expected count ≥5) or Fisher exact test (categorical variables with lowest expected count <5). The three subgroups of women not using n-3 LCPUFA supplements, using CLO capsules and L-CLO (Paper III) were compared using ANOVA with the Tukey's post-hoc test (continuous and normally distributed variables), Kruskal-Wallis with Bonferroni correction (continuous, non-normally distributed variables), or chi-square test (categorical variables).

Associations between characteristics, FA proportions and concentrations in RBC, dietary intake, other lifestyle and pregnancy outcome were assessed by calculating Pearson's product-moment correlation coefficients and/or Kendall nonparametric correlation coefficients (continuous variables), and the Mann-Whitney *U* test for correlation (categorical variables). Significantly associated variables were combined in linear regression models. Interaction between the independent variables was tested by calculating the correlation of their products and the residuals between the model and the data (188). A logistic regression model was designed for high birthweight and L-CLO intake in early pregnancy in the whole Cohort 1. The odds of healthy women giving birth to an infant of ≥4500 g after a healthy pregnancy were calculated with 95% confidence intervals (95%CI).

Three different methods were used to analyze the RBC FA data from Cohort 1. One method included all the RBC FA data unadjusted (unadjusted-all, U-A), another included unadjusted FA data from RBC samples considered not peroxidized (unadjusted-not peroxidized, U-NP), and the third included all the RBC FA data adjusted for total FA concentration (adjusted-all, A-A). Since data analyzed by the U-A method were non-normally distributed, Wilcoxon signed-rank test was used for comparison of mean proportions of each FA in RBC between data analyzed by the U-A and A-A methods. Paired t-test was used to compare mean proportions of each FA in RBC between data analyzed by he U-NP and A-A methods. Due to multiple comparisons, Bonferroni correction was used. The effect of storage time and added antioxidant, and interaction between storage time and added antioxidant in the RBC FA data from Cohort 3 were evaluated with ANOVA for repeated measures and Tukey-Kramer post-hoc test.

Statistical analyses on the data from Cohorts 1 and 2 were performed using the SPSS program (SPSS 11.0 for Windows), and on the data from Cohort 3 using the SAS program (SAS for Windows, V8). The level of significance was set at $P<0.05$ unless otherwise stated, and two-tailed P values were used.

# RESULTS

## 14 Papers I to III and unpublished results

### 14.1 Relationship between dietary intake of n-3 LCPUFA and n-3 LCPUFA in RBC (Cohorts 1 and 2)

The prevalence of n-3 LCPUFA supplement use (liquid or capsules) was over 40% early in pregnancy (Paper I: Table 1, Paper II: Table 1), over 50% late in pregnancy (Paper I: Table 1), and 38% among the non-pregnant women (Paper III: Table 1). PUFA intake and PUFA proportions in RBC according to use of n-3 LCPUFA supplements can be seen in Table 2 (pregnant women early in pregnancy) and in Paper III: Table 1 and 2 (non-pregnant women). As expected, pregnant and non-pregnant women using L-CLO had higher (P<0.001) intake of DHA and total n-3 PUFA and lower (P<0.001) n-6/n-3 PUFA ratio than women not using n-3 LCPUFA supplements. Pregnant and non-pregnant women using L-CLO had higher (P<0.001) proportion of DHA and total n-3 PUFA and lower (P<0.001) n-6/n-3 PUFA ratio in RBC compared with women not using n-3 LCPUFA supplements. Women using CLO capsules were intermediate in all these variables.

**Table 2 PUFA intake and PUFA proportions in RBC from pregnant women early in pregnancy according to use of n-3 LCPUFA supplements.**

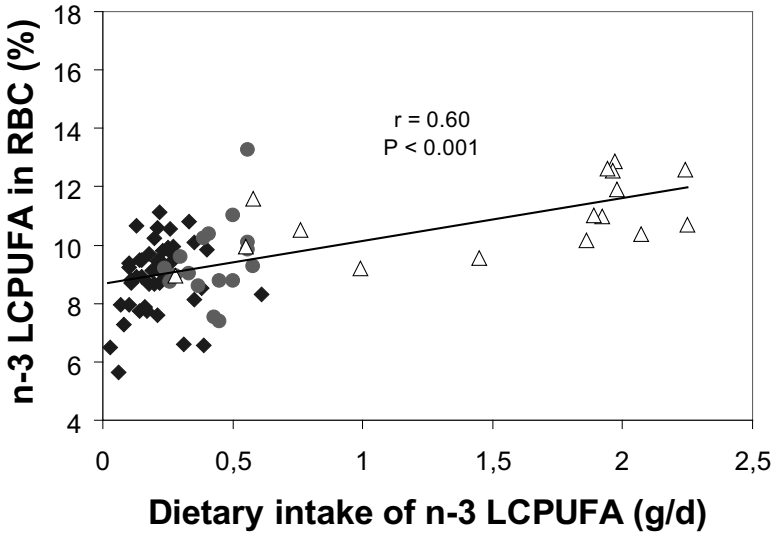
| | No n-3 LCPUFA supplements *n* =49 | Cod liver oil capsules *n* =18 | Liquid cod liver oil *n* =16 | Main effects P |
|---|---|---|---|---|
| Dietary intake | | | | |
| DHA (mg/d) | 127.8 ± 70.7[a] | 255.6 ± 64.2[b] | 926.9 ± 314.9[c] | <0.001 |
| n-3 PUFA (mg/d) | 1581.0 ± 1069.1[a] | 1623.9 ± 760.9[a] | 3160.6 ± 1249.1[b] | <0.001 |
| n-6 PUFA (mg/d) | 6731.6 ± 4093.8 | 6707.8 ± 3976.8 | 7520.0 ± 3488.8 | 0.772 |
| n-6/n-3 PUFA | 4.45 ± 0.80[a] | 4.04 ± 0.72[a] | 2.44 ± 0.77[b] | <0.001 |
| RBC proportions | | | | |
| DHA (%) | 5.70 ± 0.84[a] | 6.00 ± 0.95[a] | 7.06 ± 0.64[b] | <0.001 |
| n-3 PUFA (%) | 8.87 ± 1.19[a] | 9.42 ± 1.34[a] | 11.08 ± 1.16[b] | <0.001 |
| n-6 PUFA (%) | 24.32 ± 1.71 | 25.04 ± 1.89 | 23.80 ± 1.39 | 0.102 |
| n-6/n-3 PUFA | 2.80 ± 0.40[a] | 2.71 ± 0.39[a] | 2.18 ± 0.31[b] | <0.001 |

Values are reported as mean ± SD.

[a,b,c]Means with different superscripts are significantly different (P<0.05).

Figure 11 shows the correlation between the dietary intake of n-3 LCPUFA and the proportion of n-3 LCPUFA in RBC from (A) women early in pregnancy and (B) non-pregnant women. The relationship between dietary intake of n-3 LCPUFA and proportion of n-3 LCPUFA in RBC was strong and positive (P<0.001) in both the cohorts.

**A)**




**B)**

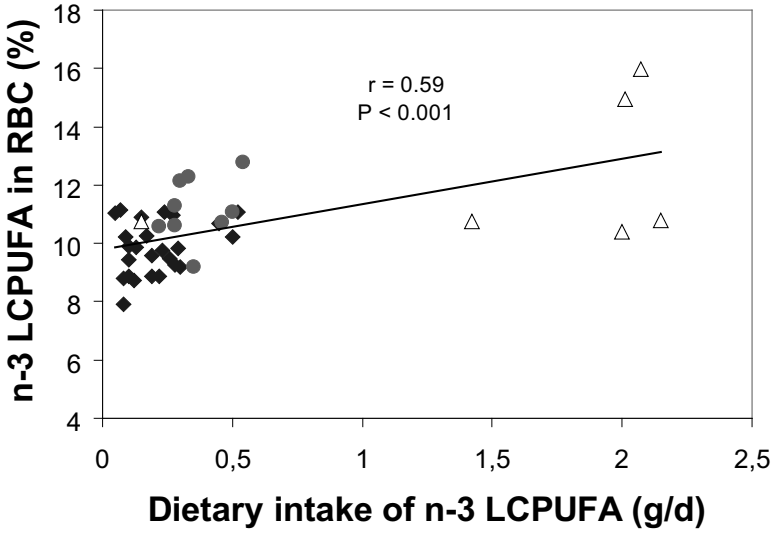



**Fig. 11 Relationship between the proportion of n-3 LCPUFA in RBC and the dietary intake of n-3 LCPUFA (A) early in pregnancy and (B) among non-pregnant women. Black diamonds represent the women using no n-3 LCPUFA supplements (*n*=55 and *n*=25), grey circles those using cod liver oil capsules (*n*=13 and *n*=9) and white triangles those using liquid cod liver oil (*n*=15 and *n*=6) early in pregnancy and among non-pregnant women, respectively.**

There was a positive (P≤0.002) correlation between total intake of DHA and intake of the antioxidant vitamins A and E, but not vitamin C, in Cohorts 1 and 2. A positive (P≤0.030) correlation was found between DHA in RBC and intake of the vitamins A and E (from food and supplements), but this correlation disappeared when adjusted for total DHA intake (data not shown).

## 14.2 Use of L-CLO and pregnancy outcome (whole Cohort 1)

Pregnant women using L-CLO before 11$^{th}$ to 15$^{th}$ week of pregnancy gave birth to 139 g heavier infants than those who did not use L-CLO (P=0.026) (Paper I: Table 2). These women were getting more energy from their diet (P=0.032), and they had higher intake of protein (P=0.015), fat (P=0.018) and alcohol (P=0.005), than women not using L-CLO. Nevertheless, the women using L-CLO early in pregnancy were not gaining more weight during pregnancy.

L-CLO use early in pregnancy was positively correlated with birthweight after adjusting for duration of gestation and other confounding factors (Paper I: Table 3). After adjustment for all other factors, the neonatal birthweight of pregnant women using L-CLO early in pregnancy was 132.1 g (95%CI 18.3 to 246.0) higher than that of women not using L-CLO. No interaction between independent variables was found. The model expressed 35.2% of the variation in birthweight. The pregnant women who used L-CLO early in pregnancy were 11 times (95%CI 3.3 to 36.7) more likely to give birth to an infant of 4500 g or more after a healthy pregnancy and delivery, than the women not using L-CLO (Paper I: Table 4). Use of CLO capsules or fish intake were not related to pregnancy outcome and no correlation was found between n-3 LCPUFA intake (from food or supplements) later in pregnancy and pregnancy outcome.

## 14.3 n-3 LCPUFA in RBC and pregnancy outcome (Cohort 1)

A negative correlation (r=-0.23, P=0.041) was found between the proportion of n-3 LCPUFA in RBC early in pregnancy and placental weight to birthweight ratio (*n*=77, data not shown). This was attributed to a negative, but non-significant trend between the proportion of n-3 LCPUFA in RBC and placental weight, while no correlation was seen between n-3 LCPUFA in RBC and birthweight. In a linear regression model predicting placental weight, n-3 LCPUFA in RBC early in pregnancy was

negatively correlated with placental weight, adjusted for birthweight (Paper II: Table 4). According to the model, a 100 g increase in birthweight increased the placental weight by 19 g, adjusted for n-3 LCPUFA in RBC. With each 1% increase in the proportion of n-3 LCPUFA in RBC, the placental weight decreased by 14.6 g, adjusted for birthweight. Birthweight contributed 46.7% of the variation in placental weight. By adding the proportion of n-3 LCPUFA in RBC, the total contribution reached 49.2% (95%CI 32 to 64). Age, body mass index, alcohol intake, occupation, education, parity, cohabitation, nausea, pelvic pain, physical activity, use of n-3 LCPUFA supplements or smoking had no effect on the relationship. No correlation was found between the proportion of n-3 LCPUFA in RBC and other pregnancy outcome variables. No relationship was seen between n-3 LCPUFA in RBC late in pregnancy and pregnancy outcome. When all the RBC samples were included and adjusted for total FA concentration (*n*=98) the correlations were similar (data not shown).

## 14.4 Lifestyle factors affecting DHA in RBC (Cohorts 1 and 2)

Table 3 shows linear regression models to predict the proportion of DHA in RBC from 83 women early in pregnancy and 48 women late in pregnancy, and from 40 non-pregnant women (See also Paper II: Table 3 (model for predicting the proportion of total n-3 LCPUFA in RBC from 77 women in early pregnancy) and Paper III: Table 3). For every g/day increase in total intake of DHA, the proportion of DHA in RBC increased by 1.77% of total FA (95%CI 1.27 to 2.28) early in pregnancy, by 2.89% of total FA (95%CI 1.82 to 3.96) late in pregnancy, and by 1.87% of total FA (95%CI 1.07 to 2.67) among non-pregnant women with all other variables constant. When the proportion of AA in RBC increased by 1% of total FA, the proportion of DHA increased by 0.20% of total FA (95%CI 0.06 to 0.34) early in pregnancy and by 0.81% of total FA (95%CI 0.46 to 1.16) late in pregnancy, but there was no correlation found between AA and DHA in RBC in non-pregnant women.

**Table 3 Multiple regression analysis to predict the proportion of DHA in RBC from women early and late in pregnancy and from non-pregnant women.**

| | Week of pregnancy | | | | Month post-partum | |
|---|---|---|---|---|---|---|
| | 11th to 15th *n* =83 | | 34th to 37th *n* =48 | | 18th *n* =40 | |
| | B | P | B | P | B | P |
| (Constant) | 2.53 | 0.032 | -4.09 | 0.062 | 5.89 | <0.001 |
| Dietary intake of DHA (g/day) | 1.77 | <0.001 | 2.89 | <0.001 | 1.87 | <0.001 |
| AA in RBC (%) | 0.20 | 0.007 | 0.81 | <0.001 | | |
| Smoking | -0.52 | 0.003 | | | | |
| Gestational length at entry (d) | 0.02 | 0.017 | | | | |
| Alcohol intake (percentile) | | | 0.34 | 0.017 | | |
| Oral contraceptive use | | | | | 0.54 | 0.040 |
| Physical activity | | | | | 0.16 | 0.025 |
| Storage time at -20°C (weeks) | | | | | -0.10 | 0.005 |
| Model as a whole | Adj. $R^2$=0.433 | P<0.001 | Adj. $R^2$=0.423 | P<0.001 | Adj. $R^2$=0.478 | P<0.001 |

Reference categories:
Smoking in 11th to 15th week of pregnancy: 1=do not smoke, 2=do smoke.
Alcohol intake: 1=up to 25th perc., 2=from 25th to 50th perc., 3=from 50th to 75th perc., 4=from 75th perc.
Physical activity: 1=no physical activity, 2=yes, but less than once a week, 3=yes, once a week, 4=yes, twice a week, 5=yes, three times a week, 6=yes, four times a week or more often.
B, unstandardized coefficient.

Several lifestyle and other interfering factors were significantly associated with the proportion of DHA in RBC (Table 3). According to the models, smoking decreased the proportion of DHA in RBC by 0.52% of total FA (95%CI -0.86 to −0.19) early in pregnancy, while higher quartile of alcohol intake increased the proportion of DHA in RBC by 0.34% of total FA (95%CI 0.06 to 0.61) late in pregnancy, adjusted for dietary intake of DHA and the proportion of AA in RBC. A one-week increase in gestational length at entry increased the proportion of DHA in RBC by 0.14% of total FA (95%CI 0.003 to 0.03), adjusted for DHA intake, smoking and AA in RBC. Among the non-pregnant women the proportion of DHA in RBC increased by 0.54% of total FA (95%CI 0.03 to 1.06) with oral contraceptive use and by 0.16% of total FA (95%CI 0.02 to 0.31) with more frequent physical activity, independent of total intake of DHA and storage time at -20°C (Table 3 and Paper III: Table 3). Even though the antioxidant BHT was added to the RBC samples from the non-pregnant women before storage, storage time at -20°C had a small negative effect on the proportion of DHA in RBC. Storage for one week at −20°C decreased the proportion of DHA in RBC by 0.10% of total FA with all other variables constant. Other confounders that were tested, such as age, body weight, parity, occupation, education, cohabitation, nausea or pelvic pain among the pregnant

women, or dietary and supplement intake of natural antioxidants had no impact on the proportion of DHA in RBC. No interaction between independent variables was found. The models expressed 43.3%, 42.3% and 47.8% of the variation in proportion of DHA in RBC from the women early and late in pregnancy and from the non-pregnant women, respectively. When all the RBC samples from the pregnant women were included and adjusted for total FA concentration (*n*=101 early in pregnancy, *n*=121 late in pregnancy), the same lifestyle factors fitted the regression models, and the effect showed the same direction as when only RBC samples that were considered not lipid peroxidized were included (data not shown).

# 15 Paper IV and V and unpublished results

## 15.1 Stability of RBC PUFA during storage (Cohort 3)

RBC samples from healthy non-pregnant women were stored at -20°C for two, four, nine or 17 weeks, with or without added antioxidant. The proportion of DHA in RBC had decreased after nine weeks storage at -20°C without added antioxidant compared with baseline. (Paper IV: Table 1). After 17 weeks storage, the proportions of all SFA and MUFA were higher, and that of all n-6 and n-3 PUFA lower than at baseline.

The concentrations of AA and DHA in RBC had decreased after four weeks storage at -20°C without added antioxidant compared with baseline (Paper IV: Fig. 1). After nine weeks storage, the concentrations of LA and 18:1n-9 had also decreased, and after 17 weeks, the concentrations of both 16:0 and 18:0 were lower than at baseline. As seen in Paper IV: Table 1 and Fig. 1**,** addition of the antioxidant BHT preserved the FA composition, the total FA concentration and the concentration of individual FA in RBC during 17 weeks storage at -20°C.

There was a strong, positive correlation between the change in total concentration of FA in RBC during 17 weeks of storage at -20°C without BHT and the proportion of DHA at baseline without BHT (Paper IV: Fig. 2). Age and other FA proportions in RBC were not related to the change in total concentration of FA during 17 weeks storage at -20°C without BHT.

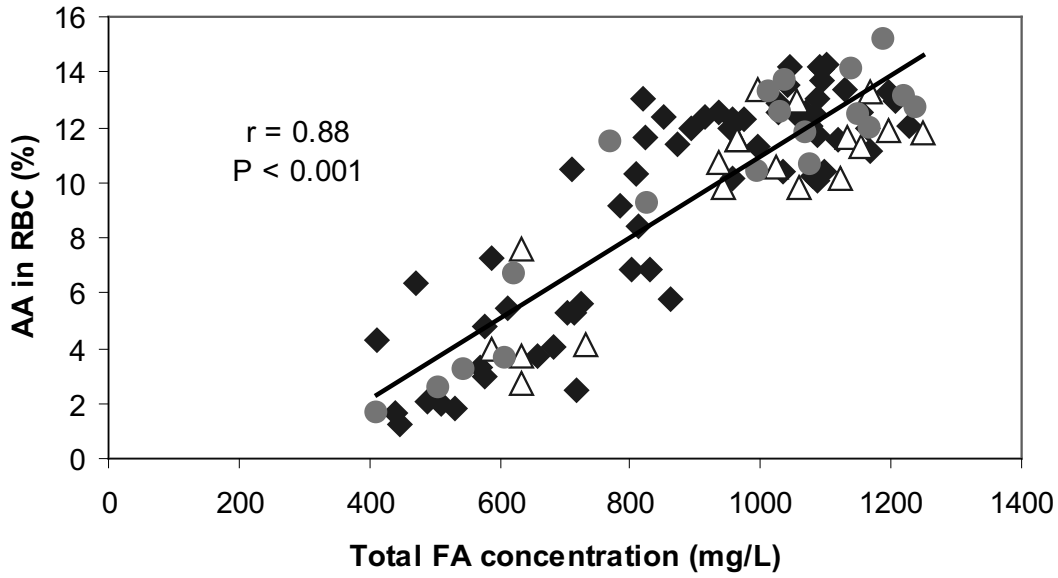
## 15.2 Analysis of FA data from partly peroxidized RBC lipids (Cohort 1)

No antioxidant was added to the RBC samples from the pregnant women in Cohort 1 before they were stored at -20°C. The RBC data were analyzed by three different data analysis methods (Paper V). One of the data analysis methods included all the RBC FA data unadjusted (U-A, *n*=101 early in pregnancy and *n*=121 late in pregnancy), another included unadjusted FA data from RBC samples considered not lipid peroxidized (U-NP, *n*=67 early in pregnancy and *n*=47 late in pregnancy), and the third included all the RBC FA data adjusted for total FA concentration (A-A, *n*=101 early in pregnancy and *n*=121 late in pregnancy). In Paper V only RBC samples with a value for total FA concentration early in pregnancy were included.

### 15.2.1 All the data unadjusted (U-A method)

The distribution of the proportions of AA and DHA was bimodal in the RBC samples from the women early (Paper V: Fig. 1a,b) and late (data not shown) in pregnancy. Reflection and/or inverse or logarithmic transformation did not normalize the distribution. Total FA concentration of RBC correlated negatively with the proportion of each SFA and MUFA, and positively with the proportion of each n-6 and n-3 PUFA early (Fig. 12 and Paper V: Table 2) and late (data not shown) in pregnancy.

**A)**




**B)**

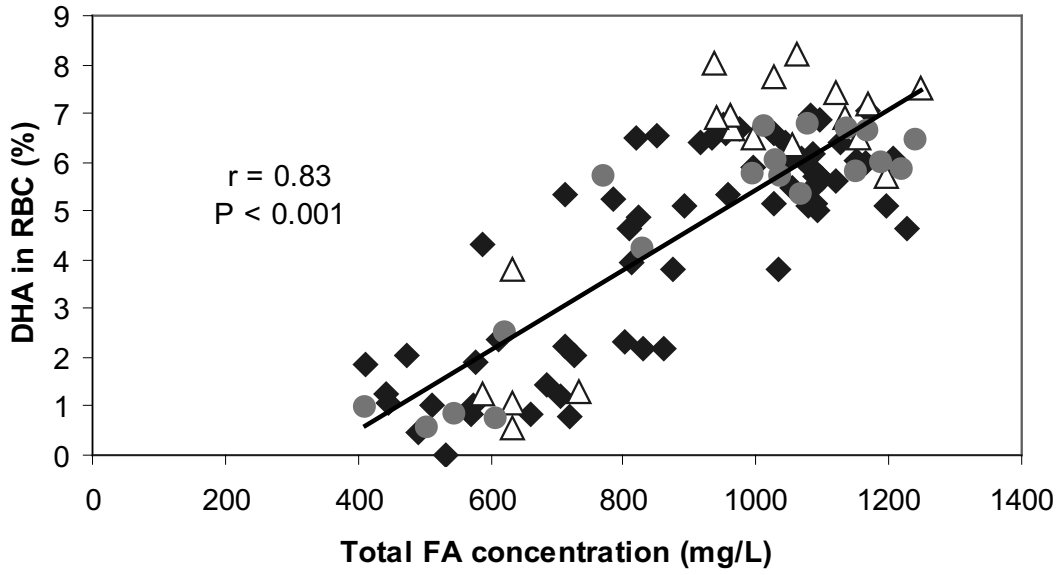



**Fig. 12 Relationship between A) the proportion of AA and total FA concentration of RBC early in pregnancy and B) the proportion of DHA and total FA concentration of RBC early in pregnancy. Black diamonds represent the women using no n-3 LCPUFA supplements (*n*=63), grey circles those using cod liver oil capsules (*n*=19) and white triangles those using liquid cod liver oil (*n*=19).**

Figure 13 shows the relationship between the proportions of AA and DHA in RBC early in pregnancy. AA and DHA in RBC were strongly and positively correlated (P<0.001), and the higher mode of the bimodal distribution of DHA was made up of the same RBC samples as the higher mode of the bimodal distribution of AA. The range of AA proportion was only 1 to 7% in the lower mode. The results for the second time point of pregnancy were similar (data not shown).

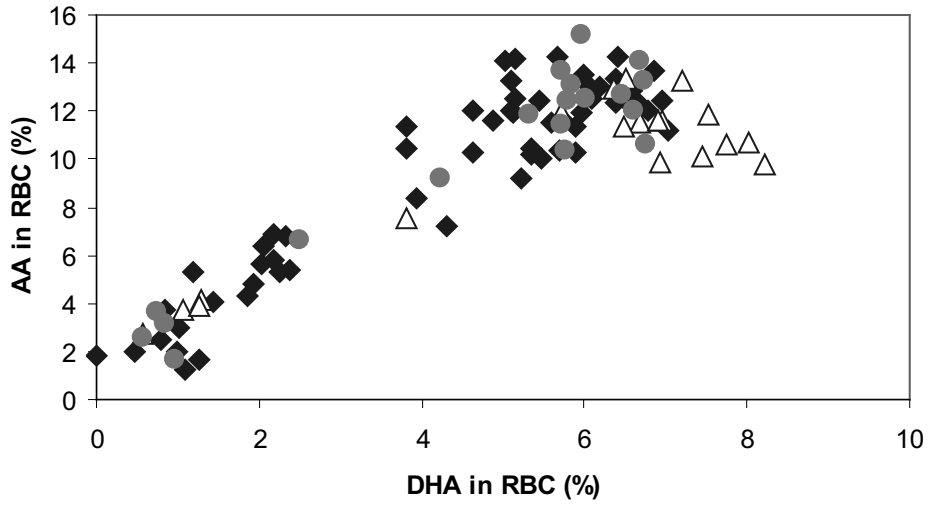



**Fig. 13 Relationship between the proportions of AA and DHA in RBC early in pregnancy. Black diamonds represent the women using no n-3 LCPUFA supplements (*n*=63), grey circles those using cod liver oil capsules (*n*=19) and white triangles those using liquid cod liver oil (*n*=19).**

15.2.2 Exclusion of peroxidized RBC samples (U-NP method)

RBC samples in the lower mode of the bimodal distribution of the proportion of AA were considered lipid peroxidized and excluded from analysis by the unadjusted not peroxidized (U-NP) method. The limit between the higher and lower mode was at 9.5% of total FA early in pregnancy (Paper V: Fig. 1a) and at 9% of total FA late in pregnancy (data not shown). RBC samples from 67 women early in pregnancy and 47 women late in pregnancy were considered not lipid peroxidized and included in the U-NP method. The proportions of AA and DHA in data analyzed by the U-NP method were normally distributed (Paper V: Fig. 1c,d). The correlation between the total FA concentration of RBC and the proportion of each FA early in pregnancy was weaker in data analyzed by the U-NP method than in data analyzed by the U-A method, or even absent (Paper V: Table 2).

The women who were excluded from the U-NP method were of shorter stature, and had higher BMI, systolic blood pressure and parity than the women included, but there were no differences in age or in total dietary intake of DHA, fish, or CLO (liquid or capsules) (Paper V: Table 1). No difference was found in any other dietary intake variable between the women included and excluded from the U−NP method. Similar results were obtained from the second time point of pregnancy (data not shown).

### 15.2.3 Modified adjustment method (A-A method)

All the RBC FA data were adjusted for total FA concentration by regressing each RBC FA proportion on the total FA concentration of RBC by the adjusted-all (A-A) method. A constant (the FA proportion referring to the mean total FA concentration of the higher mode of the bimodal distribution of AA) was added to the residuals of the regression line. FA proportions were thus standardized to the mean total FA concentration of RBC samples not considered lipid peroxidized. The RBC proportions of AA and DHA were approximated to normal distribution after the adjustment (Paper V: Fig. 1e,f). Similar results were seen for late pregnancy (data not shown).

## 15.3 Comparison of the three data analysis methods (Cohort 1)

The mean proportions of all PUFA were lower, and those of all SFA and MUFA higher in data analyzed by the U-A method compared to the A-A method (Paper V: Table 3). There was no difference in the mean proportion of any FA in RBC between the U-NP and the A-A methods. Similar results were obtained in the second time point of pregnancy (data not shown).

Linear regression models predicting the proportion of DHA in RBC early in pregnancy (Paper V: Table 4) show that the unstandardized B coefficient predicting the change in the proportion of DHA in RBC for each g increase in dietary intake of DHA was fairly similar in data analyzed by all the three methods (from 1.50% of total FA in the A-A model to 1.62% of total FA in the U-NP model). This means that after adjustment for the proportion of AA in RBC, the correlation between dietary intake of DHA and the proportion of DHA in RBC was similar in the three models. When the standardized β coefficient for the proportion of AA in RBC and

dietary DHA intake is compared within each model, it can be seen that in the U-A model the relative contribution of AA in RBC is much stronger than the contribution of dietary DHA (0.89 vs. 0.23, respectively). In the U-NP model, the contribution of AA in RBC was non-significant, and the contribution of dietary DHA was fairly strong (0.67). In the A-A model the contribution of AA in RBC was stronger than the contribution of dietary DHA (0.75 vs. 0.40), but not as strong as in the U-A model. Both independent variables, the proportion of AA in RBC and dietary DHA intake, had a positive relationship with the proportion of DHA in RBC in all of the models. The U-A model expressed 87% of the variation in DHA in RBC, the U-NP model 37.8% and the A-A model 56.9%. Similar relations were seen for the second time point of pregnancy (data not shown).

## 16 Data presented in each Paper

Paper I includes data on maternal anthropometry, dietary intake, lifestyle factors, and pregnancy outcome from the whole Cohort 1. Paper II includes data on maternal anthropometry, dietary intake, lifestyle factors, FA composition in RBC, and pregnancy outcome from the women in Cohort 1. Paper II only includes data from RBC samples considered not peroxidized. Paper III includes data on anthropometry, dietary intake, lifestyle factors and FA composition in RBC from the women in Cohort 2. Paper IV includes data on FA composition in RBC from the women in Cohort 3. Paper V includes data on maternal anthropometry, dietary intake and FA composition in RBC from the women in Cohort 1, before and after the FA data were adjusted for total FA concentration.

# DISCUSSION

## 17 General discussion

The results of the studies presented in this thesis demonstrate, as expected, a positive correlation between total intake of n-3 LCPUFA and n-3 LCPUFA in RBC among the pregnant women early and late in pregnancy and among the non-pregnant women. The prevalence of n-3 LCPUFA supplement use was relatively high in both the cohorts, and women using n-3 LCPUFA supplements had higher proportion of n−3 PUFA and lower n-6/n-3 PUFA ratio in RBC, compared to those who did not. Intake of n-3 LCPUFA and their RBC level was compared with pregnancy outcome, and the results demonstrate that use of L-CLO early in pregnancy was positively correlated with birthweight after correction for duration of gestation and other confounding factors. Furthermore, the proportion of n-3 LCPUFA in RBC early in pregnancy was negatively correlated with placental weight adjusted for birthweight. Special attention was paid to DHA, the longest and most unsaturated n-3 LCPUFA in membranes, and the relationship with lifestyle after adjustment for total DHA intake. The results demonstrate that the proportion of DHA in RBC was negatively correlated with smoking early in pregnancy, and positively correlated with alcohol consumption late in pregnancy. Oral contraceptive use and physical activity were positively correlated with DHA in RBC from non-pregnant women. We also investigated the stability of PUFA in RBC during storage. The results demonstrate that PUFA composition and total FA concentration were stable in washed venous RBC from healthy non-pregnant women during four weeks storage at -20°C without addition of antioxidant. Addition of the antioxidant BHT did preserve FA proportions and concentrations of the RBC lipids for at least 17 weeks at -20°C. An adjustment method designed for nutritional data analysis was modified for use in the FA data analysis of the partly peroxidized RBC lipids from the pregnant women. There was no difference in mean proportions of any SFA, MUFA or PUFA in RBC from the pregnant women, between the unadjusted data from RBC samples considered not peroxidized, and the adjusted data from all the RBC samples.

## 17.1 Relationship between dietary intake of n-3 LCPUFA and n-3 LCPUFA in RBC

Our findings, that the n-3 LCPUFA level in RBC reflects dietary and supplement intake of n-3 LCPUFA among pregnant women early and late in pregnancy and among non-pregnant women, are consistent with published data which reported a higher level of n-3 LCPUFA in RBC of supplemented pregnant women (116) and a positive association between dietary intake and RBC level of n-3 LCPUFA (157, 159-161). The semi-quantitative FFQ that the pregnant and the non-pregnant women filled in covered food intake for the last three months. The FA composition of RBC membrane mirrors dietary intake of FA for the preceding three to four months, since RBC circulate in the blood for 120 days, and when mature they are not able to synthesize FA. The study demonstrates an agreement between n-3 LCPUFA in RBC of pregnant and non-pregnant women and total n-3 LCPUFA intake, as measured with a semi-quantitative FFQ.

The higher proportion of DHA and total n-3 LCPUFA in RBC of women in Cohort 1 with longer gestation at entry, is in agreement with the study of Otto et al. (189). The reason for the increase and the mechanisms involved are not evident. However, it may be a physiological adoption of pregnancy designed to optimize the delivery of n-3 LCPUFA to the developing fetus.

## 17.2 The n-6/n-3 PUFA ratio in diet and RBC

Imbalance in dietary intake of n-6 and n-3 PUFA changes the ratio of n-6 to n-3 PUFA in membranes (35). High n-6/n-3 PUFA ratio in membranes has been associated with increased risk of several common diseases (36-41). Among the women in Cohorts 1 and 2, it was the total intake of n-3 LCPUFA, but not ALA or n−6 PUFA that determined the n-6/n-3 PUFA ratio in RBC (data not shown). Forty-six early pregnant women (59.7%) and 25 non-pregnant women (62.5%) were not using n-3 LCPUFA supplements. The mean n-6/n-3 PUFA intake ratio of these 46 pregnant and 25 non-pregnant women was however considerably lower (4.46:1 in both groups) than has been found among women in Central Europe (7-12:1 (189-191)). The low n-6/n-3 PUFA intake ratio among the pregnant and non-pregnant women not using n-3 LCPUFA supplements in Iceland compared to that among the

women in the Central European countries (189-191) can be explained by a low intake of n-6 PUFA (6.7 and 5.7 g/d vs. 11-13 g/d, respectively), while the n-3 PUFA intake (1.6 and 1.3 g/d vs. 1.3-1.7 g/d, respectively) and intake of DHA (128 and 138 mg/d vs. 90-140 mg/d, respectively) were in the same range in Iceland as in the Central European countries. Vegetables, grains and vegetable oils are the main sources of n−6 PUFA. In 2002, intake of vegetables and vegetable oils was lower in Iceland than in many other countries, while intake of grains was similar to that in neighbouring countries (9). In accordance with the Dietary Survey of The Icelandic Nutrition Council 2002 (9), the fish intake of the women was moderate (28 ± 20 g/d in both groups). Furthermore, the fish commonly consumed in Iceland is low in fat (9).

One of the aforementioned Central European studies reported the FA composition in RBC from women in the prepregnant state and in the 10$^{th}$ week of pregnancy (189). The n-6/n-3 PUFA ratio in RBC from the pregnant and non-pregnant women not using n-3 LCPUFA supplements in Iceland was considerably lower than in RBC of the pregnant and prepregnant women in the Netherlands (189) (2.80:1 and 2.82:1 vs. 4.56:1 and 5.06:1, respectively). Interestingly, the low n-6/n-3 PUFA ratio in RBC from the women in Iceland was not due to a lower proportion of n-6 PUFA (24.32% and 27.45% vs. 26.85% and 27.38%, respectively), but rather to a much higher proportion of DHA (5.70% and 6.11% vs. 3.43% and 2.94%, respectively) and the other n-3 PUFA. High intake of the n-6 PUFA LA has been shown to inhibit the incorporation of n-3 LCPUFA in cell membranes of human subjects (31), which may explain why similar intake of n-3 LCPUFA among the women in Iceland and the Netherlands results in a much higher proportion of n-3 LCPUFA in RBC from the women in Iceland.

It is common in Iceland to use L-CLO, though today many prefer CLO capsules. The mean DHA intake was 7.3 (Cohort 1) and 6.5 (Cohort 2) times higher among women using L-CLO than among women not using n-3 LCPUFA supplements. Similarly, the total n-6/n-3 PUFA intake ratio was 45% (Cohort 1) and 46% (Cohort 2) lower among women using L-CLO compared with women not using n-3 LCPUFA supplements. The PUFA composition of RBC reflected the dietary intake with a 24% (Cohorts 1 and 2) higher mean proportion of DHA and a 22% (Cohort 1) and 25% (Cohort 2) lower n-6/n-3 PUFA ratio in RBC from women using

L-CLO, compared with RBC from women not using n-3 LCPUFA supplements. The women using CLO capsules were intermediate in all of these variables.

The mean daily DHA intake of the women not using n-3 LCPUFA supplements in Cohorts 1 and 2 did not reach the minimum daily 200 mg DHA intake recommended for pregnant and lactating women (32), which shows the importance of dietary counselling for women in the early stages of pregnancy. Even though the mean daily DHA intake of the women using n-3 LCPUFA supplements was considerably higher than the previously mentioned recommendation, 4 pregnant and 5 non-pregnant women using CLO capsules actually did not reach the minimum recommended. All of the women using L-CLO had DHA intake well above the recommended minimum, and the n-6/n-3 PUFA intake ratio among them was near the ratio assumed for preagricultural humans (33).

Increasing the n-3 LCPUFA intake and decreasing the n-6/n-3 PUFA intake ratio may be important for the health of women (43-46). Furthermore, in the case of pregnant and lactating women, it may also be important for the health of the neonates (102, 192-194). However, there are indications that the n-6 LCPUFA AA plays a role in intrauterine growth (195-198), and concern has been expressed that increased intake of n-3 LCPUFA during pregnancy might compromise the AA status of mother and fetus (118). The correlation between AA and DHA in RBC was positive among the healthy pregnant women in Cohort 1 who gave birth to healthy and high birthweight neonates. Furthermore, other studies have found plasma and tissue contents of AA to be relatively stable during pregnancy, even though the women were supplemented with n-3 LCPUFA (199, 200). In conclusion, the total n-6/n-3 PUFA intake ratio was lower among the pregnant and non-pregnant women in our study than has been found among women in several Western countries. This might have a positive effect on the health of the women and their offspring.

## 17.3 Pregnancy outcome in relation to n-3 LCPUFA

It has been hypothesized that dietary intake of marine n-3 LCPUFA during pregnancy might increase birthweight by prolonging gestation (201-205). Higher birthweight, independent of duration of gestation, has been associated with fish intake during pregnancy in observational studies (99, 119-121). However, intervention studies supplementing women with n-3 LCPUFA from week 15 of

pregnancy or even later have not found increased birthweight after adjusting for duration of gestation (110-113, 115, 116). The findings from the whole Cohort 1 showed that intake of L-CLO before week 15 of pregnancy was positively related to birthweight after correction for duration of gestation and other confounding factors. Women using L-CLO early in pregnancy differed in a few variables from women not using L-CLO, but only alcohol consumption will be mentioned here. Women using L-CLO consumed more alcohol early in pregnancy (1.8 g/d) than women not using L-CLO (0.8 g/d). However, while use of L-CLO had a positive relationship to birthweight, the relationship between alcohol intake and birthweight was negative. Both variables entered the multiple regression model for birthweight, showing that L-CLO use had a positive impact on birthweight, after adjustment for alcohol consumption. The most probable reason for the lack of relationship between intake of CLO capsules and birthweight is that the amount of n-3 LCPUFA in the common daily dose of CLO capsules (0.18 g) is 10 times less than in the common 10 mL spoon of L-CLO used daily (1.8 g).

Mean birthweight has increased in some countries in the past decade, possibly due to a change in lifestyle and an increase in maternal obesity (206-208). Earlier research focused on the risk of giving birth to a low birthweight infant (<2500 g) (57, 209). Recently macrosomia, defined as birthweight over 4500 g, or over two SD above the mean birthweight by gestational age (210) has gained attention, since it also increases the risk of obstetric and perinatal complications (210, 211). The women in the whole Cohort 1 who took L-CLO early in pregnancy were 11 times more likely to give birth to an infant of 4500 g or more after a normal pregnancy and delivery. Birthweight of term infants in Iceland is among the highest in the world, and it has been high at least since the beginning of the twentieth century (99, 212-215). Even in this high birthweight population, a larger size at birth is related to a low prevalence of common adult diseases (215-217).

We are aware of only one study where placental weight was related to n-3 LCPUFA (119). Olsen et al. (119) found placental weight, birthweight and head circumference of term neonates to be positively correlated with maternal fish intake in the third trimester of pregnancy among non-smoking women. There was no relationship between the proportion of n-3 LCPUFA in RBC in the third trimester of pregnancy and pregnancy outcome among the women in Cohort 1, but placental weight at delivery might be programmed early in pregnancy and further affected by

dietary intake in third trimester. The reason for placental weight relating positively to fish intake in the study of Olsen et al. (119) and negatively to the proportion of n-3 LCPUFA in RBC among the women in Cohort 1 is not clear. The relationship between the proportion of n-3 LCPUFA in RBC and placental weight did not reach significance unless we divided placental weight with birthweight, or adjusted the placental weight for birthweight with multiple regression. Placental weight and birthweight are positively correlated (51, 52). Therefore it is important to look at the ratio between the two, or to adjust one to the other when investigating the relationship of these variables with a third one. In the study of Olsen et al. (119) it was not mentioned whether the placental weight increased or decreased relative to the birthweight as fish intake increased. Furthermore, fish contains many other substances than n-3 LCPUFA that might affect placental weight in a positive way.

The findings among the women in Cohort 1 that L-CLO intake in the first trimester of pregnancy was positively correlated with birthweight, and that the proportion of n-3 LCPUFA in RBC in the first trimester was negatively correlated with placental weight adjusted for birthweight indicate that n-3 LCPUFA are among the nutrients programming placental and fetal growth. High birthweight in combination with low placental weight have been related to lower risk of hypertension and cardiovascular disease in adulthood (56, 57, 59, 60). Our conclusion is that for healthy women without pregnancy complications, regular intake of L-CLO early in pregnancy is to be considered as positive for the health of the offspring.

## 17.4 Pregnancy outcome in relation to tobacco and alcohol use

Maternal smoking (52, 128-130, 143, 144) and alcohol intake (133-137, 218) have been related to fetal growth retardation. Among the women in the whole Cohort 1, smoking throughout pregnancy and alcohol intake early in pregnancy were related to a decrease in birthweight, after adjustment for L-CLO intake and other confounding factors. The reason for slower growth of fetuses of women who smoke could be either inefficient placental transfer of oxygen and nutrients or direct growth-retarding effects of chemical substances in tobacco smoke. The pregnant women consumed less alcohol than the non-pregnant women, or 0.88±1.61 g/d early in pregnancy, 0.82±1.92 g/d late in pregnancy vs. 1.94±1.92 g/d among the non-pregnant women

(P<0.0083, data not shown). The reason is probably increased health awareness of women during pregnancy, and general knowledge about the negative effects of alcohol on fetal development. However, even small amounts of alcohol had a negative relationship to birthweight. The evidence of fetal growth retardation due to moderate maternal alcohol intake has been supported earlier (133, 137). In conclusion, the use of tobacco and alcohol should be avoided throughout pregnancy, as the study indicates that their use is related to lower birthweight of the neonate.

## 17.5 n-3 LCPUFA in RBC in relation to tobacco and alcohol use

When LCPUFA status of smokers and non-smokers has been compared, some studies found no difference (160, 219), while one study found lower LCPUFA status in men who smoked than in those who did not (220). Further, neonates of smoking women had lower LCPUFA status than neonates of non-smoking women (142). The lower proportion of DHA and total n-3 LCPUFA found in RBC of smoking compared with non-smoking women in Cohort 1 was still present after adjustment for n-3 LCPUFA intake. Smoking has been shown to increase lipid peroxidation (141, 221), and there is also evidence that biosynthesis of n-3 LCPUFA is enhanced in smoking men and non-pregnant women, as if to compensate for the peroxidative loss (222, 223). Our results indicate that the biosynthesis of n-3 LCPUFA does not compensate fully for the peroxidative loss of these important FA in smoking women under the high demand of pregnancy.

Stark *et al*. recently found frequent and high intake of alcohol among African-American women to be associated with lower proportion of DHA in both plasma and RBC (224). However, alcohol consumption of the pregnant women in Cohort 1 late in pregnancy was associated with higher proportion of DHA in RBC that could not be explained by different DHA intake. A study on rat hepatocytes *in vitro* has shown that PUFA synthesis is stimulated at low ethanol levels and inhibited at high ethanol levels (225). This might explain the contradiction between our results and the results of Stark *et al*. (224), supported by the fact that the only alcoholic beverage that fitted the regression model in our study was light beer with ≤ 2.25% ethanol (data not shown).

In conclusion, the findings indicate that smoking has a negative impact on maternal n-3 LCPUFA status, as assessed by RBC level. Even though light beer

consumption in the later half of pregnancy might stimulate biosynthesis of DHA, alcohol consumption cannot be recommended at any stage of pregnancy, since its use, even at low level, was associated with lower birthweight of the neonate. Moreover, other studies have found alcohol consumption in pregnancy to be associated with multiple congenital anomalies and the fetal alcohol syndrome (138, 139).

## 17.6 DHA in RBC in relation to oral contraceptive use and physical activity

Biosynthesis of DHA is higher in women (11) than men (10). The higher proportion of DHA in RBC from women in Cohort 2 using oral contraceptives than in RBC from those who did not, could not be explained by different dietary intake of DHA. Previous studies on women taking 30-35 μg ethynyloestradiol/d as oral contraceptives have found a non-significant trend towards higher biosynthesis of DHA compared with women not using oral contraceptives (11, 145). Oestrogen therapy with an intake of 100 μg/ethynyloestradiol/d resulted in a significant increase in DHA status of male-to-female transsexuals (145). The ethynyloestradiol dose in the oral contraceptives used by the women in Cohort 2 was 20-50 μg/d. As far as we know our study is the first to show that low-dose oestrogen treatment with oral contraceptives is able to stimulate biosynthesis of DHA.

Physically active women usually lead a healthier lifestyle than sedentary women, often including healthier dietary habits. However, the higher DHA status in RBC of physically active women in Cohort 2 was not due to higher fish intake or intake of n-3 LCPUFA supplements. One study found both exercise and training to be related to a lower content of phosphatidylserine in RBC membrane and a lower proportion of DHA in the phosphatidylserine lipid class (226). The effect was explained by increased oxidative stress and other studies have confirmed that heavy endurance exercise increases both oxidative stress (146, 147) and hemolysis (148). Physical activity was practiced twice a week or more often by 37.5% of the women in Cohort 2, but no information on exercise intensity was gathered, nor was their biochemical antioxidant status assessed. The body has a mechanism meeting the exercise-induced oxidative stress by increasing antioxidant activity (146) and fluidity of the RBC membranes (149, 150). Increased fluidity can be achieved by

decreasing the ratio of cholesterol to PL or increasing the level of unsaturation in the membrane (151, 152). Heavy endurance exercise has been found to increase the n-3 LCPUFA EPA and DPA in RBC (149, 227), and training also seems to increase the DHA level in skeletal muscle PL (228, 229). Our results are in accordance with these studies, finding more frequent physical exercise (cycling, swimming or other physical activity) of the women in Cohort 2 to be related to a higher proportion of DHA in RBC. Increased liver biosynthesis of DHA and increased incorporation of DHA into RBC membranes of physically active people might serve to increase the fluidity of the membranes and counteract the exercise-induced hemolysis.

In conclusion, the findings indicate that oral contraceptive use and physical activity have a positive impact on the DHA status, as assessed by RBC level, of non-pregnant women of reproductive age.

## 17.7 Stability of RBC PUFA during storage

Storage time at -20°C had a small negative impact on the proportion of DHA in RBC samples from the women in Cohort 2. The final concentration of BHT (10-20 mg/L) added to the RBC samples before the samples were frozen may not have been high enough to protect the RBC lipids completely from peroxidation during storage. The RBC samples from the women in Cohort 2 were stored for a different length of time, 15 weeks at the most.

The PUFA composition and total FA concentration of RBC samples from the healthy non-pregnant women in Cohort 3 were stable for four weeks at -20°C without addition of antioxidant, which supports the findings reported previously (171). The antioxidant BHT has previously been shown to preserve FA composition of RBC samples for one year at -70°C and for two years at -80°C (171, 172). The addition of BHT at a final concentration of 42 mg/L to the RBC samples from the women in Cohort 3 did preserve FA proportions and concentrations for at least 17 weeks at -20°C. Studies on vulnerability of n-3 and n-6 PUFA in RBC to peroxidation have been inconsistent, some showing similar (230-232), others increased (152, 177), and yet others decreased (233) peroxidation of RBC lipids high in n-3 PUFA compared to n-6 PUFA. The baseline proportion of DHA in RBC samples from the women in Cohort 3 was positively correlated with the change in total FA concentration of RBC without BHT over a period of 17 weeks at -20°C,

meaning that the higher the proportion of DHA at baseline, the less the decrease in total FA concentration of RBC was during these 17 weeks. However, DHA deteriorated faster than the n-6 PUFA, since it took 9 weeks for the proportion of DHA, but 17 weeks for the n-6 PUFA to be lower than at baseline. The possibility that RBC samples high in DHA at baseline are also high in antioxidants such as α−tocopherol or antioxidative enzymes naturally present in the RBC cannot be excluded. A positive association has been found between the proportion of n-3 PUFA in RBC and concentration of α-tocopherol in plasma from women in Iceland (234), and vitamin E has been shown to prevent lipid peroxidation of n-3 PUFA in animal RBC (235). However, hemolysis is one of the factors inducing lipid peroxidation of PUFA during storage (154), and RBC high in n-3 PUFA have been shown to be more resistant to hemolysis (152, 153). High proportion of DHA at baseline might therefore also be the factor protecting the RBC samples from lipid peroxidation.

The decrease in total FA concentration of venous RBC from the healthy non-pregnant women during 17 weeks of storage without addition of BHT was 35 ± 15%, and in half the women, the decrease was 60% (over 500 mg/L). In those samples the baseline proportion of DHA was under 7%. A similar decrease in total FA concentration has been found in capillary RBC with low DHA content at baseline after only four weeks at -20°C (171). The loss of total FA concentration of the RBC samples from Cohort 3 was due not only to lipid peroxidation of PUFA, as the concentration of SFA also decreased. Hemolysis causing PL degradation forming lysophospholipids that are not fully extracted by organic solvents might explain the loss of total FA concentration. Addition of BHT before storage of the RBC at -20°C prevented the degradation, probably by contributing to maintenance of the integrity of the membrane.

The extent of lipid peroxidation and PL degradation, was highly variable between the 12 women in Cohort 3, as indicated by a high variation in PUFA proportions and total FA concentration, even though storage time and other conditions were similar. Therefore, total FA concentration might be a better indicator of the extent of RBC PUFA peroxidation than storage time. In conclusion the findings indicate that high content of DHA in venous RBC of healthy women is associated with less deterioration of lipid FA content during storage at low temperature without addition of antioxidant.

## 17.8 Modified adjustment method

It was impossible to accurately trace storage time of the RBC samples from the pregnant women in Cohort 1, but total FA concentration of each RBC sample was known, and it correlated strongly with the proportion of each FA in the RBC. Therefore, it was decided to use the total FA concentration of RBC as the adjustment factor for the adjusted-all (A-A) data analysis method.

The limit between lipid peroxidized and not lipid peroxidized RBC samples has been drawn at 10% of AA (157, 236). The distribution of the proportions of AA and DHA in RBC from the pregnant women in Cohort 1 was bimodal, while it was normal in RBC from the non-pregnant women in Cohort 2 (data not shown). The limit between the higher and lower mode was at 9.5% early and 9.0% late (data not shown) in pregnancy. The FA composition of RBC samples analyzed by the unadjusted-not peroxidized (U-NP) method was considered to be reliable, since the distributions of the proportions of AA and DHA in data analyzed by the U-NP method were normal, total FA concentration was relatively high (≥710 mg/L) and so were the proportions of AA and DHA (>9.5% and >3.5%, respectively). Furthermore, the correlation between the FA proportions in RBC and total FA concentration was weaker in data analyzed by the U-NP method than in all the data unadjusted (U-A). The correlation was in fact absent for n-3 PUFA, which supports the results from Cohort 3, which showed that RBC samples with a high DHA content at baseline might be better protected against PUFA peroxidation and PL degradation during inappropriate storage conditions.

Pathology has been shown to affect stability of RBC PUFA during storage (173, 174). RBC samples from women excluded from the U-NP method had by definition low proportions of both AA (≤9.5%) and DHA (≤3.5%). Interestingly, these women were shorter and had higher BMI, systolic blood pressure and parity than the women included in the U-NP method. Most likely the pregnant women excluded from the U-NP method had a relatively low proportion of DHA in their RBC at baseline and less protection against PUFA peroxidation and PL degradation than the women with higher proportion of DHA in RBC at baseline. A low proportion of DHA in RBC should reflect a low total intake of n-3 LCPUFA (159-161), and a trend towards lower total DHA intake was seen among women excluded from the U-NP method than those included, though it did not reach significance.

The modified adjustment method used on the RBC data from the pregnant women was considered reliable because total FA concentration was strongly correlated to all RBC FA proportions in data analyzed by the U-A method, and the stronger the correlation between an adjustment factor and the variable it is meant to adjust, the more reliable the adjustment is. Secondly the distributions of the proportions of AA and DHA in data analyzed by the A-A method were approximated to normal distribution.

To our knowledge, no one has previously attempted to adjust FA data from partly peroxidized RBC lipids, but the residual adjustment method that was modified is well known in nutrition science (178-181). Using the A-A method makes it possible to include all the participants in the data analysis, and since no difference was observed in mean proportions of any PUFA, SFA or MUFA between the A-A and U-NP methods, the A-A method seems to be valid. However, the U-NP analysis method probably gave the most reliable RBC FA data from the pregnant women in Cohort 1. The U-NP method included only the more true, unadjusted FA data from RBC samples considered not peroxidized, and the correlation between the proportion of DHA in RBC and total DHA intake was better than in the other two models. However, the participants included in the U-NP method were not quite representative of the whole group, since height, BMI, blood pressure and parity differed between them and the participants excluded from the U-NP method, which causes a selection bias. On the other hand, adjustment is always limited by uncertainty, since the adjusted FA values are not true, but estimated. It was decided to use the U-NP method to analyze the RBC data from the pregnant women in Cohort 1 in Paper II and in the thesis. In conclusion, the stability of RBC lipids might depend on individual physiological condition as well as on the storage condition of the RBC samples. Furthermore, the study supported the use of an FA data adjustment method for partly peroxidized RBC lipids, so that all the participants could be included in the analysis.

## 17.9 Relationship between AA and DHA in RBC

The proportions of AA and DHA in RBC of the pregnant women in Cohort 1 were found to be strongly positively correlated at both time points of pregnancy when analyzed by the U-A method. These FA were also positively correlated early and

late (data not shown) in pregnancy when analyzed by the A-A method. When the data was analyzed by the U-NP method, the correlation was borderline significant early in pregnancy but highly significant late in pregnancy. In contrast, no correlation was seen between the proportions of AA and DHA in RBC of the non-pregnant women. A positive relationship between the proportions of AA and DHA has been seen before in maternal plasma PL and RBC in the third trimester of pregnancy, and in term and preterm infants at birth (237). A physiological mechanism regulating the ratio between AA and DHA in RBC membranes during pregnancy might be important to prevent the membrane from becoming too rigid and minimizing the resistance to RBC flow through the placental microcirculation (151). At the same time, it would be important to balance the production of eicosanoids derived from AA and EPA that affect blood flow. It has been shown that DHA is retroconverted to EPA in the human body (238, 239). DHA in the membrane could then serve as a reservoir for EPA and eicosanoid synthesis.

## 17.10 Advantages and disadvantages of the study

The study was prospective, observational research, which gives it both strengths and limitations. Observational studies can only measure associations between exposure and outcome, which makes them vulnerable to methodological problems, and they can never prove causality (240). However, the information obtained by observational studies is necessary for the planning of intervention studies (240).

The prospective observational design avoids most of the potential sources of methodological bias associated with retrospective case-control studies (241). Because the dietary information from the pregnant women was collected before birth, the pregnancy outcome cannot have affected the recall of the diet during pregnancy. Prospective studies also provide the opportunity to repeat dietary assessment over time (241). An additional strength of our study is that we had already collected information on diet, lifestyle and FA composition of RBC in the $11^{th}$ to $15^{th}$ week of pregnancy. Since the questionnaires cover the preceding 3 months, and RBC circulate in the blood for 3-4 months, the data can be considered to cover diet, lifestyle and RBC PUFA composition from the beginning of pregnancy. The controlled trials done so far have supplemented women with n-3 LCPUFA after the $15^{th}$ week of pregnancy (110-113, 115, 116).

Bias is one of four main reasons for associations in observational studies (240). Selection bias was our main concern. When comparing the 77 women included in Paper II (with a valid RBC sample, dietary and lifestyle information early in pregnancy and placental weight recorded) with the 47 women excluded because of an RBC sample considered peroxidized, there was no significant difference in the dietary or lifestyle variables used, or in pregnancy outcome. However, when comparing the 67 women included in the U-NP method in Paper V (with a valid RBC sample and a value for total concentration of FA in RBC, and dietary and lifestyle information early in pregnancy), with the 34 women excluded because of a RBC sample considered peroxidized, there was a significant difference in several variables, indicating a selection bias. The findings therefore cannot be generalized for those who were excluded (240). Recall/reporting bias cannot be ruled out, since dietary intake was self-reported, as were physical activity, alcohol intake and smoking. Smoking was not confirmed by cotinine or other biochemical measurement.

Another reason for associations in observational studies are confounding factors (240). In our analyses we adjusted for the confounding variables we had measured, but the correlations found may be confounded by some uncontrolled factors. It would have been interesting to include body composition of the neonates, such as arm circumference and skinfolds, as fetal programming of lean body mass has been indicated (242-244). Paternal factors are involved in skeletal growth of the fetus (245), but paternal factors were not monitored in the study. Measurement of antioxidant status or activity in RBC would have given weight to our results, and so would FA composition in cord blood or vessel walls have done. Even though maternal PUFA composition of RBC early in pregnancy has been found to be strongly correlated with neonatal PUFA composition of RBC (246), PUFA status of maternal RBC can never be seen as more than an estimate of fetal PUFA status.

Women with chronic or gestational diabetes were excluded from the study, but it would have been interesting to measure glucose tolerance. Mild glucose intolerance, below the threshold for diagnosis of gestational diabetes, may affect pregnancy outcome (247), and possibly also the proportion of n-3 LCPUFA in RBC (248). Furthermore, the small size of the cohorts made it harder to detect significant differences and confounding with statistical tests.

The third main reason for associations in observational studies is chance (240). In our analyses, the level of significance was $P<0.05$. This means that there was a 5% chance that we rejected a null hypothesis (no effect) that was actually true. The fourth reason for association in observational studies is cause, but further research in the form of clinical trials is the only way to confirm whether the associations seen are causal.

Our intention was to compare an equal number of pregnant and non-pregnant women of reproductive age. All the pregnant women that made an appointment for a first visit to the Center of Prenatal Care in Reykjavik during the study period were invited to participate in the study. They all decided to join, leaving us with full data from 141 women without antenatal or intrapartum complications. However, we ended up with full data from only 40 healthy non-pregnant women. One reason for their reluctance to participate may have been that while blood samples from the pregnant women were collected along with routine blood samples at the Center of Prenatal Care, the non-pregnant women had to come specially to the Center of Prenatal Care for blood collection. Furthermore, taking care of an 18-month old infant is a demanding job that might have left the mothers less willing to fill out the time-consuming questionnaires. Since technical accidents made a considerable part of the RBC samples from Cohort 1 unusable, we ended up comparing 83 pregnant with 40 non-pregnant women.

The women in Cohort 2 were mothers of 18-month old children. DHA is transported from the mothers blood to breast milk during lactation (249, 250). The proportion of DHA in plasma and RBC PL of unsupplemented mothers has been shown to decrease after delivery, and more so among breastfeeding women (251). Twelve months after birth, the proportion of DHA in maternal plasma PL had normalized (252), even though maternal stores do not seem to be replenished, since both maternal DHA status during pregnancy and at delivery, and neonatal DHA status have been shown to decrease with parity (253). In Iceland the frequency (95%) and duration (7.5-8.5 months) of breast-feeding is high, and 6.8% of women are still breastfeeding their children to some degree at 18 to 24 months post-partum (213). The frequency of breastfeeding among the women in Cohort 2 was 96.7% and the duration $8.4 \pm 4.2$ months (mean $\pm$ SD) (data not shown). None of the women were still breastfeeding at 18 months post-partum. We assume the results from Cohort 2 can be generalized for non-pregnant women of reproductive age in Iceland.

# 18 Conclusions

The n-3 LCPUFA DHA plays an important role in fetal development, and the placenta transports DHA in concentrated amounts from the maternal to the fetal blood. The first weeks of pregnancy are at least as important for fetal development as the later half of pregnancy. It is therefore important that women of reproductive age have a reservoir of n-3 LCPUFA when they become pregnant.

The results of this study show that L-CLO intake early in pregnancy and a relatively high n-3 LCPUFA status, as assessed by RBC level, are related to a healthy increase in birthweight and a decrease in placental weight. High birthweight in combination with a low placental weight have in turn been related to a lower risk of hypertension and cardiovascular disease in adult life. For healthy women without pregnancy complications, regular intake of L-CLO early in pregnancy could be important for the health of the offspring.

The total n-6/n-3 PUFA intake ratio was lower among the pregnant and non-pregnant women in our study than has been found among women in many Western countries, which might have a positive effect on the health of the women and their offspring. The mean daily DHA intake of the pregnant and non-pregnant women not using n-3 LCPUFA supplements did not reach the minimum daily 200 mg intake recommended for pregnant and lactating women, which shows the importance of dietary counselling for women in the early stages of pregnancy or even preconceptionally.

Even though light beer consumption in the later half of pregnancy might stimulate biosynthesis of DHA, alcohol consumption cannot be recommended at any stage of pregnancy, since its use, even at a low level, was associated with lower birthweight of the neonate. Smoking was also associated with lower birthweight, and the findings of the study indicate that smoking early in pregnancy has a negative impact on maternal n-3 LCPUFA status, as assessed by RBC level. Use of tobacco and alcohol should therefore be avoided throughout pregnancy.

Non-pregnant women who used oral contraceptives had a higher level of DHA in RBC than those who did not, and there are indications that oestrogen in the pills elevates the biosynthesis of DHA. Physical activity of the non-pregnant women was also related to higher DHA in RBC. Increased biosynthesis and/or incorporation

of DHA in RBC membranes of physically active people might serve to increase the fluidity of the membranes and counteract exercise-induced hemolysis.

The findings of the study indicate that a high content of DHA in RBC of healthy women is associated with less deterioration of lipid FA content during storage at low temperature without addition of antioxidant. The stability of RBC lipids might depend on the physiological condition of the individual as well as on storage condition of the RBC samples. Additionally, the study supports the use of a FA data adjustment method for partly peroxidized RBC lipids, so that all the participants can be included in the analysis.

The diet of humans must contain n-3 PUFA, as these are essential FA. Including n-3 LCPUFA in the diet of women in the periconceptional period is especially important, since n-3 LCPUFA status in the beginning of pregnancy is associated with the outcome of pregnancy. Optimal outcome of pregnancy has been related to improved health of the offspring later in life.

# 19 Future perspectives

It is important to confirm the associations found in the study between n-3 LCPUFA in diet and RBC, lifestyle and pregnancy outcome, with larger cohorts and with randomized controlled trials. Follow-up study on pregnant women and their neonates through lactation and on through the school years to adulthood would be very interesting. In such a follow-up study, it would be possible to relate free-living habitual L-CLO use of the mother, not only with the growth of the offspring, but also with the mental and physical development of the infant and schoolchild and with health in adult life.

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# PAPERS I-V

# Paper I

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# Relationship between dietary intake of cod liver oil in early pregnancy and birthweight

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**Objective** To investigate the possible association between birth outcome and marine food and cod liver oil intake of healthy women in early (prior to 15 weeks of gestation) pregnancy.

**Design** An observational study.

**Setting** Free-living conditions in a community with traditional fish and cod liver oil consumption.

**Population** Four hundred and thirty-five healthy pregnant Icelandic women without antenatal and intrapartum complications.

**Methods** Dietary intake of the women was estimated with a semi-quantitative food frequency questionnaire (FFQ) covering food intake together with lifestyle factors for the previous three months. Questionnaires were filled out at between 11 and 15 weeks and between 34 and 37 weeks of gestation. The estimated intake of marine food and cod liver oil was compared with birthweight by linear and logistic regression controlling for potential confounding.

**Main outcome measures** Birthweight, cod liver oil intake, lifestyle factors (alcohol, smoking).

**Results** Fourteen percent of the study population used liquid cod liver oil in early pregnancy. Regression analysis shows that these women gave birth to heavier babies ($P < 0.001$), even after adjusting for the length of gestation and other confounding.

**Conclusions** Maternal intake of liquid cod liver oil early in pregnancy was associated with a higher birthweight. Higher birthweight has been associated with a lower risk of diseases later in life and maternal cod liver oil intake might be one of the means for achieving higher birthweight.

## INTRODUCTION

Observational studies have found higher birthweight among women with moderate to high intake of marine food, rich in $n-3$ long-chain polyunsaturated fatty acids ($n-3$ LCPUFA) during pregnancy, compared with women with low intake of marine food.[1–4] Controlled intervention trials where cod liver oil was given to pregnant rats[5] and studies on pregnant women from week 15,[6] week 18,[7] week 20,[8,9] week 30[10] or in the third trimester[11] did not show increased birthweight after adjusting for gestational length. It has therefore been speculated that increased birthweight is either caused by other fish chemicals or $n-3$ LCPUFA assert their effect before 15 weeks of pregnancy.[12] Another explanation for the difference between the results of observational studies and interventional trials is that observational studies can be influenced by a variety of biases that are minimised or eliminated in controlled trials.

Birthweight in Iceland is among the highest in the world.[1,13–15] Fish intake in Iceland has been high[16] but has decreased substantially the last decade.[17] Liquid cod liver oil intake has been, and still is, a substantial source of vitamin D during the long, dark winter season in Iceland.[17]

The aim of this study was to investigate the relationship between dietary intake of marine food and cod liver oil in early pregnancy and birth outcome in healthy and low risk pregnant Icelandic women.

## METHODS

In this observational study, the participants were healthy and low risk pregnant women, who attended a routine first visit at the Center of Prenatal Care in Reykjavik, Iceland from 1999 to 2001. The inclusion criteria were absence of pre-eclampsia, hypertension or diabetes mellitus. Of the 549 women enrolled in the research group, 113 were excluded for one or more reasons. These included women whose personal data could not be found or who moved abroad before giving birth ($n = 8$), had a miscarriage or stillbirth ($n = 17$), twins or triplets ($n = 5$), a preterm birth

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**Table 1.** Maternal daily intake of fish and number of pregnant women consuming liquid cod liver oil. Values are expressed as mean [SD] or *n* (%).

| | Week of pregnancy | |
|---|---|---|
| | 11th to 15th | 34th to 37th |
| | $n = 436$ | $n = 358$ |
| Fish (g/day) | 29 [28] | 27 [20] |
| Liquid cod liver oil | 63 (14.4) | 62 (17.3) |
| Concentrated cod liver oil capsules | 134 (30.7) | 123 (34.4) |

($\leq$259 days) ($n = 17$), hypertension/pre-eclampsia ($n = 62$) or gestational diabetes mellitus ($n = 4$). A total of 436 healthy women with no complications and giving birth to full-term babies completed the study. The National Bioethics Committee of Iceland and the Icelandic Data Protection Commission approved the study. All women gave their informed consent for participation in the study.

A semi-quantitative food frequency questionnaire (FFQ) covering food intake for the last three months and a questionnaire on other lifestyle factors were mailed to the participants two weeks before their first visit to the clinic at between 11 and 15 weeks of gestation and again two weeks before a visit at between 34 and 37 weeks of gestation. The women were asked to fill in the questionnaires at home and bring them to the clinic visits or return them by mail. The semi-quantitative FFQ was developed at the Icelandic Nutrition Council to assess the entire diet over the previous three-month period. FFQs were scanned and food and nutrient intake were calculated, using the Icelandic Nutrition Database (ISGEM) and a nutrient and food calculating programme (ICEFOOD). The intake of each type of $n-3$ LCPUFA from the whole diet was calculated along with the intake of lean fish, oily fish, liquid cod liver oil, concentrated cod liver oil capsules and other supplements containing $n-3$ LCPUFA. The FFQ has been validated against the percentage of $n-3$ LCPUFA in red blood cells of healthy pregnant women (Magnusardottir AR, unpublished data) and for some nutrients.[18] The questionnaire can be obtained from the authors on request.

From the lifestyle questionnaire and medical records at the Center of Prenatal Care, information was gathered on age, height, education, parity, cohabitation, degree of nausea, duration of nausea and pelvic pain. Information on physical activity, body weight, smoking, blood pressure, haemoglobin and gestational length was gathered at both pregnancy time points. Information on body weight, blood pressure and haemoglobin at the last visit before delivery was also available. Weight gain from initial visit at between 11 and 15 weeks of gestation until the last weighing before delivery was calculated to estimate the pregnancy weight gain.

Gestational length was estimated from an early ultrasound scan, the date of the last menstrual period or if uncertain, the best clinical judgement. Data on birth outcome (gestational age at birth, birthweight, birth length, head circumference and placental weight) were collected from maternity records at the Department of Obstetrics and Gynaecology, National University Hospital in Reykjavik, Iceland.

The association between the dietary intake of marine foods and supplements (concentrated cod liver oil capsules and liquid cod liver oil) containing $n-3$ LCPUFA and birth outcome was assessed by calculating Pearson's product–moment correlation coefficients and/or Kendall non-parametric correlation coefficients, or in case of binary data,

**Table 2.** Maternal and neonatal characteristics for women consuming liquid cod liver oil in early pregnancy *versus* non-consumers. Values are presented as mean (SD), unless otherwise indicated.

| | Liquid cod liver oil | | *P* |
|---|---|---|---|
| | No ($n = 373$) | Yes ($n = 63$) | |
| **Infant anthropometry** | | | |
| Gestational age (days) | 282.2 (8.0) | 282.9 (8.2) | 0.525 |
| Birthweight (g) | 3759 (457) | 3898 (519) | 0.026 |
| Birth length (cm) | 52.0 (2.0) | 52.5 (2.0) | 0.089 |
| Head circumference (cm) | 35.8 (1.3) | 35.9 (1.6) | 0.584 |
| Placenta (g) | 724 (148) | 728 (143) | 0.845 |
| Gender—boys (%) | 52.5 | 50.8 | 0.797 |
| **Maternal intake in first trimester** | | | |
| Energy (kJ/day) | 8521 (3074) | 9417 (3020) | 0.032 |
| Protein (g/day) | 78.5 (28.7) | 88.3 (34.1) | 0.015 |
| Fat (g/day) | 74.4 (34.7) | 85.4 (31.3) | 0.018 |
| Saturated fat (g/day) | 30.8 (15.4) | 34.4 (13.2) | 0.079 |
| Monounsaturated fat (g/day) | 20.8 (10.1) | 24.6 (9.8) | 0.006 |
| Polyunsaturated fat (g/day) | 9.1 (4.5) | 10.9 (4.8) | 0.003 |
| Carbohydrate (g/day) | 259.3 (93.9) | 276.7 (95.4) | 0.181 |
| Added sugar (g/day) | 64.7 (44.8) | 62.6 (41.9) | 0.728 |
| Alcohol (g/day) | 0.8 (1.4) | 1.8 (2.5) | 0.005 |
| Fish (g/day) | 28.3 (28.4) | 35.6 (26.4) | 0.056 |
| Concentrated cod liver oil capsules (yes, %)[a,*] | 33.0 | 17.5 | 0.014 |
| **Maternal anthropometry** | | | |
| Age (years) | 27.8 (4.9) | 29.6 (4.6) | 0.008 |
| Height (cm) | 167.7 (5.9) | 168.5 (5.6) | 0.335 |
| BMI at first visit (kg/m$^2$) | 24.3 (4.2) | 24.2 (3.2) | 0.801 |
| Weight gain during pregnancy (kg) | 13.7 (4.7) | 13.5 (4.6) | 0.695 |
| Haemoglobin first visit (g/dL) | 12.3 (0.8) | 12.2 (0.8) | 0.254 |
| Haemoglobin last visit (g/dL) | 11.8 (0.9) | 11.8 (0.8) | 0.715 |
| **Smoking ($n = 354$, %)*** | | | |
| Non-smoker | 63.6 | 76.9 | 0.155 |
| Only smoking in early pregnancy | 21.2 | 15.4 | |
| Smoking throughout pregnancy | 15.2 | 7.7 | |
| **Parity ($n = 436$, %)*** | | | |
| First child | 49.2 | 31.7 | 0.036 |
| Second child | 31.7 | 41.3 | |
| Third child or more | 19.1 | 27.0 | |

[a] % of participants in each group, consuming concentrated cod liver oil capsules during the first trimester.

* $\chi^2$ statistics.

**Table 3.** Linear regression model for birthweight.

| | Regression coefficient | 95% CI | | *P* |
|---|---|---|---|---|
| | | Lower bound | Upper bound | |
| (Constant) | −3408.7 | −5173.1 | −1644.4 | <0.001 |
| Gender of infant | −90.9 | −169.6 | −12.1 | 0.024 |
| Gestational age (days) | 20.6 | 15.4 | 25.8 | <0.001 |
| Mother's height (cm) | 8.5 | 1.6 | 15.4 | 0.016 |
| BMI at first visit (kg/m$^2$) | 22.6 | 12.7 | 32.6 | <0.001 |
| Haemoglobin at last visit (g/dL) | −70.4 | −114.7 | −26.0 | 0.002 |
| Alcohol consumption in first trimester (g/day) | −27.6 | −53.2 | −2.1 | 0.034 |
| Parity | 90.1 | 37.9 | 142.2 | 0.001 |
| Smoking during pregnancy | −76.4 | −131.1 | −21.7 | 0.006 |
| Liquid cod liver oil in first trimester (yes/no) | 132.1 | 18.3 | 246.0 | 0.023 |
| Weight gain during pregnancy (kg) | 24.7 | 15.8 | 33.6 | <0.001 |

Dependent variable: birthweight (g).
Reference categories: Gender: 0 = males, 1 = females; Parity: 0 = first child, 1 = second child, 2 = third child or more; Smoking: 0 = non-smoking, 1 = only smoked during first trimester, 2 = smoking throughout pregnancy; Cod liver oil: 0 = no intake 1 = intake.
$n = 350$.
Adjusted $R^2 = 0.352$.
$P < 0.001$.

the Mann–Whitney nonparametric test for correlation. Liquid cod liver oil intake was treated as binary data, because almost every subject took a common dose of 10 mL/day. To control for potential confounding, significantly associated variables were combined in linear regression models with backward stepwise regression. Interaction between the independent variables was tested by calculating the correlation of their products and the residuals between the model and the data.[19]

We also designed a logistic regression model for high birthweight and liquid cod liver oil intake in early pregnancy in the same manner. The odds of healthy women giving birth to an infant of ≥4500 g after a healthy pregnancy was calculated with 95% confidence intervals (95% CI), considering the same confounding factors as in the linear regression model. Stepwise backwards elimination was used for selecting confounding. The level of significance was set at $P = 0.05$ and two-tailed $P$ values were used. All statistical analyses were performed using the SPSS programme (SPSS 11.0 for Windows, SPSS, Chicago).

## RESULTS

Table 1 presents daily intake of fish (g), and percentage of pregnant women consuming liquid cod liver oil and concentrated cod liver oil capsules at between 11 and 15 weeks and between 34 and 37 weeks of gestation. More women chose to use concentrated cod liver oil capsules than liquid cod liver oil at both time points (30.7% *vs* 14.4% and 34.4% *vs* 17.3%, respectively).

Table 2 presents maternal and neonatal characteristics for the 436 participants included in the study. The participants were split into two groups: women consuming liquid cod liver oil in the beginning of pregnancy ($n = 63$) *versus* non-consumers ($n = 373$). The women consuming liquid cod liver oil gave birth to 139 g heavier infants ($P = 0.026$). These women were getting more energy (kJ/day) from their diet ($P = 0.032$), and they had higher intake of protein ($P = 0.015$), fat ($P = 0.018$) and alcohol ($P = 0.005$), compared with the women not consuming liquid cod liver oil. Despite that, the women consuming liquid cod liver oil were not gaining more weight ($P = 0.695$). They were also almost two years older ($P = 0.008$) and fewer of them were primiparous, or 31.7% *versus* 49.2%, respectively ($P = 0.036$).

Liquid cod liver oil intake in early pregnancy was related to higher birthweight ($r = 0.107$, $P = 0.026$), whereas concentrated cod liver oil capsules and fish consumption were not. No relationship was found between birthweight and fish or cod liver oil later in pregnancy (data not shown).

Table 3 shows a linear regression model for birthweight. The regression coefficients show changes in birthweight (g) related to one unit change in each variable included in the model. Liquid cod liver oil intake in early pregnancy was still significantly and positively correlated ($P = 0.023$) with birthweight after adjustment for gestational age at birth and other confounding [gender of infant, mother's height (cm), body mass index (BMI) at first visit (kg/m$^2$), haemoglobin at last visit (g/dL), alcohol consumption (g/day) in first trimester, parity and smoking during pregnancy, and maternal weight gain (kg) during pregnancy]. The regression model expressed 35.2% ($R^2$ adjusted) of the variation in birthweight. Only 350 mothers were included in the regression model as 85 women did not fill out their food frequency and lifestyle questionnaires at the second time point, and therefore information on their smoking habits in late pregnancy were lacking. There was no significant difference in any of the other variables included in the

**Table 4.** Logistic regression model for high birthweight and liquid cod liver oil intake in early pregnancy. Odds of giving birth to an infant of ≥4500 g and confounding factors.

| Birthweight ≥ 4500 g | Odds ratio | 95% CI | | *P* |
|---|---|---|---|---|
| | | Lower bound | Upper bound | |
| Liquid cod liver oil in first trimester (yes/no) | 10.99 | 3.29 | 36.70 | <0.001 |
| Gestational age (days) | 1.14 | 1.05 | 1.24 | 0.003 |
| BMI at first visit (kg/m$^2$) | 1.22 | 1.07 | 1.39 | 0.003 |
| Alcohol consumption in first trimester (g/day) | 0.28 | 0.10 | 0.80 | 0.017 |
| Weight gain during pregnancy (kg) | 1.15 | 1.03 | 1.28 | 0.013 |

$n = 350$.

regression model between the 85 women excluded *versus* women included in the regression model.

Energy percent (E%) from PUFA was also found to correlate positively with birthweight ($r = 0.065$, $P = 0.042$), and E% from added sugar correlated inversely with birthweight ($r = -0.078$, $P = 0.015$) (data not shown). Other factors related to birthweight were diastolic and systolic blood pressure at the first visit to the prenatal clinic, diastolic blood pressure at the last visit, maternal education, maternal work, maternal age and cohabitation with the father of the unborn child. Maternal intake of macronutrients, fish and concentrated cod liver oil capsules were also taken into consideration as potential confounding factors. However, none of these factors remained significant in the final regression model (data not shown).

Table 4 presents a logistic regression model for high birthweight and liquid cod liver oil intake in early pregnancy. Healthy women were 11 times more likely to give birth to an infant of 4500 g or more after a healthy pregnancy if they used liquid cod liver oil (95% CI 3.3–36.7, $P < 0.001$), after correcting for confounding factors. Alcohol consumption in the first trimester (g/day) had a negative association to high birthweight (OR = 0.28, 95% CI = 0.10–0.80, $P = 0.017$).

## DISCUSSION

In the present study, maternal intake of liquid cod liver oil in early pregnancy was related to the birthweight of the offspring. The amount of $n-3$ LCPUFA in the common daily dose of concentrated cod liver oil capsules (0.18 g) is 10 times less than in a 10-mL spoon of liquid cod liver oil (1.8 g). We would suggest that this difference in the amount of ingested $n-3$ LCPUFA is the most probable reason for the lack of relationship between intake of concentrated cod liver oil capsules and birthweight. Intervention studies supplementing with $n-3$ LCPUFA did not show increased birthweight after adjusting for gestational age, but they had all been supplementing $n-3$ LCPUFA from week 15 or later.[6–11] Docosahexaenoic acid (DHA) is the main $n-3$ LCPUFA in food and in the body. It is believed to play a crucial role for the development of the central nervous system.[6] During the first 10 weeks of pregnancy, the proportion of DHA in red blood cells and plasma increases without a simultaneous increase in DHA intake.[20] Adequate maternal DHA stores are therefore important to allow their transport to blood early in pregnancy, fulfilling the increased need of the fetus for DHA in this critical period of development. If the maternoplacental nutrient supply does not meet the fetal nutrition demand, fetal adaptations are invoked. According to the Barker hypothesis,[21] several serious diseases in later life including coronary heart disease, hypertension and type 2 diabetes mellitus originate from impaired intrauterine growth and development, and may be the consequences of fetal programming. If $n-3$ LCPUFA intake early in pregnancy is related to larger offspring, and larger offspring is related to a lower risk of these diseases, cod liver oil intake in early pregnancy could be important for the health of the infant in adult life. Deficiency of $n-3$ LCPUFA in the perinatal period has been associated with higher blood pressure later in life.[22]

In the present study, women consuming liquid cod liver oil in early pregnancy did not differ from their non-consuming counterparts in anthropometric measures, but their dietary intake in the first trimester was different. The women who consumed liquid cod liver oil consumed more energy (9417 [3020] kJ *vs* 8521 [3074] kJ, $P = 0.032$), and their intake of protein, total fat, mono- and polyunsaturated fatty acids as well as alcohol consumption was higher. Nonetheless, they did not gain more weight and their BMI was not different from the non-consuming mothers. This is difficult to explain. One explanation might be an effect of the cod liver oil intake on weight gain itself. For example, a study from Wang *et al.* suggests that $n-3$ PUFA are less likely to cause obesity than other fats.[23] However, the higher birthweight of infants born to the liquid cod liver oil consuming mothers without simultaneous increase in maternal weight gain is even more interesting, because maternal weight gain is one of the major determinants of birthweight.[14,24] Pregnancy weight gain is, however, still controversial and too much weight gain may have negative consequences for both infants and mothers.[25] In our study, we looked at the self-reported maternal pre-pregnancy weight, weight at first visit and weight at the end of pregnancy as well as weight gain during pregnancy and found the last one to have the highest impact on birth size. Each kilogram of maternal weight gain led to a 25-g increase in

birthweight. We also considered net weight gain (weight of fetus and placenta subtracted from total weight gain) and proportional weight gain (gained weight/weight at first prenatal visit), but these variables did not correlate with birthweight (data not shown). It is likely that weight gain in pregnancy, caused by a larger fetus and placenta, predicts birthweight better than weight gain due to excessive maternal fat accumulation. This is in accordance with Langhoff-Roos *et al.*,[26] who have shown that lean body mass, but not fat mass, is strongly correlated with infant birthweight. Factors, other than liquid cod liver oil, included in the regression model have been linked to birthweight in earlier studies.[27–32] Smoking and alcohol consumption are lifestyle factors that should be avoided throughout pregnancy. According to our study as well as others, there is a negative relationship between smoking and birthweight, but if the mother stops smoking early in pregnancy the birthweight is higher than among women who continue smoking.[33] In our study, even small amounts of alcohol had a negative relationship with birthweight. The evidence of neonatal functional damage due to alcohol, even at very low doses, has been supported earlier.[34]

Healthy women were 11 times more likely to give birth to an infant of 4500 g or more after a healthy pregnancy if they used liquid cod liver oil during the first trimester. Research on the benefits of higher birthweight has been focussing on the risk of giving birth to low birthweight infants (<2500 g).[35,36] However, macrosomia, which is defined as having a fetal weight of above the 90th centile, a birthweight above 4000 g or 4500 g, or a birthweight of over two standard deviations above the mean birthweight by gestational age,[37] has been considered to be not desirable because of the increased risk of obstetric and perinatal complications.[32,37] Mean birthweight has increased in the past decade in some countries, and the main reason is thought to be the mothers' changed lifestyle and increasing obesity.[30,38,39] Birthweight of term infants in Iceland is very high (3779 [469] g in our data), compared with other countries and has been so for a long time.[1,13–15] Birgisdottir *et al.*[40] collected data from people born in Iceland 1914–1935, and the mean birthweight was 3820 (600) g for boys and 3680 (500) g for girls. Icelandic studies show that even in this high birthweight population, a larger size at birth is related to low prevalence of common adult diseases (e.g. glucose intolerance and hypertension).[40,41]

Cod liver oil is not only a rich source of $n-3$ LCPUFA, but is also a good source of vitamins A, D and E. All cod liver oil in Iceland is purified and controlled for dioxin and dioxin-like compounds. Vitamin A is essential for the normal embryonic development, but excessive exposure to retinol may result in congenital malformations. According to Nordic recommendations, retinol intake during pregnancy should therefore be limited to 3000 μg/day.[42] In our study, the mean intake of retinol was 1284 (723) μg/day for the women not consuming cod liver oil and 2499 (1293) μg/day for the women consuming cod liver oil early in pregnancy (unpublished results). For the latter group, at the 75th centile, retinol intake was above the recommended 3000 μg maximum. However, since the completion of the study, the retinol content of Icelandic cod liver oil has been lowered, from 2400 to 460 μg in the common 10-mL dosage,[17] resulting in much lower retinol intakes.

We conclude that for healthy Icelandic women without pregnancy complications, regular intake of liquid cod liver oil early in pregnancy is to be considered as positive for the infants' health. It is important to confirm the association between liquid cod liver oil intake in early pregnancy and birthweight further with randomised controlled trials.

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# Paper II

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**ORIGINAL ARTICLE**

# Red blood cell n-3 polyunsaturated fatty acids in first trimester of pregnancy are inversely associated with placental weight

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**Abstract**

*Objective*. To investigate pregnancy outcome in relation to red blood cell (RBC) level of long-chain n-3 polyunsaturated fatty acids (PUFA) in the first trimester of pregnancy and the influence of lifestyle factors on the RBC level of long-chain n-3 PUFA. *Design and setting*. Observational study in a community with traditional fish and cod liver oil consumption. *Population*. Seventy-seven healthy pregnant women. *Methods.* The PUFA composition of RBC was measured in the 11th to 15th week of pregnancy. The women answered food frequency and lifestyle questionnaires. Information on pregnancy outcome was collected from birth records. *Main outcome measures*. Placental weight, long-chain n-3 PUFA in diet and RBC, smoking. *Results.* Of all the pregnancy outcome variables tested, placental weight was the only one associated with long-chain n-3 PUFA in RBC. Inverse association was found between the proportion of long-chain n-3 PUFA in RBC and placental weight, adjusted for birthweight ($p = 0.035$). The proportion of long-chain n-3 PUFA in RBC was positively related to long-chain n-3 PUFA intake ($p < 0.001$) and negatively related to smoking ($p = 0.011$). *Conclusion.* The human fetus relies on maternal supply and placental delivery of long-chain n-3 PUFA for optimal development and function, particularly of the central nervous system. Given the importance of dietary n-3 PUFA during pregnancy, further studies are warranted to investigate the relationship between placental weight, maternal long-chain n-3 PUFA status and smoking.

**Key words:** *n-3 Polyunsaturated fatty acids, cigarette smoking, red blood cells, placental weight, pregnancy outcome*

**Abbreviations:** *AA: arachidonic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, FAME: fatty acid methyl esters, FFQ: food-frequency questionnaire, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, RBC: red blood cells, SFA: saturated fatty acids*

## Introduction

The long-chain n-3 polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA; 22:6n-3) represents the longest and the most unsaturated fatty acid commonly found in biological systems. Fatty acids are the building blocks of membranes and DHA is the major n-3 PUFA constituent in neuronal membranes (1). Humans must obtain DHA from their diet, or from liver conversion of the respective dietary precursors, α-linolenic acid (18:3n-3) from plant products and eicosapentaenoic acid (EPA; 20:5n-3) of marine origin (2). The fetus relies on maternal supply and placental delivery of DHA for normal development and function of the central nervous system (3).

Fish and cod liver oil are rich in long-chain n-3 PUFA and maternal fish intake has been found to be positively correlated with both placental weight and birthweight among non-smoking women (4). The nutritional status of women around conception and in the first trimester of pregnancy, when fetal organs

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are developing with a high rate of cell division, is as important as the nutritional status in the second and third trimesters of pregnancy (5). In a prospective, observational study on healthy pregnant Icelandic women, we found liquid cod liver oil intake before week 15 of pregnancy to be positively correlated with birthweight, adjusted for duration of gestation, smoking and other confounding factors (6). However, there is limited comprehensive published data on the effect of maternal n-3 PUFA status in early pregnancy on pregnancy outcome.

PUFA are susceptible to oxidative damage (7) and cigarette smoking has been shown to increase lipid peroxidation (8). Agostoni and colleagues (9) have recently reported that maternal smoking is associated with a reduction in long-chain PUFA pools in neonates and this may have structural and functional consequences. If this is the case, it is plausible that one of the reasons for a negative impact of smoking on birth outcome (10) may be an insufficiency of long-chain PUFA during the critical period of fetal growth and development.

The PUFA composition of red blood cell (RBC) membrane lipids is a good indicator of dietary PUFA intake over the previous few months (11) and it has been suggested that maternal RBC lipids act as a source of long-chain PUFA for placental transfer to the fetus (12). The aim of the present study was to investigate pregnancy outcome in relation to RBC level of long-chain n-3 PUFA in the first trimester of pregnancy and the influence of lifestyle factors on the RBC level of long-chain n-3 PUFA.

## Material and methods

The study is a part of a larger project on dietary intake of healthy and low-risk, pregnant Icelandic women and outcome of pregnancy (6). The participants in the present study were 77 singleton pregnant women. They were enrolled at a routine first visit in the 11th to 15th week of pregnancy to the Center of Prenatal Care in Reykjavik, Iceland. Blood samples were collected and the women filled in a semi-quantitative food-frequency questionnaire (FFQ) and a questionnaire on other lifestyle factors at study entry. Women with previous history of and/or diagnosed with preeclampsia, hypertension, intrahepatic cholestasis, diabetes mellitus (gestational or chronic), anemia, polyhydramnion, oligohydramnion or preterm birth ($<37$ weeks) were excluded. The National Bioethics Committee of Iceland approved the study and the Icelandic Data Protection Commission was notified of the processing of personal data. The women gave informed consent for their participation in the study.

Non-fasting venous blood samples were collected in Vacuette® tubes containing ethylene diamine tetraacetate (EDTA). RBC were isolated immediately by centrifuging whole blood at $1300 \times g$ for 10 min at 4°C and washed three times with isotonic saline solution. RBC total lipids were extracted with isopropanol and chloroform (2:1, v/v) (13). The antioxidant butylated hydroxytoluene (50 mg/L) was added to the extraction medium. The fatty acids were transmethylated for 45 min at 110°C using 14% boron trifluoride/methanol (Sigma Chemical Co., St. Louis, MO). The fatty acid methyl esters (FAME) were analyzed using gas chromatograph (HP Series II 5890 A, Hewlett Packard Co., Palo Alto, CA) equipped with a flame ionization detector and a Chrompack CP-WAX 52CB column (25 m $\times$ 320 μm i.d. $\times$ 0.2 μm film thickness). The oven was programmed to have an initial temperature of 90°C for 2 min, then rising at 30°C/min to 165°C and at 3°C/min to 225°C and then held isothermal for 6 min. The injector and detector temperatures were maintained at 235 and 250°C, respectively. Hydrogen was used as the carrier gas. The FAME peaks were identified and calibrated against those of commercial standards (Sigma Chemical Co.; Nu-Chek-Prep, Elysian, MN). Instrumental control and data handling were done by HP 3365 Chemstation, Version A.02.12 (Hewlett Packard Co., Palo Alto, CA).

A semi-quantitative FFQ developed at the Icelandic Nutritional Council was used to assess the womens' entire diet over the previous three months. The FFQ were scanned and food and nutrient intake calculated, using The Icelandic Nutrition Database (ISGEM) and a nutrient and food calculating program (ICEFOOD). Total fat intake and intake of individual fatty acids from the whole diet was calculated. With regard to sources of long-chain n-3 PUFA, the intake of lean fish, oily fish, liquid cod liver oil (10 mL spoon contains 1.8 g long-chain n-3 PUFA), cod liver oil capsules (the common daily dose contains 0.18 g long-chain n-3 PUFA) and other supplements containing long-chain n-3 PUFA was assessed. The FFQ has been validated against a reference method and several nutrient biomarkers (14).

Information was gathered from a lifestyle questionnaire on age, height, body weight (at entry), education, parity, cohabitation, nausea, physical activity (cycling, swimming or other physical activity), and smoking. Non-smoking women were defined as women who had never smoked or quit smoking more than six months prior to study entry. Smoking women were defined as women who quit smoking less than six months prior to study entry and women who smoked at study entry.

Gestational length at entry was calculated from the last menstrual period, reestimated at 20th week of pregnancy with an ultrasound scan and corrected if not in accordance with the date of the last menstrual period. Data on pregnancy outcome (duration of gestation, birthweight, crown-heel length, head circumference and placental weight) were collected from maternity records at the Department of Obstetrics and Gynaecology, National University Hospital in Reykjavik, Iceland. The placenta was weighed untrimmed after washing to remove all clots and after inspection of the cotyledons and membranes for any missing tissue.

*Statistical analysis*

Fatty acid composition of RBC from women using and not using long-chain n-3 PUFA supplements, and from smoking and non-smoking women, was compared with independent *t*-test. A multiple linear regression model with the proportion of long-chain n-3 PUFA in RBC as the dependent variable and total long-chain n-3 PUFA intake (from food and supplements), smoking and gestational length at entry as independent variables was constructed for the whole study group. A multiple linear regression model with placental weight as the dependent variable and the proportion of long-chain n-3 PUFA in RBC and birthweight as independent variables was constructed for the whole study group. Age, body mass index, alcohol intake, education, parity, cohabitation, nausea and physical activity were tested as covariates in the model for n-3 PUFA in RBC and in the model for placental weight, but they had no effect on the models. Smoking, use of long-chain n-3 PUFA supplements and duration of gestation were also tested as covariates when assessing effects on placental weight. None of these variables had a significant effect and they were not included in the final model. The level of significance was set at 0.05 and two-tailed *p*-value was used. Statistical analysis was performed using the SPSS statistical software package version 15.0 (SPSS Incorporated, Chicago, USA).

## Results

Table I shows the maternal and neonatal characteristics. The prevalence of long-chain n-3 PUFA supplement intake (liquid or capsules) in first trimester of pregnancy was 40.3% ($n=31$). Half of the women used liquid cod liver oil ($n=15$), and the other half cod liver oil capsules ($n=16$). The prevalence of smoking was 39.0% ($n=30$). Ten of the smoking women were using long-chain n-3 PUFA supplements (data not shown).

Table I. Maternal and neonatal characteristics.

| | Whole study group ($n=77$) |
|---|---|
| Maternal characteristics in first trimester | |
| Age (years) | 27.4 (4.7) |
| Body mass index at entry (kg/m$^2$)* | 24.1 (3.4) |
| Smoking (*n*) | 30 (39.0) |
| Total long-chain n-3 PUFA intake (mg/day) | 530.6 (636.8) |
| DHA intake (mg/day) | 309.9 (341.1) |
| EPA intake (mg/day) | 194.3 (241.5) |
| Fish intake (g/day) | 29.2 (23.5) |
| Liquid cod liver oil (*n*) | 15 (19.5) |
| Cod liver oil capsules (*n*) | 16 (20.8) |
| Total n-6 PUFA/n-3 PUFA intake ratio | 3.98 (1.11) |
| Outcome of pregnancy | |
| Birthweight (g) | 3828 (445) |
| Crown-heel length (cm) | 52.4 (1.9) |
| Head circumference (cm) | 35.7 (1.4) |
| Placenta (g) | 726 (125) |
| Placental weight to birthweight ratio (%)# | 19.0 (2.4) |

*$n=75$ for body mass index.
#Placental weight to birthweight ratio = placental weight/birth weight $\times 100$.
PUFA: polyunsaturated fatty acids, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid.
Data are given as mean (SD) or as number (percentage).

Table II shows the fatty acid composition of RBC in first trimester of pregnancy according to use of long-chain n-3 PUFA supplements and smoking. The proportion of all individual n-3 PUFA and total n-3 PUFA was higher ($p \leq 0.002$) in RBC of the women using long-chain n-3 PUFA supplements than in RBC of the women not doing so. The proportions of 22:4n-6, total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), as well as the n-6 PUFA/n-3 PUFA ratio, were lower ($p \leq 0.006$) in RBC of the women using long-chain n-3 PUFA supplements than in RBC of the women not doing so. The proportion of DHA and total n-3 PUFA was lower ($p \leq 0.031$) and the n-6 PUFA/n-3 PUFA ratio higher ($p=0.024$) in RBC of the smoking than the non-smoking women.

Table III shows a multiple linear regression model to predict the proportion of long-chain n-3 PUFA in RBC in the whole study group. As expected, total intake of long-chain n-3 PUFA was positively correlated with the proportion of long-chain n-3 PUFA in RBC. Furthermore, according to the model, smoking decreased the proportion of long-chain n-3 PUFA in RBC by 0.71% of total fatty acids, adjusted for total long-chain n-3 PUFA intake

Table II. Fatty acid composition (% of total fatty acids) of RBC total lipids in first trimester of pregnancy according to use of long-chain n-3 PUFA supplements and smoking.

| | Long-chain n-3 PUFA supplements | | | Smoking | | |
|---|---|---|---|---|---|---|
| | No ($n=46$) | Yes ($n=31$) | $t$-test ($p$) | No ($n=47$) | Yes ($n=30$) | $t$-test ($p$) |
| 18:2n-6 | 8.36 (1.10) | 8.85 (0.91) | 0.042 | 8.43 (0.95) | 8.76 (1.18) | 0.183 |
| 20:3n-6 | 1.53 (0.30) | 1.56 (0.29) | 0.669 | 1.50 (0.27) | 1.61 (0.32) | 0.128 |
| AA (20:4n-6) | 12.00 (1.14) | 11.84 (1.28) | 0.556 | 12.07 (1.24) | 11.72 (1.10) | 0.202 |
| 22:4n-6 | 2.42 (0.35) | 2.16 (0.46) | 0.006 | 2.29 (0.44) | 2.36 (0.38) | 0.483 |
| EPA (20:5n-3) | 0.76 (0.23) | 1.14 (0.44) | <0.001 | 0.97 (0.41) | 0.84 (0.30) | 0.141 |
| 22:5n-3 | 2.41 (0.50) | 2.58 (0.33) | 0.002 | 2.48 (0.36) | 2.39 (0.28) | 0.273 |
| DHA (22:6n-3) | 5.71 (0.86) | 6.54 (0.98) | 0.001 | 6.29 (0.94) | 5.75 (1.04) | 0.022 |
| SFA | 41.20 (1.87) | 40.03 (1.50) | 0.005 | 40.60 (1.84) | 40.99 (1.76) | 0.330 |
| MUFA | 17.39 (1.05) | 16.70 (0.97) | 0.004 | 16.95 (0.96) | 17.37 (1.19) | 0.095 |
| n-6 PUFA | 24.31 (1.63) | 24.41 (1.78) | 0.803 | 24.29 (1.73) | 24.44 (1.64) | 0.718 |
| n-3 PUFA | 8.88 (1.22) | 10.27 (1.53) | <0.001 | 9.73 (1.52) | 8.98 (1.39) | 0.031 |
| n-6 PUFA/n-3 PUFA | 2.80 (0.41) | 2.44 (0.45) | <0.001 | 2.56 (0.44) | 2.80 (0.45) | 0.024 |

RBC: red blood cells, PUFA: polyunsaturated fatty acids, AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids.
Data are given as mean (SD).

and gestational length. The model explained 41.7% of the variation in the proportion of long-chain n-3 PUFA in RBC.

A negative correlation ($r=-0.23$, $p=0.041$, Pearson correlation) was found between the proportion of long-chain n-3 PUFA in RBC in first trimester of pregnancy and the placental weight to birthweight ratio in the whole study group ($n=77$, data not shown). This was attributed to a negative, but non-significant trend between the proportion of long-chain n-3 PUFA in RBC and placental weight, while no correlation was seen between long-chain n-3 PUFA in RBC and birthweight.

Table IV shows a multiple linear regression model to predict placental weight in the whole study group. According to the model, an increase in the proportion of long-chain n-3 PUFA in RBC by 1% of total fatty acids decreased the placental weight by 14.6 g, adjusted for birthweight. The model explained 49.2% of the variation in placental weight. No correlation was found between the proportion of n-3 PUFA in RBC and other pregnancy outcome variables.

## Discussion

In the present study the proportion of long-chain n-3 PUFA in RBC in the first trimester of pregnancy was inversely correlated with placental weight, adjusted for birthweight. There was also an inverse correlation between the proportion of long-chain n-3 PUFA in RBC in the first trimester of pregnancy and smoking, adjusted for long-chain n-3 PUFA intake and gestational length.

Table III. Multiple linear regression model for factors related to the proportion of long-chain n-3 PUFA in RBC (% of total fatty acids) in first trimester of pregnancy in the whole study group.

| | | 95% CI | | |
|---|---|---|---|---|
| Independent variables | Unstandardized coefficient, $B$ | Lower bound | Upper bound | $p$ |
| (Constant) | 7.91 | 5.99 | 9.84 | <0.001 |
| Total long-chain n-3 PUFA intake (g/d) | 1.41 | 1.00 | 1.83 | <0.001 |
| Smoking | −0.71 | −1.25 | −0.17 | 0.011 |
| Exact gestational length at entry (days) | 0.02 | 0.00 | 0.04 | 0.044 |

Dependent variable: Long-chain n-3 PUFA in RBC (% of total fatty acids).
Reference categories for smoking: 0 = non-smoking, 1 = smoking.
Adjusted $R^2=0.417$.
$p<0.001$.
$n=76$.
PUFA: polyunsaturated fatty acids, RBC: red blood cells, CI: confidence interval.

Table IV. Multiple linear regression model for factors related to the placental weight in the whole study group.

| Independent variables | Unstandardized coefficient, *B* | 95% CI | | *p* |
|---|---|---|---|---|
| | | Lower bound | Upper bound | |
| (Constant) | 136 | −89.0 | 361.4 | 0.232 |
| Birthweight (g) | 0.190 | 0.144 | 0.236 | <0.001 |
| Long-chain n-3 PUFA in RBC (%) | −14.6 | −28.2 | −1.06 | 0.035 |

Dependent variable: placental weight (g).
Adjusted $R^2 = 0.492$.
$p < 0.001$.
$n = 77$.
CI: confidence interval, PUFA: polyunsaturated fatty acids, RBC: red blood cells.

We are aware of only one study where placental weight was related to n-3 PUFA (4). Olsen et al. (4) found placental weight, birthweight and head circumference of term neonates to be positively correlated with maternal fish intake in the third trimester of pregnancy among non-smoking women. In our study, the third trimester level of n-3 PUFA in RBC was not related to placental weight (unpublished data). The reason for placental weight relating positively to fish intake in the study of Olsen et al. (4) and negatively to the proportion of long-chain n-3 PUFA in RBC in the present study is not clear. One possible reason could be that chemicals in fish other than long-chain n-3 PUFA affect placental weight in a positive way. In our study, the relationship between the proportion of long-chain n-3 PUFA in RBC and placental weight did not reach significance unless we divided placental weight with birthweight, or adjusted the placental weight for birthweight with multiple regression. In the study of Olsen et al. (4) it was not mentioned whether the placental weight increased or decreased relative to the birthweight as fish intake increased.

Human vascular endothelium is rich in the long-chain n-6 PUFA arachidonic acid (AA; 20:4n-6) (15), and AA accumulates in the highly vascular placenta in the first trimester of pregnancy (16). An imbalance in maternal long-chain PUFA membrane composition, with a high proportion of long-chain n-3 PUFA and a low proportion of AA, could disturb the vascular development of the placenta and possibly lead to lower placental weight. However, in the present study long-chain n-3 PUFA supplement intake was not accompanied by a lower proportion of AA in RBC and there was no correlation between the proportions of AA and n-3 PUFA in RBC. Moreover, no correlation was found between the proportion of AA in RBC and placental weight.

When PUFA status of smokers and non-smokers has been compared, some studies found no difference (17,18), while one study found lower long-chain PUFA status in smoking than non-smoking men (19). Further, neonates of smoking women had lower long-chain PUFA status than neonates of non-smoking women (9). The lower proportion of long-chain n-3 PUFA found in RBC of smoking compared with non-smoking women in the present study was still present after adjustment for total long-chain n-3 PUFA intake. Smoking has been shown to increase lipid peroxidation (8) and there is also evidence that biosynthesis of long-chain n-3 PUFA from precursors in smoking men and non-pregnant women is enhanced, as if to compensate for the peroxidative loss (20). Our results indicate that the biosynthesis of long-chain n-3 PUFA does not compensate fully for the peroxidative loss of these important fatty acids in smoking women under the high demands of pregnancy.

In the first trimester of pregnancy, the placenta and the fetal organs are developing with a high rate of cell division (21). Fetal programming is when undernutrition in early or mid-pregnancy leads to impaired growth or development of specific fetal organs, with consequences for the later health of the individual (5). A high birthweight, a low placental weight and a low placental weight to birthweight ratio have been associated with decreased risk of hypertension and cardiovascular disease in the adult human life (22,23). Associations between neonatal size and cardiovascular risk factors have been seen in the high birthweight population of Iceland (24,25). The positive relationship we found between liquid cod liver oil intake in the first trimester of pregnancy and birthweight in our previous study of the whole cohort (6), and the negative relationship between the proportion of long-chain n-3 PUFA in RBC in the first trimester and placental weight adjusted for birthweight in the present study, indicate that long-chain n-3 PUFA are

among the nutrients programming placental and fetal growth. One could speculate whether a relatively high maternal intake of long-chain n-3 PUFA in the periconceptional period might be beneficial for the later health of the developing individual, since higher n-3 PUFA status in first trimester of pregnancy was in the present study associated with lower placental weight without negative effects on birthweight.

In summary, an inverse association was found between the maternal long-chain n-3 PUFA status, as assessed by RBC level in first trimester of pregnancy, and placental weight at birth. In addition, smoking was inversely associated with the maternal long-chain n-3 PUFA level in RBC. The human fetus relies on maternal supply and placental delivery of long-chain n-3 PUFA for optimal development and function, particularly of the central nervous system. Given the importance of dietary n-3 PUFA during pregnancy, further studies are warranted to investigate the relationship between placental weight, maternal long-chain n-3 PUFA status and smoking.

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# Paper III

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# Docosahexaenoic acid in red blood cells of women of reproductive age is positively associated with oral contraceptive use and physical activity ☆

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ABSTRACT

Optimal intake of the long-chain n-3 polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) and proper balance between intake of n-6 PUFA and n-3 PUFA are important for human health. Considerable evidence exists to show that DHA has a marked benefit during pregnancy. Lifestyle factors can affect the biosynthesis of DHA from dietary precursors, incorporation into membranes and degradation. The purpose of this study was to investigate the PUFA composition of red blood cells (RBCs) from women ($n = 40$) in reproductive age, and how it is affected by diet and other lifestyle factors. Of all the lifestyle factors tested oral contraceptive use and physical activity were the ones correlated with DHA in RBCs, after adjustment for DHA intake. The findings indicate that oral contraceptive use and physical activity have a positive impact on the DHA status, as assessed by RBC level, of women in reproductive age.



## 1. Introduction

The dietary intake ratio of n-6 polyunsaturated fatty acids (PUFA) to n-3 PUFA has increased from 1–2:1 in preagricultural time to 10–20:1 in typical modern Western diet [1,2]. This change is attributed both to an increased intake of n-6 PUFA and to a decreased intake of n-3 PUFA, especially long-chain n-3 PUFA, with 20 or more carbons and 4 or more double bonds [1,2]. Docosahexaenoic acid (DHA, 22:6n-3) is the main membrane long-chain n-3 PUFA in the human body. Marine food and supplements such as cod liver oil are important sources of DHA and other long-chain n-3 PUFA. The human body is capable of biosynthesizing DHA in small amounts from the respective dietary precursors, α-linolenic acid (ALA, 18:3n-3) from plant products and eicosapentaenoic acid (EPA, 20:5n-3) of marine origin [3,4]. Biosynthesis of DHA is higher in women than men, and this has been explained by the action of oestrogen [3,4].

The PUFA composition of red blood cell (RBCs) membrane lipids is a good indicator of dietary PUFA intake over the preceding few months [5]. On the other hand, lifestyle factors such as physical activity can affect the membrane PUFA composition through oxidative stress and compensating increase in antioxidant activity [6,7]. Improving the long-chain n-3 PUFA status of women of reproductive age could be important in the prevention of several common diseases, such as major [8] and perinatal [9] depression, osteoporosis [10] and breast cancer [11]. DHA is especially important for women planning to become pregnant [12], since the fetus relies on maternal supply and placental delivery of DHA for normal development and function of the central nervous system [13]. The purpose of this study was to investigate the PUFA composition of RBC from women of reproductive age, and how it is affected by diet and other lifestyle factors.

## 2. Subjects and methods

### 2.1. Subjects

The participants in the present study were 40 healthy, non-pregnant women. They were enrolled at a visit to the Center for Child Health Services in Reykjavík, Iceland, when they came with their 18-month-old children for evaluation of their growth and general development and for vaccination. The National Bioethics

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Committee of Iceland approved the study and the Icelandic Data Protection Commission was notified of the processing of personal data. The women gave informed consent for their participation in the study.

### 2.2. Assessment of RBC total lipid FAs

Non-fasting venous blood samples were collected in Vacuette® tubes containing ethylene diamine tetra-acetate (EDTA). RBCs were isolated immediately by centrifugating whole blood at 3000 rpm for 10 min at 4 °C and washed three times with isotonic saline solution. The antioxidant butylated hydroxytoluene (BHT, dissolved in methanol) was added to the RBC samples at a final concentration of 90 μM before they were frozen to −20 °C and stored until FA analysis within 15 weeks.

RBC total lipids were extracted as described by Bligh and Dyer [14] except that isopropanol was used instead of methanol (isopropanol/chloroform 2:1, v/v). BHT (225 μM) was added to the extraction medium. The FAs were transmethylated for 45 min at 110 °C using 14% boron trifluoride/methanol (Sigma Chemical Co., St. Louis, MO). The fatty acid methyl esters (FAMEs) were analyzed using GC (Agilent 6890 N, Agilent, Palo Alto, CA) equipped with a flame ionization detector and a Chrompack CP-SIL 8CB column (25 m × 250 μm i.d. × 0.12 μm film thickness). The oven temperature was programmed to have an initial temperature of 150 °C for 4 min, then rising at 4 °C/min to 230 °C and at 20 °C/min to 280 °C and then held isothermal for 4 min. The injector and detector temperatures were maintained at 280 and 300 °C, respectively. Hydrogen was used as the carrier gas. The FAMEs peaks were identified and calibrated against those of commercial standards (Sigma Chemical Co.; Nu-Chek-Prep, Elysian, MN). Phosphatidylcholine, diheptadecanoyl (17:0) and heneicosanoic acid (21:0) methyl ester were used as internal and external standards, respectively. Each FA value was the mean of two separate RBC total lipid extractions of the same sample. The intra-assay CV for analytical variation was 1.7% for DHA and 1.3% for arachidonic acid (AA, 20:4n-6). RBC values are presented as % (by weight) of total FA with chain length from $C_{14}$ to $C_{24}$. Instrumental control and data handling were done by HP 3365 Chemstation, Version A.02.12. (Hewlett Packard Co., Palo Alto, CA).

### 2.3. Dietary and lifestyle questionnaires

A semi-quantitative food-frequency questionnaire (FFQs) and a questionnaire on other lifestyle factors were mailed to the women 2 weeks before they came with their 18-month-old children for evaluation of their development and for vaccination at the Center for Child Health Services. The participants were asked to fill in the questionnaires at home and bring them to the clinic or mail them back.

The semi-quantitative FFQ was developed at the Icelandic Nutritional Council to assess the entire diet over the preceding 3 months. The FFQ were scanned and food and nutrient intake calculated, using The Icelandic Nutrition Database (ISGEM) and a nutrient and food calculating program (ICEFOOD). Total fat intake and intake of individual FA from the whole diet were calculated. Regarding sources of long-chain n-3 PUFA, the intake of lean fish, oily fish, liquid cod liver oil, cod liver oil capsules and other supplements containing long-chain n-3 PUFA was assessed. All long-chain n-3 PUFA supplements on the Icelandic market are purified and controlled for dioxin and dioxin-like compounds. Ten milliliters spoon of liquid cod liver oil contained 1.0 g DHA, 0.7 g EPA, 2400 μg vitamin A and 2.4 mg vitamin E. The common daily dose of cod liver oil capsules contained 0.10 g DHA, 0.07 g EPA, 330 μg vitamin A and 0.255 mg vitamin E. The FFQ has been validated against a reference method and several nutrient biomarkers [15].

Information was gathered from the lifestyle questionnaire on age, height, body weight, education, parity, cohabitation, physical activity (cycling, swimming or other physical activity), smoking and oral contraceptive use (brand name).

### 2.4. Statistical analysis

Data from women using no long-chain n-3 PUFA supplements, women using cod liver oil capsules and women using liquid cod liver oil were compared with ANOVA with the Tukey's post-hoc test (continuous and normally distributed variables), Kruskal–Wallis with Bonferroni correction (continuous, non-normally distributed variables), or chi-square test (discrete variables). Pearson correlation was used to estimate the correlation between DHA in RBC on the one hand and DHA intake and intake of antioxidant vitamins on the other hand in the whole study group. Multiple linear regression model with the proportion of DHA in RBC as the dependent variable and total DHA intake (from food and supplements), oral contraceptive use, physical activity and storage time at −20 °C as independent variables was constructed for the whole study group. Age, body mass index, intake of natural antioxidants and alcohol, education, parity, cohabitation and smoking were tested as covariates, but they had no effect on the relationship. No interaction between the independent variables was found. The level of significance was set at 0.05 and two-tailed $p$-value was used. Statistical analysis was performed using the SPSS statistical software package version 15.0 (SPSS Incorporated, Chicago, USA).

## 3. Results

Characteristics, lifestyle and dietary intake of the women according to use of n-3 PUFA supplements are shown in Table 1. Fifteen women (37.5%) were using n-3 PUFA supplements, nine of them were using cod liver oil capsules and six were using liquid cod liver oil. The women using liquid cod liver oil were older than the women using no n-3 PUFA supplements. The women using no n-3 PUFA supplements had a lower intake of EPA and DHA than the women using n-3 PUFA supplements (liquid or capsules). The women using liquid cod liver oil had a lower n-6/n-3 PUFA intake ratio and higher intake of vitamin A than the women in the other subgroups.

Table 2 shows the FA composition of RBC according to use of n-3 PUFA supplements. The proportion of total n-6 PUFA was lower and the proportion of DHA was higher in RBC from women using liquid cod liver oil than in RBC from women using no n-3 PUFA supplements. The proportion of dihomo-γ-linolenic acid (DHGLA, 20:3n-6) was lower and the proportion of EPA was higher in RBC from women using liquid cod liver oil, than in RBC from women in the other two subgroups. The proportion of total n-3 PUFA was lower and the n-6/n-3 PUFA ratio was higher in RBC from women using no n-3 PUFA supplements than in RBC from women in the other two subgroups.

There was a positive ($p \leqslant 0.002$) correlation between intake of DHA and intake of the antioxidant vitamins A and E in the whole study group (data not shown). The proportion of DHA in RBC was positively correlated with intake of all individual and total long-chain n-3 PUFA ($p < 0.001$), but not with intake of ALA (data not shown). A positive ($p \leqslant 0.030$) correlation was found between DHA in RBC and total intake of the vitamins A and E (data not shown).

A multiple linear regression model to predict the proportion of DHA in RBC in the whole study group is shown in Table 3.

**Table 1**
General characteristics, lifestyle and dietary intake of the women according to use of n-3 PUFA supplements.

| | No n-3 PUFA supplements, $n = 25$ | Cod liver oil capsules, $n = 9$ | Liquid cod liver oil, $n = 6$ | $p$ for main effects |
|---|---|---|---|---|
| Anthropometry | | | | |
| Age (yr) | 28.9 ± 4.8[a] | 29.2 ± 3.9[ab] | 35.3 ± 7.1[b] | 0.023 |
| Body mass index (kg/m$^2$) | 23.8 ± 5.2 | 23.9 ± 4.1 | 23.9 ± 4.2 | 0.998 |
| Lifestyle | | | | |
| Smoking | 11 (44.0) | 2 (22.2) | 0 (0.0) | 0.089 |
| Physical activity | | | | |
| No physical activity | 12 (48.0) | 2 (22.2) | 4 (66.7) | 0.211 |
| Yes, but less than once a week | 2 (8.0) | 0 (0.0) | 1 (16.7) | |
| Yes, once a week | 3 (12.0) | 1 (11.1) | 0 (0.0) | |
| Yes, two times a week | 4 (16.0) | 3 (33.3) | 0 (0.0) | |
| Yes, three times a week | 3 (12.0) | 1 (11.1) | 1 (16.7) | |
| Yes, four times a week or more often | 1 (4.0) | 2 (22.2) | 0 (0.0) | |
| Oestrogene contraceptive use | 12 (48.0) | 1 (11.1) | 2 (33.3) | 0.143 |
| Dietary intake | | | | |
| Fish (g/d) | 27.9 ± 16.4 | 40.2 ± 16.9 | 22.5 ± 17.5 | 0.095 |
| EPA (20:5n-3, mg/d) | 66.8 ± 37.6[a] | 146.7 ± 49.0[b] | 610.0 ± 281.8[b] | <0.001 |
| DHA (22:6n-3, mg/d) | 137.6 ± 90.2[a] | 232.2 ± 74.3[b] | 890.0 ± 410.6[b] | 0.001 |
| n-3 PUFA (mg/d) | 1300.4 ± 621.1[a] | 1926.7 ± 1504.9[ab] | 2771.7 ± 782.5[b] | 0.010 |
| n-6 PUFA (mg/d) | 5657.6 ± 2722.8 | 7098.9 ± 3922.0 | 6113.3 ± 2209.2 | 0.464 |
| n-6/n-3 PUFA | 4.46 ± 0.96[a] | 4.10 ± 0.80[a] | 2.43 ± 1.35[b] | <0.001 |
| Alcohol (g/d) | 2.34 ± 2.77 | 1.34 ± 1.96 | 1.92 ± 1.04 | 0.573 |
| Vitamin A (retinol, μg/d) | 891.3 ± 620.6[a] | 1026.1 ± 324.6[a] | 2434.7 ± 852.6[b] | <0.001 |
| Vitamin E (tocopherol equivalents, mg/d) | 28.3 ± 57.8 | 17.1 ± 10.1 | 86.1 ± 109.6 | 0.166 |

Values are reported as mean ± SD or as number (percentage).
PUFA, long-chain polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.
[a,b] Means with different superscripts are significantly different ($p < 0.05$).

**Table 2**
FA composition of RBC from the women according to use of n-3 PUFA supplements.

| RBC FA (% of total FA) | No n-3 PUFA supplements, $n = 25$ | Cod liver oil capsules, $n = 9$ | Liquid cod liver oil, $n = 6$ | $p$ for main effects |
|---|---|---|---|---|
| SFA | 40.49 ± 1.53 | 39.85 ± 0.44 | 40.45 ± 0.74 | 0.430 |
| 16:0 | 19.87 ± 1.12 | 19.19 ± 0.40 | 19.76 ± 0.88 | 0.213 |
| 18:0 | 15.22 ± 0.68 | 15.23 ± 0.29 | 15.20 ± 0.65 | 0.994 |
| 22:0 | 1.35 ± 0.15 | 1.35 ± 0.16 | 1.32 ± 0.16 | 0.911 |
| 24:0 | 3.52 ± 0.39 | 3.52 ± 0.19 | 3.64 ± 0.28 | 0.709 |
| MUFA | 16.56 ± 0.97 | 16.35 ± 0.85 | 16.39 ± 1.25 | 0.827 |
| 18:1n-9 | 12.32 ± 0.83 | 12.08 ± 0.56 | 12.16 ± 0.78 | 0.707 |
| 18:1n-7 | 1.13 ± 0.23 | 1.14 ± 0.24 | 0.97 ± 0.25 | 0.324 |
| 24:1n-9 | 3.12 ± 0.31 | 3.13 ± 0.29 | 3.25 ± 0.40 | 0.659 |
| n-6 PUFA | 27.45 ± 2.08[a] | 26.68 ± 1.13[ab] | 24.98 ± 2.42[b] | 0.029 |
| 18:2n-6 | 9.55 ± 1.30 | 9.65 ± 0.80 | 9.26 ± 1.59 | 0.834 |
| 20:4n-6 (AA) | 13.53 ± 1.30 | 12.81 ± 1.02 | 12.31 ± 1.57 | 0.083 |
| 20:3n-6 (DHGLA) | 1.78 ± 0.28[a] | 1.72 ± 0.13[a] | 1.31 ± 0.17[b] | 0.001 |
| 22:4n-6 | 2.59 ± 0.41 | 2.50 ± 0.45 | 2.09 ± 0.81 | 0.099 |
| n-3 PUFA | 9.84 ± 0.90[a] | 11.18 ± 1.10[b] | 12.26 ± 2.49[b] | <0.001 |
| 20:5n-3 (EPA) | 0.88 ± 0.16[a] | 1.07 ± 0.22[a] | 1.62 ± 0.74[b] | <0.001 |
| 22:5n-3 (DPA) | 2.85 ± 0.37 | 3.20 ± 0.25 | 3.08 ± 0.52 | 0.054 |
| 22:6n-3 (DHA) | 6.11 ± 0.85[a] | 6.91 ± 0.85[ab] | 7.57 ± 1.31[b] | 0.002 |
| n-6/n-3 PUFA | 2.82 ± 0.36[a] | 2.41 ± 0.33[b] | 2.12 ± 0.52[b] | <0.001 |
| Total FA (mg/L RBC) | 1121 ± 114 | 1197 ± 86 | 1142 ± 117 | 0.210 |

Values are reported as mean ± SD.
RBC, red blood cells; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; AA, arachidonic acid; DHGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.
[a,b] Means with different superscripts are significantly different ($p < 0.05$).

As expected, total DHA intake was positively correlated with the proportion of DHA in RBC. Furthermore, oral contraceptive use increased the proportion of DHA in RBC by 0.54% of total FA and more frequent physical activity increased it by 0.16% of total FA, after adjustment for total intake of DHA and storage time at −20 °C. Even though the antioxidant BHT was added to the RBC samples before storage, storage time at −20 °C had a small negative effect on the proportion of DHA in RBC. The model explained 47.8% of the variation in proportion of DHA in RBC. Intake of the antioxidant vitamins failed to enter the model, as did age, body mass index, alcohol intake, education, parity, cohabitation and smoking.

**Table 3**
Linear regression model for the proportion of DHA in RBC in the whole study group.

| | Regression coefficient | Standard error | *p* |
|---|---|---|---|
| (Constant) | 5.89 | 0.31 | <0.001 |
| Total intake of DHA (22:6n-3, g/d) | 1.87 | 0.39 | <0.001 |
| Oral contraceptive use | 0.54 | 0.25 | 0.040 |
| Physical activity | 0.16 | 0.07 | 0.025 |
| Storage time at −20 °C (weeks) | −0.10 | 0.03 | 0.005 |

*Dependent variable*: DHA in RBC (% of total FA).
*Reference categories*: Oral contraceptive use: 1 = not using, 2 = using.
*Reference categories*: Physical activity: 1 = no physical activity, 2 = yes, but less than once a week, 3 = yes, once a week, 4 = yes, twice a week, 5 = yes, three times a week, 6 = yes, four times a week or more often.
*Model*: $n = 40$; adjusted $R^2 = 0.478$; $p < 0.001$.
DHA, docosahexaenoic acid; RBC, red blood cells.

## 4. Discussion

DHA represents the longest and the most unsaturated FA commonly found in biological systems. DHA increases the fluidity of cell membranes and takes part in various cell mechanisms [16]. Studies have shown that when $^{13}$C-labeled ALA, the precursor of DHA, is given to human subjects, endogenous biosynthesis of DHA is higher in women [4] than men [3]. Further, when women and men consumed the same rigidly controlled diet for 3 weeks, containing ALA but no long-chain n-3 PUFA, the women had a higher level of DHA in plasma cholesteryl esters than the men at the end of the study [17]. In two of these studies there was a non-significant trend towards higher synthesis of DHA in women using oral contraceptives (30–35 μg ethynyloestradiol/d) compared with those who did not [4,17]. Oestrogene therapy (100 μg oral ethynyloestradiol/d) resulted in a significant increase in the proportion of DHA in plasma cholesteryl esters of male to female transsexuals, while testosterone therapy resulted in a parallel decrease in plasma estradiol and DHA of female to male transsexuals [17]. The present study found oral contraceptive use to be related to a significantly higher proportion of DHA in RBC that could not be explained by a higher dietary intake of DHA. The ethynyloestradiol content of the pills was 20–50 μg. As far as we know our study is the first to show that a low-dose oestrogen treatment with oral contraceptives is able to stimulate biosynthesis of DHA.

One study found both exercise and training to be related to a lower content of phosphatidylserine in RBC membrane and a lower proportion of DHA in the phosphatidylserine lipid class [18]. The effect was explained by increased oxidative stress and other studies have confirmed that heavy endurance exercise increases both lipid peroxidation [19] and hemolysis [20]. In the present study, 37.5% of the women were physically active twice a week or more, but no information on the exercise intensity was gathered, nor their biochemical antioxidant status assessed. Exercise-induced oxidative stress and hemolysis is partly counteracted by increased antioxidant activity [19] and increased fluidity of the RBC membrane [7]. Increased fluidity can be achieved by decreasing the ratio of cholesterol to phospholipids (PLs) or increasing the level of unsaturation in the membrane [21,22]. Training has been found to increase the proportion of DHA in skeletal muscle PLs [23,24], and two studies have found long-chain n-3 PUFA to be higher in RBC of long-distance runners compared with controls [6,7]. Our results are in accordance with these studies, finding more frequent physical exercise (cycling, swimming or other physical activity) to be related to a higher proportion of DHA in RBC.

Increased antioxidant activity in RBC of physically active people would protect DHA in the RBC membrane from lipid peroxidation and this could lead to a higher DHA level in the RBC. As expected there was a positive association between DHA intake and total intake of the antioxidant vitamins A and E in the present study, since food and supplements high in long-chain n-3 PUFA are sources of vitamins A and E. However, the positive relationship between DHA in RBC and intake of the vitamins A and E disappeared when controlled for DHA intake. The positive relationship found in the present study between frequency of physical exercise and the proportion of DHA in RBC could therefore not be explained by different dietary and supplement intake of the vitamins A or E.

Imbalance in dietary intake of n-6 PUFA and n-3 PUFA changes the ratio of n-6 PUFA to n-3 PUFA in membranes [2]. High n-6/n-3 PUFA ratio in membranes has been associated with increased platelet aggregation [25], cardiovascular disease [26], inflammatory processes [27], and to proliferation of cancer cells [28]. Among the women in the present study it was the total intake of long-chain n-3 PUFA, but not ALA or n-6 PUFA that determined the n-6/n-3 PUFA ratio in RBC.

Twenty-five women in the present study were not using long-chain n-3 PUFA supplements or 62.5% of the whole study group. However, the n-6/n-3 PUFA intake ratio of these 25 women was considerably lower compared with the results from a study of 23 Dutch prepregnant women [29] (4.46 ± 0.96 vs. 11.31 ± 3.31, respectively). The difference can be explained by a considerably lower intake of n-6 PUFA (5.7 ± 2.7 vs. 13.2 ± 3.9 g/d) and a higher intake of DHA (137.6 ± 90.2 vs. 90 ± 131 mg/d). The Dutch study does not mention fish intake of the women or if any of them used long-chain n-3 PUFA supplements.

Vegetables, grains and vegetable oils are the main sources of n-6 PUFA. Intake of vegetables and of vegetable oils in 2002 was lower in Iceland than in many other countries, while intake of grains was similar as in the neighbouring countries [30]. In accordance with the Dietary Survey of The Icelandic Nutrition Council 2002 [30], fish intake of the women not using long-chain n-3 PUFA supplements was moderate (27.0 ± 16.4 g/d). Further, the fish commonly consumed in Iceland is low in fat [30]. However, FA analysis of food on the Icelandic market has shown that not only fish but also meat and eggs contain long-chain n-3 PUFA, since it is common to feed pigs and poultry fish meal [31,32]. Moreover, cattle and sheep in Iceland are mostly fed on grass, rich in ALA [33], which makes the meat higher in ALA and long-chain n-3 PUFA and lower in the n-6 PUFA LA, compared with meat from animals fed on grain [34]. Low vegetable and vegetable oil intake, together with moderate fish intake and the traditional livestock feeding habits probably explain the low n-6/n-3 PUFA intake ratio of the women not using long-chain n-3 PUFA supplements in the present study.

The PUFA composition of RBC reflected the dietary intake, with a higher proportion of DHA and a lower n-6/n-3 PUFA ratio in RBC of the women not using long-chain n-3 PUFA supplements in the present study, than in RBC of the Dutch women [29] (6.11 ± 0.85% vs. 2.94 ± 0.58% and 2.82 ± 0.36 vs. 5.06 ± 0.86, respectively). Comparing values for dietary intake and RBC FA proportions between different studies has to be done with care because survey and analysis methods can vary. In the former mentioned study the women answered FFQ and total PL were isolated from RBC and FAs analyzed [29]. Therefore, we assume the results of the Dutch study [29] are comparable with the present results.

It has long been common in Iceland to use liquid cod liver oil, though today many prefer cod liver oil capsules. Among the women in the present study the prevalence of long-chain n-3 PUFA supplement use (liquid or capsules) was 37.5%. The mean DHA intake was 6.5 times higher and the total n-6/n-3 PUFA

intake ratio 46% lower among the women using liquid cod liver oil compared with the women not using long-chain n-3 PUFA supplements. This was reflected in a 24% higher proportion of DHA and 25% lower n-6/n-3 PUFA ratio in RBC from the women using liquid cod liver oil compared with those not using long-chain n-3 PUFA supplements. The women using cod liver oil capsules were intermediate in all these variables.

The mean DHA intake of the women not using long-chain n-3 PUFA supplements did not reach the 200 mg/d minimum recommended for pregnant and lactating women [12], which shows the importance of dietary counseling for women in the early stages of pregnancy. Even though the mean DHA intake of the women using long-chain n-3 PUFA supplements was well over the previously mentioned recommendation, five of the women using cod liver oil capsules actually did not reach the 200 mg/d minimum. All the women using liquid cod liver oil had DHA intake well above the recommended minimum, and the n-6/n-3 PUFA intake ratio among them was near the ratio assumed for preagricultural humans [1].

Cod liver oil is not only a rich source of long-chain n-3 PUFA, but also a good source of the vitamins A, D and E. Vitamin A (retinol) is an essential nutrient, but women in reproductive age should take care not to consume retinol in excess, since excessive exposure in the early stages of pregnancy may result in congenital malformations [35]. The mean intake of retinol among the women using liquid cod liver oil in the present study was $2434.7 \pm 852.6$ μg/d and one of them reached the recommended 3000 μg/d maximum (3016.6 μg/d). However, since the completion of the study, the retinol content of long-chain n-3 PUFA supplements has been lowered (in liquid cod liver oil from 2400 to 460 μg in 10 mL dosage), and vitamin E added as an antioxidant.

The women in the present study were mothers of 18-month-old children. DHA is transported from the mothers blood to breast milk during lactation [36]. The proportion of DHA in plasma and RBC PL has been shown to decrease after delivery, and more so among breastfeeding women [37]. Twelve months after birth the proportion of DHA in maternal plasma PL had normalized [38]. In Iceland the frequency (95%) and duration (7.5–8.5 months) of breast-feeding are high, but most women have stopped breast-feeding 18 months post-partum [39]. The women in the present study breastfed their children for $8.4 \pm 4.2$ months (mean ± SD) (data not shown), which gave them on average 10 months post-lactation to normalize the FA composition of their RBC before the blood samples were collected. Therefore, we assume the results of the present study can be generalized for non-pregnant Icelandic women of reproductive age.

The limitations of the present study are mainly due to the low number of participants. Confounding can never be ruled out in observational studies, and further research in the form of clinical trials is the only way to confirm if the associations seen are causal. Recall/reporting bias can not be ruled out, since dietary intake was self-reported, and so was physical activity.

In conclusion, the total n-6/n-3 PUFA intake ratio was lower among the women in the present study than in many Western countries, which might have positive effect on their health. The findings indicate that oral contraceptive use and physical activity have a positive impact on the DHA status, as assessed by RBC level, of women in reproductive age.

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# Paper IV

# Effects of Storage Time and Added Antioxidant on Fatty Acid Composition of Red Blood Cells at –20°C

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**ABSTRACT:** The stability of PUFA in venous red blood cells (RBC) of women aged 25 to 55 years (n = 12) was investigated during storage at –20°C. The RBC sample from each participant was divided into seven portions: one baseline with the antioxidant BHT, another without BHT, samples without BHT stored for 2, 4, 9, or 17 wk, and samples with BHT stored for 17 wk. No difference was found in proportions of PUFA at baseline and after storage for 2 and 4 wk without BHT, and 17 wk with BHT. After 9 wk without BHT the proportion of 22:6n-3 in RBC was lower, and after 17 wk without BHT proportions of all PUFA were lower than at baseline. High proportion of 22:6n-3 in RBC at baseline was associated with more stable concentration of total FA in RBC without BHT during 17 wk. The findings indicate that PUFA in RBC from healthy women are stable at –20°C for 4 wk without BHT and for at least 17 wk with BHT.

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Red blood cell (RBC) membrane lipids are a good indicator of dietary fat intake (1,2), as well as a biomarker of disease risk (3,4,5). RBC membrane is rich in PUFA, and a mature RBC is filled with hemoglobin containing iron, which might induce production of free radicals and lipid peroxidation of RBC membrane lipids (6).

Some centers for health service have access to only a –20°C or –30°C freezer, and human studies have shown that venous RBC stored at –20°C without addition of antioxidant had unchanged FA composition after 4 wk compared to baseline (7). Storage of RBC samples for more than 4 wk before FA analysis is often unavoidable, but lower proportion of long-chain PUFA has been found after 6 mon compared to baseline (8). The stability of PUFA in RBC may be affected by the physiological or pathological state of the individual. PUFA in RBC from healthy individuals were more stable during storage at –20°C than PUFA in RBC from patients with schizophrenia (9) and autism (10). Addition of the antioxidant BHT to RBC samples before freezing has been shown to preserve the PUFA composition for 1 yr at –50°C (7) and for 2 yr at –80°C (8) in healthy individuals.

The present study was undertaken to investigate at –20°C whether PUFA composition of RBC is stable for more than 4 wk without addition of antioxidant, and if addition of BHT preserves the PUFA content even longer.

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Abbreviations: MUFA, monounsaturated FA; RBC, red blood cells; SFA, saturated FA.

## MATERIALS AND METHODS

*Subjects.* Nonfasting venous blood samples from healthy women ($n$ = 12) aged 25 to 55 yr were collected in tubes containing EDTA. RBC were isolated immediately by centrifuging whole blood at 1300 × $g$ for 10 min at 4°C, and washed three times with isotonic saline solution. The RBC sample from each participant was divided into seven portions: one baseline sample with and another without addition of the antioxidant BHT, samples without BHT stored at –20°C for 2, 4, 9, or 17 wk, and one sample with BHT stored at –20°C for 17 wk. BHT dissolved in methanol (500 mg/L) was added to RBC sample at a final concentration of 42 mg/L. No methanol was added to the RBC samples without BHT. Baseline RBC samples with or without BHT were lipid-extracted within 3 h of collection, and FA were analyzed.

*Analysis of RBC total lipid fatty acids.* RBC total lipids were extracted as described by Bligh and Dyer (11) except isopropanol was used instead of methanol (isopropanol/chloroform 2:1, v/v). BHT (50 mg/L) was added to the extraction medium. The FA were transmethylated for 45 min at 110°C using 14% boron trifluoride/methanol (Sigma Chemical Co., St. Louis, MO). The FAME were analyzed using GC (Agilent 6890 N, Agilent, Palo Alto, CA) equipped with a Chrompack CP-SIL 8CB column (25 m × 250 μm i.d. × 0.12 μm film thickness). The oven temperature was programmed to have an initial temperature of 150°C for 4 min, then rising 4°C/min to 230°C, then 20°C/min to 280°C, and then held isothermal for 4 min. The injector and detector temperatures were maintained at 280°C and 300°C, respectively. Hydrogen was used as the carrier gas. The FAME peaks were identified and calibrated against those of commercial standards (Sigma Chemical Co.; Nu-Chek-Prep, Elysian, MN). PC diheptadecanoyl (17:0) and heneicosanoic acid (21:0) methyl ester were used as internal and external standards, respectively. The intra- and interassay CV for analytical variation (measurement error) were, respectively, 1.13% and 1.46% for 18:2n-6, 2.09% and 1.95% for 20:4n-6, 2.38% and 2.08% for 22:6n-3, 1.29% and 1.09% for 16:0, 1.78% and 0.85% for 18:0, and 0.91% and 0.92% for 18:1n-9.

*Statistical analysis.* The effect of storage time and added antioxidant, and interaction between storage time and added antioxidant, were evaluated with the ANOVA for repeated measures and Tukey–Kramer *post-hoc* test in the SAS program (Mixed procedure in SAS for Windows, V8). $P < 0.05$ was considered significant. Multiple linear regression was used to evaluate the association of age or proportion of indi-

**TABLE 1**
**Effects of Storage Time at –20°C and Addition of the Antioxidant BHT on FA Proportions (% of total FA) in RBC Total Lipids of Healthy Women[a]**

| | | | Storage time at –20°C | | | | |
|---|---|---|---|---|---|---|---|
| FA | Baseline + BHT ($n$ = 8) | Baseline ($n$ = 12) | 2 wk ($n$ = 11) | 4 wk ($n$ = 12) | 9 wk ($n$ = 11) | 17 wk ($n$ = 12) | 17 wk + BHT ($n$ = 11) |
| Total SFA | 40.47 ± 0.68 | 39.34 ± 0.95 | 40.00 ± 0.96 | 42.78 ± 1.64 | 44.38 ± 2.68[b] | 50.43 ± 8.72[b] | 40.42 ± 2.24 |
| 16:0 | 20.02 ± 0.99 | 19.46 ± 0.87 | 20.06 ± 0.80 | 21.37 ± 1.20 | 21.91 ± 1.48 | 26.01 ± 4.81[b] | 20.09 ± 1.07 |
| 18:0 | 15.25 ± 0.64 | 14.85 ± 0.77 | 15.01 ± 0.86 | 16.29 ± 1.50[b] | 16.49 ± 1.73[b] | 17.02 ± 2.06[b] | 15.51 ± 1.79 |
| 22:0 | 1.44 ± 0.20 | 1.34 ± 0.19 | 1.28 ± 0.21 | 1.36 ± 0.14 | 1.58 ± 0.15 | 1.99 ± 0.65[b] | 1.29 ± 0.15 |
| 24:0 | 3.70 ± 0.46 | 3.43 ± 0.42 | 3.33 ± 0.42 | 3.48 ± 0.26 | 4.09 ± 0.47 | 5.09 ± 1.55[b] | 3.28 ± 0.42 |
| Total MUFA | 16.91 ± 0.60 | 16.70 ± 0.46 | 16.92 ± 0.77 | 17.25 ± 0.83 | 17.74 ± 0.65[b] | 19.51 ± 1.72[b] | 16.50 ± 0.88 |
| 18:1n-9 | 12.34 ± 0.78 | 12.09 ± 0.55 | 12.38 ± 0.45 | 12.53 ± 0.54 | 12.53 ± 0.58 | 13.87 ± 1.20[b] | 12.00 ± 0.75 |
| 18:1n-7 | 1.01 ± 0.08 | 1.06 ± 0.15 | 1.08 ± 0.18 | 1.19 ± 0.18 | 1.22 ± 0.14[c] | 1.27 ± 0.13[c] | 1.14 ± 0.25 |
| 24:1n-9 | 3.56 ± 0.51 | 3.44 ± 0.35 | 3.33 ± 0.30 | 3.35 ± 0.36 | 3.81 ± 0.36 | 4.18 ± 0.63[b] | 3.25 ± 0.33 |
| Total n-6 PUFA | 26.69 ± 1.79 | 25.62 ± 3.07 | 25.25 ± 3.18 | 24.54 ± 2.91 | 23.03 ± 2.92 | 17.42 ± 6.14[b] | 25.03 ± 3.93 |
| 18:2n-6 | 9.08 ± 1.03 | 9.12 ± 1.10 | 9.16 ± 1.10 | 9.00 ± 0.99 | 8.50 ± 0.94 | 7.25 ± 1.83[b] | 8.96 ± 1.24 |
| 20:4n-6 | 13.63 ± 1.29 | 12.81 ± 1.80 | 12.44 ± 1.97 | 12.05 ± 1.83 | 11.30 ± 1.63 | 8.10 ± 3.19[b] | 12.50 ± 2.33 |
| 20:3n-6 | 1.67 ± 0.38 | 1.53 ± 0.39 | 1.52 ± 0.42 | 1.47 ± 0.40 | 1.36 ± 0.36 | 0.92 ± 0.66[b] | 1.49 ± 0.42 |
| 22:4n-6 | 2.30 ± 0.37 | 2.15 ± 0.63 | 2.13 ± 0.65 | 2.02 ± 0.57 | 1.87 ± 0.56 | 1.16 ± 0.81[b] | 2.08 ± 0.67 |
| Total n-3 PUFA | 11.16 ± 1.61 | 11.54 ± 2.71 | 11.24 ± 2.99 | 10.66 ± 2.73 | 9.97 ± 2.65[b] | 7.20 ± 4.40[b] | 11.38 ± 2.06 |
| 20:5n-3 | 1.21 ± 0.48 | 1.51 ± 1.12 | 1.49 ± 1.16 | 1.40 ± 1.03 | 1.35 ± 1.03 | 0.91 ± 1.21[b] | 1.48 ± 0.97 |
| 22:5n-3 | 2.85 ± 0.32 | 2.87 ± 0.51 | 2.76 ± 0.53 | 2.66 ± 0.54 | 2.50 ± 0.51 | 1.71 ± 1.05[b] | 2.84 ± 0.43 |
| 22:6n-3 | 7.11 ± 0.98 | 7.16 ± 1.24 | 6.99 ± 1.46 | 6.60 ± 1.34 | 6.12 ± 1.23[b] | 4.57 ± 2.30[b] | 7.06 ± 0.88 |
| Total FA[d] | 1052 ± 192 | 1178 ± 139 | 1122 ± 126 | 1058 ± 150 | 949 ± 196[b] | 766 ± 200[b] | 1082 ± 144 |

[a]Values are reported as mean ± SD ($n$ = 8 to 12).
[b]Significant interaction between weeks and BHT, $P < 0.05$, compared with baseline without BHT; ANOVA for repeated measures and Tukey–Kramer *post-hoc* test.
[c]Significant effect of weeks, $P < 0.05$, compared with baseline without BHT; ANOVA for repeated measures and Tukey–Kramer *post-hoc* test.
[d]mg/L red blood cells (RBC). MUFA, monounsaturated FA; SFA, saturated FA.

vidual FA in RBC lipids at baseline with the change in concentration of total FA in RBC lipids after 17 wk of storage at –20°C without added antioxidant.

## RESULTS

The FA composition of RBC at baseline and stored at –20°C for 2, 4, 9, or 17 wk with or without BHT are shown in Table 1. RBC without BHT contained lower proportion of 22:6n-3 after 9 wk, and higher proportions of all saturated FA (SFA) and monounsaturated FA (MUFA), and lower proportions of all n-6 and n-3 PUFA after 17 wk than at baseline. The concentration of total FA in RBC without BHT was lower after 9 wk of storage than at baseline (Table 1).

Figure 1 shows the concentrations of six main FA in RBC at all time points with or without BHT as a percentage of the concentration at baseline without BHT. In RBC without BHT the concentrations of 20:4n-6 and 22:6n-3 were lower after 4 wk, and the concentrations of 18:2n-6 and 18:1n-9 were lower after 9 wk of storage than at baseline. After 17 wk the concentrations of 16:0 and 18:0 were also lower than at baseline. Addition of BHT preserved the FA composition and concentration of total FA (Table 1) and prevented the decrease in concentrations of individual FA in RBC during 17 wk of storage (Figure 1).

The relationship between change in concentration of total FA in RBC after 17 wk of storage without BHT and the proportion of 22:6n-3 at baseline without BHT is shown in Figure 2. The two variables were strongly associated. Multivariate model analysis showed that age and other FA proportions in RBC at baseline had no effect on the change in total FA during 17 wk without BHT.

## DISCUSSION

In the present study the PUFA composition and total FA concentration were stable in washed venous RBC from healthy women during 4 wk storage at –20°C without addition of antioxidant, which supports the findings reported previously (7). However, after 9 wk the proportion of 22:6n-3 and total FA concentration was lower, and after 17 wk without addition of antioxidant all PUFA proportions were lower than at baseline. On the other hand, addition of BHT did preserve FA proportions and concentrations of venous RBC for at least 17 wk at –20°C. BHT has been shown to preserve FA composition of RBC for at least 1 yr at lower temperature (7,8).

Studies on vulnerability of n-3 and n-6 PUFA in RBC to peroxidation have been inconsistent, some showing similar (12,13,14), others increased (15,16), and yet others decreased (17) peroxidation of RBC lipids high in n-3 PUFA compared

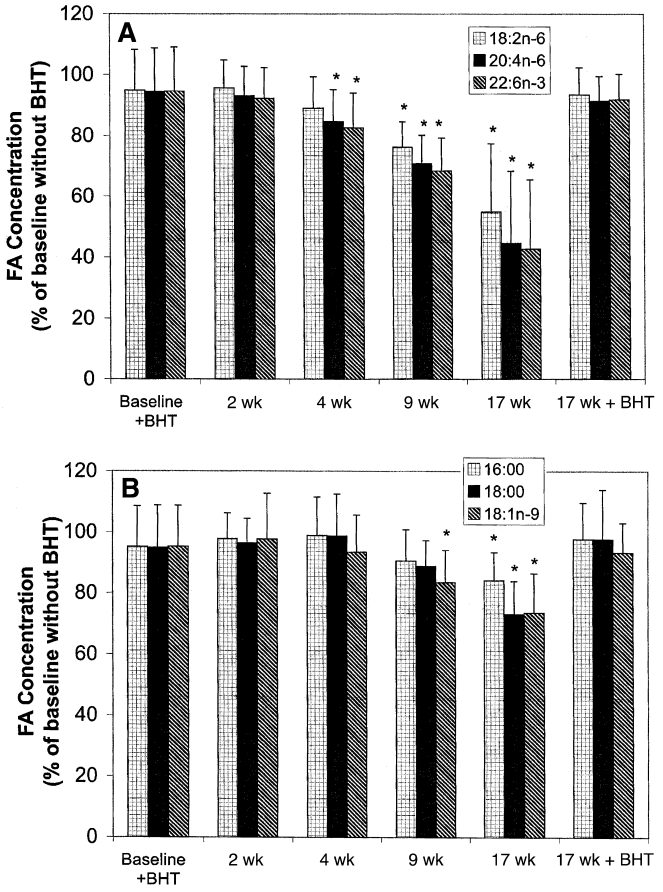


**FIG. 1.** Concentrations (% of baseline without BHT) of (A) 18:2n-6, 20:4n-6, and 22:6n-3 and (B) 16:0, 18:0, and 18:1n-9 in RBC of healthy women at baseline with BHT, and after 2, 4, 9, or 17 wk storage at –20°C with or without BHT. See Table 1 for number of subjects in each time point. $^*P < 0.05$, compared with baseline without BHT, ANOVA for repeated measures and Tukey–Kramer *post-hoc* test.

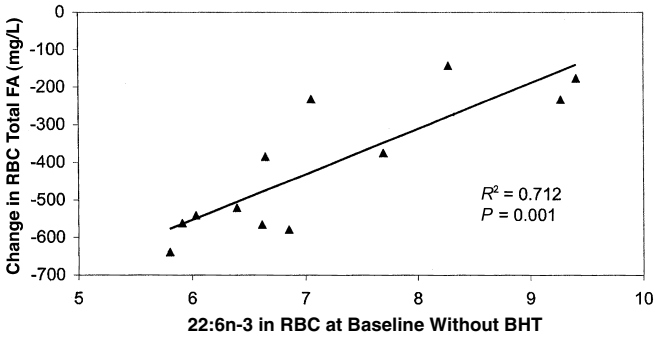


**FIG. 2.** Correlation between change in total FA concentration (mg/L) in red blood cells (RBC) of healthy women ($n = 12$) from baseline to 17 wk at –20°C without BHT and proportion (% of total FA) of 22:6n-3 in RBC at baseline without BHT.

with n-6 PUFA. In the present study, the proportion of 22:6n-3 at baseline was positively correlated to the change in concentration of total FA in RBC without BHT during 17 wk at –20°C (Fig. 2), even though n-3 PUFA deteriorated faster than n-6 PUFA (Table 1). A positive association has been found between proportion of n-3 PUFA in RBC and concentration of α-tocopherol in plasma of Icelandic women (18), and vitamin E has been shown to prevent lipid peroxidation of n-3 PUFA in animal RBC (19). Therefore we cannot exclude the possibility that antioxidants such as α-tocopherol or antioxidative enzymes naturally present in RBC may be confounding factors, and explain the correlation observed in our study. However, RBC high in n-3 PUFA have also been shown to be more resistant to hemolysis (16,20), which is one of the factors inducing lipid peroxidation of PUFA during cold storage (21). In the present study the decrease in total FA concentration during 17 wk storage without BHT was 35 ± 15% (mean ± SD), and in half the women the decrease was about 60% (over 500 mg/L, Fig. 2), where the baseline proportion of 22:6n-3 was under 7%.

In capillary RBC with low 22:6n-3 content at baseline, the decrease was 45% after 4 wk at –20°C without BHT (7). The present study indicates that the loss of RBC total FA concentration was not due only to lipid peroxidation of PUFA, as the concentration of SFA was also decreased (Fig. 1). One of the explanations for loss of total FA concentration might be that the extraction of lysophospolipids and partly degraded PL, which increase with hemolysis, was incomplete by the solvent used. Addition of BHT before storage of the RBC at –20°C prevented the degradation, probably by contributing to maintenance of the integrity of the membrane.

The findings in this study indicate that high content of 22:6n-3 in venous RBC of healthy women is associated with less deterioration of lipid FA content during storage at low temperature without addition of antioxidant. Whether RBC membranes with high content of 22:6n-3 are more resistant to deterioration in storage, because of α-tocopherol or other natural antioxidants, remains to be investigated.

## ACKNOWLEDGMENTS

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# Paper V

# A method for adjustment of peroxidized fatty acid data: the relationship between *n*-3 polyunsaturated fatty acids in diet and red blood cells from women in early pregnancy

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**Abbreviations:** A-A, adjusted all; FAME, fatty acid methyl esters; L-CLO, liquid cod liver oil; PL, phospholipids; RBC, red blood cells; U-A, unadjusted all; U-NP, unadjusted not peroxidized.

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**Running title**: FA data adjustment method for peroxidized RBC

**Key words: Adjustment method, Peroxidation, Polyunsaturated fatty acids, Red blood cells.**

**Abstract**

Marine *n*-3 PUFA level of red blood cell (RBC) lipids is a well-recognized biomarker of dietary intake of *n*-3 PUFA, as well as of disease risk. In the present study an adjustment method designed for nutritional data analysis was modified to use for FA data analysis of partly peroxidized RBC lipids from women in early pregnancy (*n* 101). The bimodal distribution of the proportions of 20 : 4*n*-6 and 22 : 6*n*-3 found in RBC of all the unadjusted data was normalized by the modified adjustment method (*n* 101). The RBC samples in the higher mode of the bimodal distribution of the proportion of 20 : 4*n*-6 (>9.5 g/100g of total FA) were considered not lipid peroxidized (*n* 67). No difference was found between the FA proportions of the adjusted data and the unadjusted data from RBC considered not peroxidized. The correlation between dietary intake of 22 : 6*n*-3 and proportion of 22 : 6*n*-3 in RBC was stronger in the adjusted data than in all the unadjusted data. The women whose RBC samples were considered peroxidized were shorter, had higher body mass index, blood pressure and parity than those whose samples were considered not peroxidized. The study demonstrated that the stability of RBC lipids might depend on individual physiological condition as well as on storage condition of the RBC samples. The study also supported the use of a FA data adjustment method for partly peroxidized RBC lipids, such that all the participants could be included in the analysis.

## Introduction

The PUFA level of red blood cell (RBC) lipids is a well-recognized biomarker of dietary PUFA intake[1-3], as well as of disease risk[4, 5]. PUFA in RBC are at risk of peroxidation because haemoglobin in the RBC contains oxidation inducing iron[6]. The risk of lipid peroxidation increases considerably if the RBC samples are stored without addition of antioxidant for weeks or months at -20°C[7-9]. However, it has been suggested that the stability of PUFA in RBC stored at inappropriate storage conditions depends on the status of antioxidants naturally present in the RBC[10] and also on the pathological state of the individual[11, 12]. Even though it is expected that RBC high in PUFA are sensitive to free radicals and lipid peroxidation, RBC with high *n*-3 PUFA content have been shown to be resistant to haemolysis[13, 14], which is one of the factors inducing peroxidation of PUFA during cold storage[15]. Haemolysis also causes phospholipid (PL) degradation forming lysophospholipids not fully extracted by organic solvents, leading to a loss in total FA concentration[7, 9].

In a recent study on stability of RBC PUFA stored at -20°C without addition of antioxidant the extent of lipid peroxidation and PL degradation was very variable between individuals even though storage time and other conditions were similar[9]. The change in total FA concentration of RBC lipids during 17 weeks of storage was positively correlated to the baseline proportion of the *n*-3 PUFA, 22 : 6*n*-3, indicating that RBC samples with a high 22 : 6*n*-3 content at baseline might be better protected against FA peroxidation and PL degradation[9]. Since the change in total FA concentration of RBC lipids had a stronger correlation with the baseline proportion of 22 : 6*n*-3 than with storage time, total FA concentration of RBC lipids might be a better indicator of the extent of RBC PUFA peroxidation than storage time.

In studies examining the relationship between diet and disease outcome, energy adjusted nutrient intake has been used instead of excluding low energy reporters[16, 17]. This method includes all participants in the data analysis, instead of excluding participants known to be under-reporting total energy intake. It has been indicated that energy adjustment where all participants are included, may be the preferred method for reducing the influence of reporting bias, because low energy reporters are unevenly distributed across employment grades, which causes selection bias if they are excluded[18, 19].

In the present study one third of the RBC samples from women in early pregnancy were considered lipid peroxidized due to inappropriate storage conditions. The objective of this study was to modify an adjustment method designed for nutritional data analysis to use for analysis of the RBC FA data, such that all the participants could be included in the data analysis.

**Subjects and experimental procedures**

*Subjects*

The study is a part of a larger project on dietary intake of healthy, pregnant Icelandic women and outcome of pregnancy[20-23]. The participants in the present study were 176 healthy, low-risk, singleton pregnant women. They were enrolled at a routine first visit at week 12.9 (SD 2.1) of pregnancy to the Center of Prenatal Care in Reykjavik, Iceland, in the years 1999 and 2000. The women gave blood samples and filled out a semi-quantitative FFQ covering food intake and supplemental use for the last three months. Of the 176 women enrolled, a total of 141 healthy women showed no pregnancy-induced complications and gave birth to full-term babies. Three women did not fill out the FFQ and one blood sample was lost. Data on total FA concentration of RBC were lacking from 36 women because of a technical accident. Full data were available from 101 healthy women. The National Bioethics Committee of Iceland and the Icelandic Data Protection Commission approved the study. The women gave informed consent for their participation in the study.

*RBC total lipid FA*

Non-fasting venous blood samples were collected in Vacuette® tubes containing EDTA. RBC were isolated immediately by centrifuging whole blood at 1300 x *g* for 10 min at 4°C and washed three times with isotonic saline solution. RBC samples were stored at -20°C without addition of antioxidant until FA analysis within 20 weeks.

RBC total lipids were extracted as described by Bligh & Dyer[24] except isopropanol was used instead of methanol (isopropanol/chloroform 2:1, v/v). BHT (225 μM) was added to the extraction medium. The FA were transmethylated for 45 min at 110°C using 14% boron trifluoride/methanol (Sigma Chemical Co., St. Louis, MO). The fatty acid methyl esters (FAME) were analyzed using GC (HP Series II 5890 A, Hewlett Packard Co., Palo Alto, CA) equipped with a flame ionization detector and a Chrompack CP-WAX 52CB column (25 m x 320 μm i.d. x 0.2 μm film thickness). The oven temperature was programmed to have an initial temperature of 90°C for 2 min, then rising at 30°C/min to 165°C and at 3°C/min to 225°C and then held isothermal for 6 min. The injector and detector temperatures were maintained at 235°C and 250°C, respectively. Hydrogen was used as the carrier gas. The FAME peaks were identified and calibrated against those of commercial standards (Sigma Chemical Co.; Nu-Chek-Prep, Elysian, MN). Phosphatidylcholine diheptadecanoyl (17 : 0) and heneicosanoic acid (21 : 0) methyl ester were

used as internal and external standards, respectively. Recovery of 17 : 0 was 83.0 (SD 5.8) %. Each FA value was the mean of two separate RBC total lipid extractions of the same sample. The intra-assay CV for analytical variation (measurement error based on 50 measurements) was 5.2% for 22 : 6*n*-3, 3.9% for 20 : 4*n*-6 and 5.5% on average for all the FA analyzed. RBC values are presented as g/100g of total FA with chain length from $C_{14}$ to $C_{24}$. Instrumental control and data handling were done by HP 3365 Chemstation, Version A.02.12. (Hewlett Packard Co.).

*Diet and other interfering covariates*

A semi-quantitative FFQ was mailed to the pregnant women two weeks before a visit to the Center for Prenatal Care at week 12.9 (SD 2.1) of pregnancy. The participants were asked to fill in the questionnaire at home and bring it to the clinic or mail it back. The semi-quantitative FFQ was developed at the Icelandic Nutritional Council to assess the entire diet over the previous three months. The FFQ were scanned and food and nutrient intake calculated, using The Icelandic Nutrition Database (ISGEM) and a nutrient and food calculating program (ICEFOOD). Total fat intake and intake of individual FA from the whole diet was calculated, and so was the intake of lean fish, oily fish, liquid cod liver oil (L-CLO), CLO capsules and other supplements containing marine *n*-3 PUFA. The common daily dose of L-CLO and CLO capsules gave 0.98 g and 0.10 g of 22 : 6*n*-3, respectively. The FFQ has been validated against a reference method and several nutrient biomarkers[25]. Information was gathered on age, height, body weight, parity and blood pressure from medical and birth records.

*Modified adjustment method for RBC FA data*

Dietary intake of many nutrients is strongly, positively correlated with total caloric intake. With this in consideration Walter Willett and coworkers[16, 17] did design an adjustment method where nutrient intake was adjusted for total energy intake, instead of excluding low energy reporters. In the present study the distributions of the proportions of 20 : 4*n*-6 and 22 : 6*n*-3 in RBC in all the data unadjusted (*n* 101) were bimodal (Fig. 1 (a,b)), and a strong correlation was found between all RBC FA proportions and total FA concentration of RBC (Table 2). The RBC samples in the lower mode of the bimodal distribution of the proportion of 20 : 4*n*-6 (≤9.5 g/100g of total FA) were considered peroxidized. Instead of excluding those RBC samples Walter Willetts adjustment method was modified to use for all the RBC FA data. By the modified A-A (adjusted-all) method (*n* 101) the RBC FA data were adjusted for total FA concentration by calculating the equation for the regression line between each RBC FA proportion and total FA concentration ($y = a + b*x$,

where y is the predicted FA proportion for a given total FA concentration, x; a and b are constants). The residuals between the regression line and each FA proportion value were found by subtracting the predicted FA proportions from the obtained proportions, y' – y = y' – a – b*x (where y' is the obtained value and y is the predicted value). The residuals were either negative or positive, and their mean was zero. A constant was added to the residuals to convey the sense of actual FA proportion values. The constant Willett and coworkers used was the nutrient intake referring to the mean caloric intake of the cohort, assuming that the mean caloric intake of the cohort was the same as the average, true caloric intake of the population. In the present study the constant added to the residuals of the regression line was the FA proportion referring to the mean total FA concentration of the higher mode of the bimodal distribution of the proportion of 20 : 4*n*-6, or 1047.08 mg/L, Fig. 1 (a), Table 3). The constant was calculated from the equation for the regression line, $y_k = a + b*x_m$ (where $y_k$ is the constant and $x_m$ is 1047.08 mg/L). The sum of the residual and the constant gave the residually adjusted FA proportion value, which was thus standardized to the mean total FA concentration of RBC samples not considered peroxidized.

*Statistical analysis*

Three different methods were used to analyze the RBC FA data. One included all the RBC FA data unadjusted (U-A, *n* 101), another included unadjusted FA data from RBC samples considered not peroxidized (U-NP, *n* 67), and the third included all the RBC FA data adjusted for total FA concentration (A-A, *n* 101). Characteristics and dietary marine *n*-3 PUFA intake of the pregnant women, whose data were included in the U-NP and those whose data were excluded from the U-NP analysis method due to RBC lipids considered peroxidized, were compared with independent t-test (continuous and normally distributed) or Mann-Whitney U test (discrete or non-normally distributed). Histograms of the proportions of 20 : 4*n*-6 and 22 : 6*n*-3 in RBC data analyzed by the three methods were drawn and Kolmogorov-Smirnoff test was used to assess if the distribution was normal. Pearson correlation was used to assess the correlation between the total FA concentration of RBC lipids and the proportion of each RBC FA in data analyzed by the U-A and the U-NP methods. Since data analyzed by the U-A method was non-normally distributed, Wilcoxon signed-rank test was used for comparison of mean proportions of each FA in RBC between data analyzed by the U-A and A-A methods. Paired t-test was used to compare mean proportions of each FA in RBC between data analyzed by he U-NP and A-A methods. Due to multiple comparisons Bonferroni correction was used. Multiple linear regression models for each analysis method were constructed combining the proportion of 22 : 6*n*-3 in RBC with dietary intake of 22 : 6*n*-3 and the proportion of 20 : 4*n*-6 in RBC. Log contrast models, which have been recommended instead of

habitual regression models for analysis of proportions[26], gave similar associations as the habitual models. We chose to use the habitual regression models, since they are easier to interpret. The level of significance was set at 0.05 and two-tailed *P*-value was used. Statistical analysis was performed using the SPSS statistical software package version 14.0 (SPSS Incorporated, Chicago, USA).

## Results

### *Characteristics*

Age, height, BMI, blood pressure, dietary marine *n*-3 PUFA intake and parity of all the women in the study (*n* 101), of the women included in the unadjusted-not peroxidized (U-NP) analysis method (*n* 67), and of the women excluded from the U-NP method (*n* 34) are shown in Table 1. The women were on average 28.0 (SD 5.2) years old and their BMI was 25.2 (SD 4.3) kg/m$^2$. Their fish intake was 27.9 (SD 21.1) g/d and 33.7% of them took marine *n*-3 PUFA supplements (liquid or capsules). The women who were excluded from the U-NP method were of shorter stature, had higher BMI, systolic blood pressure and parity than the women included, but there were no differences in age or in total dietary intake of 22 : 6*n*-3, fish, or CLO (liquid or capsules). No difference was found in any other dietary intake variable between these two subgroups of women.

**Table 1.** Characteristics and dietary marine *n*-3 PUFA intake of all the subjects, of subjects included in the U-NP method, and of subjects excluded from the U-NP method

| | Subjects | | | | | | |
|---|---|---|---|---|---|---|---|
| | All (*n* 101) | | Included in U-NP (*n* 67) | | Excluded from U-NP (*n* 34) | | *P** |
| | Mean | SD | Mean | SD | Mean | SD | |
| Age (years) | 28.0 | 5.2 | 27.5 | 4.8 | 28.9 | 6.0 | 0.250 |
| Height (cm) | 168.3 | 5.7 | 169.5 | 5.5 | 165.9 | 5.5 | 0.001 |
| BMI at entry (kg/m$^2$) | 25.2 | 4.3 | 24.1 | 3.5 | 27.3 | 5.0 | 0.002 |
| Systolic blood pressure at entry (mm Hg) | 113.2 | 10.9 | 111.1 | 9.3 | 117.2 | 12.7 | 0.023 |
| Diastolic blood pressure at entry (mm Hg) | 65.9 | 8.3 | 64.9 | 7.7 | 67.9 | 9.0 | 0.167 |
| Dietary intake of marine n-3 PUFA | | | | | | | |
| Total 22 : 6*n*-3 (mg/d) | 293.3 | 337.0 | 327.3 | 360.4 | 226.2 | 278.1 | 0.084 |
| Fish (g/d) | 27.9 | 21.1 | 27.5 | 20.5 | 28.8 | 22.5 | 0.749 |
| | % | | % | | % | | |
| L-CLO | 13.9 | | 16.4 | | 8.8 | | 0.299 |
| CLO capsules | 19.8 | | 20.9 | | 17.6 | | 0.700 |
| Pregnant with third child or more | 22.8 | | 16.4 | | 35.3 | | 0.033 |

L-CLO, liquid cod liver oil; U-NP, unadjusted not-peroxidized.
*Significant difference between subjects included in U-NP and subjects excluded from U-NP at $P<0.05$ (independent t-test for continuous and normally distributed variables and Mann-Whitney U test for discrete or non-normally distributed variables).

*Unadjusted-all (U-A) method*

The distribution of 20 : 4*n*-6 and 22 : 6*n*-3 proportions in RBC in all the data unadjusted (*n* 101) was bimodal (Fig. 1 (a,b)). Reflection and/or inverse or logarithmic transformation did not normalize the distribution.

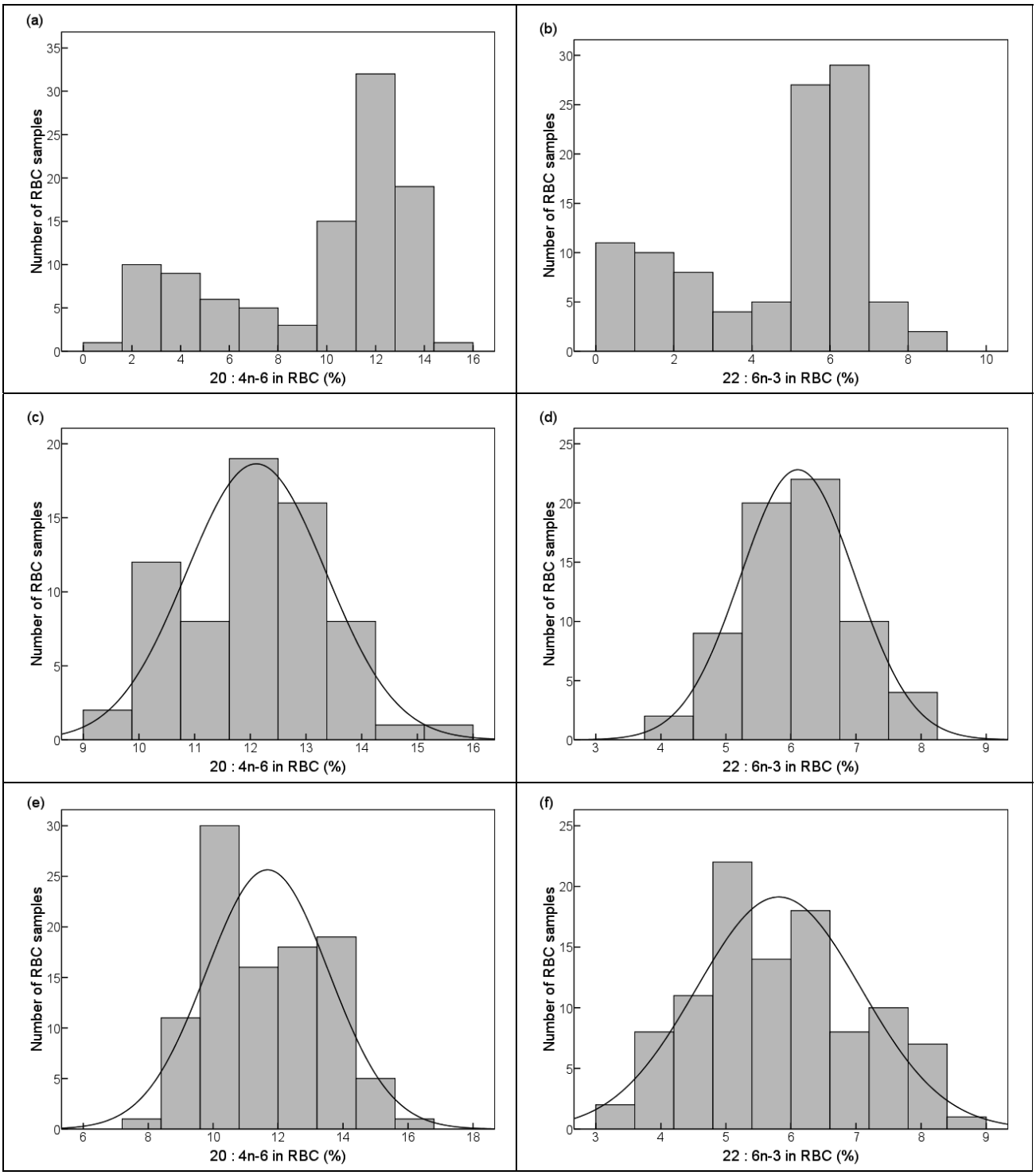


**Fig. 1. Distribution of the proportions of 20 : 4*n*-6 and 22 : 6*n*-3 in red blood cells of women at week 12.9 (SD 2.1) of pregnancy in data analyzed by the U-A (unadjusted-all) method (a and b, *n* 101), the U-NP (unadjusted-not peroxidized) method (c and d, *n* 67), and the A-A (adjusted-all) method (e and f, *n* 101).**

The correlation between total FA concentration of RBC and proportion of each FA in RBC is shown in Table 2. In data analyzed by the U-A method total FA concentration of RBC correlated negatively with the proportion of each SFA and MUFA, and positively with the proportion of each *n*-6 PUFA and *n*-3 PUFA. The proportion of 20 : 4*n*-6 in RBC was strongly and positively correlated with the proportion of 22 : 6*n*-3 in RBC, and the proportions of all major PUFA were strongly and negatively correlated with the proportions of all major SFA and MUFA (data not shown).

**Table 2.** Correlation between RBC FA proportions (g/100g of Total FA) and total FA concentration (mg/L) of RBC

| | Analysis method | | |
|---|---|---|---|
| | U-A (*n* 101)* | U-NP (*n* 67) | |
| FA | *r*† | *r*† | *P*‡ |
| Total SFA | -0.89 | -0.60 | <0.001 |
| 14 : 0 | -0.69 | -0.18 | 0.157 |
| 16 : 0 | -0.88 | -0.55 | <0.001 |
| 18 : 0 | -0.83 | -0.20 | 0.104 |
| 20 : 0 | -0.74 | 0.03 | 0.787 |
| 22 : 0 | -0.77 | -0.18 | 0.144 |
| 24 : 0 | -0.81 | -0.20 | 0.100 |
| Total MUFA | -0.83 | -0.34 | 0.004 |
| 18 : 1*n*-9 | -0.85 | -0.39 | 0.001 |
| 18 : 1*n*-7 | -0.52 | -0.27 | 0.028 |
| 24 : 1*n*-9 | -0.66 | -0.12 | 0.353 |
| Total n-6 PUFA | 0.90 | 0.49 | <0.001 |
| 18 : 2*n*-6 | 0.75 | 0.37 | 0.002 |
| 20 : 4*n*-6 | 0.88 | 0.29 | 0.018 |
| 20 : 3*n*-6 | 0.76 | 0.07 | 0.595 |
| 22 : 4*n*-6 | 0.83 | 0.16 | 0.200 |
| Total n-3 PUFA | 0.84 | 0.16 | 0.202 |
| 20 : 5*n*-3 | 0.69 | 0.13 | 0.289 |
| 22 : 5*n*-3 | 0.84 | 0.09 | 0.489 |
| 22 : 6*n*-3 | 0.83 | 0.14 | 0.245 |

U-A, unadjusted-all; U-NP, unadjusted-not peroxidized
RBC, red blood cells.
*All correlations were significant at $P<0.001$.
†Pearson correlation coefficient.
‡Correlations were significant at $P<0.05$.

*Unadjusted-not peroxidized (U-NP) method*

RBC samples in the higher mode of the bimodal distribution of the proportion of 20 : 4*n*-6 (Fig. 1 (a)) were considered not peroxidized and included in the U-NP method (*n* 67). The higher mode of the bimodal distribution of 22 : 6*n*-3 (Fig. 1 (b)) was made up of the same RBC samples as the higher mode of the bimodal distribution of 20 : 4*n*-6. The proportions of 20 : 4*n*-6 and 22 : 6*n*-3 in

data analyzed by the U-NP method were normally distributed (Fig. 1 (c,d)), and the correlation between the total FA concentration of RBC and the proportion of each FA was weaker than in data analyzed by the U-A method or absent (Table 2).

*Adjusted-all (A-A) method*

All the RBC FA data (*n* 101) were adjusted for total FA concentration by regressing each RBC FA proportion on the total FA concentration of RBC. A constant (the FA proportion referring to the mean total FA concentration of the higher mode of the bimodal distribution of 20 : 4*n*-6, or 1047.08 mg/L, Fig. 1 (a), Table 3), was added to the residuals of the regression line. FA proportions were thus standardized to the mean total FA concentration of RBC samples not considered peroxidized. The proportions of 20 : 4*n*-6 and 22 : 6*n*-3 in RBC were approximated to normal distribution after the adjustment (Fig. 1 (e,f)).

*Comparison between analysis methods*

The mean proportions of individual FA and total SFA, MUFA, *n*-6 PUFA and *n*-3 PUFA in RBC data analyzed by the three methods are shown in Table 3. The mean total FA concentration (mg/L) of RBC samples analyzed by the U-A and the U-NP methods is also shown. The mean proportion of all PUFA were lower and of all SFA and MUFA higher in data analyzed by the U-A method than by the A-A method. There was no difference in the mean proportion of any FA in RBC between the U-NP and the A-A methods.

**Table 3.** FA composition of RBC in data analyzed by three analysis methods*

| | Analysis method | | | | | |
|---|---|---|---|---|---|---|
| | U-A (*n* 101)† | | U-NP (*n* 67)‡ | | A-A (*n* 101) | |
| FA | Mean | SD | Mean | SD | Mean | SD |
| Total SFA | 45.80 | 8.43 | 40.47 | 1.69 | 41.22 | 3.79 |
| 14 : 0 | 0.47 | 0.17 | 0.40 | 0.08 | 0.40 | 0.12 |
| 16 : 0 | 23.41 | 5.07 | 20.27 | 1.24 | 20.71 | 2.43 |
| 18 : 0 | 15.47 | 1.51 | 14.58 | 0.61 | 14.71 | 0.84 |
| 20 : 0 | 0.43 | 0.13 | 0.36 | 0.06 | 0.38 | 0.09 |
| 22 : 0 | 1.68 | 0.58 | 1.36 | 0.31 | 1.41 | 0.37 |
| 24 : 0 | 4.33 | 1.44 | 3.51 | 0.67 | 3.62 | 0.85 |
| Total MUFA | 18.50 | 2.46 | 16.97 | 1.01 | 17.27 | 1.37 |
| 18 : 1*n*-9 | 12.45 | 1.59 | 11.49 | 0.72 | 11.64 | 0.85 |
| 18 : 1*n*-7 | 1.47 | 0.21 | 1.40 | 0.15 | 1.40 | 0.18 |
| 24 : 1*n*-9 | 4.15 | 0.88 | 3.68 | 0.53 | 3.80 | 0.66 |
| Total n-6 PUFA | 20.52 | 6.64 | 24.67 | 1.73 | 24.13 | 2.95 |
| 18 : 2*n*-6 | 7.81 | 1.84 | 8.63 | 1.07 | 8.64 | 1.22 |
| 20 : 4*n*-6 | 9.57 | 3.94 | 12.11 | 1.25 | 11.67 | 1.89 |
| 20 : 3*n*-6 | 1.30 | 0.51 | 1.56 | 0.28 | 1.54 | 0.34 |
| 22 : 4*n*-6 | 1.84 | 0.87 | 2.37 | 0.44 | 2.29 | 0.49 |
| Total n-3 PUFA | 7.22 | 3.66 | 9.55 | 1.36 | 9.09 | 1.96 |
| 20 : 5*n*-3 | 0.67 | 0.50 | 0.92 | 0.39 | 0.88 | 0.36 |
| 22 : 5*n*-3 | 1.90 | 1.00 | 2.52 | 0.43 | 2.41 | 0.55 |
| 22 : 6*n*-3 | 4.65 | 2.29 | 6.10 | 0.88 | 5.81 | 1.26 |
| Total FA (mg/L) | 905.06 | 233.95 | 1047.08 | 118.50 | --- | |

U-A, unadjusted-all; U-NP, unadjusted-not peroxidized; A-A, adjusted-all.
*Data are expressed as g/100g of total FA unless otherwise stated.
†All fatty acid proportions were significantly different from the A-A method ($P < 0.001$, Wilcoxon signed-rank test).
‡FA proportions were not significantly different from the A-A method (Paired t-test). Bonferroni correction was used because of multiple comparisons.

A multiple regression model for each analysis method, where the proportion of 20 : 4*n*-6 in RBC and dietary 22 : 6*n*-3 intake were tested against the proportion of 22 : 6*n*-3 in RBC, is shown in Table 4. The B coefficient for dietary intake of 22 : 6*n*-3 is fairly similar in all the three models (from 1.50 in the A-A model to 1.62 in the U-NP model). This means that when the proportion of 20 : 4*n*-6 in RBC is constant, an increase in dietary intake of 22 : 6*n*-3 by 1g/d will lead to about 1.5 g/100g increase in the proportion of 22 : 6*n*-3 in RBC. The B coefficient for the proportion of 20 : 4*n*-6 in RBC is much lower and in fact non-significant in the U-NP model compared with the other two models. When the β coefficient for the proportion of 20 : 4*n*-6 in RBC and dietary 22 : 6*n*-3 intake is compared within each model, it can be seen that in the U-A model the relative contribution of 20 : 4*n*-6 in RBC is much stronger than the contribution of dietary 22 : 6*n*-3 (0.89 vs. 0.23, respectively). In the U-NP model the contribution of 20 : 4*n*-6 in RBC is non-significant, and the contribution of dietary 22 : 6*n*-3 is fairly strong (0.67). In the A-A model the contribution of 20 : 4*n*-6 in RBC is stronger than the contribution of dietary 22 : 6*n*-3 (0.75 vs. 0.40), but though not as strong as in the U-A model. Both independent variables, the proportion of 20 : 4*n*-6 in RBC and

dietary 22 : 6*n*-3 intake, had a positive relationship with the proportion of 22 : 6*n*-3 in RBC in all the models. The U-A model expressed 87% of the variation in 22 : 6*n*-3 in RBC, the U-NP model 37.8% and the A-A model 56.9%.

**Table 4.** Multiple regression analysis to predict the proportion of 22 : 6*n*-3 in RBC with the analysis methods U-A, U-NP and A-A

| Analysis method<br>Dependent variable | *n* | Independent variables | B | Std. Error | β | *P* | Adj. $R^2$ | *P* for the model |
|---|---|---|---|---|---|---|---|---|
| U-A | 101 | | | | | | | |
| RBC 22 : 6*n*-3 (g/100g) | | Constant | -0.80 | 0.23 | | 0.001 | | |
| | | RBC 20 : 4*n*-6 (g/100g) | 0.52 | 0.02 | 0.89 | <0.001 | | |
| | | Dietary 22 : 6*n*-3 (g/d) | 1.59 | 0.25 | 0.23 | <0.001 | 0.870 | <0.001 |
| U-NP | 67 | | | | | | | |
| RBC 22 : 6*n*-3 (g/100g) | | Constant | 4.06 | 0.91 | | <0.001 | | |
| | | RBC 20 : 4*n*-6 (g/100g) | 0.13 | 0.07 | 0.18 | 0.088 | | |
| | | Dietary 22 : 6*n*-3 (g/d) | 1.62 | 0.25 | 0.67 | <0.001 | 0.378 | <0.001 |
| A-A | 101 | | | | | | | |
| RBC 22 : 6*n*-3 (g/100g) | | Constant | -0.47 | 0.56 | | 0.398 | | |
| | | RBC 20 : 4*n*-6 (g/100g) | 0.50 | 0.05 | 0.75 | <0.001 | | |
| | | Dietary 22 : 6*n*-3 (g/d) | 1.50 | 0.25 | 0.40 | <0.001 | 0.569 | <0.001 |

RBC, red blood cells; U-A, unadjusted-all; U-NP, unadjusted-not peroxidized; A-A, adjusted-all.

## Discussion

In the present study one third of the RBC samples from a group of women in early pregnancy were considered lipid peroxidized due to inappropriate storage conditions. An attempt was made to modify an adjustment method designed for nutritional data analysis, such that the RBC FA data from all the women could be included in the data analysis. RBC FA proportions were standardized to the mean total FA concentration of RBC samples not considered peroxidized, to meet the consequences of loss in total FA concentration and peroxidation of PUFA.

The limit between lipid peroxidized and not lipid peroxidized RBC samples has been drawn at 10 g/100g of 20 : 4*n*-6[27, 28]. In the present study the limit was drawn at 9.5 g/100g because the distribution of the proportion of 20 : 4*n*-6 was bimodal and the limit between the higher and lower mode was at 9.5 g/100g (Fig. 1 (a)). The FA composition of RBC samples analyzed by the U-NP method was considered to be reliable, since the distributions of the proportions of 20 : 4*n*-6 and 22 : 6*n*-3 in data analyzed by the U-NP method were normal (Fig. 1 (c,d)), total FA concentration was relatively high (≥710 mg/L) and so were the proportions of 20 : 4*n*-6 and 22 : 6*n*-3 (>9.5 g/100g and >3.5 g/100g, respectively). Furthermore, the correlation between the FA proportions in RBC and the total FA concentration was weaker in data analyzed by the U-NP method than in data analyzed by the U-A method (Table 2), and it was in fact absent for *n*-3 PUFA, which supports our previous study that RBC samples with a high 22 : 6*n*-3 content at baseline might be better protected against PUFA peroxidation and PL degradation during inappropriate storage conditions[9].

The combined effects of the duration of storage at -20°C[7-9], the natural pro-oxidant and antioxidant content of the RBC[10], and the *n*-3 PUFA content of the RBC[9, 13, 14] should rather have

lead to a distribution of PUFA proportions with a significant tail towards low proportions of PUFA, than to the bimodal distribution seen in the present study (Fig. 1 (a,b)). The bimodal distributions of the proportions of 20 : 4*n*-6 and 22 : 6*n*-3 can not be explained by distinct experimental conditions, since all the blood samplings and handling were carried out in the same way by the same person, and the RBC FA analyses also. Pathological state of the individual has been shown to affect stability of RBC PUFA during storage[11, 12]. Interestingly, women excluded from the U-NP method were shorter and had higher BMI, systolic blood pressure and parity than those included (Table 1). Furthermore, their RBC had by definition low proportions of both 20 : 4*n*-6 (≤9.5 g/100g) and 22 : 6*n*-3 (≤3.5 g/100g). Possibly the women excluded from the U-NP method had a relatively low proportion of 22 : 6*n*-3 in their RBC at baseline, since a low proportion of 22 : 6*n*-3 at baseline has been associated with a less protection against PUFA peroxidation and PL degradation during inappropriate storage conditions[9]. A low proportion of 22 : 6*n*-3 in RBC should reflect a low dietary intake of marine *n*-3 PUFA[1-3]. However, no significant difference was found in mean dietary intake of marine *n*-3 PUFA between the women included and excluded, probably due to high inter-individual variation in dietary intake (Table 1). Whether the different characteristics between women included and excluded from the U-NP method indicate a distinct natural antioxidant status of RBC or distinct physiological conditions, which could give rise to a bimodal distribution of the proportions of PUFA in RBC after storage at inappropriate conditions, needs further study.

A study has shown a positive correlation between the proportions of 20 : 4*n*-6 and 22 : 6*n*-3 in RBC of pregnant British and Korean women in the third trimester[29]. In RBC of the women in the present study, who were at week 12.9 (SD 2.1) of pregnancy, the proportions of 20 : 4*n*-6 and 22 : 6*n*-3 were found to be strongly, positively correlated in the U-A model. They were also positively correlated in the A-A model (Table 4), while no correlation was found between the proportions of 20 : 4*n*-6 and 22 : 6*n*-3 in RBC in the U-NP model, probably due to the relatively high and stabilized proportion of 20 : 4*n*-6. On the other hand, the correlation between the proportion of 22 : 6*n*-3 in RBC and dietary intake of 22 : 6*n*-3 was strongest in the U-NP model and weakest in the U-A model (Table 4). The weak correlation found between the proportion of 22 : 6*n*-3 in RBC and dietary intake of 22 : 6*n*-3 in the U-A model indicates that the RBC FA were partly peroxidized.

An adjustment is more reliable if the correlation between the adjustment factor (total FA concentration in the present study) and the variable it is meant to adjust (proportion of any FA in the present study) is strong. Total FA concentration was strongly correlated to all RBC FA proportions in data analyzed by the U-A method (Table 2) and therefore the adjustment method was considered to be reliable for the RBC FA data. To our knowledge, no one has previously attempted to adjust FA data from partly peroxidized RBC lipids. Willett *et al.* explained that the method they

designed was analogous, although not identical, to including both the adjustment factor and the variable it was meant to adjust as independent factors in a multivariate model with disease outcome as the dependent variable[16]. This can, however, not be recommended since including two strongly correlated, independent variables simultaneously in a multiple regression model leads to multicollinearity. Furthermore, the adjustment method used in the present study brought the sense of actual FA values with a mean and SD to the adjusted RBC FA data, which made the adjusted data better comparable with the unadjusted data. Dividing the variable meant to adjust with the adjustment factor is another approach to overcome confounding. However, when associated with disease outcome such an adjustment leads to confounding in the opposite direction[17].

In conclusion, the U-NP analysis method probably gave the most reliable RBC FA data. The U-NP method included only the more true, unadjusted FA data from RBC samples considered not peroxidized, and the correlation between the proportion of 22 : 6*n*-3 in RBC and the dietary intake of 22 : 6*n*-3 was better than in the other two models. However, the participants included in the U-NP method were not quite representative for the whole group, since several characteristics differed between them and the participants excluded from the U-NP method, which causes a selection bias. All the participants were included in the A-A method, and no difference was observed in mean proportions of any PUFA, SFA or MUFA between the A-A and U-NP methods, which supports the use of the A-A method. Furthermore, the A-A regression model predicting the proportion of 22 : 6*n*-3 in RBC resembled the U-NP regression model. Adjustment is though always limited by uncertainty, since the adjusted FA values are not true, but estimated.

The present study demonstrated that the stability of RBC lipids might depend on individual physiological condition as well as on storage condition of the RBC samples. The study also supported the use of a FA data adjustment method for partly peroxidized RBC lipids, such that all the participants could be included in the analysis.

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# APPENDICES
# I-IV

# Appendix I

Kæri þátttakandi

Þessi spurningalisti varðar mataræði þitt og neysluvenjur. Vinsamlegast merktu við með vel ydduðum blýanti í viðeigandi reit fyrir hverja fæðutegund sem er á listanum.

Þegar þú svarar spurningunum skalt þú hafa síðustu þrjá mánuði í huga. Það er þó alls ekki gert ráð fyrir að þú munir nákvæmlega allt sem þú hefur borðað! Við biðjum þig einfaldlega að áætla hversu oft á dag, í viku eða í mánuði þú borðir hverja fæðutegund eða hversu mörg stykki þú borðar af hverju fyrir sig. Mataræði flestra er misjafnt frá degi til dags og frá einni viku til annarrar. Því ert þú fyrst og fremst beðinn að áætla eftir bestu getu hvernig mataræði þitt er að öllum jafnaði. Við biðjum þig að merkja við hverja einustu fæðutegund, líka þær sem þú borðar aldrei, að öðrum kosti verða svörin ónothæf.

Í fyrstu 6 spurningunum er spurt um brauð, kökur, álegg og drykki. Þar ertu beðinn að áætla hversu margar sneiðar, stykki eða glös þú neytir af hverju fyrir sig, í mánuði, viku eða á dag. Í spurningum 7-21 ertu beðinn að áætla hversu oft þú borðar viðkomandi fæðu. Í lokin eru spurningar um hversu mikið þú borðar yfirleitt af nokkrum fæðutegundum. Leiðbeiningar og dæmi um hvernig á að fylla út eyðublaðið eru á næstu blaðsíðu.

Farið verður með allar upplýsingar sem trúnaðarmál.

Eyðublöðin verða lesin af tölvu og því skiptir miklu máli hvernig þau eru fyllt út.

**Notið vel yddaðan blýant no. 2**
Merkið greinilega í viðeigandi reit með x
Notið strokleður til leiðréttinga, ekki krassa eða strika út
Varist að brjóta upp á blöðin eða káma þau út

Bestu þakkir fyrir þátttökuna

# Dæmi um hvernig á að fylla út eyðublaðið

## Sjá spurningu 1. Brauð, kökur, kex

Hér er spurt hversu margar brauðsneiðar, kexkökur og kökusneiðar þú borðar yfirleitt í mánuði, viku eða á dag.

Segjum sem svo að Jón Jónsson borði yfirleitt tvær heilhveitibrauðsneiðar á morgnana alla virka daga, hann fái sér ævinlega samloku í hádeginu úr heilhveitibrauði og auk þess eina til tvær grófar brauðsneiðar síðdegis 5 daga vikunnar. Franskbrauð, rúgbrauð eða hrökkbrauð borðar hann hins vegar aldrei. Hann áætlar neysluna á heilhveitibrauði og grófu brauði þannig:

Hann áttar sig á því að 5 daga vikunnar borðar hann 5-6 sneiðar á dag en tvo daga vikunnar aðeins tvær sneiðar. Að jafnaði borðar hann því 4-5 sneiðar á dag. Á eyðublaðinu er hvergi hægt að merkja við 4-5 sneiðar á dag heldur annað hvort 3-4 á dag eða 5 eða fleiri. Jón metur stöðuna þannig að réttara sé að merkja við reitinn 5 eða fleiri, því það sé líklega nær hinu rétta.

Jón fær sér ævinlega vínarbrauð á sunnudagsmorgnum og merkir því í reitinn 1/viku. Pönnukökur fær hann u.þ.b. einu sinni í mánuði en borðar þá 4-5 pönnukökur og merkir því við 1/viku (4 í mánuði). Kleinur eða jólaköku fær hann sér með kaffinu flesta daga vikunnar og borðar þá gjarnan 2 kleinur eða kökusneiðar. Hann merkir því við reitinn 2 á dag. Rjóma setur hann aðeins á tvær pönnukökur í mánuði og merkir í samræmi við það. Kex borðar hann aldrei.

Útfyllt eyðublað Jóns lítur þannig út fyrir spurningu 1:

### *1. Brauð, kökur, kex*

**Hversu margar** brauðsneiðar, stykki, borðar þú á dag, í viku eða mánuði? Eitt rúnnstykki eða langloka teljast tvær brauðsneiðar. Merktu við einn reit í hverri línu, annað hvort við sneiðar á dag, í viku, í mánuði eða aldrei eftir því sem við á.

| **Fæðutegund** | **Aldrei** | **Í mánuði** sneiðar | | | **Í viku** sneiðar | | | **Á dag** sneiðar | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Færri en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða fleiri |
| Franskbrauð, hvítt brauð | ☒ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rúg-, malt-, flatbrauð | ☒ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Heilhveitibrauð, gróft brauð | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☒ |
| Hrökkbrauð, bruður, kringlur | ☒ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Vínarbrauð, smjörkaka | ☐ | ☐ | ☐ | ☐ | ☒ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Snúður, pönnukökur, vöfflur | ☐ | ☐ | ☐ | ☐ | ☒ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kleinur, kökur, annað sætabrauð | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☒ | ☐ | ☐ |
| Kex, sætt, ósætt | ☒ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rjómi á kökur, vöfflur | ☐ | ☐ | ☐ | ☒ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *1. Brauð, kökur, kex*

**Hversu margar** brauðsneiðar, stykki, borðar þú á dag, í viku eða mánuði? Eitt rúnnstykki eða langloka teljast tvær brauðsneiðar. Merktu við einn reit í hverri línu, annað hvort við sneiðar á dag, í viku, í mánuði eða aldrei eftir því sem við á.

| Fæðutegund | Aldrei | Í mánuði sneiðar | | | Í viku sneiðar | | | Á dag sneiðar | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Færri en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða fleiri |
| Franskbrauð, hvítt brauð | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rúg-, malt-, flatbrauð | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Heilhveitibrauð, gróft brauð | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hrökkbrauð, bruður, kringlur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Vínarbrauð, smjörkaka | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Snúður, pönnukökur, vöfflur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kleinur, kökur, annað sætabrauð | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kex, sætt, ósætt | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rjómi á kökur, vöfflur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *2. Með hverju er brauðið (kexið) smurt?*

**Hversu margar** brauðsneiðar eða kexkökur smyrðu á dag eða á viku með hverju feitmeti fyrir sig? Teldu samanlagðan fjölda sneiða eða stykkja af öllu brauði, hrökkbrauði eða kexi sem er smurt.

| Fæðutegund | Aldrei | Í mánuði sneiðar | | | Í viku sneiðar | | | Á dag sneiðar | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fjöldi brauðsneiða | Aldrei | Færri en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða fleiri |
| Smjör (fjöldi sneiða) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Smjörvi | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Létt og laggott | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Klípa | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Sólblóma (65%) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Létta, Létt-Sólblóma, Lína, Smyrill | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *3. Álegg. Fjöldi brauðsneiða með hverju áleggi*

Skráðu hér samanlagðan **fjölda** brauðsneiða eða kexa á dag, viku eða mánuði með hverri áleggstegund. Ef tvær eða fleiri áleggstegundir eru hafðar á einni sneið, skal merkja við allar tegundirnar.

| Fæðutegund | Aldrei | Í mánuði fjöldi | | | Í viku fjöldi | | | Á dag fjöldi | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Færri en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða fleiri |
| Ostur, 26% eða feitari | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Ostur, 17%, 11%, smurostur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Camembert, Brie, rjómaostur, gráðostur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Skinka, hangikjöt, kjötpylsa, steik | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Spægipylsa, pepperóní, rúllupylsa, beikon | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kæfa, paté | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Salat með majonesi/sýrðum rjóma | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Lax, silungur, síld | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rækjur, egg | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Grænmeti/ávextir sem álegg | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Sulta, marmelaði, hunang | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hnetusmjör | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *4. Mjólk til drykkjar.*

**Hversu mörg** mjólkurglös drekkur þú á dag, viku eða í mánuði (1 glas=2dl)
(Ekki telja með mjólk út á graut o.s.frv. eða út í kaffi),

| Fæðutegund | Aldrei | Í mánuði fjöldi glasa | | | Í viku fjöldi glasa | | | Á dag fjöldi glasa | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Færri en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða fleiri |
| Nýmjólk, kakó úr nýmjólk | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Léttmjólk, sælumjólk, kakó úr léttmjólk | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Undanrenna, fjörmjólk | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Mysa | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *5. Drykkjarvörur*

**Hversu mörg** glös, bolla o.s.frv. drekkur þú af hverjum drykk?

| **Fæðutegund** | **Aldrei** | **Í mánuði** fjöldi | | | **Í viku** fjöldi | | | **Á dag** fjöldi | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Færri en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða fleiri |
| Vatn, sódavatn, sykurlaus gosdrykkur (glas) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Ávaxtasafi, hreinn (lítil ferna) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Svali, djús (lítil ferna) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Gosdrykkur (glas) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Pilsner, maltöl (glas) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Bjór (glas) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Léttvín, borðvín (vínglas) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Millisterkt vín (sherry, púrtvín, líkjör) (glas) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Sterkt vín (sjúss) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kaffi (bolli) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Te, jurtate (bolli) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *6. Notar þú mjólk, sykur o.s.frv. í kaffi, te?*

Skráðu **fjölda** bolla með hverju fyrir sig.

| **Fæðutegund** | **Aldrei** | **Í mánuði** fjöldi | | | **Í viku** fjöldi | | | **Á dag** fjöldi | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Færri en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða fleiri |
| Mjólk, léttmjólk í kaffi, te | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rjómi, kaffirjómi í kaffi, te | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Sykur, hunang í kaffi, te | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *Athugið: Hér eftir verður spurt um tíðni, þ.e. hversu oft þú borðar tilgreindar fæðutegundir*

### *7. Morgunkorn, grautar, sýrðar mjólkurvörur*

**Hversu OFT** borðar þú þessar vörur?

| Fæðutegund | Aldrei | Í mánuði skipti | | | Í viku skipti | | | Á dag skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Hafragrautur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| All-bran, múslí, haframjöl | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Morgunkorn, (Cheerios o.þ.h.) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hrísgrjónagrautur, mjólkursúpa | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Súrmjólk, AB-mjólk, ABT-mjólk, jógúrt | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Sýrð léttmjólk, léttjógúrt, fismjólk | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Skyr, berjaskyr | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rjómaskyr, þykkmjólk, engjaþykkni | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Sykur á morgunkorn, skyr, súrmjólk, graut | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

### *8. Mjólk út á graut, morgunkorn eða skyr*

Hversu oft hefurðu þessar vörur sem útálát? (Ekki til drykkjar eða í kaffi, te)

| Fæðutegund | Aldrei | Í mánuði skipti | | | Í viku skipti | | | Á dag skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Nýmjólk | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Léttmjólk, sælumjólk | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Undanrenna, fjörmjólk | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rjómi | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rjómabland, kaffirjómi | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *9. Ávextir og ber.* **Hversu oft** borðar þú ávexti og ber?

| **Fæðutegund** | **Aldrei** | **Í mánuði** skipti | | | **Í viku** skipti | | | **Á dag** skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Epli, perur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Appelsínur, mandarínur, greip, kíví | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Bananar | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rúsínur, sveskjur, döðlur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Aðrir ávextir eða ber | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *10. Súpur, eftirréttir*

Ef um er að ræða matarmiklar súpur skal skrá kjöt, fisk, grænmeti, kartöflur o.s.frv. úr súpunni á viðeigandi stað í spurningum nr. 12 til 16 en skrá sjálfa súpuna hér.

| **Fæðutegund** | **Aldrei** | **Í mánuði** skipti | | | **Í viku** skipti | | | **Á dag** skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Súpa, tær | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Súpa, þykkt eða rjómalöguð | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Búðingur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Ís, frómas | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *11. Heitur matur. Yfirlit.* **Hversu oft** borðar þú þessar fæðutegundir eða rétti?

(Ekki telja með álegg á brauð).

| **Fæðutegund** | **Aldrei** | **Í mánuði** skipti | | | **Í viku** skipti | | | **Á dag** skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Fiskur, fiskfarsréttir | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kjöt, pylsur, hamborgari, hakkréttir | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Pizza | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Pastaréttir (**sem aðalréttur**) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Grænmetis-, bauna- og hrísgrjónaréttir (**sem aðalréttur**) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Eggjaréttir (**sem aðalréttur**) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |


***12. Fiskréttir.*** **Hversu oft** borðar þú þessa fiskrétti?

| **Fæðutegund** | **Aldrei** | **Í mánuði** skipti | | | **Í viku** skipti | | | **Á dag** skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Soðinn nýr fiskur, bakaður, glóðaður | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Steiktur fiskur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Fiskibollur, fiskibúðingur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Saltfiskur, reyktur fiskur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

***13. Kjötréttir.*** **Hversu oft** borðar þú þessa kjötrétti?

| **Fæðutegund** | **Aldrei** | **Í mánuði** skipti | | | **Í viku** skipti | | | **Á dag** skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Hakkréttur, hamborgari | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Pylsur, kjötfars, kjötbúðingur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Bjúgu | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Feitt og millifeitt kjöt (t.d. kótilettur, frampartur) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Magurt kjöt (t.d. læri, gúllas eða fita skorin burt) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Saltkjöt, hangikjöt, reykt kjöt | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Blóðmör, lifrarpylsa, lifur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kjúklingur, annað fuglakjöt | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

***14. Meðlæti.*** **Hversu oft** borðar þú þessar fæðutegundir sem **meðlæti**

| **Fæðutegund** | **Aldrei** | **Í mánuði** skipti | | | **Í viku** skipti | | | **Á dag** skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Bakaðar baunir | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Egg (soðin, steikt, hrærð) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kartöflur (soðnar, bakaðar) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kartöflur (steiktar, franskar) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hrísgrjón | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Pasta, spaghetti | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hamborgara-, pylsu-, pítubrauð | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *15. Grænmeti. Hrátt eða soðið.* Hversu oft borðar þú:

| Fæðutegund | Aldrei | Í mánuði | | | Í viku | | | Á dag | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | skipti | | | skipti | | | skipti | | | |
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Hrátt grænmeti, grænmetissalat | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Soðið grænmeti (nýtt, frosið, niðursoðið) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *16. Grænmeti.* Hversu oft borðar þú þessar **grænmetistegundir**? Teldu bæði með grænmeti sem fer í pottrétti og blandaða rétti eða borðað sér. Ekki skal telja með grænmeti sem álegg á brauð eða pizzur.

| Fæðutegund | Aldrei | Í mánuði | | | Í viku | | | Á dag | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | skipti | | | skipti | | | skipti | | | |
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Gulrætur, rófur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hvítkál, blóm-, spergil-, rauðkál | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Grænar baunir, maískorn frosin grænmetisblanda | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Laukur, púrra, hvítlaukur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Gúrka, tómatur, papríka, salat | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

## *17. Sósur og feiti.* Hér er spurt **hversu oft** þú borðar sósur og feiti, hvort heldur er með fiski, kjöti grænmeti, salati eða pasta. Telja skal með sósur í pottréttum og í bökuðum fiskréttum. Einnig skal telja með sósur með hamborgurum, pítum, frönskum kartöflum og þess háttar.

| Fæðutegund | Aldrei | Í mánuði | | | Í viku | | | Á dag | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | skipti | | | skipti | | | skipti | | | |
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Smjör, smjörvi, kalt, brætt | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Létt og laggott, Klípa, Létt smjörlíki | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Annað smjörlíki | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Flot, tólg | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Brún sósa, sósa úr tómötum, grænmeti | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rjómasósa, ostasósa, sýrður rjómi | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Mjólkurjafningur, sósa úr súrmjólk, jógúrti | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hollandais-, bernaissósa | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kokteilsósa, remúlaði, hamborgarasósa | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Salatsósa í flösku, létt sósa, létt majónes | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |


*18. Sælgæti, snakk.* **Hversu oft** borðar þú þessar vörur?

| **Fæðutegund** | **Aldrei** | **Í mánuði** skipti | | | **Í viku** skipti | | | **Á dag** skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Súkkulaði, súkkulaðikex | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Brjóstsykur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Lakkrís (1-3 stk) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Lakkrís (poki) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hlaup, Ópal, Tópas | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Poppkorn | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kartöfluflögur, skrúfur, saltkex | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Salthnetur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Harðfiskur, hákarl | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Ídýfur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Ostur, ekki sem álegg | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

*19. Tekurðu vítamín, lýsi eða önnur fæðubótarefni?*

| **Fæðutegund** | **Aldrei** | **Í mánuði** skipti | | | **Í viku** skipti | | | **Á dag** skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Fjölvítamín **með** A- og D-vítamíni | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Fjölvítamín **án** A- og D-vítamíns | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Lýsi (þorska-, ufsa-) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Lýsispillur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| E-vítamíntöflur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| B-vítamíntöflur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Fólasíntöflur (fólín, fólínsýra) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| C-vítamíntöflur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Járntöflur, járnmixtúra | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kalktöflur | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Omega-3 hylki, hákarlalýsishylki | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

***20. Máltíðir.*** Merktu við **hversu oft** þú borðar þessar máltíðir eða millibita.
Drykkur án annarrar fæðu telst hvorki máltíð né millibiti.

| **Fæðutegund** | **Aldrei** | **Í mánuði** skipti | | **Í viku** skipti | | | **Á dag** skipti | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Aldrei | Sjaldnar en 1 | 1 | 2-3 | 1 | 2-3 | 4-6 | 1 | 2 | 3-4 | 5 eða oftar |
| Morgunverður | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Millibiti að morgni | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hádegisverður | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Síðdegiskaffi, síðdegishressing | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kvöldverður | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kvöldhressing | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Millibiti að nóttu | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

***21. Hversu oft eru þessar fitutegundir notaðar til steikingar þar sem þú borðar?***
(þegar matur er steiktur á annað borð)

| **Fæðutegund** | **Veit ekki** | **Sjaldan eða aldrei** | **Stundum** | **Oftast eða alltaf** |
|---|---|---|---|---|
| Steikingasmjörlíki | ☐ | ☐ | ☐ | ☐ |
| Soja-, sólblóma-, maís-, matarolía | ☐ | ☐ | ☐ | ☐ |
| Ólífuolía | ☐ | ☐ | ☐ | ☐ |
| Smjör | ☐ | ☐ | ☐ | ☐ |

## *22. Hvað færðu þér yfirleitt mikið af hverju fyrir sig?*

Skoðaðu ljósmyndirnar af mismunandi skammtastærðum á næstu blaðsíðum og merktu við í töflunni hér á eftir hvaða skammtur er líkastur því sem þú færð þér oftast af hverri fæðu eða sambærilegri fæðu. Mundu að taka með í reikninginn ef þú færð þér yfirleitt oftar en einu sinni á diskinn, þá þarftu að áætla samanlagt magn.

| **Fæðutegund** | | | | | |
|---|---|---|---|---|---|
| | Minna en A | A | B | C | Meira en C |
| Fiskur | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kjötréttur | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kartöflur | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hrísgrjón | ☐ | ☐ | ☐ | ☐ | ☐ |
| Soðið grænmeti | ☐ | ☐ | ☐ | ☐ | ☐ |
| Grænmetissalat | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hvernig er brauðið smurt | ☐ | ☐ | ☐ | ☐ | ☐ |

## *23. Hér er spurt um algengan sósuskammt í matskeiðum (msk) eða desilítrum (dl)*

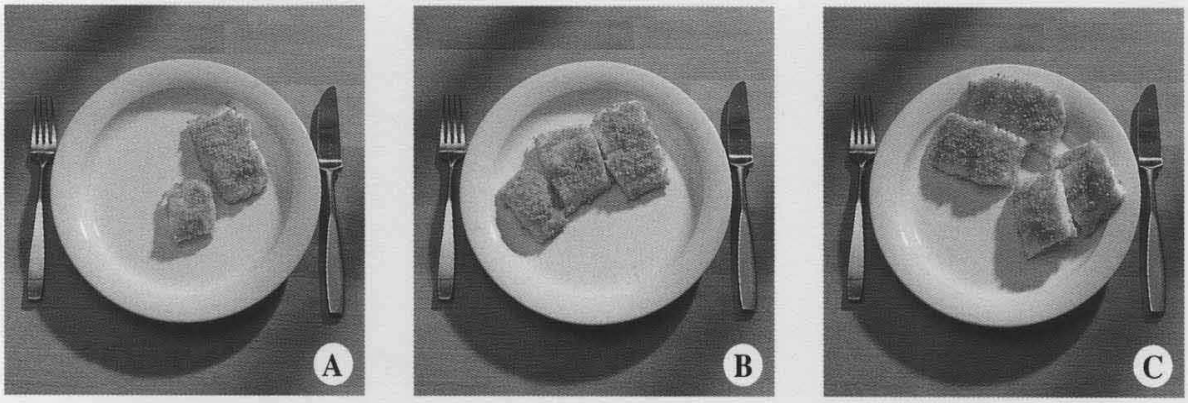
| **Fæðutegund** | | | | | |
|---|---|---|---|---|---|
| | Minna en 1 msk | 1 msk | 2-3 msk | ½-1 dl | Meira en 1 dl |
| Rjómasósa, ostasósa | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kokteilsósa, salatsósa | ☐ | ☐ | ☐ | ☐ | ☐ |
| Feiti | ☐ | ☐ | ☐ | ☐ | ☐ |

***Bestu þakkir fyrir þátttökuna!***

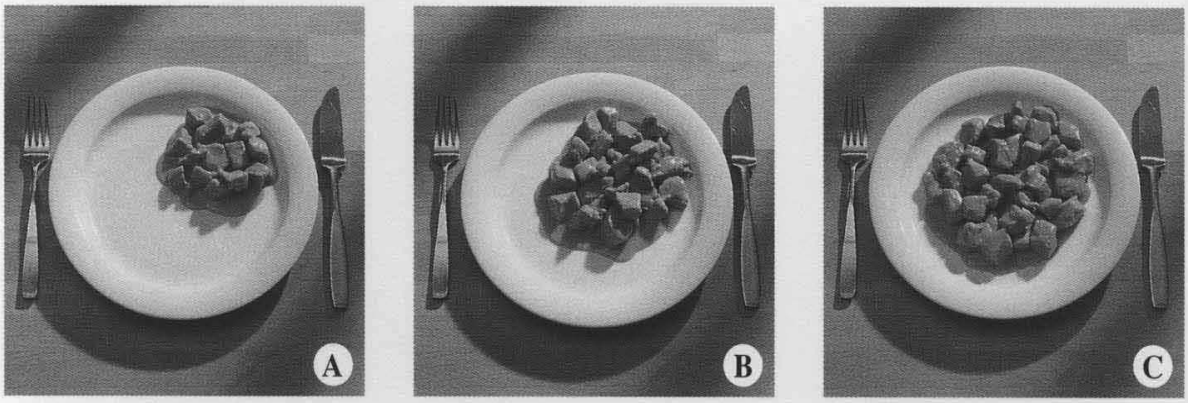
# *Veldu þá skammtastærð sem mest líkist því sem þú færð þér oftast*

***Færðu svörin inn í spurningu 22 hér að framan***

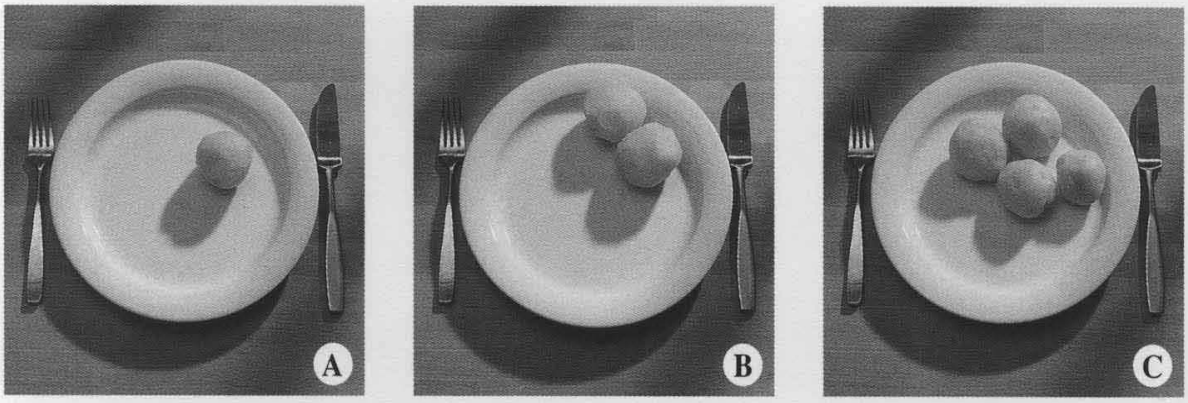
***Fiskur***



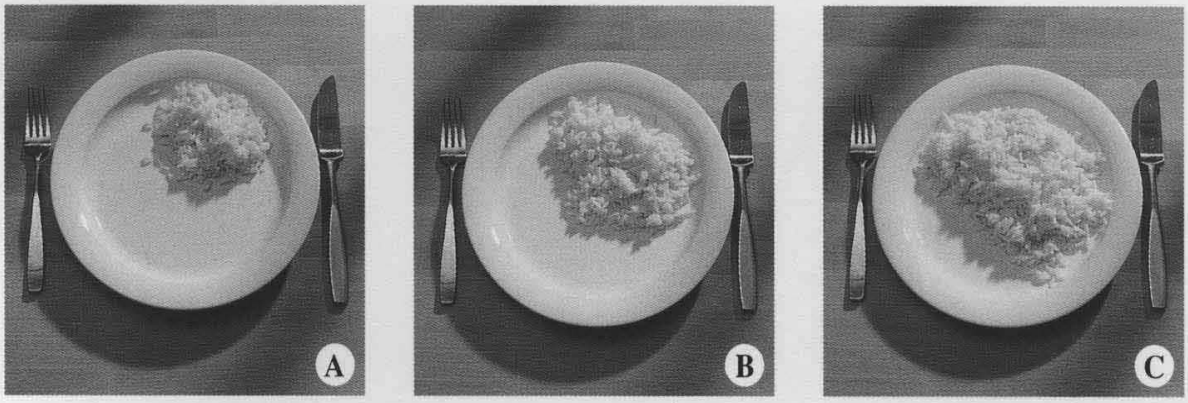

***Kjötréttur***



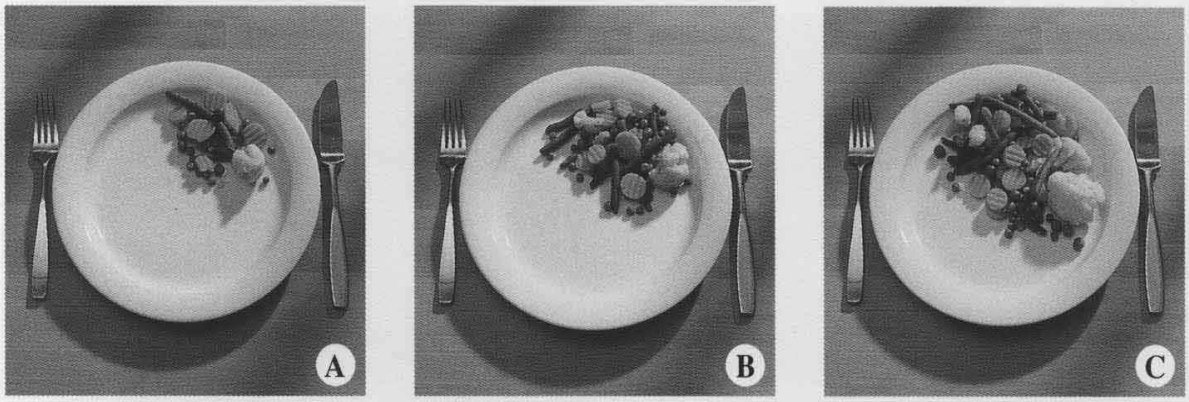

***Kartöflur***



*Hrísgrjón*

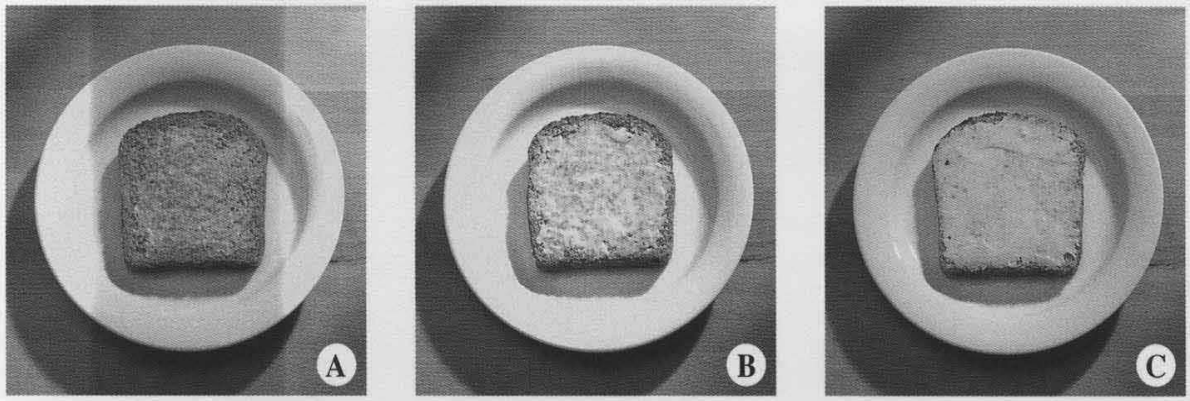



*Soðið grænmeti*




*Grænmetissalat*


*Hvernig er brauðið smurt*



# Appendix II

**Nr.....**

# Rannsókn á verðandi mæðrum
# Spurningaeyðublað I (vika 11-15)

**Vinsamlegast settu hring um viðeigandi tölustaf:**

**101. Ert þú: (ath. að það má merkja við fleiri en einn tölustaf)**

1. atvinnulaus
2. í veikindafríi eða öryrki
3. heimavinnandi
4. í námi
5. í hlutastarfi utan heimilis, starfsheiti.....
6. í fullu starfi utan heimilis, starfsheiti.....

**102. Hversu mörg ár hefur þú verið í skóla í fullu námi?**

1. 10 ár eða minna (grunnskóli)
2. 11-14 ár (framhaldsskóli)
3. 15-17 ár
4. 18 ár eða meira

**103. Býrð þú...**

1. ein (eða ein með börnum)
2. með maka eða sambýlismanni (með eða án barna)
3. hjá foreldrum (með eða án barna)
4. með öðrum en foreldrum eða sambýlismanni

**104. Hefur þú fætt barn?**

1. nei
2. já, ein fæðing
3. já, tvær fæðingar
4. já, þrjár fæðingar
5. já, fjórar fæðingar
6. já, fimm fæðingar
7. já, sex fæðingar eða fleiri

**105. Hefur þú greinst með einhver af neðangreindum einkennum á meðgöngu:**

1. hef ekki verið barnshafandi
2. skert sykurþol eða sykursýki á meðgöngu
3. meðgöngueitrun eða hækkaðan blóðþrýsting á meðgöngu
4. ófullnægjandi þyngdaraukningu á meðgöngu
5. ekkert ofangreint

**106. Notar þú einhver lyf að staðaldri, getnaðarvarnarpillan þar meðtalin?**

1. nei
2. já, nefnið tegundir .....
   .....

**107. Tekur þú lýsi, vítamín, járn eða önnur steinefni?**

1. nei
2. já (nefnið tegundir eða heiti) .....
.....

**108. Tekur þú einhverjar næringarblöndur, t.d. Herbalife, Nature's own, Nupolet eða þess háttar?**

1. nei
2. já, einu sinni í viku eða sjaldnar, nefnið tegund.....
3. já, tvisvar til sex sinnum í viku, nefnið tegund.....
4. já, um það bil einu sinni á dag, nefnið tegund.....
5. já, tvisvar á dag eða oftar, nefnið tegund.....

**109. Tekur þú einhver önnur fæðubótarefni eða hollustuefni, t.d. Q10, ginseng eða annað þess háttar einu sinni í viku eða oftar?**

1. nei
2. já (nefnið tegundir eða heiti) .....
.....

**110. Hefur þú fundið fyrir meðgönguógleði á þessari meðgöngu?**

1. nei
2. já, en ég kasta aldrei upp vegna ógleðinnar
3. já, og ég kasta stundum upp vegna ógleðinnar
4. já, og ég kasta upp daglega vegna ógleðinnar

**111. Hefur þú fundið fyrir óvenju sterkri löngun í ákveðnar fæðutegundir, sætindi eða drykki eftir að þú varðst barnshafandi?**

1. nei
2. já (nefnið það sem þið hafið oftast eða mesta löngun í ).....

**112. Hefur mataræðið breyst eftir að þú varðst barnshafandi? (má merkja við fleiri en eitt atriði)**

1. nei
2. já, ég borða meira
3. já, ég borða minna
4. ég reyni að velja hollari mat
5. ég borða meira af sætindum
6. ég borða meira af grænmeti og ávöxtum
7. ég drekk meiri mjólk
8. ég hef óbeit á mat sem mér þótti áður góður

**113. Hefurðu stundað líkamsrækt, t.d. leikfimi, sund eða aðra íþrótt, síðastliðinn mánuð?**

1. nei
2. já, en sjaldnar en einu sinni í viku
3. já, einu sinni í viku
4. já, tvisvar í viku
5. já, þrisvar í viku
6. já, fjórum sinnum í viku eða oftar

**114. Hverjar eru reykingavenjur þínar?**

1. ég hef aldrei reykt að staðaldri
2. ég hætti að reykja fyrir meira en sex mánuðum síðan
3. ég hætti að reykja fyrir minna en sex mánuðum síðan
4. ég reyki núna, en þó ekki daglega
5. ég reyki núna, 1-5 sígarettur á dag
6. ég reyki núna, 6-10 sígarettur á dag
7. ég reyki núna, 11-20 sígarettur á dag
8. ég reyki núna, meira en 20 sígarettur á dag

**115. Hefurðu verið frædd um áhrif reykinga á fóstrið?**

1. nei
2. já, í skóla
3. já, af starfsfólki heilsugæslu/mæðraskoðunar
4. já, af öðrum en heilbrigðisstarfsfólki eða skóla

**116. Hefurðu endurskoðað áfengisneyslu þína eftir að þú varðst barnshafandi?**

1. nei
2. já

**117. Hefurðu verið frædd um áhrif áfengis á fóstrið?**

1. nei
2. já, í skóla
3. já, af starfsfólki heilsugæslu/mæðraskoðunar
4. já, af öðrum en heilbrigðisstarfsfólki eða skóla

**118. Hefurðu greinst með einhvern neðangreindra sjúkdóma? (Settu hring utan um viðeigandi tölustafi)**

1. sykursýki eða skert sykurþol
2. skjaldkirtilssjúkdóm
3. háþrýsting
4. flogaveiki
5. nýrnasjúkdóm
6. meltingar- eða lifrarsjúkdóm

**119. Ertu á sérstöku fæði vegna sjúkdóms eða sjúkdómsáhættu?**

1. nei
2. já

**120. Hvað ertu gömul? .....ára**

**Nr.....**

# Rannsókn á verðandi mæðrum
## Spurningaeyðublað II (vika 34-37)

**Vinsamlegast settu hring um viðeigandi tölustaf:**

**201. Hefurðu fundið fyrir meðgönguógleði á þessari meðgöngu?**

5. nei
6. já, í eina viku eða skemur
7. já, í tvær til sex vikur
8. já, í sjö til tólf vikur
9. já, í meira en þrjá mánuði

**202. Hefur mataræðið breyst eftir að þú varðst barnshafandi? (settu hring um viðeigandi tölustaf, ath að það má merkja við fleiri en einn tölustaf)**

9. nei
10. já, ég borða meira
11. já, ég borða minna
12. ég reyni að velja hollari mat
13. ég borða meira af sætindum
14. ég borða meira af grænmeti og ávöxtum
15. ég drekk meiri mjólk
16. ég hef óbeit á mat sem mér þótti áður góður

**203. Hefurðu stundað líkamsrækt, t.d. leikfimi, sund eða aðra íþrótt, síðastliðinn mánuð?**

7. nei
8. já, en sjaldnar en einu sinni í viku
9. já, einu sinni í viku
10. já, tvisvar í viku
11. já, þrisvar í viku
12. já, fjórum sinnum í viku eða oftar

**204. Reykirðu núna?**

9. nei
10. já, en ég reyki ekki daglega
11. já, ég reyki 5 sígarettur á dag eða minna
12. já, 6-10 sígarettur á dag
13. já, 11-20 sígarettur á dag
14. já, meira en 20 sígarettur á dag

**205. Tekurðu einhverjar næringarblöndur, t.d. Herbalife, Nature's own, Nupolet eða þess háttar?**

6. nei
7. já, einu sinni í viku eða sjaldnar, nefnið tegund.....
8. já, tvisvar til sex sinnum í viku, nefnið tegund.....
9. já, um það bil einu sinni á dag, nefnið tegund.....
10. já, tvisvar á dag eða oftar, nefnið tegund.....

**206. Tekurðu einhver önnur fæðubótarefni eða hollustuefni, t.d. Q10, ginseng eða annað þess háttar?**

3. nei
4. já, nefnið tegund.....

**207. Ertu á sérstöku fæði vegna sjúkdóms eða sjúkdómsáhættu?**

3. nei
4. já, hvaða fæði.....

**208. Notarðu einhver lyf að staðaldri?**

3. nei
4. já, hvaða lyf? .....
.....

**209. Hefurðu fundið fyrir bakverk eða grindarverk síðustu þrjá mánuði?**

3. nei
4. já, en ég hef ekki þurft að vera frá vinnu eða skóla vegna bakverkja
5. já, og ég hef þurft að vera frá vinnu eða skóla vegna bakverkja

**210. Hefurðu verið frædd um áhrif áfengis á fóstrið?**

5. nei
6. já, í skóla
7. já, af starfsfólki heilsugæslu/mæðraskoðunar
8. já, af öðrum en heilbrigðisstarfsfólki eða skóla

**211. Hvenær fékkstu fræðslu um áhrif áfengis á fóstrið?**

1. fékk aldrei slíka fræðslu
2. fékk fræðslu áður en ég varð barnshafandi
3. eftir að ég varð barnshafandi
4. bæði áður og eftir að ég varð barnshafandi

**Fyllist út af ljósmóður:**

212. Dagsetning.....(ár, mánuður, dagur)
213. Meðgöngulengd.....vikur
214. Þyngd.....kg
215. Blóðþrýstingur
efri mörk.....
neðri mörk.....
216. Hemoglobin.....

# Appendix III

**Nr:.....**

# Rannsókn á heilsu kvenna á barnseignaaldri
# Spurningaeyðublað

**Vinsamlegast settu hring um viðeigandi tölustaf:**

**501. Ert þú: (ath. að það má merkja við fleiri en einn tölustaf)**

1. atvinnulaus
2. í veikindafríi eða öryrki
3. heimavinnandi
4. í námi
5. í hlutastarfi utan heimilis, starfsheiti.....
6. í fullu starfi utan heimilis, starfsheiti.....

**502. Hversu mörg ár hefur þú verið í skóla í fullu námi?**

1. 10 ár eða minna (grunnskóli)
2. 11-14 ár (framhaldsskóli)
3. 15-17 ár
4. 18 ár eða meira

**503. Býrð þú...**

1. ein (eða ein með börnum)
2. með maka eða sambýlismanni (með eða án barna)
3. hjá foreldrum (með eða án barna)
4. með öðrum en foreldrum eða sambýlismanni

**504. Hefur þú fætt barn?**

1. nei
2. já, ein fæðing
3. já, tvær fæðingar
4. já, þrjár fæðingar
5. já fjórar fæðingar
6. já fimm fæðingar
7. já sex fæðingar eða fleiri

**505. Hefur þú greinst með einhver af neðangreindum einkennum á meðgöngu:**

1. hef ekki verið barnshafandi
2. skert sykurþol eða sykursýki á meðgöngu
3. meðgöngueitrun eða hækkaðan blóðþrýsting á meðgöngu
4. ófullnægjandi þyngdaraukningu á meðgöngu
5. ekkert ofangreint

**506. Notar þú einhver lyf að staðaldri, getnaðarvarnarpillan þar meðtalin?**

1. nei
2. já, nefnið tegundir .....
   .....

**507. Tekur þú lýsi, vítamín, járn eða önnur steinefni?**

1. nei
2. já (nefnið tegundir eða heiti).....
   .....

**508. Tekur þú einhverjar næringarblöndur, t.d. Herbalife, Nature´s own, Nupolet eða þess háttar?**

1. nei
2. já, einu sinni í viku eða sjaldnar, nefnið tegund.....
3. já, tvisvar til sex sinnum í viku, nefnið tegund.....
4. já, um það bil einu sinni á dag, nefnið tegund.....
5. já, tvisvar á dag eða oftar, nefnið tegund.....

**509. Tekur þú einhver önnur fæðubótarefni eða hollustuefni, t.d. Q10, ginseng eða annað þess háttar einu sinni í viku eða oftar?**

1. nei
2. já (nefnið tegundir eða heiti).....
   .....

**510. Hefurðu stundað líkamsrækt, t.d. leikfimi, sund eða aðra íþrótt, síðastliðinn mánuð?**

1. nei
2. já, en sjaldnar en einu sinni í viku
3. já, einu sinni í viku
4. já, tvisvar í viku
5. já, þrisvar í viku
6. já, fjórum sinnum í viku eða oftar

**511. Reykirðu núna?**

1. nei
2. já, en ég reyki ekki daglega
3. já, ég reyki 5 sígarettur á dag eða minna
4. já, 6-10 sígarettur á dag
5. já, 11-20 sígarettur á dag
6. já, meira en 20 sígarettur á dag

**512. Hefurðu greinst með einhvern neðangreindra sjúkdóma? (Settu hring um viðeigandi tölustafi)**

1. sykursýki eða skert sykurþol
2. skjaldkirtilssjúkdóm
3. háþrýsting
4. flogaveiki
5. nýrnasjúkdóm
6. meltingar- eða lifrarsjúkdóm

**513. Ertu á sérstöku fæði vegna sjúkdóms eða sjúkdómsáhættu?**

1. nei
2. já

**514. Hvað ertu gömul?.....ára**

**515. Hver er hæð þín í cm?.....cm**

**516. Hver er þyng þín í kg?.....kg**

# Appendix IV

**Nr þátttakanda**: _____

**Úr fæðingarskýrslum**

**301** Dagsetning fæðingar_____(dag,mán,ár)

**301b** kyn barns □ strákur □ stelpa

**302** meðgöngulengd _____dagar **eða** _____vikur og dagar

**303** þyngd móður í síðustu skoðun _____kg

**303b** meðgögnulengd þegar síðasta vigtun fór fram _____dagar (eða vikur og dagar)

**aukaspurn:**

- hæð móður:_____cm
- venjuleg þyngd móður: _____kg
- var þyngd í fyrstu skoðun frábrugðin venjulegri þyngd? _____kg _____vika

**304** Sjúkdómar á meðgöngu □nei$_1$ □já$_2$

**305** Ef já einhver neðantalinna

□ 1: háþrýstingur
□ 2: meðgöngueitrun
□ 3: sykursýki
□ 4: annað, hvað: _____

- síðasta blóðþrýstingsmæling: _____ (systóla/díastóla)
- síðasta hemóglóbínmæling: _____

**306** Fæðingarþyngd barns _____g

**307** Lengd barns _____cm

**308** Ummál höfuðs _____cm

**309** Afdrif barns

□ 1: heilbrigt

□ 2: öndunarörðugleikar

□ 3: hjartagalli

□ 4: annað, hvað:_____

**310** Þyngd fylgju _____g

**Athugasemdir:** (t.d. ef hár blóðþrýstingur eða lágt hb án þess að sjúkdómsgreining, ef mikill bjúgur, sykur eða
albúmen í þvagi)