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High-throughput fatty acid analyses in plasma and serum glycerophospholipids and in plasma total lipids: Method development, validation, and application in clinical trials and epidemiological studies

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1. Introduction

The dietary intake of polyunsaturated fatty acids (PUFA) has a significant effect on blood and tissue PUFA content (1, 2). Tissue availability of PUFA, which depends on both diet and metabolic turnover, has a major impact on human health (3-5). Adequate fatty acid (FA) intake is very important in every stage of life, but particularly for the fetus and neonate to enable optimal visual and cognitive development (6, 7). Biomarkers of FA status are widely used in observational studies, as they reflect a combination of dietary intake and metabolism. Associations of FA status with current and future health indicators have been demonstrated (8, 9). Epidemiological and clinical studies have revealed associations between FA and cardiovascular diseases, diabetes, and certain types of cancer (10-12).

Analysis of FA composition in different blood and lipid fractions seems to be a valuable biomarker to assess the FA status in humans (8-10). Depending on the scientific question the FA composition can be determined in adipose tissue, erythrocytes, plasma, platelets, whole blood, and specific cells or tissues. Adipose tissue, as the main storage compartment for FA in humans, is considered a good long term biomarker for FA intake, because of the slow turnover time (8, 13). However, in large clinical trials and epidemiological studies, blood collection is easier and less invasive than tissue collection.

The most convenient way to asses FA composition is whole blood analysis, because separation of plasma and lipid fractions is not required and dried blood spots have been shown as suitable for analysis (14, 15). This simplifies study logistics considerably as the

need of immediate sample freezing is avoided. However, the procedure is not yet well established (14, 16, 17) and data interpretation seems more difficult, because different influencing factors have to be considered. A crucial influencing factor is hematocrit, the proportion of blood volume that is occupied by red blood cells. The hematocrit depends on gender (18) and age, e.g. hematocrit is higher in neonates than adults and decreases during the first months of life (19). The hematocrit may be altered by factors such as hypertension (18), pulmonary and cardiac diseases, and pregnancy (20). Variation of the hematocrit may lead to misinterpretation of whole blood FA data because the FA composition of plasma and red blood cells, the main components of whole blood, differs significantly (21).

Very specific information on FA status can be obtained by analyzing the FA composition in an individual lipid fraction of serum or plasma, which minimizes the influence of factors such as postprandial state and individual lipid metabolism. Conventional methods consist of several analytical steps and are therefore cumbersome and time-consuming. Typically a skilled chemist requires 2 days for analyzing 10-20 samples (22). For studies focusing on long chain polyunsaturated fatty acids (LC-PUFA) it seems most promising to analyze plasma phospholipids (PL), because they contain higher percentages of LC-PUFA and are less sensitive to short term variation than plasma non esterified fatty acids (NEFA), triacylglycerides (TAG) and cholestryl esters (CE) (23, 24). For the determination of PL FA in biological samples Bondia-Pons et al. (25) presented an optimized method for lipid extraction and lipid separation using solid phase extraction instead of thin layer chromatography. Their sample preparation technique is easier than those of established methods, but it is still very time-consuming.

1. INTRODUCTION

The term phospholipid is used for all lipids with a phosphorus-containing polar head group. PL can be further subdivided in glycerophospholipids (GP) and phosphospingolipids (SM), based on their glycerole or sphingoid base backbone, respectively. The major PL fractions in plasma are glycerophosphocholines and SM (26, 27). While LC-PUFA percentages are high in glycerophosphocholines and other GP, LC-PUFA contribute less than 5 % to the FA esterified to sphingosine in SM (26, 28). The contribution of SM to total plasma PL varies widely in healthy controls and in patients suffering from coronary artery disease (27). Thus, a further source of variation in LC-PUFA percentages can be eliminated by determining selectively the FA composition of GP, while excluding SM from FA analysis. One objective of this dissertation was to develop a high-throughput method for the analysis of GP FA composition in plasma and serum.

Although analysis of FA composition in defined lipid fraction is considered optimal, analysis of total plasma lipids can be a suitable alternative, if fasted blood samples are available and precise quantification of intervention effects is not needed. The plasma total FA pool represents a mixture of all plasma lipid fractions that contain FA moieties, in particular CE, NEFA, PL, and TAG. The FA composition is typical for each lipid class, and different FA compete for the incorporation in individual plasma lipid classes. Thus, analysis of the total FA pool offers the opportunity to determine overall changes in plasma FA status. It has to be considered that variation in the contribution of lipid fractions modify total plasma FA composition even if FA composition within the fraction does not change. Nevertheless, if methods are robust, total lipid FA analysis can provide valuable information in a cost effective way.

1. INTRODUCTION

Lepage and Roy (29) developed the first direct transesterification method to assess total FA composition in plasma. They performed transesterification with acetyl chloride in methanol-benzol (4:1) at 100 °C for 1h, followed by neutralization and centrifugation. Masood, Stark and Salem (30) presented in 2005 a simplified version of the original method of Lepage and Roy. In 2008 Masood and Salem (22) published a modified version of this method, which enables a half automated sample preparation, but the reaction time is very long and large amounts of solvents are used.

A further objective of this dissertation was to simplify the assessment of total lipid FA composition in plasma with as few sample preparation steps as possible to enable its application in large studies. Furthermore, to confine consumable, reagent and solvent requirements to a minimum and perform all preparation steps in one vial.

Clinical and epidemiological studies showed that an adequate availability of PUFA is necessary for normal growth as well as normal visual, cognitive and immune functions in infants and children (31-33). Monitoring of FA status may be indicated in children with modified dietary FA intake (e.g. in some patients with parenteral nutrition), or with disease related alterations of absorption or metabolism (e.g. in patients with short bowel syndrome, inflammatory bowel disease, metabolic liver disease), and or with interventions such as fish oil supplementation in children with phenylketonuria or other inborn errors of amino acid metabolism to monitor the intervention effects (6, 33-36). Monitoring of FA status in individual patients or in populations depends on the availability of reference values obtained in healthy children to allow interpretations of the results obtained. Thus, the availability of reference values is a prerequisite for interpreting FA status of individuals. The newly developed high-throughput method was used to analyze the GP FA composition in 1326 serum samples

obtained from 951 children aged 2 and 6 years who participated in a birth cohort study. The data were used to establish reference values for GP FA composition in children.

The concentrations of individual FA in plasma and tissues do not evolve independently, but rather mutually influence each other as they compete for incorporation into lipids. Consequently the percentage FA composition of specific compartments is widely used for the description of FA status. Although analyses of FA status are successfully applied for the evaluation of dietary intake (13), FA status is influenced by both diet and endogenous metabolism. In several studies strong associations were found between variants in the human genes *fatty acid desaturase 1 (FADS1)* and *fatty acid desaturase 2 (FADS2)* and blood levels of PUFA (37-39). The importance of endogenous metabolism is even greater for LC-PUFA than for the saturated and monounsaturated FA which can be synthesized de novo by human metabolism (40).

Another important aspect, when evaluating a biomarker, is temporal variability, which depends on the turnover of the compartment. Thus, the composition of plasma lipids varies within days or weeks, whereas significant changes of FA composition in adipose tissue are only observed after months (13). As plasma lipids can change within short periods, an investigation of the tracking of FA status over years yields information on longer term changes of dietary habits and life style factors. In adults it has been shown that FA composition of plasma CE and PL showed a high tracking with coefficients of correlation between FA percentages up to 0.83 (41, 42). Similar values were observed in total plasma PL of a small group of Portuguese children (n = 26), but this observation might depend very much on the given cultural and socioeconomic situation of that sample (43). The aim of this work was to

reevaluate these finding of Guerra et al. in a population-based sample of children considering additionally the potential influence of different *FADS* gene variants.

This work is intended to provide new sophisticated methods for sensitive high-throughput FA analysis in human blood plasma and serum. The goal is to develop markedly simplified approaches to preanalytical sample preparation, which currently is a very time consuming and hence expensive factor, limiting the number of subjects that can be studied for clinical and research questions. The newly developed methodological approaches should allow for fast and cost effective yet sensitive and precise measurements from small sample volumes, which should allow the application in large clinical studies even in infants and young children. The objectives of this work are as follows:

- Development of a method for high-throughput analysis of GP FA composition in plasma.
- Validation of the suitability to analyze GP FA composition in serum with the new method.
- Determination of GP FA compositions in children to provide reference values.
- Investigation of the tracking of serum GP FA concentrations and percentage composition in children.
- Evaluation of the influence of *FADS* polymorphisms on tracking of FA in children.
- Development of a method for high-throughput analysis of plasma total lipid FA composition.

1. INTRODUCTION

This dissertation is based on the following published or submitted articles:

1. **Review:**

   Glaser C, Heinrich J, Koletzko B. Role of *FADS1* and *FADS2* polymorphisms in polyunsaturated fatty acid metabolism. Metabolism 2010, 59:993-9.

2. **Research article:**

   Glaser C, Demmelmair H, Koletzko B. High-throughput analysis of fatty acid composition of plasma glycerophospholipids. J Lipid Res 2010, 51:216-21.

3. **Research article:**

   Glaser C, Demmelmair H, Sausenthaler S, Herbarth O, Heinrich J, Koletzko B. Fatty acid composition of serum glycerophospholipids in children. J Pediatr 2010, 157:826-31.

4. **Research article:**

   Glaser C, Demmelmair H, Rzehak P, Klopp N, Heinrich J, Koletzko B. Influence of *FADS* polymorphisms on tracking of serum glycerophospholipid fatty acid concentrations and percentage composition in children. submitted.

5. **Research article:**

   Glaser C, Demmelmair H, Koletzko B. High-throughput analysis of total plasma fatty acid composition with direct in situ transesterification. PLoS ONE 2010, 5(8):e12045.

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2. Publication 1: “Role of *FADS1 FADS 2* polymorphisms in polyunsaturated fatty acid metabolism”

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Role of *FADS1* and *FADS2* polymorphisms in polyunsaturated fatty acid metabolism

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Abstract

Tissue availability of polyunsaturated fatty acids (PUFAs) depends on dietary intake and metabolic turnover and has a major impact on human health. Strong associations between variants in the human genes fatty acid desaturase 1 (*FADS1*, encoding Δ-5 desaturase) and fatty acid desaturase 2 (*FADS2*, encoding Δ-6 desaturase) and blood levels of PUFAs and long-chain PUFAs (LC-PUFAs) have been reported. The most significant associations and the highest proportion of genetically explained variability (28%) were found for arachidonic acid (20:4n-6), the main precursor of eicosanoids. Subjects carrying the minor alleles of several single nucleotide polymorphisms had a lower prevalence of allergic rhinitis and atopic eczema. Therefore, blood levels of PUFAs and LC-PUFAs are influenced not only by diet, but to a large extent also by genetic variants common in a European population. These findings have been replicated in independent populations. Depending on genetic variants, requirements of dietary PUFA or LC-PUFA intakes to achieve comparable biological effects may differ. We recommend including analyses of *FADS1* and *FADS2* polymorphism in future cohort and intervention studies addressing biological effects of PUFAs and LC-PUFAs.
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1. Introduction

The polyunsaturated fatty acid (PUFA) status has a major impact on human health and has been shown to be associated with outcomes such as early visual, cognitive, and motor development [1,2]; mental health and psychiatric disorders [3]; cardiovascular disease mortality [4]; immunologic and inflammatory responses; as well as related diseases such as allergies [5,6]. These and other biological effects of PUFAs are suggested to be mediated to a large extent by the availability of the n-6 long-chain PUFA (LC-PUFA) arachidonic acid (AA, 20:4n-6) and the n-3 LC-PUFAs eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3).

Long-chain PUFAs are precursors of eicosanoids and similar mediators (eg, docosanoids) and hence can influence inflammatory processes (Fig. 1) [7-9]. The key link between PUFAs and inflammatory processes are the eicosanoids, which are derived from 20-carbon PUFAs. The main precursor of eicosanoids is AA. Arachidonic acid–derived eicosanoids have important roles in sensitization to allergens and in allergic inflammation. It has been hypothesized that there is a link between high dietary intake of n-6 PUFAs and atopic disease [9]. The n-3 LC-PUFAs inhibit AA incorporation into cell membranes and AA metabolism to eicosanoids. The n-3 LC-PUFA EPA acts as a substrate for the generation of alternative eicosanoids. Thus, it is hypothesized that inflammatory diseases (eg, atopy and obesity) are associated with a higher ratio of n-6 PUFAs to n-3 PUFAs [10]. Individual eicosanoids have different biological effects and can act in different ways depending upon their specific action. In general, AA-derived eicosanoids have mainly proinflammatory effects, whereas EPA-derived eicosanoids are rather less inflammatory. Furthermore, recent studies have identified a novel group of mediators termed *E*- and *D-series resolvins* formed from

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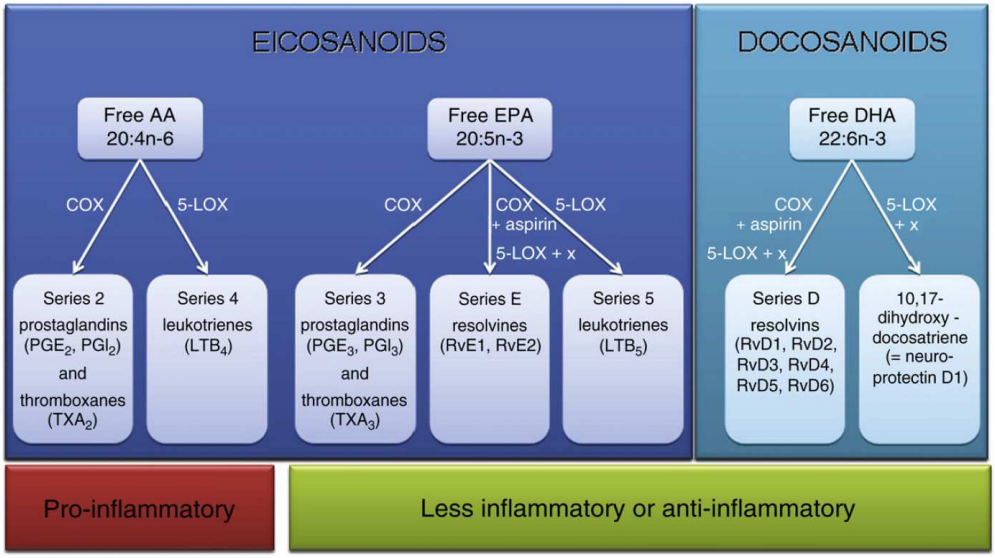

Fig. 1. Outline of eicosanoid and docosanoid pathways with free AA, EPA, and DHA, released from cell membranes by phospholipase A_2, as substrates for cyclooxygenase (COX), 5-lipoxygenase (5-LOX), and other reactions (x). Free AA is a substrate for potent inflammatory eicosanoids such as prostaglandin (PG) E_2, thromboxane (TX) A_2, and leukotriene (LT) B_4. Free EPA may compete with AA as a substrate and lead to less potent inflammatory eicosanoids such as PGE_3, TXA_3, and LTB_5. Anti-inflammatory E-series resolvins (RvE) and D-series resolvins (RvD) are formed by a series of reactions involving COX (acting in the presence of aspirin) and 5-LOX from EPA and DHA, respectively. A further potent anti-inflammatory mediator, termed *neuroprotectin D1*, is synthesized from DHA by several steps including 5-LOX.

EPA and DHA, respectively. Together with neuroprotectin D1, a mediator formed from DHA via several reactions, these mediators appear to exert strong inflammation resolving effects [9,10].

Long-chain PUFAs are provided by the diet, but can also be synthesized in human metabolism from the precursor essential fatty acids, linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), by the action of desaturases and elongases (Fig. 2). The Δ-5 and Δ-6 desaturases are considered the rate-limiting enzymes in the formation of LC-PUFAs [11-13]. It is hypothesized that both desaturases play a key role in inflammatory diseases. This is strengthened by functional studies in mice, where selective Δ-5 and Δ-6 desaturase inhibitors showed marked anti-inflammatory effects [14,15].

Regulatory mechanisms of Δ-5 and Δ-6 desaturases have been scarcely examined in human tissue. The human desaturase complementary DNAs were first cloned in 1999 [16,17]. In 2000, they were identified as *FADS1* and *FADS2* in the human genome [18]. Both genes, *FADS1* and *FADS2*, are oriented head-to-head and localized in a cluster on chromosome 11 (11q12-13.1). Linkage was previously reported between or nearby the human chromosomal region 11q12-13.1 and complex diseases such as asthma [19], atopy [20,21], bipolar disorders [22], osteoarthritis [23], and type 1 diabetes mellitus [24].

2. PUFA metabolism and dietary intake

Fatty acids are aliphatic compounds comprising a carboxyl group and a hydrocarbon chain of varying length and degree of saturation. Natural fatty acids commonly have straight chains of an even number of 4 to 28 carbon atoms. Saturated fatty acids have no double bonds in the acyl chain, whereas unsaturated fatty acids contain at least one double bond. Fatty acids containing 2 or more double bonds are referred to as *PUFAs*. Polyunsaturated fatty acids are classified in 2 principal families, the n-6 (or ω-6) and the n-3 (or ω-3) families, according to the position of the terminal double bond. The parent fatty acids of these families, LA and ALA, cannot be synthesized in mammals; they must be provided by the diet and are therefore defined as essential fatty acids.

Linoleic acid and ALA serve as substrates for other important fatty acids (Fig. 2). By insertion of additional double bonds into the acyl chain and by elongation of the acyl chain, LC-PUFAs are synthesized endogenously from LA and ALA. Both fatty acids have analogous reaction pathways catalyzed by the same enzymes. Therefore, a competition exists between both fatty acid families for metabolism. Linoleic acid and ALA can be converted by Δ-6 desaturation to γ-linolenic acid (GLA, 18:3n-6) and stearidonic acid (SA, 18:4n-3), respectively. This step is

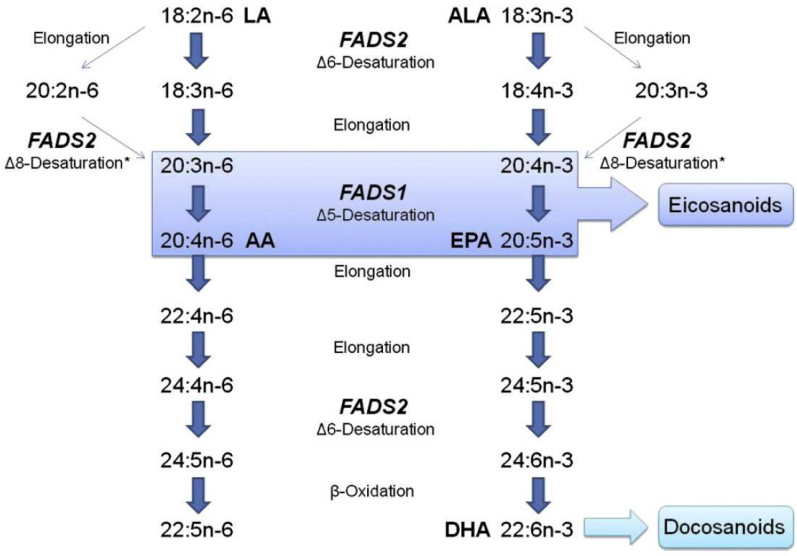

Fig. 2. Pathways for LC-PUFA synthesis from n-6 (left) and n-3 (right) essential fatty acids by enzymatic desaturation and chain elongation (*alternative pathways via Δ-8 desaturase demonstrated in mammals by Park et al 2009 [25]).

rate limiting and is followed by elongation of GLA to dihomo-γ-linolenic acid (DGLA, 20:3n-6) and of SA to eicosatetraenoic acid (ETA, 20:4n-3). In addition to these common pathways, Park et al [25] reported an alternative pathway via elongation of LA and ALA to n-6 eicosadienoic acid (EDA, 20:2n-6) and n-3 eicosatrienoic acid (20:3n-3), followed by a Δ-8 desaturation of these PUFAs to DGLA and ETA, respectively. Both PUFAs can be further elongated, leading to the production of AA and EPA.

A further important LC-PUFA is DHA, the end-product of the n-3 family. The conversion of ALA to DHA requires several elongation and desaturation steps, all taking place in the endoplasmatic reticulum. However, the last step requires a compartmental translocation to peroxisomes, the unique place for β-oxidation of LC-PUFAs [26,27]. This restriction may explain why the conversion rate of docosapentaenoic acid (DPA, 22:5n-3), the elongation product of EPA, to DHA is low, as shown in humans using ^{13}C-labeled precursors [28].

In Western diets, PUFAs comprise up to 20% of dietary fat. In most cases, LA and ALA contribute more than 95% of dietary PUFA intake [7,9]. Linoleic acid is the primary PUFA, found in significant quantities in many vegetable oils (eg, corn, safflower, soybean, and sunflower oil) and in products made from such oils (eg, margarines). α-Linolenic acid is found in green plant tissues, in some common oils (eg, flaxseed, rapeseed, and soybean oil) and in nuts [7,9]. Over the last 40 years, LA intake increased markedly in Western countries because of an increased popularity of cooking oils and margarines in these countries. Although ALA intake changed rather slightly over this time, consumption of both PUFAs exceeds minimal requirements needed to prevent essential fatty acid deficiency. The increased LA intake has changed the ratio of n-6 to n-3 PUFAs in the diet. This ratio increased markedly and is today estimated at 5 to 20 in Western countries [7,9].

In contrast to consumption of LA and ALA, dietary intake of LC-PUFAs is markedly lower. Arachidonic acid is typically provided by meat, eggs, and offal. Intakes of AA are typically in the range of 50 to 500 mg/d [7,9]. Eicosapentaenoic acid and DHA are found in marine foods, especially in fatty sea fish (eg, herring, mackerel, salmon, and tuna). One oily sea fish meal can provide 1.5 to 3.5 g of n-3 LC-PUFAs. In the absence of oily sea fish consumption, intakes of n-3 LC-PUFAs are very low, at approximately less than 100 mg/d [7,9].

3. Indications for interindividual variations in PUFA metabolism

Several studies indicate interindividual variations in the capacity for endogenous formation of LC-PUFAs. In 1988, Koletzko et al [29] found a significant correlation between n-6 and n-3 LC-PUFA contents in mature breast milk of 15 German women. Because n-6 and n-3 LC-PUFAs originate from different dietary sources, it was assumed that some women had a higher ability to synthesize and secret milk n-6 and n-3 LC-PUFAs than others.

Innis et al [30] observed a positive correlation of DHA and n-6 DPA in erythrocyte phosphatidylethanolamine of 84 Canadian preschool children. This was in contrast to results

obtained in animals, where DHA and n-6 DPA were inversely correlated. The authors concluded that the positive relationship obtained in children may reflect different activity levels of desaturases between individuals affecting the conversion of n-6 and n-3 fatty acids.

Guerra et al [31] studied the tracking of different plasma phospholipid fatty acids over 3 years of 28 children at 24, 36, and 60 months of age. They found a marked change of dietary fatty acid intake over time; and intakes of saturated fatty acid, monounsaturated fatty acid, and PUFAs showed no correlation for the 3 time points. In contrast, significant correlations were obtained for n-6 LC-PUFAs, n-6/n-3 LC-PUFA ratio, AA/LA ratio, and DHA/ALA ratio over time. This significant tracking points to an influence of individual endogenous fatty acid metabolism on plasma concentrations of LC-PUFAs. Similar findings were observed by Moilanen et al [32,33] in serum cholesteryl ester fatty acid compositions of Finnish youth.

4. Evaluation of the effects of *FADS1* and *FADS2* polymorphisms on LC-PUFA status in humans

Some 7 years after the human desaturases were first cloned in 1999 [16,17], Schaeffer et al [34] performed an analysis of 18 single nucleotide polymorphisms (SNPs) of the *FADS1 FADS2* gene cluster in 727 white subjects participating in the European Community Respiratory Health Survey I. To explore genetic determinants of PUFA metabolism, the fatty acid composition in serum phospholipids of these 727 participants was analyzed.

Association analysis of SNPs with fatty acids showed that the *FADS1 FADS2* cluster and the n-6 and n-3 fatty acids were highly associated except for n-6 DPA and DHA. Carriers of the minor alleles of 11 SNPs (rs174544, rs174553, rs174556, rs174561, rs3834458, rs968567, rs99780, rs174570, rs2072114, rs174583, and rs174589) showed enhanced levels of the n-6 fatty acids LA, EDA, and DGLA and of the n-3 fatty acid ALA, and decreased levels of the n-6 fatty acids GLA, AA, and adrenic acid (22:4n-6) and of the n-3 fatty acids EPA and DPA [34].

Analysis of reconstructed haplotypes indicated highly significant associations between the haplotypes and the fatty acid levels, which remained also significant after correction for multiple testing. Virtually all haplotypes carrying minor alleles were associated with increased levels of LA, EDA, DGLA, and ALA and with decreased levels of GLA, AA, adrenic acid, EPA, and n-3 DPA. These findings were in line with the findings of the SNP analysis. For n-6 DPA and n-3 DHA, no significant associations were achieved, probably because the effect of desaturation activity on their serum concentration was diminished by the indirect synthesis via peroxisomal β-oxidation. The most significant associations and the highest proportion of genetically explained variability (28%) were found for AA, the main precursor of eicosanoids [34].

Subjects carrying the minor alleles of several SNPs had a lower prevalence of allergic rhinitis and atopic eczema, whereas no association were found for genotypes or haplotypes with total or specific immunoglobulin E levels [34].

Further evidence for the role of *FADS* polymorphisms was provided by Rzehak et al [35], who studied a subgroup of Bavarian adults participating in the Bavarian Nutrition Survey II (163 subjects for plasma and 535 subjects for erythrocyte fatty acids). They confirmed the associations between *FADS1 FADS2* haplotypes and serum phospholipid PUFA levels. In addition, associations of haplotypes with PUFAs in erythrocyte membranes were established, particularly for the n-6 fatty acids AA and DGLA [35].

Malerba et al [36] analyzed 13 SNPs located in the *FADS1 FADS2* gene cluster and reported associations between SNPs and serum phospholipid and erythrocyte total fatty acids in 658 Italian adults participating in the Verona Heart Project. Minor allele homozygotes and heterozygotes of the studied Italian adults were associated with higher levels of LA, EDA, and ALA and lower levels of AA. No significant association were observed for SA, EPA, and DHA [36].

Analogous results were reported by Xie and Innis [37] in a cohort of 69 pregnant women from Canada. They analyzed 4 SNPs in the *FADS1 FADS2* gene cluster and associated them with plasma phospholipid and erythrocyte membrane fatty acids compositions. They showed that carriers of the minor alleles of the 4 analyzed SNPs had higher LA and lower AA levels in plasma phospholipids and erythrocyte membranes. Furthermore, they showed that genetic variants of *FADS1* and *FADS2* influenced breast milk essential fatty acids in pregnancy and lactation [37].

A study that aimed at identifying a common *FADS2* gene promoter polymorphism as potential modulator of the effect of ALA on myocardial infarction was performed by Baylin et al [38]. They evaluated the effect of the polymorphism on adipose tissue PUFA concentrations in 1820 control subjects of their Costa Rican study population.

A common deletion in the *FADS2* promoter was found to be associated with GLA, AA, EPA, ETA, and EDA. In agreement with previous findings [34], carriers of the minor deletion allele showed enhanced desaturase substrate levels and decreased desaturase product levels. Furthermore, they analyzed plasma fatty acids in a subsample of 196 controls and found analogous associations as reported before [34].

5. Genomewide association studies identified *FADS* polymorphisms as genetic contributors to PUFA concentrations

Gieger et al [39] determined associations between the genotype of 284 men (55-79 years old) from Augsburg who participated in the KORA study (Cooperative Health Research in the Region of Augsburg, Southern Germany)

with 363 metabolites measured in serum samples of these participants. They found strong associations between the SNP rs174548, located on the *FADS1* gene, and a number of plasma glycerophospholipid concentrations. This SNP explained up to 10% of the observed variance of certain glycerophospholipid species in plasma. Carriers of the minor allele of rs174548 were shown to have the lowest levels of glycerophospholipid species containing PUFAs with 4 and more double bonds. Arachidonic acid was found to be significantly reduced with increasing copy number of the minor allele. Concentrations of glycerophospholipid species containing PUFAs with 3 or less double bonds showed a positive association with the *FASD1* genotype [39].

In the InCHIANTI study (Invecchiare in Chianti, aging in the Chianti area, Tuscany, Italy), 1075 Italian adults were genotyped [40]. The strongest evidence for association with plasma PUFA concentrations was observed in a region of chromosome 11. In the analysis of AA, the SNP with the most significant association was rs174537 near *FADS1*. Homozygotes carrying only the minor alleles had lower AA levels compared with the major allele homozygotes. The SNP rs174537 was found to account for 18.6% of the additive variance in AA concentrations. These effects were further confirmed in an independent sample of 1076 subjects participating in the GOLDN study (Genetics of Lipid Lowering Drugs and Diet Network, white men and women from the United States) in erythrocyte total fatty acids [40].

6. *FADS2* polymorphism and breastfeeding effects on cognitive development

Caspi et al [41] studied the association between breastfeeding and later intelligence quotient (IQ) development in 2 independent birth cohorts: the Dunedin Multidisciplinary Health and Development Study (1037 children from Dunedin, New Zealand) and the Environmental Risk Longitudinal Twin Study (2232 children from England and Wales). They found that the effect of breastfeeding had a significant effect on cognitive development in both cohorts, whereas genetic polymorphism in the *FADS2* gene (rs174575) had no significant effect in the 2 total study populations. Further analyses revealed that rs174575 polymorphisms interacted with breastfeeding in predicting the IQ in both cohorts (Fig. 3). In both cohorts, breastfed children carrying the C allele had a marked IQ advantage over children not breastfed, whereas breastfeeding had no influence on the IQ in GG homozygotes. Caspi et al [41] were able to rule out potential confounding of the gene-environment interaction due to gene-exposure correlation, intrauterine growth differences, social class differences, and maternal cognitive ability. These observations raise the hypothesis that breastfeeding might have beneficial effects on later cognitive achievements due to its supply of LC-PUFAs, which were not contained in conventional infant formulas in the past, in subpopulations of infants with genetically determined metabolic conversion activity of LC-PUFA synthesis. This question deserves further exploration.

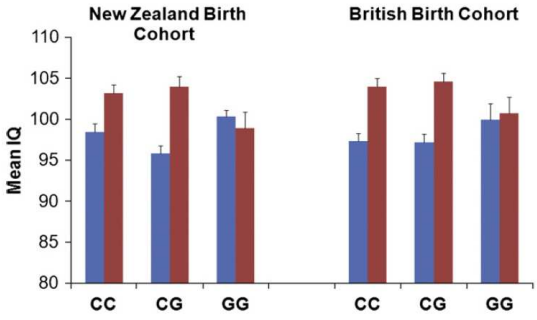

Fig. 3. Association between breastfeeding and IQ moderated by a genetic polymorphism (rs174575) in the *FADS2* gene studied by Caspi et al [41] in 2 independent birth cohorts. Breastfed children (■) carrying the C allele showed an IQ-point advantage relative to children not breastfed (■). Breastfeeding had no effect on IQ of GG homozygotes (adapted from Caspi et al 2007 [41]).

7. Conclusion

FADS1 and *FADS2* gene polymorphisms are likely to be important factors contributing to the variability in PUFA levels in serum phospholipids as well as in erythrocyte membranes [34-37,39,40]. The n-6 LC-PUFA AA as well as its precursors LA, GLA, and DGLA showed strong associations with polymorphisms and statistically reconstructed haplotypes of *FADS1* and *FADS2* [34,35]. In free-living individuals with self-selected diets, the reconstructed haplotypes explain a major proportion of the variation in serum phospholipid and erythrocyte membrane contents. Up to 28% of variation of blood level AA is due to genetic variation, whereas the value is in the order of 10% for the precursor fatty acids of AA [34]. For n-3 fatty acids, smaller percentage values are found. This could be due to a larger degree of variation in dietary intakes of the precursor ALA primarily from vegetable oils as well as of the products EPA and DHA primarily from marine foods.

These data demonstrate that blood and tissue levels of the essential fatty acids LA and ALA, as well as their biologically active LC-PUFA derivatives, are influenced not only by diet, but to a large extent also by genetic variants common in European and Canadian populations. Thus, in relation to genetic variants, population subgroups may have different requirements of dietary PUFA or LC-PUFA intakes to achieve comparable biological effects.

More needs to be known about the associations between fatty acid availability, PUFA metabolism, and genetic variants. Therefore, we recommend including analyses of *FADS1* and *FADS2* polymorphism in future cohort and intervention studies addressing biological effects of PUFAs and LC-PUFAs. We believe investigations in the generation

of specific eicosanoid and docosanoid mediators from PUFAs in association with PUFA status and genetic variants are of large interest and would help to understand more about the link between PUFAs and inflammation and the impact of PUFA metabolism on human health.

Acknowledgment

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2. PUBLICATION 1

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3. Publication 2: “High-throughput analysis of fatty acid composition of plasma glycerophospholipids”

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methods

High-throughput analysis of fatty acid composition of plasma glycerophospholipids[S]

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Abstract Plasma FA composition, a marker of FA status and dietary intake, is associated with health outcomes on a short- and long-term basis. Detailed investigation of the relationships between plasma FA composition and health requires the analysis of large numbers of samples, but manual sample preparation is very cumbersome and time consuming. We developed a high-throughput method for the analysis of FAs in plasma glycerophospholipids (GPs) with increased sensitivity. Sample preparation requires two simple steps: protein precipitation and subsequent base catalyzed methyl ester synthesis. Analysis of GP FAs is performed by gas chromatography. Coefficients of variation for FAs contributing more than 1% to total FAs are below 4%. Compared with the established reference method, results of the new method show good agreement and very good correlations ($r > 0.9$). The new method reduces the manual workload to about 10% of the reference method. Only 100 µl plasma volume is needed, which allows for the analysis of samples from infants. The method is well suitable for application in large clinical trials and epidemiological studies.—Glaser, C., H. Demmelmair, and B. Koletzko. High-throughput analysis of fatty acid composition of plasma glycerophospholipids. *J. Lipid Res.* 2010. 51: 216–221.

Supplementary key words essential fatty acids • fatty acids • fatty acid methyl esters • high-throughput • glycerophospholipids • phospholipids

The FA composition of cellular and plasma lipids is of major importance for many biological functions. Although limited by different turnover rates and widely differing contribution of individual lipid classes to total pools, there is generally a reasonable correlation between FA composition of cellular and of plasma lipids (1). In clinical studies and epidemiological observations, only blood is easily accessible for analysis and can be assessed in large numbers of subjects (2). Many studies have aimed at investigating the relationship between FA status and cardiovascular disease (3). Furthermore, the availability of n-3 long-chain polyunsaturated fatty acids (LC-PUFAs) has been related to mental development in infants (4) as well as to the attenuation of the decline of mental performance in the elderly (5). The n-6 LC-PUFA dihomo-γ-linolenic acid and arachidonic acid and the n-3 LC-PUFA eicosapentaenoic acid are precursors of eicosanoids with different biological effects (6). The determination of FA status has a pivotal role in clinical trials and cross-sectional studies (7–9) and is a valuable biomarker for the quality of consumed dietary fat (10).

Conventional methods for analyzing the FA composition in biological samples consist of several analytical steps and are, therefore, cumbersome and time consuming. Typically, a skilled chemist requires 2 days for analyzing 10–20 samples (11). In the last years, different approaches are employed to overcome this disadvantage. Fast gas chromatographic techniques were introduced and optimized for fatty acid methyl ester (FAME) analyses and quantification (11, 12), but sample preparation has been the time- and cost-expensive factor. Therefore, new approaches focus on simplifying and reducing sample preparation steps like lipid extraction, lipid separation, and FAME synthesis. Masood and Salem (11) developed a robotic transesterification method for the analysis of FAs from total plasma lipids. Akoto et al. (13) used direct thermal desorption to analyze the FA composition of whole blood and total plasma lipids. Methods for analysis of FAs composition of total plasma or whole blood lipids provide valuable information and are cost-effective alternatives to the complex and time-consuming analysis of individual lipid fractions (1, 14–16). However, analyses of FA composition of individual lipid fractions are more suitable for the sensitive detection of intervention effects or metabolic relationships (17).

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Abbreviations: CE, cholesteryl ester; CV, coefficient of variation; FAME, fatty acid methyl ester; GC, gas chromatography; GP, glycerophospholipid; LC-PUFA, long-chain PUFA; PhL, phospholipid; TAG, triacylglycerol.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one table and two figures.

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We aimed to develop a method that is both sensitive, especially for the measurement of changes in plasma LC-PUFA content, and cost effective. For studies focusing on LC-PUFAs, it seems most promising to analyze plasma phospholipids (PhLs), because they contain higher percentages of LC-PUFAs and are less sensitive to short term variation than plasma triacylglycerols (TAGs) and cholesteryl esters (CEs) (18, 19). For the determination of PhL FA in biological samples, Bondia-Pons et al. (12) presented an optimized method for lipid extraction and lipid separation using solid phase extraction instead of TLC. Their sample preparation technique is easier than those of established methods, but it is still very time consuming.

The term phospholipid is used for all lipids with a phosphorus-containing polar head group. PhLs can be further subdivided in glycerophospholipids (GPs) and SMs, based on their glycerole or sphingoid base backbone, respectively. The major PhL fractions in plasma are glycerophosphocholines and SMs (20, 21). Although LC-PUFA percentages are high in glycerophosphocholines and other GPs, LC-PUFAs contribute less than 5% to the FAs esterified to sphingosine in SMs (20, 22). The contribution of SMs to total plasma PhL varies widely in healthy controls and in patients suffering from coronary artery disease (21). Thus, a further source of variation in LC-PUFA percentages can be eliminated by determining selectively the FA composition of GPs while excluding SMs from FA analysis.

To the best of our knowledge, no method has been described for determination of plasma GP FAs, avoiding cumbersome lipid extraction steps and preparative chromatographic isolation of lipid fractions. We developed a high-throughput method for the determination of the FA composition of plasma GPs by eliminating labor intensive procedures and compared our newly developed method with an established reference method.

MATERIALS AND METHODS

Reagents and samples

Solvents were obtained in the highest available purity from Merck KGaA (Darmstadt, Germany). Methanolic HCl (3 N) and sodium methoxide (25 wt. % in methanol) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Two internal standards were applied. For internal standard A, pentadecanoic acid, cholesteryl pentadecanoate, tripentadecanoin, and 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (Sigma-Aldrich) were dissolved in methanol/chloroform (35:15). The internal standard B consisted of 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine dissolved in methanol. For the determination of the efficiency of base catalyzed transesterification octadecane (Sigma-Aldrich) dissolved in methanol was used as internal standard. To prevent FA oxidation, 2 g/l 2.6-di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene, BHT, Sigma-Aldrich) was added to each internal standard. GLC-85, containing 32 fatty acid methyl esters, (Nu-Check Prep, Inc., Elysian, MN) was applied as external standard. SM (chicken egg yolk, ≥98% TLC) was purchased from Sigma-Aldrich. A mixture of sodium carbonate, sodium hydrogen carbonate, and sodium sulfate (1:2:2, Merck KGaA) was applied as buffer for neutralization after acid catalyzed transesterification. Thirty-three blood samples from healthy volunteers (fasting and nonfasting) were collected in EDTA-containing vacutainers (Sarstedt AG and Co., Nümbrecht, Germany). The plasma was separated by centrifugation (900 g, 5 min) and stored at −20°C until analysis.

New method

A total of 100 µl of plasma, 100 µl of internal standard B, and 0.6 ml methanol (precooled to 5°C) were combined in glass tubes and shaken for 30 s. The precipitated proteins were separated from the methanolic phase by centrifugation at 900 *g* for 5 min. The methanolic supernatant, which contained mainly polar lipids, was transferred into another glass tube. Twenty-five µl sodium methoxide solution were added to the supernatant, then the tubes were shaken while selective synthesis of methyl esters from GP FAs proceeded at room temperature. The reaction was stopped after 3 min by adding 75 µl methanolic HCl. FAMEs were extracted by adding 300 µl hexane and shaking the tubes for 30 s. The upper hexane phase, which contains the extracted GP FAMEs, was transferred into a 2 ml vial. The extraction was repeated and combined extracts were dried under nitrogen flow at room temperature. The dry residue was taken up in 50 µl hexane (containing 2 g/l BHT) for GC analysis.

To evaluate lipid compositions in the methanolic supernatant after plasma protein precipitation and to compare the recovery of PhLs in the methanolic supernatant with the the recovery of PhLs in Folch extracts (reference method), the supernatant was deposited on a TLC plate. Lipid classes were separated by TLC and FAs bound in the different lipids were converted to FAMEs by acid catalyzed transesterification (see reference method).

To optimize base catalyzed transesterification and FAME extraction, a model sample containing 100 µl water (representing plasma), 100 µl internal standard B, and 100 µl octadecane standard (not participating in the reactions) was applied. The ratio of the peak areas of methyl pentadecanoate to octadecane was used as indicator for transesterification as well as for extraction efficiency.

Reference method

Folch extraction. To 250 µl of plasma, 100 µl of internal standard A was added, the lipids were extracted according to a modified Folch method (23, 24) using chloroform/methanol (2:1, v/v), and the extracts were washed two times with NaCl solution (2% in water). The extracts were dried at 30°C under reduced pressure and taken up in 400 µl chloroform/methanol (1:1) for application on the TLC plate.

Lipid fraction separation by TLC, acid catalyzed transesterification. N-heptane, diisopropyl ether, and acetic acid (60:40:3) were used as mobile phase for the separation of PhLs, NEFAs, TAGs, and CEs (24). The corresponding bands were scraped from the TLC plate, transferred into glass tubes and 1.5 ml methanolic HCl was added. The closed tubes were shaken for 30 s and heated to 85°C for FAME synthesis (45 min). After cooling to room temperature, samples were neutralized with carbonate buffer. For methyl ester extraction, 1 ml hexane was added. After centrifugation at 900 *g* for 5 min, the upper hexane phase was transferred into a further glass tube. The extraction was repeated and combined extracts were taken to dryness under nitrogen flow at room temperature. The dry residue was taken up in 50 µl hexane (containing 2 g/l BHT) for GC analysis.

Chromatography

Individual FAMEs were quantified by GC with flame ionization detection. GC analysis was carried out on a BPX 70 column (25 m × 0.22 mm, 0.25 µm film, SGE, Weiterstadt, Germany) using an Agilent 5890 series II gas chromatograph (Agilent, Waldbronn,

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Germany) with an optimized temperature program starting at 150°C. Without initial hold, temperature was increased by 2.5°C per min to 180°C and then with 1.5°C per min to 200°C followed by an 1-min isothermal period, allowing a total run time of only 26.33 min. The pressure program (carrier gas He) started with an initial column head pressure of 0.9 bar, which was increased by 0.02 bar per min to 1.2 bar, with 0.05 bar per min to 1.5 bar and 0.1 bar per min to the final pressure of 2.0 bar.

Data quantitation

Individual FAMEs were identified by comparison with authentic standards. For each FAME, the response relative to pentadecanoic acid methyl ester (internal standard) was determined using GLC-85 as external standard. EZChrom Elite version 3.1.7 (Agilent) was used for peak integration.

Statistical analysis

For FAs with a chain length between 14 and 24 carbon atoms, the results were expressed as absolute concentrations (mg/l plasma) and as percentages (% wt/wt). The FA data were presented as mean ± SD. As a measure of analytical precision, coefficients of variation (CV) expressed as percentages were used. Correlations were evaluated using the two-sided Spearman test and paired *t*-tests were used for comparisons between mean values ($P < 0.05$ was considered statistically significant). All statistical analyses were performed with SPSS for Windows, Version 15.0.1 (SPSS Inc., Chicago, IL).

RESULTS

Individual FAMEs were analyzed by GC. For specific analyses of GP FA compositions, a new method was developed, which allowed the selective formation of FAMEs from GPs. This was achieved by two simple steps (**Fig. 1**): *1*) Protein precipitation with methanol: separation of polar lipids [PhLs (GPs + SMs) and NEFAs], which were dissolved in the methanolic supernatant, from non polar lipids [TAGs and CEs], which were almost quantitatively precipitated with the protein; and *2*) base catalyzed transesterification at room temperature: FAMEs were formed from GPs and the very small quantities of TAGs, (which were not precipitated with the protein), whereas no FAMEs were formed from NEFAs or FAs bound in SMs and CEs under these conditions.

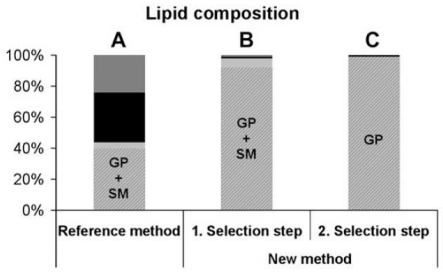

Fig. 1. Lipids recovered from a plasma sample (estimated from total FAs in corresponding fractions) with different analysis methods or at different steps of the method, respectively. A: lipid composition of a plasma extract obtained by the reference method [solid dark gray, CEs (cholesteryl esters); striped gray, GPs (glycerophospholipids) (+ SM); light gray, NEFAs, and black, TAGs (triacylglycererols)]; all lipid fractions were extracted. B: Lipid composition after the first step of the new method (protein precipitation with methanol), mainly polar lipids were dissolved in the methanolic supernatant. C: Lipid composition after the second step of the new method (base catalyzed transesterification), only GP and TAG fatty acids were transesterified to fatty acid methyl esters.

To examine the efficiency of the first separation step, we compared the lipid composition of 16 different plasma samples obtained by the reference method with the lipid composition in the methanolic supernatant. The lipid composition was estimated by the sum of FAs determined in the individual lipid fractions. According the reference method, PhLs contributed 37.7%–54.6%, NEFAs 1.3%–3.7%, TAGs 15.4%–35.8%, and CEs 23.6%–32.4% to total extracted lipids. The lipid composition in the methanolic supernatant after protein precipitation and TLC was 90.9%–96.8% PhLs, 1.3%–6.3% NEFAs, 0.9%–2.5% TAGs, and 0.8%–2.0% CEs. The use of 7 vols of methanol to volume of plasma was found to be optimal for dissolving PhLs quantitatively. The recovery of total PhLs (n = 16) in the methanolic supernatants was found to be 88.1% ± 6.6% (mean ± SD) compared with the reference method (Folch extraction). Direct addition of internal standard B to the plasma enabled correction for the loss of PhLs, thus 101.0% ± 2.6% of PhLs were correctly determined in the methanolic supernatants.

The total PhL (GP + SM) FA concentration for these 16 samples was, on average, 1317.4 mg/l (1054.2 mg/l–1908.3 mg/l), according to the reference method. The new method identified in total 1229.9 mg/l (970.4 mg/l–1836.3 mg/l) FAs in plasma GPs.

Hydrolysis of methyl esters was a concern, as water (from plasma sample) was present during base catalyzed transesterification. Therefore, we studied reaction yields in a methanolic solution containing 100 µl of water and 100 µl of internal standard B. Reaction times between 3 min and 10 min ensured complete transesterification of GP FAs. The recovery of the internal standard B was 99.1% ± 0.8% (mean ± SD) relative to the octadecane standard in eight independent analyses.

After base catalyzed transesterification, FAMEs were extracted twice with 300 µl hexane. To evaluate extraction efficiency, samples were reextracted with 1 ml hexane. These extracts contained less than 1% of the total FAMEs recovered with the previous extractions.

Storage of the GC ready derivatives for one month at −20°C revealed no significant alterations in FA concentrations (data not shown).

Intra-assay reproducibilities (n = 8) obtained by the analysis of PhLs with the reference method were compared with those obtained by the analysis of GPs with the new method. FA concentrations (mg/l) and compositions (% wt/wt) were comparable, but concentrations of the saturated FAs C20:0, C22:0, and C24:0 and of the monounsaturated FA C24:1n-9 were below the quantification level in GPs (**Table 1**). GP total FA concentration was about 10% lower than in PhLs, whereas some individual FAs showed higher concentrations. For GPs, CVs for all FAs were found to be below 4%; C18:3n-3, contributing only 0.21% to total FAs, had the highest CV (3.8%).

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TABLE 1. Intra-assay reproducibility ($n = 8$) of fatty acid concentration (mg/l) and composition (%) in phospholipids (PL) obtained by the reference method and in glycerophospholipids (GP) obtained by the new method, mean (CV)

	FA Concentration (mg/l)		FA Composition (% wt./wt.)	
FA	PhL	GP	PhL	GP
Saturated FA				
C14:0	5.24 (2.4)	7.95 (3.2)	0.40 (3.0)	0.65 (3.4)
C16:0	368.36 (0.8)	354.78 (0.7)	27.96 (0.3)	28.85 (0.6)
C17:0	5.37 (1.3)	4.93 (1.4)	0.41 (0.7)	0.40 (0.6)
C18:0	185.00 (1.5)	168.46 (1.1)	14.04 (0.9)	13.70 (0.7)
C20:0	6.67 (1.4)	ND	0.51 (1.1)	ND
C22:0	15.64 (1.5)	ND	1.19 (1.4)	ND
C24:0	14.11 (2.7)	ND	1.07 (2.6)	ND
Monounsaturated FA				
C16:1n-7	9.85 (1.5)	14.05 (2.0)	0.75 (1.7)	1.14 (1.8)
C18:1n-7	19.88 (1.4)	20.69 (1.0)	1.51 (0.6)	1.68 (0.6)
C18:1n-9	143.35 (1.0)	156.59 (1.3)	10.88 (0.5)	12.73 (0.7)
C20:1n-9	2.35 (1.5)	2.26 (2.3)	0.18 (1.6)	0.18 (2.6)
C24:1n-9	28.89 (1.2)	ND	2.19 (0.8)	ND
n-9 PUFA				
C20:3n-9	2.64 (2.9)	2.76 (1.9)	0.20 (2.3)	0.22 (1.8)
n-6 PUFA				
C18:2n-6	248.15 (1.1)	251.05 (1.4)	18.84 (0.8)	20.41 (0.7)
C18:3n-6	1.73 (8.0)	2.15 (2.5)	0.13 (8.5)	0.17 (2.4)
C20:2n-6	3.78 (1.7)	3.96 (1.9)	0.29 (1.5)	0.32 (1.3)
C20:3n-6	43.25 (1.3)	41.59 (1.3)	3.28 (0.6)	3.38 (0.7)
C20:4n-6	133.09 (1.2)	124.89 (1.3)	10.10 (0.4)	10.15 (0.8)
C22:4n-6	5.41 (1.5)	4.64 (2.6)	0.41 (1.4)	0.38 (2.0)
C22:5n-6	4.08 (3.2)	3.70 (2.5)	0.31 (3.2)	0.30 (2.1)
n-3 PUFA				
C18:3n-3	1.92 (2.5)	2.59 (3.8)	0.15 (2.1)	0.21 (3.7)
C20:5n-3	10.50 (1.3)	9.99 (1.6)	0.80 (0.6)	0.81 (0.7)
C22:5n-3	12.00 (1.1)	10.69 (1.5)	0.91 (0.6)	0.87 (0.9)
C22:6n-3	46.22 (1.9)	42.15 (1.4)	3.51 (1.1)	3.43 (0.9)
Total FA				
	1317.42 (0.9)	1229.85 (0.9)		

Inter-assay reproducibility (**Table 2**) of the new method was obtained by analyzing 49 aliquots of one plasma sample over a period of 4 months. CVs for all FAs contributing more than 1% to total FAs were found to be below 4%. Values of C18:3n-6, contributing 0.10% to total FAs, showed the highest CV (10.9%). The FA contents remained constant over 4 months.

FA concentrations and percentage contributions in PhLs obtained by the reference method were correlated with those in GPs obtained with the new method (see supplementary Table I). For concentrations of all analyzed FAs (except C14:0 and C18:3n-6) in PhLs and GPs, correlation coefficients higher than 0.9 ($P < 0.0001$) were observed. Both C14:0 and C18:3n-6 showed very low concentrations, and their contribution to total FAs in PhLs and GPs was below 1%, respectively. Percentage contributions of most FAs in PhLs and in GPs correlated with $r > 0.9$ ($P < 0.0001$). Only for C14:0, C20:1n-9, C22:4n-9, and C18:3n-3, lower r-values, between 0.76 and 0.89 ($P \leq 0.001$), were observed.

DISCUSSION

The new method enabled rapid, precise, and reproducible analysis of FA composition of plasma GPs

Plasma proteins were precipitated by adding methanol to plasma. TAGs and CEs dissolve poorly in polar solvents such as methanol. NEFAs showed better solubility in methanol. Due to their amphiphilic character, PhLs were easily soluble in methanol. The advantage of methanol precipitation combined with subsequent base catalyzed transesterification was that TAGs and CEs were dissolved in methanol only in negligible amounts, contributing less than 2.5% to the total FA in the methanolic supernatant, and NEFAs, SM FAs, as well as CE FAs were not converted to FAMEs by reaction with sodium methoxide at room temperature, as previously shown (25–27). Thus, only two simple steps were required to convert selectively GP FAs to FAMEs for GC analysis.

In an alkaline milieu, hydrolysis of synthesized methyl esters must be inhibited by neutralization of the reaction mixture, thus, the reaction was stopped after 3 min by adding methanolic HCl to the mixture. A reaction time of 3 min was sufficient for complete transesterification of GP FAs, but also a reaction time of 10 min yielded complete transesterification without saponification. This was in agreement with previous observations, which showed base catalyzed transesterification to be about 1,500 times faster than saponification (25, 26).

Results showed that C20:0, C22:0, C24:0, and C24:1n-6 were present in PhLs but not in GPs, as these FAs were mainly bound in SMs and, therefore, not transesterified to FAMEs. This was in agreement with the previously shown high abundance of these FAs in SMs, as about 95% of the SM FAs were saturated or monounsaturated (22). High percentages of LC-PUFAs were found in GPs, especially glycero-

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TABLE 2. Inter-assay reproducibility ($n = 49$) of fatty acid concentrations (mg/l) and compositions (% wt./wt.) in glycerophospholipids analyzed over a time period of 4 months with at least two measurements per week with the new method

	FA Concentration		FA Composition	
FA	Mean	CV	Mean	CV
Saturated FA				
C14:0	7.29	3.5	0.60	3.2
C16:0	378.91	2.1	31.32	1.5
C17:0	4.63	3.3	0.38	2.6
C18:0	167.85	4.0	13.87	2.7
C20:0	ND	ND	ND	ND
C22:0	ND	ND	ND	ND
C24:0	ND	ND	ND	ND
Monounsaturated FA				
C16:1n-7	12.18	2.9	1.01	2.3
C18:1n-7	21.46	3.0	1.77	1.7
C18:1n-9	152.91	3.0	12.64	1.2
C20:1n-9	2.16	6.8	0.18	5.4
C24:1n-9	ND	ND	ND	ND
n-9 PUFA				
C20:3n-9	2.39	7.8	0.20	7.2
n-6 PUFA				
C18:2n-6	244.26	2.8	20.19	1.0
C18:3n-6	1.26	10.9	0.10	10.5
C20:2n-6	4.48	4.4	0.37	3.3
C20:3n-6	38.68	4.0	3.20	2.4
C20:4n-6	108.31	4.3	8.95	3.0
C22:4n-6	3.68	9.5	0.30	8.7
C22:5n-6	4.14	7.6	0.34	6.7
n-3 PUFA				
C18:3n-3	2.73	4.2	0.23	3.3
C20:5n-3	7.19	7.6	0.59	6.6
C22:5n-3	7.90	7.1	0.65	6.1
C22:6n-3	37.56	3.8	3.10	2.8
Total FA				
	1221.34	2.5		

phosphoethanolamine and glycerophosphoserine but also in glycerophosphocholine (22, 28). Total GP FA concentration was about 10% lower than PhL FA concentrations because SM FAs were not transesterified and, therefore, were not assessed with the new method. Some individual FAs showed higher concentrations in GPs, which could be due to small contaminations with TAGs. Furthermore, Folch extraction has been shown to be nonquantitative in extracting lipids from plasma (29).

Results for GP FAs could not be directly compared with results for PhL FAs because PhL FAs contained additional SM FAs and so differing FA compositions were obtained. Nevertheless, comparison of results for PhL FAs obtained by the reference method with results for GP FAs obtained by the new method showed high correlations.

The LC-PUFA content of plasma glycerophosphocholine, which was by far the most abundant plasma GP, has been found to be similar to red blood cell glycerophosphocholine LC-PUFA content (17). Thus, it can be expected that GP FA composition was representative for the FA status in a variety of physiological and pathological conditions. As shown by the very good correlation between the FA composition of PhLs obtained by the reference method and the FA composition of the GP obtained by the new method, the small amount of TAGs contributing to the analyzed FAs did not disturbed the results. Furthermore, the percentage contribution of individual FAs to total FAs in TAGs and PhLs correlated and, thus, the influence of those residues on compositional results was limited (30).

Our new sample preparation technique enables the analysis of FA composition of plasma glycerophospholipids from more than 50 samples per day. Throughput is limited by the running time of the GC, which takes 26.33 min for each sample. It provides results equivalent to the analysis of PhL FAs by the reference method and is especially suitable for the determination of long-chain unsaturated fatty acids, which are mainly bound in glycerophospholipids. The easy handling allows reproducible and robust analyses and enables the use of FA concentrations as biomarkers in large clinical and epidemiological studies.

The new method for the analysis of glycerophospholipid fatty acid composition in plasma described in this article is in patent-pending status. The presented data are part of a PhD thesis accomplished by Claudia Glaser at the Medical Faculty of the Ludwig-Maximilians-University of Munich.

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Table S1

Correlations (n = 16. P < 0.0001, except for * P = 0.001) between fatty acid concentrations (mg/l) and compositions (% wt/wt) in phospholipids (PL) obtained by the reference method and in glycerophospholipids (GP) obtained with the new method

FA	*PL vs. GP*	
	FA concentration	FA composition
Saturated FA		
C14:0	0.841	0.835
C16:0	1.000	0.918
C17:0	0.958	0.956
C18:0	0.933	0.985
C20:0	ND	ND
C22:0	ND	ND
C24:0	ND	ND
Monounsaturated FA		
C16:1n-7	0.924	0.962
C18:1n-7	0.974	0.970
C18:1n-9	0.976	0.968
C20:1n-9	0.913	0.804
C24:1n-9	ND	ND
n-9 PUFA		
C20:3n-9	0.978	0.999
n-6 PUFA		
C18:2n-6	0.974	0.956
C18:3n-6	0.885	0.938
C20:2n-6	0.975	0.963
C20:3n-6	0.974	0.982
C20:4n-6	0.944	0.950
C22:4n-6	0.915	0.760*
C22:5n-6	0.979	0.996
n-3 PUFA		
C18:3n-3	0.916	0.888
C20:5n-3	0.995	0.985
C22:5n-3	0.950	0.940
C22:6n-3	0.915	0.946
Total FA		
	0.974	

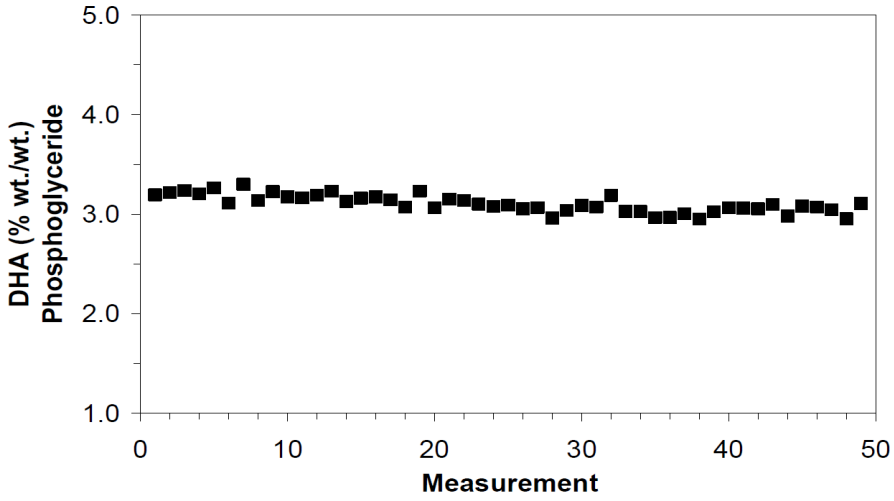

Fig. S1

Inter-assay reproducibility *(n = 49)* of the percentage contribution of DHA to total FA (% wt./wt.) in plasma gycerophospholipids analyzed over a time period of 4 month with at least 2 measurements per week with the new method

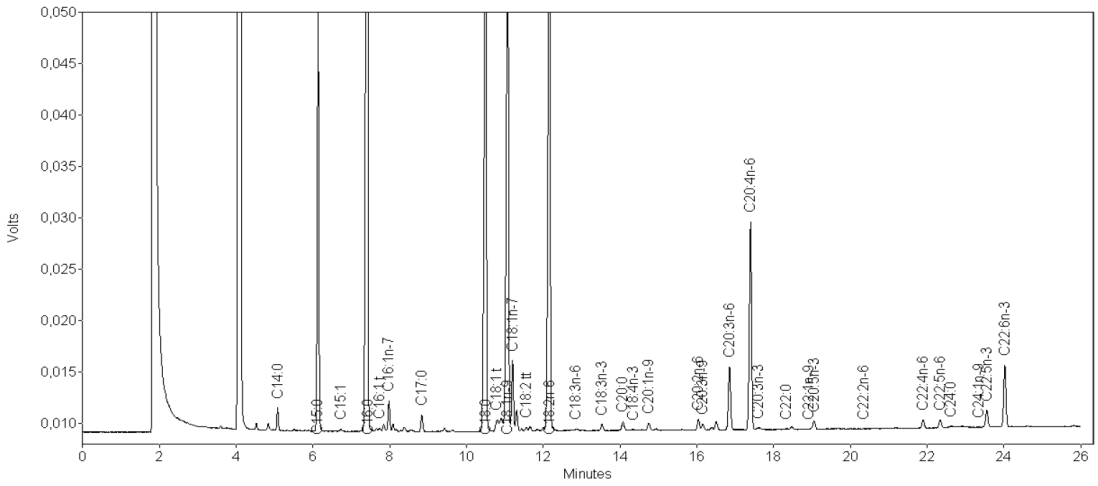

Fig. S2

Representative gas chromatogram of FAME obtained from plasma glycerophospholipids (GP) by using the new method; Longer chain saturated FA (20:0, 22:0, 24:0) and longer chain monounsaturated FA (24:1n-9) contribute only small amounts to plasma GP, and the peaks of these FAME prepared are below the detection limit

4. Publication 3: “Fatty acid composition of serum glycerophospholipids in children”

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Fatty Acid Composition of Serum Glycerophospholipids in Children

Claudia Glaser, MSc, Hans Demmelmair, PhD, Stefanie Sausenthaler, PhD, Olf Herbarth, Prof, Joachim Heinrich, PhD, and Berthold Koletzko, Prof

Objective To determine reference values for fatty acid (FA) composition of serum glycerophospholipids (GPs) in children with a new high-throughput method.

Study design The GP FA composition of 1326 serum samples obtained from a cohort of 951 children at 2 and 6 years, participating in the German Influences of Lifestyle Related Factors on the Immune System and the Development of Allergies in Childhood (LISA) study, was analyzed with a new high-throughput method. Only 2 simple preparation steps were necessary to obtain fatty acid methyl esters selectively from serum GPs. The FA status was determined by separating and quantifying the fatty acid methyl esters with high-resolution capillary gas chromatography.

Results FA values in serum GPs were in very good agreement with other published values in serum or plasma phospholipids for most of the analyzed FAs. No major age and sex differences in GP FA composition were observed.

Conclusion The serum GP FA values obtained from children aged 2 and 6 years may serve as reference values in clinical practice (eg, for monitoring and improving therapeutic interventions). Furthermore, they can serve as a reference point for interpreting FA values in clinical and epidemiological studies. *(J Pediatr 2010;157:826-31).*

An adequate availability of polyunsaturated fatty acid (PUFA) is necessary for normal growth, normal visual, and cognitive and immune functions in infants and children.[1-3] Monitoring of fatty acid (FA) status may be indicated in children with modified dietary FA intake, with disease-related alterations of absorption or metabolism (eg, patients with short bowel syndrome, inflammatory bowel disease, or metabolic liver disease), or with interventions such as fish oil supplementation in children with phenylketonuria or other inborn errors of amino acid metabolism.[3-7] Monitoring of FA status in individual patients or in populations depends on the availability of reference values obtained in healthy children to allow interpretations of the results obtained.

Plasma FA composition is an accepted biomarker of FA status and dietary intake.[8] Phospholipids are the predominant lipid class in fasting plasma, comprising more than half of plasma lipid total FAs, and the most of plasma arachidonic acid (AA) and docosahexaenoic acid (DHA) are found in phospholipids (PLs).[9] Compared with non-esterified FAs (NEFAs) and triacylglycerols (TAGs), FA composition of PLs is less prone to short-term variation.[8,10,11] Therefore, analyses of plasma PLs are widely used for assessment of FA status, and ample data on human plasma PL FA composition are available.[4,12-15]

The major PL fractions in plasma are glycerophosphocholines and phosphosphingolipids (SMs).[11,16,17] The high PUFA and long chain polyunsaturated fatty acid (LC-PUFA) content in glycerophospholipids (GPs) lead us to asses human FA status by analyzing the percentage contribution of FAs to plasma or serum GPs. Analyses of GP FA composition can be performed quickly and cost effectively with a newly developed high-throughput method that provides highly sensitive and precise results.[18]

Because the availability of reference values is a prerequisite for interpreting FA status of individuals, we aimed at establishing reference values for GP FA composition in children. With a new high-throughput method, we analyzed 1326 serum samples obtained from 951 children aged 2 and 6 years who participated in the Influences of Lifestyle Related Factors on the Immune System and the Development of Allergies in Childhood (LISA) birth cohort study. We explored the potential influence of age and sex on serum GP FA compositions and compared the results with published values of plasma and serum PL FA compositions in children.

AA	Arachidonic acid
DHA	Docosahexaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
GC	Gas liquid chromatography
GP	Glycerophospholipid
LC-PUFA	Long chain polyunsaturated fatty acid
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
SM	Phosphosphingolipid
TAG	Triacylglycerol

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The presented data are part of a PhD thesis by C.G.

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Methods

Samples from participants of the prospective German birth cohort study LISA (Influences of lifestyle-related factors on the immune system and the development of allergies in childhood) were analyzed. The study design of this population-based, birth cohort study has been described.[19,20] In brief, 3097 healthy full-term neonates were recruited between November 1997 and January 1999 in 4 German cities, Munich (n = 1467), Leipzig (n = 976), Wesel (n = 348), and Bad Honnef (n = 306). Excluded were neonates with at least 1 of the exclusion criteria: preterm birth (<37 gestational week), low birth weight (<2500 g), congenital malformation, symptomatic neonatal infection, antibiotic medication, and hospitalization or intensive medical care during neonatal period. In addition to non-consent to participate in this study, newborns from mothers with immune-related diseases such as autoimmune disorders, diabetes mellitus, hepatitis B, long-term medication use, or abuse of drugs and alcohol and newborns from parents with a nationality other than German and parents who were not born in Germany were excluded because the primary goal of the LISA study was to examine several aspects of the immune system and their determinants. To minimize variation of those immune function variables, we did not include children of non-German ancestries. Thus, overall, 55% of all newborns were included.

We collected questionnaire data on family history of atopy, parental education, and health problems during pregnancy, smoking of the mother during pregnancy, and mothers' exposure to environmental tobacco smoke during pregnancy at home. The cohort was followed up at the ages of 6, 12, and 18 months and 2, 4, and 6 years. Venous blood samples were collected from children at 2 and 6 years. Ethical approval for the study was obtained from the local ethics committee of the Bavarian Board of Physicians. Written consent for participation to the study was given by the parents.[21,22] For this analysis on FA measurements in GPs, serum samples from children from Munich were used. Serum samples from 737 children (412 boys, 325 girls) at the age of 2 years and of 589 children (330 boys, 259 girls) at the age of 6 years were available. The origin of the samples is shown in detail in Supplementary **Figure 1** (available at www.jpeds.com). Serum samples both at the age of 2 and 6 years were available for analysis in 375 children (213 boys, 162 girls).

Laboratory Analyses

In the LISA study, venous blood samples were collected in serum separator tubes. Samples were centrifuged and serum was frozen in plastic vials and stored at –80°C until analysis. The analysis of GP composition was performed as described.[18] In brief, 100 μL of serum (or plasma, cf. below) 100 μl of internal standard (1,2-dipentadecanoyl-sn-glycero-3-phosphocholine dissolved in methanol), and 0.6 mL methanol (precooled to 5°C) were combined in glass tubes and shaken for 30 seconds. Samples were centrifuged, and the supernatant was transferred to another glass tube. Sodium methoxide solution (25 μL) was added, tubes were shaken, and synthesis of methyl esters proceeded at room temperature. The reaction was stopped after 3 minutes by adding 75 μl methanolic HCl. Fatty acid methyl esters (FAMEs) were extracted twice by adding 300 μL hexane each time. Extracts were combined, dried under nitrogen flow at room temperature, and taken up in 50 μL hexane (containing 2 g/L butylated hydroxytoluol) for gas liquid chromatography (GC) analysis.

This sample preparation technique enables the analysis of FA composition of serum GPs from >50 samples per day; in contrast, 5 to 10 samples can be analyzed per day with conventional methods.[18,23] Furthermore, solvent consumption is drastically reduced. In summary, the high-throughput method reduces manual workload and costs no more than 10% of conventional methods.[18]

Individual FAMEs were quantified by using GC with flame ionization detection and identified by comparison with authentic standards (GLC-569B, Nu-Check Prep, Elysian, Minnesota; cis-5,8,11-eicosatrienoic acid methyl ester, Sigma-Aldrich, Taufkirchen, Germany; methyl vaccenate [11c], methyl octadecatetraenoate [6c, 9c, 12c, 15c], and methyl brassidate [13tr], Larodan Fine Chemicals AB, Malmö, Sweden). With these standards, we identified these trans FAs in our chromatogram as trans-9-C16:1 (methyl palmitelaidate), trans-9-C18:1 (methyl elaidate), trans-9, trans-12-C18:2 (methyl linoelaidate) and trans-13-C22:1 (methyl brassidate). Because our interest is focused on PUFAs, the GC program used was not optimized for the separation of isomeric trans FAs. Thus, we cannot exclude that different isomeric trans FAs coelute together as a single peak. Therefore we used the general designations C16:1t, C18:1t, C18:2tt, and C22:1t in the result section.

Response relative to pentadecanoic acid methyl ester (internal standard) was determined with GLC-85 (Nu-Check Prep, Elysian, Minnesota) as an external standard. EZChrom Elite version 3.1.7 (Agilent, Waldbronn, Germany) was used for peak integration. Results were expressed as concentration (mg/L) and percentage (% wt/wt) of all FAs detected with a chain length between 14 and 24 carbon atoms.

The applied method for analyzing the FA composition was developed with EDTA plasma samples.[18] To ensure the applicability of this method to serum samples, venous blood samples from 8 volunteers aged between 25 and 46 years were simultaneously collected in serum separator tubes (Sarstedt AG & Co., Nümbrecht, Germany) and in EDTA plasma tubes (Sarstedt AG & Co.). The analytical procedure was identical for serum and plasma samples.

For quality assurance, aliquots of a control serum were stored in plastic vials at –80°C. One control serum aliquot was analyzed with every sample batch (serum samples that were processed simultaneously).

Data Presentation and Statistical Analyses

FAs with ≥2 double bonds were defined as PUFAs, and FAs with ≥20 carbon atoms and ≥2 double bonds were defined as LC-PUFAs.

The FA data were presented as medians and interquartile ranges (ranges from 1st-3rd quartile) because FAs with

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very low concentrations, such as trans FAs, showed skewed distributions.

For comparisons between plasma and serum GP FA compositions, linear regression analysis was performed. Intra-subject comparisons were performed with the 2-sided Wilcoxon rank test. The 2-sided Mann-Whitney rank test was used for comparisons in FA values obtained from independent samples. For all statistical analyses, SPSS software for Windows version 15.0.1 (SPSS, Chicago, Illinois) was used.

Results

Comparison of Plasma and Serum

We compared the GP FA composition of 8 volunteers in plasma and serum with linear regression. Similar medians and interquartile ranges were obtained in plasma and serum (Table I). Most FAs showed high correlation coefficients (r^2 >0.80) for plasma and serum results. Slopes and intercepts of the linear regression curves were close to 1 and 0, respectively, for most FAs. Lower r^2 values were found for FA with very small concentrations that showed higher dispersions (eg, C16:1t [r^2 = 0.43] and C20:3n-3 [r^2 = 0.70]). These FAs contributed only 0.02 % and 0.06 % to total FAs in GPs, respectively. Fatty acids with very low concentrations showed higher dispersions, and therefore poorer linear regressions were obtained. For n-3 and n-6 PUFAs, correlation coefficients (r^2) between 0.70 and 1.00 were obtained.

Table I. Comparison in glycerophospholipid fatty acid composition (% wt/wt, median, and interquartile ranges) of serum and plasma samples from 8 individuals with linear regression

	Plasma		Serum				
FA	Median	IQR	Median	IQR	r^2	Slope	Intercept
Saturated FA							
C14:0	0.61	0.09	0.62	0.14	0.986	1.1	−0.05
C16:0	28.74	2.32	29.11	2.20	0.996	1.0	−0.72
C17:0	0.33	0.05	0.34	0.05	0.931	0.8	0.08
C18:0	11.87	1.11	11.96	1.42	0.986	1.0	0.32
∑ SFA	43.07	1.72	43.46	1.57	0.983	0.9	4.05
Trans FA							
C16:1t	0.02	0.01	0.02	0.01	0.425	0.6	0.01
C18:1t	0.11	0.03	0.11	0.03	0.923	0.7	0.05
C18:2tt	0.05	0.04	0.05	0.03	0.919	0.9	0.01
C22:1t	0.13	0.10	0.13	0.10	0.860	0.9	0.02
∑ ***trans***	0.26	0.18	0.29	0.17	0.931	0.9	0.05
Cis monounsaturated FA							
C15:1n-5	0.06	0.04	0.06	0.04	0.972	1.0	0.01
C16:1n-7	0.89	0.39	0.91	0.41	0.990	1.0	−0.01
C18:1n-7	1.58	0.28	1.57	0.28	0.997	1.0	0.03
C18:1n-9	11.78	1.08	11.89	1.01	0.847	0.7	3.25
C20:1n-9	0.16	0.08	0.16	0.07	0.972	0.9	0.02
∑ MUFA	14.16	1.71	14.19	1.31	0.846	0.7	3.86
n-9 PUFA							
C20:3n-9	0.17	0.06	0.17	0.06	0.976	0.9	0.01
n-6 PUFA							
C18:2n-6	22.27	4.22	22.21	3.88	0.997	0.9	1.37
C18:3n-6	0.11	0.11	0.11	0.11	1.000	1.0	−0.01
C20:2n-6	0.41	0.09	0.42	0.07	0.989	1.0	0.02
C20:3n-6	3.29	0.59	3.29	0.76	0.977	1.1	−0.26
C20:4n-6	9.64	2.73	9.33	2.83	0.982	1.0	−0.56
C22:4n-6	0.31	0.07	0.26	0.06	0.819	0.8	0.04
C22:5n-6	0.30	0.14	0.30	0.14	0.998	1.0	−0.01
∑ n-6 LC-PUFA	13.85	3.81	13.43	3.84	0.986	1.0	−0.77
∑ n-6 PUFA	36.47	2.71	36.25	2.63	0.944	1.0	0.01
n-3 PUFA							
C18:3n-3	0.23	0.17	0.23	0.19	0.992	1.0	−0.01
C20:3n-3	0.06	0.01	0.06	0.01	0.701	0.7	0.02
C20:5n-3	0.64	0.68	0.62	0.66	0.999	1.0	0.01
C22:5n-3	0.72	0.45	0.71	0.43	0.990	0.9	0.01
C22:6n-3	4.02	1.32	3.98	1.30	0.993	1.0	−0.07
∑ n-3 LC-PUFA	5.57	2.01	5.57	1.95	0.995	1.0	0.11
∑ n-3 PUFA	5.82	2.05	5.81	2.00	0.996	1.0	0.09

Quality Assurance

Comparable and constant values were observed for GP FA compositions of aliquots from control serum in the whole analyzing period of 129 days (Figure 2; available at www.jpeds.com). For clarity, Figure 2 shows only the most abundant FAs contributing at least 0.5 % to total FAs in GPs. For the other analyzed FAs, comparable results were obtained (data not shown). For all analyzed FAs, no outliers or any other deviations were found.

Age Differences in FA Composition

We compared GP FA composition of serum samples from 375 children obtained at two different times (2 years and 6 years). Wilcoxon rank tests revealed differences for most of the analyzed FAs ($P < .05$) in both points (Figure 3). However, FA values differed only slightly with overlapping interquartile ranges, and no consistent overall trend was observed.

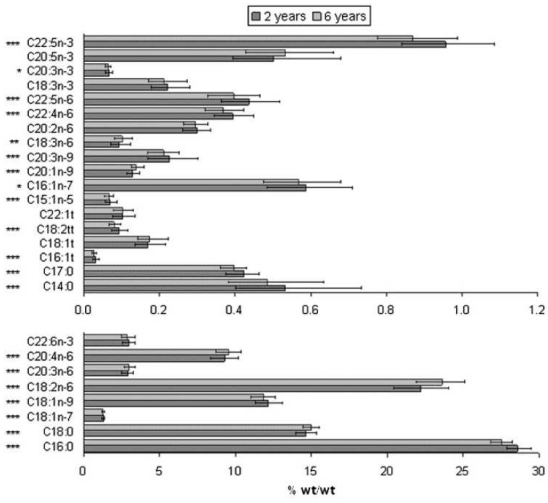

Figure 3. Glycerophospholipid fatty acid composition (% wt/wt, median, and interquartile ranges) of serum samples from 357 children obtained at 2 points (2 and 6 years). Stars indicate significant differences of FA values in both points (***P < .001, **P < .01, *P < .05).

Sex Differences in FA Composition

We categorized the distribution of FAs in GPs by sex for both age groups (Table II). The group sizes were similar, with 412 boys and 325 girls at 2 years and 330 boys and 259 girls at 6 years. Medians and interquartile ranges of the groups were similar. With the 2-sided Mann-Whitney rank tests, statistically significant differences between boys and girls were revealed only for very few FAs (2 years: C18:3n-3; 6 years: C20:3n-6, C22:4n-6, C22:5n-6).

Reference Values

The FA values were considered appropriate to generate reference values, because no major age- and sex-related differences were observed. We combined the data of serum sample analyses of 737 children at age 2 years and of 589 children at 6 years. For establishing reference values, we did not use all 1326 samples because samples from 375 children samples were obtained at both points. Thus, from one randomly determined half (188) of these children, we excluded the samples collected at the age of 2 years and from the other half (187) at 6 years. The remaining samples were combined with the samples from the children who had only provided a sample at 1 point (2 years: 362; 6 years: 214). Thus, FA values from 951 samples obtained from 951 different children (2 years: 549l 6 years: 402) were included for calculating medians and interquartile ranges (Table III).

For most of the analyzed FAs, median and mean values are similar because their data are normally distributed (eg, C18:0; Figure 4, available at www.jpeds.com). In contrast, some FAs, such as C18:1t, which were found only in small quantities, show a right-skewed distribution (Figure 4).

Discussion

We conclude that both plasma and serum can be used to determine GP FA composition with our method. Serum

Table II. Glycerophospholipid fatty acid composition (% wt/wt, median and interquartile ranges) of serum samples from 737 children aged 2 years and 589 children aged 6 years, grouped by sex and the differences between the sexes

FA	Boys 2 years (412)	Girls 2 years (325)	Difference 2 years	Boys 6 years (330)	Girls 6 years (259)	Difference 6 years
Saturated FA						
C14:0	0.56 (0.35)	0.55 (0.34)	−0.003	0.49 (0.26)	0.49 (0.25)	−0.005
C16:0	28.59 (1.57)	28.62 (1.78)	0.037	27.52 (1.49)	27.50 (1.40)	−0.029
C17:0	0.42 (0.10)	0.43 (0.09)	0.007	0.40 (0.07)	0.40 (0.07)	−0.001
C18:0	14.69 (1.36)	14.81 (1.41)	0.117	14.97 (1.23)	15.04 (1.22)	0.071
∑ SFA	44.17 (1.42)	44.45 (1.51)	0.274*	43.43 (1.18)	43.42 (1.43)	−0.005
Trans FA						
C16:1t	0.03 (0.02)	0.03 (0.02)	0.001	0.03 (0.01)	0.03 (0.01)	0.0004
C18:1t	0.20 (0.09)	0.17 (0.08)	−0.037	0.18 (0.09)	0.17 (0.07)	−0.001
C18:2tt	0.10 (0.05)	0.10 (0.04)	0.001	0.08 (0.03)	0.08 (0.03)	0.0005
C22:1t	0.12 (0.07)	0.10 (0.06)	−0.014	0.10 (0.05)	0.10 (0.06)	0.001
∑ *trans*	0.43 (0.18)	0.42 (0.17)	−0.012	0.40 (0.14)	0.41 (0.13)	0.006
Cis monounsaturated FA						
C15:1n-5	0.07 (0.03)	0.07 (0.03)	0.001	0.07 (0.02)	0.07 (0.03)	0.0004
C16:1n-7	0.58 (0.27)	0.59 (0.22)	0.006	0.57 (0.21)	0.57 (0.21)	0.003
C18:1n-7	1.29 (0.20)	1.30 (0.20)	0.012	1.26 (0.18)	1.27 (0.20)	0.011
C18:1n-9	12.32 (1.78)	12.09 (1.77)	−0.236	11.84 (1.66)	11.86 (1.73)	0.015
C20:1n-9	0.13 (0.03)	0.13 (0.03)	−0.005	0.14 (0.03)	0.14 (0.03)	−0.003
∑ MUFA	14.37 (1.79)	14.17 (1.82)	−0.199	13.92 (1.80)	13.90 (1.89)	−0.011
n-9 PUFA						
C20:3n-9	0.25 (0.14)	0.22 (0.11)	−0.025	0.21 (0.09)	0.20 (0.09)	−0.007
n-6 PUFA						
C18:2n-6	22.25 (3.75)	21.96 (3.48)	−0.296	23.31 (3.34)	23.77 (3.16)	0.458
C18:3n-6	0.11 (0.05)	0.09 (0.04)	−0.017	0.10 (0.04)	0.10 (0.05)	−0.003
C20:2n-6	0.30 (0.07)	0.30 (0.08)	0.001	0.30 (0.07)	0.29 (0.07)	−0.005
C20:3n-6	2.93 (0.69)	2.88 (0.68)	−0.047	3.03 (0.68)	2.95 (0.68)	−0.077†
C20:4n-6	9.19 (1.82)	9.19 (1.75)	−0.001	9.54 (1.79)	9.35 (1.65)	−0.192
C22:4n-6	0.40 (0.10)	0.39 (0.11)	−0.014	0.38 (0.09)	0.36 (0.09)	−0.014†
C22:5n-6	0.44 (0.15)	0.42 (0.16)	−0.027	0.42 (0.13)	0.38 (0.13)	−0.035*
∑ n-6 LC-PUFA	13.18 (2.13)	13.29 (1.91)	0.107	13.75 (2.22)	13.47 (1.99)	−0.283†
∑ n-6 PUFA	35.71 (3.14)	35.51 (2.76)	−0.200	37.18 (2.55)	37.23 (2.35)	0.052
n-3 PUFA						
C18:3n-3	0.25 (0.11)	0.22 (0.09)	−0.030†	0.22 (0.10)	0.21 (0.11)	−0.002
C20:3n-3	0.07 (0.02)	0.07 (0.02)	0.002	0.06 (0.01)	0.06 (0.01)	−0.001
C20:5n-3	0.54 (0.28)	0.50 (0.24)	−0.046	0.52 (0.21)	0.53 (0.22)	0.004
C22:5n-3	0.98 (0.25)	0.95 (0.24)	−0.028	0.88 (0.21)	0.85 (0.21)	−0.023
C22:6n-3	3.02 (0.96)	2.92 (0.86)	−0.097	2.87 (0.91)	2.81 (0.80)	−0.067
∑ n-3 LC-PUFA	4.49 (1.09)	4.53 (0.94)	0.043	4.38 (1.15)	4.30 (0.97)	−0.075
∑ n-3 PUFA	4.75 (1.05)	4.76 (0.93)	0.006	4.60 (1.11)	4.49 (1.00)	−0.102

*$P < .01$.
†$P < .05$.

Table III. Glycerophospholipid fatty acid composition (% wt/wt, median, and interquartile ranges) of serum samples obtained from 951 children aged 2 years (549) or 6 years (402)

FA	Median	IQR
Saturated FA		
C14:0	0.53	0.31
C16:0	28.17	1.67
C17:0	0.41	0.08
C18:0	14.89	1.31
∑ SFA	43.95	1.59
Trans FA		
C16:1t	0.03	0.02
C18:1t	0.17	0.09
C18:2tt	0.09	0.04
C22:1t	0.11	0.06
∑ *trans*	0.42	0.15
Cis monounsaturated FA		
C15:1n-5	0.07	0.03
C16:1n-7	0.58	0.23
C18:1n-7	1.28	0.19
C18:1n-9	12.03	1.77
C20:1n-9	0.14	0.03
∑ MUFA	14.11	1.88
n-9 PUFA		
C20:3n-9	0.22	0.11
n-6 PUFA		
C18:2n-6	22.70	3.64
C18:3n-6	0.10	0.05
C20:2n-6	0.30	0.07
C20:3n-6	2.94	0.72
C20:4n-6	9.22	1.86
C22:4n-6	0.38	0.10
C22:5n-6	0.42	0.14
∑ n-6 LC-PUFA	13.33	2.14
∑ n-6 PUFA	36.27	2.99
n-3 PUFA		
C18:3n-3	0.22	0.10
C20:3n-3	0.06	0.02
C20:5n-3	0.51	0.24
C22:5n-3	0.92	0.24
C22:6n-3	2.90	0.91
∑ n-3 LC-PUFA	4.44	1.08
∑ n-3 PUFA	4.68	1.02

samples were obtained by keeping venous blood at room temperature for at least 30 minutes for clotting before centrifugation and freezing serum. Although some lipolysis remains active at room temperature during the clotting period, the percentage composition of GP FAs in serum and plasma is similar (Table I).

When stored at –80°C, plasma or serum samples have been reported to be stable for as long as 4 years.[11] Zeleniuch-Jacquotte et al[24] showed that by storing serum samples for as long as 12 years at –80°C, PUFAs were well preserved from oxidative degradation. For our investigations, serum samples were frozen directly after sampling and stored at –80°C until analysis. Analyses of control serum aliquots and earlier investigations[18] show that the newly developed high-throughput method for analyzing GP FA composition is robust and provides reproducible results.

There was little effect of age on FA composition of serum GPs, although the Wilcoxon rank test is very sensitive because of the large number of samples and the small interquartile ranges. We assume that dietary preferences and food patterns changed considerably from the age of 2 years to 6 years. At the age of 2 years, children tend to have a more stable food pattern, whereas older children should have a greater day-to-day variability of food intake. Guerra et al[13] showed that dietary fat intakes changed considerably in children from 2 years of age to school age, with an increasing percentage of dietary energy contributed by fat. However, our results show only very minor differences in the two investigated points and no consistent trend. Age-related effects on serum GP FA compositions between 2- and 6-year-old children were negligible and without physiological relevance. Our findings agree with those of Decsi and Koletzko,[4] who found no major differences in plasma PL FA composition for children from the age of 1 to 15 years, whereas infants showed a divergent FA pattern.

No major sex differences in GP FA compositions were observed, which agrees with other publications[4,25] on the FA composition of plasma lipid classes (PLs, TAGs, and cholesteryl esters) and erythrocyte lipids.

Plasma or serum GP FA composition is considered a good biomarker for dietary FA intake and body status, especially for PUFAs.[18] Fatty acid composition of human plasma or serum total PLs and individual PL classes have been reported, but we found no data on the FA composition of plasma or serum total GPs.

Our median FA values in serum GPs are in very good agreement with published values in serum or plasma PLs for most of the analyzed FAs.[4,12-15] We found differences for the values of these saturated and monounsaturated FAs: C20:0, C22:0, C24:0, and C24:1n-6. These FAs showed a high abundance in SMs, but not in GPs.[18,26] In epidemiological or clinical studies, these FAs are often poorly assessed, because they occur only in minor amounts in PLs, can be synthesized endogenously, and vary widely from day to day within an individual.[27] For trans FAs, we found lower values in serum GPs than Decsi and Koletzko[4] in plasma PLs. This could be caused by the different fractions that were analyzed or to a reduced trans FA exposure with time. In the human diet, trans FAs originate from meat and milk of ruminant animals, but are also derived from commercially processed food (eg, hydrogenation of oils).[28,29] Trans FA exposure of infants and children decreased considerable during the last 2 decades,[25] which may explain why our trans FA results in children are lower than those published 15 years ago.

We present the reference values for serum GP FA status in children. When applying these data to other populations, regional life style, and in particular dietary habits should be considered. The method used is robust and provides results of excellent quality, which are reproducible in a long period of analysis. The method is suitable for plasma and for serum samples and reveals comparable results, thus serum and plasma samples are equally suitable for the evaluation of FA status. The method enables high throughput analyses. Presently, there is no other high throughput method available for these analyses. Studies with high numbers of subjects become more easily feasible with this high-throughput

method, such as studies exploring associations of PUFA status and genetic polymorphisms of FA metabolism[30,31] or studies on the relationship of FA status with clinically relevant outcomes such as cognitive development of children[32] or allergic disease risk in adults.[33] ■

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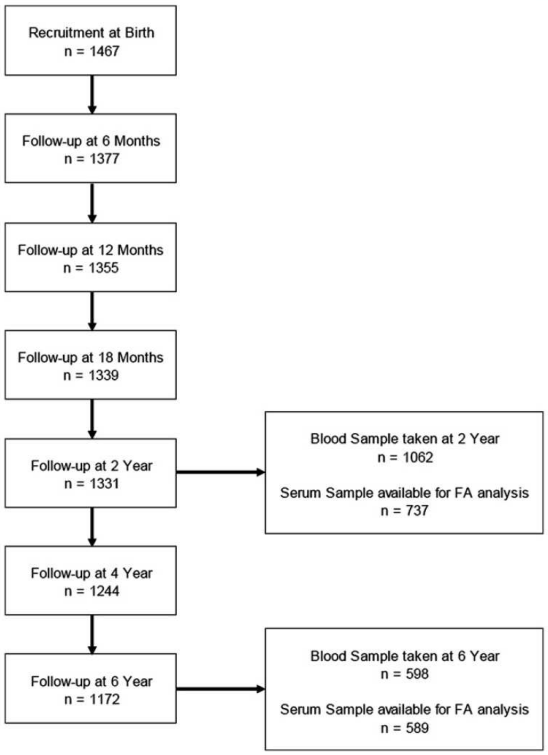

Figure 1. Outline of the LISA study subgroup Munich.

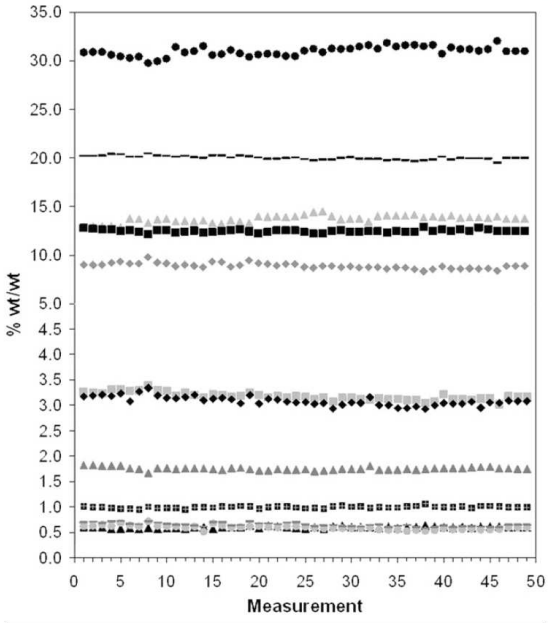

Figure 2. Glycerophospholipid fatty acid composition (% wt/wt) of the control serum measured in the whole analyzing period of 129 days together with each of all 49 sample batches. For clarity only, the most abundant fatty acids, contributing at least 0.5 % to total fatty acids in glycerophospholipids, are shown (C16:0, C18:2n-6, C18:0, C18:1n-9, C20:4n-6, C20:3n-6, C20:5n-3, C18:1n-7, C16:1n-7, C22:5n-3, C22:6n-3, C14:0).

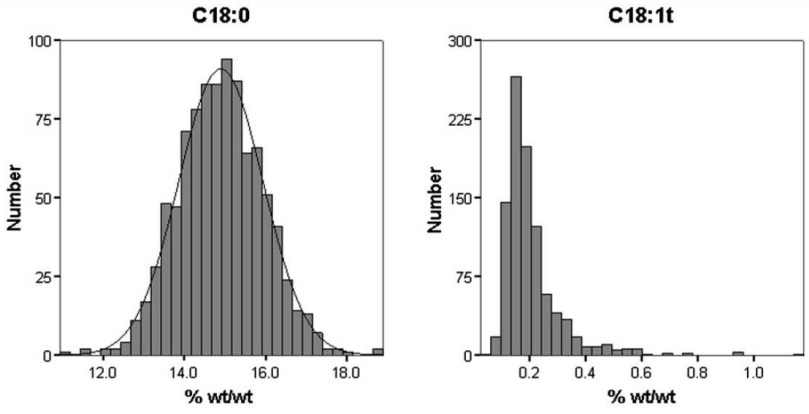

Figure 4. Distribution of stearic acid (C18:0) and elaidic acid (C18:1t) in serum samples obtained from 951 children aged 2 years (549) or 6 years (402).

5. Publication 4: “Influence of *FADS* polymorphisms on tracking of serum glycerophospholipid fatty acid concentrations and percentage composition in children”

5. PUBLICATION 4

Influence of *FADS* polymorphisms on tracking of serum glycerophospholipid fatty acid concentrations and percentage composition in children

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Short title: Association of *FADS* variants and FA tracking

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Abstract *(222 words)*

Background: Tracking of fatty acid (FA) contribution to plasma or serum lipids over time was shown in children and adults. However, the potential role of *FADS* gene variants has not been investigated.

Methods and Principal Findings: Serum GP FA composition of 331 children aged 2 and 6 years, participating in an ongoing birth cohort study, was analyzed. Correlation coefficients were estimated to describe FA tracking over 4-years and to assess the influence of *FADS* variants on tracking. We found low to moderate tracking (r = 0.12 - 0.49) of FA compositions and concentration between 2 and 6 years. Concentration changes of total monounsaturated FA and total saturated FA over time correlated closely (r = 0.79) but percentage values were unrelated (r = -0.02). Tracking for n-6 long chain polyunsaturated fatty acid (LC-PUFA) concentrations was lower in subjects homozygous for the major allele of *FADS* variants and higher in carriers of at least one minor allele, whereas for total n-3 LC-PUFA concentrations and compositions this was vice versa. For individual n-3 PUFA inconsistent results were found.

Conclusions and Significance: Serum GP FA composition shows low to moderate tracking over 4-years with a higher tracking for LC-PUFA metabolites than for their precursor FA. Serum PUFA levels and their tracking seem to be more influenced by lipid and lipoprotein metabolism than by FA specific pathways.

Key Words: arachidonic acid, children, docosahexaenoic acid, *FADS1 FADS2* gene cluster, polyunsaturated fatty acids

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Introduction

Biomarkers of fatty acid (FA) status are widely used in observational studies. They reflect a combination of dietary intake and metabolism. Associations of FA status with current and future health indicators have been demonstrated [1-2]. Epidemiological and clinical studies have revealed associations between FA and cardiovascular diseases, diabetes, and certain types of cancer [3-5].

The concentrations of individual FA in plasma and tissues do not evolve independently, but rather mutually influence each other. As individual FA are preferentially partitioned into specific lipid pools [6], the percentage FA composition of specific compartments is widely used to describe FA status. Although analyses of FA status are successfully applied for the evaluation of dietary intake [7], FA status is influenced by both diet and endogenous metabolism. In several studies strong associations were found between variants in the human genes *fatty acid desaturase 1 (FADS1)* and *fatty acid desaturase 2 (FADS2)* and blood levels of polyunsaturated fatty acids (PUFA) [8-13]. These associations clearly indicate an influence of endogenous metabolism on the blood levels of essential FA. The importance of endogenous metabolism is even greater for long chain polyunsaturated fatty acids (LC-PUFA) than for the saturated and monounsaturated FA which can be synthesized de novo by human metabolism [14].

FA status and in particular the balance of n-3 and n-6 LC-PUFA have been related to long term health [15-16]. LC-PUFA have been demonstrated in all lipid compartments considered so far as indicative of human FA status. Their concentrations are highest in glycerophospholipids (GP), which suggests their suitability for describing sensitively FA status including LC-PUFA status. Due to the differences of LC-PUFA concentrations in different

compartments (e.g. plasma lipid fractions), analysis of defined, purified fractions is mandatory in order to avoid misleading results.

Another important aspect when evaluating a biomarker is temporal variability, which depends on the turnover of the compartment. The composition of plasma lipids varies within days or weeks, whereas significant changes of FA composition in adipose tissue are only observed after months [7]. As plasma lipid composition may change within short periods, an investigation of the tracking of plasma lipid FA status over years yields information on longer term changes of dietary habits and life style factors. In adults it has been shown that FA composition of plasma cholesteryl esters and phospholipids showed a high degree of tracking, with coefficients of correlation between FA percentages up to 0.83 [17-18]. Similar values were observed in total plasma phospholipids of a small group of Portuguese children (n = 26). However, this observation might be very much influenced by the given cultural and socioeconomic conditions of this particular group of children [19].

We aimed to reevaluate the findings of Guerra et al. [19] in a larger, population-based sample of children. Furthermore, we aimed to investigate the influence of polymorphisms in the *FADS* gene cluster on FA tracking which has not been investigated so far.

According to current knowledge, the carriers of the major alleles of the 5 studied SNPs (rs174545, rs174546, rs174556, rs174561, rs3834458) should have a higher conversion of LA and ALA to their corresponding derivates than carriers of at least one minor allele [8,11-12]. We hypothesized that carriers of the major allele would show a greater stability of LC-PUFA values over time and therefore show a greater degree of LC-PUFA tracking than carriers of at least one minor allele.

Methods

Ethics statement

For this study, approval by the respective local Ethics Committees (Bavarian General Medical Council, University of Leipzig, Medical Council of North-Rhine-Westphalia) and written informed consent from the families (parents) of participants were obtained.

Study design and population

The LISA study ("Influences of Lifestyle related Factors on the Immune System and the Development of Allergies in Childhood") is an ongoing population-based birth cohort study of unselected newborns. The design of this study has been described elsewhere before [20-21]. Between November 1997 and January 1999, 3097 healthy full-term newborns were recruited in Munich (n=1467), Leipzig (n=976), Wesel (n=306), and Bad Honnef (n=348). Neonates were excluded if at least one of the following exclusion criteria was present: preterm birth (maturity < 37 gestational week), low birth weight (< 2500 g), congenital malformation, symptomatic neonatal infection, antibiotic medication, hospitalisation or intensive medical care during neonatal period. In addition to lack of consent to participate in this study, newborns from mothers with immune-related diseases such as autoimmune disorders, diabetes, hepatitis B, long-term medication or abuse of drugs and alcohol, and newborns from parents with a nationality other than German and parents who were not born in Germany were excluded.

We collected questionnaire data on family history of atopy, parental education and health problems during pregnancy, smoking of the mother during pregnancy and mothers' exposure to environmental tobacco smoke during pregnancy at home. The cohort was followed up at

the ages of 6, 12 and 18 months, and 2, 4, 6 and 10 years. Blood samples were collected at birth (cord blood) and from the children at 2, 6 and 10 years.

For the present analyses, only data from the 2- and 6-year follow-up of study center Munich (n=1331 and n=1172, respectively) were included. Serum samples both at the age of 2 and 6 years were available for analysis in 375 children (213 boys, 162 girls). Both genotyping data and fatty acid data were available for 331 children. **Figure 1** shows the origin of the samples.

Fatty acid analysis

Venous blood samples were collected in serum separator tubes and centrifuged. Serum was frozen in plastic vials and stored at -80 °C until analysis. Glycerophospholipid FA were analyzed as described earlier [22]. Briefly, 100 µl of internal standard (1,2-dipentadecanoyl-sn-glycero-3-phosphocholine dissolved in methanol) and 0.6 ml methanol were added to 100 µl of serum, and samples were shaken for 30 s. After centrifugation the supernatant was transferred into another glass tube. Twenty-five µl of sodium methoxide solution were added and tubes were shaken for synthesis of fatty acid methyl esters (FAME). The transesterification was stopped after 3 min by adding 75 µl methanolic HCl. FAME were extracted twice with 300 µl hexane each. Extracts were combined and dried under nitrogen flow at room temperature. The residue was taken up in 50 µl hexane (containing 2 g/l butylated hydroxy toluene) and analyzed by gas chromatography (GC).

We used GC with flame ionization detection for quantifying the FAME. Individual FAME were identified by comparison with authentic standards (GLC-569B, Nu-Check Prep, Inc., Elysian, MN, USA; *cis*-5,8,11-eicosatrienoic acid methyl ester, Sigma-Aldrich, Taufkirchen, Germany; methyl vaccenate (11c), methyl octadecatetraenoate (6c, 9c, 12c, 15c) and methyl brassidate (13tr), Larodan Fine Chemicals AB, Malmö, Sweden). We used the general designations

C16:1t, C18:1t, C18:2tt and C22:1t in the results section because we cannot exclude that different isomeric *trans* FA coelute.

As external standard GLC-85 (Nu-Check Prep, Inc., Elysian, MN, USA) was used for determining the response relative to pentadecanoic acid methyl ester (internal standard). For peak integration EZChrom Elite version 3.1.7 (Agilent, Waldbronn, Germany) was used.

Genotyping

Genomic DNA was extracted from EDTA blood using standard methods and amplified by using REPLI-g UltraFast technology (Qiagen™). Five variants of the *FADS1 FADS2* gene cluster (rs174545, rs174546, rs174556, rs174561, rs3834458) were typed, which have been previously shown to be in strong linkage disequilibrium with each other [8,11]. SNPs were selected based on previous publications [8,11-12]. Applying the tagger server program (http://www.broadinstitute.org/mpg/tagger/) in combination with HapMap we found that with the 3 SNPs rs174545, rs174546 and rs174556 27 SNPs between basepair positions 61234329 and 61372379 of *FADS1 FADS2* could be tagged. The efficiency was 10.7 fold even though the two further SNPs rs174561 and rs3834458 could not be included as these are not included in the HapMap database. Genotyping of SNPs was realized with the iPLEX (Sequenom, San Diego, CA, USA) method by means of matrix assisted laser desorption ionization-time of flight mass spectrometry method (MALDI-TOF MS, Mass Array; Sequenom) according to the manufacturer's instructions. Standard genotyping quality control included 10 % duplicate and negative samples. Genotyping discordance rate was below 0.3 %.

Statistical analysis

For the studied subpopulation allele frequencies and Fisher's exact test of Hardy-Weinberg-Equilibrium were conducted with procedure "proc allele" of the statistical software module SAS/GENETICS of SAS version 9.1.3. Lewontin's D' and pairwise-squared correlations r^2 were calculated with the software JLIN [23] to examine linkage disequilibrium.

FA data are presented as medians and interquartile ranges (IQR, ranges from the 1st to the 3rd quartile), since FA with very low concentrations showed skewed distributions.

Level of tracking between FA data obtained at the two time points was estimated by Spearman correlation. This is the simplest way for continuous outcome variables to asses tracking between two measurements [24]. Furthermore, Spearman correlation coefficients were calculated for the single SNPs applying an additive model where homozygous minor allele carriers were coded as 2, heterozygous coded as 1, and homozygous major allele carriers coded as 0 (= reference category). Spearman correlations were performed using the statistical software PASW Statistics, version 18.0.0.

Results

Baseline characteristics for the total study population and for the studied subpopulation of the LISA Munich cohort are listed in **Table 1**. Weight, height and BMI are comparable between the total and the subpopulation. However, the percentage of boys, fully breastfeeding and high maternal education is somewhat higher and the proportion of maternal smoking during pregnancy is slightly lower in the studied subpopulation.

Information regarding position, possible functional region, genotyping frequencies and *P*-values of Fisher's exact test for violation of Hardy-Weinberg-Equilibrium for the five analyzed SNPs of the *FADS1 FADS2* gene cluster are given in **Table 2**. The minimum *P*-value for any of the five analyzed SNPs was 0.69 (rs174556 and rs174561).

Lewontin's D' and pairwise-squared correlations r^2 for the studied subpopulation are shown in **Figure 2**. Lewontin's D' ranged between 0.97 and 1.0 and the pairwise-squared correlations r^2 ranged between 0.87 and 1.0, confirming that all five SNPs are in high linkage disequilibrium.

Fatty acid composition of serum GP

Five FA, namely the saturated FA palmitic (C16:0) and stearic acid (C18:0), the monounsaturated FA oleic acid (C18:1n-9) and the two n-6 PUFA linoleic acid (LA, C18:2n-6) and arachidonic acid (AA, C20:4n-6), showed the highest concentrations in serum GP and accounted for more than 85 % of total GP FA (**Supporting Table 1**). Up to 45 % of all serum GP FA were saturated, ~40 % were polyunsaturated, and ~15 % were monounsaturated. *Trans* FA were found in minor quantities, contributing less than 0.5 % to total GP FA. The abundance of n-6 PUFA was ~7 times higher than that of n-3 PUFA in GP.

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Concentrations of total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA) and PUFA were higher at 6 years compared to 2 years, while the concentration of total *trans* FA did not differ with time. Percentage values did not differ significantly between both time points. However, the PUFA/SFA ratio increased from 0.92 at 2 years to 0.96 at 6 years. N-6 PUFA showed higher values at 6 years compared to 2 years, while n-3 PUFA values remained constant. The n6/n3 ratio increased from 7.5 to 8.2 over time.

Changes in concentrations of individual FA or groups of FA were positively correlated with each other for most FA (data not shown), e.g. changes of LA concentrations from 2 to 6 years were highly correlated with changes of the concentrations of total SFA (0.71***), total MUFA (0.49***), C18:3n-6 (0.30***), C20:3n-6 (0.25***), C20:4n-6 (0.41***) and C18:3n-3 (0.43***). For percentage values changes in LA were negatively correlated with the changes in the percentage values of total SFA (-0.50***), total MUFA (-0.40***), C20:3n-6 (-0.45***), C20:4n-6 (-0.34***), C20:5n-3 (-0.35***), and C22:6n-3 (-0.37***). Besides the correlation with LA, total SFA and total MUFA percentage values were not correlated with other FA percentage values (data not shown). The changes of total SFA concentrations over time were highly correlated with the changes of total MUFA concentrations (r = 0.79***, **Figure 3a**), whereas no correlation was observed between the changes of total SFA and total MUFA percentages over the four year period (r = -0.02, **Figure 3b**).

Tracking

For most FA the percentage contributions and concentrations (**Supporting Table 1**) at the age of 2 years were significantly correlated to those at 6 years. FA concentrations were stronger correlated between both time points than FA percentage contributions. The highest correlations between both time points were observed for the n-6 LC-PUFA dihomo-gamma-linolenic acid (DGLA, C20:3n-6), AA and docosapentaenoic acid (DPA, C22:5n-6) for both, concentrations and percentage values.

Moderate tracking ($0.6 > r > 0.4$) was observed for the percentage values of four FA (C17:0, C20:3n-6, C20:4n-6, and C22:5n-6) and for the concentrations of six FA (C17:0, C18:0, C20:3n-6, C20:4n-6, C22:5n-6, and C22:5n-3), respectively. The majority of all analyzed FA showed low or no tracking ($r < 0.4$).

Concentrations of SFA in serum GP showed a higher degree of tracking than their corresponding percentage values. Interestingly, total SFA percentage values showed no correlation whereas single SFA showed low to moderate tracking. For n-6 PUFA moderate correlations between both time points were observed, with the highest correlations for DGLA, and weaker correlations for their precursor FA LA and gamma-linoleic acid (GLA, C18:3n-6). Tracking of n-3 PUFA was markedly lower than that of n-6 PUFA, with lower tracking rates for the precursor n-3 PUFA compared to their longer chain derivates. Similar findings were observed for n-9 FA. Percentage values of mead acid (20:3n-9) were higher correlated between both time points ($r = 0.39$) than those of oleic acid ($r = 0.22$), a precursor of mead acid. *Trans* FA and MUFA had weak correlations.

The ratio of the n-6 PUFA AA/LA showed a moderate degree of tracking ($r = 0.41$), and the ratio remained practically constant over the 4 year period with 0.42 ± 0.13 at 2 years, and 0.40 ± 0.11 at 6 years, respectively. There was an increasing trend over time for the n-3

PUFA ratios EPA/ALA (2 years: 2.31 ± 1.27; 6 years: 2.50 ± 1.51) and DHA/ALA (2 years: 12.72 ± 8.23; 6 years: 13.58 ± 8.34). Compared to the n-6 PUFA ratio of AA/LA somewhat lower tracking levels were observed for the n-3 PUFA ratios EPA/ALA (r = 0.20) and DHA/ALA (r = 0.20), respectively.

Influence of FADS polymorphisms on tracking

We found that tracking of n-6 LC-PUFA concentrations (except for C22:4n-6) was higher for homozygous and heterozygous carriers of minor alleles in the five analyzed SNPs than for homozygous carriers of major alleles (**Supporting Table 2**). In contrast, tracking of n-6 LC-PUFA compositions showed no clear trend. For total n-3 LC-PUFA concentrations and compositions, minor allele carriers had a lower tracking compared to homozygous major allele carriers but individual n-3 LC-PUFA showed no consistent trend. Tracking of C20:3n-3 and EPA was lower in minor allele carriers than in carriers homozygous for the major allele, whereas results for C22:5n-3 were inconsistent. For DHA tracking was comparable between the 3 groups in all analyzed SNPs.

Discussion

Fatty acid status

We found increasing serum GP total FA concentrations over the 4-year follow-up. The children studied had higher serum PUFA concentrations at the age of 6 than at 2 years, whereas SFA and MUFA concentrations showed only a slight increase over time. Hence, the PUFA/SFA ratio increased with age. These observations point towards a change in food habits with a shift to higher proportions of polyunsaturated dietary fat. Our findings are in accordance with observations from Guerra et al. [19] who found an increase of plasma phospholipid PUFA/SFA ratio in 26 Portuguese children from 2 to 5 years of age.

Plasma GP n-6 PUFA concentrations increased during the follow-up, which likely reflects a higher intake of foods providing LA or AA (e.g. vegetable oils, eggs, meat and meat products) at older age [25]. In contrast, plasma concentrations of n-3 PUFA remained almost constant over time. This is presumably attributable to a low consumption of n-3 PUFA in the diets of most German children. The main dietary sources of n-3 LC-PUFA are fish and other seafood. Children who like to eat fish and whose parents pay attention to a regular fish intake are likely to maintain such habits over time, and a relatively high n-3 LC-PUFA level will persist; whereas children who tend not to eat fish retain a low n-3 LC-PUFA level. Our findings suggest that only few children change their food habits with regard to fish intake over time.

The strong positive correlation between the changes in the concentrations of total MUFA and total SFA, points towards a strong influence of lipid metabolism on FA concentrations. This assumption is strengthened by the absence of a correlation between changes in the percentage values of total MUFA and total SFA, as well as previous findings of Moilanen et al. [26] who reported a significant association between serum cholesteryl ester FA composition and various serum lipids.

LA values seem to have a strong influence on the percentage composition of serum GP FA, whereas the impact of SFA and MUFA on FA composition is very low. The correlations between the changes in FA percentages over the 4-year period indicate that the changes in LA mainly affected SFA and MUFA. We found negative correlations of LA with C20:3n-6, C20:4n-6, C20:5n-3 and C22:6n-3, which was in accordance with previous findings [27].

Tracking

Tracking of FA percentage values in plasma or serum phospholipids and cholesteryl esters has previously been reported in children and adults [17-19,27], but tracking of FA concentrations has not been investigated before. Our results revealed tracking rates of similar magnitude compared to findings of Guerra et al. [19] for total SFA (0.02/-0.02, our results/Guerra et al.) and total MUFA (0.22/0.25), whereas n-6 LC-PUFA (0.35/0.63) and n-3 LC-PUFA (0.23/0.31) showed a somewhat lower tracking in our study. This might be explained by the different study settings. Guerra et al. examined tracking of plasma phospholipid FA in 26 Portuguese children between the age of 2 and 5 years, whereas we examined tracking of FA in serum GP (a subgroup of phospholipids) in a larger group of 331 German children between the ages of 2 and 6 years. Previous publications show that tracking of plasma cholesteryl ester FA is higher than that of plasma phospholipid FA, and that FA tracking might be higher in adults than in children [17-18,28]. However, independent from the studied subjects and the analyzed lipid fraction, our results are consistent with previously published data on FA tracking in that n-6 LC-PUFA showed greater tracking than n-3 LC-PUFA, and that LA and ALA have a lower degree of tracking than their LC-PUFA derivates.

We also determined tracking of FA concentrations, which turned out to be somewhat greater than the tracking of FA percentages, particularly for SFA. This further supports that lipid

metabolism might have a significant influence on serum GP FA concentrations. The high tracking of C17:0, which reflects the intake of dairy fats, might indicate a stable dietary milk and dairy product intake. Patterson et al. [29] reported a tracking correlation coefficient of 0.3 for dietary intakes of milk and yoghurt in Swedish children over a period of 6 years.

Our hypothesis that carriers of the major allele show higher tracking than carriers of at least one minor allele could be confirmed only for serum GP total n-3 LC-PUFA levels. For individual n-3 PUFA this trend is not consistent. In contrast to our expectation, tracking of total n-6 LC-PUFA percentage and concentration values was lower in subjects homozygous for the major allele than in subjects carrying one minor allele. However, tracking values were comparable between carriers homozygous for the major allele and carriers homozygous for the minor allele, except for SNP rs3834458, here tracking values of subjects homozygous for the minor allele were comparable with theses of subjects carrying one minor and one major allele.

We assume that the major portion of serum GP n-6 LC-PUFA levels are contributed by endogenous synthesis via the desaturase/elongase pathway, which was shown to be largely influenced by genetic variants in the *FADS1 FADS2* gene cluster [11]. Linoleic acid, the n-6 LC-PUFA precursor, is the most abundant PUFA in diet and serum lipids. Serum GP LA values are largely determined by dietary intake. In our study we found an increase of LA levels over time and only slight tracking for LA values. Thus, subjects with higher LA conversion rates have a higher variability in n-6 LC-PUFA levels depending on available LA and therefore tend to show a lower tracking. The hypothesized association of higher tracking with higher conversion intensity can only be observed for very low precursor levels, as is the case for n-3 PUFA.

In conclusion, the present study documents low to moderate tracking of serum GP FA composition over a period of 4-years. The highest tracking was observed for n-6 LC-PUFA. Furthermore, LC-PUFA metabolites have a higher tracking than their precursor FA. We found that *FADS1 FADS2* gene variants modulate tracking of serum GP PUFA levels. However, results are inconsistent and the influence of general lipid and lipoprotein metabolism seems to be more pronounced than the influence of FA specific pathways. As a consequence FA concentrations show a higher degree of tracking than FA percentage values.

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The method for the analysis of GP FA composition in serum described in this article is in patent pending status.

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FIGURE LEGEND

Figure 1. Analyzed serum samples and genetic measurements from the Munich LISA cohort.

Figure 2. Pair wise linkage disequilibrium measured by Lewontin's D' and r^2 for the common five single nucleotide polymorphisms (SNP) in the studied subpopulation (n=331) of the LISA Munich cohort.

Figure 3. Changes of total monounsaturated fatty acids between 2 and 6 years (delta MUFA, mg/l) show a high correlation (r = 0.79) with changes of total saturated fatty acids (delta SFA, mg/l) (a), while changes of corresponding percentage values (%) are unrelated (b, r = -0.02).

Table 1. Baseline characteristics of the total study population and the studied subpopulation of the LISA Munich cohort.

	Total population			Studied subpopulation		
	n	mean or %	SD	n	mean or %	SD
% boys	1467	52.7		331	55.6	
% girls	1467	47.3		331	44.4	
birth weight (kg)	1466	3.4	0.4	331	3.4	0.4
birth length (cm)	1445	52.1	2.4	326	52.0	2.3
% fully breastfed for at least 4 months	1371	68.7		329	71.4	
% high maternal education	1457	63.9		328	66.5	
% maternal smoking during pregnancy	1458	14.2		328	11.0	

Table 2. Characteristics of the five analyzed variants in *FADS1 FADS2* gene of the studied subpopulation (n= 331).

dbSNP	Position	Possible functional region (on chromosome 11)	Alleles (major/minor)	Number (%) of subjects with genotype			*P*-value (Fischer's exact test)
	bp		1/2	11	12	22	
rs174545	61325882	*FADS1 UTR-3*	C/G	157 (47.6)	139 (42.1)	34 (10.3)	0.70
rs174546	61326406	*FADS1 UTR-3*	C/T	158 (47.7)	139 (42.0)	34 (10.3)	0.70
rs174556	61337211	*FADS1 intron 2*	C/T	166 (50.5)	138 (41.9)	25 (7.6)	0.69
rs174561	61339284	*FADS1 intron 1*	T/C	166 (50.5)	138 (41.9)	25 (7.6)	0.69
rs3834458	61351497	*FADS2 5' flanking*	T/Del	154 (47.1)	142 (43.4)	31 (9.5)	0.90

Note: Del indicates deletion; SNP build 131 accessed 27 April 2010, Map to Genom Build 36.3,

11 = homozygous major, 12 = heterozygous, 22 = homozygous minor

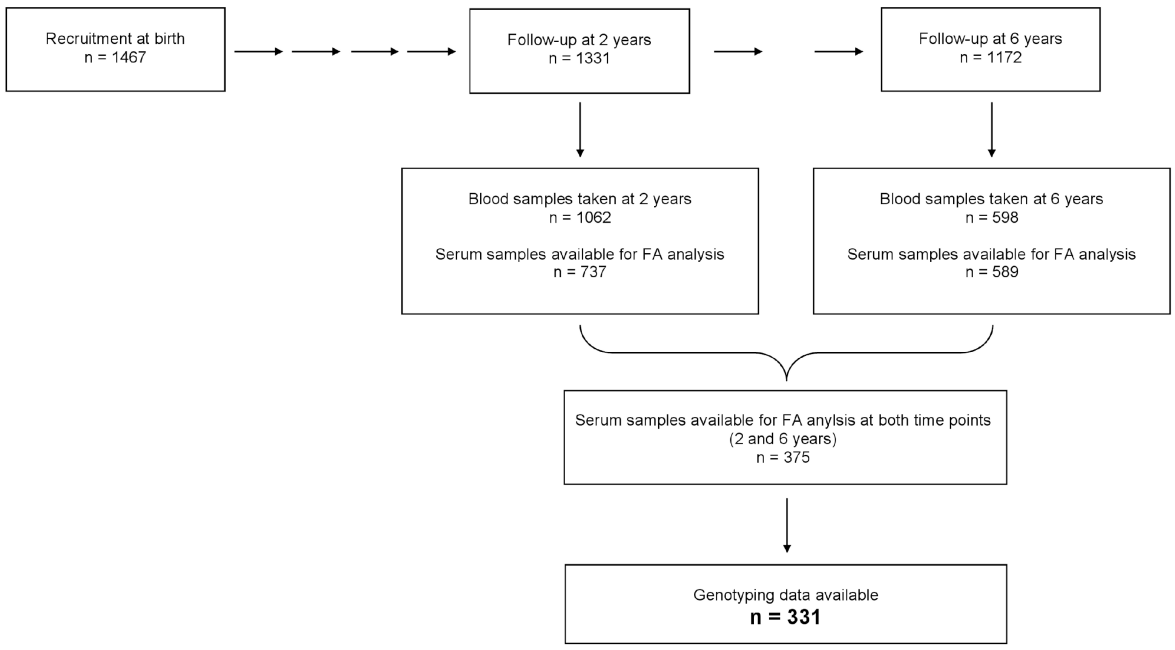

Recruitment at birth
n = 1467
Follow-up at 2 years
n = 1331
Follow-up at 6 years
n = 1172
Blood samples taken at 2 years
n = 1062
Serum samples available for FA analysis
n = 737
Blood samples taken at 6 years
n = 598
Serum samples available for FA analysis
n = 589
Serum samples available for FA anylsis at both time points
(2 and 6 years)
n = 375
Genotyping data available
n = 331

Figure 1

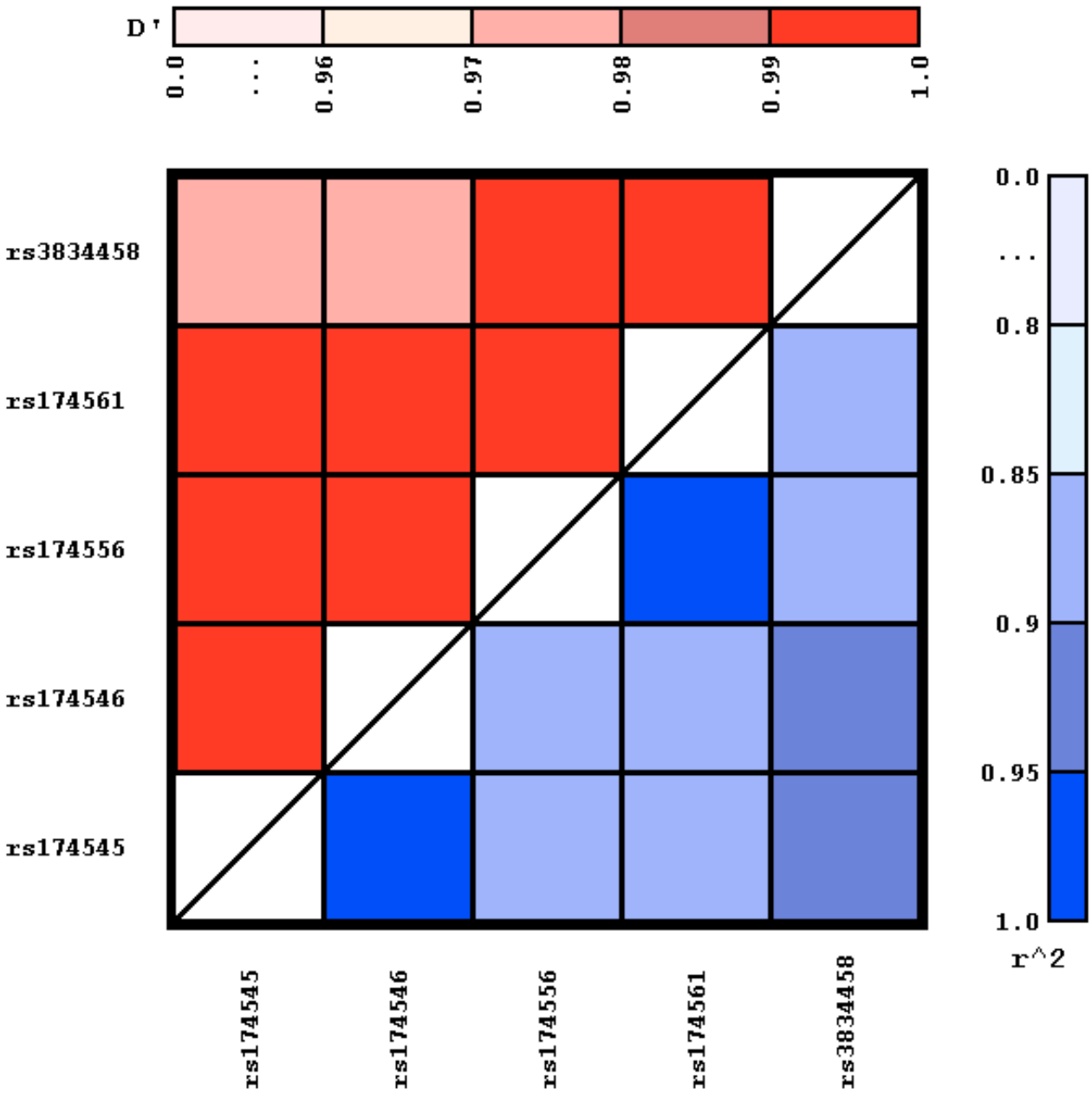
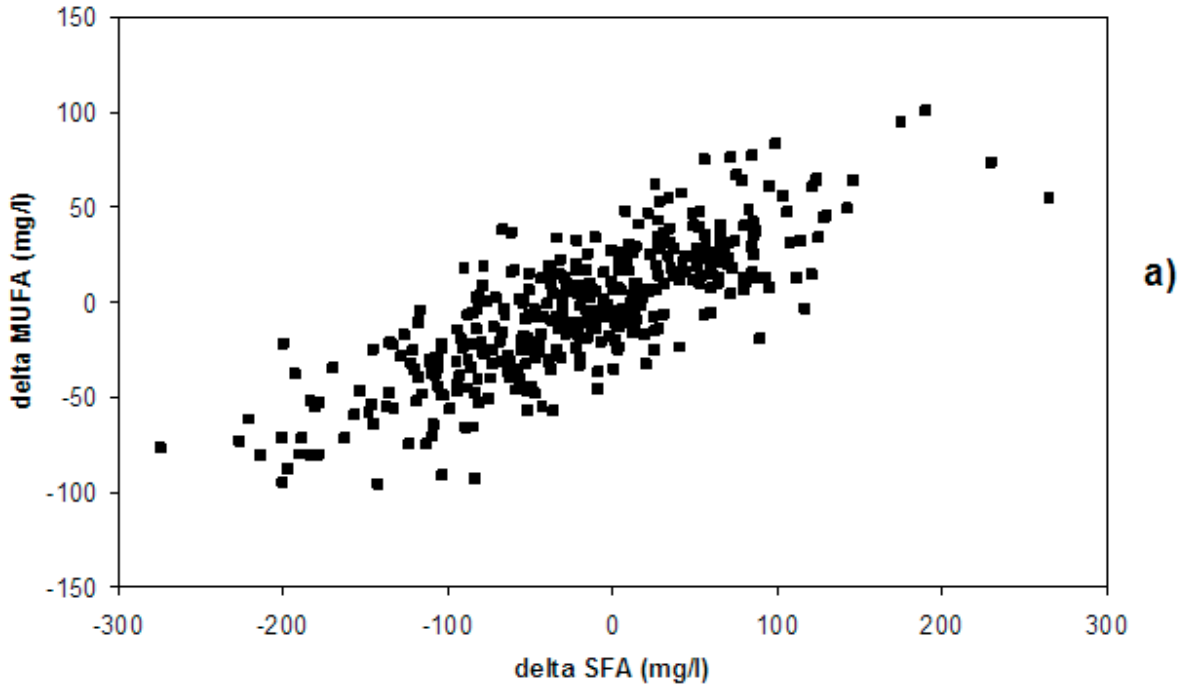

D'
0.0
...
0.96
0.97
0.98
0.99
1.0
rs3834458
rs174561
rs174556
rs174546
rs174545
rs174545
rs174546
rs174556
rs174561
rs3834458
0.0
...
0.8
0.85
0.9
0.95
1.0
r^2

Figure 2


150
100
50
0
-50
-100
-150
-300
-200
-100
0
100
200
300
delta MUFA (mg/l)
delta SFA (mg/l)
a)

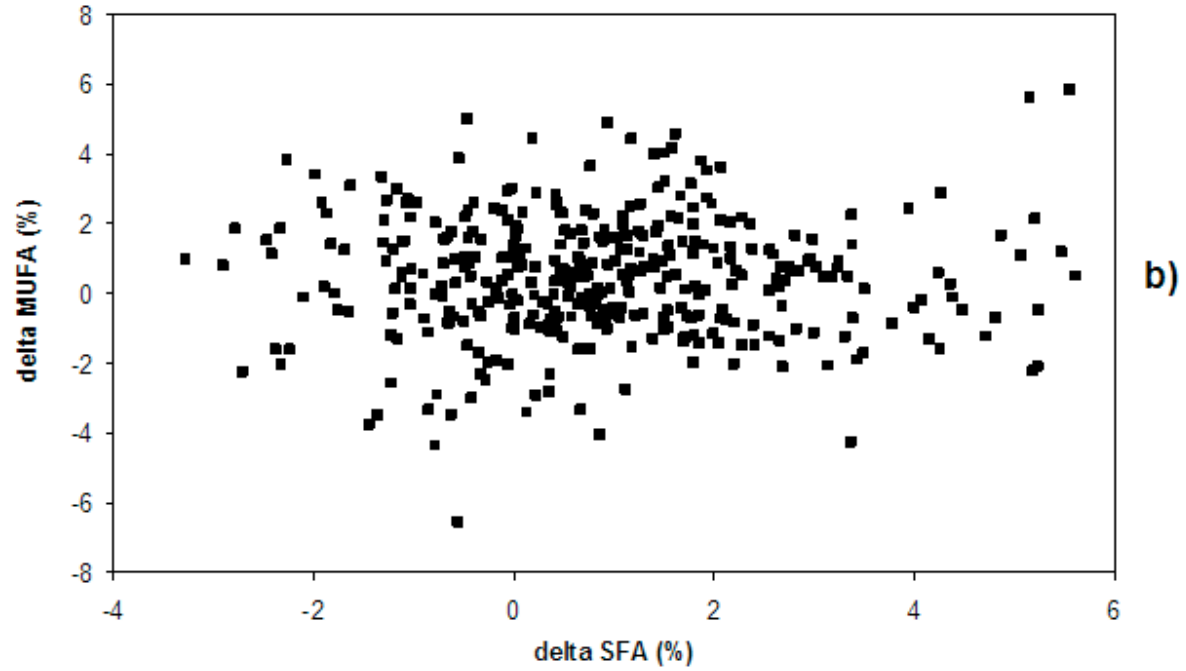

8
6
4
2
0
-2
-4
-6
-8
-4
-2
0
2
4
6
delta MUFA (%)
delta SFA (%)
b)

Figure 3

Supporting Table 1. Tracking of serum glycerophospholipid fatty acids between both time points of the studied subpopulation (n= 331) estimated by Spearman correlations.

Fatty acid	Composition (%)				Concentration (mg/l)			
			Spearman				Spearman	
	2 years	6 years	R	(Significance)	2 years	6 years	R	(Significance)
Saturated FA								
C14:0	0.52 (0.33)	0.48 (0.25)	0.15	(0.006)	5.9 (3.8)	5.7 (3.5)	0.17	(0.002)
C16:0	28.56 (1.59)	27.47 (1.42)	0.28	(< 0.001)	326.1 (58.3)	331.1 (67.0)	0.40	(< 0.001)
C17:0	0.42 (0.09)	0.40 (0.07)	0.42	(< 0.001)	4.8 (1.3)	4.7 (1.3)	0.45	(< 0.001)
C18:0	14.68 (1.36)	15.07 (1.14)	0.33	(< 0.001)	165.3 (36.9)	180.3 (43.1)	0.47	(< 0.001)
Sum SFA	44.13 (1.45)	43.43 (1.33)	0.02	(0.763)	506.6 (91.8)	517.2 (113.4)	0.42	(< 0.001)
Trans FA								
C16:1t	0.03 (0.02)	0.03 (0.01)	0.26	(< 0.001)	0.4 (0.1)	0.3 (0.1)	0.29	(< 0.001)
C18:1t	0.17 (0.08)	0.17 (0.09)	0.18	(< 0.001)	1.9 (1.0)	2.1 (1.0)	0.19	(< 0.001)
C18:2tt	0.09 (0.04)	0.08 (0.04)	0.28	(< 0.001)	1.1 (0.5)	1.0 (0.5)	0.33	(< 0.001)
C22:1t	0.10 (0.05)	0.10 (0.05)	0.20	(< 0.001)	1.1 (0.8)	1.2 (0.7)	0.2	(< 0.001)
Sum *trans*FA	0.42 (0.16)	0.41 (0.14)	0.22	(< 0.001)	4.8 (2.1)	4.9 (1.8)	0.26	(< 0.001)
Cis monounsaturated FA								
C15:1n-5	0.07 (0.03)	0.07 (0.03)	0.28	(< 0.001)	0.8 (0.4)	0.8 (0.2)	0.32	(< 0.001)
C16:1n-7	0.58 (0.23)	0.55 (0.21)	0.31	(< 0.001)	6.3 (3.3)	6.6 (2.8)	0.31	(< 0.001)
C18:1n-7	1.29 (0.21)	1.26 (0.19)	0.33	(< 0.001)	14.4 (3.4)	15.2 (3.3)	0.36	(< 0.001)
C18:1n-9	12.09 (1.77)	11.84 (1.64)	0.22	(< 0.001)	136.6 (37.9)	139.7 (36.8)	0.25	(< 0.001)
C20:1n-9	0.13 (0.04)	0.14 (0.03)	0.12	(0.031)	1.5 (0.6)	1.7 (0.6)	0.23	(< 0.001)
Sum MUFA	14.17 (1.76)	13.88 (1.79)	0.22	(0.603)	160.9 (42.2)	165.0 (41.7)	0.27	(< 0.001)
n-9 PUFA								
C20:3n-9	0.23 (0.13)	0.21 (0.08)	0.39	(< 0.001)	2.5 (1.7)	2.5 (1.1)	0.34	(< 0.001)
n-6 PUFA								
C18:2n-6	22.22 (3.59)	23.60 (3.38)	0.27	(< 0.001)	252.7 (63.3)	282.8 (78.7)	0.32	(< 0.001)
C18:3n-6	0.09 (0.05)	0.10 (0.05)	0.29	(< 0.001)	1.0 (0.7)	1.2 (0.7)	0.25	(< 0.001)

C20:2n-6	0.30 (0.08)	0.30 (0.06)	0.22	(< 0.001)	3.4 (1.0)	3.5 (1.0)	0.31	(< 0.001)
C20:3n-6	2.89 (0.79)	2.99 (0.70)	0.49	(< 0.001)	32.4 (9.9)	36.2 (10.6)	0.47	(< 0.001)
C20:4n-6	9.35 (1.86)	9.54 (1.60)	0.43	(< 0.001)	104.4 (25.3)	114.8 (24.9)	0.45	(< 0.001)
C22:4n-6	0.40 (0.11)	0.37 (0.11)	0.31	(< 0.001)	4.5 (1.4)	4.5 (1.2)	0.40	(< 0.001)
C22:5n-6	0.44 (0.16)	0.40 (0.15)	0.45	(< 0.001)	5.0 (2.0)	4.7 (1.8)	0.48	(< 0.001)
Sum n-6 LC-PUFA	13.43 (2.12)	13.59 (2.05)	0.35	(< 0.001)	150.2 (31.2)	162.7 (32.9)	0.40	(< 0.001)
n-3 PUFA								
C18:3n-3	0.22 (0.10)	0.21 (0.10)	0.19	(< 0.001)	2.6 (1.3)	2.6 (1.5)	0.21	(< 0.001)
C20:3n-3	0.07 (0.02)	0.06 (0.01)	0.13	(0.019)	0.8 (0.3)	0.8 (0.2)	0.27	(< 0.001)
C20:5n-3	0.50 (0.29)	0.53 (0.23)	0.26	(< 0.001)	5.8 (3.3)	6.4 (3.5)	0.26	(< 0.001)
C22:5n-3	0.96 (0.24)	0.87 (0.23)	0.32	(< 0.001)	11.2 (3.4)	10.6 (3.1)	0.44	(< 0.001)
C22:6n-3	2.97 (0.83	2.84 (0.88)	0.27	(< 0.001)	33.8 (10.4)	34.8 (11.4)	0.37	(< 0.001)
Sum n-3 LC-PUFA	4.53 (0.89)	4.34 (1.14)	0.23	(< 0.001)	51.0 (13.9)	53.1 (14.4)	0.38	(< 0.001)
Total FA					1138.4 (217.9)	1197.6 (244.9)	0.40	(< 0.001)

Note: FA compositions (%, wt./wt.) and concentrations (mg/l) at 2 and 6 years are presented as median (interquartile range).

Supporting Table 2. Influence of *FADS1 FADS2* gene variants on tracking of serum glycerophospholipid PUFA levels between both time points of the studied subpopulation (n = 331) estimated by Spearman correlations.

		n-6 PUFA								n-3 PUFA					
		C18:2n-6	C18:3n-6	C20:2n-6	C20:3n-6	C20:4n-6	C22:4n-6	C22:5n-6	Sum n-6 LC-PUFA	C18:3n-3	C20:3n-3	C20:5n-3	C22:5n-3	C22:6n-3	Sum n-3 LC-PUFA
rs174545															
Compositions															
11 n = 157	R	0.24	0.27	0.17	0.48	0.27	0.34	0.45	0.27	0.07	0.21	0.23	0.30	0.27	0.29
	(Significance)	(0.003)	(0.001)	(0.034)	(<0.001)	(0.001)	(<0.001)	(<0.001)	(0.001)	(0.405)	(0.010)	(0.004)	(<0.001)	(0.001)	(<0.001)
12 n = 139	R	0.28	0.26	0.16	0.47	0.40	0.30	0.43	0.38	0.35	0.06	0.19	0.23	0.26	0.18
	(Significance)	(0.001)	(0.002)	(0.066)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.516)	(0.029)	(0.006)	(0.002)	(0.039)
22 n = 34	R	0.16	0.32	0.33	0.34	0.40	0.03	0.34	0.27	0.001	0.08	0.18	0.51	0.27	0.16
	(Significance)	(0.354)	(0.061)	(0.059)	(0.053)	(0.020)	(0.864)	(0.050)	(0.125)	(0.994)	(0.647)	(0.319)	(0.001)	(0.128)	(0.359)
Concentrations															
11 n = 157	R	0.31	0.24	0.20	0.42	0.38	0.39	0.48	0.35	0.13	0.30	0.27	0.46	0.38	0.45
	(Significance)	(<0.001)	(0.002)	(0.010)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.097)	(<0.001)	(0.001)	(<0.001)	(<0.001)	(<0.001)
12 n = 139	R	0.33	0.20	0.33	0.45	0.44	0.42	0.46	0.43	0.33	0.23	0.17	0.34	0.38	0.34
	(Significance)	(<0.001)	(0.016)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.006)	(0.043)	(<0.001)	(<0.001)	(<0.001)
22 n = 34	R	0.27	0.23	0.55	0.47	0.41	0.26	0.54	0.38	-0.09	0.18	0.17	0.51	0.34	0.22
	(Significance)	(0.120)	(0.190)	(0.001)	(0.005)	(0.016)	(0.138)	(0.001)	(0.027)	(0.601)	(0.314)	(0.337)	(0.002)	(0.048)	(0.213)
rs174546															
Compositions															
11 n = 158	R	0.24	0.27	0.17	0.48	0.27	0.35	0.46	0.27	0.07	0.20	0.23	0.29	0.27	0.28
	(Significance)	(0.003)	(0.001)	(0.036)	(<0.001)	(0.001)	(<0.001)	(<0.001)	(0.001)	(0.373)	(0.012)	(0.003	(<0.001)	(0.001)	(<0.001)
12 n = 139	R	0.28	0.26	0.16	0.47	0.40	0.30	0.43	0.38	0.35	0.06	0.19	0.23	0.26	0.18
	(Significance)	(0.001)	(0.002)	(0.066)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.516)	(0.029)	(0.006)	(0.002)	(0.039)
22 n = 34	R	0.16	0.32	0.33	0.34	0.40	0.03	0.34	0.27	0.001	0.08	0.18	0.51	0.27	0.16
	(Significance)	(0.354)	(0.061)	(0.059)	(0.053)	(0.020)	(0.864)	(0.050)	(0.125)	(0.994)	(0.647)	(0.319)	(0.002)	(0.128)	(0.359)
Concentrations															
11 n = 158	R	0.31	0.24	0.20	0.43	0.39	0.39	0.49	0.35	0.14	0.31	0.27	0.46	0.38	0.45
	(Significance)	(<0.001)	(0.002)	(0.011)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.082)	(<0.001)	(0.001)	(<0.001)	(<0.001)	(<0.001)

12 n = 139	R	0.33	0.20	0.33	0.45	0.44	0.42	0.46	0.43	0.33	0.23	0.17	0.34	0.38	0.34
	(Significance)	(<0.001)	(0.016)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.006)	(0.043)	(<0.001)	(<0.001)	(<0.001)
22 n = 34	R	0.27	0.23	0.55	0.47	0.41	0.26	0.54	0.38	-0.09	0.18	0.17	0.51	0.34	0.22
	(Significance)	(0.120)	(0.190)	(0.001)	(0.005)	(0.016)	(0.138)	(0.001)	(0.027)	(0.601)	(0.314)	(0.337)	(0.002)	(0.048)	(0.213)
rs174556															
Compositions															
11 n = 166	R	0.22	0.26	0.15	0.45	0.29	0.35	0.44	0.28	0.06	0.15	0.18	0.28	0.26	0.26
	(Significance)	(0.004)	(0.001)	(0.050)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.463)	(0.049)	(0.021)	(<0.001)	(0.001)	(0.001)
12 n = 138	R	0.28	0.28	0.19	0.49	0.38	0.29	0.43	0.37	0.37	0.11	0.26	0.27	0.27	0.16
	(Significance)	(0.001)	(0.001)	(0.026)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.192)	(0.002)	(0.001)	(0.002)	(0.055)
22 n = 25	R	0.32	0.38	0.41	0.27	0.40	0.08	0.46	0.29	0.03	0.11	0.09	0.51	0.28	0.19
	(Significance)	(0.116)	(0.064)	(0.042)	(0.186)	(0.051)	(0.692)	(0.021)	(0.160)	(0.882)	(0.602)	(0.669)	(0.009)	(0.168)	(0.371)
Concentrations															
11 n = 166	R	0.30	0.24	0.22	0.44	0.41	0.41	0.49	0.37	0.12	0.30	0.21	0.43	0.39	0.44
	(Significance)	(<0.001)	(0.002)	(0.005)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.126)	(<0.001)	(0.006)	(<0.001)	(<0.001)	(<0.001)
12 n = 138	R	0.34	0.21	0.33	0.44	0.42	0.40	0.45	0.41	0.35	0.25	0.25	0.39	0.37	0.35
	(Significance)	(<0.001)	(0.012)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.003)	(0.004)	(<0.001)	(<0.001)	(<0.001)
22 n = 25	R	0.20	0.35	0.60	0.46	0.42	0.28	0.63	0.38	-0.05	0.18	0.07	0.48	0.38	0.25
	(Significance)	(0.330)	(0.089)	(0.001)	(0.020)	(0.036)	(0.181)	(0.001)	(0.062)	(0.822)	(0.380)	(0.729)	(0.016)	(0.058)	(0.235)
rs174561															
Compositions															
11 n = 166	R	0.22	0.26	0.15	0.45	0.29	0.35	0.44	0.28	0.06	0.15	0.18	0.28	0.26	0.26
	(Significance)	(0.004)	(0.001)	(0.050)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.463)	(0.049)	(0.021)	(<0.001)	(0.001)	(0.001)
12 n = 138	R	0.28	0.28	0.19	0.49	0.38	0.29	0.43	0.37	0.37	0.11	0.26	0.27	0.27	0.16
	(Significance)	(0.001)	(0.001)	(0.026)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.192)	(0.002)	(0.001)	(0.002)	(0.055)
22 n = 25	R	0.32	0.38	0.41	0.27	0.40	0.08	0.46	0.29	0.03	0.11	0.09	0.51	0.28	0.19
	(Significance)	(0.116)	(0.064)	(0.042)	(0.186)	(0.051)	(0.692)	(0.021)	(0.160)	(0.882)	(0.602)	(0.669)	(0.009)	(0.168)	(0.371)
Concentrations															
11 n = 166	R	0.30	0.24	0.22	0.44	0.41	0.41	0.49	0.37	0.12	0.30	0.21	0.43	0.39	0.44
	(Significance)	(<0.001)	(0.002)	(0.005)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.126)	(<0.001)	(0.006)	(<0.001)	(<0.001)	(<0.001)
12 n = 138	R	0.34	0.21	0.33	0.44	0.42	0.40	0.45	0.41	0.35	0.25	0.25	0.39	0.37	0.35
	(Significance)	(<0.001)	(0.012)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.003)	(0.004)	(<0.001)	(<0.001)	(<0.001)
22 n = 25	R	0.20	0.35	0.60	0.46	0.42	0.28	0.63	0.38	-0.05	0.18	0.07	0.48	0.38	0.25
	(Significance)	(0.330)	(0.089)	(0.001)	(0.020)	(0.036)	(0.181)	(0.001)	(0.062)	(0.822)	(0.380)	(0.729)	(0.016)	(0.058)	(0.235)

rs3834458

Compositions															
11 n = 154	R	0.25	0.29	016	0.46	0.27	0.36	0.45	0.27	0.06	0.19	0.25	0.29	0.27	0.28
	(Significance)	(0.002)	(<0.001)	(0.045)	(<0.001)	(0.001)	(<0.001)	(<0.001)	(0.001)	(0.478)	(0.016)	(0.001	(<0.001)	(0.001)	(<0.001)
12 n = 142	R	0.26	0.25	0.16	0.46	0.41	0.33	0.43	0.38	0.33	0.04	0.17	0.23	0.26	0.16
	(Significance)	(0.002)	(0.003)	(0.054)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.637)	(0.047)	(0.005)	(0.002	(0.062)
22 n = 31	R	0.28	0.38	0.31	0.37	0.44	0.03	0.40	0.34	0.10	0.19	0.18	0.51	0.26	0.12
	(Significance)	(0.125)	(0.037)	(0.086)	(0.042)	(0.014)	(0.883)	(0.026)	(0.065)	(0.593)	(0.308)	(0.328)	(0.003)	(0.157)	(0.523)
Concentrations															
11 n = 154	R	0.29	0.26	0.20	0.41	0.38	0.39	0.48	0.35	0.13	0.30	0.27	0.45	0.37	0.43
	(Significance)	(<0.001)	(0.001)	(0.013)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.107)	(<0.001)	(0.001)	(<0.001)	(<0.001)	(<0.001)
12 n = 142	R	0.34	0.20	0.34	0.45	0.45	0.43	0.46	0.43	0.32	0.22	0.17	0.35	0.39	0.34
	(Significance)	(<0.001)	(0.019)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.007)	(0.041)	(<0.001)	(<0.001)	(<0.001)
22 n = 31	R	0.23	0.31	0.53	0.51	0.47	0.34	0.61	0.45	-0.02	0.22	0.15	0.49	0.36	0.18
	(Significance)	(0.218)	(0.088	(0.002)	(0.004)	(0.007)	(0.065)		(0.011)	(0.913)	(0.235)	(0.410)	(0.005)	(0.046)	(0.338)

Note: Spearman correlation coefficients were calculated for the single SNPs applying an additive model where homozygous minor allele carriers were coded as 2, heterozygous coded as 1, and homozygous major allele carriers coded as 0 (= reference category). 11 = homozygous major, 12 = heterozygous, 22 = homozygous minor

6. Publication 5: “High-throughput analysis of total plasma fatty acid composition with direct in situ transesterification”

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High-Throughput Analysis of Total Plasma Fatty Acid Composition with Direct *In Situ* Transesterification

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Abstract

Background: Plasma fatty acid (FA) composition reflects dietary intake and endogenous turnover and is associated with health outcomes on a short and long term basis. The total plasma FA pool represents the composition of all FA containing lipid fractions. We developed a simplified and affordable high-throughput method for the analysis of total plasma FA composition, suitable for large studies.

Methodology/Principal Findings: The total lipid FA from 100 µl plasma is transferred in situ into methyl esters, avoiding initial extraction and drying steps. The fatty acid methyl esters are extracted once and analyzed by gas chromatography. For the new direct in situ transesterification method optimal, reaction parameters were determined. Intra-assay analysis (n = 8) revealed coefficients of variation below 4% for FA contributing more than 1% to total FA.

Conclusions/Significance: The results show good agreement with FA concentrations obtained by a reference method. The new direct in situ transesterification method is robust and simple. Sample preparation time and analysis costs are reduced to a minimum. This method is an economically and ecologically superior alternative to conventional methods for assessing plasma FA status in large studies.

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Introduction

Tissue availability of polyunsaturated fatty acids, which depends on both diet and metabolic turnover, has a major impact on human health [1–3]. An adequate supply is very important in every stage of life, but particularly for the fetus and neonate to enable optimal visual and cognitive development [4,5].

Analysis of fatty acid (FA) composition in different blood and lipid fractions seems to be a valuable biomarker to assess the FA status in humans [6–8]. Depending on the scientific question, the FA composition can be determined in adipose tissue, erythrocytes, plasma, platelets, whole blood, and other cells or tissues.

The most convenient way to asses FA composition is in whole blood, because separation of plasma and lipid fractions is not required and dried blood spots have been shown as suitable for analysis [9,10]. However, the procedure is not yet well established [9,11,12] and data interpretation seems more difficult, because different influencing factors have to be considered. A crucial influencing factor is the hematocrit, which depends on gender [13] and age, e.g. hematocrit is higher in neonates than adults and decreases during the first months of life [14]. The hematocrit may be altered by factors such as hypertension [13], pulmonary and cardiac diseases, and pregnancy [15]. Variation of hematocrit may lead to misinterpretation of whole blood FA data because the FA composition of plasma and red blood cells, the main components of whole blood, differs significantly [6].

Analytical precision can be improved by analyzing the FA composition in plasma glycerophospholipids, which contain high percentages of e.g. docosahexaenoic acid and hence are a sensitive biomarker for long chain polyunsaturated FA body status [6]. The analysis of plasma glycerophospholipid FA composition can be performed with a high-throughput methodology [16] in large studies. In addition to this very sensitive method focusing on a specific plasma lipid fraction, it could be revealing to analyze the plasma total FA pool, which represents a mixture of all plasma lipid fractions that contain FA moieties, in particular cholesteryl esters, nonesterified FA, phospholipids, and triglycerides. The FA composition is typical for each lipid class, as specific FA are preferentially partitioned into different plasma lipid pools [17]. Thus, analysis of the total FA pool offers the opportunity to determine overall changes in plasma FA status. However, to assess total FA composition in large studies, methods have to be robust and affordable.

Lepage and Roy [18] developed the first direct transesterification method to assess total FA composition in plasma. Masood, Stark and Salem [19] presented in 2005 a simplified version of the original method of Lepage and Roy. In 2008 Masood and Salem [20] published a modified version of this method, which enables a half automated sample preparation.

We aimed to simplify the assessment of total FA composition in plasma with as few sample preparation steps as possible to enable

its application in large studies. Furthermore, we tried to confine sample volume, consumable, reagent, and solvent requirements to a minimum and perform all preparation steps in one vial. We compared our method with a reference method and examined the reliability and limitations of the new method.

Materials and Methods

Reagents and biological material

We used anonymous leftover plasma samples that were originally obtained from patients of the Dr. von Hauner Children's Hospital for clinical diagnostics. Five anonymous patient samples were pooled. This pooled sample was aliquoted, stored, and used for analysis of intra-assay reproducibility and the influence of different reaction conditions. Another five anonymous patient samples were pooled to obtain the sample used for storability analysis. For each of the sixteen samples used for comparison analysis of the new with the reference method, five anonymous patient samples were pooled. The ethical committee of the University of Munich Medical Faculty approved this procedure and approved that no informed consent was needed in this case.

Analytical-grade chloroform, hexane, methanol, and water were purchased from Merck KGaA (Darmstadt, Germany) and methanolic HCl (3 N) was obtained from Sigma-Aldrich (Taufkirchen, Germany). Pentadecanoic acid, cholesteryl pentadecanoate, tripentadecanoin, and 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (Sigma-Aldrich) were dissolved in methanol/chloroform (35:15) and used as internal standard. To prevent FA oxidation 2 g/l 2,6-di-*tert*-butyl-*p*-cresol (Sigma-Aldrich) was added to the internal standard. The external standard (GLC-85), containing 32 fatty acid methyl esters (FAME), was purchased from Nu-Check Prep, Inc. (Elysian, MN, USA). A mixture of sodium carbonate, sodium hydrogen carbonate and sodium sulfate (1:2:2, Merck KGaA) was applied as buffer for neutralization after acid catalyzed transesterification.

New direct in situ transesterification method

We developed a method for the analysis of total plasma FA compositions requiring small plasma (100 µl), reagent (1.5 ml), and solvent volumes (0.6 ml) and few sample preparation steps. According to the newly developed method, 100 µl of plasma, 100 µl of internal standard and 1.5 ml methanolic HCl (3 N, containing 2 g/l 2,6-di-*tert*-butyl-*p*-cresol) were combined in closed glass tubes. The samples were shaken for 30 s and heated to 85°C for 45 min. After cooling to room temperature, 0.5 ml hexane (containing 2 g/l 2,6-di-*tert*-butyl-*p*-cresol) was added and the tubes were shaken for 30 s. Phase separation occurred after approx. 1 min at room temperature. For storage and gas chromatographic (GC) analysis an aliquot of the upper hexane phase was transferred into 2 ml vials. A schematic diagram of the procedure is given in Figure 1.

Reference method

We analyzed the total plasma FA composition according to a reference method to compare the results with that obtained by the new method. For this purpose, we used the standard procedure established in our laboratory, which is based on Folch extraction. In detail, 100 µl of internal standard was added to 250 µl plasma and lipids were extracted by a modified Folch method [21] using chloroform/methanol (2:1, v/v). The extract was washed two times with NaCl solution (2% in water) and subsequently dried at 30°C under reduced pressure. For methyl ester synthesis the extract was taken up in 400 µl chloroform/methanol and 1.5 ml methanolic HCl (3 N) were added. The tubes were closed, shaken for 30 s, and heated to 85°C for 45 min. Samples were neutralized with carbonate buffer after cooling to room temperature. 1 ml hexane was added for FAME extraction. After centrifugation at 900×g for 5 min the upper hexane phase was transferred into a further glass tube and the extraction was repeated. The extracts were combined, taken to dryness under nitrogen flow at room temperature, and taken up in 50 µl hexane (containing 2 g/l 2,6-di-*tert*-butyl-*p*-cresol) for GC analysis.

Chromatography

The individual FAME were quantified by GC with flame ionization detection. GC analysis was performed on an Agilent 5890 series II GC (Agilent, Waldbronn, Germany) using a BPX 70 column (25 m×0.22 mm, 25 µm film, SGE, Weiterstadt, Germany). The temperature program started with an initial temperature of 150°C, which was increased with 2.5°C/min to 180°C and 1.5°C/min to 200°C followed by an 1 min isothermal period. Helium was used as carrier gas, starting with a column head pressure of 0.9 bar, which was increased with 0.02 bar/min to 1.2 bar, with 0.05 bar/min to 1.5 bar, and 0.1 bar/min to the final pressure of 2.0 bar.

Data quantitation

Peak integration was performed using EZChrom Elite version 3.1.7. Individual FAME were identified using authentic standards for comparisons. We used a FAME mixture (GLC-85) as external standard, which was analyzed directly by GC and used to

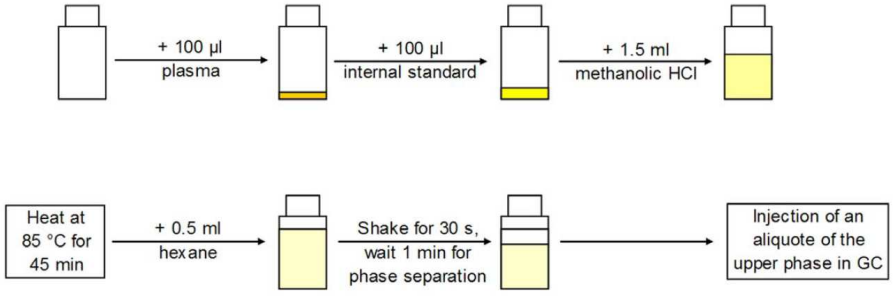

Figure 1. Schematic diagram of the procedure according the direct in situ transesterification method to assess the total fatty acid composition in a plasma sample (GC, gas chromatography).
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determine the response of each FAME relative to pentadecanoic acid methyl ester.

Method comparison and statistical analysis

Results were expressed as absolute plasma concentrations (mg/l) and as percentage values (% wt/wt), and FA data were presented as mean. Intra-assay reproducibility was obtained by analyzing 8 aliquots of a pooled plasma sample at an interval of 1 week. Coefficients of variation (CV, %) were used to express analytical precision. The statistical differences between both methods were obtained by analyzing 16 different plasma samples (pooled) and paired t-test was used for comparison between the mean values. All statistical analyses were performed with SPSS for Windows, Version 15.0.1 (SPSS Inc., Chicago, IL, USA).

Results

Intra-assay reproducibility obtained by the analysis of 8 aliquots of one plasma sample at an interval of 1 week was determined for both methods. Coefficients of variation (Table 1) for all FA contributing more than 1% of total FA were below 4% with the direct in situ transesterification method. Analysis of C22:0, contributing 0.34% of total FA, showed the highest CV (8%).

Furthermore, we analyzed the FA concentrations of sixteen different pooled plasma samples (Table S1). The measured total FA concentrations for these samples were 2715.9 mg/l (range 1851.1 mg/l–5409.8 mg/l) with the reference method and 2913.2 mg/l (range 1936.0 mg/l–5865.0 mg/l) with the direct in situ transesterification method. Absolute plasma concentrations (mg/l)

Table 1. Intra-assay *(8 independent measurements of 1 sample)* reproducibility of total lipid fatty acid (FA) concentrations (mg/l) and compositions (% wt/wt) obtained by analysis according to the reference and the direct in situ transesterification method (CV, coefficient of variation; PUFA, polyunsaturated fatty acid).

FA	*Reference method*				*Direct in situ transesterification method*			
	FA concentration		**FA composition**		**FA concentration**		**FA composition**	
	Mean	**CV**	**Mean**	**CV**	**Mean**	**CV**	**Mean**	**CV**
Saturated FA								
C14:0	51.90	3.0	1.53	3.5	70.90	3.7	1.87	3.6
C16:0	901.81	2.6	26.54	1.2	975.65	1.7	25.76	0.7
C17:0	11.48	2.9	0.34	2.3	11.55	3.9	0.30	3.2
C18:0	276.75	5.9	8.14	4.2	278.15	1.5	7.34	0.7
C20:0	10.39	3.4	0.31	1.8	8.60	4.3	0.23	4.1
C22:0	21.54	2.9	0.63	2.2	16.15	7.9	0.43	8.0
C24:0	17.53	4.4	0.52	4.2	15.10	7.0	0.40	7.4
Monounsaturated FA								
C14:1	3.99	7.9	0.12	8.7	6.45	4.0	0.17	3.7
C16:1n-7	93.56	1.4	2.75	1.3	107.40	3.1	2.84	2.9
C18:1n-7	65.49	2.1	1.93	0.9	69.40	3.2	1.83	2.9
C18:1n-9	839.26	2.1	24.70	0.9	914.55	1.3	24.14	0.3
C20:1n-9	7.31	3.5	0.22	3.1	7.10	5.8	0.19	5.7
C24:1n-9	31.16	3.5	0.92	3.1	31.35	5.7	0.83	5.4
n-9 PUFA								
C20:3n-9	4.48	2.6	0.13	0.8	5.90	4.8	0.16	4.9
n-6 PUFA								
C18:2n-6	719.04	1.8	21.16	0.7	836.60	1.4	22.09	0.3
C18:3n-6	14.91	6.0	0.44	7.0	18.00	2.4	0.48	1.4
C20:2n-6	6.01	4.4	0.18	5.0	6.40	5.8	0.17	5.2
C20:3n-6	47.86	2.4	1.41	1.5	58.50	1.7	1.54	1.1
C20:4n-6	172.53	2.3	5.08	1.7	219.50	1.1	5.79	1.0
C22:4n-6	6.78	4.7	0.20	5.2	8.50	7.0	0.22	7.0
C22:5n-6	4.74	9.5	0.14	10.1	6.50	7.2	0.17	7.8
n-3 PUFA								
C18:3n-3	13.83	1.9	0.41	1.2	17.20	1.8	0.45	1.2
C20:5n-3	18.41	9.5	0.54	9.6	21.55	2.9	0.57	2.2
C22:5n-3	13.38	3.6	0.39	3.4	17.15	3.4	0.45	3.0
C22:6n-3	43.78	2.8	1.29	2.8	59.80	1.9	1.58	1.6
Total FA								
	3397.89	2.1			3787.95	1.3		

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were significantly higher for most of the analyzed FA with the direct in situ transesterification method compared to the reference method. There were no significant differences in the concentrations for the saturated FA C17:0, C20:0, and C22:0, for the monounsaturated FA C14:1n-5 and C20:1n-9, and for the polyunsaturated FA C20:2n-6, C22:4n-6, C22:5n-6, C18:3n-3, and C20:5n-3. The concentration of C18:3n-6 was higher obtained with the reference methods compared to the direct in situ transesterification method. For all other FA, particularly docosahexaenoic acid, absolute plasma concentrations were higher with the direct in situ transesterification method than with the reference method. However, both methods revealed very similar percentage FA values (% wt/wt).

We assessed the effects of reaction conditions on the determined FA in more detail (Table S2). Both, longer reaction times (90 min instead of 45 min) as well as an increase of the reaction temperature from 85°C to 100°C led to higher concentration yields for C20:0, C22:0, C24:0, and C24:1n-9 (up to 40%). No further increase in concentrations was obtained with prolonging reaction times beyond 90 min. However, the effect on percentage FA compositions is very small, because of the low abundance of these four FA (<1% of total FA).

Storage of the GC ready derivatives for one month at −20°C did not lead to appreciable alterations of FA concentrations (Table 2).

Discussion

The new analysis method allows for rapid, precise, and reproducible analysis of total plasma FA. Significantly higher

Table 2. Fatty acid (FA) values obtained with the new direct in situ transesterification method for a plasma sample analyzed by gas chromatography directly after sample preparation, then stored for 1 month at −20°C and analyzed again (PUFA, polyunsaturated fatty acid).

FA	*FA concentration (mg/l)*				**FA composition (% wt/wt)**			
	Start	**4 weeks**	**Difference**		**Start**	**4 weeks**	**Difference**	
			mg/l	**%**			**% wt/wt**	**%**
Saturated FA								
C14:0	57.40	57.60	0.20	0.3	1.66	1.66	0.00	0.1
C16:0	896.20	899.20	3.00	0.3	25.86	25.88	0.02	0.1
C17:0	10.80	10.80	0.00	0.0	0.31	0.31	0.00	−0.3
C18:0	258.40	263.40	5.00	1.9	7.46	7.58	0.13	1.7
C20:0	8.10	8.40	0.30	3.7	0.23	0.24	0.01	3.4
C22:0	14.80	15.60	0.80	5.4	0.43	0.45	0.02	5.1
C24:0	13.90	14.80	0.90	6.5	0.40	0.43	0.02	6.2
Monounsaturated FA								
C14:1n-5	5.40	5.40	0.00	0.0	0.16	0.16	0.00	−0.3
C16:1n-7	99.60	97.80	−1.80	−1.8	2.87	2.81	−0.06	−2.1
C18:1n-7	65.80	67.20	1.40	2.1	1.90	1.93	0.04	1.9
C18:1n-9	860.80	857.00	−3.80	−0.4	24.84	24.66	−0.17	−0.7
C20:1n-9	8.20	7.80	−0.40	−4.9	0.24	0.22	−0.01	−5.1
C24:1n-9	27.20	29.40	2.20	8.1	0.78	0.85	0.06	7.8
n-9 PUFA								
C20:3n-9	5.00	5.20	0.20	4.0	0.14	0.15	0.01	3.7
n-6 PUFA								
C18:2n-6	762.60	758.00	−4.60	−0.6	22.00	21.82	−0.19	−0.9
C18:3n-6	15.60	15.40	−0.20	−1.3	0.45	0.44	−0.01	−1.5
C20:2n-6	6.00	6.20	0.20	3.3	0.17	0.18	0.01	3.1
C20:3n-6	52.60	52.60	0.00	0.0	1.52	1.51	0.00	−0.3
C20:4n-6	187.80	192.00	4.20	2.2	5.42	5.53	0.11	2.0
C22:4n-6	7.60	7.40	−0.20	−2.6	0.22	0.21	−0.01	−2.9
C22:5n-6	4.50	4.80	0.30	6.7	0.13	0.14	0.01	6.4
n-3 PUFA								
C18:3n-3	15.00	14.60	−0.40	−2.7	0.43	0.42	−0.01	−2.9
C20:5n-3	16.60	17.20	0.60	3.6	0.48	0.50	0.02	3.4
C22:5n-3	16.00	15.60	−0.40	−2.5	0.46	0.45	−0.01	−2.7
C22:6n-3	50.00	51.20	1.20	2.4	1.44	1.47	0.03	2.1
Total FA								
	3465.90	3474.60	8.70	0.3				

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absolute plasma concentrations for most of the analyzed FA were obtained by direct in situ transesterification compared to our reference method, presumably because losses due to non-quantitative extraction are avoided by the direct in situ transesterification method as previously noted by other authors [18,22]. Of importance, the addition of an internal standard dissolved in an organic solvent to the plasma sample obviously is not fully adequate for the extraction of lipoprotein bound lipids.

Absolute plasma FA concentration of C20:0 obtained by direct in situ transesterification for 45 min at 85°C was lower than concentrations found with the reference method. Longer reaction times and/or higher temperatures enabled more complete direct in situ transesterification of C20:0, C22:0, C24:0, and C24:1n-9. These FA are mainly found in the phospholipid fraction of human plasma [23]. The concentrations of these FA in glycerophospholipids are below the quantification level, whereas they show a high abundance in sphingomyelin [23]. Masood et al. [19] obtained a decline of 40% of certain FA (C20:0, C22:0, C24:0 and C24:1n-9) with transesterification at 75°C for 60 min–90 min with their open-tube method compared to the direct transesterification method of Lepage and Roy [18]. They considered that more extreme conditions such as higher temperatures and longer heating times were required for complete transesterification of sphingomyelin FA, because of their less reactive amide bonds. Quantitative transesterification of these FA would require at least duplication of reaction time. However, the benefit of exact determination of the concentrations of C20:0, C22:0, and C24:0 seems small for most clinical and research applications, which must be weighed against the loss of productivity and the increasing risk of FA degradation or isomerization during derivatization [24–26]. Moreover, for most applications the percentage FA compositions are of interest, which are constant at the different reaction conditions, because the amounts of C20:0, C22:0, and C24:0 are too small to alter the entire composition.

Different lipoproteins contain distinct amounts of cholesteryl esters, phospholipids, and triglycerides, which have an individual FA composition. Therefore, the FA composition obtained by direct in situ transesterification in plasma reflects the total plasma FA composition given by the composition of different lipoproteins and the non-esterified FA.

In conclusion, the newly developed direct in situ transesterification method presented here, enables analysis of total plasma FA concentrations and percentage contributions from only 100 µl of plasma. The method is robust and simple, because lipid extraction and washing steps as well as neutralization, centrifugation and drying steps are avoided and only one FAME extraction step is required. Sample preparation can be performed in one vial, preparation time is reduced to a minimum, and only small volumes of reagents and solvents are necessary. Thus, the method is economically and ecologically superior to conventional methods, and it is well suitable for the application in large clinical trials and epidemiological studies.

Supporting Information

Table S1 Differences between the reference and the direct in situ transesterification method for total lipid fatty acid concentrations (mean, mg/l) and compositions (mean, % wt/wt) obtained by the analysis of 16 different plasma samples (pooled).
Found at: doi:10.1371/journal.pone.0012045.s001 (0.10 MB DOC)

Table S2 Influence of different reaction conditions on the determined fatty acid (FA) concentrations (mg/l) and compositions (%) (PUFA, polyunsaturated fatty acid).
Found at: doi:10.1371/journal.pone.0012045.s002 (0.10 MB DOC)

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Author Contributions

Conceived and designed the experiments: CG. Performed the experiments: CG. Analyzed the data: CG. Contributed reagents/materials/analysis tools: BK. Wrote the paper: CG. Provided additional expertise: HD BK. Reviewed and revised the manuscript: HD BK. Obtained funding and acted as guarantor: BK.

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Table S1: Differences between the reference and the direct in situ transesterification method for total lipid fatty acid concentrations (mean, mg/l) and compositions (mean, % wt/wt) obtained by the analysis of 16 different plasma samples (pooled).

FA	*Reference method*		*Direct in situ transesterification method*		*Difference*					
					FA concentration			*FA composition*		
	FA concentration	*FA composition*	*FA concentration*	*FA composition*	*Absolute*	*Percentage*	*Significance*	*Absolute*	*Percentage*	*Significance*
Saturated FA										
C14:0	36.7	1.3	40.0	1.3	3.31	9.0	<0.001	0.02	1.8	0.098
C16:0	658.5	23.7	710.6	23.9	52.02	7.9	<0.001	0.15	0.6	0.062
C17:0	9.0	0.3	9.2	0.3	0.27	3.0	0.171	-0.01	-4.1	0.866
C18:0	212.8	8.0	233.1	8.2	20.33	9.6	<0.001	0.18	2.3	0.007
C20:0	8.0	0.3	7.8	0.3	-0.23	-2.9	0.288	-0.03	-10.2	<0.001
C22:0	16.6	0.6	17.4	0.6	0.82	4.9	0.172	-0.01	-1.9	0.375
C24:0	14.3	0.5	16.1	0.6	1.80	12.7	0.002	0.03	5.2	0.045
Monounsaturated FA										
C14:1n-5	2.6	0.1	2.6	0.1	0.04	1.7	0.756	-0.004	-4.7	0.278
C16:1n-7	72.3	2.6	75.4	2.5	3.17	4.4	<0.001	-0.07	-2.7	0.001
C18:1n-7	52.1	1.9	56.2	1.9	4.05	7.8	<0.001	0.01	0.3	0.360
C18:1n-9	612.7	22.1	667.8	22.5	55.06	9.0	<0.001	0.38	1.7	<0.001
C20:1n-9	5.4	0.2	5.5	0.2	0.09	1.7	0.479	-0.01	-5.9	0.002
C24:1n-9	28.5	1.1	31.6	1.1	3.06	10.7	0.001	0.03	3.0	0.036
n-9 PUFA										
C20:3n-9	5.4	0.2	6.1	0.2	0.71	13.2	<0.001	0.01	6.3	<0.001
n-6 PUFA										
C18:2n-6	632.8	23.6	669.5	23.3	36.74	5.8	<0.001	-0.32	-1.4	0.001
C18:3n-6	12.3	0.4	11.8	0.4	-0.51	-4.2	0.012	-0.05	-10.9	<0.001
C20:2n-6	5.4	0.2	5.6	0.2	0.14	2.5	0.375	-0.01	-5.5	0.034
C20:3n-6	42.5	1.6	46.1	1.6	3.63	8.6	<0.001	0.02	1.0	0.017

C20:4n-6	185.8	7.1	196.7	7.0	10.87	5.8	<0.001	-0.11	-1.5	0.014
C22:4n-6	7.7	0.3	7.9	0.3	0.25	3.2	0.076	-0.01	-3.7	0.030
C22:5n-6	7.4	0.3	7.7	0.3	0.24	3.3	0.103	-0.01	-3.6	0.022
n-3 PUFA										
C18:3n-3	15.2	0.6	15.3	0.5	0.15	1.0	0.329	-0.04	-6.5	<0.001
C20:5n-3	14.2	0.5	13.7	0.5	-0.51	-3.6	0.067	-0.05	-10.2	<0.001
C22:5n-3	13.5	0.5	13.9	0.5	0.42	3.1	0.036	-0.02	-4.2	0.002
C22:6n-3	44.3	1.7	45.7	1.6	1.41	3.2	0.018	-0.07	-4.1	<0.001
Total FA										
	2715.9	-	2913.2	-	197.34	7.3	<0.001	-	-	-

Table S2: Influence of different reaction conditions on the determined fatty acid (FA) concentrations (mg/l) and compositions (%) (PUFA, polyunsaturated fatty acid).

	Concentrations (mg/l)						*Compositions (%)*					
FA	*Transesterification at 85 ℃*			*Transesterification at 100 ℃*			*Tranesterification at 85 ℃*			*Transesterifica tion at 100 ℃*		
	45 min	*60 min*	*90 min*	*45 min*	*60 min*	*90min*	*45 min*	*60 min*	*90 min*	*45 min*	*60 min*	*90 min*
Saturated FA												
C14:0	68.4	73.2	66.0	68.0	67.6	66.8	1.9	2.0	1.9	1.9	1.9	1.9
C16:0	959.6	977.2	934.0	966.0	958.0	964.0	26.8	27.0	26.8	26.8	26.9	26.9
C17:0	11.2	11.6	11.2	11.2	11.6	11.6	0.3	0.3	0.3	0.3	0.3	0.3
C18:0	276.8	276.8	268.8	276.4	273.2	276.0	7.7	7.6	7.7	7.7	7.7	7.7
C20:0	7.6	7.6	8.4	9.6	8.8	9.2	0.2	0.2	0.2	0.3	0.2	0.3
C22:0	13.6	14.0	16.0	16.8	17.2	18.0	0.4	0.4	0.5	0.5	0.5	0.5
C24:0	12.4	16.0	17.2	17.2	17.2	18.4	0.3	0.4	0.5	0.5	0.5	0.5
Monounsaturated FA												
C14:1n-5	4.0	4.0	4.0	4.0	3.6	4.0	0.1	0.1	0.1	0.1	0.1	0.1
C16:1n-7	107.6	109.2	103.2	106.8	105.6	105.2	3.0	3.0	3.0	3.0	3.0	2.9
C18:1n-7	68.0	70.4	67.6	69.6	69.2	69.2	1.9	1.9	1.9	1.9	1.9	1.9
C18:1n-9	852.8	866.8	837.6	861.2	854.0	860.0	23.8	23.9	24.1	23.9	23.9	24.0
C20:1n-9	6.8	6.8	6.8	6.8	6.8	7.2	0.2	0.2	0.2	0.2	0.2	0.2
C24:1n-9	25.6	28.4	32.4	34.4	35.6	36.4	0.7	0.8	0.9	1.0	1.0	1.0
n-9 PUFA												
C20:3n-9	6.0	5.6	5.2	5.2	5.6	5.2	0.2	0.2	0.1	0.1	0.2	0.1
n-6 PUFA												
C18:2n-6	771.2	777.6	740.4	769.6	760.4	763.6	21.6	21.5	21.3	21.4	21.3	21.3
C18:3n-6	16.8	16.8	15.6	16.8	16.4	16.4	0.5	0.5	0.4	0.5	0.5	0.5
C20:2n-6	6.0	5.6	5.6	5.6	5.6	5.6	0.2	0.2	0.2	0.2	0.2	0.2
C20:3n-6	52.4	51.6	49.6	51.6	50.4	51.2	1.5	1.4	1.4	1.4	1.4	1.4
C20:4n-6	194.0	191.2	184.0	191.6	188.8	189.2	5.4	5.3	5.3	5.3	5.3	5.3

C22:4n-6	6.8	6.8	7.2	6.8	7.2	6.8	0.2	0.2	0.2	0.2	0.2	0.2
C22:5n-6	6.4	6.4	4.8	6.0	6.4	5.2	0.2	0.2	0.1	0.2	0.2	0.1
n-3 PUFA												
C18:3n-3	15.6	15.6	14.8	15.6	15.6	15.2	0.4	0.4	0.4	0.4	0.4	0.4
C20:5n-3	18.4	17.2	16.8	17.6	17.6	16.8	0.5	0.5	0.5	0.5	0.5	0.5
C22:5n-3	17.0	17.0	14.4	14.4	14.8	15.2	0.5	0.5	0.4	0.4	0.4	0.4
C22:6n-3	52.0	50.8	48.0	50.8	49.6	50.0	1.5	1.4	1.4	1.4	1.4	1.4
Total FA												
	3577.0	3624.2	3479.6	3599.6	3566.8	3586.4						

7. Summary

Objectives: Clinical and epidemiological studies show that the plasma FA composition, a marker of FA status and dietary intake, is associated with health outcomes on a short and long term basis. Detailed investigation of the relationships between plasma FA composition and health requires the analysis of high numbers of samples, which is often limited by laborious sample preparation procedures. The objective of this work was to develop high-throughput methods which enable simple, precise and inexpensive analysis of plasma and serum GP FA composition and plasma total lipid FA composition. For serum GP FA composition, reference values for children were provided using the newly developed methodology. The tracking behavior of serum GP FA composition and concentrations in children was investigated. Furthermore, the potential influence of *FADS* gene variants on tracking, which has not been investigated so far, was determined.

Methods: For the analysis of GP FA composition only protein precipitation and subsequent base catalyzed methyl ester synthesis are required. The plasma total lipid FA are in situ transferred into methyl esters, avoiding initial extraction and drying steps. Identical gas chromatographic conditions can be applied for both methods. The GP FA composition of 1326 serum samples obtained from a cohort of 951 children at 2 and 6 years, participating in an ongoing birth cohort study, was analyzed with the newly developed high-throughput method. Data were used to provide reference values. From 331 children samples were available at both time points (2 and 6 years) and used to assess tracking. Spearman

correlation coefficients were estimated to describe tracking of FA compositions and concentrations over 4-years and to assess the influence of *FADS* gene variants (rs174545, rs174546, rs174556, rs174561, rs3834458) on FA tracking.

Results: Coefficients of variation for FA contributing more than 1 % to total FA were below 4 % for both methods. The results showed good agreement with FA values obtained by standard reference methods. FA values in serum GP were in very good agreement with other published values in serum or plasma PL for most of the analyzed FA. No major age and gender differences in GP FA composition were observed. For most FA compositions and concentrations at 2 and 6 years were significantly correlated, with correlation coefficients from 0.12 to 0.49. FA concentrations correlated more closely than FA compositions. Concentration changes of total monounsaturated FA and total saturated FA over time correlated closely ($r = 0.79$) but percentage values were unrelated ($r = -0.02$). Tracking for n-6 LC-PUFA concentrations was lower in subjects homozygous for the major allele of *FADS* variants, and higher in carriers of at least one minor allele, whereas for n-3 LC-PUFA this was vice versa.

Conclusions: The newly developed methods enable the precise quantification of 25 FA (including arachidonic, eicosapentaenoic and docosahexaenoic acid) while reducing the manual work load to about 10 % compared to standard reference methods. Samples from infants can be assessed as the required plasma volume is very low. The methods are simple, robust and well suitable for the application in large clinical trials and epidemiological studies. The serum GP FA values obtained from children aged 2 and 6 years may serve as reference values in clinical routine, e.g. for monitoring and improving therapeutic interventions. Furthermore, they can serve as a reference for interpreting FA values in clinical and epidemiological studies. Serum GP FA composition shows tracking over 4-years particularly

for n-6 PUFA, with a higher tracking for LC-PUFA metabolites than for their precursor PUFA. Lipid and lipoprotein metabolism seem to have a stronger influence on serum FA levels and tracking than FA specific metabolic pathways.

8. Zusammenfassung

Ziele: Klinische und epidemiologische Studien zeigen, dass die Plasma-Fettsäure(FS)-Zusammensetzung, ein Marker für den Fettsäurestatus und die Ernährung, sowohl mit der kurz- als auch mit der langfristigen Verbesserung des Gesundheitszustandes in Zusammenhang steht. Detaillierte Untersuchungen der Zusammenhänge zwischen der Plasma-FS-Zusammensetzung und der Gesundheit erfordern Analysen großer Probenzahlen, welche oftmals durch aufwendige Probenaufarbeitungsprozeduren begrenzt sind. Ziel dieser Arbeit war die Entwicklung von Hochdurchsatzmethoden, die eine einfache, präzise und kostengünstige Analyse der Plasma- und Serum-Glycerophospholipid(GP)-FS-Zusammensetzung und der Plasma Gesamtlipid-FS-Zusammensetzung ermöglichen. Für die Serum-GP-FS-Zusammensetzung wurden Referenzwerte für Kinder mit Hilfe der neuentwickelten Methode ermittelt. Das Spurhalten der Serum-GP-FS-Zusammensetzung und –Konzentrationen wurde in Kindern untersucht. Weiterhin wurde der potentielle Einfluss von *FADS*-Genvarianten auf das Spurhalten untersucht, was bisher nicht erforscht ist.

Methoden: Zur Analyse der GP-FS-Zusammensetzung werden nur eine Fällung der Proteine und eine anschließende basenkatalysierte Methylestersynthese benötigt. Die Plasma-Gesamtlipid-FS werden in situ in Methylester überführt, wobei vorangehende Extraktions- und Trocknungsschritte vermieden werden. Identische gaschromatographische Bedingungen können für beide Methoden verwendet werden. Die GP-FS-Zusammensetzung von 1326 Serumproben, erhalten aus einer Kohorte von 951 Kindern bei 2 und 6 Jahren, die in einer

fortlaufenden Geburtskohortenstudie teilnehmen, wurde mit der neuentwickelten Hochdurchsatzmethode analysiert. Die Daten wurden zur Bereitstellung von Referenzwerten verwendet. Von 331 Kindern waren Proben von beiden Zeitpunkten (2 und 6 Jahre) erhältlich und wurden verwendet, um das Spurhalten zu bestimmen. Spearmans Korrelations-koeffizienten wurden ermittelt, um das Spurhalten der FS-Zusammensetzung und -Konzentrationen über 4 Jahre und den Einfluss von *FADS*-Genvarianten (rs174545, rs174546, rs174556, rs174561, rs3834458) auf das Spurhalten zu bestimmen.

Ergebnisse: Variationskoeffizienten der Fettsäuren, welche mehr als 1 % zu den Gesamtfettsäuren beitrugen, lagen unter 4 % bei beiden Methoden. Die Ergebnisse zeigten eine gute Übereinstimmung mit FS-Werte gemessen mit Standard-Referenzmethoden. Die Fettsäurewerte in Serum-GP waren in sehr guter Übereinstimmung mit anderen publizierten Werten in Serum- oder Plasma-Phospholipiden für die meisten analysierten FS. Es wurden keine wesentlichen Alters- und Geschlechtsunterschiede in der GP-FS-Zusammensetzung beobachtet. Die meisten FS-Zusammensetzungen und –Konzentrationen bei 2 und 6 Jahren korrelierten signifikant mit Korrelationskoeffizienten von 0,12 bis 0,49. Die FS-Konzentrationen waren stärker korreliert als die FS-Zusammensetzungen. Die Konzentrationsänderungen über die Zeit der gesamten einfach ungesättigten FS und der gesamten gesättigten FS korrelierten stark (r=0,79), wohingegen die prozentualen Werte nicht zusammenhingen (r=-0,02). Das Spurhalten der von n-6 LC-PUFA Konzantrationen war geringer in Probanden welche homozygot für das Hauptallel der *FADS*-Varianten waren und größer in Trägern von mindestens einem Unterallel, während dies für n-3 LC-PUFA umgekehrt war.

8. ZUSAMMENFASSUNG

Schlussfolgerungen: Die neuentwickelten Methoden erlauben die präzise Quantifizierung von 25 FS (einschließlich Arachidon-, Eicosapentaen- und Dokosahexaensäure) während der manuelle Arbeitsaufwand auf ungefähr 10 % reduziert wurde im Vergleich zu Standard-Referenzmethoden. Proben von Kleinkindern können untersucht werden, da das benötigte Plasmavolumen sehr klein ist. Die Methoden sind einfach, robust und sehr gut einsetzbar in großen klinischen und epidemiologischen Studien. Die Serum-GP-FS Werte erhalten von Kindern im Alter von 2 und 6 Jahren können als Referenzwerte in der klinische Routine, z.B. zur Überwachung und Verbesserung therapeutischer Eingriffe dienen. Weiterhin können sie als Referenzen zur Interpretation von FS Werten in klinischen und epidemiologischen Studien dienen. Die Serum-GP-FS-Zusammensetzung zeigt Spurhalten über 4 Jahre besonders für n-6 mehrfach ungesättigte FS mit einem höheren Spurhalten für die langkettigen Metabolite als für ihre Vorläufer FS. Der Lipid- und Lipoproteinmetabolismus scheint einen stärkeren Einfluss auf die Serum-FS Werte und das Spurhalten zu haben als FS spezifische Stoffwechselwege.

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10. Publications and presentations

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