FADS genes –

Key genetic regulators of polyunsaturated fatty acid levels

Dissertation

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If you can imagine it, you can achieve it.
If you can dream it, you can become it.

William Arthur Ward

Table of contents

Summary

The composition of long-chain polyunsaturated fatty acids (LC-PUFAs) in human tissues is essential for maintaining metabolic functions and health. Various complex diseases such as psychiatric disorders, metabolic syndrome, cardiovascular disease, and allergies have been associated with LC-PUFA levels. Analysis of the factors that determine the fatty acid composition in human tissues will help to understand and prevent the development of fatty acid related complex diseases. In addition to diet, one important factor for the determination of LC-PUFA levels in human tissues is the desaturation pathway of fatty acids. In this pathway, LC-PUFAs are synthesized endogenously from precursor essential fatty acids (linoleic acid and alpha-linolenic acid) by consecutive desaturation and chain-elongation. The rate-limiting enzymes in this reaction cascade are the delta-5 and delta-6 desaturases (D5D and D6D) encoded by the genes *FADS1* and *FADS2* (fatty acid desaturase 1 and 2), respectively. These genes build a gene cluster on chromosome 11 together with a third desaturase gene, *FADS3*. In various genetic association studies, single nucleotide polymorphisms (SNPs) in the *FADS* gene cluster have been found to be significantly associated with LC-PUFA and lipid levels in different tissues.

The sufficient supply with LC-PUFAs during pregnancy and lactation is considered highly important for proper development and health in prenatal life and infancy. For that reason, the analysis of genetically determined inter-individual differences in maternal fatty acid levels during pregnancy and lactation is of special interest to understand and consequently optimize the infant's fatty acid supply. The first aim of this thesis was therefore to analyze associations of *FADS* genotypes with fatty acid levels in maternal blood and breast milk - body fluids of particular importance for fatty acid supply of the infant - in two large cohorts of pregnant and lactating women, respectively. Although the results of all association studies on fatty acid levels show an important role of the *FADS* gene cluster in the regulation of fatty acid composition, the causative variants have not been identified until now, which is essential for understanding the molecular basis of individual fatty acid levels. The second aim was thus to identify functional relevant variants for the regulation of fatty acid levels by *in vitro* studies.

Association analysis of 17 SNPs spanning the complete *FADS* gene cluster with maternal blood fatty acid levels was performed in the largest cohort investigated on this subject so far, including more than 4,000 pregnant women from the Avon Longitudinal Study of Parents and Children (ALSPAC). Minor alleles of the majority of the analyzed *FADS* polymorphisms were positively associated with desaturase substrates and negatively associated with desaturase products and product/substrate ratios ($p<0.001$), which was independent of sociodemographic confounders and dietary habits. Negative associations were also shown for docosahexaenoic acid (DHA) ($p<0.001$), which is considered particularly important for neuronal development.

Associations of eight *FADS* polymorphisms with the concentration of breast milk fatty acids were analyzed in 772 (1.5 months postpartum) and 463 (6 months postpartum) lactating mothers from the Ulm Birth Cohort Study. SNPs rs174547 and rs174556 were negatively associated with arachidonic acid levels and the 20:4n-6 / 20:3n-6 ratio estimating D5D activity at both time points (p-values ranged from 0.0002 to 0.0064). Longitudinal analysis of the *FADS* genotype effect on the time course of fatty acid levels resulted in associations for dodecanoic, cis-15-tetracosenoic, and trans-9-octadecenoic acid ($p = 0.0186$, 0.0022, and 0.0032, respectively).

Two polymorphisms in the *FADS2* gene promoter were selected for functional analysis based on bioinformatic prediction of putative regulatory regions. By luciferase reporter gene assays, it was shown that the presence of the minor T allele of polymorphism rs968567 in the *FADS2* gene promoter leads to increased promoter activity. Furthermore, an influence of this SNP on the binding affinity of transcription factor ELK1 to the *FADS2* gene promoter was demonstrated by DNA-affinity purification and subsequent immunoblotting.

In conclusion, the results obtained during this thesis clearly support an effect of *FADS* genotypes on the fatty acid composition of maternal body fluids, which are important for the child's fatty acid supply during pregnancy and lactation. Whether maternal *FADS* genotypes have a direct effect on the child's fatty acid status awaits further investigation. One of the associated polymorphisms was found to be functionally relevant, offering first insights into allele-dependent regulatory mechanisms of desaturase activity. This provides the basis for further functional studies to completely understand the genetically determined molecular mechanisms leading to inter-individual differences in fatty acid metabolism.

Zusammenfassung

Die Zusammensetzung langkettiger mehrfach ungesättigter Fettsäuren (long-chain polyunsaturated fatty acids, LC-PUFAs) in Humangeweben ist essentiell für die Aufrechterhaltung wichtiger Stoffwechselfunktionen sowie für die Gesundheit. Zahlreiche komplexe Erkrankungen (zum Beispiel psychische Störungen, metabolisches Syndrom, kardiovaskuläre Erkrankungen und Allergien) wurden mit der Zusammensetzung von LC-PUFAs im menschlichen Körper assoziiert. Die Analyse derjenigen Faktoren, die die Fettsäurezusammensetzung in Humangeweben beeinflussen, wird zum Verständnis und zur Prävention von Fettsäure-assoziierten komplexen Erkrankungen beitragen. Neben der Ernährung ist der Desaturase-Stoffwechselweg ein entscheidender Faktor für die Zusammensetzung von LC-PUFAs in Geweben. LC-PUFAs werden hierbei durch die aufeinanderfolgende Desaturierung und Kettenverlängerung essentieller Vorläuferfettsäuren (Linolsäure und Alpha-Linolensäure) endogen synthetisiert. In dieser Reaktionskaskade stellen die Delta-5- und Delta-6-Desaturasen (D5D und D6D), kodiert durch die Gene *FADS1* und *FADS2* (fatty acid desaturase 1 and 2), die geschwindigkeitsbestimmenden Enzyme dar. Zusammen mit einem dritten Desaturase-Gen (*FADS3*) bilden *FADS1* und *FADS2* ein Gencluster auf Chromosom 11. Zahlreiche genetische Assoziationsstudien zeigten eine signifikante Assoziation verschiedener Einzelnukleotidaustausche (single nucleotide polymorphisms, SNPs) im *FADS*-Gencluster mit LC-PUFA- und Lipidkonzentrationen in verschiedenen Geweben.

Auch für die pränatale und frühkindliche Entwicklung und Gesundheit gilt die ausreichende Versorgung mit LC-PUFAs als entscheidend. Daher ist die Analyse genetisch determinierter interindividueller Unterschiede der maternalen Fettsäurekonzentration in der Schwangerschaft und Stillzeit für das Verständnis und die dadurch mögliche Optimierung der frühkindlichen Fettsäureversorgung von großer Bedeutung. Das erste Ziel dieser Arbeit war daher die Assoziationsanalyse von *FADS*-Genotypen mit Fettsäurekonzentrationen in maternalem Blut sowie Muttermilch - beides wichtige Körperflüssigkeiten bzw. Sekrete für die frühkindliche Versorgung mit Fettsäuren - in zwei großen Kohortenstudien bestehend aus schwangeren bzw. stillenden Frauen. Alle bisherigen Assoziationsstudien bestätigen eine wichtige Rolle des *FADS*-Genclusters in der Regulierung der Fettsäurezusammensetzung. Die

kausalen Varianten wurden bisher jedoch noch nicht identifiziert, was für das Verständnis der molekularen Ursachen von individuellen Fettsäurespiegeln essentiell ist. Das zweite Ziel dieser Arbeit bestand deshalb darin, funktionell relevante Varianten mit Hilfe von *in vitro*-Studien zu identifizieren.

Die Assoziation zwischen 17 SNPs im *FADS*-Gencluster und Fettsäurekonzentrationen in maternalem Blut wurde in der bisher größten Kohorte zu diesem Thema analysiert, bestehend aus über 4000 schwangeren Frauen aus der „Avon Longitudinal Study of Parents and Children“ (ALSPAC). Die seltenen Allele der meisten analysierten *FADS*-Polymorphismen waren konsistent positiv mit den Substraten der Desaturase-Reaktionen assoziiert ($p<0,001$). Die gleichen Allele zeigten negative Assoziationen mit den Produkten der Desaturase-Reaktionen, sowie dem Produkte/Substrate-Verhältnis ($p<0,001$). Alle Assoziationen waren unabhängig von soziodemographischen Variablen und Ernährungsgewohnheiten. Negative Assoziationen konnten ebenso für Docosahexaensäure beobachtet werden ($p<0,001$), einer Fettsäure, der spezielle Bedeutung bei der neuronalen Entwicklung zugesprochen wird.

Die Assoziation zwischen acht *FADS*-Genvarianten und Fettsäurekonzentrationen in Muttermilch wurde in 772 (1,5 Monate nach der Geburt) bzw. 463 (6 Monate nach der Geburt) stillenden Müttern der Ulmer Säuglingsstudie untersucht. SNPs rs174547 and rs174556 zeigten negative Assoziationen mit Arachidonsäure und der D5D-Aktivität (Verhältnis Arachidonsäure zu Dihomo-gamma-linolensäure) an beiden Zeitpunkten (p-Werte zwischen 0,0002 und 0,0064). Eine longitudinale Analyse des Genotyp-Effekts auf den zeitlichen Verlauf der Fettsäurekonzentrationen in Muttermilch ergab Assoziationen mit Dodecansäure, Cis-15-Tetracosensäure und Trans-9-Octadecensäure ($p = 0,0186$, 0,0022 und 0,0032).

Basierend auf bioinformatischen Vorhersagen von regulatorischen Regionen wurden zwei Polymorphismen im *FADS2*-Genpromoter für funktionelle Analysen ausgewählt. Mittels Luciferase-Reporter-Assays konnte gezeigt werden, dass das seltene T-Allel des *FADS2*-Promoterpolymorphismus rs968567 eine erhöhte Promoteraktivität verursacht. Ausserdem wurde mit Hilfe von DNA-Affinitätsaufreinigung und anschließendem Immunblot ein Einfluss dieses SNPs auf die Bindungsaffinität des Transkriptionsfaktors ELK1 an den *FADS2*-Promoter nachgewiesen.

Zusammenfassend konnte in dieser Arbeit ein deutlicher Effekt von *FADS*-Polymorphismen auf die Fettsäurezusammensetzung maternaler Körperflüssigkeiten, die für

die frühkindliche Fettsäureversorgung wichtig sind, gezeigt werden. Die Analyse des Einflusses von maternalen *FADS*-Polymorphismen auf den frühkindlichen Fettsäurestatus ist Aufgabe zukünftiger Studien. Für einen der assoziierten Polymorphismen konnte im Rahmen dieser Arbeit eine funktionelle Relevanz gezeigt werden, wodurch erste Einblicke in allel-abhängige Regulationsmechanismen der Desaturase-Aktivität ermöglicht wurden. Dies ist Grundlage für weitere funktionelle Studien, um die genetisch determinierten molekularen Mechanismen, die zu interindividuellen Unterschieden im Fettsäuremetabolismus führen, komplett aufzuklären.

Abbreviations

AA	arachidonic acid, 20:4n-6
ADHD	attention deficit/hyperactivity disorder
ALA	alpha-linolenic acid, 18:3n-3
ALSPAC	Avon Longitudinal Study of Parents and Children
AUS	Australia
BCL6	B-cell lymphoma 6 protein
BMI	body mass index
CAD	coronary artery disease
cm	centimeters
CRC	Costa Rica
D5D	delta-5 desaturase
D6D	delta-6 desaturase
DHA	docosahexaenoic acid, 22:6n-3
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DPA	docosapentaenoic acid, 22:5n-3
ELK1	Ets like gene 1
EPA	eicosapentaenoic acid, 20:5n-3
FADS	fatty acid desaturase
FFQ	food frequency questionnaire
GBR	Great Britain
GER	Germany
GLA	gamma-linolenic acid, 18:3n-6
GWAS	genome-wide association study
HDL	high-density lipoprotein cholesterol
HOMA	homeostasis model assessment
HWE	Hardy-Weinberg equilibrium
IgE	immunoglobulin E

ITA	Italy
IQ	intelligence quotient
kg	kilograms
KORA	Cooperative Health Research in the region of Augsburg
LA	linoleic acid, 18:2n-6
LC-PUFA	long-chain polyunsaturated fatty acid
LD	linkage disequlilibrium
LDL	low-density lipoprotein cholesterol
m	meters
µl	microliters
MALDI-TOF	matrix assisted laser desorption ionization – time of flight
mg	milligrams
MI	myocardial infarction
mRNA	messenger ribonucleic acid
N	number of subjects
NED	Netherlands
NZL	New Zealand
PCR	polymerase chain reaction
PPARα	peroxisome proliferator-activated receptor alpha
PUFA	polyunsaturated fatty acid
RBC	red blood cells
RNA	ribonucleic acid
SAP	shrimp alkaline phosphatase
SD	standard deviation
SNP	single nucleotide polymorphism
SREBP	sterol regulatory element-binding protein
STAT1	signal transducer and activator of transcription 1
STAT3	signal transducer and activator of transcription 3

Index of fatty acids

Common name	Common abbreviation	Lipid name	Chemical name
linoleic acid	LA	18:2n-6	all-cis-9,12-octadecadienoic acid
gamma-linolenic acid	GLA	18:3n-6	all-cis-6,9,12-octadecatrienoic acid
eicosadienoic acid	-	20:2n-6	all-cis-11,14-eicosadienoic acid
dihomo-gamma-linolenic acid	DGLA	20:3n-6	all-cis-8,11,14-eicosatrienoic acid
arachidonic acid	AA	20:4n-6	all-cis-5,8,11,14-eicosatetraenoic acid
adrenic acid	-	22:4n-6	all-cis-7,10,13,16-docosatetraenoic acid
docosapentaenoic acid, osbond acid	-	22:5n-6	all-cis-4,7,10,13,16-docosapentaenoic acid
tetracosatetraenoic acid	-	24:4n-6	all-cis-9,12,15,18-tetracosatetraenoic acid
tetracosapentaenoic acid	-	24:5n-6	all-cis-6,9,12,15,18-tetracosapentaenoic acid
alpha-linolenic acid	ALA	18:3n-3	all-cis-9,12,15-octadecatrienoic acid
stearidonic acid	-	18:4n-3	all-cis-6,9,12,15-octadecatetraenoic acid
eicosatrienoic acid	ETE	20:3n-3	all-cis-11,14,17-eicosatrienoic acid
eicosatetraenoic acid	ETA	20:4n-3	all-cis-8,11,14,17-eicosatetraenoic acid
eicosapentaenoic acid	EPA	20:5n-3	all-cis-5,8,11,14,17-eicosapentaenoic acid
docosapentaenoic acid, clupanodonic acid	DPA	22:5n-3	all-cis-7,10,13,16,19-docosapentaenoic acid
docosahexaenoic acid	DHA	22:6n-3	all-cis-4,7,10,13,16,19-docosahexaenoic acid
tetracosapentaenoic acid	-	24:5n-3	all-cis-9,12,15,18,21-tetracosapentaenoic acid
nisinic acid	-	24:6n-3	all-cis-6,9,12,15,18,21-tetracosahexaenoic acid
palmitoleic acid	-	16:1n-7	cis-9-hexadecenoic acid
vaccenic acid	-	18:1n-7	cis-11-octadecenoic acid
elaidic acid	-	t18:1n-9	trans-9-octadecenoic acid
nervonic acid	-	24:1n-9	cis-15-tetracosenoic acid
lauric acid	-	12:0	dodecanoic acid
myristic acid	-	14:0	tetradecanoic acid
palmitic acid	-	16:0	hexadecanoic acid

1 Introduction

The composition of polyunsaturated fatty acids (PUFAs) in phospholipids has been associated with early visual, cognitive and motor development (1, 2), mental health and psychiatric disorders (3-5), metabolic syndrome (6-8), cardiovascular disease mortality (9, 10), immunological and inflammatory responses (11) as well as related diseases such as allergies (12-14), suggesting that PUFAs have a major impact on human health.

The biological effects of PUFAs are assumed to be mediated by the availability of long-chain polyunsaturated fatty acids (LC-PUFAs) with $\geq$20 carbon atoms and $\geq$3 double bonds, such as the omega-6 LC-PUFA arachidonic acid (AA; 20:4n-6), and the omega-3 LC-PUFAs eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (for a detailed description of fatty acid nomenclature see Index of fatty acids, page X). These fatty acids have several important functions in human metabolism, such as regulating the integrity and fluidity of cell membranes, acting as second messengers in intracellular signaling pathways, or regulating transcription. Also, LC-PUFAs are precursors for eicosanoids and docosanoids, which play an important role in inflammatory processes (15, 16).

The exact mechanisms how the individual composition of PUFAs and LC-PUFAs contributes to the development of complex diseases have not been revealed until now. Analysis of the factors that influence the composition of fatty acids in human tissues and determination of the optimal fatty acid status for maintenance of human health will help in the prevention of fatty acid related complex diseases. One important endogenous factor for the determination of PUFA and LC-PUFA levels in humans is the desaturation pathway of fatty acids, which will be presented in the first chapter of this thesis.

1.1 Endogenous production of LC-PUFAs from fatty acid precursors is mediated by delta-5 and delta-6 desaturase

Because of its significant effect on blood and tissue LC-PUFA contents, sufficient dietary supply with LC-PUFAs is pivotal in every stage of human life (17). Arachidonic acid is contained especially in meats and eggs, and marine foods are important sources for EPA and DHA (18). In addition to the dietary supply of pre-formed LC-PUFAs, they can also be synthesized endogenously from the precursor essential fatty acids linoleic acid (LA or 18:2n-6) and alpha-linolenic acid (ALA or 18:3n-3), which mainly originate from vegetable oils, by consecutive desaturation and chain-elongation. The rate-limiting enzymes in this reaction cascade are the delta-6 (D6D) and delta-5 desaturase (D5D). They are membrane-bound proteins with amino-terminal cytochrome *b5* domains carrying heme-binding motifs, two-membrane-spanning domains, three His-box motifs, and consist of 444 amino acids (19). The human delta-5 desaturase shares 61% amino acid identity and 75% similarity to the human delta-6 desaturase (20). Both enzymes are expressed in the majority of human tissues, with highest levels in liver and to a smaller amount in brain, heart and lung (19, 20). The conversion of both omega-3 and omega-6 precursors to their respective LC-PUFA products is catalyzed by D6D and D5D in a reaction cascade (21). In the first step, delta-6 desaturase converts LA to gamma-linolenic acid (GLA or 18:3n-6) in the omega-6 pathway and ALA to stearidonic acid (18:4n-3) in the omega-3 pathway by inserting an additional *cis* double bond at position 6 of the fatty acid chains. After an elongation step (resulting in dihomo-gamma-linolenic acid (20:3n-6) and eicosatetraenoic acid (20:4n-3), respectively), delta-5 desaturase catalyzes the formation of arachidonic acid (20:4n-6) and eicosapentaenoic acid (20:5n-3), which can be further elongated and desaturated, again with the help of delta-6 desaturase (15, 22-24). AA and EPA serve as substrates for eicosanoid synthesis, whereas DHA is the precursor for docosanoid production. Figure 1 gives an overview of the desaturation pathway in humans.

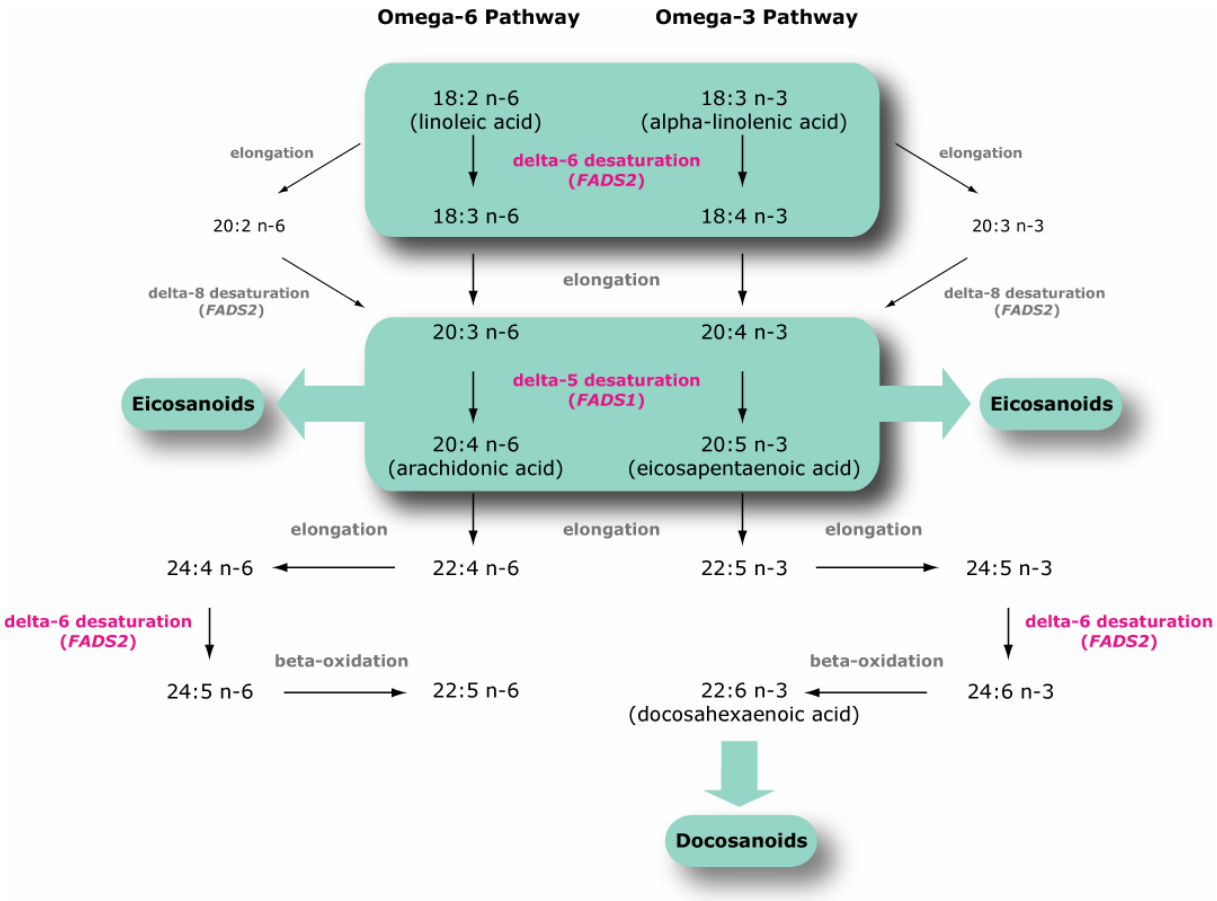

Figure 1: Omega-6 and omega-3 pathway for LC-PUFA synthesis from essential fatty acids by enzymatic desaturation and chain elongation. Modified from (25).

1.2 Delta-5 and delta-6 desaturase are encoded by *FADS1* and *FADS2*

The importance of the fatty acid desaturation pathway for LC-PUFA levels in human tissues led to great efforts in the characterization of the desaturase encoding genes rapidly at the beginning of the genomic era. Cloning of the human desaturases was first reported in 1999 (19, 20) and the corresponding genes (*FADS1* for delta-5 desaturase and *FADS2* for delta-6 desaturase) were mapped to chromosome 11q12-13.1 of the human genome in 2000 (26), which shows conserved synteny to the mouse genomic region containing the murine *fads1* and *fads2* genes on chromosome 19 (15). The two human genes are arranged in a head-to-head orientation and build a gene cluster together with a third desaturase gene, *FADS3* (see Figure 2). It is assumed that these three genes have arisen evolutionary from gene duplication, due to their similar exon/intron organization (12 exons and 11 introns) and a high degree of sequence homology (26). Whereas the function of the delta-5 and delta-6 desaturase is well known, the function of the protein product of the *FADS3* gene is still unidentified. Recently, several alternative splice forms of *FADS3* were identified and a tissue- or PUFA-specific role of *FADS3* in LC-PUFA synthesis was hypothesized (27-30).

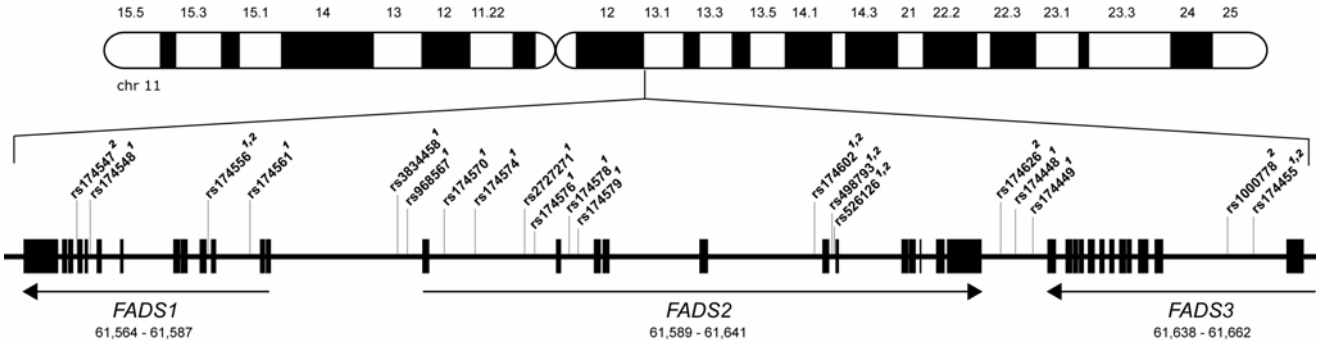

Figure 2: Schematic structure of the human fatty acid desaturase gene cluster located on chromosome 11. The genomic position is given below the gene names for each of the three *FADS* genes (NCBI database Build 37.1). The genomic location of polymorphisms analyzed in the two described association studies performed during this thesis is also depicted in this picture (1 denotes SNPs that were analyzed in the ALSPAC study, 2 denotes SNPs that were analyzed in the Ulm Birth Cohort).

1.3 *FADS* gene cluster polymorphisms are associated with LC-PUFA and lipid levels in different tissues

The important function of the delta-5 and delta-6 desaturase in the synthesis of LC-PUFAs made the desaturase encoding genes perfect candidate genes for association studies of genetic polymorphisms with PUFA and LC-PUFA levels in human tissues. All association studies on fatty acid levels that were published up to date reported significant associations of *FADS* genotypes with PUFA and LC-PUFA levels.

In the first study, 18 selected single nucleotide polymorphisms (SNPs) in and around the *FADS1* and *FADS2* genes were analyzed by Schaeffer et al. (31) for association with fatty acids in serum phospholipids in 727 German probands of the European Community Respiratory Health Survey I (ECRHS I). Significant results were obtained for 11 SNPs, which are all located in the same linkage disequilibrium (LD) block, and all analyzed fatty acids (p-values $< 1.0 \times 10^{-13}$) except for docosapentaenoic acid (22:5n-6) and DHA (22:6n-3). Carriers of the minor alleles of these 11 SNPs (rs174544, rs174553, rs174556, rs174561, rs3834458, rs968567, rs99780, rs174570, rs2072114, rs174583, and rs174589) had enhanced levels of 18:2n-6, 20:2n-6, 20:3n-6, and 18:3n-3 and decreased levels of 18:3n-6, 20:4n-6, 22:4n-6, 20:5n-3, and 22:5n-3. Haplotype analyses were in line with the findings of the single SNP analysis. The variability in fatty acid levels explained by the genetic variants for the 11 analyzed SNPs varied from exceptionally high for arachidonic acid (28.5%) to low for 22:5n-6 and 22:6n-3 (1-3%). This first association study on *FADS* polymorphisms with fatty acid levels in serum phospholipids clearly showed a significant association with an accumulation of desaturase substrates and a decline of desaturase products due to the minor alleles of the associated SNPs. The authors concluded that this might be the case as a result of a decline in the transcriptional levels or in the conversion rates of the desaturases in subjects carrying the minor alleles. These associations have been replicated in several independent studies, which showed associations of *FADS* genotypes with fatty acids in tissues including serum, plasma, erythrocyte membrane phospholipids, adipose tissue, and breast milk (32-37). In addition to the observed association in adults, some studies recently investigated the relationship between

FADS genotypes and fatty acid levels in adolescents (38) and young children (39) and also reported significant associations.

A recent genome-wide association study (GWAS) on plasma fatty acid concentrations in 1,075 subjects identified SNP rs174537 in the 5' region of *FADS1* as top hit for the genetic determination of arachidonic acid levels (p-value = 5.95×10^{-46}) (40). Individuals homozygous for the minor allele of rs174537 had lower concentrations of 20:4n-6 compared to the homozygous carriers of the major allele. The SNP accounted for 18.6% of the variance in the concentration of arachidonic acid. Furthermore, the SNP was associated with altered levels of 20:2n-6 (p-value = 6.78×10^{-9}) and 20:5n-3 (p-value = 1.04×10^{-14}). Additionally, several genome-wide association studies (GWAS) on complex lipid traits in Caucasian populations reported associations of *FADS* polymorphisms with serum phosphatidylcholines (41, 42), and the blood lipid parameters LDL (40, 43-46), HDL (43, 44, 47), total cholesterol (40, 43-45) and triglycerides (47). An association of the *FADS1* SNP rs174547 with plasma lipid levels has as well been observed in Asian populations (48, 49). Taken together, the desaturation pathway seems to be highly important for lipid homeostasis in the human body.

Moreover, SNP rs174550 in *FADS1* has recently been associated with fasting glucose, the HOMA-B index, which is an estimate of beta-cell function, and risk of type 2 diabetes mellitus in a genome-wide association meta-analysis (50), with the insulinogenic index in a replication study (51), and fasting plasma glucose in a Korean population (49), suggesting that the fatty acid composition and degree of desaturation may influence glucose homeostasis. Another genome-wide association study on genetic determinants for resting heart rate found the minor allele of SNP rs174547 (*FADS1)* to be associated with a shorter heart rate interval (52), which might be due to a function of arachidonyl-CoA (the direct product of the delta-5 desaturase reaction) in calcium release. All these results highlight the importance of fatty acid desaturation and the *FADS* genes in various physiological processes.

1.4 Gene-diet interactions and the impact of *FADS* genotypes on complex phenotypes

In addition to the association of *FADS* genotypes with fatty acid and lipid levels, first results exist about the modulation of complex fatty acid related phenotypes by *FADS* genotypes and have been extensively reviewed (16, 53-56). Several studies exist, which analyzed the influence of *FADS* genetic variants on diseases such as coronary artery disease (34, 57), metabolic syndrome (58), and allergic diseases (31, 39, 59). Some, but not all of these studies have found significant associations between the analyzed SNPs and the respective disease. A summary of the existing studies is presented in Table 1.

Especially neurodevelopmental and mental outcomes are assumed to be influenced by genetic variants in the *FADS* gene cluster. First hints for associations of *FADS* polymorphisms with attention-deficit/hyperactivity disorder (ADHD) (60) and bipolar disorder (61) have been observed, which are supported by the detection of linkage peaks in the *FADS* chromosomal region in previous linkage scans on both diseases (62-65). Recent expression studies, which however did not include analysis of polymorphisms, reported an elevated *FADS2* gene expression in the prefrontal cortex of bipolar disorder patients (66) and reduced expression of *FADS1* in the prefrontal cortex of patients with major depressive disorder (67). In 2007, Caspi et al. (68) reported on a genetic variant in the *FADS2* gene modulating the association between breastfeeding and intelligence quotient (IQ) in two large birth cohorts. Previously breastfed and formula fed children differed in later IQ in both cohorts, but this effect was more pronounced and only significant in children carrying the major allele of the investigated SNP (rs174575). In contrast, children with the minor allele neither gained an advantage nor suffered a disadvantage from being fed breast milk. A recent attempt to replicate these findings in the ALSPAC cohort (69) showed differing effects from the Caspi study, with children homozygous for the minor allele exhibiting the biggest difference in IQ scores between the formula and breastfed group. Although these contradictory results require further replication, a modification of the breastfeeding effect on IQ scores by *FADS2* genotypes has been shown in both studies. This was one of the first hints that *FADS* genotypes modulate dietary effects and by this influence phenotypic outcomes.

However, the latest study analyzing the modulation of breastfeeding effects on IQ by *FADS* genotypes in adolescents failed in replicating the previous findings (70) (see Table 1).

Table 1: Studies exploring associations between *FADS* polymorphisms and fatty acid related outcomes

Study	Subjects	Analyzed SNP(s)	Analyzed outcome	Principal findings
Baylin et al. (34)	N (ca)=1694, N (co)=1694; CRC	rs3834458	nonfatal acute myocardial infarction (MI) in adults	no association between rs3834458 and MI
Martinelli et al. (57)	N (ca)=610, N (co)=266; ITA	13 SNPs in the *FADS* gene cluster	coronary artery disease (CAD) in adults	*FADS* haplotypes (including rs174545, rs174570, rs174583, and rs1000778) associated with high AA/LA ratio are also associated with higher CAD risk (p=0.02)
Truong et al. (58)	N (ca)=656, N (co)=1159; CRC	rs3834458	metabolic syndrome in adults	rs3834458 modulates effect of ALA concentration on metabolic syndrome prevalence ratio (p for interaction=0.08)
Schaeffer et al. (31)	N (total)=727, N (rhinitis)=76, N (eczema)=49; GER	18 SNPs in the *FADS* gene cluster	allergic rhinitis, atopic eczema, and IgE levels in adults	no association with IgE levels; minor alleles are protective for allergic rhinitis and atopic eczema, which is however not significant after correction for multiple testing
Rzehak et al. (39)	N (total)=333, % eczema=14.1, GER; N (total)=542, % eczema=30.6, NED	5 SNPs in *FADS1*/*FADS2*	IgE levels and eczema in first two years of life	no association with IgE levels; SNPs are significantly associated with eczema in the German ($p<0.005$), but not in the Dutch study

N = number of subjects in the study, ca = cases, co = controls, CRC = Costa Rica, ITA = Italy, GER = Germany, NED = Netherlands, GBR = Great Britain, NZL = New Zealand, AUS = Australia

Table 1: continued

Study	Subjects	Analyzed SNP(s)	Analyzed outcome	Principal findings
Singmann et al. (59)	N (total)=2718, % asthma=4, % bronchitis=29, % eczema=38, % hay fever=9; GER	5 SNPs in *FADS1*/*FADS2*	asthma, bronchitis, eczema, hay fever in children	no association with any of the tested outcomes
Brookes et al. (60)	N (ca)=180, N (co)=180; GBR	29 SNPs in the *FADS* gene cluster	attention-deficit/hyperactivity disorder (ADHD) in children and adolescents	SNP rs498793 is associated with ADHD (p=0.004)
WTCCC (61)	N (ca)=2000 (for each disease), N (co)=3000; GBR	genome-wide study	seven common diseases (bipolar disorder, coronary artery disease, Crohn's disease, hypertension, rheumatoid arthritis, type 1 diabetes, type 2 diabetes) in adults	no association of *FADS* SNPs with the analyzed diseases on the genome-wide level, but hints for association of SNP rs174548 with bipolar disorder (p=0.048), coronary artery disease (p=0.021), and Crohn's disease (p=0.027) (summarized in (41))
Caspi et al. (68)	N (Dunedin)=1037, NZL; N (E-risk)=2140, GBR	rs174575, rs1535	intelligence quotient (IQ) in children	SNP rs174575 modulates effect of breastfeeding on IQ development in both cohorts (p for interaction=0.035 and 0.018); biggest IQ difference in carriers of major allele
Steer et al. (69)	N=5934; GBR	rs174575, rs1535	intelligence quotient (IQ) in children	SNP rs174575 modulates effect of breastfeeding on IQ development (p for interaction=0.0091); biggest IQ difference in carriers of minor allele
Martin et al. (70)	N=720 twin pairs; AUS	rs174575, rs1535, rs174583	intelligence quotient (IQ) in adolescents	no interaction between the tested SNPs and breastfeeding on IQ

An interaction of *FADS* genotypes with dietary fatty acids has been shown in several studies. Moltó-Puigmarti et al. (36) reported that *FADS* gene variants modify the association between fish intake and DHA and EPA+DPA contents in human milk lipids. The contribution of DHA and EPA+DPA to total milk lipids was similar in women with low fish intake for all genotypes, whereas DHA and EPA+DPA increased with higher fish consumption in carriers of the major allele but not in homozygous minor allele carriers (p-value for interaction = 0.077 for DHA and p-value for interaction = 0.019 for EPA+DPA). Another study replicated the previously reported association between a *FADS1* polymorphism and cholesterol concentrations, but additionally showed that this association was only significant in subjects with a high dietary intake of n-3 and n-6 PUFA (71).

Overall, in addition to the association with fatty acid and lipid levels, these results indicate a role of *FADS* polymorphisms in the development of fatty acid related phenotypes by a modulation of dietary fatty acid effects, which emphasizes the central role of the *FADS* genes in fatty acid and lipid metabolism.

The proposed existence of gene-diet interactions is also especially interesting for intervention studies addressing the effects of LC-PUFAs on phenotypic outcomes. Several intervention studies showed that especially higher maternal supply with DHA during pregnancy and lactation is positively associated with better clinical outcomes, such as visual and cognitive development (72-76) and later intelligence scores in children (77, 78). Based on these results, it is recommended that pregnant and lactating women achieve an average daily intake of at least 200 mg DHA (an omega-3 LC-PUFA with high incorporation into neural tissues considered of particular significance for brain and retina function) (79), because the child is supplied by the mother with PUFAs and LC-PUFAs through placental transfer during pregnancy (17) and through breast milk during lactation (80-83). However, not all studies found significant associations between maternal LC-PUFA supplementation and developmental outcomes (e.g. (78, 84-86)). One reason for this discrepancy could be the influence of numerous possible confounders such as sex, maternal age, socioeconomic status, family size, birth order, birth weight and childhood experiences (87). Thinking of gene-diet interactions, another reason for such inconsistent and ambiguous results could be inter-

individual variations in LC-PUFA metabolism due to genetics, which might lead to imprecise study results (54). The identification of the genetic factors influencing the infant's fatty acid supply during early stages of life is therefore highly interesting to increase the accuracy of such intervention studies.

1.5 Molecular mechanisms for inter-individual differences of fatty acid levels

Although the association between *FADS* polymorphisms and fatty acid levels is very well established in several tissues, the functional relevant variants leading to an accumulation of desaturase substrates and a decline of desaturase products have not been identified until now. It is assumed that either the transcription rate of the *FADS* genes or the desaturase enzyme activity is reduced in carriers of the minor alleles. All of the associated SNPs are intronic or intergenic variants, and there are no reports on coding exonic variants in the *FADS* genes. Only a few studies have investigated the influence of *FADS* polymorphisms on *FADS* transcript levels, but these found a clear allele-dependent *FADS* mRNA expression level. In their study on polygenic dyslipidemia, Kathiresan et al. (47) analyzed the association between genotypes and RNA expression of over 39,000 profiled transcripts in 957 human liver tissue samples. The major T allele of SNP rs174547 was associated with higher transcript levels of *FADS1* and *FADS3* (p-values = 5.0×10^{-35} and 1.0×10^{-8}, respectively). Another genome-wide association study on global gene expression in lymphoblastoid cell lines (88) confirmed these results by showing that those alleles that were associated with higher desaturase product levels (major alleles) also associate with an increased expression level of *FADS1*. However, the real causative variants cannot be inferred from these results because of a high degree of linkage disequilibrium (LD) between SNPs in the *FADS* gene cluster. This means that the observed effects might virtual be caused by other polymorphisms, which are correlated with the analyzed SNP. Therefore, functional *in vitro* studies are needed to identify the real functional causal variant(s).

1.6 Aims of the thesis

Inter-individual differences in fatty acid levels and composition have been associated with several disorders and diseases in adulthood but also with developmental outcomes in early infancy. It is therefore of highest interest to elucidate the factors and mechanisms that influence the infant fatty acid supply during pregnancy and lactation for achievement of an optimal infant fatty acid status. The first objective of the present thesis was therefore to analyze whether maternal *FADS* polymorphisms are associated with fatty acid levels in blood and breast milk of pregnant and lactating women, which are body fluids of particular importance for fatty acid supply of the child. For this purpose, the genotype effect on maternal red blood cell (RBC) fatty acid levels in pregnant women in the largest cohort analyzed so far including more than 4000 women from the Avon Longitudinal Study of Parents and Children (ALSPAC) was determined. Additionally, the association between *FADS* genotypes and breast milk fatty acid levels in lactating mothers at 1.5 and 6 months of lactation was analyzed in 772 (1.5 months) and 463 (6 months) women of the Ulm Birth Cohort Study.

Although the results of all association studies on fatty acid levels show an important role of the *FADS* gene cluster in the regulation of fatty acid composition, the causative variants have not been identified until now, which is essential for understanding the molecular basis of individual fatty acid levels. The second objective of this thesis was thus to analyze the molecular basis for inter-individual differences in fatty acid levels by identification of functional relevant variants that modify the efficiency of *FADS* gene transcription. For this purpose, potential functional relevant SNPs were chosen using bioinformatic prediction tools and the effects of the chosen SNPs on transcription activity and protein binding were analyzed using *in vitro* techniques in cell culture based systems (e.g. reporter gene assays, electrophoretic mobility shift assays, DNA-affinity purification).

2 Subjects and methods

Subjects and methods are described in the attached papers (see Appendix A1-A3). The following section gives additional information about the studies analyzed in this thesis and some further information on the methods used.

2.1 The Avon Longitudinal Study of Parents and Children (ALSPAC)

2.1.1 General description

The Avon Longitudinal Study of Parents and Children (ALSPAC) was designed to analyze the ways in which the physical and social environments interact over time with genetic inheritance to affect health, behavior and development in infancy and childhood (89).

Avon is situated in England, about 120 miles due west of London. The study area includes the major city Bristol as well as surrounding areas including small towns, villages and farming communities. Pregnant women resident in the study area with an expected delivery date between 1 April 1991 and 31 December 1992 were asked to participate in the study. In total, almost 14,000 women were included in the study, which corresponds to estimated 85% of the eligible population in the study area (90).

Environmental influences that were considered for analysis include psychosocial conditions, features of parenting strategies, the diet and lifestyle of the family, pollutants (air, water, and food), housing circumstances, health behavior of the family, medical and dental care, the types of day care and the schooling that the child receives. Phenotypic outcomes that were measured in this study include growth, onset of obesity, respiratory function, traits relevant to adult-onset diseases, infections, motor and mental ability, educational achievements, sexual development, accidents and injuries, atopic diseases, mood behavior,

and temperament (90). Because it was anticipated that the outcomes depend on complex interactions between the environment and variation in the genetic make-up, DNA was collected from roughly 10,000 mothers and their children for genomic analysis (91).

2.1.2 Specific study methods and characteristics

In the presented study that was conducted during this thesis, 18 SNPs in the *FADS* gene cluster were genotyped in DNA samples from 9,763 mothers. SNP selection was done based on linkage disequilibrium information provided by the HapMap database (http://hapmap.ncbi.nlm.nih.gov/, HapMap data Rel24/phaseII Nov 08, on NCBI36 assembly, dbSNP b126), which recognizes redundancy between near-by markers and selects so called tag SNPs covering most of the genetic information in the region of interest (92), and on results from previous association studies.

Genotyping was performed using iPLEX^{TM} Gold chemistry (Sequenom) under standard conditions and a MALDI-TOF (matrix assisted laser desorption ionization – time of flight) based allele detection method. Briefly, the SNP-containing regions of interest were amplified by polymerase chain reaction (PCR) from genomic DNA using specific primers. After deactivation of remaining deoxynucleotides (dNTPs) by shrimp alkaline phosphatase (SAP), a single base extension reaction followed using mass-modified dideoxynucleotide terminators. In this reaction, the extension primer, which anneals immediately upstream of the polymorphic site of interest, is elongated dependent on the template sequence. After removal of salt ions by ion exchange and transfer of the extension reaction onto a silicon chip covered with a 3-hydroxypicolinic acid matrix surface, the allele-specific difference in mass between extended primers was measured using MALDI-TOF mass spectrometry. Identification of SNP alleles was done based on the resulting mass spectra with MassARRAY Typer Version 4.0.5 (Sequenom). A detailed overview of the genotyped SNPs and the used oligonucleotides is given in Appendix B1 and B2.

Additionally, relative concentrations of 40 fatty acids in maternal RBC samples have been measured by gas-liquid-chromatography in a subset of 4,457 women. 6,711 samples that were obtained at varying times from these women (between 4th and 44th week of pregnancy) were analyzed in total. For a short description of the procedure used for fatty acid analysis, see Appendix A1.

For the main analysis, linear regression was used to investigate the associations between *FADS* genotypes and log transformed fatty acid levels of 6,711 samples including also longitudinal analysis to take account of the repeat measurements during pregnancy and applying an additive genetic model. The validity of the main analysis was explored by adjusting for seven potential socio-demographic confounders: multiple pregnancy (singleton/multiple), parity (primiparous/multiparous), maternal smoking at gestation week 32 (yes/no), gestational age at blood sampling, maternal age, maternal pre-pregnancy body mass index (BMI), and a measure of family adversity calculated from 18 items including teenage pregnancy, low education, financial hardship, poor living conditions, partner conflict, substance abuse, maternal anxiety or depressive symptoms and criminal behavior of either parent. Including these confounders into the analysis, a total number of 3,625 women resulting in 5,468 observations due to repeat fatty acid measurements during pregnancy remained for adjusted analysis. A detailed description of demographics of the samples for adjusted analysis can be found in Table 2. Additionally, adjustment was performed for 81 dietary variables obtained from a Food Frequency Questionnaire (FFQ) completed around the 32nd week of gestation by the mothers to exclude possible confounding by differential LC-PUFA intake. Questions on the FFQ included the number of portions of specific foods per week, such as the frequency of eating meat, sausages, white fish, oily fish, pasta, salad, etc. Also, specific questions regarding the use of fats were asked, such as the frequency of using butter, margarine or sunflower oil.

Table 2: Demographics of the ALSPAC population in adjusted analysis.

		N	%
Multiple births	Singletons	5416	99.05
	Twins	52	0.95
Multiparity	0	2537	46.40
	1	1980	36.21
	2	698	12.76
	3+	253	4.63
Maternal smoking	Yes	934	17.08
	No	4534	82.92

	Mean	SD
Gestational age at blood sampling (weeks)	26.87	8.15
Maternal age (years)	28.57	4.59
Maternal BMI (kg/m²)	23.05	3.80
Family adversity	1.19	1.55

SD: standard deviation. Total N (number of samples) = 3625 mothers.
Numbers refer to a total number of 5468 observations.

2.2 The Ulm Birth Cohort Study

2.2.1 General description

The Ulm Birth Cohort Study was designed to investigate factors that are important for healthy child development and originally aimed to analyze intrafamilial transmission of *Helicobacter pylori* infection (93).

The city of Ulm is situated in the south of Germany. All women who came to the Department of Gynecology and Obstetrics at the University of Ulm (the major department of obstetrics in the study area) for the delivery of their baby between November 2000 and November 2001 were recruited for the study. Recruitment was done during an average stay of five days in hospital after delivery. Overall, 1066 families were included in the study (94), which corresponds to 67% of all 1593 eligible families (93).

Standardized interviews were conducted by trained personnel during hospitalization including questions about housing and living conditions, lifestyle factors, medical history, and health status during pregnancy. Additionally, anthropometric and laboratory data were collected from the mothers' pregnancy health charts using a standardized form.

2.2.2 Specific study methods and characteristics

At 1.5 months and 6 months postpartum, mothers were asked whether they were still breastfeeding and milk samples of affirmative mothers were collected by a trained nurse (N=772 and 463, respectively). Table 3 shows the general characteristics of mothers included in the present study.

Table 3: General characteristics of the Ulm Birth Cohort separated by duration of breastfeeding.

		All mothers	Mothers not breastfeeding 6 months postpartum	Mothers still breastfeeding 6 months postpartum
			Number (%)	
Number of subjects		772	309	463
School education before graduation (years)	≥12	333 (43.13)	111 (35.92)	222 (47.95)
	10	287 (37.18)	117 (37.86)	170 (36.72)
	≤ 9	143 (18.52)	73 (23.62)	70 (15.12)
	no graduation	6 (0.78)	5 (1.62)	1 (0.22)
	missing	3 (0.39)	3 (0.97)	0 (0.00)
Maternal smoking (ever 100 cigarettes in their lifetime)	yes	343 (44.43)	148 (47.90)	195 (42.12)
	no	428 (55.44)	160 (51.78)	268 (57.88)
	missing	1 (0.13)	1 (0.32)	0 (0.00)
Maternal smoking (during pregnancy)	yes	65 (8.42)	38 (12.30)	27 (5.83)
	no	707 (91.58)	271 (87.70)	436 (94.17)
	missing	0 (0.00)	0 (0.00)	0 (0.00)
Maternal smoking (1.5 months postpartum)	yes	53 (6.87)	34 (11.00)	19 (4.10)
	no	716 (92.75)	275 (89.00)	441 (95.25)
	missing	3 (0.39)	0 (0.00)	3 (0.65)
Maternal smoking (6 months postpartum)	yes	67 (8.68)	44 (14.24)	23 (4.97)
	no	671 (86.92)	232 (75.08)	439 (94.82)
	missing	34 (4.40)	33 (10.68)	1 (0.22)
			Mean (SD)	
Age (years)		31.29 (4.76)	30.18 (4.98)	32.20 (4.44)
Maternal height (cm)		166.59 (6.38)	166.59 (6.41)	166.59 (6.38)
Maternal pre-pregnancy BMI		23.03 (3.86)	23.41 (4.15)	22.78 (3.65)

SD: standard deviation. Number of subjects refers to all mothers where demographic data were available. Anthropometric data were available for 770 (age and height) and 746 (weight) mothers.

The fatty acid profile of the breast milk samples was measured by gas-liquid chromatography as described in detail previously (95). In total, 26 saturated, monounsaturated, n-3, n-6, and trans fatty acids were successfully analyzed.

300 µl of the remaining breast milk samples were used for DNA extraction with the High Pure PCR Template Preparation Kit (Roche). Genotyping of 12 selected SNPs (based on linkage disequilibrium information using the HapMap database and on results from former studies) was performed as described above and identification of SNP alleles was done based on the resulting mass spectra with MassARRAY Typer Version 3.4 (Sequenom). A detailed overview of the genotyped SNPs and the used oligonucleotides is given in Appendix B3 and B4.

Linear regression analysis applying an additive genetic model was used to analyze associations between genotypes and fatty acid levels at the two time points separately. For the longitudinal analysis of fatty acid levels by genotype over time a Generalized Estimating Equation Regression model (GEE model) was applied to account for correlations between repeated measurements over time including those mothers that were breastfeeding at both time points. The model can be written down as $E(y|x) = \beta_0 + \beta_1$ TIME $+ \beta_2$ SNP $+ \beta_3$ TIME x SNP, where TIME = 0 at time point 1.5 months and TIME = 1 at time point 6 months. The variable 'SNP' was coded as 0, 1, and 2, dependent on the number of minor alleles. β_0 indicates the intercept (mean fatty acid level in homozygous carriers of the major allele at 1.5 months), and β_1, β_2, and β_3 indicate the β coefficients (main effect of change of fatty acid levels over time, main effect of genotype with additive change in the outcome level with each minor allele copy, or the interaction of both, respectively) for the respective variables. Regression and GEE analyses where performed by statistical software SAS/STAT 9.1.3 procedures 'proc regression' or 'proc genmod' (96). Allele frequencies and Fisher's exact test for Hardy-Weinberg-Equilibrium were calculated by module SAS/Genetics (97).

2.3 The KORA F3 Study

2.3.1 General description

The KORA (Cooperative Health Research in the region of Augsburg) study is a population based study that examines the health of thousand of citizens in and around the city of Augsburg (located in the south of Germany) to investigate the effects of the environment, behavior, and genes. Focus of the study is the investigation of the development and course of chronic diseases, in particular myocardial infarction and diabetes mellitus. For this purpose, data on individual health behavior (e.g. smoking, diet, or exercise) and environmental factors (e.g. air pollution, environmental noise) as well as biomaterials such as DNA and RNA have been collected.

The KORA study consists of different surveys (S1-S4), in which over 18,000 people participated in total (see Figure 3). The surveys S3 (examined in 1994-1995) and S4 (examined in 1999-2001) comprised individuals aged 25-74 years and have been followed up later (F3, examined in 2004/2005 and F4, examined in 2006-2008) (98). In KORA F3, a total number of 3006 subjects participated, comprising individuals aged 35-79 years (99).

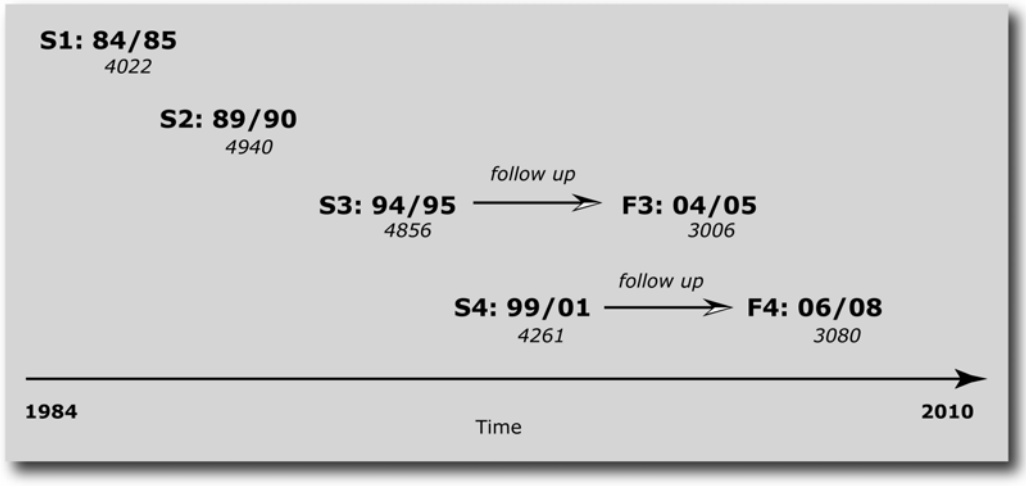

Figure 3: Schematic presentation of the four different KORA surveys and their follow-up. Italic numbers indicate number of subjects that participated in the surveys.

2.3.2 Specific study methods and characteristics

A subgroup of 1644 participants of the KORA F3 study was chosen for SNP genotyping using the Affymetrix Gene Chip Human Mapping 500K Array Set. Additionally, genome-wide mRNA expression levels in peripheral blood of 322 selected fasting KORA F3 probands (170 men and 152 women, age 51-79 years) were measured using the Illumina Human-6 v2 Expression BeadChip. A detailed description of the genotyping procedure and gene expression analysis can be found elsewhere (100).

In the present study, genotype and mRNA expression data from 322 KORA F3 samples were used to calculate Pearson's correlation coefficients for the correlation between transcription factor mRNA expression using a linear regression model either independent of the *FADS* genotype or stratified by *FADS* genotype.

3 Results and Discussion

The results of this thesis are based on three articles that were published in conjunction with this thesis (see Appendix A1-A3) and will be summarized and discussed in the following section.

3.1 *FADS* polymorphisms influence amounts of RBC fatty acids in pregnant women

The results of this project that was conducted during this thesis have been published in the American Journal of Clinical Nutrition (Koletzko B, Lattka E et al., 2011, *AJCN*, 93: 211-219) and the complete publication can be found in the appendix of this thesis (Appendix A1).

18 SNPs spanning the complete *FADS* gene cluster (including *FADS1*, *FADS2* and *FADS3*) were genotyped in the ALSPAC study. One SNP was excluded from further analysis because alleles could not be discriminated. Two SNPs showed deviations from Hardy-Weinberg equilibrium, however, due to the large sample size these departures were considered minor and it was considered important to report results for these two SNPs as well. 17 SNPs were therefore analyzed for their association with RBC fatty acid levels in the so far largest candidate gene study on *FADS* polymorphisms and fatty acid levels including data from 4,457 pregnant women (mean gestational age at blood sampling 26.8 ± 8.2 weeks). The genomic location of the analyzed SNPs is depicted in Figure 2.

3.1.1 Summary of results

Overall, the minor alleles of most of the analyzed SNPs (including *FADS3* polymorphisms) were positively associated with precursor PUFAs with two or three double bonds, and negatively associated with LC-PUFAs with four or more double bonds, such as arachidonic acid, as well as with the major product/substrate ratios of the n-6 pathway (AA to LA ratio) and the n-3 pathway (EPA to ALA ratio). These results are in accordance with results from previous studies. Associations in terms of the explained variability and the size of the regression coefficient (measured in terms of standard deviations) were strongest for the n-6 metabolite 20:3n-6, followed by the n-6 FA 20:4n-6, 22-carbon fatty acids, 20:2n-6 and 18:2n-6. Only five of the 17 SNPs showed evidence of an association with 18:3n-6. With respect to n-3 fatty acids, associations were generally weaker than for n-6 fatty acids. However, interestingly, 14 of the 17 analyzed SNPs showed highly significant associations with DHA levels ($p < 0.001$). Regression coefficients (measured in terms of standard deviations) indicated a relative decline of arachidonic acid levels of 7.9%-15.5% (DHA: 6.2%-9.7%) of total fatty acid levels per copy of the respective minor alleles. R^2 values reflecting the variability of fatty acid levels explained by the genetic variants ranged from 0.14% (18:3n-6) to 5.61% (20:3n-6) and reached 1.13% for arachidonic acid and 0.51% for DHA (see Table 4). Similar, although weaker, associations were observed for the *FADS3* SNP rs174455.

All associations remained significant after correction for multiple testing using the false discovery rate method. Also, the associations observed in the main analysis were stable towards controlling for seven potential socio-demographic confounders as well as 81 dietary variables derived from food frequency questionnaires completed by the mothers during pregnancy. By including these co-variables into the analysis, up to 12.1% of the variance in fatty acid amounts could be explained (see Table 4).

Table 4: Maximum R^2 across the 17 genetic variants for each fatty acid in unadjusted analyses (reflecting the genetic association) and in adjusted analyses (including the effect of confounders). N = number of subjects in the analysis.

The genetically explained variability of RBC fatty acid levels was rather low in this study compared to other studies, which measured serum and plasma FA, but R^2 values were in the same range as reported in another recent study on RBC fatty acids (101).

By inclusion of sociodemographic and dietary co-variables into the analysis, up to 12.1% of the variance in RBC fatty acid levels could be explained. In the case of 20:3n-6, most of the explained variance comes from the genetic association, whereas in the case of e.g. 20:4n-6 and 22:6n-3, a smaller part of the explained variance is due to the genetic association. This shows that both factors – genetics and environment – play an important role in the determination of RBC fatty acid levels.

	Unadjusted	Adjusted
Omega-6		
18:2	0.64%	11.72%
18:3	0.14%	2.38%
20:2	0.72%	10.38%
20:3	5.61%	9.17%
20:4	1.13%	7.04%
22:4	1.21%	5.99%
22:5	1.30%	5.41%
Omega-3		
18:3	0.38%	6.54%
20:5	0.27%	12.10%
22:5	0.15%	8.12%
22:6	0.51%	6.04%
Ratios		
AA to LA	3.28%	8.13%
EPA to ALA	1.37%	9.45%
N	6711	5468

As a control, association analyses were repeated for saturated and monounsaturated fatty acids, which are synthesized in other pathways independent of desaturation reactions. As expected, no associations were observed for any of the tested saturated fatty acids. For monounsaturated fatty acids there were also no significant associations except for cis-9-hexadecenoic acid (16:1n-7).

3.1.2 Discussion

In the presented study, increased levels of desaturase substrates (such as linoleic acid and alpha-linolenic acid) and decreased levels of desaturase products (such as arachidonic acid and adrenic acid) were observed, confirming previous observations in smaller studies and suggesting a decline in desaturase expression or activity due to the polymorphisms. Most of the analyzed SNPs, independent of whether they are located in *FADS1*, *FADS2*, or in the intergenic region, were associated with substrates and products of both desaturase reactions (D5D and D6D). It was not possible to ascribe the observed associations with D5D (D6D) substrates and products specifically to those SNPs that are located in *FADS1* (*FADS2*) as was also the case in the previous studies. This lies in the fact that most of the analyzed polymorphisms are in linkage disequilibrium and possibly also because the two desaturase reactions are dependent of each other, since there exist however not fully identified feedback mechanisms in the regulation of LC-PUFA synthesis (15, 102, 103).

The genetically explained variability was rather low in this study compared to other studies (1.13% for AA levels in this study versus 28.5% and 18.6% in previous studies (31, 40)), which might be explained by the fact that in this study RBC FA were measured compared to serum and plasma FA in the previous studies. A recent study by Zietemann et al. (101) reported a genetically explained variance of RBC AA levels of 2.6%. These and our results suggest that RBC fatty acid levels are less influenced by *FADS* genotypes than plasma or serum phospholipid FA levels, the reason of which remains speculative. The associations, however, remained stable after adjustment for socio-demographic confounders and dietary habits, which supports the validity of the results.

A further substantiation of the observed associations was provided by an additional association analysis of saturated and monounsaturated fatty acid levels, which showed no associations after adjustment for multiple comparisons as expected. In case of significant findings for these fatty acid groups, all other associations would have been in risk of being considered as false positive results. This risk was eliminated by the negative findings for the tested saturated and monounsaturated fatty acids. The only fatty acid that showed consistent associations with most of the analyzed *FADS* SNPs was cis-9-hexadecenoic acid (16:1n-7). A possible explanation for this association might be a correlation of this fatty acid with n-6 or n-3 PUFAs, as was observed for 22:4n-6 ($r = -0.31$).

The function of the *FADS3* protein product has not been revealed until now. However, the observed significant associations of *FADS3* SNPs with desaturase substrates and products in this study suggest a role of this gene in the desaturation pathway as well. Future functional studies on the activity of the *FADS3* protein product will further contribute to the complete elucidation of the desaturation pathway.

Transfer of LC-PUFAs from the mother to the fetus is mediated by placental transfer during pregnancy (17, 104, 105) and is of special importance because desaturation activity in the fetal liver was shown to be low before birth (106). The observed positive effects of LC-PUFAs on fetal visual, cognitive, and brain development are primarily attributed to the transfer of omega-3 LC-PUFAs and especially to DHA. Besides the mother's dietary intake, the mother's *FADS* genotype has been considered as determinant of the child's supply with these omega-3 LC-PUFAs.

However, whereas the associations of *FADS* polymorphisms with n-6 LC-PUFAs such as arachidonic acid are well established, only a few studies reported significant associations with DHA levels so far (32, 35, 36, 39), all other studies did not find such associations. First results exist about the association of *FADS* genotypes with brain-related phenotypes such as intelligence development (68, 69) and attention-deficit/hyperactivity disorder (60). These effects are thought to be in part mediated by DHA availability in the brain, but it is unclear how *FADS* genotypes modulate these phenotypes, if not by direct association with DHA levels.

In the present study, which is the largest cohort that analyzed associations between *FADS* genotypes and RBC fatty acid levels so far, a clearly significant association of *FADS* SNPs with DHA levels was observed. One explanation for the missing association in preliminary studies might be a lack of statistical power. Moreover, the presented study might reflect a higher rate of DHA synthesis in pregnant women compared to men or non-pregnant women, which might increase the likelihood of detecting SNP effects on desaturase activity as found in this study. The biological relevance of genotype-dependent maternal DHA levels for developmental child outcomes has to be analyzed in future studies.

By inclusion of sociodemographic and dietary co-variables into the analysis, up to 12.1% of the variance in RBC fatty acid levels could be explained in this study (see Table 4). In the case of 20:3n-6, most of the explained variance comes from the genetic association (5.61% in unadjusted analysis vs. 9.17% in adjusted analysis), whereas in the case of e.g. 20:4n-6 and 22:6n-3, a smaller part of the explained variance is due to the genetic association (1.13% vs. 7.04% and 0.51% vs. 6.04%). This shows that both factors – genetics and environment – play an important role in the determination of individual fatty acid levels. Future interaction analyses between heritable and environmental factors should aim at determining the biological relevance for the variance in fatty acid levels and fatty acid related disorders of these two factors, and how they influence each other. Also, the identification of those factors determining the still missing part of explained variance in RBC fatty acid levels is a task for future studies.

3.2 *FADS* polymorphisms influence amounts of breast milk fatty acids in lactating women

The results of the second study that was conducted during this thesis have been published in the American Journal of Clinical Nutrition (Lattka E, Rzehak P et al., 2011, *AJCN*, 93: 382-391) and the complete publication can be found in the appendix of this thesis (Appendix A2).

FADS polymorphisms (12 in total) were genotyped in the Ulm Birth Cohort study. One polymorphism was excluded from further analysis due to the inability to discriminate between alleles and three additional SNPs were excluded due to deviation from Hardy-Weinberg equilibrium. Finally, the influence of eight SNPs in the *FADS* gene cluster on breast milk fatty acid levels of 772 mothers 1.5 months postpartum and in a subset of 463 mothers that were still breastfeeding 6 months after birth was analyzed. In an additional longitudinal analysis, the effect of *FADS* polymorphisms on fatty acid levels over the duration of lactation was analyzed in mothers breastfeeding at two time points, 1.5 and 6 months after giving birth. The genomic location of the analyzed SNPs is depicted in Figure 2.

3.2.1 Summary of results

Linear regression analysis of each of the eight *FADS* SNPs with each of the measured n-6 and n-3 fatty acids separately at both time points of lactation resulted in significant associations for arachidonic acid levels with five of the eight analyzed SNPs at both investigated time points before correction for multiple testing. Considering correction for multiple testing ($p < 0.001$), SNPs rs174547 and rs174556 remained significant at 6 months postpartum. For all associated SNPs, carriers of the minor alleles had lower levels of AA in breast milk compared to carriers of the major alleles. For the 20:4n-6 / 20:3n-6 ratio, which is a measure of D5D activity, associations remained essentially the same. In contrast, the ratio 18:3n-6 / 18:2n-6, which approximates D6D activity, was not significant for any of the SNPs

at either of the two time points, as was also the case for all other n-6, n-3, saturated, monounsaturated, and trans fatty acids after correction for multiple testing.

Previous studies showed that the milk contents of fat and most fatty acids change during lactation (107, 108). Therefore, the influence of *FADS* genotypes on the timely change of fatty acid levels was investigated in a longitudinal analysis. Mothers breastfeeding at both time points (1.5 and 6 months) were investigated (complete case approach). Using this model, no genotype-specific difference regarding the timely course of milk arachidonic acid levels or the ratio estimating D5D activity (20:4n-6 / 20:3n-6) was observed.

Longitudinal analysis of all other fatty acids revealed no significant associations with *FADS* genotypes over time after correction for multiple testing. However, associations before correction for multiple testing were observed for dodecanoic acid (12:0), cis-15-tetracosenoic acid (24:1n-9) and trans-9-octadecenoic acid (t18:1n-9) (see Figure 4). Homozygous carriers of the minor allele of SNP rs174626 exhibited a remarkable increase in dodecanoic acid levels over the duration of breastfeeding, whereas the dodecanoic acid levels remained rather stable in homozygous carriers of the major allele (p-value for interaction (SNP x Time) = 0.0186). A similar effect was observed for tetradecanoic acid (14:0) and the same SNP, as well as for the association between SNP rs526126 and the timely change of hexadecanoic acid (16:0) levels. An increase of the monounsaturated fatty acid cis-15-tetracosenoic acid over lactation was observed in carriers of the major allele of SNP rs174547, whereas the level in homozygous minor allele carriers was stable (p-value for interaction = 0.0022). Similar effects were observed for three other SNPs (rs174556, rs174626, and rs174455). For trans-9-octadecenoic acid, only carriers of the major allele of the *FADS3* SNP rs174455 showed a remarkable decrease of this fatty acid over the breastfeeding period (p-value for interaction = 0.0032). A similar trend, although not statistically significant, was observed for SNP rs174626.

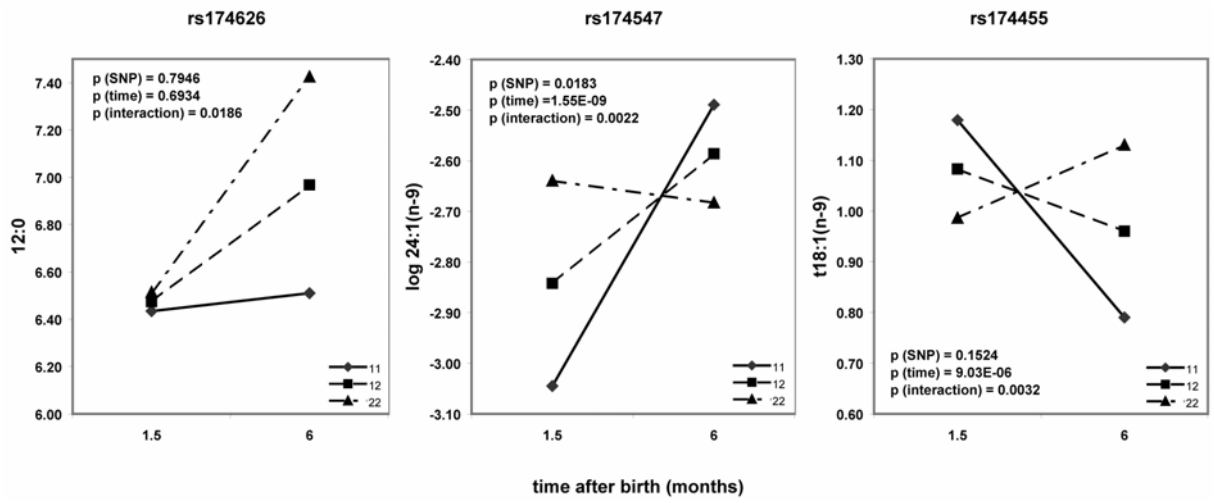

Figure 4: Association analysis of the *FADS* genotype effect over time using a Generalized Estimating Equation regression model (interaction SNP x Time) resulted in associations for the three fatty acids dodecanoic acid (12:0), cis-15-tetracosenoic acid (24:1n-9), and trans-9-octadecenoic acid (t18:1n-9). Homozygous carriers of the minor allele of SNP rs174626 exhibited a remarkable increase in dodecanoic acid levels over the duration of breastfeeding, whereas the dodecanoic acid levels remained rather stable in homozygous carriers of the major allele (p-value for interaction (SNP x Time) = 0.0186). An increase of the monounsaturated fatty acid cis-15-tetracosenoic acid over lactation was observed in carriers of the major allele of SNP rs174547, whereas the level in homozygous minor allele carriers was rather stable (p-value for interaction = 0.0022). For trans-9-octadecenoic acid, only carriers of the major allele of the *FADS3* SNP rs174455 showed a remarkable decrease of this fatty acid over the breastfeeding period (p-value for interaction = 0.0032). These results suggest an influence of the fatty acid desaturation pathway on the biosynthesis of saturated and monounsaturated fatty acids in lactating women. 11: homozygous major allele, 12: heterozygous, 22: homozygous minor allele.

3.2.2 Discussion

In the presented study, a significant association between maternal *FADS* genotypes and milk arachidonic acid levels at different time points of lactation was observed. However, in contrast to other studies (35, 36) no associations with other n-6 or n-3 fatty acids were observed. This might be due to differing time points of breast milk sample collection, differing dietary intake of fatty acids among the participants of the different studies, or different statistical methods and differing stringency in correcting p-values for multiple comparisons.

One of the novelties of the presented study is the availability of fatty acid data at two time points of lactation. In a longitudinal analysis, the time course of AA levels was independent of the *FADS* genotype in the conducted complete case approach comprising 463 mothers that were breastfeeding at both time points. However, AA amounts were markedly higher in carriers of the major alleles at both investigated time points compared to minor allele carriers, suggesting lower desaturase expression rates or enzyme activity in minor allele carriers. It is not clear whether this can be attributed to an altered synthesis rate in the mammary gland itself, or whether it is due to lower desaturase activity in other tissues such as the liver and consequently diminished import into the mammary gland. Tracer studies suggest a role of the mammary gland itself in LC-PUFA synthesis (109, 110) and it is known that non-human mammary gland expresses D5D and D6D (111, 112). Further studies will be needed to understand the role of the human mammary gland in fatty acid synthesis and the influence of the *FADS* genotype.

Although a function of the D5D and D6D in the biosynthesis of saturated and monounsaturated fatty acid has not been reported, Xie and Innis previously found an association of *FADS* SNP rs174553 with breast milk tetradecanoic acid (14:0) and cis-11-octadecenoic acid (18:1n-7) levels. In the presented study, a genotype-dependent longitudinal change of breast milk dodecanoic acid (12:0), cis-15-tetracosenoic acid (24:1n-9) and trans-9-octadecenoic acid (t18:1n-9) levels was observed. In the ALSPAC cohort, an association of *FADS* genotypes with RBC cis-9-hexadecenoic acid (16:1n-7) levels was seen (see Appendix

A1), which was also seen in the presented study in breast milk, however, only when not corrected for multiple testing. These associations are rather unexpected, because the biosynthesis of saturated and monounsaturated fatty acids takes place in other pathways independent of the desaturation pathway. One possible explanation for these unexpected associations might be the regulation of other fatty acid levels by PUFAs by a modification of gene expression through binding to nuclear receptors such as PPARα (113, 114). Another explanation could be a possible use of LC-PUFAs for energy production and storage of saturated and monounsaturated fatty acids as was shown in pregnant and lactating non-human primates (115). A third hypothesis relates to the fact that such associations have only been described in pregnant and lactating women so far. One might therefore additionally speculate that these women are especially sensitive to genetic influences on their fatty acid metabolism, because their primary target during pregnancy and lactation is the optimal supply of their child. Disturbances in the desaturation pathway due to *FADS* polymorphisms might possibly be compensated by changes in other pathways of the fatty acid or lipid metabolism. Future studies should aim at replicating these findings, analyze whether these associations can also be found in non-pregnant, non-lactating women and men, and investigate the interaction between the different pathways of fatty acid biosynthesis.

The association of SNP rs174455 with trans-9-octadecenoic acid over time in the present study was not a less surprising result because this fatty acid is exclusively nutritional. One might speculate that lactating women exhibit a differential eating behavior dependent on the genotype over the breastfeeding period. Another explanation might be correlations between trans fatty acids and long-chain polyunsaturated fatty acids in human breast milk as reported recently (95). Future studies will be required to replicate and explain these findings.

Adequate LC-PUFA supply of the neonate can be provided by breastfeeding, which supplies the child with pre-formed LC-PUFA, and is therefore regarded as the preferred method of feeding during the first six months of life (116). In addition to the positive effects of LC-PUFAs on visual and cognitive development, there are also indications that early exposure to dietary LC-PUFAs is related to the development of atopic diseases (117, 118) and protects from high blood pressure and cardiovascular risk in later childhood, which is also

thought to persist into adulthood (119, 120), although controversial data also exist (121). In the presented study, it was shown that the maternal *FADS* genotype influences the fatty acid composition of breast milk. Whether the differing fatty acid levels between the maternal genotype groups have any influence on the breast-fed infant cannot be inferred from this study and needs further investigation.

3.3 Functional relevance of *FADS2* promoter polymorphism rs968567

The results of the third project performed during this thesis have been published in the Journal of Lipid Research (Lattka E, Eggers S et al., 2010, *J Lipid Res*, 51: 182-191) and the complete publication can be found in the appendix of this thesis (Appendix A3).

The objective of this study was to analyze the molecular basis for inter-individual differences in fatty acid levels by identification of functional relevant variants that modify the efficiency of *FADS* gene transcription. The effects of bioinformatically selected potential functional relevant SNPs on transcription activity and protein binding were analyzed using *in vitro* techniques in cell culture based systems.

3.3.1 Summary of results

Bioinformatic analyses of polymorphisms in the *FADS* gene cluster were performed prior to selection of the most promising SNPs for further functional analysis. Important regulatory regions were identified using the UCSC Genome Browser (http://genome.ucsc.edu), evolutionary conserved regions were identified by ECR browser (http://ecrbrowser.dcode.org) and Vista Browser (http://pipeline.lbl.gov/cgi-bin/gateway2), and differential transcription factor binding was tested using the Genomatix MatInspector software (http://www.genomatix.de). Among several candidate SNPs, two interesting polymorphisms in the *FADS2* gene promoter were chosen for analysis: rs3834458 (T/Del) and rs968567 (C/T). Both polymorphisms are located in human-mouse conserved regions, and additionally rs968567 is located in a predicted CpG island. The Genomatix MatInspector prediction of transcription factors showed that both polymorphisms are located in proximity to predicted binding sites for transcription factors that are involved in desaturase gene transcription (SREBP and PPARα). Furthermore, polymorphism rs3834458 is located in a predicted BCL6 binding site, which is only present when the sequence contains the major T allele and lost when the deletion mutation is present. For SNP rs968567, three additional binding sites were

predicted when the sequence contains the minor T allele: ELK1, STAT1 and STAT3, which are not present for the major C allele.

To determine the functional effects of the two selected polymorphisms on transcriptional regulation, luciferase reporter gene assays were conducted in three different human cell lines (HepG2, hepatocellular carcinoma; HEK293, embryonic kidney; and HeLa, cervical cancer) to measure allele-specific promoter activity. The replacement of the rs968567 major C allele by the minor T allele resulted in a two to three-fold increase of luciferase activity in full-length as well as truncated constructs (see Figure 5). This effect was statistically significant in HepG2 ($p<2.0E-05$) and HeLa ($p<1.0E-6$) cells, but not in HEK293 cells, which might be due to cell line specific expression of the involved transcription factors. Luciferase activity was slightly lower for all constructs containing the rs3834458 minor deletion mutation compared to the constructs containing the major T allele, however, this was not statistically significant. Altogether, the results indicate a strong role of polymorphism rs968567 for promoter activity in different cell lines, but no role of polymorphism rs3834458.

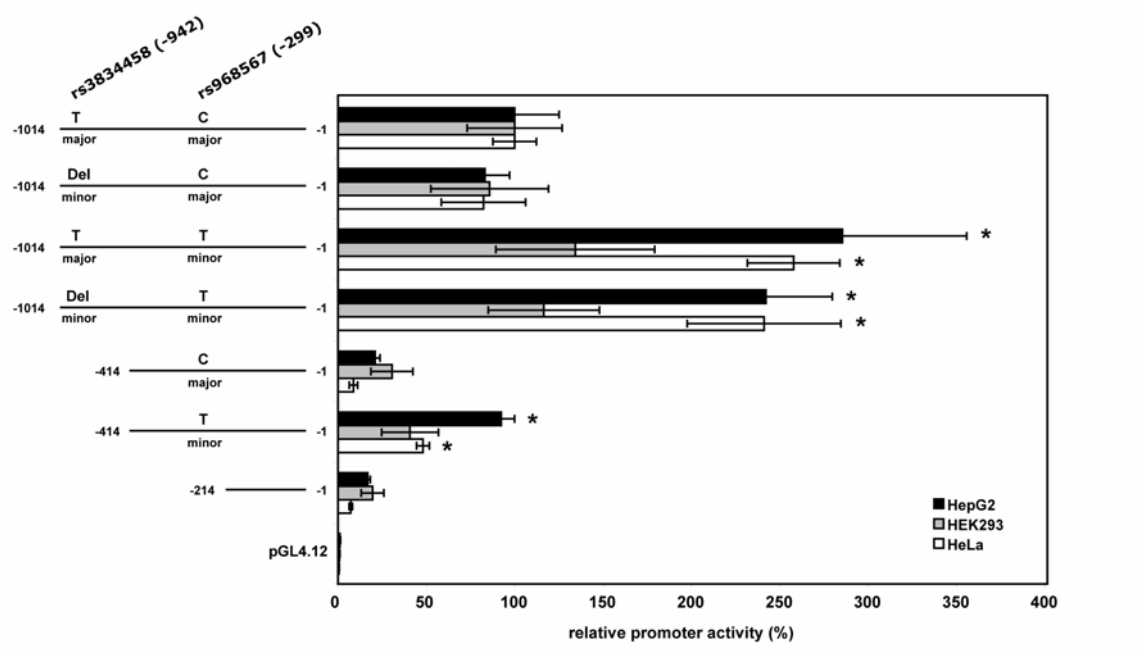

Figure 5: Relative promoter activity of different constructs of human *FADS2* gene promoter in three different human cell lines. Values represent the mean of three independent experiments performed in triplicates. Promoter activity of the major/major construct was used as reference and set at 100%. Asterisks denote statistically significant results calculated by t-test. Numbers indicate the relative position to the *FADS2* translation start site. The replacement of the rs968567 major C allele by the minor T allele resulted in a statistical significant two to three-fold increase of luciferase activity in full-length as well as truncated constructs in HepG2 and HeLa cells. Luciferase activity was slightly lower for all constructs containing the rs3834458 minor deletion mutation compared to the constructs containing the major T allele, however, this was not statistically significant. These results indicate a strong role of polymorphism rs968567 for promoter activity in two different cell lines, but no role of polymorphism rs3834458.

The next experiment was conducted to test whether the polymorphisms effect the DNA-binding ability of nuclear proteins. HeLa nuclear protein extracts were subjected to binding to oligonucleotides representing the region surrounding SNP rs968567 with either the major C allele or the minor T allele, and DNA-protein complexes were analyzed by electrophoretic mobility shift assays. Differential band intensities dependent on the allele were observed, indicating that the rs968567 minor T allele increases binding affinity of the tested promoter region for at least two protein complexes. The same experiment was conducted with oligonucleotides containing the major and minor alleles of the rs3834458 polymorphism. No significant difference of competing effects between oligonucleotides was observed pointing towards no allele-specific binding of nuclear factors in case of this polymorphism.

Because a significant impact on promoter activity and binding of nuclear protein complexes was observed only for SNP rs968567 and not for polymorphism rs3834458, only the region surrounding SNP rs968567 was chosen for further characterization. Prediction of transcription factor binding sites in this region resulted in three binding sites when the rs968567 minor T allele was present in the sequence: ELK1, STAT1 and STAT3. To test whether expression of any of these transcription factors is correlated to *FADS2* gene expression, regression analysis between *FADS2* peripheral blood mRNA expression levels and expression levels of these three transcription factors in 322 subjects was conducted and revealed a statistically significant association between mRNA expression levels of *FADS2* and *ELK1*. No significant p-values were obtained for the correlation of *FADS2* with *STAT1* and *STAT3* expression levels. The association between *FADS2* and *ELK1* expression was furthermore dependent on the rs968567 genotype, with a much higher effect size in homozygous minor allele carriers.

ELK1 protein binding to the predicted *FADS2* promoter sequence was tested by performing DNA affinity purification of nuclear proteins from HeLa nuclear extract using biotinylated oligonucleotides representing the region surrounding SNP rs968567 with either the major C allele or the minor T allele. The supernatant and wash fractions containing unbound proteins as well as the elution fraction with the bound proteins were immunoblotted and a specific antibody against human ELK1 was used to detect the presence of ELK1 protein

in the fractions. The appearance of ELK1 in the elution fraction of the minor T allele, which was lacking in the elution fraction of the major C allele, confirmed binding of ELK1 to the *FADS2* promoter sequence *in vitro* exclusively when the minor T allele was present (see Figure 6).

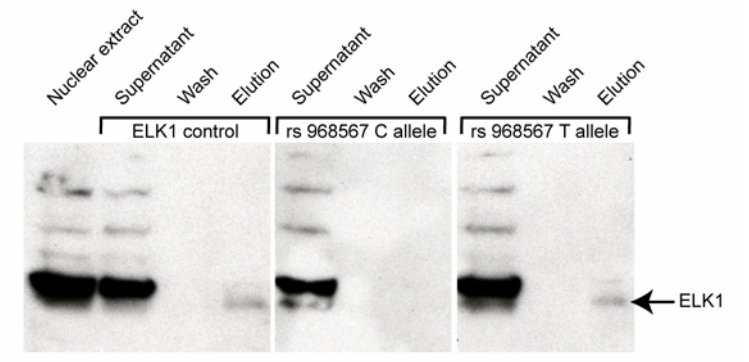

Figure 6: Detection of human ELK1 protein in DNA affinity purification fractions by immunoblotting. The first lane represents crude HeLa nuclear extract and verifies the presence of ELK1 protein in HeLa cells. The supernatant and wash fractions contain all unbound proteins, whereas the elution fraction contains all oligonucleotide binding proteins. A specific band at 62 kDa corresponds to the ELK1 protein (indicated by an arrow). The appearance of ELK1 in the elution fraction of the minor T allele, which was lacking in the elution fraction of the major C allele, confirmed binding of ELK1 to the *FADS2* promoter sequence *in vitro* exclusively when the minor T allele was present.

3.3.2 Discussion

Numerous studies have reported associations of several SNPs in the *FADS* gene cluster with PUFA/LC-PUFA level and composition in different human tissues (31-42). However, the causative functional variant(s) are not known up to date due to the presence of linkage disequilibrium (LD) between SNPs in the *FADS* gene cluster. The real functional variant(s) could therefore cause associations of all other SNPs being in high LD, and cannot be directly identified by association studies for this reason. Functional approaches are therefore needed to determine the effect of the associated SNPs on the molecular level and by this identify the causative variant(s).

This study aimed to analyze the influence of two selected polymorphisms in the promoter region of the human delta-6 desaturase gene on transcription regulation. Results from the association studies of the *FADS* gene cluster with LC-PUFA levels and composition suggested a decline in desaturase transcription or conversion rate due to the minor alleles of the associated polymorphisms, because desaturase reaction products were diminished and substrates accumulated (31-34, 41, 42). Functional analyses of one associated polymorphism (rs3834458) showed a (however non-significant) decrease of promoter activity due to the minor allele, which seems consistent with the association data. Yet, a statistically significant increase of promoter activity was measured due to the minor allele of rs968567, which is at first glance contradictory to the association results. However, results from a recent association study on *FADS* genotypes and D5D and D6D activities estimated by serum fatty acid ratios (20:4n-6/20:3n-6 and 20:3n-6/18:2n-6, respectively) in adolescents revealed that the minor allele of rs968567 is significantly associated with higher D6D activity (38), which is in line with the results from the functional analysis. The minor alleles of most of the other analyzed SNPs were associated with lower D5D activity. Based on these results, the authors suggested that genetic variability in LC-PUFA levels explained by *FADS* gene cluster polymorphisms is explained by both higher D6D activity and lower D5D activity. The identification of the functional relevant polymorphisms causing diminished D5D activity is a task for future studies.

In this study, it was shown that SNP rs968567 modulates *FADS2* promoter activity and ELK1 was identified as a putative new regulator of *FADS2* gene transcription in an allele-specific manner. ELK1 is a member of the ETS domain family of transcription factors, was first cloned in 1989 (122) and is primarily known for its role in the transcriptional regulation of immediate early genes including *c-fos* (123) and *egr-1* (124) by forming ternary complexes with serum response factor on the serum response elements of gene promoters (125). A role of ELK1 in lipid metabolism has not been reported until now. Further experiments will be required to completely characterize its role in *FADS2* gene expression *in vivo*.

During this thesis, a functional relevant polymorphism for *FADS2* promoter activity was identified. Future studies should aim at the analysis of the effect of this SNP *in vivo* as well as the identification of additional functional variants in the *FADS* gene cluster, especially in *FADS1*. Although present data point at an individual genetic regulation of desaturase activities on the transcriptional level, it cannot be excluded that there exist not yet identified exonic variants in the *FADS* gene cluster, the identification of which might also be a task for future studies.

4 Conclusion and outlook

The supply with PUFAs and LC-PUFAs during pregnancy by placental transfer and during lactation by breast milk is considered essential for children's health and normal body function. In this thesis, polymorphisms in the *FADS* gene cluster were identified as important regulators of fatty acid composition in RBC phospholipids of pregnant women and breast milk of lactating women. These new results show that, in addition to the regulation of fatty acid status by dietary supply, a genetic reason for inter-individual differences in fatty acid composition of these body fluids exists. The supply of the child with key PUFAs and LC-PUFAs might therefore be related to the maternal *FADS* genotype. The second attempt of this thesis was to characterize the molecular basis of the inter-individual differences in fatty acid metabolism. A single nucleotide polymorphism in the *FADS2* gene promoter was newly identified to be functionally relevant for *FADS2* promoter activity and transcription factor binding. These results offer first insights into allele-dependent regulation mechanisms of desaturase activities.

The observed variability in maternal fatty acid levels due to *FADS* genetic variants might be the reason for ambiguous results in intervention studies examining the relationship between maternal fatty acid supplementation and child outcomes such as cognitive development. Future studies addressing the biological effects of PUFAs and LC-PUFAs should therefore aim at including the analysis of *FADS* variants as important genetic factors for differential fatty acid metabolism to enhance the sensitivity of such studies. Some studies exist, which have shown that *FADS* genotypes modulate dietary effects on fatty acid and lipid levels as well as on cognitive outcomes. Future gene-nutrition interaction studies should aim at studying the inherited basis of individual differences to nutrient response ('nutrigenetics'). This might help to better understand the mechanisms leading to complex fatty acid related phenotypes and it might enable us to determine individual genotype-dependent nutritional requirements for optimal fatty acid supply in all stages of human life. Association analyses of *FADS* genotypes with complex outcomes such as cardiovascular diseases or allergy have

shown weak or inconsistent results. Inclusion of dietary data into these association analyses might provide the missing link to understand the development of such diseases.

The results from this thesis suggest a role of the *FADS3* gene product in the desaturation of fatty acids, and detailed functional characterization of the respective protein will be a task for future studies. Also, analysis of the genetically determined molecular mechanisms leading to inter-individual differences in fatty acid metabolism and individual differences in nutrient response is essential to completely understand the regulation of desaturase activity. Further functional studies are therefore needed to explore these mechanisms in detail. Knowledge about the molecular mechanisms will also shed light on the mechanisms leading to the development of fatty acid related disorders. In addition to the identified functional relevant SNP in the *FADS2* gene promoter, several other potential functional SNPs are present in the *FADS* gene cluster, which await further investigation. The identification of additional yet unknown potential functional relevant variants might be enabled by the 1000 Genomes project. In this project, the genomes of 628 individuals have been recently sequenced (release November 2010) and the aim is to sequence about 2,500 individuals in order to find most genetic variants that have frequencies of at least 1% in the populations studied.

Rapidly evolving techniques in the fields of transcriptomics, metabolomics, or epigenomics might complement results obtained in functional analysis. Genotype-dependent transcriptome and metabolome profiles in different tissues (with or without nutritional intervention) would contribute to a more global understanding of the molecular mechanisms leading to fatty acid related diseases. Especially critical stages for the fatty acid supply of the child during pregnancy and lactation could be identified by parallel metabolic profiling of maternal and fetal cord blood during pregnancy and maternal breast milk and child blood during lactation, respectively. Epigenetic regulation of desaturase activity has not been analyzed until now, but might in the future also further contribute to get a complete picture of desaturase regulation and individual fatty acid metabolism.

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Specific contributions

Publication 1: Koletzko, B., **E. Lattka**, S. Zeilinger, T. Illig, and C. Steer. 2011. Genetic variants of the fatty acid desaturase gene cluster predict amounts of red blood cell docosahexaenoic and other polyunsaturated fatty acids in pregnant women: findings from the Avon Longitudinal Study of Parents and Children. *Am J Clin Nutr.* **93**: 211-219.

Eva Lattka was involved in the designing of the study (gene and SNP selection), was responsible for all genetic experimental parts of the study (assay design, genotyping by MALDI-TOF MS and genotype calling), and contributed to interpretation of results and writing of the manuscript.

Publication 2: **Lattka, E.**, P. Rzehak, É. Szabó, V. Jakobik, M. Weck, M. Weyermann, H. Grallert, D. Rothenbacher, J. Heinrich, H. Brenner, T. Decsi, T. Illig, and B. Koletzko. 2011. Genetic variants in the *FADS* gene cluster are associated with arachidonic acid concentrations of human breast milk at 1.5 and 6 mo postpartum and influence the course of milk dodecanoic, tetracosenoic and trans-9-octadecenoic acid concentrations over the duration of lactation. *Am J Clin Nutr.* **93:** 382-391.

Eva Lattka was involved in the designing of the study (gene and SNP selection), was responsible for all genetic experimental parts of the study (assay design, genotyping by MALDI-TOF MS and genotype calling), performed statistical data analysis and wrote the manuscript.

Publication 3: **Lattka, E.**, S. Eggers, G. Möller, K. Heim, M. Weber, D. Mehta, H. Prokisch, T. Illig, and J. Adamski. 2010. A common FADS2 promoter polymorphism increases promoter activity and facilitates binding of transcription factor ELK1. *J Lipid Res.* **51**: 182-191.

Eva Lattka was responsible for the design of the study, performed all bioinformatic and experimental parts of the study, and wrote the manuscript.

Scientific communications

Original papers

Standl, M., S. Sausenthaler, **E. Lattka**, S. Koletzko, C.-P. Bauer, H.-E. Wichmann, A. von Berg, D. Berdel, U. Krämer, B. Schaaf, S. Röder, O. Herbarth, N. Klopp, B. Koletzko, and J. Heinrich for the GINIplus and LISAplus Study Group. *FADS* gene variants modulate the effect of dietary fatty acid intake on allergic diseases in children. Submitted.

Lattka, E., P. Rzehak, É. Szabó, V. Jakobik, M. Weck, M. Weyermann, H. Grallert, D. Rothenbacher, J. Heinrich, H. Brenner, T. Decsi, T. Illig, and B. Koletzko. 2011. Genetic variants in the *FADS* gene cluster are associated with arachidonic acid concentrations of human breast milk at 1.5 and 6 mo postpartum and influence the course of milk dodecanoic, tetracosenoic and trans-9-octadecenoic acid concentrations over the duration of lactation. *Am J Clin Nutr.* **93:** 382-391.

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Posters

Lattka E*, Eggers S, Möller G, Heim K, Weber M, Mehta D, Prokisch H, Adamski J, Illig T (2009) "Functional analysis of fatty acid desaturase gene cluster polymorphisms". Presentation at the "XVI Lipid Meeting Leipzig", Leipzig, Germany, December 10th-12th 2009.

Lattka E*, Möller G, Adamski J, Illig T (2008) "Functional analysis of polymorphisms in the human *FADS* gene cluster". Presentation at the "3rd ESF Functional Genomics Conference", Innsbruck, Austria, October 1st-4th 2008.

Lattka E, Möller G*, Glöckner C J, Bogumil R, Baes M, Adamski J (2007) " Identification of regulated proteins in MFP-2 knockout mouse". Presentation at the "VII European Symposium of The Protein Society – From Proteins to Proteome", Stockholm, Sweden, May 12th-16th 2007.

* presenting author

Oral presentations

"Functional analysis of fatty acid desaturase (*FADS*) gene cluster polymorphisms". European Human Genetics Conference, Gothenburg, Sweden, June 12th-15th 2010.

"Genes and nutrition: The impact of fatty acid desaturase (*FADS*) polymorphisms on long-chain polyunsaturated fatty acid levels". The Power of Programming, International Conference on Developmental Origins of Health and Disease, Munich, Germany, May 6th-8th 2010.

"Follow up of GWA signals by functional studies: How does rs7927894 on chromosome 11q13 contribute to atopic dermatitis development?" Frühjahrsakademie der Gesellschaft für Genetik 2010: Genetic Epidemiology – Status quo, Where to go, Wittenberg, Germany, April 28th-30th 2010

"From association to function: Functional impact of polymorphisms on the human delta-6 desaturase (*FADS2*) gene promoter". 1st Annual Meeting NGFN-Plus and NGFN-Transfer in the Program of Medical Genome Research, Neuherberg, Germany, December 12th-13th 2008.

Appendix

Appendix A

Appendix A1.

Koletzko, B., E. Lattka, S. Zeilinger, T. Illig, and C. Steer. 2011. Genetic variants of the fatty acid desaturase gene cluster predict amounts of red blood cell docosahexaenoic and other polyunsaturated fatty acids in pregnant women: findings from the Avon Longitudinal Study of Parents and Children. *Am J Clin Nutr.* **93**: 211-219.

Genetic variants of the fatty acid desaturase gene cluster predict amounts of red blood cell docosahexaenoic and other polyunsaturated fatty acids in pregnant women: findings from the Avon Longitudinal Study of Parents and Children[1–3]

Berthold Koletzko, Eva Lattka, Sonja Zeilinger, Thomas Illig, and Colin Steer

ABSTRACT

Background: Blood and tissue long-chain polyunsaturated fatty acid (LC-PUFA) amounts, which have been associated with early development and lifelong health, depend on dietary intake and endogenous conversion of precursor fatty acids (FAs) by the enzymes Δ^5-desaturase and Δ^6-desaturase. Polymorphisms in the desaturase encoding genes *FADS1* and *FADS2* have been associated with several n−6 (omega-6) and n−3 (omega-3) FAs and especially with arachidonic acid (AA) amounts. Associations with docosahexaenoic acid (DHA), which is considered particularly important for brain and retina development, are hardly existent.

Objective: We explored the relation between *FADS* gene cluster polymorphisms and red blood cell (RBC) FA amounts in >4000 pregnant women participating in the Avon Longitudinal Study of Parents and Children.

Design: Linear regression analysis of 17 single nucleotide polymorphisms (SNPs) in the *FADS* gene cluster was conducted with RBC phospholipid FAs from 6711 samples from 4457 women obtained throughout pregnancy (mean ± SD gestational age: 26.8 ± 8.2 wk).

Results: Independent of dietary effects, the minor alleles were consistently positively associated with precursor FAs and negatively associated with LC-PUFAs and product:substrate ratios of the n−6 (AA:linoleic acid ratio) and n−3 (eicosapentaenoic acid:α-linolenic acid ratio) pathways. In contrast to previous studies, we also showed significant inverse associations with DHA. Similar but weaker associations were shown for the *FADS3* SNP rs174455.

Conclusions: *FADS* genotypes influence DHA amounts in maternal RBC phospholipids and might affect the child's DHA supply during pregnancy. It is highly likely that a gene product of *FADS3* has a desaturating activity. *Am J Clin Nutr* 2011;93:211–9.

INTRODUCTION

The content of polyunsaturated fatty acids (PUFAs) in blood and tissues is associated with the occurrence of metabolic syndrome and cardiovascular diseases, immunologic and inflammatory responses, and related diseases such as allergies, early visual, cognitive and motor development, and mental health and psychiatric disorders (1, 2). These effects of PUFAs are thought to be primarily mediated by tissue contents of long-chain polyunsaturated fatty acids (LC-PUFAs) with ≥20 carbon atoms and ≥3 double bonds, such as arachidonic acid (AA; 20:4n−6), eicosapentaenoic acid (EPA; 20:5n−3), and docosahexaenoic acid (DHA; 22:6n−3) (3, 4) (**Figure 1**). LC-PUFAs are indispensible components of cell membranes and modulate their integrity and fluidity, they act as second messengers in intracellular signaling pathways or regulate transcription, and they serve as precursors for the synthesis of eicosanoids and docosanoids, which are potent regulators of inflammatory processes (8). LC-PUFA contents in human blood and tissue are determined by their dietary intakes from animal lipids contained in meat, eggs, fish, and human milk and by the intake of the essential fatty acids linoleic acid (LA; 18:2n−6) and α-linolenic acid (ALA; 18:3n−3) primarily from vegetable oils and the subsequent endogenous conversion of these precursors into LC-PUFAs mediated by the enzymes Δ^5-desaturase and Δ^6-desaturase. Genetic association studies of single nucleotide polymorphisms (SNPs) in the desaturase encoding genes *FADS1* and *FADS2* showed significant associations between these SNPs and fatty acid amounts in serum phospholipids with an extraordinarily high

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[2] Core support for the Avon Longitudinal Study of Parents and Children study is currently provided by the UK Medical Research Council, the Wellcome Trust, and the University of Bristol. Financial support for this study was obtained from the Commission of the European Communities, specific RTD Programme "Quality of Life and Management of Living Resources," within the 7th Framework Programme NUTRIMENTHE, FP7-212652. Further support was granted by the Kompetenznetz Adipositas (Competence Network for Adiposity) funded by the Federal Ministry of Education and Research (01GI0826) and by the Munich Center of Health Sciences. The assays of the maternal blood samples were carried out and funded by Scotia at the instigation of the late David Horrobin. Support was also received from a Freedom to Discover Award of the Bristol-Myers-Squibb Foundation (New York, NY) (to BK) and by NOAA (to CS).

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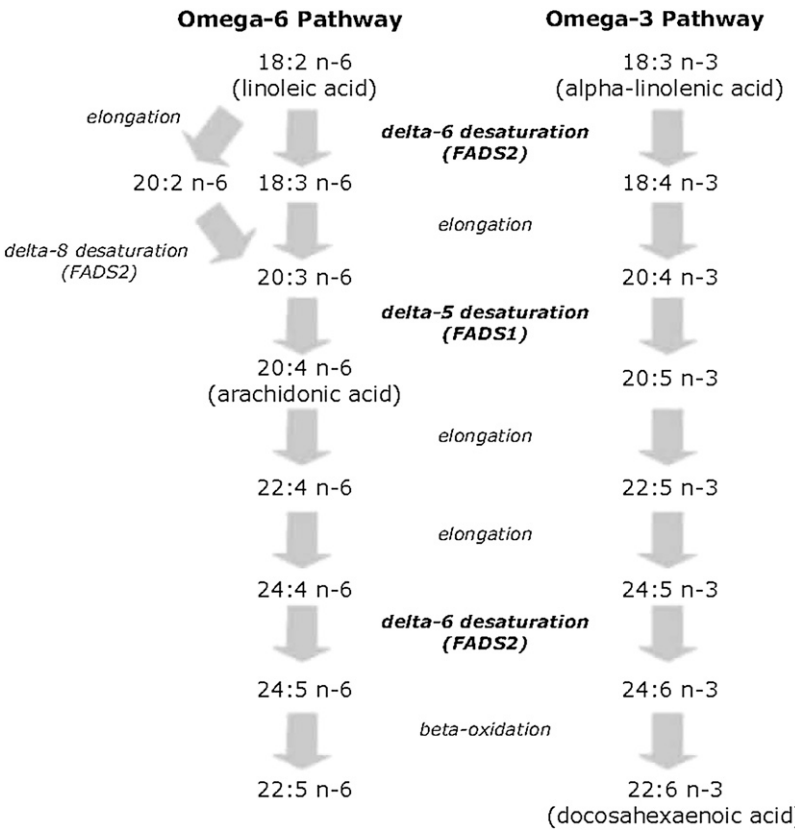

FIGURE 1. The mammalian pathway of n−6 and n−3 long-chain polyunsaturated fatty acid synthesis. Modified and redrawn from references 5–7.

genetically explained variance for AA amounts ≤28.5% (5). Carriers of the minor alleles showed increased amounts of desaturase substrates and reduced amounts of desaturase products, which suggests a decline in desaturase activity because of the polymorphisms. These results were replicated in several independent candidate gene studies that included between 69 and 1820 human subjects (9–13) and in a recent genome-wide association study (14). All studies agreed on the strong association of *FADS1* and *FADS2* genotypes with several n−6 and n−3 fatty acids; however, reports on an association with DHA amounts are hardly existent. DHA is an n−3 LC-PUFA with high incorporation into neural tissues that are considered of particular importance for brain and retina function (15). Only 2 studies reported an association of single *FADS* SNPs with DHA amounts (10, 13); all other studies did not find such associations. There are first indications for an association of *FADS* genotypes with brain-related phenotypes such as intelligence development (16) and attention deficit hyperactivity disorder (ADHD) (17). These effects are thought to be mediated, in part, by DHA availability in the brain, but the question remains how *FADS* genotypes modulate these phenotypes, if not by direct association with DHA amounts. It seems possible that the preliminary studies were too small to detect consistent associations between *FADS* SNPs and DHA amounts. Therefore, we explored the relation between polymorphisms of the *FADS* gene cluster including *FADS3*, the function of which is still unclear, and red blood cell PUFA amounts in a large cohort of >4000 pregnant women participating in the Avon Longitudinal Study of Parents and Children (ALSPAC).

SUBJECTS AND METHODS

Subjects

The ALSPAC (http://www.alspac.bris.ac.uk) is a multipurpose birth cohort study based at Bristol University (Bristol, United Kingdom) and involving over 14,000 pregnancies in the Avon area of England in the early 1990s, the children from which have been followed through childhood (18). Blood samples that had been taken at times of diagnostic venipunctures for clinical care were used with up to 6 samples per woman. Ethical approval for the study was obtained from the ALSPAC Law and Ethics Committee and the local research ethics committees.

Analysis of fatty acids in red blood cell phospholipids

At least one blood sample each was analyzed for fatty acids from 5144 mothers during pregnancy, of whom 4136 mothers had at least one sample taken after 20 wk of pregnancy. Blood samples were collected in heparin-containing tubes and centrifuged at 1500 × *g* for 15 min at 4°C to separate the red cells, which were then stored at −20°C until 1993 and subsequently at −70°C. Frozen red blood cell samples were shipped to the laboratories of Scotia Pharmaceuticals in Canada in 1996 for fatty acid composition analysis as previously described (19). Briefly, lipids from thawed red blood cells were extracted with chloroform and methanol, extracted lipids were redissolved in chloroform (100 μL), and phospholipids were isolated by thin-layer chromatography with a mixture of hexane:diethyl ether: acetic acid (80:20:1; vol:vol:vol). Fatty acid methyl esters were prepared by incubation with 120 g boron trifluoride/L in

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methanol at 90°C for 30 min. Fatty acid methyl esters were taken up in hexane, and the relative amounts of 40 fatty acids were measured by gas-liquid-chromatography and expressed as the weight percentage of all measured fatty acids. Fatty acid methyl esters were identified by comparison with authentic standards. Fatty acid amounts below the limit of detection of the assay (0.01%) were recoded to one-half that value.

Genetic analyses

Genomic DNA was extracted from whole blood samples as previously reported (20). SNPs genotyped in this study were chosen on the basis of a minor allele frequency >10% and linkage disequilibrium information provided by HapMap (http://hapmap.ncbi.nlm.nih.gov/). SNPs with $r^2 > 0.8$ were considered to have a high degree of coinheritance in Europeans and, therefore, were selected as tagSNPs. Twelve tagSNPs (rs174576, rs174579, rs174448, rs2727271, rs174634, rs174449, rs968567, rs526126, rs174455, rs174602, rs498793, and rs174570) located in the genomic region spanning *FADS1*, *FADS2*, and *FADS3* were selected for genotyping together with 6 additional SNPs (rs174556, rs174561, rs3834458, rs174548, rs174574, and rs174578) that have already been associated with fatty acid amounts in previous studies. Five nanograms of genomic DNA was subjected to polymerase chain reaction amplification followed by the genotyping procedure by using iPLEX chemistry (Sequenom, San Diego, CA) according to the manufacturer's protocol and a matrix-assisted laser desorption/ionization time-of-flight–based allele-detection method. The procedure has been described in detail elsewhere (12). Genotyping failure rates ranged from 1.1% to 4.1%. Error rates on the basis of 790 duplicate samples ranged from 0% to 0.64%.

Statistics and confounding

All SNPs, except SNP rs174634, passed all quality criteria, and genotypes were used for statistical analyses. Linear regression analysis was used to investigate the associations of *FADS* polymorphisms with fatty acid amounts. In addition, product:substrate ratios for AA:LA and EPA:ALA were also analyzed. Because of the skewness of the data, log transformations were applied. Outcomes were standardized to have a variance of one to produce more comparable effect sizes. Genetic variants were modeled with the assumption of a linear relation with the number of copies of the minor allele. Although genetic effects are unlikely to be confounded with sociodemographic variables (21), the validity was explored in analyses adjusting for 7 confounders as follows: multiple pregnancy (singleton or multiple), parity (primiparous or multiparous), maternal smoking at 32 wk gestation (yes or no), gestation, maternal age, maternal prepregnancy body mass index, and a measure of family adversity, with the latter 4 variables being used as continuous variables. In addition, to exclude possible confounding by differential LC-PUFA intake from the diet, we simultaneously adjusted for 81 dietary variables obtained from a food-frequency questionnaire completed around the 32nd wk of gestation by the mothers. To avoid complications of allele frequencies and effect sizes varying with ethnicity, analyses were restricted to mothers of white ethnic origin. Longitudinal analyses were used to take account of the repeat measurements during pregnancy (xtreg, Stata version 11.0; StataCorp LP, College Station, TX).

RESULTS

Some 6711 samples (91% of total samples) from 4457 women (55% of women genotyped) were obtained at varying times (between the 4th and 44th wk) throughout pregnancy (mean ± SD gestational age at blood sampling: 26.8 ± 8.2 wk). Only 3% of samples were taken in the first trimester, whereas 33% and 64% of samples were taken in the second and third trimesters, respectively.

The composition of red blood cell lipid fatty acids revealed amounts of essential fatty acids similar to a previous report in Dutch pregnant women (22) but with somewhat lower amounts of EPA (0.24% in the ALSPAC cohort compared with 0.39% in midpregnancy in the Netherlands) and DHA (2% compared with 3.9% in the Netherlands) (**Table 1**) perhaps because of a higher habitual fish consumption in the Netherlands.

Genetic associations

Genetic variants were generally in Hardy-Weinberg equilibrium although disequilibrium existed for rs174579 ($P < 0.001$) and rs174570 ($P = 0.001$) (*see* supplemental Table 1 under "Supplemental data" in the online issue.). In general, heterozygotes were underrepresented in this sample, although the reasons for these departures remained unclear. Possible explanations associated with genotyping problems, such as poor cluster separation, were not applicable for these data. Differential fetal survival may provide an alternative explanation (23). But overall, these departures were considered minor, with the

TABLE 1
Polyunsaturated fatty acids (weight % of total fatty acids) in red blood cell lipids of pregnant women of white ethnic origin (6711 blood samples from 4457 women)[1]

Fatty acid	Values
Omega-6	
18:2	11.15 (9.62–12.59)
18:3	0.02 (0.005–0.03)
20:2	0.26 (0.22–0.30)
20:3	1.38 (1.05–1.69)
20:4	6.09 (3.89–8.36)
22:4	0.93 (0.53–1.40)
22:5	0.22 (0.14–0.32)
Omega-3	
18:3	0.14 (0.10–0.18)
20:5	0.24 (0.16–0.36)
22:5	0.66 (0.35–1.05)
22:6	2.01 (1.24–3.05)
Ratios	
AA to LA	0.52 (0.38–0.70)
EPA to ALA	1.83 (1.33–2.53)

[1] All values are medians; interquartile ranges in parentheses. AA, arachidonic acid; LA, linoleic acid; EPA, eicosapentaenoic acid; ALA, α-linolenic acid. Sample reflects observations where genetic data are present for at least one polymorphism. All fatty acids and ratios showed evidence of skewness ($P < 0.001$), although the interquartile ranges suggested that the deviations from symmetry were minor for some outcomes.

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significance reflecting the large sample size. It was considered important to report results for these 2 SNPs, but nevertheless, if these departures reflected genotype misclassifications, any associations would be biased toward the null. Hence, reported results would tend to underestimate the true effect sizes.

The minor alleles of variants in the *FADS* genes were positively associated with the precursor PUFAs with 2 or 3 double bonds and negatively associated with the LC-PUFAs with ≥4 double bonds and major product-to-substrate ratios of the n−6 pathway (AA-to-LA ratio) and the n−3 pathway (EPA-to-ALA ratio) (**Table 2**). Associations of *FADS* and intergenic SNPs were strongest in terms of the variability explained and the sizes of the regression coefficients (measured in terms of SDs) for the n−6 metabolite 20:3n−6 followed by the n−6 fatty acid 20:4n−6, 22-carbon metabolites, 20:2n−6, and 18:2n−6. Only 5 of the 17 SNPs showed evidence of an association with 18:3n−6. With respect to n−3 fatty acids, associations were generally weaker than for n−6 fatty acids. Regression coefficients for DHA (22:6n−3) tended to be lower than for 20:4n−6, and regression coefficients for 18:3n−3 tended to be lower than those for 18:2n−6 with the regression coefficients for these n−3 fatty acids being typically one-half for those of the corresponding n−6 fatty acids. Overall, the strongest n−3 associations were observed for DHA. Only 3 of the genetic variants were associated with 22:5n−3. Associations for the *FADS3* SNP rs174455 were weaker than for all *FADS1* and some *FADS2* SNPs but were in the same direction. Perhaps because of the number of SNPs, *FADS2* variants showed greater heterogeneity in results than *FADS1*, with only dihomo-γ-linolenic acid (20:3n−6) showing consistent associations. In contrast, the 3 *FADS1* variants only failed to show consistent associations for γ-linolenic acid (18:3n−6) and docosapentaenoic acid (22:5n−3).

In general, R^2 values that reflected the variability of fatty acid amounts explained by the genetic variants ranged from very low for 22:5n−3 (0.15%) or 18:3n−6 (0.14%) to moderate for 20:3n−6 (5.61%) (**Table 3**). The variability of AA amounts explained by the analyzed polymorphisms reached 1.13% and was 0.51% for DHA amounts.

Adjusted analyses

An adjusted analysis was performed for 81 dietary variables obtained from the food-frequency questionnaire completed around the 32nd wk of gestation by mothers and for 7 potential sociodemographic confounders (*see* supplemental Table 2 under "Supplemental data" in the online issue for the demographics of the sample in the adjusted analysis). Because of missing data, these analyses were restricted to a maximum of 5468 observations. Overall, the associations observed in unadjusted analyses were resilient to controlling for these factors (*see* supplemental Figure S1 under "Supplemental data" in the online issue).

Multiple comparisons

In the main analyses, hypotheses relating to 17 genetic effects for 13 outcomes were being tested. Of these 221 comparisons, 48 comparisons had nominal $P > 0.05$, 173 comparisons had nominal $P < 0.05$, 156 comparisons had nominal $P < 0.01$, 138 comparisons had nominal $P < 0.001$, and 110 comparisons had nominal $P < 0.0001$. With the use of the false discovery rate method of adjusting for multiple comparisons (24), the adjusted 5% critical P value was 0.039. All of the 173 nominally significant P values were also less than this adjusted critical value.

The distribution of the observed P values differed markedly from that expected if all 221 comparisons were null (**Figure 2**). In particular, 78% of observed P values were less than the 5% significance level compared with 5% (by definition) for the null scenario. Even with the use of the overly conservative Bonferroni correction, 53% of tests would have remained significant. A more powerful method, the false discovery rate, suggested a revised critical value of 0.039. The minor adjustment to the nominal value of 0.05 reflected the early divergence in the distributions of observed and expected P values.

The strength of these associations was further illustrated when analyses were repeated for saturated and monounsaturated fatty acids. These fatty acids were not expected to show any associations with genetic variants in the *FADS* genes. In general, no associations after adjustment for multiple comparisons were observed (*see* supplemental Figure S2 under "Supplemental data" in the online issue). Of all saturated and monounsaturated fatty acids, only 16:1n−7 showed consistent and strong associations with most of the analyzed SNPs (*see* supplemental Table 3 under "Supplemental data" in the online issue).

DISCUSSION

The results of this largest available cohort study on the relation between *FADS* polymorphisms and PUFA-status markers showed a consistent association of the minor alleles of the tested SNPs in the *FADS1 FADS2* gene cluster with increased amounts of desaturase substrates, such as 18:2n−6, 20:2n−6, 20:3n−6, and 18:3n−3, decreased amounts of desaturase products such as 20:4n−6 and 22:4n−6, and lower values of the major product-to-substrate ratios of the n−6 pathway (AA-to-LA ratio) and the n−3 pathway (EPA-to-ALA ratio). These data confirmed previous observations in smaller studies (9–13) and suggested a decline in desaturase expression or activity because of the polymorphisms. The prevalence of these minor alleles is relatively high and ranged from 11% to 40% of the population (*see* supplemental Table 1 under "Supplemental data" in the online issue); hence, one would expect a considerable public health relevance of these genetic variants that modulate the effects of environmental exposures on human health, although the genetically explained variability of the fatty acid amounts was relatively low in our study compared with in others and ranged from 0.14% for 18:3n−6 to 5.61% for 20:3n−6. Schaeffer et al (6) reported a genetically explained variability of 28.5% for AA amounts and Tanaka et al (14) showed a variability of 18.6%. In our study, the variability of AA amounts explained by the 17 analyzed genetic variants was 1.13%. The reason for this may lie in the fact that these 2 former studies analyzed fatty acids in serum and plasma, whereas we analyzed red blood cell fatty acids. A recent study by Zietemann et al (25) reported a genetically explained variance for red blood cell AA amounts of 2.6%. These and our results suggest that red blood cell fatty acid amounts are less influenced by *FADS* genotypes than are plasma or serum phospholipid fatty acid amounts, the reason of which remains speculative. Nevertheless, the associations remained stable despite adjustments for sociodemographic

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TABLE 2
Regression coefficients from analyses of log n−6 and n−3 fatty acid amounts as a percentage of total fatty acids on *FADS* single nucleotide polymorphisms[1]

	FADS1			Intergenic		*FADS2*	
	rs174548	rs174556	rs174561	rs3834458	rs968567	rs174570	rs174574
Omega-6							
18:2	0.110 (0.020)***	0.112 (0.020)***	0.108 (0.020)***	0.106 (0.019)***	0.018 (0.024)	0.150 (0.027)***	0.113 (0.019)***
18:3	−0.047 (0.020)*	−0.036 (0.020)	−0.035 (0.020)	−0.037 (0.020)	0.012 (0.024)	−0.079 (0.027)**	−0.042 (0.019)*
20:2	0.104 (0.021)***	0.110 (0.021)***	0.104 (0.021)***	0.116 (0.020)***	0.011 (0.025)	0.172 (0.028)***	0.115 (0.020)***
20:3	0.336 (0.020)***	0.347 (0.021)***	0.345 (0.021)***	0.316 (0.020)***	0.403 (0.025)***	0.089 (0.029**	0.305 (0.020)***
20:4	−0.154 (0.021)***	−0.151 (0.021)***	−0.155 (0.021)***	−0.151 (0.020)***	−0.131 (0.025)***	−0.129 (0.029)***	−0.147 (0.020)***
22:4	−0.148 (0.020)***	−0.151 (0.020)***	−0.154 (0.020)***	−0.140 (0.020)***	−0.144 (0.025)***	−0.100 (0.028)***	−0.134 (0.020)***
22:5	−0.141 (0.021)***	−0.134 (0.021)***	−0.137 (0.021)***	−0.140 (0.020)***	−0.085 (0.025)***	−0.166 (0.028)***	−0.146 (0.020)***
Omega-3							
18:3	0.079 (0.021)***	0.084 (0.021)***	0.081 (0.021)***	0.075 (0.020)***	0.013 (0.025)	0.071 (0.028)*	0.073 (0.020)***
20:5	−0.074 (0.021)***	−0.069 (0.021**	−0.074 (0.021)***	−0.075 (0.021)***	−0.091 (0.026)***	−0.037 (0.029)	−0.068 (0.020)***
22:5	−0.039 (0.021)	−0.036 (0.021)	−0.039 (0.021)	−0.036 (0.021)	−0.059 (0.026)*	−0.015 (0.029)	−0.035 (0.020)
22:6	−0.092 (0.021)***	−0.092 (0.021)***	−0.096 (0.021)***	−0.097 (0.020)***	−0.089 (0.025)***	−0.073 (0.028)**	−0.092 (0.020)***
Ratios							
AA to LA	−0.262 (0.021)***	−0.259 (0.021)***	−0.262 (0.021)***	−0.256 (0.020)***	−0.175 (0.025)***	−0.255 (0.028)***	−0.254 (0.020)***
EPA to ALA	−0.165 (0.020)***	−0.165 (0.020)***	−0.166 (0.020)***	−0.161 (0.020)***	−0.111 (0.025)***	−0.117 (0.028)***	−0.151 (0.019)***
n	6565	6524	6560	6558	6666	6583	6554

	FADS2						
	rs2727271	rs174576	rs174578	rs174579	rs174602	rs498793	rs526126
Omega-6							
18:2	0.155 (0.028)***	0.108 (0.019)***	0.111 (0.019)***	0.053 (0.022)*	0.080 (0.023)***	−0.025 (0.018)	−0.011 (0.024)
18:3	−0.056 (0.029)	−0.044 (0.020)*	−0.043 (0.019)*	−0.010 (0.022)	−0.015 (0.023)	−0.001 (0.019)	−0.013 (0.024)
20:2	0.164 (0.030)***	0.115 (0.020)***	0.112 (0.020)***	0.057 (0.023)*	0.080 (0.024)***	−0.019 (0.019)	−0.017 (0.025)
20:3	0.115 (0.031)***	0.304 (0.020)***	0.304 (0.020)***	0.330 (0.023)***	0.118 (0.024)***	−0.056 (0.020)**	0.168 (0.025)***
20:4	−0.114 (0.030)***	−0.144 (0.020)***	−0.146 (0.020)***	−0.120 (0.023)***	−0.037 (0.024)	0.041 (0.020)*	−0.079 (0.025)**
22:4	−0.086 (0.029)**	−0.131 (0.020)***	−0.135 (0.019)***	−0.126 (0.022)***	−0.032 (0.023)	0.033 (0.019)	−0.083 (0.025)***
22:5	−0.126 (0.030)***	−0.147 (0.020)***	−0.145 (0.020)***	−0.111 (0.023)***	−0.035 (0.024)	0.049 (0.019)*	−0.068 (0.025)**
Omega-3							
18:3	0.123 (0.030)***	0.069 (0.020)***	0.070 (0.020)***	0.028 (0.023)	0.063 (0.024)**	0.007 (0.019)	−0.008 (0.025)
20:5	−0.017 (0.030)	−0.064 (0.021)**	−0.064 (0.020)**	−0.066 (0.023)**	−0.012 (0.024)	0.031 (0.020)	−0.067 (0.025)**
22:5	0.006 (0.031)	−0.037 (0.021)	−0.039 (0.020)	−0.054 (0.023)*	0.015 (0.024)	0.021 (0.020)	−0.072 (0.025)**
22:6	−0.052 (0.030)	−0.091 (0.020)***	−0.093 (0.020)***	−0.088 (0.023)***	−0.034 (0.024)	0.045 (0.019)*	−0.092 (0.025)***
Ratios							
AA to LA	−0.241 (0.030)***	−0.248 (0.020)***	−0.252 (0.020)***	−0.183 (0.023)***	−0.096 (0.024)***	0.065 (0.020)***	−0.093 (0.025)***
EPA to ALA	−0.152 (0.029)***	−0.144 (0.020)***	−0.144 (0.019)***	−0.101 (0.022)***	−0.082 (0.023)***	0.026 (0.019)	−0.063 (0.024)**
n	6569	6481	6574	6556	6591	6555	6595

(Continued)

The American Journal of Clinical Nutrition

TABLE 2 *(Continued)*

	Intergenic		*FADS3*																	
	rs174448	rs174449	rs174455																	
Omega-6																				
18:2	0.070 (0.019)***	0.065 (0.019)***	0.059 (0.019)**																	
18:3	−0.025 (0.019)	−0.027 (0.019)	−0.018 (0.020)																	
20:2	0.070 (0.020)***	0.069 (0.020)***	0.062 (0.020)**																	
20:3	0.216 (0.020)***	0.218 (0.020)***	0.196 (0.020)***																	
20:4	−0.095 (0.020)***	−0.103 (0.020)***	−0.099 (0.020)***																	
22:4	−0.096 (0.019)***	−0.103 (0.020)***	−0.096 (0.020)***																	
22:5	−0.097 (0.020)***	−0.103 (0.020)***	−0.102 (0.020)***																	
Omega-3																				
18:3	0.043 (0.020)*	0.043 (0.020)*	0.032 (0.020)																	
20:5	−0.043 (0.020)*	−0.050 (0.020)*	−0.051 (0.021)*																	
22:5	−0.033 (0.020)	−0.035 (0.020)	−0.035 (0.020)																	
22:6	−0.076 (0.020)***	−0.082 (0.020)***	−0.062 (0.020)**																	
Ratios																				
AA to LA	−0.164 (0.020)***	−0.169 (0.020)***	−0.160 (0.020)***																	
EPA to ALA	−0.093 (0.019)***	−0.101 (0.020)***	−0.088 (0.020)***																	
n	6623	6583	6569																	

[1] SEs are in parentheses. AA, arachidonic acid; LA, linoleic acid; EPA, eicosapentaenoic acid; ALA, α-linolenic acid. Fatty acids and ratios were standardized to have a variance of one. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

confounders and dietary habits. By including these covariables into the analysis, we were able to explain ≤12.1% of the variance in fatty acid amounts (*see* supplemental Table 2 under "Supplemental data" in the online issue).

The strength of the observed associations with n−6 and n−3 fatty acids was further substantiated when analyses were repeated for saturated and monounsaturated fatty acids. These fatty acids were not expected to show any associations with genetic variants in the *FADS* genes because they are synthesized in different pathways. In general, no associations after adjustment for multiple comparisons were observed (*see* supplemental Figure S2 under "Supplemental data" in the online issue). However, 16:1n−7 showed consistent and strong associations with most of the analyzed SNPs (*see* supplemental Table 3 under "Supplemental data" in the online issue), the reason of which was not immediately obvious. The association might have possibly reflected a correlation of 16:1n−7 with 22:4n−6 (r = –0.31).

In contrast to all previous studies cited, we also showed a consistent significant association of the rare SNP alleles with lower amounts of DHA (22:6n−3) in red blood cell phospholipids of pregnant women. Schaeffer et al (5) had reported a similar trend in female and male adults, which, however, failed to reach significance. Two other studies reported isolated weak associations of individual *FADS* SNPs with DHA amounts in serum and breast milk (10, 13). The missing consistent association in previous studies might potentially be due to a lack of statistical power. Moreover, it may reflect a higher rate of DHA synthesis in pregnant women compared with in men or nonpregnant women. The conversion of ALA to EPA and DHA in women was reported to be substantially greater than in a comparable study of men of similar age (26, 27). It has been suggested that estrogen may increase the activity of the desaturation pathway because DHA synthesis was shown to be almost 3 times greater in women who used an oral contraceptive pill that contained 17-ethynylestradiol than in women who did not (26). DHA in plasma cholesteryl esters increased by >40% in male-to-female transsexuals who were given 17-ethynylestradiol, whereas the supplementation of testosterone decreased DHA amounts by >20% in female-to-male transsexuals (28). Thus, exposure to female sex hormones seemed to stimulate DHA synthesis, which appeared to be considerably more active in women and, particularly, in pregnant women exposed to enhanced hormone amounts. This might reflect protective biological mechanisms that contribute to meeting the high fetal demands for DHA. A higher rate of endogenous synthesis in pregnancy could increase the likelihood of detecting effects of SNPs that affect desaturase activity, as shown in this study. Although Xie et al (13) also investigated plasma and red blood cell membrane fatty acids of pregnant women, the reason for the missing association with DHA in their study might have been the very small number of 69 subjects.

Although the genetically explained variability of red blood cell DHA amounts was rather low in this study (0.51%), a modulation of DHA status during pregnancy by frequently occurring *FADS* genotypes may be of major relevance for child outcomes. Several cohort and randomized control studies showed positive (29–34) but also null associations (34–37) between LC-PUFA intake and status in the pre- and postnatal period and developmental outcomes in early childhood. For example, Malcolm et al (29)

TABLE 3
Maximum R^2 across the 17 genetic variants for each fatty acid in unadjusted analyses (reflecting the genetic association) and in adjusted analyses (including the effect of confounders)[1]

	Unadjusted	Adjusted
Omega-6		
18:2	0.64	11.72
18:3	0.14	2.38
20:2	0.72	10.38
20:3	5.61	9.17
20:4	1.13	7.04
22:4	1.21	5.99
22:5	1.30	5.41
Omega-3		
18:3	0.38	6.54
20:5	0.27	12.10
22:5	0.15	8.12
22:6	0.51	6.04
Ratios		
AA to LA	3.28	8.13
EPA to ALA	1.37	9.45
n	6711	5468

[1] AA, arachidonic acid; LA, linoleic acid; EPA, eicosapentaenoic acid; ALA, α-linolenic acid.

provided fish oil during pregnancy and showed that the DHA status of the infants at birth was related to improved visual development at 2.5 and 6 mo of age. Another study reported that eye-hand coordination at the age of 2.5 y was improved in infants whose mothers received high-dose fish oil during pregnancy (30). Trials that investigated the effect of direct infant LC-PUFA supplementation after birth via formulae on potential long-term benefits such as stereoacuity, vision, and intelligence quotient (IQ) did not report significant findings (35–37). Although these contradictory results exist, a sufficient availability of DHA during the perinatal brain growth spurt is widely considered mandatory for normal cognitive, visual, and motor development (32). An average intake ≥200 mg DHA/d for pregnant and lactating women has been recommended (38) because maternal DHA status has a direct effect on DHA availability to the fetus (39, 40), which in turn has been linked to short- and long-term developmental child outcomes (32, 33, 41). Future studies on the association between *FADS* genotypes, DHA amounts, and early childhood development will be needed to evaluate the biological relevance of genotype-dependent DHA amounts. In 2007, Caspi et al (16) reported that a genetic variant in the *FADS2* gene (rs174575) modulated the association between breastfeeding and IQ in 2 large birth cohorts. In both cohorts, previously breastfed and formula-fed children differed in later IQ, but this effect was more pronounced and only significant in children carrying the rs174575 major C allele (IQ point advantage: 6.35 and 7.91, respectively; $P < 0.001$), whereas children with the minor G allele neither gained an advantage nor suffered a disadvantage from being fed breast milk. A recent attempt to replicate these findings in the ALSPAC population showed different results that those of the Caspi study (16), with GG children exhibiting the biggest difference in IQ scores between the formula and breastfed group. These contradictory results require further replication; however, a modification of the breastfeeding effect on IQ scores by *FADS2* genotypes was shown in both studies, even though the direction of effects differed (42). Brookes et al (17) showed an association of SNP rs498793 in the *FADS2* gene with ADHD in 180 ADHD cases compared with control subjects. It is tempting to speculate that genetic heterogeneity in fatty acid metabolism may be one of the reasons besides differing study design and variable quality for the apparent inconsistent results of different studies that investigated effects of a perinatal supply of DHA sources on developmental outcome (43, 44).

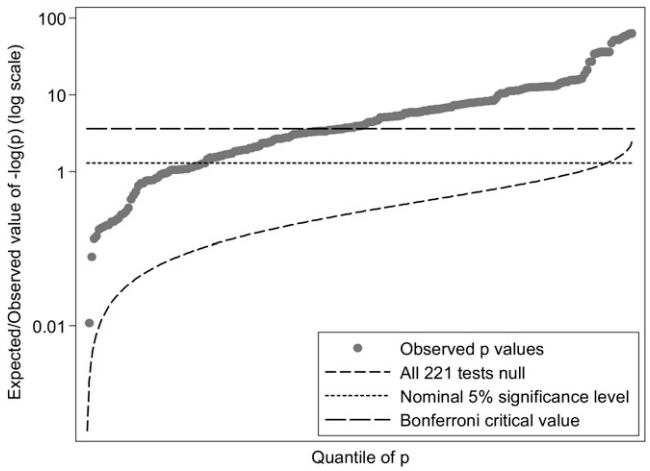

FIGURE 2. Quantile-quantile plot of 221 tests of significance (13 fatty acid outcomes × 17 genetic variants) reported in Table 2. The distribution of the observed *P* values differed markedly from that expected if all 221 comparisons were null. In particular, 78% of observed *P* values were less than the 5% significance level compared with 5% (by definition) for the null scenario. Even with the use of the overly conservative Bonferroni correction, 53% of tests would have remained significant. A more powerful method, the false discovery rate, suggested a revised critical value of 0.039. The minor adjustment to the nominal value of 0.05 reflected the early divergence in the distributions of observed and expected *P* values.

The genes *FADS1* and *FADS2* encoding for the enzymes Δ^5-desaturase and Δ^6-desaturase that are important in the desaturation and elongation pathway of n−6 and n−3 LC-PUFA biosynthesis, respectively, were mapped to chromosome 11q12-13.1 of the human genome in the year 2000 (45). This region shows conserved synteny to the mouse genomic region that contains the murine *fads1* and *fads2* genes on chromosome 19 (46). The 2 human genes are arranged in a head-to-head orientation and build a gene cluster together with a third desaturase gene, *FADS3*, which shows a high degree of sequence homology (47). Protein products of the *FADS3* gene have only recently been identified, but their function is not yet clear (48). Martinelli et al (11) reported the minor allele of rs1000778 mapped in the *FADS3* gene to be weakly associated with both the arachidonic:linoleic ratio and the eicosapentaenoic:α-linolenic ratio at $P <$ 0.05. In this study, we found an association of the minor allele of rs174455 mapped in the *FADS3* gene with higher amounts of the n−6 precursor fatty acids 18:2n−6, 20:2n−6, and 20:3n−6 as well as lower amounts of the LC-PUFA product fatty acids 20:4n−6, 22:4n−6, and 22:5n−6. Moreover, a significant negative association with DHA (22:6n−3) was shown. The strength of the association was comparable with the average of those observed for the *FADS2* SNPs studied. Therefore, it is highly likely that a gene product of *FADS3* has a desaturating activity, which, however, has to be confirmed in functional studies.

Supplemental Material can be found at:

In conclusion, this study shows that genetic variants common in the population have effects on pregnant women's blood amounts of PUFAs and LC-PUFAs, including the amounts of DHA considered particularly important for fetal development. Therefore, future studies on the relation between n−3 fatty acid supply and developmental outcomes should aim at inclusion of such genetic analyses to evaluate the biological relevance of genotype-dependent fatty acid amounts.

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The authors' responsibilities were as follows—BK: wrote the first version of the manuscript and acted as the guarantor; EL, SZ, and TI: performed the genetic analyses; CS: performed the statistical data evaluation; and all authors: contributed to the interpretation of the results and to the writing of the manuscript. None of the authors had a conflict of interest.

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Supplemental Material can be found at:

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Supplemental material

Supplementary Figure titles and legends

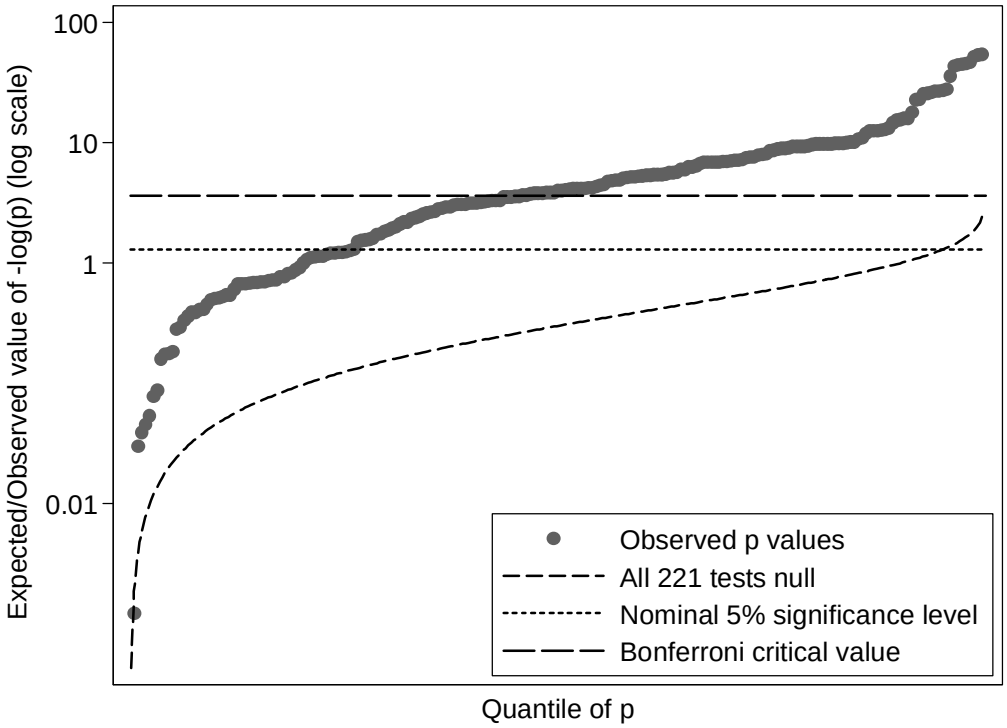
Supplementary Figure S1: Q-Q plot of adjusted analysis for 81 dietary variables and seven potential socio-demographic confounders.

Despite adjustment, the distribution of the observed p values still differs markedly from that expected if all 221 comparisons were null. In particular, 74% of observed p values were less than the 5% significance level compared to 5% (by definition) for the null scenario (compared to 78% in the unadjusted analysis). Using the overly conservative Bonferroni correction, 54% of tests would have remained significant (compared to 53% in the unadjusted analysis).

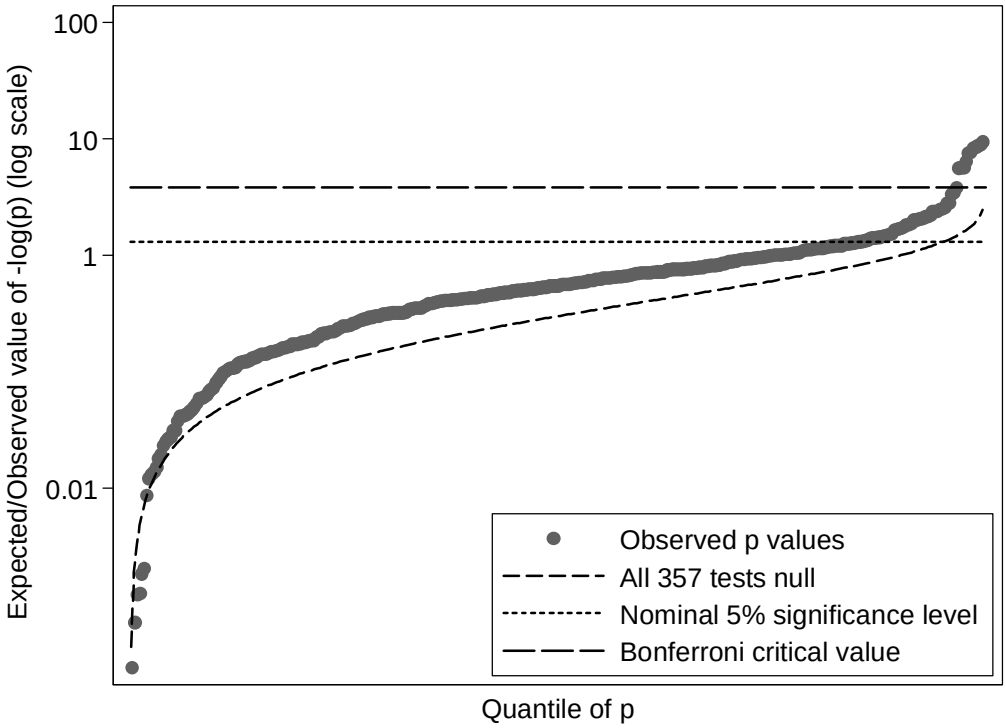
Supplementary Figure S2: Q-Q plot of 357 tests on saturated and mono-unsaturated fatty acids (21 fatty acids x 17 genetic variants)

The distribution of the observed p values was much closer to that expected if all 357 comparisons were null compared to the corresponding results for polyunsaturated fatty acids. However there was still some evidence of associations. In particular, 15% of observed p values were less than the 5% significance level compared to 5% (by definition) for the null scenario. Using the conservative Bonferroni correction, 11 (3%) of tests remained significant. A more powerful method, the False Discovery Rate, suggested a revised critical value of 0.0023 leading to 16 results below this criterion. Of these results, 13 related to 16:1n7.

Supplementary Figure S1:



Supplementary Figure S2:



Supplementary Table 1: Minor allele frequencies for the 17 polymorphisms

	Minor Allele	N	Frequency	HWE
rs174548	C	7835	30.56%	0.083
rs174556	A	7802	29.90%	0.049
rs174561	G	7824	29.73%	0.070
rs3834458	Deletion	7834	33.12%	0.339
rs968567	T	7973	17.03%	0.272
rs174570	T	7868	12.79%	0.001
rs174574	A	7839	34.14%	0.183
rs2727271	T	7850	11.73%	0.379
rs174576	A	7722	34.30%	0.196
rs174578	A	7846	34.32%	0.092
rs174579	T	7833	21.53%	<0.001
rs174602	G	7874	20.29%	0.319
rs498793	T	7837	39.44%	0.144
rs526126	G	7869	18.16%	0.790
rs174448	C	7900	36.15%	0.596
rs174449	C	7867	35.16%	0.785
rs174455	C	7862	34.51%	0.209

Data restricted to the availability of maternal fatty acid data and to mothers of white ethnic origin. N denotes the number of mothers genotyped rather than the number of observations used in the main analyses.

Supplementary Table 2: Demographics of sample in adjusted analyses (N=5468 observations)

	N	%
Multiple births	52	0.95%
Multiparity	2957	54.08%
Smokers	934	17.08%
	Mean	SD
Gestational age at blood sampling (wks)	26.87	8.15
Maternal age (yrs)	28.57	4.59
Maternal BMI (kg/m^2)	23.05	3.80
Family adversity	1.19	1.55

The sample reflects those observations where genetic data was present for at least one polymorphism and present for all 7 demographic variables and 81 dietary variables. Maternal blood samples were obtained throughout pregnancy (gestation range 4w – 44w). Maternal age reflected the age at birth of the ALSPAC child. Maternal BMI was derived from self-reported pre-pregnancy weight and height. The family adversity score was calculated from 18 items of adversity including teenage pregnancy, low education, financial hardship, poor living conditions, partner conflict, substance abuse, maternal anxiety or depressive symptoms and criminal behavior of either parent.

The 81 dietary variables (data not shown) were obtained from a Food Frequency Questionnaire completed around the 32nd gestation week by the mothers. The questions covered a range of food groups including meat, poultry, fish, milk and other dairy products, fruit, vegetables, breakfast cereals, bread, biscuits, cakes, desserts and confectionery. These questions also included lifestyle choices such as ‘eating the fat on meat’ and use of butter, margarine or vegetable oils as a spread on bread or for cooking.

Supplementary Table 3: Regression coefficients from analyses of log saturated and monounsaturated fatty acid levels as a percentage of total fatty acid levels on *FADS* polymorphisms.

	FADS1			Intergenic		FADS2	
	rs174548	rs174556	rs174561	rs3834458	rs968567	rs174570	rs174574
Saturated							
12:0	0.025 (0.019)	0.028 (0.019)	0.026 (0.019)	0.026 (0.019)	0.040 (0.023)	-0.011 (0.026)	0.034 (0.019)
13:0	0.023 (0.019)	0.030 (0.019)	0.029 (0.019)	0.026 (0.019)	0.038 (0.023)	-0.003 (0.026)	0.024 (0.018)
14:0	0.022 (0.021)	0.020 (0.021)	0.019 (0.021)	0.021 (0.020)	0.021 (0.026)	0.014 (0.028)	0.031 (0.020)
15:0	0.016 (0.021)	0.018 (0.021)	0.022 (0.021)	0.010 (0.020)	0.008 (0.025)	0.031 (0.028)	0.019 (0.020)
16:0	0.032 (0.020)	0.032 (0.020)	0.037 (0.020)	0.024 (0.020)	0.046 (0.025)	0.004 (0.028)	0.022 (0.019)
17:0	0.012 (0.020)	0.010 (0.020)	0.015 (0.020)	0.024 (0.020)	0.009 (0.025)	0.034 (0.028)	0.015 (0.020)
18:0	-0.031 (0.020)	-0.035 (0.020)	-0.038 (0.020)	-0.017 (0.019)	0.017 (0.024)	-0.032 (0.027)	-0.024 (0.019)
19:0	0.012 (0.019)	0.006 (0.019)	0.009 (0.019)	0.003 (0.019)	0.005 (0.023)	0.004 (0.026)	0.002 (0.019)
20:0	0.003 (0.019)	0.004 (0.019)	0.002 (0.019)	0.014 (0.019)	0.024 (0.023)	-0.001 (0.026)	0.010 (0.019)
21:0	0.023 (0.020)	0.022 (0.020)	0.027 (0.020)	0.036 (0.019)	0.039 (0.024)	0.010 (0.027)	0.034 (0.019)
22:0	0.040 (0.019)*	0.038 (0.019)	0.040 (0.019)*	0.040 (0.019)*	0.061 (0.023)**	0.018 (0.026)	0.035 (0.019)
23:0	0.022 (0.019)	0.022 (0.019)	0.019 (0.019)	0.028 (0.019)	0.063 (0.023)**	-0.018 (0.026)	0.024 (0.018)
24:0	-0.028 (0.020)	-0.027 (0.020)	-0.021 (0.020)	-0.025 (0.019)	0.001 (0.024)	-0.031 (0.027)	-0.021 (0.019)
Mono-unsaturated							
14:1n5	-0.012 (0.019)	-0.008 (0.019)	-0.011 (0.019)	-0.017 (0.019)	0.001 (0.023)	-0.047 (0.026)	-0.022 (0.018)
16:1n7	0.126 (0.021)***	0.129 (0.021)***	0.132 (0.021)***	0.122 (0.021)***	0.070 (0.026)**	0.108 (0.029)***	0.114 (0.020)***
17:1n7	-0.032 (0.019)	-0.029 (0.019)	-0.032 (0.019)	-0.026 (0.019)	-0.047 (0.023)*	0.007 (0.026)	-0.020 (0.019)
18:1n1	0.031 (0.019)	0.030 (0.019)	0.028 (0.019)	0.018 (0.019)	0.030 (0.023)	0.002 (0.026)	0.030 (0.019)
20:1n9	0.046 (0.020)*	0.046 (0.020)*	0.042 (0.020)*	0.058 (0.020)**	-0.021 (0.024)	0.097 (0.027)***	0.050 (0.019)**
21:1n9	0.050 (0.019)**	0.040 (0.019)*	0.039 (0.019)*	0.046 (0.019)*	0.008 (0.023)	0.054 (0.026)*	0.052 (0.018)**
22:1n9	0.027 (0.019)	0.026 (0.019)	0.024 (0.019)	0.031 (0.019)	0.001 (0.023)	0.049 (0.026)	0.031 (0.018)
23:1n9	0.000 (0.025)	0.001 (0.025)	-0.000 (0.025)	0.013 (0.024)	0.028 (0.030)	0.000 (0.034)	0.016 (0.024)
N	6565	6524	6560	6558	6666	6583	6554

* p<0.05 ** p<0.01 *** p<0.001
Fatty acids and ratios have been standardized to have a variance of one. Standard errors are reported in parentheses.
The omega-9 fatty acid (18:1) was excluded due to its association with a metabolic pathway involving the FADS genes.

Supplementary Table3: continued

	FADS2						
	rs2727271	rs174576	rs174578	rs174579	rs174602	rs498793	rs526126
Saturated							
12:0	0.038 (0.028)	0.031 (0.019)	0.032 (0.018)	0.025 (0.021)	-0.006 (0.022)	-0.025 (0.018)	0.025 (0.023)
13:0	0.018 (0.027)	0.025 (0.018)	0.032 (0.018)	0.021 (0.021)	-0.020 (0.022)	-0.040 (0.018)*	0.017 (0.023)
14:0	0.041 (0.030)	0.029 (0.020)	0.036 (0.020)	0.020 (0.023)	-0.007 (0.024)	-0.005 (0.019)	0.022 (0.025)
15:0	0.054 (0.030)	0.014 (0.020)	0.018 (0.020)	-0.008 (0.023)	-0.033 (0.024)	-0.007 (0.019)	-0.024 (0.025)
16:0	0.010 (0.029)	0.017 (0.020)	0.025 (0.019)	0.028 (0.022)	-0.026 (0.023)	0.006 (0.019)	0.033 (0.024)
17:0	0.012 (0.029)	0.017 (0.020)	0.012 (0.020)	0.007 (0.022)	-0.013 (0.023)	-0.003 (0.019)	0.041 (0.024)
18:0	-0.074 (0.028)**	-0.017 (0.019)	-0.021 (0.019)	0.015 (0.022)	-0.010 (0.023)	-0.036 (0.018)*	0.026 (0.024)
19:0	0.012 (0.028)	0.009 (0.019)	0.001 (0.019)	0.000 (0.021)	-0.010 (0.022)	-0.013 (0.018)	0.004 (0.023)
20:0	-0.022 (0.028)	0.006 (0.019)	0.007 (0.019)	0.025 (0.021)	0.009 (0.022)	-0.035 (0.018)*	0.010 (0.023)
21:0	0.016 (0.028)	0.037 (0.019)	0.035 (0.019)	0.032 (0.022)	0.027 (0.023)	-0.016 (0.018)	0.009 (0.024)
22:0	0.003 (0.028)	0.035 (0.019)	0.037 (0.019)*	0.052 (0.021)*	-0.011 (0.022)	-0.051 (0.018)**	0.020 (0.023)
23:0	-0.031 (0.027)	0.022 (0.019)	0.029 (0.018)	0.056 (0.021)**	-0.007 (0.022)	-0.036 (0.018)*	0.013 (0.023)
24:0	-0.037 (0.028)	-0.020 (0.019)	-0.020 (0.019)	-0.002 (0.021)	-0.031 (0.022)	-0.024 (0.018)	0.006 (0.024)
Mono-unsaturated							
14:1n5	-0.012 (0.027)	-0.019 (0.019)	-0.025 (0.018)	-0.015 (0.021)	-0.021 (0.022)	0.000 (0.018)	0.018 (0.023)
16:1n7	0.143 (0.030)***	0.114 (0.021)***	0.119 (0.020)***	0.073 (0.023)**	0.052 (0.024)*	-0.007 (0.020)	0.076 (0.025)**
17:1n7	-0.014 (0.028)	-0.020 (0.019)	-0.036 (0.019)	-0.029 (0.021)	-0.001 (0.022)	0.026 (0.018)	-0.023 (0.023)
18:1n1	0.019 (0.028)	0.030 (0.019)	0.032 (0.018)	0.031 (0.021)	0.016 (0.022)	-0.000 (0.018)	-0.014 (0.023)
20:1n9	0.100 (0.029)***	0.047 (0.020)*	0.051 (0.019)**	0.003 (0.022)	0.056 (0.023)*	-0.031 (0.019)	-0.029 (0.024)
21:1n9	0.080 (0.027)**	0.053 (0.019)**	0.058 (0.018)**	0.015 (0.021)	0.047 (0.022)*	-0.041 (0.018)*	0.003 (0.023)
22:1n9	0.033 (0.027)	0.024 (0.018)	0.033 (0.018)	0.007 (0.021)	-0.025 (0.022)	-0.030 (0.018)	0.019 (0.023)
23:1n9	-0.035 (0.036)	0.015 (0.024)	0.016 (0.024)	0.042 (0.027)	0.025 (0.028)	0.030 (0.023)	0.016 (0.030)
N	6569	6481	6574	6556	6591	6555	6595

Supplementary Table3: continued

	Intergenic		FADS3
	rs174448	rs174449	rs174455
Saturated			
12:0	0.020 (0.018)	0.028 (0.019)	0.026 (0.019)
13:0	0.007 (0.018)	0.004 (0.018)	-0.015 (0.018)
14:0	-0.013 (0.020)	-0.010 (0.020)	-0.002 (0.020)
15:0	-0.037 (0.020)	-0.031 (0.020)	-0.019 (0.020)
16:0	0.023 (0.019)	0.020 (0.019)	0.017 (0.020)
17:0	0.019 (0.019)	0.033 (0.020)	0.026 (0.020)
18:0	-0.021 (0.019)	-0.019 (0.019)	-0.019 (0.019)
19:0	-0.012 (0.018)	-0.011 (0.019)	-0.015 (0.019)
20:0	-0.015 (0.018)	-0.019 (0.019)	-0.018 (0.019)
21:0	0.002 (0.019)	-0.000 (0.019)	-0.003 (0.019)
22:0	0.013 (0.018)	0.007 (0.019)	0.015 (0.019)
23:0	-0.017 (0.018)	-0.024 (0.018)	-0.013 (0.019)
24:0	-0.008 (0.019)	-0.016 (0.019)	-0.002 (0.019)
Mono-unsaturated			
14:1n5	-0.012 (0.018)	-0.020 (0.018)	-0.027 (0.019)
16:1n7	0.101 (0.020)***	0.096 (0.020)***	0.097 (0.020)***
17:1n7	-0.032 (0.018)	-0.029 (0.019)	-0.024 (0.019)
18:1n1	-0.007 (0.018)	-0.020 (0.019)	-0.017 (0.019)
20:1n9	0.018 (0.019)	0.015 (0.019)	0.007 (0.019)
21:1n9	0.035 (0.018)	0.030 (0.018)	0.038 (0.019)*
22:1n9	0.001 (0.018)	-0.005 (0.018)	0.004 (0.018)
23:1n9	0.009 (0.023)	0.005 (0.024)	0.007 (0.024)
N	6623	6583	6569

Appendix A2.

Lattka, E., P. Rzehak, É. Szabó, V. Jakobik, M. Weck, M. Weyermann, H. Grallert, D. Rothenbacher, J. Heinrich, H. Brenner, T. Decsi, T. Illig, and B. Koletzko. 2011. Genetic variants in the *FADS* gene cluster are associated with arachidonic acid concentrations of human breast milk at 1.5 and 6 mo postpartum and influence the course of milk dodecanoic, tetracosenoic and trans-9-octadecenoic acid concentrations over the duration of lactation. *Am J Clin Nutr*. **93:** 382-391.

Genetic variants in the *FADS* gene cluster are associated with arachidonic acid concentrations of human breast milk at 1.5 and 6 mo postpartum and influence the course of milk dodecanoic, tetracosenoic, and *trans*-9-octadecenoic acid concentrations over the duration of lactation[1–4]

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ABSTRACT

Background: Breastfeeding is considered an optimal nutritional source of n−6 (omega-6) and n−3 (omega-3) fatty acids (FAs) for the proper visual and cognitive development of newborn children. In addition to maternal nutrition as an important regulator of FA concentrations, first results exist on an association of breast-milk FAs with single nucleotide polymorphisms (SNPs) in the *FADS* gene cluster, which encodes the rate-limiting enzymes in the elongation-desaturation pathway of long-chain polyunsaturated fatty acids (LC-PUFAs).

Objective: We analyzed the influence of *FADS* SNPs on breast-milk FA concentrations and their time course during lactation in the Ulm Birth Cohort study, which comprised 772 nursing mothers at 1.5 mo after giving birth, and in a subset of 463 mothers who were still breastfeeding at 6 mo postpartum.

Design: We conducted linear regression analysis of 8 *FADS* SNPs with FA concentrations at both time points separately and assessed the genotype effect over time in a longitudinal analysis by using a generalized estimating equation regression model.

Results: We observed significant associations of *FADS* genotypes with arachidonic acid (AA) concentrations and the 20:4n−6/20:3n−6 ratio at both time points but no association of *FADS* SNPs with the time course of AA concentrations. A longitudinal analysis of FAs other than LC-PUFAs by genotype over time showed associations for dodecanoic acid, *cis*-15-tetracosenoic acid, and *trans*-9-octadecenoic acid.

Conclusions: Maternal *FADS* genotypes are associated with breast-milk AA concentrations and might therefore influence the supply of this FA for children. Furthermore, our data indicate an interrelation between the LC-PUFA pathway and saturated and monounsaturated FAs. *Am J Clin Nutr* 2011;93:382–91.

INTRODUCTION

The supply of the newborn infant with n−6 and n−3 fatty acids by breastfeeding is considered highly beneficial for child health and development. Lipids in human milk are not only an important energy source for the infant but are also considered important for visual and cognitive development (1). Arachidonic acid (AA) and especially docosahexaenoic acid (DHA) are thought to be important long-chain polyunsaturated fatty acids (LC-PUFAs) for developmental processes. AA and DHA are essential membrane constituents, especially in the brain and retina, and AA serves as a precursor to prostaglandins and leukotrienes. In addition to effects on visual and cognitive development, there are also indications that early exposure to dietary LC-PUFAs protects individuals from high blood pressure and cardiovascular risk in later childhood (2), even though controversial data have emerged (3). Moreover, the fatty acid supply with breast milk has been associated with the development of atopic diseases in several studies (4–6).

[1] From the Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Epidemiology, Neuherberg, Germany (EL, PR, HG, JH, and TI); the Institute of Medical Informatics, Biometrics, and Epidemiology, Ludwig-Maximilians University Munich, Oberschleissheim, Germany (PR); the Department of Paediatrics, University of Pécs, Pécs, Hungary (PR, ÉS, VJ, and TD); the Division of Clinical Epidemiology and Aging Research, German Cancer Research Center, Heidelberg, Germany (M Weck, M Weyermann, and HB); and the Division of Metabolic and Nutritional Medicine, Dr von Hauner Children's Hospital, University of Munich Medical Centre, Munich, Germany.

[2] This article does not necessarily reflect the views of the Commission of the European Communities and in no way anticipates the future policy in this area.

[3] Supported by the Commission of the European Communities specific RTD Programme Quality of Life and Management of Living Resources within the 6th Framework Programmes EARNEST (Food-CT-2005-007036) and EURRECA (FP6-036196-2) and within the 7th Framework Programme NUTRIMENTHE (FP7-212652), the German Federal Ministry of Education and Research (01GS0485), the Kompetenznetz Adipositas (Competence Network Obesity) funded by the Federal Ministry of Education and Research (01GI0826), the Munich Center of Health Sciences, and a Freedom to Discover Award (to BK) from the Bristol-Myers-Squibb Foundation (New York, NY). The Ulm Birth Cohort Study was supported by the Deutsche Forschungsgemeinschaft (the German Research Foundation (grants BR 1704/3-1, 3-2, and 3-3).

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The concentrations of long-chain n−6 and n−3 fatty acids in breast milk are highly dependent on the mother's dietary habits (7–9) and are similar to dietary effects on blood fatty acid concentrations (10–12). In addition to their dietary supply, LC-PUFAs can also be endogenously derived from the precursor essential fatty acids linoleic acid (18:2n−6) and α-linolenic acid (18:3n−3) by consecutive desaturation and chain elongation as originally described by Sprecher (13) and Sprecher et al (14). The rate-limiting enzymes in this reaction cascade are the δ-6 desaturase (D6D) and δ-5 desaturase (D5D). A detailed overview of the pathway was shown elsewhere (15, 16). The human desaturase-encoding genes (*FADS1* for D5D and *FADS2* for D6D) are arranged in a head-to-head orientation and build a gene cluster on chromosome 11 together with a third desaturase gene, *FADS3*, the function of which has not yet been revealed. In the past few years, numerous genetic association studies have shown that single nucleotide polymorphisms (SNPs) in the *FADS* gene cluster are associated with n−6 and n−3 fatty acid concentrations in serum, plasma, erythrocyte membranes, and adipose tissue (17–21). Carriers of the minor alleles of the significantly associated SNPs had enhanced concentrations of desaturase substrates and decreased concentrations of desaturase products, which led to the hypothesis that there was a decline in the transcriptional levels or conversion rates of desaturases in minor allele carriers. Associations of *FADS* polymorphisms with fatty acid concentrations in human breast milk have been investigated in 2 previous studies (22, 23). Both of these studies reported significant associations with various fatty acids; however, the study size was rather small in both cases. Also, these studies did not investigate how *FADS* genotypes influence the timely change of fatty acid concentrations over the duration of lactation.

The aim of the current study was to analyze the influence of *FADS* genotypes on breast-milk fatty acid concentrations in a substantially larger German birth cohort that comprised 772 mothers who were breastfeeding their children at 1.5 mo after birth. In addition, breast-milk fatty acid measurements from a subset of 463 nursing mothers at 6 mo postpartum were available. We initially investigated the effect of 8 SNPs in the *FADS* gene cluster on fatty acid concentrations at both time points separately. Because it is known that the concentrations of several fatty acids change during the duration of lactation (7, 24), we also analyzed whether the polymorphisms had an influence on the increase or decrease of fatty acid concentrations during lactation.

SUBJECTS AND METHODS

Study population

Women were recruited during their stay at the Department of Gynecology and Obstetrics at the University of Ulm after delivery of their infants between November 2000 and November 2001. To obtain a birth cohort of healthy and mature infants, exclusion criteria were delivery before 32 gestational weeks, birth weight <2500 g, and transfer to pediatric care immediately after delivery. Also, women with no understanding of the German, Turkish, or Russian language and all women who left the hospital immediately after birth were excluded. In total, 1066 families were included into this study. Participation was voluntary, and written informed consent was obtained in each case. Detailed information on characteristics of study subjects can be obtained elsewhere (25–28). The study was approved by the ethics boards of the University of Ulm and the physicians' boards of the states of Baden-Württemberg and Bavaria.

Data and sample collection

Standardized interviews in German, Turkish, or Russian were conducted by trained interviewers during the hospitalization of mothers after delivery. They included detailed questions about living and housing conditions, lifestyle factors, medical histories, and health status during pregnancy. Furthermore, anthropometric data before and during pregnancy were collected from the pregnancy health charts of mothers ("Mutterpass") by using a standardized form. All participating mothers were contacted 6 wk postpartum and asked if they were breastfeeding at that time. A total of 1024 (96%) mothers were successfully contacted again, and 786 (76.7%) mothers were still breastfeeding their infants. For the collection of milk samples, a trained nurse visited all women who were still breastfeeding and collected 10 mL manually expressed human milk from both breasts before feeding. In some cases, milk was collected with the help of a breast pump. Samples were immediately cooled and frozen at −80°C for ≤24 h. From 786 breastfeeding mothers, 769 (97.8%) milk samples were successfully collected. The women who were breastfeeding after 6 wk were contacted again at 6 mo postpartum and asked if they were still breastfeeding. Milk samples of 98% of mothers who were still breastfeeding were collected successfully by using the same procedure as previously described (n = 463).

Fatty acid analyses

Fatty acids were analyzed by using the procedure previously described (25). In brief, fatty acids were extracted from 100 μl milk, and fatty acid methyl esters were measured by high-resolution capillary gas-liquid chromatography with a 60-m cyanopropyl column and a flame ionization detector. The peak identification was confirmed by comparison with weighted standards. In total, 26 saturated, monounsaturated, n−3, n−6, and *trans* fatty acids with chain lengths between 10 and 24 carbons were measured and used for analyses.

SNP selection and genotyping

Genetic analysis of samples from study participants in an anonymous manner was approved by the ethics committee of the Bayerische Landesärztekammer (the Bavarian Board of Physicians). Ten tagging SNPs in the genomic region spanning *FADS1*, *FADS2*, and *FADS3* were selected by using the HapMap project homepage (http://www.hapmap.org), and 2 additional SNPs were selected based on results of a former association study (17). The genomic DNA of all mothers was extracted from 300 μl breast milk with a High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland) by using the protocol for DNA extraction from whole blood. A total of 5 μL DNA were subjected to polymerase chain reaction amplification followed by a genotyping procedure with the MassARRAY system and iPLEX chemistry as suggested by the manufacturer (Sequenom). The procedure was previously described in detail (18).

Statistical analyses

Genotype frequencies, allele frequencies, and the Hardy-Weinberg equilibrium were calculated with the statistical software module SAS/Genetics (SAS version 9.1.3; SAS Institute Inc, Cary, NC) by using the proc allele procedure. Deviations from the Hardy-Weinberg equilibrium were tested by using Fisher's exact test. To examine the linkage disequilibrium, Lewontin's D' and pairwise squared correlations r^2 were calculated with the software JLIN (version 1.6.0) (29).

The normal distribution of fatty acids was tested by Kolmogorov-Smirnov tests and evaluated by box plots and quantile-quantile plots (by using the proc univariate procedure of SAS software, version 9.1.3; SAS Institute Inc). Several severely skewed fatty acids (18:3n−6, 22:4n−6, 20:5n−3, 22:6n−3, 22:0, 24:0, 22:1n−9, and 24:1n−9) were log transformed to better approximate the normal distribution for further analysis.

We conducted a linear regression analysis of each of the 8 *FADS* SNPs with each of the measured n−6, n−3, monounsaturated, saturated, and *trans* fatty acids as continuous outcome variables separately at both time points of lactation (1.5 and 6 mo). In addition, D6D and D5D desaturation indexes were calculated as 18:3n−6/18:2n−6 and 20:4n−6/20:3n−6, respectively. We applied an additive model where homozygous minor allele carriers were coded as 2, heterozygous subjects were coded as 1, and homozygous major allele carriers were coded as 0, with the assumption of a linear relation between the fatty acid outcomes and number of minor alleles. For correction for multiple testing, the number of effective loci was calculated with the spectral decomposition method software SNPSpD (Queensland Institute of Medical Research, Herston, Australia; http://genepi.qimr.edu.au/general/daleN/SNPSpd/). For the 8 analyzed SNPs, the number of effective loci was calculated as 6.8269, which resulted in a reduced significance threshold of 0.05/6.8269 = 0.0073. To additionally account for the number of fatty acids in each tested fatty acid group, the significance threshold required to keep the type I error rate at 5% was further reduced to 0.001 (which corresponded to 0.05/6.8269 × 8 analyzed n−6 fatty acids). This threshold was calculated for the group of n−6 fatty acids and was also applied for all other tested fatty acid groups. For longitudinal analysis of fatty acid concentrations by genotype over time between 1.5 and 6 mo of breastfeeding, a generalized estimating equation regression model was applied to account for the correlated data structure (30) in a complete case analysis (which included only those mothers who were breastfeeding at both time points).

RESULTS

General characteristics of the study sample are presented in **Table 1**. Generally, women had a mean age of 31.29 ± 4.76 y, a mean height of 166.59 ± 6.38 cm, and a mean prepregnancy body mass index (in kg/m^2) of 23.03 ±3.86. Most women had

TABLE 1
General characteristics of the sample[1]

	All mothers	Mothers not breastfeeding 6 mo postpartum	Mothers still breastfeeding 6 mo postpartum
No. of subjects	772	309	463
School education before graduation [*n* (%)]			
≥12 y	333 (43.13)	111 (35.92)	222 (47.95)
10 y	287 (37.18)	117 (37.86)	170 (36.72)
≤9 y	143 (18.52)	73 (23.62)	70 (15.12)
No graduation	6 (0.78)	5 (1.62)	1 (0.22)
Missing	3 (0.39)	3 (0.97)	0 (0.00)
Maternal smoking [*n* (%)]			
Ever smoked 100 cigarettes during lifetime			
Yes	343 (44.43)	148 (47.90)	195 (42.12)
No	428 (55.44)	160 (51.78)	268 (57.88)
Missing	1 (0.13)	1 (0.32)	0 (0.00)
During pregnancy			
Yes	65 (8.42)	38 (12.30)	27 (5.83)
No	707 (91.58)	271 (87.70)	436 (94.17)
Missing	0 (0.00)	0 (0.00)	0 (0.00)
1.5 mo postpartum			
Yes	53 (6.87)	34 (11.00)	19 (4.10)
No	716 (92.75)	275 (89.00)	441 (95.25)
Missing	3 (0.39)	0 (0.00)	3 (0.65)
6 mo postpartum			
Yes	67 (8.68)	44 (14.24)	23 (4.97)
No	671 (86.92)	232 (75.08)	439 (94.82)
Missing	34 (4.40)	33 (10.68)	1 (0.22)
Age (y)	31.29 ± 4.76[2]	30.18 ± 4.98	32.20 ± 4.44
Maternal height (cm)	166.59 ± 6.38	166.59 ± 6.41	166.59 ± 6.38
Maternal prepregnancy BMI (kg/m^2)	23.03 ± 3.86	23.41 ± 4.15	22.78 ± 3.65

[1] Number of subjects refers to all mothers for whom demographic data were available. Anthropometric data were available for 770 (age and height) and 746 (weight) mothers.
[2] Mean ± SD (all such values).

a high education and were nonsmokers. When women were separated into those who were not breastfeeding after 6 mo postpartum and those who were still breastfeeding, women who were still breastfeeding after 6 mo were of higher age, had a higher education, lower body mass index, and less frequently smoked (ever smoked 100 cigarettes during their lifetime as well as during pregnancy and lactation).

Genotyping was successful for all 12 selected SNPs, except for rs174553, for which alleles could not be discriminated. The minor allele frequencies for the successfully genotyped SNPs ranged from 12% to 44% and matched those reported in the Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP/). The overall mean genotyping success rate was 92.1%. The distribution of genotypes of 8 SNPs was consistent with the Hardy-Weinberg equilibrium, whereas 3 SNPs showed a deviation from the Hardy-Weinberg equilibrium (rs174561, $P = 0.0042$; rs2072114, $P = 0.0039$; and rs174627, $P = 0.0179$) and, therefore, were excluded from further analysis. Eight SNPs were included in the final analysis, and the characteristics of these SNPs are listed in **Table 2**, including their position on chromosome 11, their location, and their genotype and allele frequencies. Genotype and allele frequencies did not differ between the group of women who were not breastfeeding at 6 mo postpartum and those women who were still breastfeeding at 6 mo postpartum.

Fatty acid concentrations and CVs at time points of 1.5 and 6 mo with fatty acids expressed as the percentage of weight divided by the weight of total fatty acids are shown in **Table 3**. Some fatty acids had higher CVs compared with others at the 1.5-mo time point (ie, 18:3n−6, 18:3n−6/18:2n−6, 20:5n−3, 22:5n−3, 22:6n−3, and 24:1n−9).

Association of *FADS* SNPs with n−6 and n−3 fatty acids in breast milk

Significant associations were observed for milk AA (20:4n−6) concentrations with SNPs rs174547 and rs174556 at 6 mo after birth ($P < 0.001$; **Table 4**). Before correction for multiple testing, both SNPs also showed significant associations with AA at the 1.5-mo time point ($P = 0.0031$ and 0.0025). Three additional SNPs (rs174626, rs1000778, and rs174455) were associated with AA concentrations at both investigated time points; however, this occurred without taking multiple testing into account ($P = 0.0022$–0.0090). For all associated SNPs, carriers of the minor alleles had lower concentrations of AA in breast milk compared with those of carriers of the major alleles. SNPs rs174602, rs498793, and rs526126 did not show significant associations, even without correction for multiple testing. When we looked at the 20:4n−6/20:3n−6 ratio, which was a measure of the D5D activity, associations remained essentially the same. Sensitivity analysis that excluded all potential outliers defined as the mean ± (1.5 × the interquartile range) confirmed the significant results obtained in the original analysis. Significant associations with other fatty acids were not observed, except for 22:4n−6, which showed an association with rs1000778 ($P = 0.0007$) 6 mo after birth; however, this association was not stable in the outlier sensitivity analysis. The ratio 18:3n−6/18:2n−6, which approximated the D6D activity, was not significant for any SNPs at either of the 2 time points. For n−3 fatty acids, no significant associations were observed after correction for multiple testing (*see* supplemental Table S1 under "Supplemental data" in the online issue).

Associations of *FADS* genotypes with saturated, monounsaturated, and *trans* fatty acids

Because Xie and Innis (22) reported an association of *FADS* polymorphisms with the saturated fatty acid 14:0 and the monounsaturated fatty acid 18:1n−7, we tested associations of the genotyped SNPs with all measured saturated fatty acids (10:0, 12:0, 14:0, 16:0, 18:0, 20:0, 22:0, and 24:0), monounsaturated fatty acids (16:1n−7, 18:1n−9, 22:1n−9, and 24:1n−9), and *trans* fatty acids (*t*-16:1, *t*-18:1n−9, and *tt*-18:2n−6) in our study.

After correction for multiple testing, no significant associations were observed for saturated, monounsaturated, and *trans* fatty acids (*see* supplemental Tables S2–S4 under "Supplemental data" in the online issue for summary of results of saturated, monounsaturated, and *trans* fatty acids).

TABLE 2
Characteristics of 8 analyzed polymorphisms in the *FADS* gene cluster region[1]

					No. of subjects at 1.5/6 mo with				
					Genotype[2]			Allele[2]	
dbSNP	Position (bp)	Gene	Alleles (major/minor) 1/2	No. at 1.5/6 mo	11	12	22	1	2
rs174547	61327359	*FADS1*	T/C	716/423	353/208 (49)	294/178 (41)	69/37 (10)	1000/594 (70)	432/252 (30)
rs174556	61337211	*FADS1*	C/T	714/424	393/236 (55)	270/161 (38)	51/27 (7)	1056/633 (74)	372/215 (26)
rs174602	61380990	*FADS2*	A/G	714/423	463/276 (65)	214/127 (30)	37/20 (5)	1140/679 (80)	288/167 (20)
rs498793	61381281	*FADS2*	G/A	701/415	253/146 (36)	334/203 (48)	114/66 (16)	840/495 (60)	562/335 (40)
rs526126	61381461	*FADS2*	C/G	718/421	482/279 (67)	213/129 (30)	23/13 (3)	1177/687 (82)	259/155 (18)
rs174626	61393633	Intergenic *FADS2/3*	T/C	710/419	234/137 (33)	326/195 (46)	150/87 (21)	794/469 (56)	626/369 (44)
rs1000778	61411881	*FADS3*	G/A	714/425	406/244 (57)	261/157 (37)	47/24(7)	1073/645 (75)	355/205 (25)
rs174455	61412693	*FADS3*	A/G	711/417	311/182 (44)	302/184 (42)	98/51 (14)	924/548 (65)	498/235 (35)

[1] dbSNP, Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP/); bp, base pairs.
[2] Numbers in parentheses indicate genotype or allele frequencies at 1.5 mo (%).

TABLE 3
Raw fatty acid (FA) concentrations in breast milk of mothers at 1.5 and 6 mo postpartum[1]

	1.5 mo (n = 769)		6 mo (n = 463)	
	Mean ± SD	CV	Mean ± SD	CV
n−6 FA				
18:2n−6	11.02 ± 4.06	36.85	11.65 ± 4.14	35.59
18:3n−6	0.17 ± 0.29	172.02	0.18 ± 0.10	55.83
20:2n−6	0.23 ± 0.11	45.54	0.25 ± 0.09	33.86
20:3n−6	0.33 ± 0.21	63.01	0.33 ± 0.17	52.55
20:4n−6	0.44 ± 0.24	52.92	0.54 ± 0.29	54.63
22:4n−6	0.08 ± 0.08	96.62	0.13 ± 0.10	75.65
18:3n−6/18:2n−6	0.02 ± 0.03	203.63	0.02 ± 0.01	58.49
20:4n−6/20:3n−6	1.46 ± 0.80	54.69	1.76 ± 1.02	58.01
n−3 FA				
18:3n−3	0.79 ± 0.46	58.21	0.89 ± 0.45	50.49
20:3n−3	0.05 ± 0.04	81.90	0.06 ± 0.04	67.05
20:5n−3	0.06 ± 0.07	114.81	0.08 ± 0.06	74.88
22:5n−3	0.15 ± 0.19	127.85	0.20 ± 0.12	62.88
22:6n−3	0.22 ± 0.23	107.76	0.25 ± 0.16	64.91
Saturated FA				
10:0	2.23 ± 0.86	38.50	2.16 ± 1.21	56.02
12:0	6.61 ± 2.35	35.56	6.89 ± 2.18	31.64
14:0	7.19 ± 2.02	28.10	7.49 ± 2.06	27.50
16:0	22.38 ± 2.94	13.13	23.26 ± 2.98	12.80
18:0	8.15 ± 2.25	27.58	8.37 ± 2.14	25.56
20:0	0.28 ± 0.13	45.29	0.26 ± 0.09	35.12
22:0	0.16 ± 0.11	68.19	0.16 ± 0.09	54.35
24:0	0.16 ± 0.16	97.47	0.12 ± 0.08	70.73
Monounsaturated FA				
16:1n-7	2.72 ± 0.90	33.14	2.58 ± 0.68	26.56
18:1n−9	30.89 ± 3.84	12.42	29.74 ± 3.83	12.88
22:1n−9	0.07 ± 0.05	76.95	0.10 ± 0.11	108.57
24:1n−9	0.10 ± 0.11	113.44	0.09 ± 0.06	60.22
trans FA				
t-16:1	0.43 ± 0.23	53.17	0.42 ± 0.23	54.26
t-18:1n−9	1.16 ± 0.94	81.26	0.95 ± 0.90	94.49
tt-18:2n−6	0.40 ± 0.35	88.59	0.29 ± 0.14	48.26

[1] Values are presented as the percentage of weight divided by the weight of total FAs.

Association of SNPs with the timely change in fatty acid concentrations during lactation

Milk contents of fat and of most fatty acids change during lactation (7, 24). Therefore, we asked whether *FADS* genotypes influence the change in fatty acid concentrations from 1.5 to 6 mo of lactation and investigated the interaction between SNPs and the time effect on fatty acid concentrations. We used a complete case design that included only those mothers who were breastfeeding at both investigated time points to avoid problems of a generalized estimating equation regression models with missing data.

FADS SNPs were not associated with the timely change in AA concentrations during lactation

With the use of a longitudinal model, we examined whether *FADS* genotypes modulated the change of AA concentrations over time in women who were breastfeeding at both investigated time points. There was no significant association of any of the tested SNPs with AA concentrations or the ratio (20:4n−6/20:3n−6) that estimated the D5D activity over time accounting for multiple testing.

Longitudinal analysis indicated a role of *FADS* polymorphisms in the regulation of monounsaturated, saturated, and *trans* fatty acid concentrations

In addition to the longitudinal analysis for AA, we also analyzed the time course of all other measured fatty acids dependent on the *FADS* genotypes in women who were breastfeeding at both investigated time points. Although we did not observe any significant associations below the significance threshold of 0.001, we observed some significant associations before correction for multiple testing. We observed time-genotype interactions for 12:0 (dodecanoic acid) and SNP rs174626 (P for interaction = 0.0186) with a difference in dodecanoic acid concentrations between the 3 genotype groups only at the 6-mo time point (**Figure 1**). Homozygous carriers of the minor allele exhibited a remarkable increase in dodecanoic acid concentrations over the duration of breastfeeding. This effect was also visible in heterozygous subjects, although it was less pronounced. The dodecanoic acid concentrations in homozygous carriers of the major allele remained rather stable during the lactation period. A similar effect was observed for 14:0 (tetradecanoic acid) and the same SNP (P for interaction = 0.0287) as well as for the association between SNP rs526126 and the timely

TABLE 4
Results of linear regression analysis of 8 *FADS* single nucleotide polymorphisms with n−6 fatty acid concentrations and ratios that estimated desaturase activity in human breast milk after 1.5 and 6 mo of lactation[1]

	18:2 n−6		log 18:3 n−6		20:2 n−6		20:3 n−6		20:4 n−6		log 22:4 n−6		log 18:3 n−6/18:2 n−6		20:4 n−6/20:3 n−6	
	1.5 mo	6 mo	1.5 mo	6 mo	1.5 mo	6 mo	1.5 mo	6 mo	1.5 mo	6 mo	1.5 mo	6 mo	1.5 mo	6 mo	1.5 mo	6 mo
rs174547																
Intercept	11.0726	11.6758	−2.2459	−1.8670	0.2293	0.2566	0.3308	0.3369	0.4709	0.5966	−3.0741	−2.2208	−4.5820	−4.2717	1.5756	1.9080
$\beta \pm$ SE	−0.0559 ± 0.2342	0.1145 ± 0.3193	−0.0274 ± 0.05314	−0.0520 ± 0.0460	0.0053 ± 0.00605	−0.0035 ± 0.0065	0.0029 ± 0.0111	−0.0050 ± 0.0136	−0.0391 ± 0.0132	−0.0838 ± 0.0226	0.0767 ± 0.0653	−0.1149 ± 0.0539	−0.0266 ± 0.0538	−0.0595 ± 0.0484	−0.1682 ± 0.0459	−0.2168 ± 0.0791
P	0.8116	0.7201	0.6060	0.2589	0.3836	0.5903	0.7914	0.7150	0.0031	0.0002[2]	0.2405	0.0334	0.6206	0.2196	0.0003[2]	0.0064
n	713	423	713	423	713	423	713	423	713	423	713	423	713	423	713	423
rs174556																
Intercept	11.1261	11.7266	−2.2365	−1.9180	0.2301	0.2554	0.3361	0.3310	0.4711	0.5893	−3.0494	−2.2477	−4.5800	−4.3276	1.5459	1.9382
$\beta \pm$ SE	−0.1029 ± 0.2454	−0.1479 ± 0.3185	−0.0408 ± 0.0565	0.0238 ± 0.0489	0.0020 ± 0.0061	−0.0039 ± 0.0067	0.0020 ± 0.0127	0.0062 ± 0.0141	−0.0424 ± 0.0140	−0.0808 ± 0.0237	0.0470 ± 0.0689	−0.1214 ± 0.0562	−0.0333 ± 0.0571	0.0335 ± 0.0510	−0.1430 ± 0.0485	−0.3127 ± 0.0821
P	0.6752	0.6425	0.4699	0.6266	0.7445	0.5608	0.8751	0.6603	0.0025	0.0007[2]	0.4949	0.0312	0.5595	0.5109	0.0033	0.0002[2]
n	711	424	711	424	711	424	711	424	711	424	711	424	711	424	711	424
rs174602																
Intercept	10.7271	11.5937	−2.2228	−1.8648	0.2242	0.2547	0.3350	0.3320	0.4508	0.5661	−3.0672	−2.2634	−4.5293	−4.2655	1.4893	1.8202
$\beta \pm$ SE	0.6490 ± 0.2548	0.1749 ± 0.3399	−0.0447 ± 0.0598	−0.1022 ± 0.0502	0.0125 ± 0.0064	−0.0034 ± 0.0073	0.0033 ± 0.0137	0.0030 ± 0.0151	−0.0130 ± 0.0151	−0.0496 ± 0.0255	0.0713 ± 0.0737	−0.1295 ± 0.0600	−0.1100 ± 0.0601	−0.1144 ± 0.0531	−0.0628 ± 0.0522	−0.0999 ± 0.0889
P	0.0111	0.6072	0.4549	0.0423	0.0495	0.6361	0.8065	0.8439	0.3900	0.0528	0.3336	0.0314	0.0676	0.0317	0.2293	0.2614
n	711	423	711	423	711	423	711	423	711	423	711	423	711	423	711	423
rs498793																
Intercept	11.1477	11.6647	−2.3002	−1.8739	0.2319	0.2517	0.3304	0.3346	0.4325	0.5176	−3.0070	−2.3044	−4.6439	−4.2840	1.4182	1.6705
$\beta \pm$ SE	−0.1205 ± 0.2224	0.0399 ± 0.2883	0.0440 ± 0.0509	−0.0248 ± 0.0439	−0.0022 ± 0.0055	0.0039 ± 0.0062	0.0005 ± 0.0107	−0.0002 ± 0.0129	0.0152 ± 0.0127	0.0359 ± 0.0217	−0.0549 ± 0.0628	0.0123 ± 0.0512	0.0509 ± 0.0515	−0.0232 ± 0.0459	0.0650 ± 0.0447	0.1298 ± 0.0757
P	0.5881	0.8900	0.3874	0.5721	0.6941	0.5271	0.9631	0.9855	0.2330	0.0990	0.3830	0.8101	0.3238	0.6124	0.1463	0.0868
n	699	415	699	415	699	415	699	415	699	415	699	415	699	415	699	415
rs526126																
Intercept	11.1388	11.9742	−2.2721	−1.9055	0.2330	0.2562	0.3366	0.3327	0.4501	0.5629	−3.0409	−2.3125	−4.6146	−4.3352	1.4994	1.7916
$\beta \pm$ SE	−0.3531 ± 0.2768	−0.7557 ± 0.3604	0.0529 ± 0.0647	0.0011 ± 0.0538	−0.0091 ± 0.0069	−0.0047 ± 0.0077	0.0026 ± 0.0148	0.0069 ± 0.0161	−0.0091 ± 0.0162	−0.0382 ± 0.0273	0.0260 ± 0.0796	−0.0083 ± 0.0644	0.0747 ± 0.0655	0.0580 ± 0.0570	−0.0990 ± 0.0556	−0.0353 ± 0.0952
P	0.2026	0.0366	0.4135	0.9841	0.1865	0.5467	0.8584	0.6672	0.5737	0.1621	0.7438	0.8978	0.2547	0.3097	0.0751	0.7106
n	715	421	715	421	715	421	715	421	715	421	715	421	715	421	715	421
rs174626																
Intercept	10.9464	11.7751	−2.2119	−1.8793	0.2308	0.2597	0.3433	0.3349	0.4753	0.6000	−3.0177	−2.2295	−4.5429	−4.2991	1.5830	1.9351
$\beta \pm$ SE	0.0350 ± 0.2067	−0.1573 ± 0.2707	−0.0493 ± 0.0488	−0.0265 ± 0.0410	−0.0018 ± 0.0052	−0.0060 ± 0.0058	−0.0084 ± 0.0111	−0.0009 ± 0.0122	−0.0345 ± 0.0122	−0.0583 ± 0.0205	−0.0312 ± 0.0597	−0.0890 ± 0.0478	−0.0507 ± 0.0491	−0.0085 ± 0.0432	−0.1343 ± 0.0421	−0.1712 ± 0.0714
P	0.8656	0.5614	0.3132	0.5182	0.7345	0.3057	0.4489	0.9436	0.0047	0.0047	0.6015	0.0634	0.3019	0.8446	0.0015	0.0168
n	707	419	707	419	707	419	707	419	707	419	707	419	707	419	707	419
rs1000778																
Intercept	11.1477	11.8562	−2.2227	−1.8621	0.2346	0.2551	0.3376	0.3313	0.4674	0.5832	−3.0174	−2.2189	−4.5695	−4.2789	1.5276	1.9089
$\beta \pm$ SE	−0.0984 ± 0.2512	−0.2635 ± 0.3385	−0.0615 ± 0.0572	−0.0735 ± 0.0484	−0.0058 ± 0.0064	−0.0045 ± 0.0068	−0.0018 ± 0.0130	0.0030 ± 0.0143	−0.0378 ± 0.0143	−0.0745 ± 0.0242	−0.0240 ± 0.0697	−0.1951 ± 0.0569	−0.0496 ± 0.0580	−0.0568 ± 0.0514	−0.1159 ± 0.0494	−0.2649 ± 0.0839
P	0.6954	0.4367	0.2827	0.1294	0.3649	0.5093	0.8920	0.8347	0.0086	0.0022	0.7313	0.0007	0.3933	0.2698	0.0193	0.0017
n	711	425	711	425	711	425	711	425	711	425	711	425	711	425	711	425
rs174455																
Intercept	10.9748	11.7296	−2.2338	−1.8838	0.2282	0.2513	0.3399	0.3321	0.4700	0.5924	−3.0700	−2.2658	−4.5676	−4.2947	1.5548	1.9349
$\beta \pm$ SE	0.1078 ± 0.2202	−0.0590 ± 0.2917	−0.0297 ± 0.0506	−0.0197 ± 0.0430	0.0039 ± 0.0055	0.0049 ± 0.0062	−0.0031 ± 0.0115	0.0043 ± 0.0130	−0.0330 ± 0.0126	−0.0635 ± 0.0218	0.0473 ± 0.0620	−0.0602 ± 0.0523	−0.0353 ± 0.0511	−0.0155 ± 0.0457	−0.1328 ± 0.0434	−0.2243 ± 0.0760
P	0.6245	0.8397	0.5580	0.6471	0.4802	0.4361	0.7845	0.7388	0.0090	0.0038	0.4453	0.2504	0.4902	0.7352	0.0023	0.0033
n	708	417	708	417	708	417	708	417	708	417	708	417	708	417	708	417

[1] Intercept, mean fatty acid concentration in homozygous carriers of the major allele; β, change in the fatty acid concentration with each minor allele copy; *n*, number of subjects used in analysis. Uncorrected *P* values are shown.

[2] $P < 0.001$ was significant with correction for multiple testing.

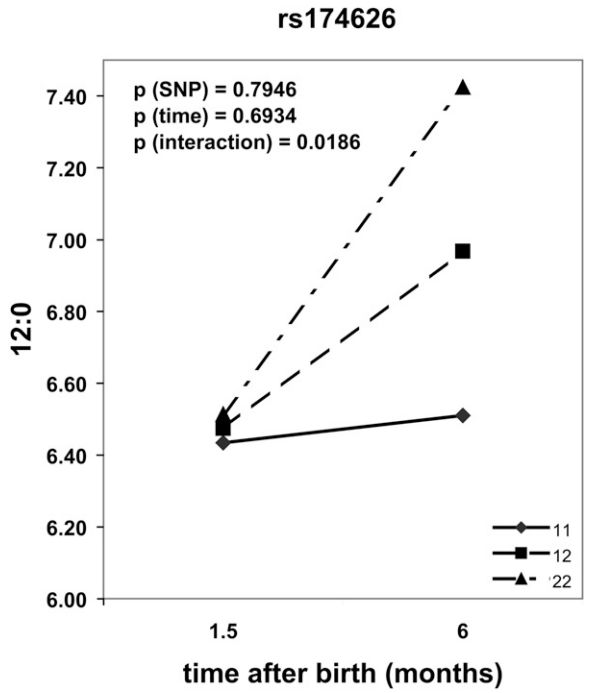

FIGURE 1. Longitudinal association analysis of single nucleotide polymorphism (SNP) rs174626 with dodecanoic acid (12:0) concentration by using a generalized estimating equation regression model. Results were based on 418 subjects. β-Coefficients (±SEs) were as follows: 0.0399 ± 0.1532 for SNP, 0.0762 ± 0.1931 for time, and 0.4172 ± 0.1751 for interaction. 11, homozygous major allele; 12, heterozygous; 22, homozygous minor allele.

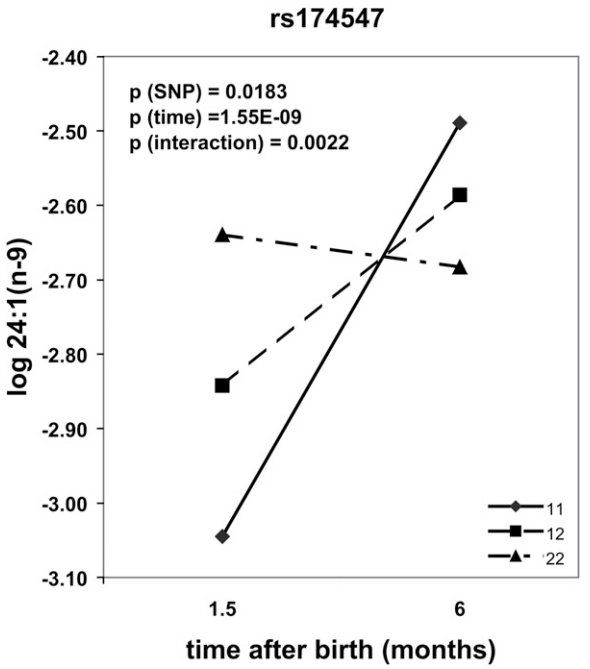

FIGURE 2. Longitudinal association analysis of single nucleotide polymorphism (SNP) rs174547 with log-transformed *cis*-15-tetracosenoic acid (log 24:1n−9) concentration by using a generalized estimating equation regression model. Results are based on 422 subjects. β-Coefficients (±SEs) were as follows: 0.2026 ± 0.0837 for SNP, 0.5555 ± 0.0850 for time, and −0.2994 ± 0.0946 for interaction. 11, homozygous major allele; 12, heterozygous; 22, homozygous minor allele.

change of 16:0 (hexadecanoic acid) concentrations (P for interaction = 0.0194).

Another interaction between time and genotype was observed for *cis*-15-tetracosenoic acid (24:1n−9) and SNP rs174547 (P for interaction = 0.0022). Carriers of the major allele showed an increase of this fatty acid over the lactation duration, whereas the concentrations in homozygous carriers of the minor allele were stable (**Figure 2**). Similar effects were observed for SNP rs174556 (P = 0.0059), which was in a high linkage disequilibrium with rs174547, and for SNPs rs174626 (P = 0.0455) and rs174455 (P = 0.0059).

The third fatty acid that showed a genotype-dependent change over time was *trans*-9-octadecenoic acid (*t*-18:1n−9) (P for interaction = 0.0032). Only carriers of the major allele of SNP rs174455 showed a remarkable decrease of this fatty acid over the breastfeeding period (**Figure 3**). A similar trend was observed for SNP rs174626; however, the P value for the interaction was not significant (P for interaction = 0.0835).

All reported associations remained significant before correction in a sensitivity analysis that excluded all potential outliers [defined as the mean ± (1.5 × the interquartile range)].

DISCUSSION

In the current study, we analyzed the effect of 8 SNPs in the *FADS* gene cluster on breast-milk fatty acids concentrations of breastfeeding women after 1.5 and 6 mo of lactation in a birth cohort that was larger than in previous studies. *FADS* genotypes were consistently associated with breast-milk AA concentrations, but not with other n−6 or n−3 fatty acids. The time course of AA concentrations during lactation was independent of the *FADS* genotype. Furthermore, our results suggested a relation between the n−6/n−3 fatty acid pathway and concentrations and time course of saturated, monounsaturated, and *trans* fatty acids.

In contrast to previous reports (22, 23), we showed no associations with n−6 or n−3 fatty acids except for an association with AA, which was significant at both investigated time points. There are several potential reasons for this discrepancy between the results of our study and the 2 previous studies. Breast-milk samples in the previous studies were collected 1 mo postpartum, whereas our first time point of collection was 1.5 mo after birth. The biggest changes in milk fatty acid concentrations occur during the first month of breastfeeding (31, 32), and possibly the genetic effect on breast-milk composition is more pronounced during this early stage of lactation. Also, the maternal dietary fatty acid intake is known to affect the fatty acid composition of breast milk (33, 34), which might modulate the strength of the genetic effect on milk fatty acid concentrations. In a recent study, associations between *FADS* genotypes and cholesterol concentrations were only observed in subjects with high intakes of n−3 LC-PUFAs, whereas this effect was not present in the low-intake group (35). In the study of Moltó-Puigmartí et al (23), the difference in breast-milk DHA concentrations between genotype groups was more pronounced in people with a higher number of fatty fish portions per week. In our study, we investigated a German study population from the area of Ulm in south Germany where, typically, a relatively low amount of sea fish is consumed, whereas in the 2 previous studies, Canadian (22) and Dutch (23) populations were investigated, which were

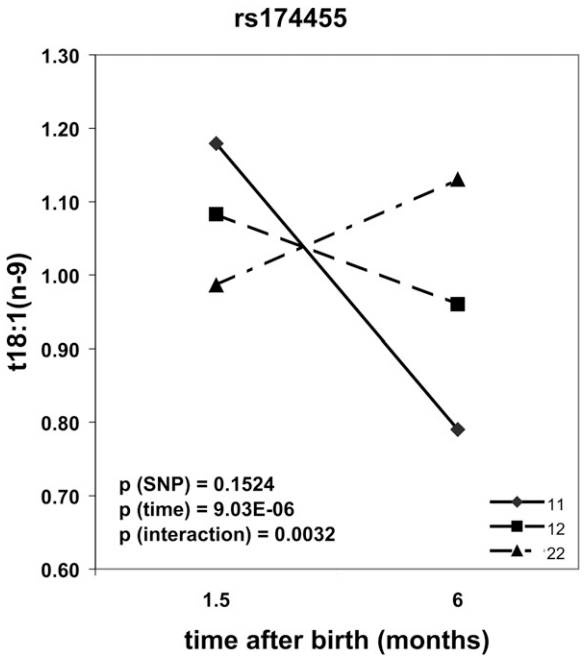

FIGURE 3. Longitudinal association analysis of single nucleotide polymorphism (SNP) rs174455 with *trans*-9-octadecenoic acid (t18:1n−9) concentration by using a generalized estimating equation regression model. Results are based on 416 subjects. β-Coefficients (±SEs) were as follows: −0.0965 ± 0.0668 for SNP, −0.3892 ± 0.0844 for time, and 0.2666 ± 0.0873 for interaction. 11, homozygous major allele; 12, heterozygous; 22, homozygous minor allele.

likely to differ in their dietary habits, especially regarding fish consumption. Furthermore, the lack of an association with n−3 fatty acids might have been caused by more imprecise measurements leading to higher CVs (Table 3) of the quite low abundant longer-chain n−3 fatty acids such as 22:6n−3 compared with the more abundant n−6 fatty acids. Furthermore, we used a more conservative statistical approach by correcting our *P* values for multiple testing to reduce the number of significant results obtained by chance. Such a correction was not reported by the 2 previous studies.

To our knowledge, one of the strengths and novelties of our study is the availability of fatty acid data at 2 time points of lactation (1.5 and 6 mo). Therefore, we conducted a longitudinal analysis to detect differences in time courses of fatty acid concentrations dependent on the *FADS* genotype. The time course of AA concentrations was independent of the *FADS* genotype in our complete case approach that comprised 463 mothers who were breastfeeding at both investigated time points. However, AA concentrations were markedly higher in carriers of the major alleles at both investigated time points compared with in carriers of the minor alleles, which suggested lower D5D expression rates or enzyme activity in minor allele carriers. It is not clear whether this was attributed to an altered synthesis rate in the mammary gland itself or whether it was due to a lower D5D activity in other tissues such as the liver and, consequently, diminished the import into the mammary gland. In several tracer studies in humans and animals, it was suggested that the mammary gland plays an important role in the synthesis of LC-PUFA itself (33, 36). In addition, it is known that the mammary gland expresses D6D and D5D (37, 38). Further studies are needed to understand the role of the mammary gland in fatty acid synthesis and the influence of the *FADS* genotype. Moreover, whether the different AA concentrations of the 3 genotype groups had any influence on the breast-fed infant could not be inferred from this study and needs further investigation.

To our knowledge, a function of the D6D and D5D in the biosynthesis of saturated and monounsaturated fatty acids has not been reported. Therefore, the reason for the association of *FADS* genotypes with the longitudinal change of dodecanoic, *cis*-15-tetracosenoic, and *trans*-9-octadecenoic acid concentrations, was not immediately apparent. It has been shown that polyunsaturated fatty acids are able to regulate pathways involved in lipid, energy, and carbohydrate metabolism by modifying gene expression in different tissues through binding to nuclear receptors such as peroxisome proliferator–activated receptor α (39, 40). In addition, in tracer studies in nonhuman primates, it was previously shown that n−6 and n−3 fatty acids could be oxidized, and their carbons could be recycled to saturated and monounsaturated fatty acids, which were detected in milk and other tissues of the animals (41). The authors argued that pregnant and lactating nonhuman primates use excess LC-PUFA from the diet for energy production and storage of saturated and monounsaturated fatty acids for later use. However, the mechanism that caused the apparent relation between n−6 or n−3 LC-PUFAs and saturated and monounsaturated fatty acids remained unclear, and our findings need to be replicated. The association of SNP rs174455 with *trans*-9-octadecenoic acid over time with major allele carriers that showed a decrease of this fatty acid in contrast to homozygous minor allele carriers was not less surprising because the source of *t*-18:1n−9 was exclusively nutritional. It was recently shown that concentrations of *trans* fatty acids decrease during the duration of lactation, possibly because of a decreased maternal dietary intake of *trans* fatty acids during the lactation period, and the concentration of *trans* fatty acids was inversely related to AA and other LC-PUFA concentrations (24, 25). We saw a decrease of *t*-18:n−9 concentrations only in carriers of the major allele of rs174455, which might suggest a differential eating behavior dependent on the genotype. Further replication including dietary data are needed.

To our knowledge, this is the largest study on *FADS* genotypes and breast-milk fatty acid concentrations [n = 772 at 1.5 mo and n = 463 at 6 mo compared with n = 54 in the study by Xie and Innis (22) and n = 309 in the study by Moltó-Puigmartí et al (23)] and the only one that additionally genotyped SNPs in *FADS3*. The biological function of the protein product of *FADS3* has not been completely clarified, but because of high a homology between all 3 *FADS* genes, a function in the desaturation pathway has been suggested. In the current study, SNPs in *FADS3* showed significant associations with AA before correction, which corroborated a functional role of *FADS3* in the fatty acid desaturation. In contrast to the 2 previous studies that analyzed the breast-milk fatty acid composition at one single time point only, we measured fatty acid concentrations at 1.5 and 6 mo of lactation and performed a longitudinal analysis of fatty acid concentrations dependent on the genotype.

Although, compared with previous studies, the availability of fatty acid data at 2 different time points was a clear strength of this study, it would be desirable to include even more time points to study the exact time course of fatty acid concentrations during

lactation, which might not be linear as assumed in our study. The longitudinal analysis included only those women who were breastfeeding at both investigated time points, and the observed associations might have been specific for this special group of women. Whether the associations can also be observed in women breastfeeding, eg, until the fourth month postpartum requires additional studies. Moreover, more subjects might be required for a longitudinal analysis to not lose too much power because of the problem of missing cases. Another limitation of our study was the lack of nutritional data to test the interaction between genes and diet on the course of fatty acid concentrations in human breast milk, which might be a task in a future study. Because fatty acid data were expressed as a percentage of total fatty acids, one might assume that the percentage change of low abundant fatty acids was highly influenced by changes in high-abundant fatty acids such as 18:2n−6. However, if the contribution of major fatty acids markedly increased by 50%, from 10% of total fatty acids to 15% of total fatty acids, the relative contribution of all other fatty acids would be expected to be equally lowered by less than a relative 5% (eg, from 0.1% to 0.095% of total fatty acids). This means that a minor change of a low-abundant fatty acid would require a very high change of a high-abundant fatty acid, which makes the assumption that low-abundant fatty acids are very much influenced by major fatty acids unlikely.

In conclusion, we showed the clear influence of *FADS* polymorphisms on breast-milk AA concentrations at 1.5 and 6 mo postpartum. The time course of AA concentrations during lactation was not influenced by the *FADS* genotype. The effect of *FADS* polymorphisms on saturated, monounsaturated, and *trans* fatty acid concentrations in human breast milk awaits further investigation.

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The authors' responsibilities were as follows—EL: wrote the first version of the manuscript and was responsible for the design, writing, and final content; M Weck, M Weyermann, DR, and HB: initialized and conducted the Ulm Birth Cohort Study; ÉS, VJ, and TD: performed DNA extraction and fatty acid analyses; EL, HG, and TI: performed genetic analyses; EL, PR, and HG: analyzed data; PR, JH, TI, and BK: contributed to the interpretation of results; and all authors: contributed to the writing of the manuscript and read and approved the final manuscript. None of the authors had a conflict of interest.

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Online supplemental material

Supplemental Table S1: Association analysis of eight *FADS* SNPs with n-3 fatty acid concentrations in human breast milk after 1.5 months and 6 months of lactation.

	18:3 n-3		**20:3 n-3**		**log 20:5 n-3**	
	1.5	6	1.5	6	1.5	6
	months		months		months	
rs174547						
Intercept	0.7648	0.9322	0.0479	0.0647	-3.2749	-2.8879
beta (SE)	0.0390 (0.0261)	-0.0676 (0.0335)	0.0014 (0.0023)	-0.0016 (0.0033)	-0.0065 (0.0592)	-0.0846 (0.0627)
p value	0.1352	0.0442	0.5477	0.6211	0.9124	0.1779
N	713	423	713	423	713	423
rs174556						
Intercept	0.7806	0.9059	0.0464	0.0627	-3.2665	-2.7443
beta (SE)	0.0292 (0.0280)	-0.0380 (0.0343)	0.0032 (0.0022)	0.0012 (0.0034)	-0.0181 (0.0619)	-0.0304 (0.0676)
p value	0.2969	0.2689	0.1420	0.7157	0.7695	0.6531
N	711	424	711	424	711	424
rs174602						
Intercept	0.7596	0.8967	0.0487	0.0635	-3.2800	-2.7357
beta (SE)	0.0731 (0.0294)	-0.0109 (0.0387)	0.0005 (0.0026)	-0.0002 (0.0037)	-0.0071 (0.0656)	-0.0408 (0.0726)
p value	0.0132	0.7782	0.8460	0.9466	0.9139	0.5740
N	711	423	711	423	711	423
rs498793						
Intercept	0.8099	0.9227	0.0518	0.0639	-3.3029	-2.8267
beta (SE)	-0.0220 (0.0249)	-0.0306 (0.0322)	-0.0047 (0.0022)	-0.0002 (0.0031)	0.0145 (0.0562)	0.1041 (0.0591)
p value	0.3772	0.3427	0.0309	0.9375	0.7968	0.0789
N	699	415	699	415	699	415
rs526126						
Intercept	0.7767	0.9045	0.0504	0.0627	-3.2691	-2.7613
beta (SE)	0.0366 (0.0318)	-0.0284 (0.0413)	-0.0036 (0.0028)	0.0030 (0.0039)	-0.0468 (0.0717)	0.0173 (0.0774)
p value	0.2503	0.4915	0.1944	0.4481	0.5137	0.8238
N	715	421	715	421	715	421
rs174626						
Intercept	0.7730	0.8968	0.0472	0.0629	-3.2241	-2.6690
beta (SE)	0.0211 (0.0240)	-0.0038 (0.0306)	0.0015 (0.0021)	0.0007 (0.0029)	-0.0728 (0.0531)	-0.0941 (0.0577)
p value	0.3808	0.9003	0.4717	0.8214	0.1712	0.1038
N	707	419	707	419	707	419
rs1000778						
Intercept	0.8140	0.9058	0.0481	0.0630	-3.2495	-2.7370
beta (SE)	-0.0403 (0.0285)	-0.0281 (0.0369)	0.0001 (0.0022)	-0.0006 (0.0034)	-0.0471 (0.0632)	-0.0352 (0.0693)
p value	0.1574	0.4466	0.9643	0.8585	0.4563	0.6119
N	711	425	711	425	711	425
rs174455						
Intercept	0.7731	0.8863	0.0467	0.0619	-3.2689	-2.7735
beta (SE)	0.0282 (0.0249)	0.0064 (0.0324)	0.0028 (0.0022)	0.0023 (0.0031)	-0.0145 (0.0553)	0.0175 (0.0619)
p value	0.2579	0.8435	0.1868	0.4675	0.7934	0.7776
N	708	417	708	417	708	417

Uncorrected p-values are shown. Considering correction for multiple testing, a p-value below 0.001 is defined as significant.

Online supplemental material

Supplemental Table S1: continued

	22:5 n-3		log 22:6 n-3	
	1.5	6	1.5	6
	months		months	
rs174547				
Intercept	0.1452	0.2113	-2.1165	-1.5197
beta (SE)	0.0114 (0.0110)	-0.0190 (0.0095)	0.1231 (0.0637)	-0.0163 (0.0458)
p value	0.3028	0.0460	0.0535	0.7230
N	713	423	713	423
rs174556				
Intercept	0.1453	0.2032	-2.1094	-1.5600
beta (SE)	0.0116 (0.0117)	-0.0107 (0.0100)	0.1356 (0.0671)	0.0195 (0.0483)
p value	0.3215	0.2851	0.0436	0.6868
N	711	424	711	424
rs174602				
Intercept	0.1460	0.2010	-2.1171	-1.5273
beta (SE)	0.0130 (0.0125)	-0.0105 (0.0106)	0.1495 (0.0715)	-0.0189 (0.0521)
p value	0.2986	0.3226	0.0368	0.7163
N	711	423	711	423
rs498793				
Intercept	0.1599	0.1978	-1.9704	-1.5383
beta (SE)	-0.0120 (0.0105)	0.0017 (0.0090)	-0.0909 (0.0605)	0.0062 (0.0435)
p value	0.2526	0.8496	0.1334	0.8871
N	699	415	699	415
rs526126				
Intercept	0.1435	0.1992	-2.0590	-1.5536
beta (SE)	0.0200 (0.0133)	-0.0060 (0.0113)	-0.0080 (0.0776)	0.0465 (0.0553)
p value	0.1336	0.5976	0.9181	0.4004
N	715	421	715	421
rs174626				
Intercept	0.1468	0.2163	-2.0552	-1.5070
beta (SE)	0.0050 (0.0102)	-0.0211 (0.0084)	-0.0099 (0.0585)	-0.0333 (0.0414)
p value	0.6268	0.0126	0.8663	0.4221
N	707	419	707	419
rs1000778				
Intercept	0.1561	0.2005	-2.0380	-1.5224
beta (SE)	-0.0125 (0.0117)	-0.0078 (0.0102)	-0.0076 (0.0682)	-0.03248 (0.0496)
p value	0.2828	0.4454	0.9116	0.5125
N	711	425	711	425
rs174455				
Intercept	0.1395	0.1963	-2.0978	-1.5658
beta (SE)	0.0139 (0.0103)	0.0012 (0.0092)	0.0653 (0.0607)	0.0338 (0.0440)
p value	0.1783	0.8931	0.2824	0.4423
N	708	417	708	417

Online supplemental material

Supplemental Table S2: Association analysis of eight *FADS* SNPs with saturated fatty acid concentrations in human breast milk after 1.5 and 6 months of lactation.

	10:0		**12:0**		**14:0**	
	1.5	6	1.5	6	1.5	6
	months		months		months	
rs174547						
Intercept	2.2334	2.0532	6.7296	6.7971	7.2879	7.3948
beta (SE)	0.0047 (0.0482)	0.1059 (0.0857)	-0.1380 (0.1335)	0.1575 (0.1668)	-0.1321 (0.1152)	0.1585 (0.1580)
p value	0.9223	0.2175	0.3016	0.3455	0.2519	0.3164
N	713	423	713	423	713	423
rs174556						
Intercept	2.2491	2.1063	6.7861	6.9129	7.2945	7.4380
beta (SE)	-0.0004 (0.0509)	0.0826 (0.0906)	-0.2446 (0.1412)	-0.0048 (0.1747)	-0.1830 (0.1210)	0.1236 (0.1636)
p value	0.9930	0.3626	0.0836	0.9781	0.1309	0.4503
N	711	424	711	424	711	424
rs174602						
Intercept	2.2840	2.0563	6.8165	6.8342	7.3539	7.3918
beta (SE)	-0.0547 (0.0542)	0.1844 (0.0960)	-0.3007 (0.1509)	0.1964 (0.1866)	-0.3114 (0.1295)	0.2335 (0.1731)
p value	0.3133	0.0553	0.0466	0.2933	0.0164	0.1782
N	711	423	711	423	711	423
rs498793						
Intercept	2.2053	2.0834	6.6390	6.8816	7.1381	7.3819
beta (SE)	0.0417 (0.0462)	0.0607 (0.0840)	0.0205 (0.1274)	-0.0089 (0.1572)	0.0819 (0.1087)	0.0912 (0.1463)
p value	0.3675	0.4701	0.8721	0.9549	0.4516	0.5334
N	699	415	699	415	699	415
rs526126						
Intercept	2.2591	2.1080	6.6530	6.8045	7.1991	7.3256
beta (SE)	-0.0157 (0.0587)	0.0065 (0.1001)	0.0493 (0.1633)	0.2525 (0.1985)	0.0717 (0.1401)	0.4488 (0.1848)
p value	0.7893	0.9485	0.7627	0.2040	0.6091	0.0156
N	715	421	715	421	715	421
rs174626						
Intercept	2.2232	1.9709	6.7669	6.5103	7.2787	7.2455
beta (SE)	0.0286 (0.0439)	0.1740 (0.0751)	-0.0887 (0.1219)	0.4571 (0.1480)	-0.0637 (0.1042)	0.2735 (0.1374)
p value	0.5142	0.0211	0.4672	0.0021	0.5412	0.0471
N	707	419	707	419	707	419
rs1000778						
Intercept	2.2653	2.1191	6.7885	6.9117	7.2344	7.5304
beta (SE)	-0.0546 (0.0515)	0.0607 (0.0941)	-0.2947 (0.1429)	0.0135 (0.1777)	-0.0904 (0.1237)	-0.0511 (0.1679)
p value	0.2888	0.5187	0.0395	0.9396	0.4652	0.7611
N	711	425	711	425	711	425
rs174455						
Intercept	2.2392	2.0856	6.8082	6.8149	7.2868	7.4734
beta (SE)	0.0077 (0.0458)	0.0358 (0.0799)	-0.2002 (0.1262)	0.0887 (0.1581)	-0.1051 (0.1087)	-0.0276 (0.1488)
p value	0.8668	0.6545	0.1131	0.5753	0.3340	0.8531
N	708	417	708	417	708	417

Uncorrected p-values are shown. Considering correction for multiple testing, a p-value below 0.001 is defined as significant.

Online supplemental material

Supplemental Table S2: continued

	16:0		18:0		20:0	
	1.5	6	1.5	6	1.5	6
	months		months		months	
rs174547						
Intercept	22.4481	23.0683	8.1703	8.3258	0.2836	0.2610
beta (SE)	-0.0631 (0.1670)	0.2250 (0.2234)	-0.1167 (0.1268)	0.0935 (0.1635)	-0.0043 (0.0063)	0.0054 (0.0071)
p value	0.7055	0.3144	0.3581	0.5679	0.4949	0.4458
N	713	423	713	423	713	423
rs174556						
Intercept	22.3505	22.9870	8.1340	8.2074	0.2807	0.2561
beta (SE)	0.0081 (0.1767)	0.4312 (0.2336)	-0.0563 (0.1341)	0.2725 (0.1697)	0.0043 (0.0079)	0.0127 (0.0074)
p value	0.9636	0.0656	0.6746	0.1091	0.5842	0.0863
N	711	424	711	424	711	424
rs174602						
Intercept	22.5579	23.2011	8.2492	8.4115	0.2923	0.2632
beta (SE)	-0.4122 (0.1870)	-0.0063 (0.2477)	-0.3041 (0.1438)	-0.0730 (0.1848)	-0.0200 (0.0083)	0.0019 (0.0080)
p value	0.0278	0.9797	0.0349	0.6932	0.0166	0.8099
N	711	423	711	423	711	423
rs498793						
Intercept	22.2153	23.2659	8.1954	8.2417	0.2777	0.2608
beta (SE)	0.2138 (0.1599)	-0.0163 (0.2109)	-0.0859 (0.1215)	0.1438 (0.1514)	0.0034 (0.0060)	0.0034 (0.0067)
p value	0.1816	0.9384	0.4798	0.3431	0.5721	0.6098
N	699	415	699	415	699	415
rs526126						
Intercept	22.4027	23.0431	8.1231	8.2500	0.2840	0.2567
beta (SE)	0.0016 (0.2031)	0.4388 (0.2652)	-0.0026 (0.1549)	0.2401 (0.1949)	-0.0023 (0.0089)	0.0148 (0.0085)
p value	0.9937	0.0987	0.9868	0.2188	0.7987	0.0802
N	715	421	715	421	715	421
rs174626						
Intercept	22.4169	23.1532	8.1772	8.2250	0.2893	0.2551
beta (SE)	-0.0134 (0.1519)	0.0465 (0.2012)	-0.0486 (0.1169)	0.1600 (0.1480)	-0.0062 (0.0068)	0.0096 (0.0064)
p value	0.9297	0.8173	0.6773	0.2803	0.3640	0.1361
N	707	419	707	419	707	419
rs1000778						
Intercept	22.2155	23.0240	8.1455	8.2182	0.2862	0.2575
beta (SE)	0.2736 (0.1802)	0.3764 (0.2379)	-0.0617 (0.1365)	0.2380 (0.1737)	-0.0048 (0.0080)	0.0078 (0.0075)
p value	0.1294	0.1143	0.6517	0.1713	0.5494	0.2961
N	711	425	711	425	711	425
rs174455						
Intercept	22.3967	22.9966	8.2011	8.2123	0.2878	0.2560
beta (SE)	-0.0153 (0.1594)	0.2876 (0.2119)	-0.1329 (0.1211)	0.2087 (0.1580)	-0.0077 (0.0070)	0.0101 (0.0068)
p value	0.9236	0.1755	0.2730	0.1873	0.2695	0.1399
N	708	417	708	417	708	417

Online supplemental material

Supplemental Table S2: continued

	log 22:0		log 24:0	
	1.5	6	1.5	6
	months		months	
rs174547				
Intercept	-1.9629	-1.9287	-2.2008	-2.2545
beta (SE)	-0.0280 (0.0275)	0.0328 (0.0369)	0.0531 (0.0491)	-0.0880 (0.0467)
p value	0.3095	0.3751	0.2799	0.0601
N	713	423	713	423
rs174556				
Intercept	-1.9820	-1.9457	-2.1983	-2.2538
beta (SE)	0.0069 (0.0286)	0.0621 (0.0389)	0.0515 (0.0510)	-0.1158 (0.0490)
p value	0.8089	0.1110	0.3137	0.0186
N	711	424	711	424
rs174602				
Intercept	-1.9625	-1.9196	-2.2224	-2.3011
beta (SE)	-0.0292 (0.0300)	0.0136 (0.0419)	0.1018 (0.0542)	-0.0077 (0.0520)
p value	0.3302	0.7462	0.0604	0.8827
N	711	423	711	423
rs498793				
Intercept	-2.0076	-1.8547	-2.2229	-2.3400
beta (SE)	0.0340 (0.0258)	-0.0786 (0.0362)	0.0546 (0.0465)	0.0290 (0.0441)
p value	0.1882	0.0259	0.2410	0.5111
N	699	415	699	415
rs526126				
Intercept	-1.9685	-1.9464	-2.1479	-2.3011
beta (SE)	-0.0004 (0.0325)	0.0715 (0.0446)	-0.0678 (0.0588)	-0.0118 (0.0558)
p value	0.9895	0.1096	0.2488	0.8326
N	715	421	715	421
rs174626				
Intercept	-1.9661	-1.9561	-2.1685	-2.2309
beta (SE)	-0.0183 (0.0250)	0.0447 (0.0338)	-0.0229 (0.0442)	-0.0906 (0.0420)
p value	0.4650	0.1870	0.6046	0.0315
N	707	419	707	419
rs1000778				
Intercept	-1.9709	-1.9525	-2.1520	-2.2491
beta (SE)	-0.000007 (0.0285)	0.0658 (0.0399)	-0.0324 (0.0524)	-0.1393 (0.0496)
p value	0.9998	0.0998	0.5371	0.0052
N	711	425	711	425
rs174455				
Intercept	-1.9836	-1.9793	-2.1707	-2.2384
beta (SE)	0.0079 (0.0257)	0.0923 (0.0357)	-0.0073 (0.0456)	-0.0898 (0.0450)
p value	0.7570	0.0101	0.8732	0.0464
N	708	417	708	417

Online supplemental material

Supplemental Table S3: Association analysis of eight *FADS* SNPs with monounsaturated fatty acid concentrations in human breast milk after 1.5 months and 6 months of lactation.

	16:1 n-7		**18:1 n-9**		**log 22:1 n-9**	
	1.5	6	1.5	6	1.5	6
	months		months		months	
rs174547						
Intercept	2.6248	2.5306	30.8589	30.1004	-2.9595	-2.4666
beta (SE)	0.1667 (0.0513)	0.0487 (0.0509)	-0.0023 (0.2172)	-0.6420 (0.2905)	0.0393 (0.0449)	-0.0663 (0.0467)
p value	0.0012	0.3400	0.9916	0.0276	0.3826	0.1566
N	713	423	713	423	713	423
rs174556						
Intercept	2.6458	2.5486	30.8876	30.0891	-2.9630	-2.5164
beta (SE)	0.1464 (0.0539)	0.0417 (0.0544)	-0.0358 (0.2289)	-0.5793 (0.3086)	0.0642 (0.0458)	-0.0158 (0.0492)
p value	0.0067	0.4434	0.8757	0.0611	0.1618	0.7484
N	711	424	711	424	711	424
rs174602						
Intercept	2.6646	2.5800	30.7289	30.0478	-2.9672	-2.5145
beta (SE)	0.1290 (0.0574)	-0.0232 (0.0580)	0.2200 (0.2454)	-0.6555 (0.3287)	0.0518 (0.0495)	-0.0238 (0.0532)
p value	0.0250	0.6890	0.3702	0.0468	0.2961	0.6549
N	711	423	711	423	711	423
rs498793						
Intercept	2.6417	2.4798	30.8528	29.9509	-2.8941	-2.5009
beta (SE)	0.0891 (0.0495)	0.1064 (0.0484)	0.0220 (0.2047)	-0.2130 (0.2703)	-0.0395 (0.0419)	-0.0035 (0.0447)
p value	0.0727	0.0284	0.9144	0.4311	0.3456	0.9383
N	699	415	699	415	699	415
rs526126						
Intercept	2.6772	2.5718	30.8862	30.1249	-2.9332	-2.5338
beta (SE)	0.1212 (0.0619)	0.0238 (0.0624)	-0.2194 (0.2629)	-0.8547 (0.3503)	-0.0007 (0.0530)	0.0330 (0.0565)
p value	0.0505	0.7023	0.4042	0.0151	0.9887	0.5599
N	715	421	715	421	715	421
rs174626						
Intercept	2.6739	2.6063	30.9878	30.5277	-2.9099	-2.4924
beta (SE)	0.0503 (0.0467)	-0.0433 (0.0458)	-0.1702 (0.1982)	-0.8163 (0.2627)	-0.0334 (0.0399)	-0.0281 (0.0422)
p value	0.2815	0.3444	0.3909	0.0020	0.4029	0.5062
N	707	419	707	419	707	419
rs1000778						
Intercept	2.6766	2.5769	30.7702	29.8300	-2.9394	-2.5201
beta (SE)	0.0703 (0.0552)	-0.0141 (0.0545)	0.2131 (0.2312)	-0.1562 (0.3147)	0.0118 (0.0471)	-0.0300 (0.0501)
p value	0.2033	0.7954	0.3569	0.6199	0.8025	0.5504
N	711	425	711	425	711	425
rs174455						
Intercept	2.7128	2.5858	30.8124	30.2903	-2.9688	-2.5322
beta (SE)	0.0105 (0.0490)	-0.0199 (0.0494)	0.0543 (0.2056)	-0.6535 (0.2799)	0.0367 (0.0421)	0.0262 (0.0448)
p value	0.8310	0.6875	0.7918	0.0200	0.3838	0.5588
N	708	417	708	417	708	417

Uncorrected p-values are shown. Considering correction for multiple testing, a p-value below 0.001 is defined as significant.

Online supplemental material

Supplemental Table S3: continued

	log 24:1 n-9	
	1.5	6
	months	
rs174547		
Intercept	-2.9749	-2.4894
beta (SE)	0.1237 (0.0631)	-0.0968 (0.0488)
p value	0.0505	0.0482
N	713	423
rs174556		
Intercept	-2.9741	-2.5055
beta (SE)	0.1260 (0.0655)	-0.0830 (0.0513)
p value	0.0548	0.1066
N	711	424
rs174602		
Intercept	-2.9727	-2.5384
beta (SE)	0.1302 (0.0710)	-0.0340 (0.0544)
p value	0.0672	0.5326
N	711	423
rs498793		
Intercept	-2.8314	-2.5889
beta (SE)	-0.0982 (0.0605)	0.0622 (0.0458)
p value	0.1047	0.1752
N	699	415
rs526126		
Intercept	-2.9143	-2.5383
beta (SE)	0.0045 (0.0762)	-0.0151 (0.0576)
p value	0.9535	0.7926
N	715	421
rs174626		
Intercept	-2.9250	-2.4570
beta (SE)	0.0112 (0.0574)	-0.1081 (0.0436)
p value	0.8459	0.0135
N	707	419
rs1000778		
Intercept	-2.9109	-2.4990
beta (SE)	-0.0066 (0.0677)	-0.1034 (0.0513)
p value	0.9228	0.0444
N	711	425
rs174455		
Intercept	-2.9757	-2.5017
beta (SE)	0.1049 (0.0589)	-0.0626 (0.0465)
p value	0.0751	0.1795
N	708	417

Online supplemental material

Supplemental Table S4: Association analysis of eight *FADS* SNPs with trans fatty acid concentrations in human breast milk after 1.5 months and 6 months of lactation.

	t 16:1		t 18:1 n-9		tt 18:2 n-6	
	1.5	6	1.5	6	1.5	6
	months		months		months	
rs174547						
Intercept	0.4290	0.4247	1.1720	0.9094	0.3833	0.2884
beta (SE)	0.0008 (0.0131)	-0.0132 (0.0170)	-0.0500 (0.0531)	0.0239 (0.0677)	0.0102 (0.0196)	0.0023 (0.0107)
p value	0.9496	0.4361	0.3469	0.7238	0.6043	0.8308
N	713	423	713	423	713	423
rs174556						
Intercept	0.4290	0.4296	1.1631	0.8853	0.3869	0.2824
beta (SE)	-0.0010 (0.0137)	-0.0170 (0.0183)	-0.0444 (0.0557)	0.0338 (0.0706)	0.0055 (0.0204)	0.0083 (0.0113)
p value	0.9395	0.3524	0.4256	0.6322	0.7896	0.4645
N	711	424	711	424	711	424
rs174602						
Intercept	0.4187	0.4310	1.1530	0.9139	0.4000	0.2770
beta (SE)	0.0235 (0.0146)	-0.0269 (0.0193)	-0.0353 (0.0593)	0.0033 (0.0764)	-0.0124 (0.0223)	0.0242 (0.0119)
p value	0.1090	0.1636	0.5512	0.9659	0.5790	0.0436
N	711	423	711	423	711	423
rs498793						
Intercept	0.4175	0.3985	1.1737	0.9348	0.3991	0.2785
beta (SE)	0.0141 (0.0126)	0.0243 (0.0163)	-0.0275 (0.0510)	-0.0177 (0.0633)	-0.0022 (0.0192)	0.0127 (0.0101)
p value	0.2617	0.1373	0.5897	0.7802	0.9081	0.2098
N	699	415	699	415	699	415
rs526126						
Intercept	0.4220	0.4242	1.1204	0.8769	0.3791	0.2824
beta (SE)	0.0157 (0.0158)	-0.0173 (0.0206)	0.0218 (0.0634)	0.0609 (0.0803)	0.0393 (0.0240)	0.0097 (0.0128)
p value	0.3207	0.4009	0.7305	0.4487	0.1014	0.4479
N	715	421	715	421	715	421
rs174626						
Intercept	0.4212	0.4180	1.1414	0.7796	0.3831	0.2832
beta (SE)	0.0060 (0.0119)	0.0027 (0.0156)	0.0040 (0.0484)	0.1472 (0.0609)	0.0127 (0.0180)	0.0028 (0.0097)
p value	0.6128	0.8638	0.9341	0.0160	0.4816	0.7752
N	707	419	707	419	707	419
rs1000778						
Intercept	0.4289	0.4253	1.1354	0.8511	0.3930	0.2842
beta (SE)	-0.0027 (0.0140)	-0.0108 (0.0186)	0.0068 (0.0560)	0.1277 (0.0721)	0.0025 (0.0212)	0.0056 (0.0115)
p value	0.8469	0.5600	0.9039	0.0773	0.9061	0.6228
N	711	425	711	425	711	425
rs174455						
Intercept	0.4399	0.4113	1.1750	0.7900	0.4053	0.2715
beta (SE)	-0.0156 (0.0124)	0.0095 (0.01655)	-0.0580 (0.0498)	0.1701 (0.0646)	-0.0156 (0.0189)	0.0212 (0.0102)
p value	0.2090	0.5668	0.2449	0.0088	0.4091	0.0392
N	708	417	708	417	708	417

Uncorrected p-values are shown. Considering correction for multiple testing, a p-value below 0.001 is defined as significant.

Appendix A3.

Lattka, E., S. Eggers, G. Möller, K. Heim, M. Weber, D. Mehta, H. Prokisch, T. Illig, and J. Adamski. 2010. A common FADS2 promoter polymorphism increases promoter activity and facilitates binding of transcription factor ELK1. *J Lipid Res.* **51**: 182-191.

A common *FADS2* promoter polymorphism increases promoter activity and facilitates binding of transcription factor ELK1

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Abstract Fatty acid desaturases (FADS) play an important role in the formation of omega-6 and omega-3 highly unsaturated fatty acids (HUFAs). The composition of HUFAs in the human metabolome is important for membrane fluidity and for the modulation of essential physiological functions such as inflammation processes and brain development. Several recent studies reported significant associations of single nucleotide polymorphisms (SNPs) in the human *FADS* gene cluster with HUFA levels and composition. The presence of the minor allele correlated with a decrease of desaturase reaction products and an accumulation of substrates. We performed functional studies with two of the associated polymorphisms (rs3834458 and rs968567) and showed an influence of polymorphism rs968567 on *FADS2* promoter activity by luciferase reporter gene assays. Electrophoretic mobility shift assays proved allele-dependent DNA-binding ability of at least two protein complexes to the region containing SNP rs968567. One of the proteins binding to this region in an allele-specific manner was shown to be the transcription factor ELK1 (a member of ETS domain transcription factor family). These results indicate that rs968567 influences *FADS2* transcription and offer first insights into the modulation of complex regulation mechanisms of *FADS2* gene transcription by SNPs.—Lattka, E., S. Eggers, G. Moeller, K. Heim, M. Weber, D. Mehta, H. Prokisch, T. Illig, and J. Adamski. **A common *FADS2* promoter polymorphism increases promoter activity and facilitates binding of transcription factor ELK1. *J. Lipid Res.* 2010.** 51: **182–191.**

Supplementary key words delta-6 desaturase • fatty acid metabolism • desaturation • single nucleotide polymorphism

Fatty acids are among other metabolites essential components of the human metabolome. In cells, phospholipids containing highly unsaturated fatty acids (HUFAs) such as arachidonic acid (all-*cis*-5,8,11,14-eicosatetraenoic acid or C20:4n-6) and docosahexaenoic acid [22:6(ω-3), all-*cis*-docosa-4,7,10,13,16,19-hexaenoic acid or C22:6n-3] have a positive effect on the fluidity of cell membranes. On the molecular level, HUFAs fulfill several other central functions like acting as second messengers in intracellular signaling pathways or regulating transcription. On the physiological level, HUFAs are important for brain development, acquisition of cognitive behaviors, and development of visual functions in early life. In addition, HUFAs are precursors for eicosanoids (leukotriens and prostaglandins), which play an important role in inflammatory processes (1).

The production of HUFAs from dietary fatty acids includes several desaturation and elongation steps. The desaturases involved in this reaction cascade, delta-6 desaturase and delta-5 desaturase, are the rate-limiting enzymes. Both are expressed in the majority of human tissues, with highest levels in liver and to a smaller amount in brain, heart, and lung (2, 3). Delta-6 desaturase inserts a double bond at position 6 and after an elongation step, delta-5 desaturase inserts an additional double bond at

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Abbreviations: C20:4n-6 or arachidonic acid, all-*cis*-5,8,11,14-eicosatetraenoic acid; C22:6n-3 or docosahexaenoic acid, 22:6(ω-3), all-*cis*-docosa-4,7,10,13,16,19-hexaenoic acid; DIP, deletion/insertion polymorphism; ELK1, member of ETS domain transcription factor family; EMSA, electrophoretic mobility shift assay; FADS, fatty acid desaturase; HUFA, highly unsaturated fatty acid; LD, linkage disequilibrium; PPAR, peroxisome proliferator activated receptor; SNP, single nucleotide polymorphism; SREBP, sterol regulatory element binding protein; TFBS, transcription factor binding site.

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position 5 of the elongated fatty acid chain. These conversions result in the formation of either arachidonic acid (C20:4n-6) in the omega-6 pathway or of eicosapentaenoic acid (C20:5n-3) in the omega-3 pathway. These molecules are either converted into eicosanoids or further elongated and desaturated, again with the help of the delta-6 desaturase (1). The importance of delta-6 desaturase for the formation of HUFAs and their influence on membrane integrity and fluidity was shown in a recent study by Stoffel et al. (4) who generated a *fads2* $^{-/-}$ (fatty acid desaturase) mouse. In this animal model, membrane polarity of Sertoli and ovarian follicle cells was completely disturbed due to the lack of HUFAs in knockout mice caused by the delta-6 desaturase deficiency. Furthermore, both male and female mice were infertile and eicosanoid synthesis was disturbed. However, the administration of a HUFA-rich diet (either C20:4n-6 or C20:5n-3/C22:6n-3) enabled the *fads2* $^{-/-}$ mice to overcome the genetic defect, restored the fatty acid pattern in membrane lipids, and rescued spermatogenesis as well as normal follicle development. Similarly, eicosanoid synthesis was restored by administration of arachidonic acid. Similar effects were observed in another *fads2* $^{-/-}$ mouse by Stroud et al. (5).

These studies showed that the level and composition of HUFAs in the body highly depends on the conversion rate of the delta-6 desaturase, which is in turn regulated by supply with dietary fatty acids and hormone signaling. The effect of dietary fatty acids on desaturase transcription regulation is mediated by two transcription factors, sterol regulatory element binding protein (SREBP1) and peroxisome proliferator activated receptor (PPARA) (6). The feedback regulation mechanisms by which dietary fatty acids act on SREBP1 processing and stability, which in turn influence *FADS2* gene expression, have been investigated intensively (7–11). The induction of desaturases by PPARA was shown to occur both by indirect and direct mechanisms (12–15). Besides the mediation of fatty acid effects, SREBP1 may also mediate the insulin effect on *FADS2* gene expression, as was observed in experimentally induced diabetic rats (16, 17).

Although dietary and hormonal influences seem to play an important role in transcription regulation of delta-6 desaturase, genetic factors are important as well for influencing the level and composition of HUFAs in human tissues. Of special interest is the *FADS* gene cluster on chromosome 11, with a head-to-head orientation of the *FADS1* and *FADS2* genes, which encode the delta-5 and delta-6 desaturase, respectively. A third putative desaturase gene, *FADS3*, is located in the 6.0 kb telomeric side from the *FADS2* gene in a tail-to-tail orientation (18). Several candidate gene studies reported an association of a number of single nucleotide polymorphisms (SNPs) in the *FADS* gene cluster with fatty acid composition in human tissues (19–22). These results were strengthened recently by our study, which for the first time compared genome-wide SNP data with metabolomics data and replicated the previous findings by this new approach (23). Additionally, several genome-wide association studies meanwhile reported an association of *FADS* polymorphisms with polyunsaturated fatty acids (24) and more complex lipid traits like low-density lipoprotein, high-density lipoprotein, and triglycerides (25–27).

In the first association study (19), the minor alleles of 11 SNPs located in and around the *FADS1* and *FADS2* genes were associated with enhanced levels of desaturase substrates in serum phospholipids. In contrast, levels of desaturase products (especially arachidonic acid, with a genetically explained variance of 28%) were lower. The same significant associations were found for haplotype analyses. This observation speaks for a strong influence of the genetic variants on the activity of the desaturases. Until now, functional data on the described polymorphisms were not available. The aim of this study was to identify causative SNPs within the *FADS1/FADS2* haplotype, and we therefore performed functional analyses of polymorphisms in the *FADS2* promoter region to gain insight into regulatory mechanisms of the *FADS2* gene resulting from the presence of these polymorphisms on the transcriptional level. Based on their close proximity to the translation start site of *FADS2*, we selected the one base pair deletion/insertion polymorphism (DIP) rs3834458 (position −942) and the SNP rs968567 (−299). In addition, both polymorphisms are located in a CpG-rich region predicted to contain interesting binding sites for transcription factors known to be involved in fatty acid metabolism such as SREBP1 and PPARA.

MATERIALS AND METHODS

Bioinformatic analysis of transcription factor binding sites

The prediction of transcription factor binding sites (TFBS) in promoter sequences was performed using the Genomatix MatInspector software with standard settings for the highest matrix similarity (28). This program uses a large library of weight matrices based on known in vivo binding sites to predict TFBS in nucleotide sequences.

Plasmid constructions

To obtain constructs for luciferase assays, the *FADS2* promoter sequence from position −1014 to −1 relative to the translation start site was amplified by PCR from human genomic DNA. The PCR product was first cloned into the vector pGEM T-Easy (Promega) and then subcloned into the reporter vector pGL4.12 (Promega). Constructs containing all possible combinations of major and minor alleles of rs3834458 (T/Del, position −942) and rs968567 (C/T, position −299) were obtained by PCR mutagenesis. Truncated constructs (containing region −414 to −1 and −214 to −1) were generated by PCR from the original respective plasmids and subsequent cloning into pGL4.12. All constructs were verified by sequencing.

Luciferase reporter assays

HeLa, HEK293, and HepG2 cells were seeded at a density of 1×10^5 cells/well in 12-well plates in MEM or DMEM medium with stable L-glutamine (PAA Laboratories), respectively, containing 10% FBS (PAA Laboratories) and 1% penicillin/streptomycin (Gibco) and incubated overnight. All cell lines were transfected with 500 ng of the promoter construct per assay using FuGene6 (Roche Diagnostics) according to the manufacturer's instructions in an appropriate ratio of FuGene-DNA. For normalization, 50 ng

of the pGL4.74 vector (Promega), which constitutively expresses *Renilla* luciferase, was cotransfected. Transfected cells were incubated for 32 h at 37°C in a 5% CO_2 atmosphere. Cells were then washed once in PBS buffer before 200 µl of 1× passive lysis buffer (Promega) was added. After gentle shaking for 30 min, the plate containing the lysed cells was frozen at −80°C overnight. After thawing, luciferase activity was measured. For this, 50 µl of both Dual Luciferase Reporter Assay System reagents (Promega) was added successively to 20 µl of the lysate according to the manufacturer's instructions. Measurements were done in a Tecan GeniosPro microplate reader. Calculation of the intensity ratios of *Firefly-Renilla* luciferase activity resulted in the relative promoter activity of the constructs. The significance of difference in promoter activity between the constructs was tested by independent-samples *t*-test using the SPSS 16.0 software.

Nuclear protein extraction and electrophoretic mobility shift assays

Confluent HeLa cells grown in T75 flasks were harvested and nuclear proteins were extracted with the NE-PER® Nuclear and Cytoplasmic Extraction reagents (Pierce) according to the manufacturer's instructions. For electrophoretic mobility shift assays (EMSAs), oligonucleotides containing predicted TFBSs surrounding the DIP rs3834458 and the SNP rs968567 were designed and purchased from the company Metabion. The oligo sequences are summarized in **Table 1**. A total of 20 pmol of double-stranded oligos containing either the major or the minor allele were 5′-end labeled with γ-^{32}P-ATP (Hartmann Analytic) and T4 polynucleotide kinase (Fermentas) according to the manufacturer's protocol. Unincorporated label was separated from labeled DNA by gel filtration on G-25 columns (GE Healthcare). Binding reaction was carried out with or without different concentrations of unlabeled competitor oligonucleotides using 15 µg of nuclear extract in 1× binding buffer (20 mM Tris/HCl, pH 7.9, 50 mM NaCl, 1 mM EDTA, 10% glycerol, 0.05% NP40, 2.5 mM DTT) with 1 µg poly dI-dC (Roche Diagnostics) and 20 fmol of labeled probe in a total volume of 20 µl for 30 min at room temperature. Protein-DNA complexes were separated on 10% nondenaturing polyacrylamide gels by electrophoresis in 1× tris-borate-EDTA (TBE) buffer. The gels were dried and radioactivity was visualized by autoradiography on Kodak films.

Gene expression analysis

Correlation analysis of peripheral blood gene expression was performed in 322 KORA F3 samples with whole-genome expression profiles available. A detailed description of the KORA F3 study, which is a population-based study comprising individuals living in the region of Augsburg, has been given elsewhere (29). Gene expression analysis was performed with the Illumina Human-6 v2 Expression BeadChip as described earlier (30). Raw data from the Illumina 'Beadstudio' software were exported to R software. Data were logarithmized and normalized using the LOWESS method (31). Associations between the expressions of two genes were computed with a linear regression model. Correlations were determined using the Pearson correlation coefficient.

DNA affinity purification and immunoblotting

Oligonucleotides for DNA affinity purification contained four repeats of the predicted TFBSs surrounding SNP rs968567 to ensure maximal binding efficiency (see **Table 2**). Double-stranded oligonucleotide binding sites were constructed by annealing 26 pmol of complementary single-stranded 5′-end biotinylated oligonucleotides containing the −299 major C allele or the −299 minor T allele in annealing buffer (89.6 mM Tris-HCl, pH 9.0, 448.2 mM KCl, and 13.4 mM $MgCl_2$). Oligonucleotides with four repeats of an experimentally verified ELK1 (member of ETS domain transcription factor family) binding site were generated accordingly as positive control. Proteins binding to the oligonucleotides were purified using streptavidin-coated Dynabeads M-280 (Invitrogen). Briefly, 26 pmol of double-stranded biotinylated oligonucleotides were coupled to 250 µg (25 µl) of the streptavidin magnetic beads according to the manufacturer's protocol. A total of 50 µg of HeLa nuclear extract was applied to the DNA-magnetic beads complex and incubated in protein binding buffer (4.6 mM Tris-HCl, pH 8.0, 18.4 mM KCl, 0.02% NP-40, 0.37% glycerol, 4.8 mM DTT, 22.9 µM $ZnSO_4$ with 9.7 mM $MgCl_2$) for 10 min at room temperature. Nonspecific DNA binding was inhibited by the subsequent addition of 2.5 µg poly[d(I-C)] (Roche) and incubation for an additional 20 min. Afterwards, the supernatant containing unbound proteins was removed by use of a magnetic separator and the beads with the DNA-protein complexes were washed three times with wash buffer (9.9 mM Tris-HCl, pH 8.0, 39.6 mM KCl, 0.05% NP-40, 0.8% glycerol, 10 mM DTT, 49.5 µM $ZnSO_4$). Bound proteins were eluted from the magnetic beads by use of a high ionic strength elution buffer (9.5 mM Tris-HCl, pH 8.0, 1.9 M KCl, 0.048% NP-40, 0.76% glycerol, 10 mM DTT, 47.5 µM $ZnSO_4$, and 10 mM $MgCl_2$), separated on a 10% Tris-tricine SDS-PAGE gel, and subsequently blotted onto a polyvinylidene fluoride membrane (Pall). Incubation with ELK1 antibody (SC-355 X, Santa Cruz, 1:500 in PBS containing 0.5% milk powder) was carried out at 4°C overnight. As secondary antibody, a peroxidase-conjugated goat anti-rabbit IgG (A-6154, Sigma, 1:5000 in PBS containing 0.5% milk powder) was used with an incubation time of 1 h at room temperature. Peroxidase reaction was carried out using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) and specific ELK1 bands were visualized by exposing Kodak films.

RESULTS

Bioinformatic analyses predict the allele-dependent presence of different TFBS in the SNP-containing FADS2 promoter regions

Bioinformatic analyses using the Genomatix software predicted transcription factors with the highest core matrix similarities and revealed that our DIP of interest

TABLE 1. Oligonucleotides representing the putative TFBSs used for EMSAs

Polymorphism	Allele	Strand	Sequence (5′→3′)	Size (bp)
rs3834458	T	for	ATTCTTTTCTAAGATTGTC	19
	T	rev	GACAATCTTAGAAAAGAAT	19
	Del	for	ATTCTTTTCAAGATTGTC	18
	Del	rev	GACAATCTTGAAAAGAAT	18
rs968567	C	for	GAGGCCCTGAGCTCCCGGGGAGTTTTTACT	30
	C	rev	AGTAAAAACTCCCCGGGAGCTCAGGGCCTC	30
	T	for	GAGGCCCTGAGCTTCCGGGGAGTTTTTACT	30
	T	rev	AGTAAAAACTCCCCGGAAGCTCAGGGCCTC	30

Nucleotides representing the polymorphisms are underlined.

TABLE 2. Oligonucleotides used for DNA affinity purification of ELK1

Primer	Strand	Sequence (5′ → 3′)	Size (bp)
ELK1_control	for	*GAATAACCGGAAGTAACCGAATAACCGGAAGTAA	72
		CCGAATAACCGGAAGTAACCGAATAACCGGAAGTAACC	
	rev	*GGTTACTTCCGGTTATTCGGTTACTTCCGGTTAT	72
		TCGGTTACTTCCGGTTATTCGGTTACTTCCGGTTATTC	
rs968567_C	for	*TGAGCTCCCGGGGAGTTGAGCTCCCGGGGA	71
		GTTGAGCTCCCGGGGAGTTGAGCTCCCGGGGAGT	
	rev	*ACTCCCCGGGAGCTCAACTCCCCGGGAGCT	71
		CAACTCCCCGGGAGCTCAACTCCCCGGGAGCTCA	
rs968567_T	for	*TGAGCTTCCGGGGAGTTGAGCTTCCGGGGA	71
		GTTGAGCTTCCGGGGAGTTGAGCTTCCGGGGAGT	
	rev	*ACTCCCCGGAAGCTCAACTCCCCGGAAGCT	71
		CAACTCCCCGGAAGCTCAACTCCCCGGAAGCTCA	

Nucleotides representing the rs968567 polymorphism are underlined. The first nucleotide of each repetitive element is written in bold. An asterisk (*) represents the position of the biotinylation site.

(rs3834458, position −942) was located in close proximity to predicted SREBP1 and PPARA binding sites, with the SREBP binding element being 48 bp and the PPAR/RXR binding element 12 bp away. Several other binding sites for transcription factors were predicted for the region containing the DIP rs3834458: C/EBP-β in 6 bp distance from the DIP, and PAX4/PAX6 and BCL6 directly spanning the −942 position. Interestingly, the BCL6 binding site is present only when the sequence contains the −942 major T allele and is lost when the deletion mutation is present, because the −942 major T allele is part of the binding site core sequence of BCL6 (**Fig. 1A, C**).

The promoter region surrounding SNP rs968567 (position −299) is also predicted to contain several TFBS. Once more, a PPAR/RXR binding site is located in the neighborhood of the SNP only 12 bp away. Three additional binding sites are predicted for the sequence containing the −299 minor T allele: ELK1, STAT1, and STAT3, which are not present for the −299 major C allele (Fig. 1B). Again, the −299 minor T allele is part of the matrix core sequences of all three TFBSs (Fig. 1D).

Luciferase reporter gene assays reveal an influence of SNP rs968567 on promoter activity

To determine the functional effects of the two polymorphisms (rs3834458, T/Del, −942 and rs968567, C/T, −299) on transcriptional regulation, luciferase reporter gene assays were conducted to measure promoter activity (**Fig. 2**). Three different human cell lines (HepG2, HEK293, and HeLa) were transiently transfected with the promoter constructs or the empty reporter vector pGL4.12 as control. Three individual experiments for each construct and cell line were performed and promoter activity was measured in triplicates for each construct and experiment. Luciferase activity was slightly lower for all constructs containing the −942 minor deletion mutation compared with the constructs containing the −942 major T allele. This was a modestly not significant effect, however, with a decrease in luciferase activity of around 20% averaged over all tested cell lines and constructs. The replacement of the −299 major C allele of rs968567 by the −299 minor T allele resulted in a 2- to 3-fold increase of luciferase activity in HeLa and HepG2 cells in full-length as well as truncated constructs. This effect was statistically significant in HepG2 ($P < 2.0E-05$) and HeLa ($P < 1.0E-6$) cells but not in HEK293 cells. Altogether, the results indicate a strong regulatory function of polymorphism rs968567 in different cell lines.

EMSA demonstrates altered DNA-binding ability of nuclear proteins to the FADS2 promoter due to SNP rs968567

Next we asked if the polymorphisms effect the DNA-binding ability of nuclear proteins. HeLa nuclear protein extracts were subjected to binding to oligonucleotides representing the region surrounding SNP rs968567 with either the −299 major C allele or the −299 minor T allele, and DNA-protein complexes were analyzed by EMSA (**Fig. 3**). Specific binding of nuclear protein to the respective oligonucleotide was tested by adding increasing amounts of competing unlabeled oligonucleotide probe, containing the respective other allele. Two bands corresponding to shifted complexes showed different intensity, depending on which allele was present. Both bands showed weaker intensity when the labeled oligonucleotide with the C allele was present, whereas a higher intensity was achieved when the labeled oligonucleotide contained the T allele. Competition of labeled C allele with unlabeled T allele resulted in a significant decrease of band intensities already at low concentrations of competitor. The upper band was still visible at very high competitor concentrations, whereas the lower band vanished completely. In contrast, competition for protein binding of labeled T allele with unlabeled C allele resulted in slightly decreased band intensities only at high concentrations of competitor. At the highest competitor concentration, the lower band vanished as well, but the upper band was much stronger than in the vice versa competition experiment. These effects were observed in two independent experiments. The results indicated that the −299 T allele increased binding affinity of the tested promoter region for at least two protein complexes. The same experiment was conducted with oligonucleotides containing the major and minor alleles of the rs3834458 polymorphism. Only very weak band intensities, hinting to very weak binding of two nuclear proteins, could be observed and no significant difference of competing effects between oligonucleotides was found (data not shown).

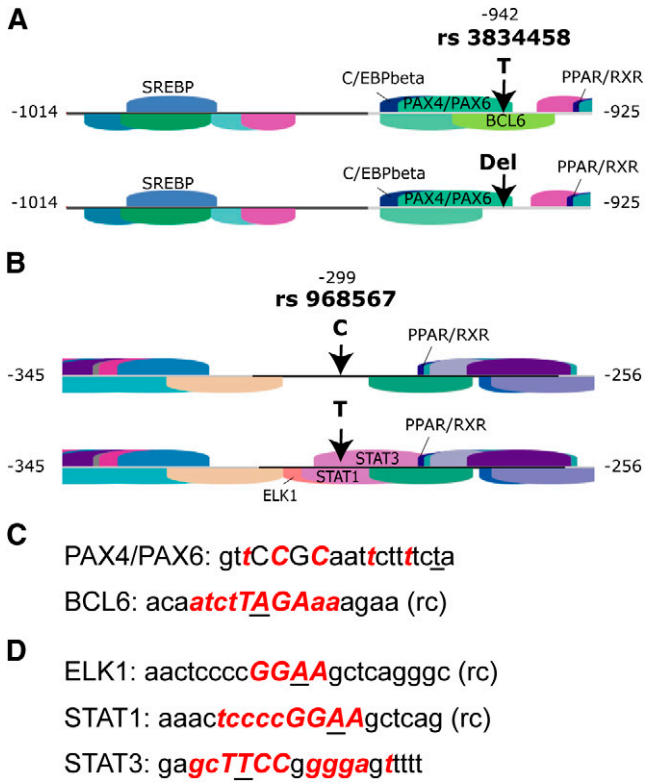

Fig. 1. Schematic presentation of location of analyzed polymorphisms in the human *FADS2* gene promoter and surrounding TFBSs modified from Genomatix MatInspector software output. A: A BCL6 binding site is predicted when the major T allele of rs3834458 is present but lacking when the deletion is present. B: The minor T allele of rs968567 leads to prediction of binding sites for ELK1, STAT1, and STAT3. In contrast, no binding sites are predicted when the major C allele is present. C and D: Sequence that was recognized by Genomatix MatInspector to contain a TFBS and also rs3834458 (C) or rs968567 (D), respectively. Nucleotides marked in bold red show a high degree of matrix conservation at this position, i.e., information content is very high. Nucleotides in capitals denote the core sequence used by MatInspector. Underlined nucleotides highlight the position of the respective polymorphism; rc means that a TFBS sequence was found for the opposite strand.

Gene expression analysis shows statistically significant association between expression levels of FADS2 and ELK1

Because we have found a significant impact on promoter activity and binding of nuclear protein complexes only for SNP rs968567 and not for DIP rs3834458, we focused on the region surrounding SNP rs968567 for further characterization. Prediction of TFBSs in this region resulted in three binding sites when the rs968567 minor T allele was present in the sequence: ELK1, STAT1, and STAT3. Regression analysis between *FADS2* whole blood mRNA expression levels and expression levels of these three transcription factors in 322 subjects revealed a statistically significant association between mRNA expression levels of *FADS2* and *ELK1* with a *P*-value of 2.29E-13 and an effect size of 0.36 (**Fig. 4A**). No significant *P*-values were obtained for the correlation of *FADS2* with *STAT1* and *STAT3* expression levels. To test the plausibility of this approach, regression analyses of *FADS2* expression levels with *PPARA* and *SREBP1*, two transcription factors already known to be involved in *FADS2* transcription regulation, were performed as positive controls. The expression levels of both transcription factors were significantly associated with *FADS2* expression (*PPARA*: $P = 4.22E\text{-}12$, effect size = 0.35 and *SREBP1*: $P = 2.93E\text{-}28$, effect size = 0.52) and by this proved the reliability of our expression data. We furthermore tested the association between *FADS2* and *ELK1* gene expression dependent on the rs968567 genotype. The effect size of association between *FADS2* and *ELK1* in homozygous carriers of the rs968567 major C allele (n = 229) was 0.3 ($P = 4.13E\text{-}8$). In heterozygous (CT) and homozygous minor T allele carriers (n = 93), it reached 0.36 ($P = 8.47E\text{-}6$), and in homozygous minor T allele carriers alone (n = 8) the effect size increased to 0.84 ($P = 0.0055$) (Fig. 4B). These results strongly point to ELK1 as a newly identified regulator of *FADS2* gene expression with a higher impact of ELK1 in carriers of the rs968567 minor T allele.

DNA affinity purification with immunoblotting reveals allele-specific binding of ELK1 to the region surrounding SNP rs968567

Our gene expression analyses in a population-based study revealed a significant association between expression levels of *FADS2* mRNA and *ELK1* mRNA in whole blood, with a higher effect size in carriers of the rs968567 minor T allele. Additionally, the Genomatix MatInspector software predicted allele-specific binding of ELK1 to the region surrounding SNP rs968567. We therefore tested the binding of ELK1 protein to the respective sequence by performing DNA affinity purification of nuclear proteins from HeLa nuclear extract using biotinylated oligonucleotides representing the region surrounding SNP rs968567 with either the −299 major C allele or the −299 minor T allele. An oligonucleotide containing an experimentally verified ELK1 binding site (32) was used as positive control. The supernatant and wash fractions containing unbound proteins as well as the elution fraction with the bound proteins were immunoblotted and a specific antibody against human ELK1 was used to detect presence of ELK1 protein in the fractions (**Fig. 5**). A specific band corresponding to ELK1 was present in the elution fraction of the positive control, showing that ELK1 from HeLa nuclear extract is able to bind to its consensus sequence under the used buffer conditions and experimental setup. The appearance of ELK1 in the elution fraction of the −299 minor T allele, which was lacking in the elution fraction of the −299 major C allele, confirms binding of ELK1 to the *FADS2* promoter sequence exclusively when the minor T allele is present.

DISCUSSION

Disorders of delta-6 desaturase activity affect essential physiological functions

Recent association studies showed an association of delta-6 and delta-5 desaturase gene polymorphisms with

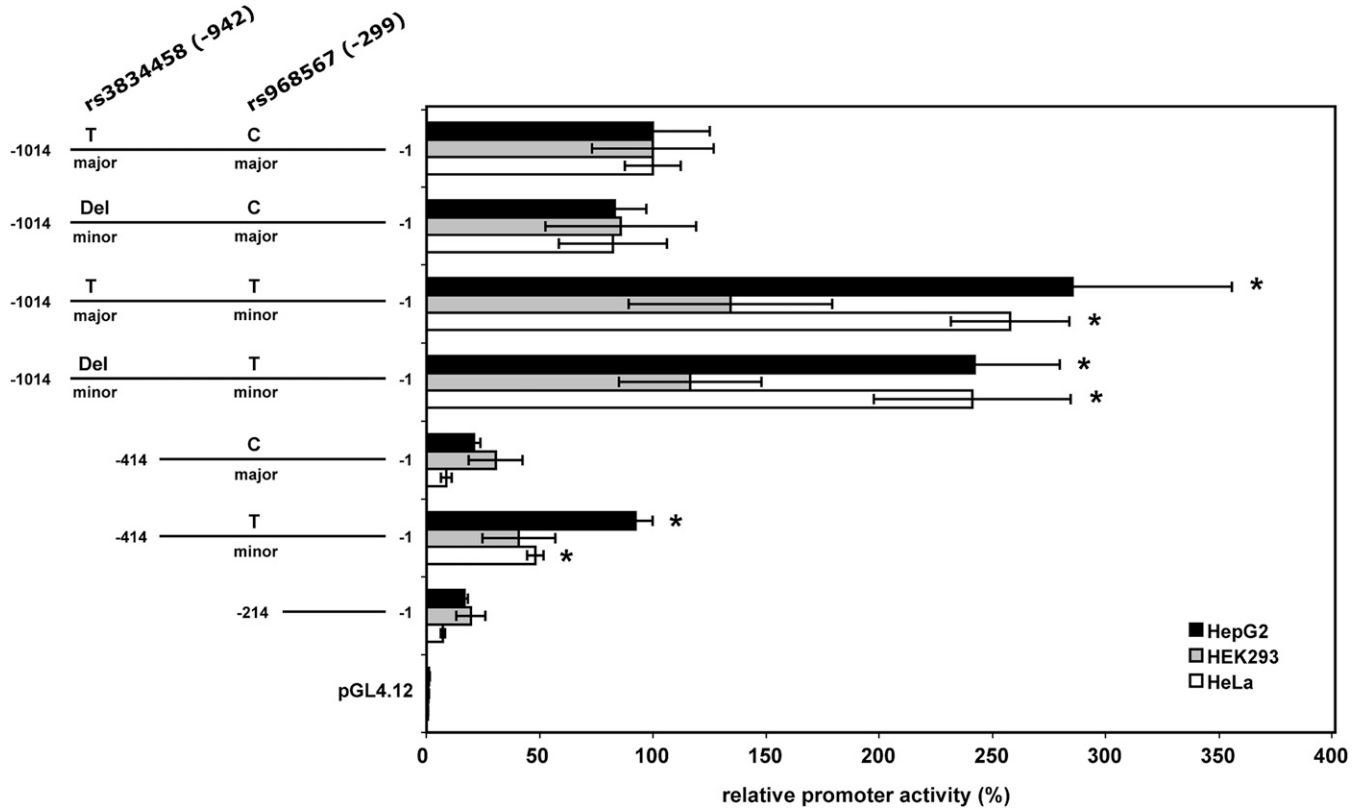

Fig. 2. Relative promoter activity of different constructs of human *FADS2* gene promoter in three different human cell lines. Values represent the mean of three independent experiments performed in triplicate. Promoter activity of the major/major construct was used as reference and set at 100%. Asterisks denote statistically significant results calculated by *t*-test. Numbers indicate the relative position to the *FADS2* translation start site.

HUFA level and composition in different human tissues accompanied by an accumulation of desaturase substrates and a decline in desaturase products (19–23). This suggests that the desaturase activity is not only regulated by nutritional and hormonal influences but also by genetic factors. The observed change of HUFA levels and composition in different human tissues due to the polymorphisms might alter several important physiological processes and is thought to modulate the development of complex diseases. The effect of *FADS* polymorphisms on brain development has been shown by Caspi et al. (33), who reported a modulation of the positive effect of breastfeeding on development of intelligence by polymorphisms in the *FADS* gene cluster in two independent birth cohorts. The importance of an intact delta-6 desaturase function on eicosanoid synthesis and membrane lipid composition was

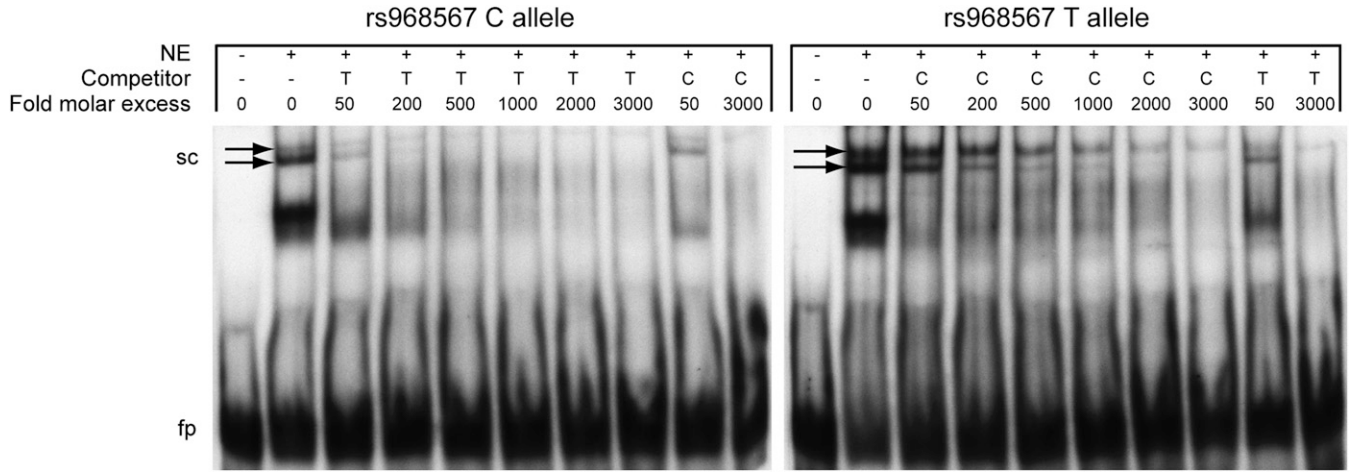

Fig. 3. Verification of molecular interactions in the human *FADS2* promoter region. Autoradiographs of competitive EMSA gels of the rs968567 polymorphism using allele-specific ^{32}P-labeled oligonucleotides. NE: nuclear extract, sc: shifted complexes, fp: free probe. Arrows indicate bands corresponding to shifted complexes and having different intensity dependent on the tested allele.

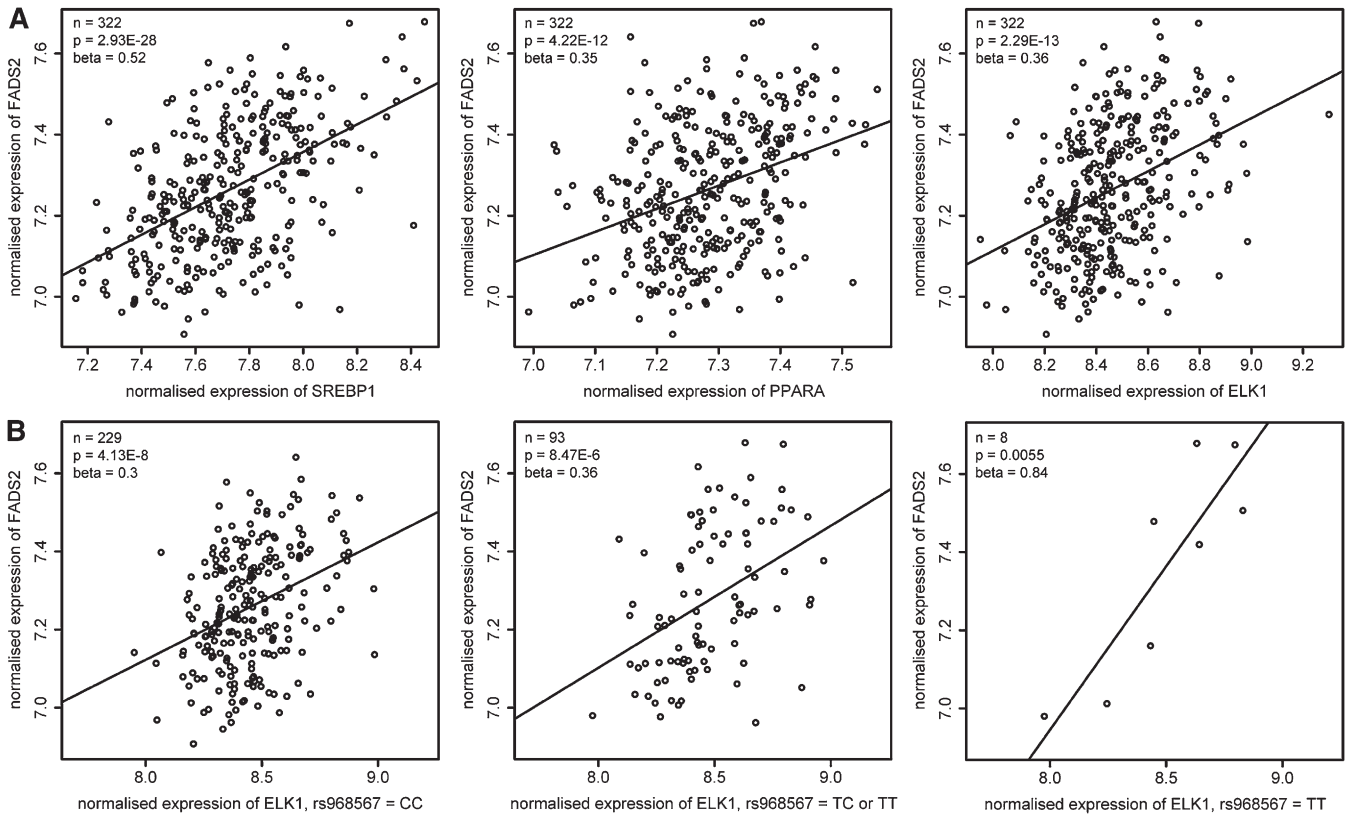

Fig. 4. Correlation analysis of *FADS2* and transcription factor mRNA expression in human whole blood samples. Axes represent normalized mRNA expression levels of the respective genes. Numbers of subjects (n), *P*-value, and effect size (β) of the association are indicated for each graph. A: Normalized expression level of *FADS2* plotted against *SREBP1* and *PPARA* as positive control and against *ELK1*. B: Normalized expression level of *FADS2* plotted against *ELK1* dependent on rs968567 genotype.

underlined by previous reports of two different *fads2* knockout mice (4, 5). The assumption that there is a direct effect of *FADS* polymorphisms on the outcome of fatty acid-related diseases has been supported by Schaeffer et al. (19), who reported an association of the *FADS* gene cluster with allergic rhinitis and atopic eczema, though without statistical significance after correction for multiple testing. Another study recently reported an association of *FADS* genotypes with inflammation and coronary artery disease (34). All these observations hint at a strong role of delta-6 desaturase in regulating fatty acid composition in human tissues to maintain health. Approaches to investigate the influence of genetic polymorphisms on the regulation of the human enzyme activity are therefore needed to understand the role of delta-6/delta-5 desaturases in the development of fatty acid-related complex diseases.

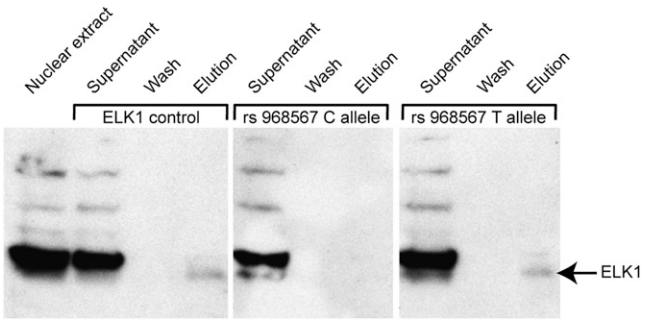

Fig. 5. Detection of human ELK1 protein in DNA affinity purification fractions by immunoblotting. The first lane represents crude HeLa nuclear extract and verifies the presence of ELK1 protein in HeLa cells. The supernatant and wash fractions contain all unbound proteins, whereas the elution fraction contains all oligonucleotide binding proteins. A specific band at 62 kDa corresponds to the ELK1 protein (indicated by an arrow).

Detection of a critical polymorphism-containing region that influences delta-6 desaturase activity

Many studies have reported associations of several SNPs in the *FADS* gene cluster with HUFA levels and composition in different human tissues and have contributed to the understanding of the influence of SNPs on the regulation of fatty acid synthesis (19–23). However, the causative functional variant(s) are not known up to date. The analysis of linkage disequilibrium (LD) structures in the *FADS* gene cluster suggests that all polymorphisms in this region are in very high LD and most of them are highly correlated. The real functional variant(s) could therefore cause associations of all other SNPs being in high LD and cannot be directly identified by association studies for this reason. Functional approaches are needed to determine the effect of the associated SNPs on the molecular level and by this identify the causative variant(s).

By performing luciferase reporter gene assays, we showed that one of the two analyzed *FADS2* promoter poly-

morphisms (rs968567) is located in a region that seems to be important for transcription regulation. While the minor deletion mutation of rs3834458 had only a little, statistically not significant effect on promoter activity, the minor T allele of rs968567 highly increased promoter activity compared with the construct containing both major alleles. The effect was the same in all three tested cell lines; however, the response in HEK293 cells was lower and not statistically significant for both polymorphisms. Because transcription regulation is tissue dependent (35), this is likely due to the tissue-specific expression pattern of involved transcription factors. To investigate if altered binding of transcription factors to the polymorphism-containing regions is the cause for the observed effects in the luciferase assays, molecular interactions were analyzed by EMSA. Bioinformatic analyses predicted several putative binding sites in the polymorphism-containing regions, and we consequently checked their functionality for protein binding. Indeed, several protein complexes were shown to bind to the regions of interest by EMSA. In the case of rs968567, a clear allele-specific binding affinity of at least two protein complexes was shown by using a competitive method. The minor T allele of rs968567 facilitated the binding in comparison to the major C allele. No differential binding affinity could be shown for the region containing rs3834458. All these observations speak for a strong influence of the rs968567 polymorphism on transcription regulation of the *FADS2* gene.

Identification of ELK1 as a potential new regulator of FADS2 gene transcription

In this study, it was shown that the *FADS2* promoter region surrounding SNP rs968567 exhibits promoter activity, which increases when the major C allele of SNP rs968567 is replaced by the minor T allele. We assumed that this effect could be caused by allele-specific differential binding affinity of transcription factors. An in silico analysis of TFBSs predicted three additional binding sites (ELK1, STAT1, and STAT3) in the sequence when the major C allele of rs968567 was replaced by the minor T allele. This was substantiated by EMSA experiments that revealed allele-specific binding of at least two nuclear protein complexes to this promoter region. Linear regression analysis of whole blood mRNA levels of the predicted transcription factors and expression levels of *FADS2* mRNA resulted in a highly significant association between *ELK1* and *FADS2*, with a much higher effect size in subjects being homozygous for the rs968567 minor T allele. We used the correlation of *PPARA* and *SREBP1* with *FADS2* as positive control, because these two transcription factors are known to activate *FADS2* transcription (1). The significant association results of our positive controls approve reliability of the expression data and substantiate the significant association between *ELK1* and *FADS2*. ELK1 is a member of the ETS domain family of transcription factors, was first cloned in 1989 (36), and is primarily known for its role in the transcriptional regulation of immediate early genes, including *c-fos* (37) and *egr-1* (38), by forming ternary complexes with serum response factor on the serum response elements of gene promoters (39). To our knowledge, a role of ELK1 in lipid metabolism has not been reported until now. We tested binding of ELK1 protein to the predicted binding site in the *FADS2* gene promoter by DNA affinity purification and subsequent immunoblotting. Specific ELK1 bands in the elution fraction were only present when the major C allele of SNP rs968567 was replaced by the minor T allele. This effect is in clear accordance with the Genomatix MatInspector prediction and identifies ELK1 as a putative new regulator of *FADS2* gene transcription in an allele-specific manner. The fact that correlation analysis between *FADS2* and *ELK1* mRNA expression gives significant results for both alleles (however, with lower effect size for the major C allele) suggests that ELK1 also binds to the *FADS2* promoter in the presence of the –299 major C allele, but with lower affinity so that we were not able to detect ELK1 protein in that case in our immunoblotting experiment. Another possibility would be an additional functional ELK1 binding site in another region of the *FADS2* gene, of which several are predicted by Genomatix MatInspector.

Controversial impact of the rs3834458 deletion polymorphism on promoter activity

Nwankwo et al. (40) published a study in 2003 that already dealt with functional investigations of the rs3834458 polymorphism. The authors aimed to identify the molecular mechanism of *FADS2* deficiency in skin fibroblasts from a patient with severe symptoms like corneal ulceration, growth failure, skin abnormalities, and photophobia previously shown to be caused by a deficiency of delta-6 desaturase (41). By sequencing the *FADS2* promoter region of DNA derived from patient fibroblasts and comparing the sequence to DNA from three healthy controls, they identified a thymidine insertion in the patient DNA, which corresponded to the T allele of rs3834458. Luciferase reporter gene assays in a mouse fibroblast cell line (NIH/3T3) with promoter sequences derived from patient (T allele present) and healthy control (T deletion) fibroblasts resulted in significantly decreased promoter activity when the T allele was present. This result could not be replicated in any of our three tested human cell lines. Possible explanations could be that Nwankwo et al. (40) used a mouse fibroblast cell line (NIH/3T3) for their assays, which might express a different set of transcription factors compared with our human cell lines or that another unrecognized polymorphism in the tested sequences caused the effect in the study of Nwankwo et al. (40).

CONCLUSION AND OUTLOOK

In this study, we showed that polymorphism rs968567 influences *FADS2* gene promoter activity and alters DNA-binding affinity of nuclear proteins. One of the proteins binding to this region in an allele-specific manner was shown to be the transcription factor ELK1. Further experiments are required to completely characterize the interaction of ELK1 with the *FADS2* gene promoter or other functional elements in the gene and its impact on *FADS2* gene expression in vivo.

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Appendix B

Appendix B1. PCR primers used for genotyping in the ALSPAC study.

SNP ID	Primer 1	Primer 2
rs174548	ACGTTGGATGATGGAAGAAAGGAGCAGTGG	ACGTTGGATGAGCCATTCTCCTGGTTATCC
rs174556	ACGTTGGATGCAACTATTGGTTGTTGAGGG	ACGTTGGATGAGACTCCCCAGAGCTACTAC
rs174561	ACGTTGGATGGCTTCCCGCAGTGTGATTTG	ACGTTGGATGACCACACATACGGACCAATC
rs3834458	ACGTTGGATGACCAAGAAAGCAGAGCAGAG	ACGTTGGATGCCTTGGATTAGAGGGCTTTG
rs968567	ACGTTGGATGAGGATGTGGAACCCGAGGC	ACGTTGGATGGGACTTTTGCCTCCAGTAAA
rs174570	ACGTTGGATGAACAGTTGGGCATCCTAGAC	ACGTTGGATGAGTGAATAGGAGAGAGGCAG
rs174574	ACGTTGGATGTACTGCACTGTGTGCCCTGT	ACGTTGGATGATCGAATGCTGCACAACTGG
rs2727271	ACGTTGGATGGATGTTAGAGCATCTCTTCC	ACGTTGGATGATAAGCTGCGTCATTGTGTG
rs174576	ACGTTGGATGATGTGACAGGCTCTCACTTG	ACGTTGGATGCATTCAACTCCCGAAAAACC
rs174578	ACGTTGGATGGTGCTTTTGTCCTGGAGGAG	ACGTTGGATGCTAGTTGTTGTCAAACCCCG
rs174579	ACGTTGGATGTTTCTGTCCTGTCTTTCCCC	ACGTTGGATGCCTGGCCAGTTTCATTTTCC
rs174602	ACGTTGGATGAGCAGATAGAAGGGATGGTG	ACGTTGGATGAGATCATGAGCACCCAGTAG
rs498793	ACGTTGGATGCTAAACTTGTTAGAAGCGGG	ACGTTGGATGACTCTCTGAGCCTCTGTGTC
rs526126	ACGTTGGATGCTGGGTTCCCCTGACCTTC	ACGTTGGATGAACTGCAGGAGAGAGACAGG
rs174448	ACGTTGGATGAGGGTGTTTGGGTCCTGCTT	ACGTTGGATGTTATGTGAGCCGAACAGGAC
rs174449	ACGTTGGATGTCCCCAGAACTGTCCGAAAC	ACGTTGGATGAGTCCCTGGAGTCAGGTCTT
rs174634	ACGTTGGATGATTGCCACATGGCCAGGAG	ACGTTGGATGTTTCAACTGCCTGCCCCCTG
rs174455	ACGTTGGATGAAATGCTGCTGGCCCCTAAG	ACGTTGGATGATAAGGTCACCCAGGAAAGG

Appendix B2. Extension primers used for genotyping in the ALSPAC study.

SNP ID	Primer
rs174548	CTTATCCTGGTTATCCAGACTCA
rs174556	GGCTTACTGTGATTACTATGACTGTGAT
rs174561	CCCCTCCCCGCCGCGGCAT
rs3834458	GACCTCTAGTCAGACAATCTT
rs968567	GGAGTAAAAACTCCCCGG
rs174570	TCAGCAAGGAGGGATGAACTTGA
rs174574	CGGTGCACTTTGCCTAGGAT
rs2727271	ACATCCCTAAGGTCTTCA
rs174576	TTTGATCCCGAAAAACCTGGTATAACTT
rs174578	CCCTGAACACAGGTGA
rs174579	TCTCCTCCATCCCTTT
rs174602	GCCGCCCATGTTCCCCAACCC
rs498793	CCATCAGGCCTGTAAC
rs526126	CAGTCGCTTAGAGGCATCCATTCCAC
rs174448	TCTCTCCTGCCCCAGAA
rs174449	GAGTACCTGGAGTCAGGTCTTAGGCTC
rs174634	TGGGAGGGCAGGCTCA
rs174455	GCTGTCCCACCATAC

Appendix B3. PCR primers used for genotyping in the Ulm Birth Cohort study.

SNP ID	Primer 1	Primer 2
rs174547	ACGTTGGATGAGACTGGAGCATAACACAAC	ACGTTGGATGGAGCCTCAGGCTAATGAGAA
rs174553	ACGTTGGATGATGGAGTGAGACAGCAGAAC	ACGTTGGATGACCATGTCAACCAGCCAGTC
rs174556	ACGTTGGATGCAACTATTGGTTGTTGAGGG	ACGTTGGATGAGACTCCCCAGAGCTACTAC
rs174561	ACGTTGGATGGCTTCCCGCAGTGTGATTTG	ACGTTGGATGACCACACATACGGACCAATC
rs2072114	ACGTTGGATGTGTTGCTGGTGAGCACTGTC	ACGTTGGATGAGAGCAGAGGGCAGTGATGA
rs174602	ACGTTGGATGAGCAGATAGAAGGGATGGTG	ACGTTGGATGAGATCATGAGCACCCAGTAG
rs498793	ACGTTGGATGACTCTCTGAGCCTCTGTGTC	ACGTTGGATGCTAAACTTGTTAGAAGCGGG
rs526126	ACGTTGGATGCTGGGTTCCCCTGACCTTC	ACGTTGGATGAACTGCAGGAGAGAGACAGG
rs174626	ACGTTGGATGGCCTTGCCCTTGAATACTTC	ACGTTGGATGCGGTTAAGCAGAGAGCAGAT
rs174627	ACGTTGGATGGTCTCTGAGGAGGAGATAAG	ACGTTGGATGGCTACCCACAAAGCTGATAC
rs1000778	ACGTTGGATGGGTCTGGGATTTGAATCCAC	ACGTTGGATGGAGTGCCGGAAATAAACAGG
rs174455	ACGTTGGATGAAATGCTGCTGGCCCCTAAG	ACGTTGGATGATAAGGTCACCCAGGAAAGG

Appendix B4. Extension primers used for genotyping in the Ulm Birth Cohort study.

SNP ID	Primer
rs174547	TGTTTTCACCTACGCA
rs174553	CAACCAGCCAGTCTAGAACCCCTG
rs174556	CTGTGATTACTATGACTGTGAT
rs174561	ACAATCGCCGTCCCCGCCGCGGCAT
rs2072114	CAGTGATGAGGCTAAGACC
rs174602	TCCCATGTTCCCCAACCC
rs498793	TCCTTGCTGTGCAGACCCCTCAGCCTG
rs526126	CTTAGAGGCATCCATTCCAC
rs174626	TAGAGAGCAGATATGAATGTC
rs174627	AGCTGATACCCTTTTATCTG
rs1000778	GCAGCATAGCATGCCCTT
rs174455	GGGCTGTCCCACCATAC

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