Evidence-based research in pediatric nutrition: focus on the supplementation of polyunsaturated fatty acids and prebiotics

Doctoral (PhD) Dissertation

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Abbreviations

LCPUFA	long-chain polyunsaturated fatty acid
LA	linoleic acid (C18:2n-6)
GLA	γ-linolenic acid (C18:3n-6)
DHGLA	dihomo-γ-linolenic acid (C20:3n-6)
AA	arachidonic acid (C20:4n-6)
ALA	α-linolenic acid (C18:3n-3)
EPA	eicosapentaenoic acid (C20:5n-3)
DPA	docosapentaenoic acid (C22:5n-3)
DHA	docosahexaenoic acid (C22:6n-3)
PL	phospholipid
TG	triacylglycerol
PC	phosphatidylcholine
CE	cholesteryl ester
PEA	phospatidylethanolamine
FFA	free fatty acids
MD	mean difference
CI	confidence interval
RCT	randomized controlled trial
RR	rate ratio
PKU	phenylketonuria
Phe	phenylalanine

HMO	human milk oligosaccharides
GOS	galacto-oligosaccharides
FOS	fructo-oligosaccharides
RTI	acute respiratory tract infections
URTI	upper respiratory tract infections
LRTI	lower respiratory tract infections
GITI	gastrointestinal infections
UTI	urinary tract infections

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1. Introduction

Evidence-based research in pediatric nutrition

Evidence-based medicine is defined as the conscientious, explicit, and reflective use of current best evidence in making decisions about the care of individual patients. It involves integrating clinical expertise with the best available external evidence from systematic research, and incorporating this into clinical decision-making (Moyer A et al. 2004).

The number of systematic reviews is increasing rapidly, also in the field of nutrition and pediatric nutrition. In nutrition, systematic reviews has been used or were considered for the formulation of dietary guidelines, the establishment of nutrient reference intakes, the formulation of clinical practice guidelines and community practice guidelines as well as for the evaluation of applications for food and supplement label health claims and for the identification of research needs and priorities (Lichtenstein et al. 2008). Systematic reviews represent a palpable support for evidence-based practitioners to weigh the strength of the evidence and, accordingly, the degree of uncertainly. However, it has to be considered that systematic reviews provide answer to a specific question, which may be only one among many needed to address an important and actual topic. In the field of nutrition, systematic reviews are essential for accurate summarizing of the evidence in the following issues (Szajewska 2013):

- baseline exposure to nutrients (either from food or from supplement intake)
- nutrient status of a population
- bioequivalence of different chemical forms of nutrients
- bioavailability of nutrients and their different chemical forms
- biological functions of a nutrient
- assessing dose-response relationships.

The nutritional role of long-chain polyunsaturated fatty acids

Long-chain-polyunsaturated fatty acids (LCPUFA) have important functions in cell membranes as indispensable building stones for human development and optimal health. Docosahexaenoic acid (DHA) and arachidonic acid (AA) are considered to be the most important functional LCPUFA. They can be provided directly from the diet or can be synthetized from their essential fatty acid precursors alpha-linolenic acid (ALA) and linoleic acid (LA).

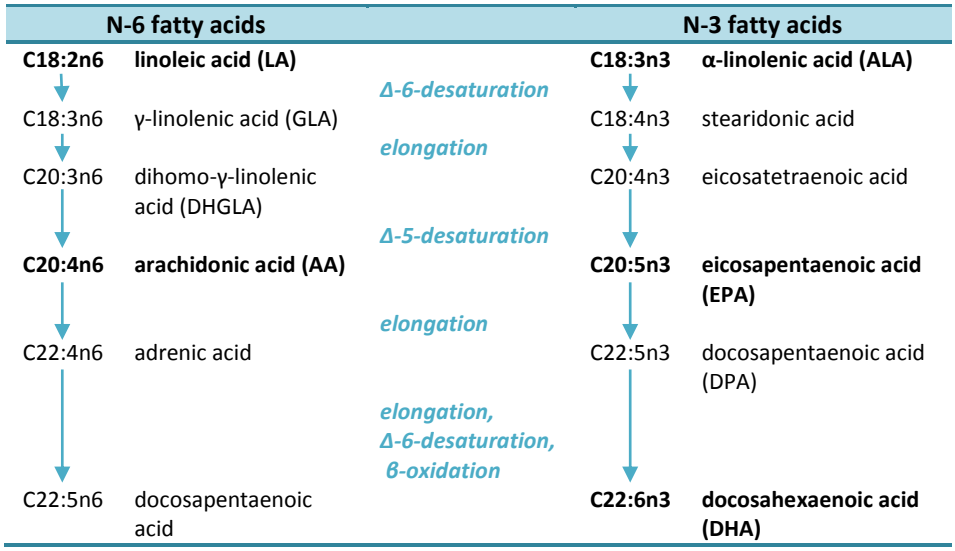

Figure 1. Metabolism of the n-6 and n-3 fatty acids

Among other enzymes, delta-6-desaturase and delta-5-desaturase are required for the formation of the longer-chain metabolites of both n-3 and n-6 series. The competitive desaturation of the n-3 and n-6 series of fatty acids by delta-6-desaturase is of major significance, because this step is considered to be the rate-limiting step of the pathway. The activity of this enzyme is modulated by hormones and by interactions of substrates and metabolic products (Burdge and Wootton 2002; Harnack et al. 2009). The fatty acid composition of cell membrane phospholipids is determined by the fatty acid composition of the diet; both, sufficient intake and the optimal n-3/n-6 fatty acid content of diet are of large importance.

Diseases, (such as diabetes mellitus or inborn errors of metabolism) where a strict metabolic control is an important part of the therapy, often result in not only insufficient supply with vitamins and minerals, but also lower amounts of saturated and polyunsaturated fatty acids are provided by the diet.

The omega-6 essential fatty acid, linoleic acid (LA; 18:2 n-6) is present in large amounts in vegetable seed oils such as sunflower seed oil, while the omega-3 essential fatty acid, alpha-linolenic acid (ALA; 18:3 n-3) is present in large concentrations in flaxseed oil and green, leafy vegetables. However, the long-chain metabolites are also present in their preformed state in the nature. The omega-6 arachidonic acid (AA; 20:4n-6) is present in lean red meat and chicken, in egg yolks, while the omega-3 PUFA, eicosapentaenoic acid (EPA or 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are synthesized in abundance by marine algae and therefore are present in concentrated form in cold water fish and in marine oils. In non-fish eaters, eggs are an important source of DHA and dairy products provide some EPA (Bradbury 2011).

The nutritional role of prebiotics

Prebiotics are selectively fermented dietary ingredients, which promote specific changes in the composition and/or activity of the gastrointestinal microbiota and thus confer benefits on the host health (Roberfroid et al. 2010). The prebiotic concept was first defined by Gibson and Roberfroid in 1995 and updated one decade later (Roberfroid et al. 2010, Gibson 2004). Accordingly, food ingredients are classified as prebiotic when they fulfill three main criteria: firstly, they escape digestion in the upper gastrointestinal tract and reach the colon intact; secondly, they are fermented by the intestinal microflora; and thirdly, they selectively stimulate the growth and/or activity of those intestinal bacteria which are associated with health and wellbeing (Moro et al. 2002, Haarman and Knol 2005, Schmelzle et al. 2003).

The role of prebiotics was investigated most extensively in the field of infant nutrition (Boehm et al 2003, Thomas et al 2010). Oligosaccharides were demonstrated to rep-

resent the most important dietary factor in human milk, promoting the development of beneficial intestinal flora (Bertino et al 2012, Barile 2013).

Prebiotics mimic the beneficial functional properties of human milk oligosaccharides: they increase stool colony counts of bifidobacteria and lactobacilli (Moro et al. 2002, Haarman and Knol 2005, Schmelzle et al. 2003); moreover, they inhibit the adhesion of pathogen organisms and interact with immune cells (Jeurink et al. 2013, Seifert and Watzl 2007). In bottle-fed infants prebiotic supplementation results in stools similar to those of breastfed neonates (i.e. leads to change in consistency to softer stools, a higher stool frequency and similar faecal pH) (Moro et al. 2002, Fanaro et al. 2005, Ashley et al 2012).

Because of their complex structure, oligosaccharides with a structure identical to that of human milk oligosaccharides (HMOs) are not yet available for supplementation purposes; however, oligosaccharides of other origin were tested for their bifidogenic effect in humans. At present galactooligosaccharides (GOS) and fructooligosaccharides (FOS) are considered the most relevant prebiotic oligosaccharides used in supplementation studies.

The main natural sources of prebiotics are chicory, Jerusalem artichoke, garlic, leek, onion and asparagus.

2. Methodical aspects of conducting a systematic review and meta-analysis

A systematic review is a review of a clearly formulated question that uses systematic and explicit methods to identify, select and critically appraise relevant research and to collect and analyze data from the studies that are included in the review (Moher et al. 2009). Its key characteristics according to the Cochrane Handbook are (Higgins and Green 2011):

- a clearly stated set of objectives with pre-defined eligibility criteria for studies
- an explicit, reproducible methodology
- a systematic search that attempts to identify all studies that would meet the eligibility criteria
- an assessment of the validity of the findings of the included studies (for example through the assessment of risk of bias)
- a systematic presentation, and synthesis, of the characteristics and findings of the included studies.

Systematic reviews and meta-analyses become increasingly important in the previous decades in health care: they are the primary source for clinicians to keep their knowledge up-to-date and they are also used as a starting point when developing clinical practice guidelines.

2.1 The PRISMA Statement

The value of systematic reviews depends on the way it was conducted, on the quality of collected studies and on the clarity of reporting. In 1996 an international group developed guideline (the QUOROM Statement = Quality of Reporting of Meta-Analyses) to optimize the reporting of meta-analyses; this guideline was revised and renamed in 2009: the PRISMA Statement (Preferred Reporting Items for Systematic reviews and Meta-Analyses) is practically a check-list which contains the items which

are important to be included when reporting a systematic review; the PRISMA statement aims to help both review authors and reviewers to improve the quality of reporting (Moher et al 2009; Liberati et al. 2009).

2.2 Framing the question and deciding on important outcomes

Framing the question covers following issues: a) the patient population, b) the intervention of interest, c) the comparator and d) the outcomes of interest has to be specified thoughtfully (Guyatt et al. GRADE 2. 2011). In systematic reviews investigating not the effect of an intervention and therefore summarizing not randomized controlled trials (RCTs), framing the question may not include all these issues. A very important decision by framing the question is to decide how widely the patients and the intervention should be defined: across the included patients and interventions the magnitude of effect on the key outcomes should be the same to avoid misleading estimates for some subpopulations or some subgroups of interventions (Guyatt et al. GRADE 2. 2011).

2.3. Searching for studies, selecting studies and collecting data

The Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE and EMBASE are the most often searched databases. Besides, trials registers are an increasingly important source of information. Both free-text and subject headings (for example Medical Subject Headings (MeSH)) should be used for search. The Cochrane Organization developed highly sensitive search strategies (filters) which facilitate the identification of randomized trials in the MEDLINE database (Lefebvre et al. 2011).

Eligibility of studies should be assessed and data extraction from study reports should be done by at least two independent reviewers. Studies, rather than reports, are the units of interest, therefore, one publication may contain more studies; on the other hand, the same study may be described and published in multiple reports (Higgins and Deeks 2011).

2.4 Assessment of the validity of the findings

A bias is a systematic error, which can lead to either underestimation or overestimation of the true intervention effect. Differences in risks of bias can help explaining the variation among results (the heterogeneity) of the studies included in a systematic review. The main types of "risk of bias" in case of a randomized controlled study are: *selection bias* (lack of allocation concealment/lack of randomization: those enrolling patients know about the group to which the next enrolled patient will be allocated), *performance bias* (the study personnel and/or included subjects are not blinded), *detection bias* (blinding of outcome assessment), *attrition bias* (incomplete outcome data addressed) and *reporting bias* (the reporting of some outcomes is incomplete or absent, while others are reported in detail in the Results section)(Higgins et al. 2011; Guyatt et al. GRADE 4. 2011).

2.5 Data synthesis and meta-analysis

Meta-analysis is the statistical combination of results from two or more separate studies. First, the type of data for the outcome measurements has to be identified. Five main types of data can be differentiated:

1. *dichotomous data*, where outcomes are one of two possible categorical responses;
2. *continuous data*, where outcomes can take any value on a numerical scale;
3. *ordinal data*, where the outcome is one of several ordered categories;
4. *counts and rates* calculated from counting the number of events that each individual experiences;
5. *time-to-event* (typically survival) *data* that analyze the time until an event occurs, but where not all individuals in the study experience the event .

Effect measures for continuous outcomes

The **mean difference (MD),** or more correctly, the difference in means, measures the absolute difference between the mean values in two groups. The **standardized mean difference (SMD)** is used when the studies all assess the same outcome but measure it in a variety of ways, for example on different scales. In this case it is necessary to standardize the results of these studies to an uniform scale before they can be combined.: $\text{SMD} = \frac{\text{Difference in mean outcome between groups}}{\text{Standard deviation of outcome among participants}}$.

Effect measures for counts and rates

The **rate ratio (RR)** compares the rate of events in the two groups by dividing one by the other. The **rate difference**, which measures the difference in rates, is used less common as a summary statistic (Deeks et al. 2011).

Meta-analysis is typically a two-stage process. In the first stage, the *effect measure* (summary statistic) is calculated for each study (for example the difference between means in case of continuous data, rate ratio in case of rates). In the second stage, a *summary (pooled) effect estimate* is calculated as a weighted average of the effects estimated in the individual studies. A weighted average is defined as $\text{weighted average} = \frac{\text{sum of (estimate} \times \text{weight)}}{\text{sum of weights}} = \frac{\sum Y_i W_i}{\sum W_i}$

where Y_i is the intervention effect estimated in the i study, W_i is the weight given to the i study, and the summation is across all studies. The bigger the weight given to the i study, the more it will contribute to the weighted average. For ratio measures (for example rate ratio), Yi is the natural logarithm of the measure.

Two types of statistical models can be used for combination of effect estimates across studies: a *random-effects meta-analysis* is preferred, in case the studies are not all estimating the same intervention effect (i.e. there is heterogeneity among studies that cannot readily be explained); while in the case each study is estimating

exactly the same quantity (i.e the effect in both magnitude and direction is the same value in every study) a *fixed-effect meta-analysis* is performed.

Heterogeneity

Heterogeneity means any kind of variability among studies in a systematic review, including clinical (variability in the participants), methodological (variability in study design and risk of bias) and statistical diversity (variability in the intervention effects).

In meta-analyses we often use the ***I^2-test*** for quantifying heterogeneity. First, a chi-squared (χ^2, or Chi^2) test is conducted to assess whether observed differences in results can be explained by chance alone. A low P value indicates heterogeneity of intervention effects. The I^2 test is a transformation of the Chi^2 test (Q):

$$I^2 = \left(\frac{Q - df}{Q}\right) \times 100\%,$$

where df is the degree of freedom for the Chi^2 test (Higgins and Thompson 2002).

I^2 test describes the percentage of variability in point estimates that is due to heterogeneity. I^2 test 0% to 40% may be interpreted as a level of heterogeneity which is not important, 30% to 60% may represent moderate, 50% to 90% may represent substantial, while 75% to 100% may represent considerable heterogeneity. The advantage of the test is that it does not depend on the number of studies included into the meta-analysis.

Subgroup analyses may be done as a means of investigating heterogeneous results (for example for different age groups of participants).

2.6 Presenting results

Forest plots are often used for visualizing results of the meta-analysis (**Figure 2**). A forest plot presents effect estimates and confidence intervals for both individual studies and meta-analyses (Lewis and Clarke 2001). The area of the block, representing point estimates, indicates the weight of that study in the meta-analysis while the horizontal line illustrates the confidence interval, usually with a 95% level of confi-

dence. The confidence interval indicates whether each intervention effect was individually statistically significant.

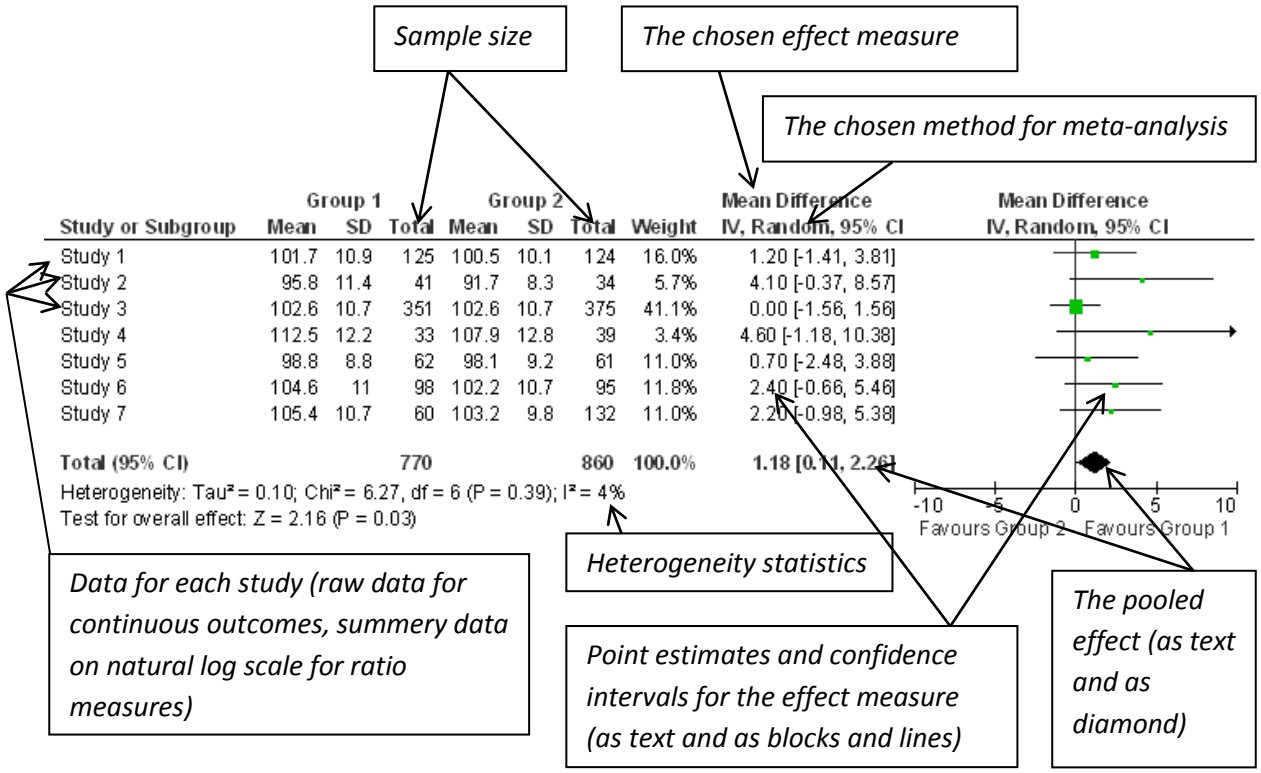

Figure 2. Details provided in a forest plot

3. Aims

1. Sex hormones may influence the activity of enzymes playing a role in the synthesis of long-chain polyunsaturated fatty acids. Our aim was to systematically review the gender-specific differences between women and men in the contribution of LCPUFAs to the fatty acid composition of the lipid pools most often reported in the literature for the characterization of fatty acid status, i.e. plasma phospholipids (PL), plasma cholesteryl esters (CE), plasma triacylglycerols (TG), total plasma lipids, erythrocyte and platelet lipids as well as subcutaneous adipose tissue from the abdomen or buttock.
2. The mainstream of phenylketonuria management is lifelong restriction of protein intake; however, this dietary restriction may be accompanied with insufficient dietary intake of long-chain polyunsaturated fatty acids. Our aim was to assess whether significant depletion of LCPUFA can be detected in phenylketonuria (PKU) patients on diet and whether LCPUFA supplementation is an effective way to increase the availability of LCPUFA in PKU patients.
3. Acute respiratory tract infections are the most common reason for people to seek medical help in developed countries. Strategies to prevent acute infections include the administration of different immunostimulants, vitamins and trace elements; recently, also probiotics were described to be effective in reducing the incidence rate of upper respiratory tract infections and that of diarrhea. In the case of probiotic supplementation, a large number (billions) of living probiotic bacteria have to be administered on a daily basis to ensure the continuous colonisation of the intestine and reach the desired health benefits; prebiotics may be an alternative and easier way to reach the same positive effects that usually are subscribed to probiotics. Our objective was to systematically review the available literature and to assess the efficacy of prebiotics in the prevention of acute infectious diseases in the pediatric age group.

4. Meta-analyses

4.1. Gender differences in long-chain polyunsaturated fatty acid status of healthy subjects: a systematic review and meta-analysis

4.1.1. Inclusion criteria

To be included into the review, a study needed to meet all of the following characteristics: 1. a study carried out in humans, 2. at least 14 participants included, 3. n–3 or n–6 LCPUFA status is reported in both males and females, 4. healthy individuals with normal weight were included, or population-based surveys was carried out in that the majority of participants were considered healthy, 5. omnivorous participants were included, 6. there was no dietary intervention (especially no lipid modified diet) or drug therapy before sample collection, 7. investigators measured at least 12 fatty acids by gas-liquid chromatography so the percentage distribution data contained the principal fatty acids of the fatty acid spectrum and presumably reflected realistic proportion of fatty acids in the given lipid fraction.

4.1.2. Search strategy

Electronic searches

Ovid MEDLINE (www.ovid.com), Scopus (www.scopus.elsevier.com), and the Cochrane Library CENTRAL database (www.thecochranelibrary.org) were searched from inception to February 2011 for studies containing LCPUFA values of both men and women (boys and girls) using text terms with appropriate truncation and relevant indexing terms. The search was in the form [n–3 LCPUFA terms] or [n–6 LCPUFA terms] and [biomarker terms] and [gender terms] and [differ*] and [human studies]. The results obtained by the full Ovid MEDLINE search strategy are shown in **Table1**. The searches of the two other databases were based also on this strategy. We did not apply any language restriction.

Table 1. Search strategy for Ovid MEDLINE from 1950 to February 2011

# ▲	Searche History	Results
1	exp Fatty Acids/	317728
2	fatty acid.mp.	70673
3	fatty acids.mp.	130870
4	omega-6.mp.	2800
5	omega6.mp.	164
6	omega-3.mp.	8847
7	omega3.mp.	345
8	PUFA.mp.	4608
9	PUFAs.mp.	1865
10	LC-PUFA.mp.	183
11	LC-PUFAs.mp.	74
12	LCPUFA.mp.	257
13	LCPUFAs.mp.	101
14	polyunsaturated.mp.	18317
15	poly-unsaturated.mp.	253
16	1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15	359501
17	exp Docosahexaenoic Acids/	4233
18	"docosahexaenoic acid".mp.	5362
19	"docosahexanoic acid".mp.	147
20	"docosahexenoic acid".mp.	32
21	DHA.mp.	6023
22	exp Eicosapentaenoic Acid/	3411
23	"eicosapentaenoic acid".mp.	5329
24	"eicosapentanoic acid".mp.	120
25	"eicosapentenoic acid".mp.	10
26	EPA.mp.	6841
27	"arachidonic acid".mp.	33150
28	"linolenic acid".mp.	5411
29	"linoleic acid".mp.	11898
30	17 or 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25 or 26 or 27 or 28 or 29	56012
31	16 or 30	371034
32	exp Plasma/	12829
33	plasma.mp.	603550
34	exp Serum/	57441
35	serum.mp.	698887
36	exp Erythrocytes/	142985
37	erythrocyte.mp.	84213
38	erythrocytes.mp.	139866

39	"red blood cell".mp.	17930
40	"red blood cells".mp.	28325
41	RBC.mp.	13721
42	"RBCs".mp.	5881
43	"red blood corpuscle".mp.	17
44	"red blood corpuscles".mp.	111
45	exp Blood Platelets/	60845
46	platelet.mp.	153311
47	platelets.mp.	85559
48	"thrombocyte".mp.	2736
49	"thrombocytes".mp.	3009
50	exp Granulocytes/	107967
51	"granulocyte".mp.	41550
52	"granulocytes".mp.	27420
53	"peripheral blood mononuclear cell".mp.	2202
54	"peripheral blood mononuclear cells".mp.	26148
55	"PBMC".mp.	13087
56	"PBMCs".mp.	6225
57	exp Lipoproteins, LDL/	37206
58	LDL.mp.	61778
59	exp Lipoproteins, HDL/	30523
60	HDL.mp.	48739
61	exp Adipocytes/	10869
62	"adipocyte".mp.	9666
63	"adipocytes".mp.	19481
64	exp Adipose Tissue/	60132
65	"adipose tissue".mp.	69064
66	32 or 33 or 34 or 35 or 36 or 37 or 38 or 39 or 40 or 41 or 42 or 43 or 44 or 45 or 46 or 47 or 48 or 49 or 50 or 51 or 52 or 53 or 54 or 55 or 56 or 57 or 58 or 59 or 60 or 61 or 62 or 63 or 64 or 65	1802378
67	31 and 66	105882
68	(male and female).mp.	3607668
69	(males and females).mp.	120913
70	(men and women).mp.	153945
71	(boys and girls).mp.	31298
72	gender.mp.	131454
73	sex.mp.	478441
74	68 or 69 or 70 or 71 or 72 or 73	3736338
75	67 and 74	23001
76	differ$.mp.	3509153
77	75 and 76	7543
78	limit 77 to humans	6309

4.1.3. Data extraction

Titles and abstracts found by the electronic and bibliographic searches were screened for inclusion by a single reviewer (SL). Because there were a large number of papers which were present in more than one databases, duplications were filtered out to compile the final list of titles and abstracts to be screened. Thereafter it was checked which of the titles and abstracts meet the predetermined eligibility criteria. If articles seemed to meet the inclusion criteria, or the title and the abstract left room for doubt, the full text of the article was evaluated by two independent reviewers (SL and KF). If the two reviewers disagreed about the eligibility, the study was discussed in detail to reach a consensus decision.

Data for each study included were extracted by a single reviewer (SL) into a Microsoft Office Excel 2007 database file. To provide a standardized format, units of measurement were recalculated to percentage contribution of LCPUFA to total fatty acid composition of the relevant tissue (% weight/weight) from original data in the publication. If it was not possible to convert data, we tried to contact the authors. If the authors couldn't be contacted (in most of the cases because of the long time elapsed since the publication of the papers), or the data were not available in the original form any more, those studies were excluded. The original forms in which fatty acid data in the included studies were expressed are shown in Table 1. In some studies the investigators measured the fatty acid composition in plasma, in other studies in serum. Because we feel that there is no major difference in the percentage fatty acid composition of plasma and serum, for the uniformity of discussion, we use the term 'plasma' throughout the text.

4.1.4. Statistical analysis

Statistical analyses were performed using the Review Manager 5.1 Software (The Cochrane Collaboration, Oxford, United Kingdom). Mean differences (MD) was used for the analysis of continuous data. The confidence interval (CI) was established at 95%. P values of less than 0.05 were considered to indicate statistical significance.

Statistical heterogeneity was assessed using the I^2 statistics (I^2 of 50% or more indicating presence of heterogeneity).

4.1.5. Results

Study inclusion

The flow diagram of the literature search for this review is shown on **Figure 3**.

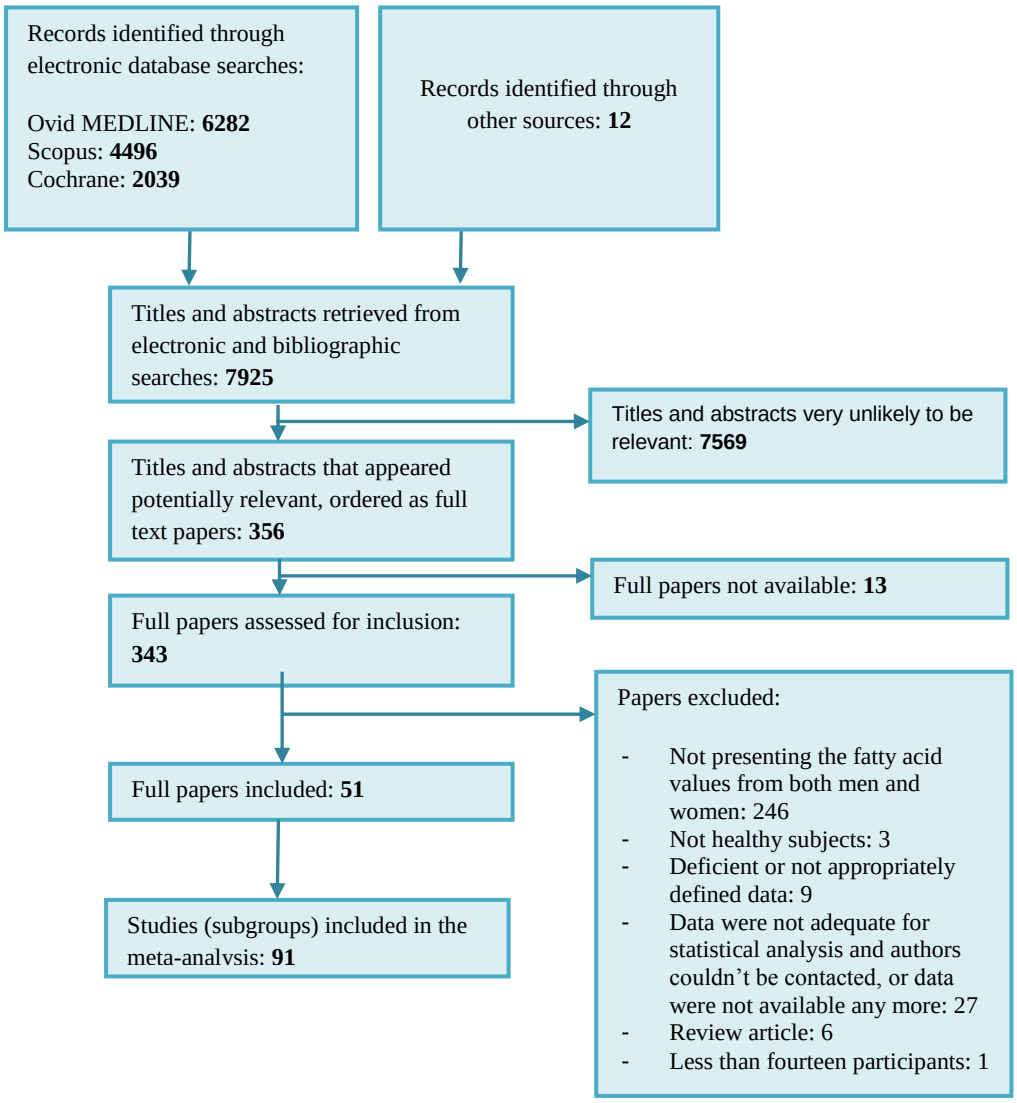

Figure 3. Flow diagram of the systematic literature search

Methods used in the studies reviewed

Among the studies reviewed there were some methodological differences in the analytical methods used for the determination of fatty acid composition of the different biomarkers. The blood samples collected were stored in a deep frozen state, but the storage temperature was different (-20ºC, -30 ºC, -40 ºC, -70 ºC or -80 ºC). Lipid extraction was carried out by chloroform and methanol in most, but not all the studies. The separation of different lipid fractions was performed by thin layer chromatography. Fatty acid analysis was carried out by gas-liquid chromatography in all studies. In some studies a packed column was used instead of capillary columns (capillary column: 34 papers, packed column: 10 papers, column type not reported: 7 papers)

Biomarkers identified

We found 11 publications analyzing plasma PLs, whereas 8 analyzed plasma CEs, 5 plasma TGs, 18 total plasma lipids, 9 total erythrocyte membrane lipids, 1 platelets and 7 adipose tissue fatty acid composition. A description of biomarkers identified in three or more studies, including also the number of studies, subgroups, participants from both gender, and the results of the primary analysis (MD, I^2) is presented in **Table 3**. We discuss in detail only these biomarkers. Descriptive data of biomarkers detected in less than three studies are presented in **Table 2**.

Fatty acids reported

In this study we focused on the following 8 PUFAs: LA, gamma-linolenic acid (GLA), dihomo-gamma-linolenic acid (DHGLA) and AA from the n-6 series and ALA, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and DHA from the n-3 series.

Table 2: Basic characteristics of included studies

First author, publication year	Participant characteristics					Biomarkers reported[1]	Fatty acids reported	Original expression of data
	Country	No. of included male subjects	No. of included female subjects	Age of males	Age of females			
Ando, 1990 [5]	Japan	31[a] 29 37	68 45 22	54.3±7.4 y 59.8±4.9 y 64.9±7.3 y	57.0±8.6 y 59.0±6.0 y 68.3±8.8 y	plasma total lipids	LA, DHGLA, AA, EPA, DHA	mg/dl (mean, SD)
Antonini, 1970 [6]	Italy	11	11	29±6.6 y	27.4±6.2 y	adipose tissue	LA	w/w% (mean, SD)
Araki, 1990 [7]	Japan	27[b] 57	110 121	20–49 y 50–79 y	20–49 y 50–79 y	plasma total lipids	LA, AA, EPA, DHA	w/w% (mean, SD)
Bakewell, 2006 [8]	United Kingdom	13	23	26±5 y	23±4 y	plasma total lipids, plasma TG, plasma FFA, plasma PC, plasma CE	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w % (mean, SD)
Bolton-Smith, 1997 [9]	Scotland	2308	2049	40–59 y	40–59 y	adipose tissue	LA, GLA, DHGLA, AA	w/w% (mean, SD)
Brouwer, 1997 [10]	The Netherlands (Curacao)	51	26	56±8 y	58±5 y	plasma CE	LA, GLA, DHGLA, AA, ALA, EPA, DHA	mol% (mean, SD)
Cheng, 2003 [11]	Taiwan	10[c] 10[d]	10 10	10–11 y 10–11 y	10–11 y 10–11 y	plasma TG	LA	w/w% (mean, SEM)
Christensen, 1999 [12]	Denmark	35	25	38±10 y	38±11 y	granulocytes, platelet	LA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
di Giuseppe, 2009 [13]	Italy, Belgium, United Kingdom	50	224	47±1.1 y	44±0.5 y	plasma total lipids, erythrocyte membrane	ALA, EPA, DPA, DHA	w/w% (geometric mean, SEM or 95% CI)
Elizondo-Montemayor, 2010 [14]	Mexico	49	51	6–12 y	6–12 y	plasma PL	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
Fernandez-Real, 2001 [15]	Spain	38	40	40.1±13.3 y	38.1±9.3 y	plasma total lipids	LA, DHGLA, AA, EPA, DHA	w/w% (mean, SD)
Geppert, 2010 [16]	United Kingdom	40	34	32.6±8.0 y	32.7±7.3 y	platelet PC, platelet PEA	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
Giltay, 2004 [17]	The Netherlands	72	71	29.6±12.9 y	27.4±10.2 y	plasma CE	LA, GLA, DHGLA, AA, ALA, EPA, DHA	w/w% (mean, 95% CI)
Glew, 2010 [18]	Nigeria	22	29	55.5±13.5 y	47.6±8.3 y	plasma PL	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)

Glew, 2002 [19]	Nigeria	37	36	14 y	13 y	plasma PL	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
Hagenfeldt, 1975 [20]	Sweden	6	8	26–35 y	23–31 y	plasma FFA	LA	w/w% (mean, SEM)
Hirai, 2005 [21]	Japan	76	76	68.6±10.6 y	67.8±11.2 y	plasma total lipids	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	μg/ml (mean, SD)
Hirai, 2000 [22]	Japan, The Netherlands	33[f] 20[g]	29 19	university students	university students	plasma total lipids	LA, AA, ALA, EPA, DHA	mg/100ml (mean, SD)
Hodge, 2007 [23]	Australia, United Kingdom, Italy, Greece	2048	2391	55±8.8 y	54.4±8.5 y	plasma PL	LA, AA, ALA, EPA, DHA	w/w% (mean, SD)
Innis, 1988 [24]	Canada	11[h] 9[h] 41[h] 14[h] 12[i]	17 12 59 13 12	11–15 y 16–20 y 21–50 >50 y 21–50 y	11–82 y 16–20 y 21–50 >50 y 21–50 y	erythrocyte PC, erythrocyte PEA	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SEM)
Iwamoto, 2002 [25]	Japan	20	20	adults	adults	plasma CE	LA, AA, EPA, DHA	mol% (mean, SEM)
Jagannathan, 1969 [26]	India	27	15	22–50 y	26–45 y	adipose tissue	LA	w/w% (mean, SEM)
Kale, 2008 [27]	India	25	21	33.6±9.7 y	35.1±8.0 y	erythrocyte membrane	LA, GLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
Karlsson, 2006 [28]	Sweden	6	9	11.3–15.4 y	11.7–14.5 y	plasma PL	LA, GLA, DHGLA, AA, ALA, EPA, DHA	mol% (mean, SD)
Kieu, 2002 [29]	South Vietnam	32[d] 40[e] 39[c]	68 58 59	47.5±5.5 y 46.6±5.0 y 46.4±4.7 y	47.5±5.2 y 47.6±6.4 y 47.2±5.9 y	plasma total lipids	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
Kuriki, 2003 [30]	Japan	15	79	45.3±10.6 y	47.2±8.1 y	plasma total lipids	LA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
Lee, 2000 [31]	Hong Kong	81	113	43.4±11.2 y	43.8±11.6 y	plasma total lipids	LA, GLA, AA, ALA, EPA, DHA	w/w% (mean, SD)
Lemaitre, 2008 [32]	Israel	112[b] 49 44	118 42 52	<45 y 45–60 y ≥60 y	<45 y 45–60 y ≥60 y	erythrocyte membrane	LA, DHGLA, AA, EPA, DHA	w/w% (mean, SD)
Lucas, 2009 [33]	Canada	127	170	18–74 y	18–74 y	plasma PL	LA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
Mamalakis, 2006 [34]	Greece	59	71	37.7±7.9 y	36.2±6.7 y	adipose tissue	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)

Mamalakis, 1998 [35]	Greece	85	59	23–69 y	23–69 y	adipose tissue	LA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
McNamara, 2010 [36]	USA	10	10	35.9±8.8 y	36.1±9.3 y	erythrocyte membrane	LA, DHGLA, AA, EPA, DPA, DHA	w/w% (mean, SEM)
Melchert, 1987 [37]	Germany	38	70	21–77 y	18–85 y	plasma total lipids, HDL	LA, GLA, DHGLA, AA, ALA, DHA	w/w% (mean, SD)
						plasma CE	LA, GLA, DHGLA, AA, ALA, EPA, DHA	
						plasma TG	LA, GLA, AA, ALA, DHA	
						plasma FFA	LA, DHGLA, AA, ALA, DHA	
Metherel, 2009 [38]	Canada	9	7	22.4±1.2 y	22.1±1.8 y	whole blood, plasma total lipids, erythrocyte membrane, fingertip prick blood	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
Mitchell, 1983 [39]	New Zealand	9	9	10–13 y	10–13 y	erythrocyte membrane	LA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
Nakamura, 1995 [40]	Japan	18[b]	13	30–39 y	30–69 y	plasma total lipids	LA, AA, EPA, DHA	w/w% (mean, SD)
		13	13	40–49 y	40–49 y			
		12	15	50–59 y	50–59 y			
		12	14	60–69 y	60–69 y			
Nikkari, 1995 [41]	Finland	41	41	43±4.3 y	40±4.3 y	plasma PL	LA, GLA, DHGLA, AA, EPA, DHA	w/w% (mean, SD)
						plasma CE	LA, GLA, DHGLA, AA, ALA, EPA, DHA	
						plasma TG	LA, AA, ALA, DHA	

Saadatian-Elahi, 2009 [42]	Greece	91[j]	100	45–64 y	45–64 y	plasma PL	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
	Spain	93	100					
	Spain	96	100					
	Spain	94	100					
	Italy	90	99					
	Italy	94	99					
	Italy	96	100					
	Germany	95	96					
	Germany	96	99					
	Netherlands	95	100					
	UK	95	100					
	Denmark	96	100					
	Sweden	100	95					
	Sweden	94	99					
Sfar, 2010 [43]	Tunesia	96	104	55.7±13.1 y	53.1±13.7 y	plasma total lipids	LA, AA, ALA, EPA, DHA	w/w% (mean, SD)
Smit, 2003 [44]	The Nether-lands	29	34	22–49 y	22–47 y	erythrocyte membrane	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	mol% (mean, SD)
Sutherland, 1995 [45]	Fiji	39[c]	44	39±16 y	36±15 y	erythrocyte membrane	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD)
		37[d]	34	38±14 y	43±18 y			
Takita, 1996 [46]	Japan	28[b]	15	20–29 y	20–29 y	plasma total lipids	LA, AA, EPA, DHA	w/w% (mean, SD)
		87	58	30–39 y	30–39 y			
		81	48	40–49 y	40–49 y			
		20	30	50–59 y	50–59 y			
		17	10	60–69 y	60–69 y			
Tavendale, 1992 [47]	Scotland	529[b]	518	40–44 y	40–44 y	adipose tissue	LA, GLA, DHGLA, AA, DPA, DHA	w/w% (mean, SD)
		508	469	45–49 y	45–49 y			
		593	479	50–54 y	50–54 y			
		555	463	55–59 y	55–59 y			
Tjønneland, 1993 [48]	Denmark	23	63	42–63 y	40–63 y	adipose tissue	LA, GLA, DHGLA, AA, ALA, EPA, DHA	w/w% (mean, SD)
Umemura, 2005 [49]	Japan	175	246	19.4±1 y	19.2±0.5 y	plasma total lipids	LA, GLA, DHGLA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SD
Vallés, 1988 [50]	Spain	49	49	16–75 y	16–75 y	plasma PL, plasma TG, plasma FFA, plasma CE, platelet PL, platelet TG, platelet FFA	LA, EPA	w/w% (mean, SD)
Warensjö, 2006 [51]	Sweden	554	295	40.6±9.1 y	40.6±9.9 y	plasma CE	LA, GLA, DHGLA, AA, ALA, EPA, DHA	w/w% (mean, SD)
Wennberg, 2011 [52]	Sweden	308	248	53±7.7 y	58±7.2 y	plasma PL	ALA, EPA, DPA, DHA	w/w% (mean, range)

Wennberg, 2007 [53]	Sweden	288	207	55.2±7.5 y	55.4±7.6 y	plasma PL, erythrocyte membrane	ALA, EPA, DPA, DHA	w/w% (median, SD)
Yamada, 2000 [54]	Japan	107[k] 78[l]	154 124	30–89 y	30–89 y	plasma total lipids	LA, AA, ALA, EPA, DPA, DHA	w/w% (mean, SEM)
Yeh, 1996 [55]	Nigeria	110[m] 126[n]	65 96	42.4±8.9 y 39.7±10.3 y	41.4±8.1 y 35.4±9.1 y	plasma total lipids	LA, GLA, DHGLA, AA, ALA, EPA, DHA	mol% (mean, SD)

Abbreviations:
[*], mean±SD; [**], range.
a, groups classified by cluster analysis; b, groups classified by age range; c, urban region; d, rural region; e, suburban region; f, Japanese group; g, Dutch group; h, Inuit group classified by age range; i, Vancouver group; j, groups classified by geographic areas (Athens, Granada, Murcia, North Spain, Ragusa/Naples, Florence, Varese/Turin, Heidelberg, Potsdam, The Netherlands, Cambridge, Denmark, Malmö, Umeå); k, fishing group; l, farming group; m, senior staff group; n, junior staff group.
LA, linoleic acid; GLA, gamma-linolenic acid; DHGLA, dihomo-gamma-linolenic acid; AA, arachidonic acid; ALA, alpha-linolenic acid;EPA, eicosapentaenoic acid; DPA,docosapentaenoic acid; DHA, docosahexaenoic acid, TG, triacylglycerol; PC, phosphatidylcholine; CE cholesteryl ester; PEA, phospatidylethanolamine; FFA, free fatty acids; PL, phospholipid.

Table 3: Primary analysis of biomarkers identified in three or more studies

		No. of individual studies	No. of participants		MD [95% CI]	Heterogenity (I^2)
			Men	Women		
total plasma lipids	**LA**	32	1594	2012	-1.55 [-1.96, -1.14][a]	40%
	GLA	11	738	881	-0.33 [-0.07, 0.00]	78%
	DHGLA	14	792	943	-0.05 [-0.17, 0.06]	92%
	AA	32	1594	2012	-0.22 [-0.37, -0.07][a]	60%
	ALA	18	1137	1614	-0.03 [-0.06, 0.00]	60%
	EPA	32	1607	2166	0.02 [-0.07, 0.11]	69%
	DPA	11	634	1118	0.06 [0.03, 0.09][b]	55%
	DHA	33	1644	2236	-0.12 [-0.22, -0.03][a]	46%
plasma phospholipids	**LA**	22	3704	4163	-0.19 [-0.43, 0.05]	50%
	GLA	19	1480	1553	0.00 [-0.00, 0.01]	36%
	DHGLA	19	1480	1553	-0.07 [-0.15, 0.01]	56%
	AA	21	3655	4114	-0.42 [-0.65, -0.18][a]	81%
	ALA	22	4052	4403	-0.00 [-0.01, 0.00]	72%
	EPA	24	4146	4493	-0.03 [-0.08, 0.02]	54%
	DPA	20	2002	2003	0.01 [-0.01, 0.04]	77%
	DHA	23	4097	4444	-0.37 [-0.51, -0.24][a]	79%
plasma cholesteryl esters	**LA**	8	805	530	-1.03 [-1.84, -0.21][a]	45%
	GLA	6	736	461	0.08 [0.03, 0.14][b]	32%
	DHGLA	6	736	461	0.06 [0.02, 0.09][b]	49%
	AA	7	756	481	0.21 [-0.24, 0.66]	79%
	ALA	6	736	461	-0.01 [-0.03, 0.00]	0%
	EPA	8	805	530	0.01 [-0.07, 0.10]	55%
	DHA	7	756	481	0.02 [-0.08, 0.04]	68%
plasma triacylglycerols	**LA**	6	128	138	0.35 [-0.81, 1.53]	57%
	AA	3	59	69	0.07 [-0.13, 0.28]	76%
	ALA	3	59	69	-0.03 [-0.20, 0.13]	0%
	DHA	3	59	69	-0.12 [-0.57, 0.32]	68%
erythrocyte membrane lipids	**LA**	10	363	371	0.05 [-0.19, 0.30]	0%
	GLA	5	139	140	-0.00 [-0.01, 0.01]	8%

	DHGLA	9	338	350	-0.02 [-0.12, 0.08]	18%
	AA	10	363	371	-0.41 [-0.86, 0.05]	64%
	ALA	8	480	577	0.01 [-0.02, 0.04]	69%
	EPA	12	701	802	0.03 [-0.00, 0.07]	27%
	DPA	9	495	590	0.14 [-0.05, 0.33]	79%
	DHA	12	698	802	-0.19 [-0.31, -0.06][a]	3%
erythrocyte phosphatidyl-choline	**LA**	5	87	113	-0.11 [-2.61, 2.38]	39%
	GLA	5	87	113	0.09 [-0.23, 0.41]	43%
	DHGLA	5	87	113	-0.18 [-0.40, 0.04]	0%
	AA	4	46	54	-1.88 [-4.05, 0.29]	0%
	ALA	3	61	88	-0.01 [-0.42, 0.40]	0%
	EPA	5	87	113	-0.41 [-0.74, -0.08][a]	0%
	DPA	5	87	113	0.32 [-0.05, 0.69]	0%
	DHA	5	87	113	-0.25 [-1.30, 0.80]	62%
erythrocyte phosphatidyl-ethanolamine	**LA**	5	87	113	0.26 [-0.58, 1.09]	0%
	GLA	4	78	101	0.02 [-0.13, 0.18]	0%
	DHGLA	5	87	113	-0.12 [-0.37, 0.13]	25%
	AA	5	87	113	0.12 [-2.08, 1.85]	38%
	ALA	5	87	113	0.08 [-0.15, 0.32]	0%
	EPA	5	87	113	0.01 [-1.10, 1.13]	30%
	DPA	5	87	113	0.25 [-0.38, 0.88]	6%
	DHA	5	87	113	0.21 [-1.16, 1.57]	0%
adipose tissue	**LA**	10	4698	4197	-0.49 [-0.60, -0.38][a]	0%
	GLA	7	4575	4112	0.03 [0.01, 0.04][b]	92%
	DHGLA	8	4660	4171	-0.04 [-0.05, -0.03][a]	93%
	AA	8	4660	4171	0.03 [0.02, 0.05][b]	74%
	ALA	3	167	193	0.01 [-0.02, 0.03]	0%
	EPA	3	167	193	0.01 [0.01, 0.02][b]	0%
	DPA	6	2329	2059	-0.01 [-0.04, 0.02]	97%
	DHA	7	2352	2122	-0.01 [-0.04, 0.01]	96%

Abbreviations: a, significantly higher ($p < 0.05$) in women than in men; b, significantly higher ($p < 0.05$) in men than in women.

Fatty acid composition of total plasma lipids

Primary analysis showed significantly higher contribution of the n-6 essential fatty acid, LA and the n-6 long-chain metabolite, AA to plasma total lipids of women compared to men (**Figure 4, Table 3**).

As to n-3 fatty acids, the values of the principal LCPUFA, DHA were significantly higher (**Figure 5**), while the values of its precursor, DPA were significantly lower in women compared to men (**Table 3**). However, with the exception of LA and DHA, considerable heterogeneity was seen among the results of the individual studies.

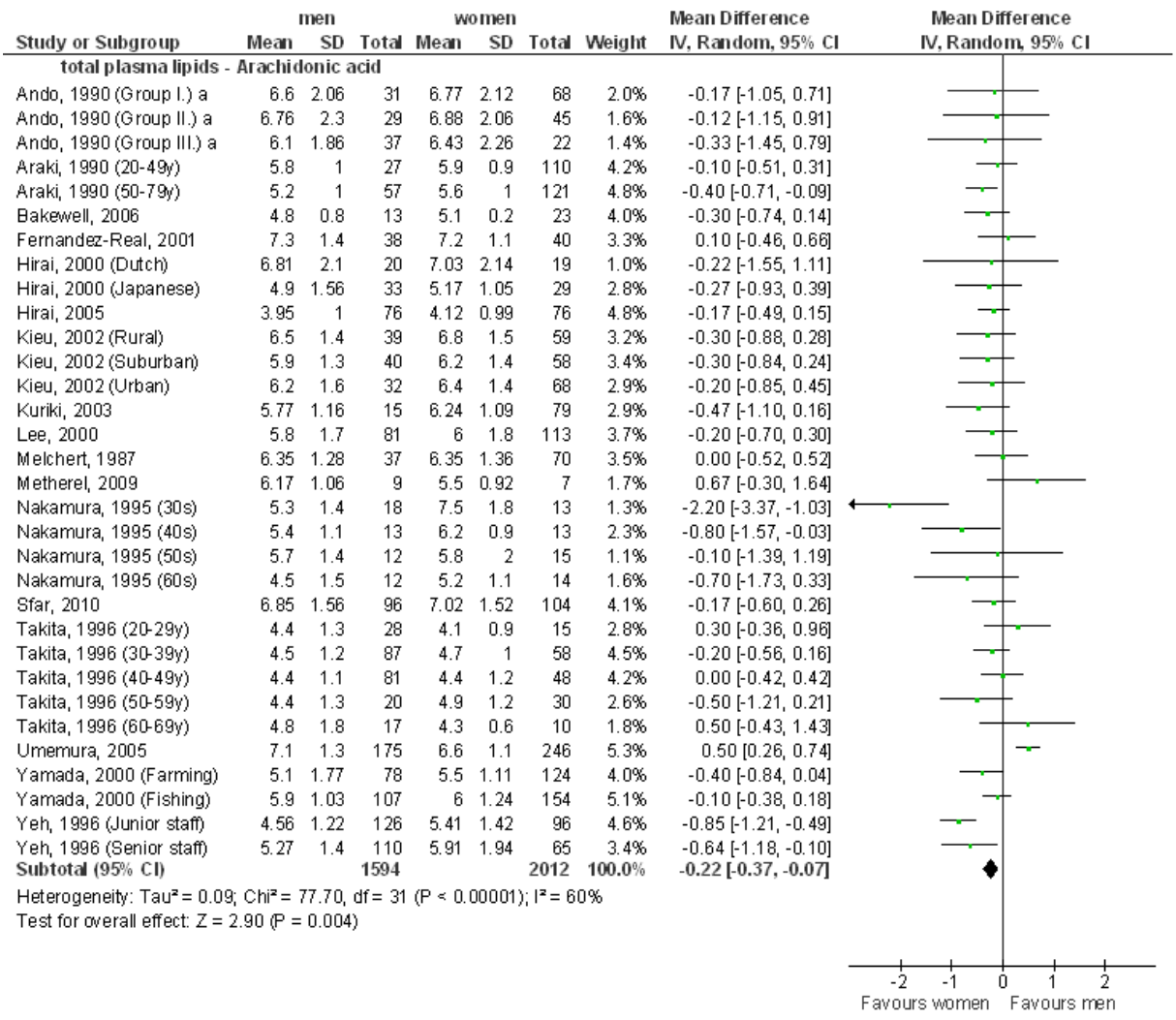

Figure 4. Mean difference in the percent contribution of AA to total plasma lipids of healthy male and female subjects

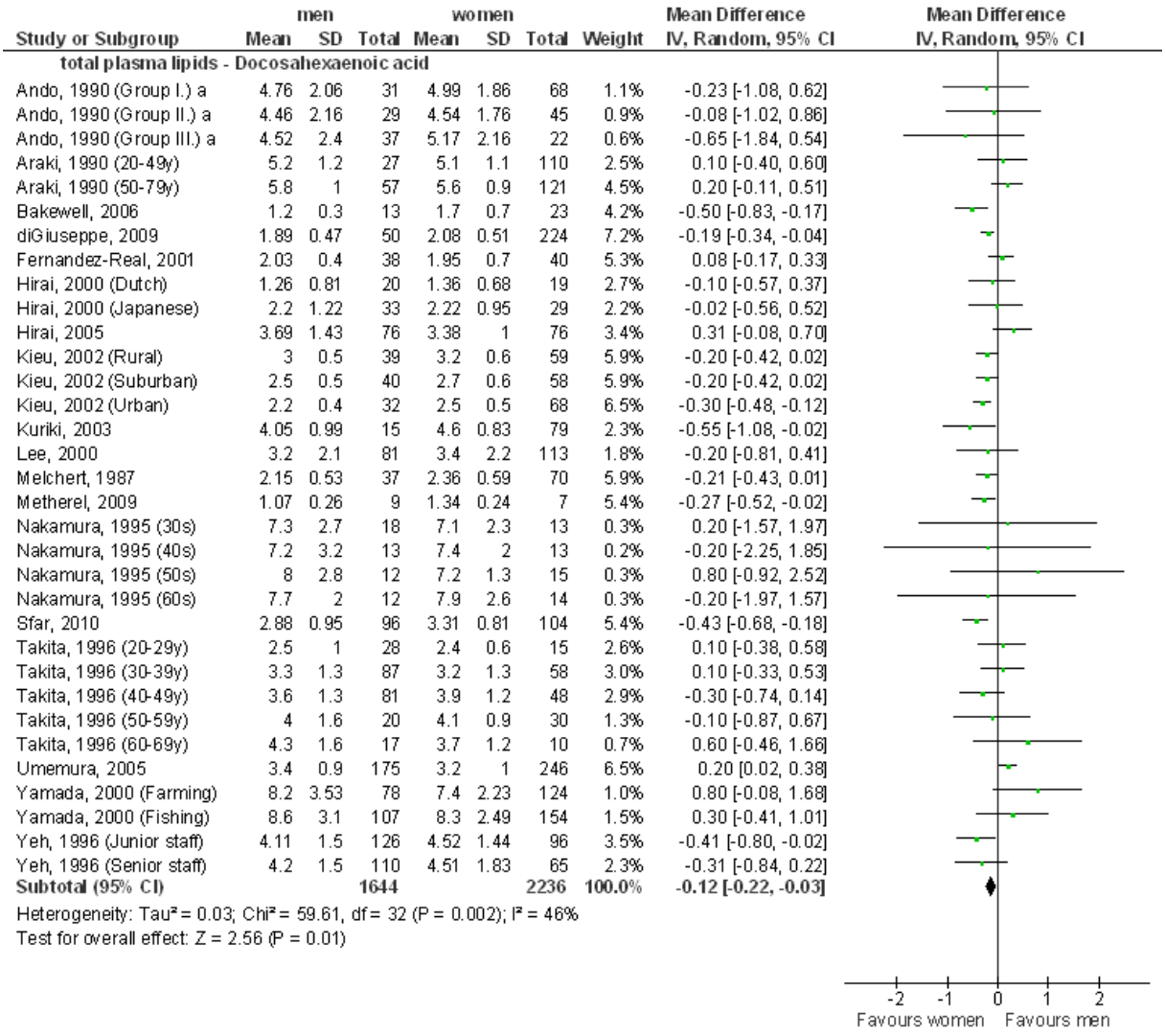

Figure 5. Mean difference in the percent contribution of DHA to total plasma lipids of healthy male and female subjects

To filter out the potential effect of age, we tried to classify the individual studies into three age categories: a) 0 to12 years, b) 13 to 50 years, and c) 51 years or more. In the case of plasma total lipids classification according to age categories yielded sufficient number of studies to evaluate the effect of gender in the two adult groups only. Among the long-chain metabolites, in the 13 to 50 years group only the values of DHA (MD: -0.16; 95% CI: -0.26, -0.06; 2418 participants; I^2 = 45%) were significantly higher in women, while the values of DPA were significantly lower in women compared to men (MD: 0.07; 95% CI: 0.03, 0.01; 1130 participants; I^2 = 67%). In the > 51 years age

group (containing postmenopausal women) the values of the n-6 LCPUFAs, DHGLA (MD: -0.14; 95% CI: -0.24, -0.04; 137 participants; I^2 = 19 %) and AA (MD: -0.25; 95% CI: -0.43, -0.08; 892 participants; I^2 = 0%) were significantly higher in women compared to men, whereas DHA values did not differ between the two genders.

To consider the potential effect of diet, we classified the studies based on the results of Meyer BJ (2011) according to fish eating habits, assigning the Inuit of Nunavik and the Japanese as high n-3 LCPUFA intake group and all other people as low n-3 LCPUFA intake group. In the high fish (n-3 LCPUFA) consuming group values of AA were significantly higher in women compared to men (MD: -0.23; 95% CI: -0.44, -0.02; 2291 participants; I^2 = 68%), while there was no significant difference in the DHA levels between the two groups (MD: -0.00; 95% CI: -0.12, 0.12; 2291 participants; I^2 = 17%). In the low fish consuming group both AA (MD: -0.24; 95% CI: -0.41, -0.07; 1383 participants; I^2 = 8%) and DHA (MD: -0.24; 95% CI: -0.31, -0.16; 1598 participants; I^2 = 11%) were significantly higher in women.

Fatty acid composition of plasma phospholipids

Plasma PL compositional data were reported for the largest number of men (n = 4097) and women (n = 4444). There was one cross-sectional study among the included articles (Saadatian-Elahi 2009), that contained 16 subgroups stratified according to geographic areas; we were able to include 14 subgroups (in one area only women were recruited, whereas another subgroup had to be excluded, because there were also vegans among the participants).

Primary analysis revealed significantly higher contribution of both AA (MD: -0.42; 95% CI: -0.65, -0.18; 7769 participants; I^2 = 81%) and DHA (MD: -0.37; 95% CI: -0.51, -0.24; 8541 participants; I^2 = 79%) to the fatty acid composition of plasma PLs in women (**Figures 6 and 7**), while in LA and ALA there was no gender difference. There were not enough studies to carry out subgroup analysis either by age or fish eating habits.

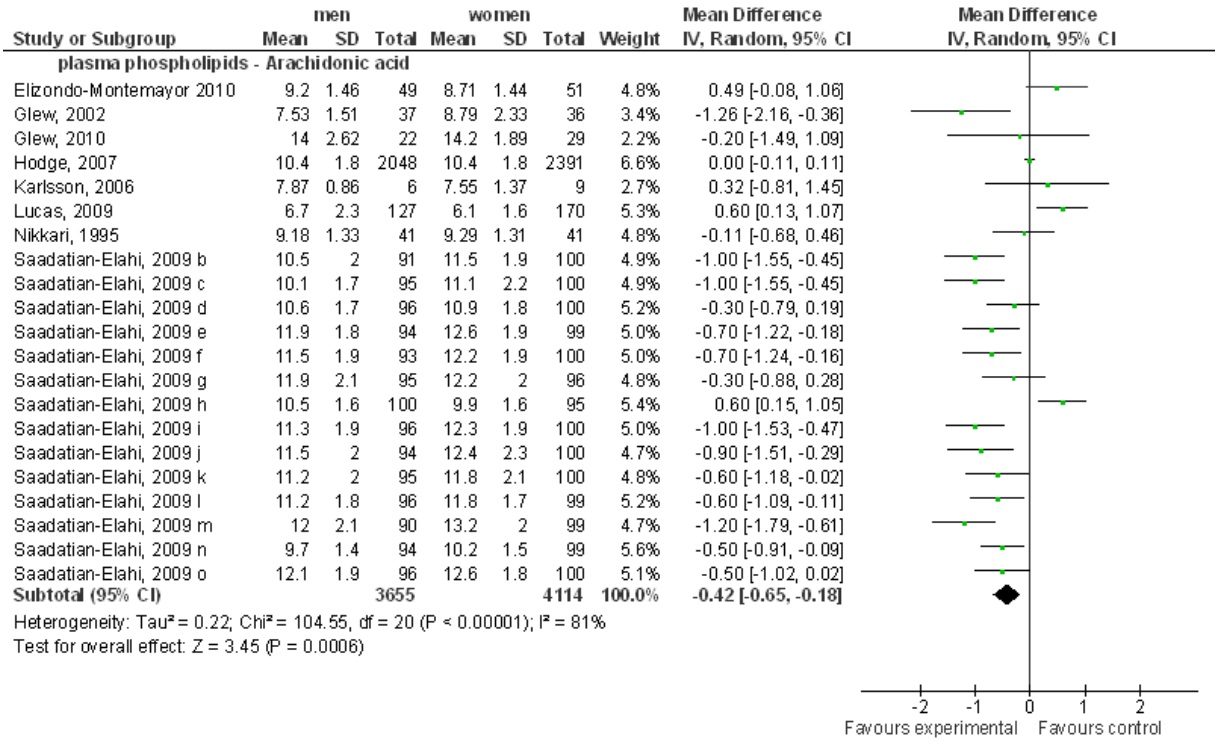

Figure 6. Mean difference in the percent contribution of AA to plasma PL of healthy male and female subjects

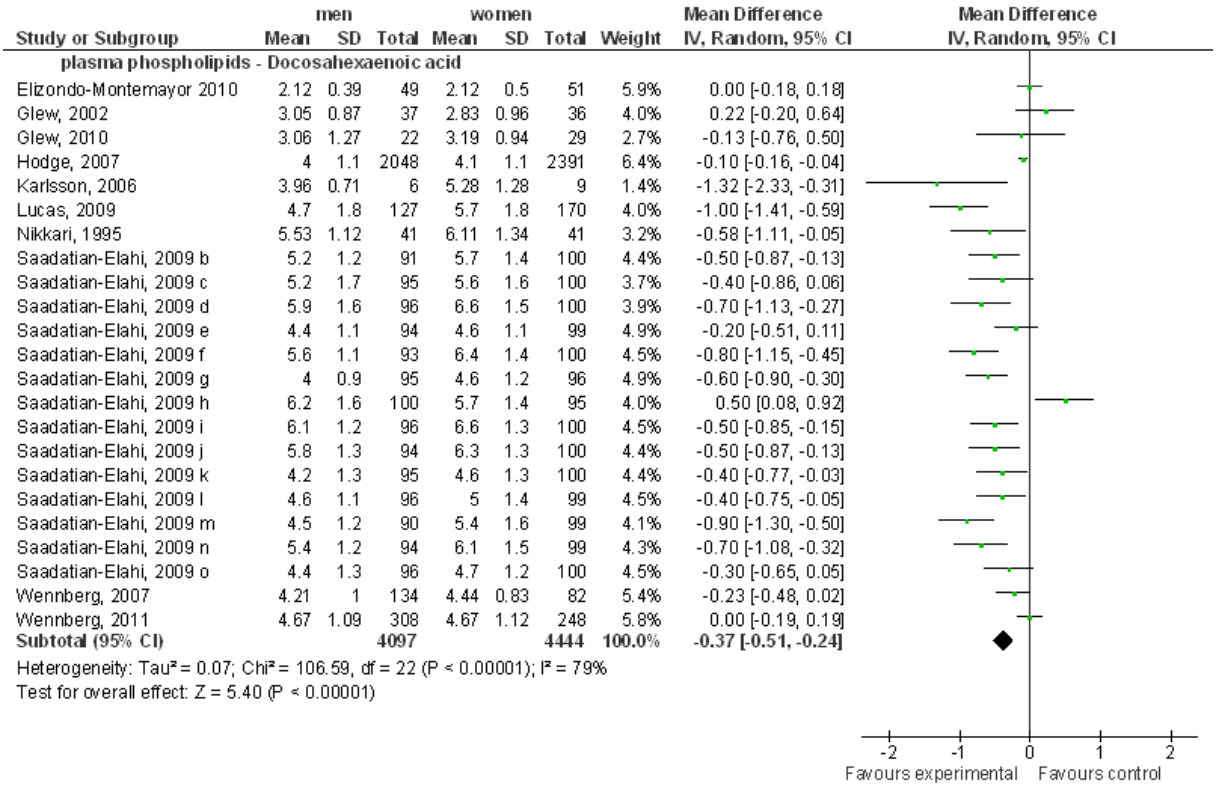

Figure 7. Mean difference in the percent contribution of DHA to plasma PL of healthy male and female subjects

Fatty acid composition of plasma cholesteryl esters

Plasma CE fatty acids were reported in 8 publications. There was no gender difference in AA and DHA values (**Table 3**), but GLA and DHGLA were found significantly higher in men as compared to women.

Fatty acid composition of plasma triacylglycerols

Five publications reported fatty acid composition of plasma TG in both men and women. The primary analysis showed no difference between the two sexes in any of the fatty acids discussed (**Table 3**).

Fatty acid composition of erythrocyte membrane total lipids

There were 9 publications reporting fatty acid composition of the erythrocyte membrane total lipids in both sexes. The primary analysis showed significant difference only in DHA values, which were higher in women than in men (Table 2). Because most of these studies were carried out in participants older than 50 years, in spite of the apparent abundance of data (12 studies with 1224 participants), there was no possibility to carry out subgroup analysis either by age or fish eating habits.

Fatty acid composition of erythrocyte phosphatidylcholine and phosphatidylethanolamine

There was one paper reporting erythrocyte phosphatidylcholine and phosphatidylethanolamine fatty acid composition in 5 subgroups [24]. In erythrocyte PC the values of EPA were significantly higher in women than in men, whereas in erythrocyte PEA there was no difference between the two sexes in any of the fatty acids discussed (**Table 3**).

Fatty acid composition of adipose tissue

In adipose tissue the values of LA and DHGLA were significantly higher in women than in men, while the values of GLA, AA, and from the n-3 series the values from EPA were significantly higher in men than in women.

4.1.6. Discussion

The endogenous synthesis of AA and DHA from their essential fatty acid precursors, LA and ALA require the contribution of elongases and desaturases. Since the elongation steps are rapid, whereas the desaturation steps are slower, the latter are considered as the rate limiting steps. In this systematic review we found higher contributions of the LCPUFAs AA and DHA to both plasma total lipids and plasma PL in women than in men. We also found significantly higher values of DHA, but not that of AA in women than in men in erythrocyte lipids. It may be assumed with good reason that the higher AA and DHA values found in plasma PLs of women may be due to the higher activity of desaturases, especially that of delta-6-desaturase.

The effects of sex hormones on essential fatty acid metabolism in humans have already been reviewed in detail by Childs et al (Childs et al. 2008). In short, human studies demonstrated that males and females differ in their ability to synthesize n-3 LCPUFA from ALA, leading to the higher circulating concentrations of DHA in women than in men (Childs et al. 2008). In the same review significant relationship between plasma and tissue fatty acid composition and circulating sex hormone concentrations was seen, suggesting that estrogen stimulates, whereas testosterone inhibits the conversion of essential fatty acids into their longer-chain metabolites. This is consistent with findings of both animal studies (Extier et al. 2010) and human stable-isotope studies, which also showed that women have a higher capacity than men to synthesize DHA from ALA (Burdge and Wootton 2002; Pawlosky et al. 2003).

We also tried to evaluate the influence of age on the gender differences in fatty acid composition of biological samples. Since previously several authors suggested that

gender differences seen in fatty acid compositional data may be due to the higher estrogen levels in women, we decided to make subgroups according to the presumably changing estrogen levels of women in the course of their life cycle (a) 0-12 years, b) 13- 50 years and c) 50-75years). In serum total lipids the higher levels of LCPUFAs in women than in men were also seen in the oldest age group, where mostly postmenopausal women were present. This finding on its own may indicate that not only the higher estrogen levels are responsible for the differences observed between sexes in LCPUFA values; e.g. beside the physiological and hormonal changes caused by ageing, different age groups can have very different dietary habits, which may also influence the composition of serum lipids. Moreover, many postmenopausal women receive estrogen supplementation therapy, which fact may further modify the picture. The data obtained in the present study indicate that gender differences in fatty acid status may be relevant also in the elderly.

Diet is considered as the major factor influencing fatty acid composition of tissues. Plasma PL fatty acid composition represents the dietary intake of fatty acids over periods of weeks or months before sample collection (Arab and Akbar 2002; Ma et al. 1995), while the rate of changes in red blood cells is slower than that seen in plasma lipids Katan et al. 1991; Glatz et al. 1989). The adipose tissue fatty acid pattern represents the diet ingested in the previous one to two and half years (Plakké et al. 1983; Beynen et al. 1980). In the vast majority of the studies included into our review the composition of diets was not investigated, so it is not possible to tell to what extent dietary fatty acids influence our results. However, there were enough studies to carry out a subgroup analysis in the total plasma lipid fraction by n-3 LCPUFA intake characteristic for the investigated populations. This analysis resulted in an appreciable decrease of the degree of heterogeneity, which confirms the important role of diet in determining LCPUFA status. In the high DHA consuming group (including Japanese and Inuit subjects) no significant difference between men and women was seen in DHA values. This observation suggests that gender is a significant potential confounding variable mainly in populations with low dietary n-3 intakes.

We think that our systematic review has some strongpoints. Firstly, we were able to identify a considerable number of studies investigating a relatively great number of women and men. Secondly, the studies included into the present review originated from a wide diversity of geographic location; consequently, the results obtained may be applied without serious geographical restriction. Thirdly, the studies analyzed were carried out during more than three decades, so the phenomena observed do not seem to be changing over time. However, there are also some weaknesses of our study. Firstly, studies included were mostly observational studies and not randomized controlled trials, so the quality control potential of the studies included into the review was limited. Secondly, in case of plasma PL 14 data sets (subgroups) originated from the same cross-sectional study, so these data influenced the statistical results notably. However, these subgroups represented 14 different geographic areas, and so there are good arguments for handling them as different studies. Thirdly, the analytical methods used by the different research groups were not standardized. However, methods of fatty acid analysis are not yet as rigorously standardized as many other laboratory methods, so any systematic review on fatty acid data faces the same difficulty. It is also important to add that there are several factors, which influence fatty acid metabolism and differ between genders (e.g. dietary fatty acid intake, alcohol ingestion, relative body fatness or level of physical activity). These factors may all contribute to the gender differences observed in fatty acid composition of different biological samples; however, the main objective of the present review was not to investigate the potential contribution of these factors, but to draw attention to the fact that gender-related differences exist.

In supplementation studies reporting fatty acid composition in serum PL, serum total lipids or erythrocyte membrane lipids, gender distribution should be regarded as significant potential confounding variable.

4.2. Long-chain polyunsaturated fatty acid status in patients with phenylketonuria as compared to healthy controls: a systematic review and meta-analysis

4.2.1. Inclusion criteria

To be included into the review, a study was required to meet the following characteristics: 1. be a study on patients of any age with PKU on low-protein diet and 2. be a case-control study reporting n–3 and/or n–6 LCPUFA status in patients and in healthy controls; or randomized controlled trial (RCT) investigating the effect of LCPUFA supplementation on LCPUFA status of patients with PKU.

4.2.2. Search strategy

Ovid MEDLINE (www.ovid.com), Scopus (www.scopus.elsevier.com), LILACS (www.bireme.br) and the Cochrane Library CENTRAL database (www.thecochranelibrary.org) were searched from inception to February 2011. The search was repeated in a reduced form in May 2012. Text terms with appropriate truncation and relevant indexing terms were used to identify articles. The search was in the form [n–3 LCPUFA terms] or [n–6 LCPUFA terms] and [PKU terms]. The Ovid MEDLINE search strategy is shown in **Table 4**. The search of the three other databases was based also on this strategy. The electronic search was supplemented with articles in the reference lists of the relevant studies and review articles. We did not apply any language restriction.

Table 4. Search strategy for Ovid MEDLINE from inception to May 2012

#	Search History	Results
1	exp Fatty Acids/	334085
2	fatty acid$.mp.	171724
3	omega-6.mp.	3218
4	omega6.mp.	231
5	omega-3.mp.	10617
6	omega3.mp.	447
7	PUFA$.mp.	6794
8	LC-PUFA$.mp.	286
9	LCPUFA$.mp.	383
10	polyunsaturated.mp.	20783
11	poly-unsaturated.mp.	306
12	exp Docosahexaenoic Acids/	4792
13	docosahexaenoic acid.mp.	6268
14	DHA.mp.	7110
15	exp Eicosapentaenoic Acid/	3740
16	eicosapentaenoic acid.mp.	6069
17	EPA.mp.	8452
18	arachidonic acid.mp.	34948
19	linoleic acid.mp.	13132
20	linolenic acid.mp.	6117
21	1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20	399834
22	exp Phenylketonurias/	5958
23	PKU.mp.	1984
24	phenylketonuria$.mp.	6700
25	hyperphenylalaninaemia.mp.	254
26	HPA.mp.	9639
27	22 or 23 or 24 or 25 or 26	16339
28	21 and 27	313

4.2.3. Data extraction

Two reviewers independently searched databases, selected studies to be included in the review and extracted data. If the two reviewers disagreed, the study was discussed in detail until they reached a consensus. Data for each study included were exported into a Microsoft Office Excel 2007 database file. To provide a standardized format, units of measurement were recalculated to percentage contribution of LCPUFA to total fatty acid composition of the relevant biomarker (% weight/weight)

from original data in the publication. When it was not possible to convert data, we contacted the authors for details.

4.2.4. Statistical analysis

Statistical analyses were performed using the Review Manager 5.1 Software (The Cochrane Collaboration, Oxford, United Kingdom), with the random-effects model. The confidence interval (CI) was established at 95%. P values of less than 0.05 were considered to indicate statistical significance. Statistical heterogeneity was assessed using the I^2 statistics (I^2 of 50% or more indicating the presence of significant heterogeneity).

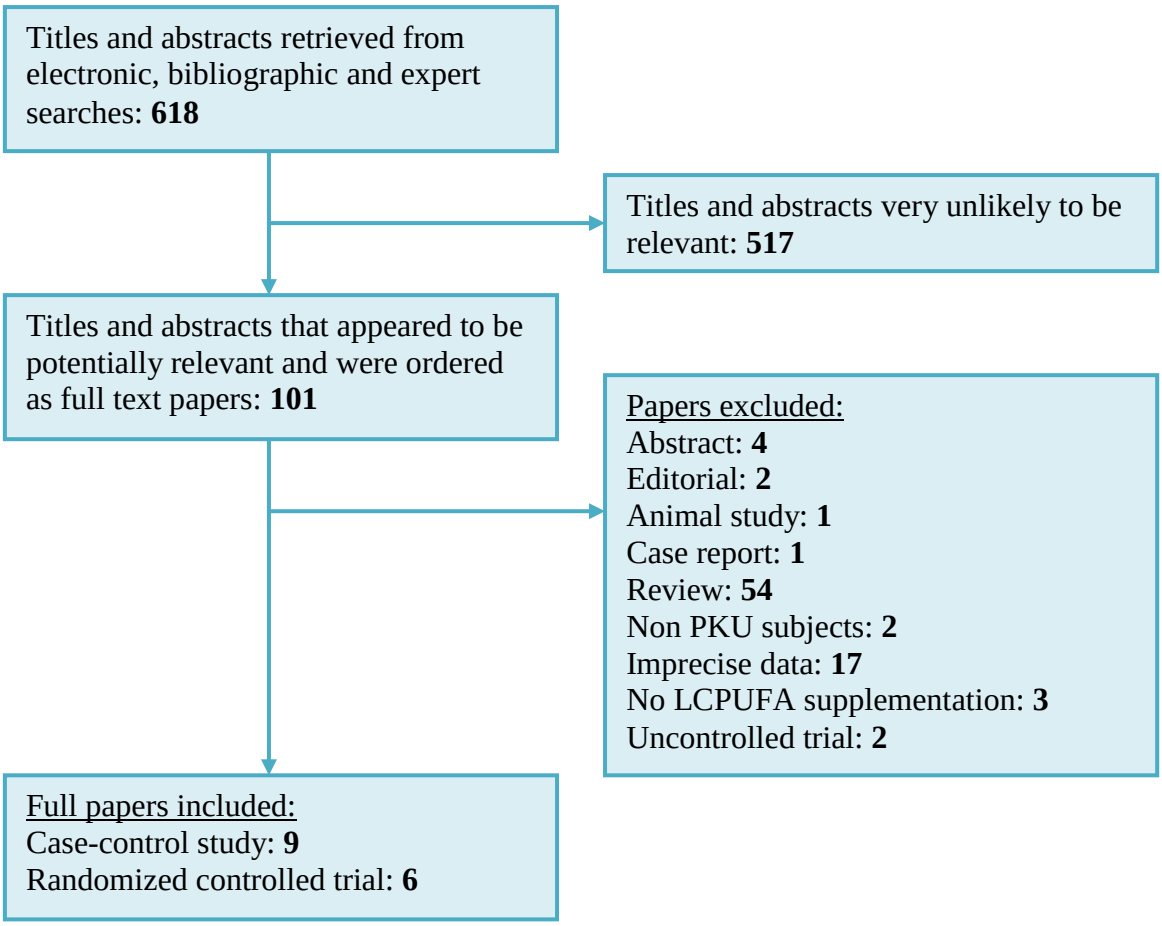

Figure 8. Flow diagram of the systematic literature search

4.2.5. Results

The flow diagram of the literature search for this review is shown in **Figure 8**. We included nine case-control studies and six RCTs in this systematic review, with a total of 713 and 273 participants, respectively.

Table 5. Basic characteristics of included case-control studies

Study	Country	Age	No. of PKU patients	No. of healthy controls	Biomarkers reported
Acosta et al 2001	USA	1 – 13 yr	28	26	P, E
Galli et al 1991	Italy	3 – 12 yr	15	12	P, PPL, PCE, E
Giovannini et al 2011	Italy	9 – 14 yr	45	45	PPL
Lage et al 2010	Spain	6 – 42 yr	47	77	P; PPL
Moseley et al 2002	USA	10 – 50 yr	27	120	P, E
Sanjurjo et al 1994	Spain	2 mo – 20 yr	40	50	P, EPL
Yi et al 2011a	USA	>12 yr	41	25	P, E
van Gool et al 2000	the Netherlands	6 mo – 25 yr	9	18	PPL, EPL

Abbreviations: P, total plasma; E, erythrocyte membrane; PKU, phenylketonuria, PPL, plasma phospholipids; PCE, plasma cholesteryl esters; EPL, erythrocyte phospholipids.

4.2.5.1. Case-control studies investigating fatty acid compositional differences between PKU patients and healthy controls

In the case-control studies included, fatty acid composition of the following 5 biomarkers was reported: total plasma lipids, plasma phospholipids, plasma cholesteryl esters, total erythrocyte membrane lipids and erythrocyte phospholipids. Basic characteristics of the studies included are presented in **Table 5**. In three papers the diagnosis of participants was classic PKU (Acosta et al. 2001; Sanjurjo et al. 1994; van Gool et al. 2000), two studies investigated also patients with moderate or mild PKU subtypes (Phe levels lower than 1200 or 600 µmol/L) (Lage et al. 2010; Vilaseca

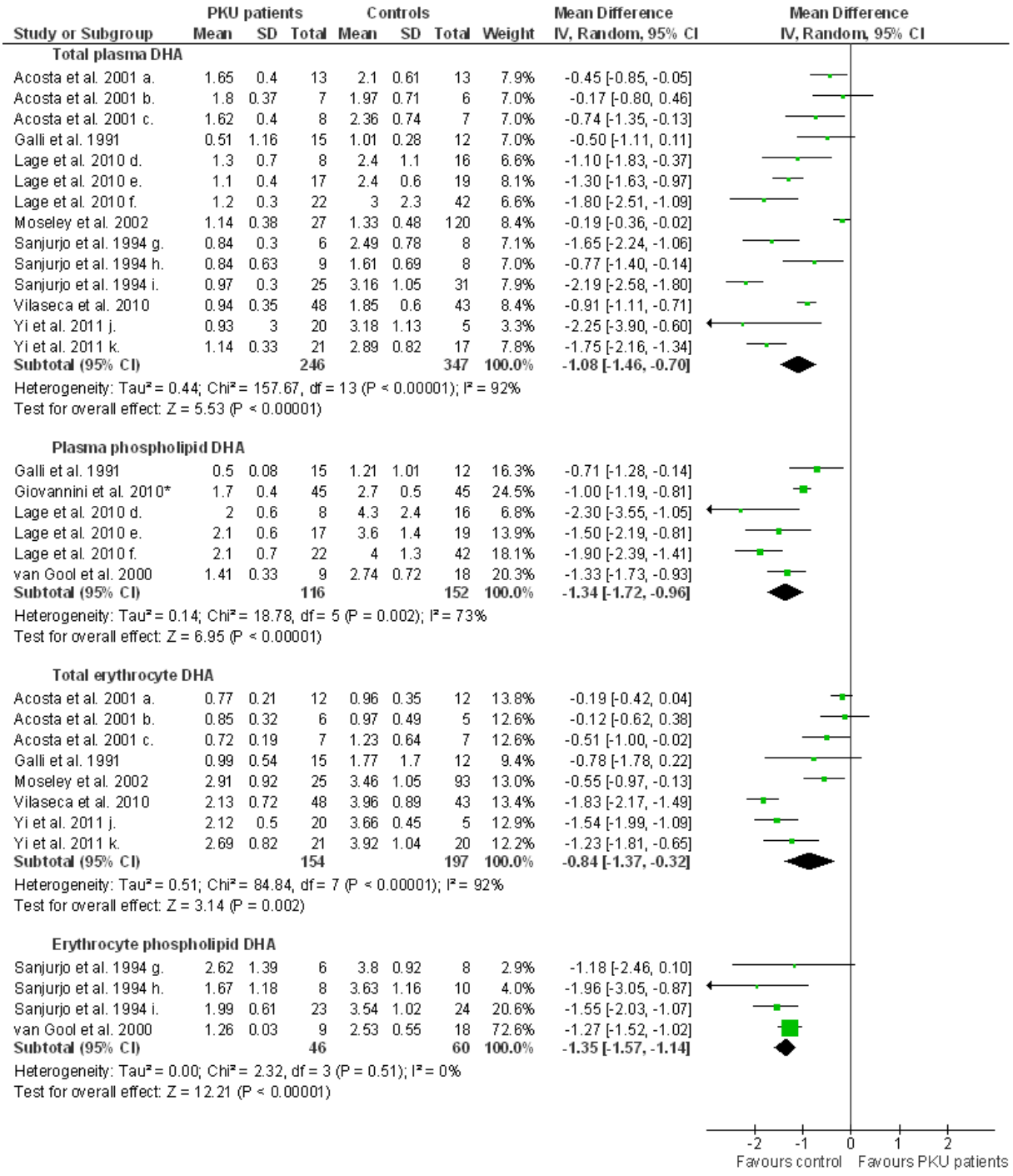

Figure 9. Case-control studies on DHA status in PKU patients versus healthy subjects, in different biomarkers

et al. 2010), whereas in four papers no further subclassification of PKU was described (Galli et al. 1991; Giovannini et al. 2011; Moseley et al. 2002; Yi et al. 2011). All patients followed amino acid-modified diets. Bad metabolic compliance of participants (Phe values higher than 600 µmol/L, in spite of dietary intervention) was reported in two of the studies included (Moseley et al. 2002; Yi et al. 2011).

In this review we focused on linoleic acid (LA) and arachidonic acid (AA) from the n-6 series and alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from the n-3 series. We conducted meta-analysis in cases of fatty acids and biomarkers used in at least three independent studies.

Fatty acid composition of total plasma lipids

Seven papers reported fatty acid composition of plasma total lipids. Analysis showed significantly lower contribution of the n-6 LCPUFA, AA (MD: -0.75; 95% CI: -1.31, -0.18; 12 studies; 596 participants; I^2 = 68%) and the n-3 LCFUFA, EPA (MD: -0.20; 95% CI: -0.27, -0.13; 11 studies; 505 participants; I^2 = 77%) and DHA (for data see **Figure 9**) to plasma total lipids of patients with PKU compared to healthy controls, while in the values of the essential precursors, LA (MD: 1.27; 95% CI: -0.23, 2.76; 12 studies; 596 participants; I^2 = 65%) and ALA (MD: 0.05; 95% CI: 0.00, 0.10; 11 studies; 569 participants; I^2 = 73%) there was no significant difference between the two groups.

Fatty acid composition of plasma phospholipids

Four papers reported fatty acid composition of plasma phospholipids. Primary analysis showed significantly lower EPA (MD: -0.41; 95% CI: -0.68, -0.14; 6 studies; 268 participants; I^2 = 94%) and DHA (**Figure 9**) values in patients with PKU than in controls. No significant differences were found between the two groups with respect to AA (MD: 0.00; 95% CI: -0.80, 0.81; 6 studies; 268 participants; I^2 = 30%), LA (MD: 2.62; 95% CI: -0.46, 5.71; 6 studies; 268 participants; I^2 = 88%) or ALA values (MD: -0.07; 95% CI: -0.17, 0.03; 5 studies; 241 participants; I^2 = 97%).

Fatty acid composition of erythrocyte membrane total lipids

Comparison of erythrocyte membrane total lipid fatty acid composition in patients with PKU and healthy controls was present in 8 studies. The primary analysis showed significantly lower EPA (MD: -0.08; 95% CI: -0.16, -0.01; 7 studies; 260 participants; I^2 = 73%) and DHA (**Figure 9**) values in patients as compared to controls; however, there was no significant difference in AA values (MD: -0.32; 95% CI: -1.07, 0.43; 8 studies; 351 participants; I^2 = 71%). There was no difference in LA values between the two groups (MD: 0.53; 95% CI: -0.18, 1.23; 8 studies; 351 participants; I^2 = 52%), whereas ALA values were significantly higher in patients than in controls (MD: 0.03; 95% CI: 0.01, 0.05; 7 studies; 324 participants; I^2 = 43%).

Fatty acid composition of erythrocyte phospholipids

Data relating to the erythrocyte phospholipid fraction were sufficient only for a meta-analysis of DHA; values were significantly lower in PKU patients than in controls (**Figure 9**).

Fatty acid composition of plasma cholesteryl esters

There were insufficient data to conduct a meta-analysis comparing fatty acid composition of plasma cholesteryl esters in subjects with PKU and controls.

4.2.5.2. Effects of LCPUFA supplementation on DHA concentrations in PKU

We included six RCTs with parallel design. In two studies participants were newborns (Agostoni et al. 2006; Koletzko et al. 2007), in three studies infants and children between 1 and 18 years (Agostoni et al. 2003; Agostoni et al. 1995; Cleary et al. 2006) were included, while one study included both children and adults (Yi et al. 2011). Five studies were carried out in Europe, one in the USA (Yi et al. 2011).

In this report we primarily focus on the effect of LCPUFA supplementation on DHA status. We found eight biomarkers used to characterize changes in DHA values. From these, only total plasma DHA was used in at least three independent studies; never-

theless, the effect of LCPUFA supplementation on every biomarker of DHA status is shown in **Figure 10**. Agostoni et al (1995) applied supplementation with 2.5–4 g fish oil (18 g EPA, 4 g DPA and 12 g DHA/100 g fatty acid) daily for 6 months; Agostoni et al (2003) used 1 capsule (37 mg AA, 27.5 mg EPA, 20 mg DPA and 40 mg DHA/0.5 g capsule) per 4 kg body weight for 1 year; Yi et al (2011) used microalgae oil capsules (10 mg/kg/day DHA) for 4.5 months of supplementation; Koletzko et al (2007) used a supplemented formula (0.46 g AA and 0.27 g DHA/100 g fatty acids) for 1 year; Agostoni et al (2006) used a supplemented formula with slightly different composition (0.7 g AA and 0.3 g DHA/100 g fatty acids) for 1 year while Cleary et al (2006) gave essential fatty acid supplemented protein substitute (17.2 g LA and 4.5 g ALA/100 g fatty acid) for 20 weeks. Although different dosages and forms of n-3 LCPUFA supplementation were used in the different studies, these were all effective in significantly increasing DHA values of different biomarkers (**Figure 10**).

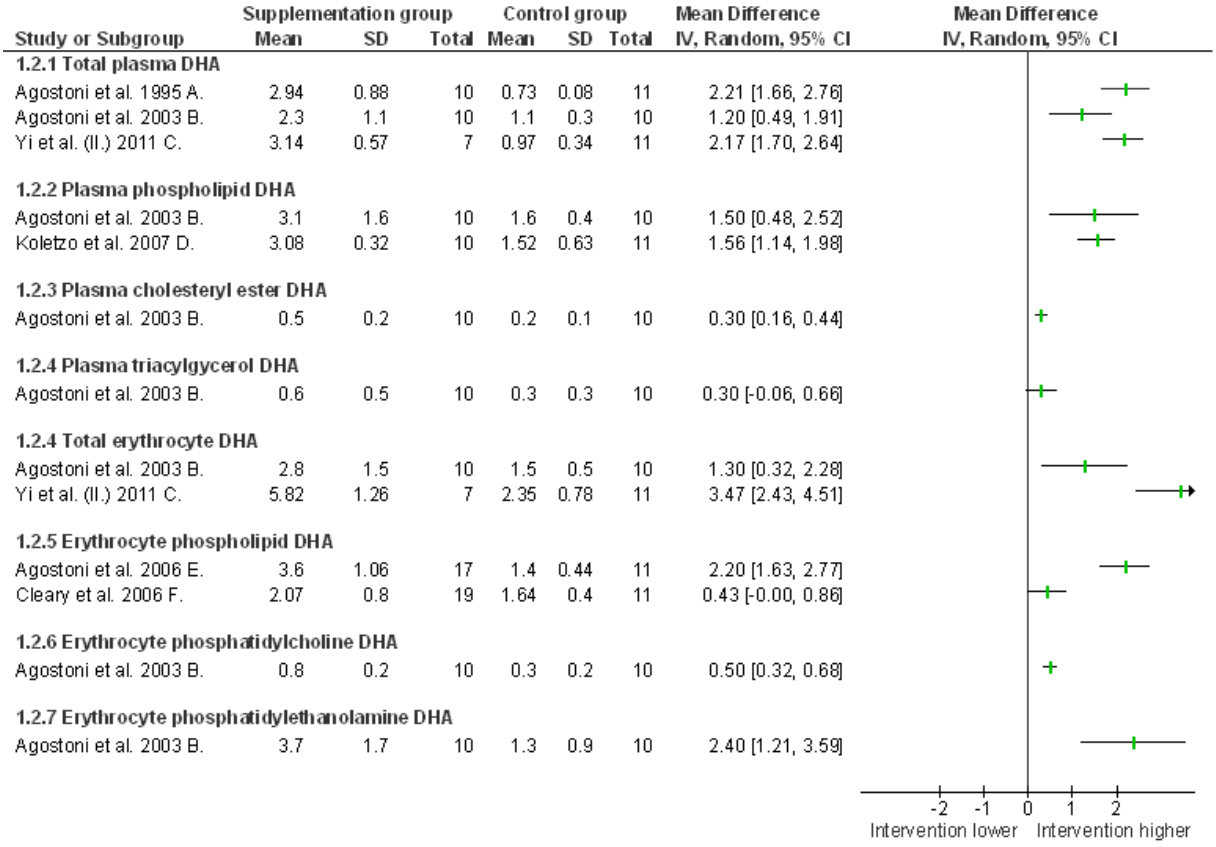

Figure 10. Randomized controlled trials on the effect of LCPUFA supplementation on DHA status in PKU

4.2.6. Discussion

The mainstream of phenylketonuria management is the restriction of dietary intake of phenylalanine, immediately after confirmation of the diagnosis. This means that patients with phenylketonuria have to consume a phenylalanine free formula and avoid foods rich in protein (e.g. meat, fish, egg, cheeses, normal bread and seeds), moreover, they have to avoid foods and drinks containing aspartame, flour, soya, beer or cream liqueurs. Low protein natural foods (potatoes, some vegetables and most cereals) can be consumed in restricted amounts (Blau et al. 2010). This strict diet may lead to the insufficient intake of some essential nutrients.

The potentially inadequate dietary LCPUFA supply of patients suffering from PKU on Phe-restricted diet has gained attention recently. There are two main sources of LCPUFA in the human organism: dietary intake and endogenous synthesis from their essential metabolites, LA and ALA (**Figure 1**). However, the endogenous synthesis of AA and DHA is limited (Calder 2012), therefore, the lack of preformed LCPUFA in the diet may lead to deficiency. Moreover, in PKU a possible inhibitory effect of Phe metabolites on endogenous DHA synthesis is suspected (Infante and Huszagh 2001).

The connection between elevated plasma Phe levels and neurological disorders seen in patients with PKU is well-established (MacDonald et al. 2011). The optimum outcome is mainly dependent on metabolic control with diet and this control varies during the patient's life. However, functional deficits may also be present in PKU patients treated early and well. Patients with PKU have lower intelligence quotients as compared to healthy controls (Brumm and Grant 2010). Subtle abnormalities in phenylketonuria include the impairment of executive abilities (such as planning, problem solving, information processing and sustained attention (Van Zutphen et al. 2007)). Besides, children with phenylketonuria have behavioral abnormalities, motor dysfunction and impaired memory (Blau et al. 2010).

LCPUFA are shown to have important role in cognitive development, in maturation of visual acuity and development of motor functions in full-term and preterm infants (Fleith and Clandinin 2005). On the basis of these considerations it has been hypothesized that LCPUFA deficiency, especially inadequate DHA supply, might contribute also to the neurological abnormalities observed in patients with PKU (Koletzko et al. 2009).

In this review we included 9 case-control studies (divided into 16 arms) and six RCTs (divided into 13 arms) in order to assess whether significant deficiency of LCPUFA can be demonstrated in patients with PKU. We also investigated whether LCPUFA status of PKU patients can be improved by supplementing their diet with LCPUFA. We found that in PKU patients on low-protein diet blood levels of the two principal n-3 LCPUFA, EPA and DHA, were consequently and significantly reduced in different blood biomarkers; whereas the values of the principal n-6 LCPUFA, AA were significantly reduced only in plasma total lipids. These data indicate that supplementation with oils containing DHA may be an effective way for improving the n-3 LCPUFA status of patients with PKU. Special dietary products for infants with PKU are usually enriched with n-3 and n-6 LCPUFA, however, supplementation should be applied routinely also after infancy. This may be achieved relatively easily since large numbers of various capsules containing fish oil are available.

A limitation of the present review was that in the different studies different dosages of LCPUFA were used for supplementation, and therefore subgroup analysis according to supplement dosage was not possible. A further limitation was the lack of description of compliance and blood Phe levels of the patients with PKU on low-protein diet; therefore, subgroup analysis evaluating the potential influence of different blood Phe levels on PUFA metabolism was not possible. We assume that insufficient LCPUFA intakes together with metabolic impairment of LCPUFA synthesis may lead to decreased LCPUFA levels seen in patients with PKU.

The data systematically reviewed here indicate that in patients with PKU n-3 LCPUFA supply is insufficient. However, data from RCTs suggest that DHA status in patients with PKU may be improved by dietary supplementation. Further studies are needed to investigate whether LCPUFA deficiency is present in all age groups, especially in adolescents and adults who usually follow more relaxed diets. Moreover, there is only limited evidence about the optimal LCPUFA supplementation dosage in the different age groups.

4.3 Prebiotics in healthy infants and children for prevention of acute infectious diseases: a systematic review and meta-analysis

4.3.1. Inclusion criteria

To be included, a study needed to meet all of the following criteria: a) randomized allocation to treatment groups (RCT); b) carried out in healthy infants or children, aged 0-18 years; c) intervention with prebiotics (prebiotics added to food in the manufacturing process or as a separate supplement) compared to controls (placebo or no supplementation); d) a supplementation time of at least two months and an observation time of at least four months; e) using one of the following supplements: oligosaccharides, galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), fructans, inulin, oligofructose; f) describing at least one of the following outcomes: incidence of overall infections (any kind of infections), incidence of all acute respiratory tract infections (RTI), incidence of upper respiratory tract infections (URTI), incidence of lower respiratory tract infections (LRTI), incidence of otitis media, incidence of gastrointestinal infections (GITI) (defined in primary studies as 3 or more liquid/semiliquid stools per day with fever, vomiting, and/or dehydration and compromised general status), incidence of diarrhea episodes, incidence of urinary tract infections (UTI), incidence of fever episodes, incidence of infections requiring antibiotic treatment. No language restrictions were applied.

4.3.2. Search Strategy

Ovid MEDLINE (www.ovid.com), Scopus (www.scopus.elsevier.com), Web of Science (www.webofknowledge.com) and the Cochrane Library CENTRAL database (www.thecochranelibrary.org) were searched from inception to Week 3, June,2013 for intervention studies using text terms with appropriate truncation and relevant indexing terms. The search was in the form: [prebiotics terms] and [search filters for children] and [search filters for randomized controlled trials]. For the Ovid MEDLINE

search we used the sensitivity- and precision-maximizing version (2008 revision) of the Cochrane Highly Sensitive Search Strategy developed for identifying randomized trials in MEDLINE (Lefebvre et al. 2011). The full search strategy for the Ovid MEDLINE database can be found in **Table 6**, search of the other databases was also based on this strategy.

Table 6. Search strategy for Ovid MEDLINE 1946 to June Week 3 2013

#	Search History	Results
1	exp Prebiotics/	632
2	prebiotic*.mp.	3213
3	exp Oligosaccharides/	71620
4	oligosaccharid*.mp.	34893
5	oligo-saccharid*.mp.	90
6	fructooligosaccharid*.mp.	490
7	galactooligosaccharid*.mp.	202
8	exp Fructans/	6464
9	(fructan or fructans).mp.	1016
10	(inulin or inulins).mp.	9338
11	(oligofructos* or oligo-fructos*).mp.	343
12	2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12	96524
13	limit 13 to "all child (0 to 18 years)"	6010
14	(child* or boy* or girl* or adolescent* or pediatric* or paediatric* or infant* or newborn* or neo-nat* or toddle* or schoolchild*).mp.	3190789
15	13 and 15	7476
16	14 or 16	7476
17	randomized controlled trial.pt.	367158
18	controlled clinical trial.pt.	87691
19	randomi#ed.ab.	318588
20	placebo.ab.	144978
21	clinical trials as topic.sh.	169978
22	randomly.ab.	187790
23	trial.ti.	115019
24	18 or 19 or 20 or 21 or 22 or 23 or 24	860275
25	exp animals/ not humans.sh.	3910958
26	25 not 26	792849
27	17 and 27	1072

4.3.3. Data extraction and management

Titles and abstracts were screened for inclusion by a single reviewer. All potentially relevant abstracts and full papers were screened for inclusion using an inclusion/exclusion form by two reviewers independently; where they disagreed the study was discussed with a third reviewer. Data for each included study were extracted by two reviewers independently. When it was necessary, units of measurement were converted to a standard form (number of episodes/person/year) to facilitate comparison across studies.

Assessment of risk of bias in the studies included

Two reviewers independently assessed the methodological quality of studies included. “Low risk of bias” meant that the study was randomized, the randomization method and allocation concealment was described, the study was double-blind, the method of blinding was described and the reasons for and numbers of dropouts were stated (or there were no dropouts) and the distribution of dropouts across comparison groups was equal. All other studies were considered as “moderate or high risk of bias”.

4.3.4 Statistical analysis

Meta-analysis was carried out according to the methodology for counts and rates described in The Cochrane Handbook (Deeks and Altman 2011). The results of the studies were expressed as a rate ratio, the ratio of the rate in the prebiotic group to the rate in the control group.

$$\text{rate ratio} = \frac{E_{prebiotic}/T_{prebiotic}}{E_{control}/T_{control}}$$

where $E_{prebiotic}$ means the events occurred during $T_{prebiotic}$ participant-years of follow-up in the prebiotic intervention group, and $E_{control}$ means the events during $T_{control}$ participant-years in the placebo group. The (natural) logarithm of the rate ratios was

combined across studies using the generic inverse-variance method. The standard error of the log rate ratio was expressed by

$$SE = \sqrt{\frac{1}{E_{prebiotic}} + \frac{1}{E_{control}}}$$

.

Assessment of heterogeneity

We carried out tests for heterogeneity using the Chi^2 test with significance being set at a p value < 0.1. We used the I^2 statistic to estimate the variation across studies. An I^2 statistic < 40% was considered to be a low level of heterogeneity, 30% to 60% a moderate level, 50% to 90 % a substantial level and 75% to 100 % a considerable level (Deeks and Altman 2011).

Data synthesis

Regardless of heterogeneity between the pooled studies, we used a random-effects model to synthesize all data.

4.3.5. Results

Study inclusion

The flow diagram of the literature search for this review is shown in **Figure 10**. Altogether 1429 titles and abstracts were identified via the electronic search, 39 of them appeared to be potentially relevant. One conference abstract could not be retrieved; all others were available for detailed assessment. Finally, 5 studies reported in 8 full-text papers and 4 conference abstracts fulfilled the inclusion criteria.

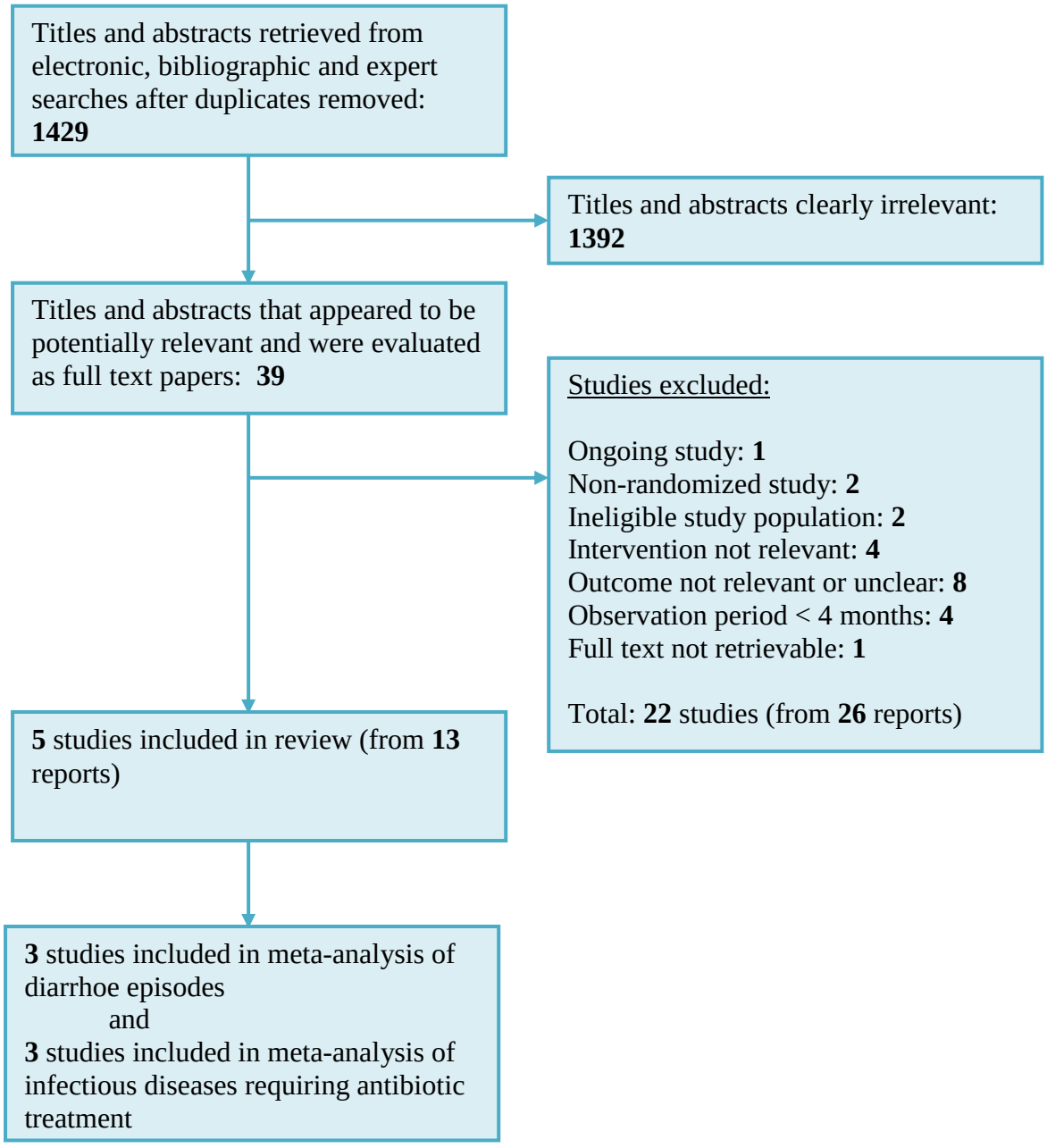

Figure 10. Flow diagram (PRISMA)

The main characteristics of the 5 included studies are described in **Table 7**.

Participants

The studies ranged in size from 140 to 830 children randomized initially. All participants were infants and children younger than two years of age at the beginning of the intervention. The countries where trials were performed are also listed in **Table 7**.

Interventions

The studies involved different types of prebiotics including GOS/FOS mixture (Arslanoglu et al. 2007, Bruzzese et al. 2009, Stuijvenberg et al. 2011), polydextrose (PDX)/GOS mixture (Ribeiro et al. 2012) or oligofructose (Duggan et al. 2003, Saavedra and Tschernia 2002). In most of the studies included the intervention and observation time was the same and ranged between 108 days and 12 months, there was only one study (Arslanoglu et al. 2008, Arslanoglu et al. 2007) in which investigators kept on observing the patients after the end of supplementation (in this study participants were followed for two years). To ensure homogenous data we included incidence data into the meta-analysis from the study mentioned above, starting from the time point the supplementation was finished.

Risk of bias in the studies included

Aspects of methodological quality of the studies included are described in **Table 8**. In two of the five studies included (Arslanoglu et al. 2008, Duggan et al. 2003, Stuijvenberg et al. 2011) method of randomization, methods of blinding and reasons for dropouts were all properly described, which did not suggest high risk of bias.

Table 7. Characteristics of included studies

Author, publication year	Participants		Intervention			Outcome
	Country	Inclusion criteria	Supplement used	Placebo used	Duration of supplementation	Relevant outcomes data available for[1]
Arslanoglu et al. (2008/2007) (34, 35)	Italy	Healthy term infants, start of formula feeding within the first two weeks of life, parental history of atopic disease	Hypoallergenic formula supplemented with 0.8 g/100 ml scGOS/lcFOS	Hypoallergenic formula supplemented with 0.8 g/100 ml maltodextrin	6 months	Incidence of physician-diagnosed infections (URTI, LRTI, otitis media, GITI, UTI), Antibiotic prescriptions, Incidence of fever episodes witnessed by the parents
Bruzzese et al. (2009) (37)	Italy	Healthy term infants, aged 15-120 days old, birth weight >2500g, start of formula feeding after at least 15 days of exclusive breastfeeding	Standard formula supplemented with 0.4 g/100 ml GOS/FOS (9:1)	Standard infant formula	12 months	Incidence of acute diarrhea, Incidence of URTI, Incidence of LRTI, Incidence of respiratory infections requiring AB therapy
Duggan et al. (2003) (38)	Peru	Healthy infants, aged 6-12 months, already consuming solid food	Rice- or oat-based cereal supplemented with 0.55 g oligofructose /15 g cereal	Non-supplemented cereal	6 months	Incidence of episodes of diarrhea

Saavedra et al. (2002) (40)	USA	Healthy infants and children, aged 4-24 months; already consuming cereals, attending daycare	cereal supplemented with 0.55 g oligofructose /15 g cereal (administered ad libitum with a minimum goal intake of 15 g cereal/day)	Non-supplemented cereal	6 months	Incidence of diarrhea by parental report, Incidence of diarrhea accompanied by fever and vomiting, Incidence of antibiotic use
Stuijvenberg et al. (2011) (43)	The Netherlands, Austria, Switzerland, Italy, Germany	Healthy term infants, aged < 8 weeks; birth weight above the 10th percentile for gestational age, without parental history of atopic disease	Standard formula supplemented with 0.8 g/100 ml GOS/FOS (9:1)	Standard infant formula	10-12 months	Incidence of fever episodes witnessed by the parents, Incidence of AB treatments reported by the parents

[1]Total number of episodes was converted into incidence of episodes, where necessary

Abbreviations: GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; scGOS, short chain GOS; lcFOS, long chain FOS; PDX, polydextrose; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; RTI, respiratory tract infection; GITI,

Table 8. Methodological quality of included studies (Risk of bias)

	Selection bias			Performance/Detection bias		Attrition bias			Reporting bias	Overall risk of bias[1]
Trial	**Study design (randomized: YES/NO/unclear)**	**Method of randomization (clearly described and appropriate: YES/NO)**	**Allocation concealment (clearly described and appropriate: YES/NO)**	**Blinding (double-blind: YES/NO/ unclear)**	**Methods of blinding appropriate (YES/NO/unclear)**	**Description of withdrawal or dropout (YES/NO)**	**Reason for drop-outs in the intervention group (Number of drop-outs)**	**Distribution of dropouts across comparison groups (Number of drop-outs/Total included)**	**All outcomes listed in Methods described in Results (YES/NO/unclear)**	
Arslanoglu et al. (2008/2007)	YES	YES "a preprepared randomization numbers table"	NO	YES	unclear	YES	- *In the first 6 months:* Reestablishment of breastfeeding (22), Move to another city (8/), Flatulence, no reason (5) - *In the 6-24 months follow up period:* Poor compliance (8)	Intervention (I): 27/129 Control (C): 26/104	YES	Moderate/ High
Bruzzese et al. (2009)	YES	YES "a random numbers table with a block design"	NO	NO "open trial"	NO	YES	Poor compliance (30), Development of cow's milk protein intolerance (3), Excluded (incomplete data) (40)	I: 73/169 C: 68/173	YES	Moderate/ High
Duggan et al. (2003)	YES	YES "random assignments (using a permuted block	YES "on enrollment of an infant, study staff opened the	YES	YES "personnel not involved in the study generated	YES	Moved (6), Refused blood sample (4), Ill with non-diarrhea	I: 12/141 C: 19/141	YES	Low

		design"	next study envelope to determine which cereal was to be dispensed to that infant"		the list of random assignments and prepared envelopes containing the cereal assignments"		illness (1), Withdrew consent (1)			
Saavedra et al. (2002)	YES	NO	NO	YES	unclear	YES	No dropouts	I: 0 C: 0	unclear	Moderate/ High
Stuijvenberg et al. (2011)	YES	YES "Time-balanced randomization was performed with the software RANCODE, with a random permuted block size of 4"	YES "only the hospital pharmacist had a copy of the randomization list with the actual treatment allocation"	YES	YES "The parents, the study physicians and the study nurses were unaware of the group allocation"	YES	Change to another formula (8), Intolerance to formula (10), Occurrence of any disease (7), Withdrawal (28)	I: 53/414 C: 42/416	YES	Low

[1] "Low risk of bias" meant that the study was randomized, the randomization method and allocation concealment was described, the study was double-blind, the method of blinding was described, the reasons for and numbers of dropouts were stated (or there were no dropouts) and the distribution of dropouts across comparison groups was equal. All other studies were considered as "moderate or high risk of bias".

Patient-relevant outcomes described in the studies included

Incidence of overall infection

The incidence of any infection during the observation period was reported only in one study (35): infants in the scGOS/lcFOS group had significantly fewer episodes of any type of infection during the 6-month supplementation-observation period (0.42 vs. 0.90 episodes/subject/year; $n_{prebiotic}$ = 102, $n_{control}$ = 104; p = 0.01; RR 0.47, 95% CI 0.32 – 0.68).

Incidence of fever episodes

The incidence of fever episodes recorded by the parents of the participating infants and children up to 24 months was examined in two studies. Saavedra et al (2002) observed a statistically significant difference in the incidence of fever with 'cold symptoms' between the intervention and the control groups (5.76 vs. 9.05 fever episodes/subject/year; $n_{prebiotic}$ = 63, $n_{control}$ = 60; p < 0.05). However, Stuijvenberg et al (2011) found no statistically significant difference in the number of fever episodes during the one-year supplementation period between the prebiotic and control group (1.79 vs. 1.78 fever episodes/subject/year; $n_{prebiotic}$ = 292, $n_{control}$ = 300; p > 0.05). The pooled effect of these two studies was not significant either (RR 0.80, 95% CI 0.51 – 1.26) (**Figure 11**) but showed a considerable level of heterogeneity (Chi^2 24.89; df = 1, P < 0.00001; I^2 statistic = 96%).

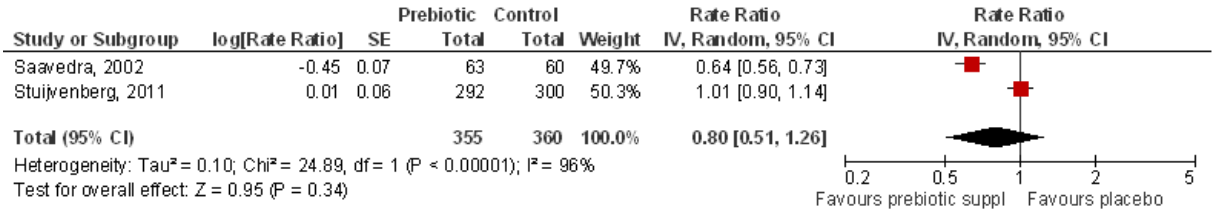

Figure 11. The rate of fever episodes (events per person/year) in infants and children supplemented with prebiotics versus placebo

Incidence of infections requiring antibiotic therapy

For three RCTs eligible count data were available on the effect of prebiotic supplementation on the incidence of infections requiring antibiotic treatment. A pooled analysis of the data from these 3 studies showed a statistically significant difference in the episode rates of acute bacterial infections (RR 0.68, 95% CI 0.61 – 0.77) (**Figure 12**). The level of heterogeneity between these studies was low (Chi^2 1.04; df = 2, P = 0.59; I^2 statistic = 0%).

Stuijvenberg et al (2011) reported their data on fever episodes requiring antibiotics as median (25th–75th percentile) and so this study was not included in the meta-analysis. It has to be noted, though, that in this study no significant difference in the median adjusted numbers of fever episodes requiring antibiotics was described (prebiotic group: 0.05 (0.05, 0.11) vs. control group: 0.05 (0.05, 0.16), median values (25th, 75th percentile)).

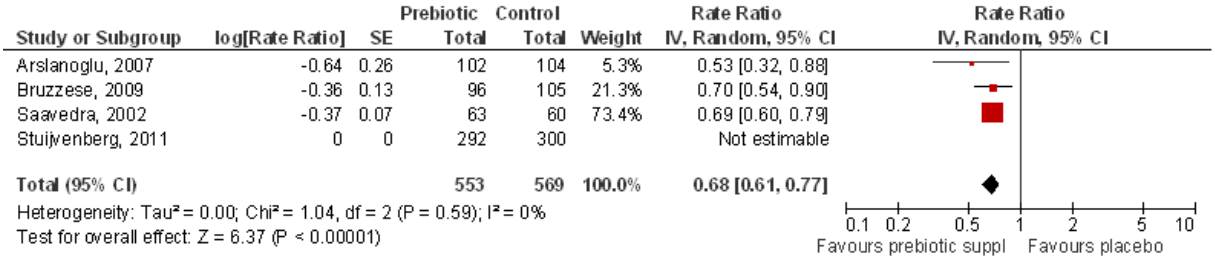

Figure 12. The rate of infections requiring antibiotic treatment (events per person/year) in infants and children supplemented with prebiotics versus placebo

Incidence of gastrointestinal infections or diarrhea

Count data on any kind of gastrointestinal infections (GITI) were available for one RCT (35); the rate of GITI was lower in the prebiotic group, however this difference was not statistically significant (0.02 vs. 0.08 episodes/subject/year; p = 0.18). One further study (Bruzzese et al. 2009) only reported that the mean number of episodes of gastroenteritis was significantly lower in the GOS/FOS group than in controls (p = 0.01).

Diarrhea episodes were reported for three RCTs (Bruzzese et al. 2009, Duggan et al. 2003, Saavedra and Tschernia 2002). The pooled effect estimate of these three studies shows no significant difference (RR 0.71, 95% CI 0.35 – 1.43) between the prebiotic supplemented and the placebo groups on the rate of diarrhea episodes (**Figure 13**), however, the level of heterogeneity among these studies was considerable (Chi^2 356.65; df = 2, P < 0.00001; I^2 statistic = 99%).

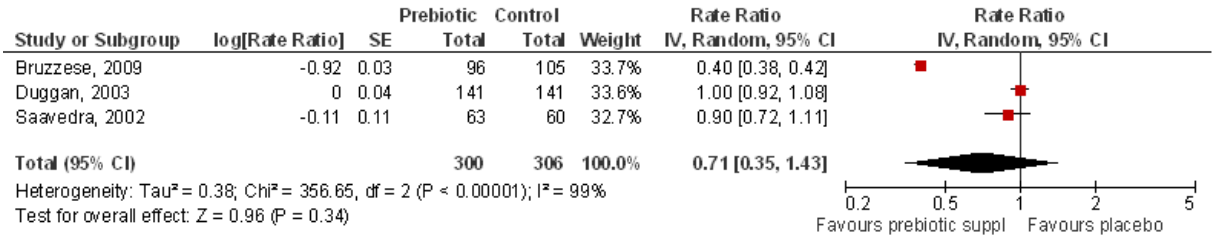

Figure 13 The rate of diarrhoea episodes (events per person/year) in infants and children supplemented with prebiotics versus placebo

Incidence of respiratory tract infections

Information on the incidence of upper respiratory tract infections (URTI) was available for 2 RCTs. Both studies (Arslanoglu et al. 2007, Bruzzese et al. 2009) only reported that the number of episodes of URTI was lower in the prebiotic group as compared to the control group, but the difference was in neither of the two studies significant.

Arslanoglu et al (2007) observed no significant difference between the prebiotic supplemented and the control group either in the rate of otitis media (0.08 vs. 0.12 episodes/subject/year; p = 0.60) or in the rate of lower respiratory tract infections (0.04 vs. 0.06 episodes/subject/year; p = 0.07).

Incidence of urinary tract infections

One report (Arslanoglu et al. 2007) provided data on the effect of prebiotic supplementation on the incidence of urinary tract infections; it showed no significant difference in the incidence between the two groups (0.04 vs. 0.14 episodes/subject/year; p = 0.26).

4.3.6. Discussion

This review was meant to investigate whether there is evidence for prebiotic oligosaccharides to have a protective effect against acute infectious diseases in infancy, and furthermore, whether or not such a protective effect is present later in childhood. Five studies investigated the relevant outcomes; however, there were only two outcomes (incidence of infections requiring antibiotic treatment and incidence of diarrhea episodes) for which eligible count data were available from three RCTs. For each further included outcome data were extracted from only one or two studies.

There is strong evidence (large sample size, low level of heterogeneity, moderate risk of bias) only for bacterial infections to have a significantly lower incidence in 0-24 month old infants and children regularly consuming prebiotics as compared to children supplemented with placebo.

Our review focused on the infection-preventive effect of prebiotics in the pediatric population; however, studies investigating the preventive efficacy of prebiotics in children aged 2-18 years are completely lacking in the literature. Based on the promising results seen in the 0-2 year-old age group it would be worth conducting such studies also in older children, especially because children newly entering a community (day nurseries, kindergartens, elementary schools) are acutely exposed to infections. Finding an effective strategy for the prevention of common infections would also decrease health expenses and thereby not only benefits for the individual but also for the entire society.

There were only two studies in which risk of bias could be labeled as low. The lack of describing allocation concealment and the method of blinding in the other three studies could potentially have biased the results favoring the prebiotic group (**Table 8**).

Most outcome measures in this review come from only one trial, therefore some effects of prebiotics may have remained hidden because of the small sample sizes for some outcomes. Future RCTs should consider the assessment of incidence data in

reference to overall infections, fever episodes, upper respiratory tract infections and urinary tract infections.

From the clinical point of view the relevant five studies included were homogenous: they all investigated infants or children aged 0-24 months, the supplementation was conducted for 6-12 months and incidence data were determined by the end of intervention.

However, when pooled analysis was performed for the outcome “Incidence of diarrhea episodes” high levels of heterogeneity was found. This may be a consequence of the different types of prebiotics used: the FOS/GOS mixture (Bruzzese 2009) seems to be more effective in the prevention of diarrhea episodes compared to oligofructose (Duggan et al. 2003, Saavedra et al. 2002). Another explanation for the heterogeneity may be the difference in the way of prebiotic administration: while in the study of Bruzzese et al the GOS/FOS mixture was added to the formula, in the other two studies cereals supplemented with oligofructose were used.

There were large differences in the baseline risk for infections among different populations: for example, the mean number of episodes/subject/year of bacterial infections was in the study of Saavedra et al (2002) 8 times higher than in the study of Bruzzese et al (2009), and 38 times higher than in the study of Arslanoglu et al (2007). Large differences were also seen in the mean number of diarrhea episodes/subject/year.

Finally, it has to be mentioned that Stuijvenberg et al (2011) reported their data on fever episodes requiring antibiotics as median values (25^{th}–75^{th} percentile) and so this study was not included in the meta-analysis. However, the rate of antibiotic use in this study was much lower than in the other three studies, indicating lower weight and therefore no determinative impact on the pooled effect estimate.

The role of prebiotics has been investigated most extensively in the field of infant nutrition (Boehm et al. 2003, Thomas et al. 2010). Oligosaccharides were demonstrated to represent the most important dietary factor in human milk, promoting the

development of beneficial intestinal flora (Bertino et al. 2012, Barile and Rastall 2013) and being the bioactive compound responsible for the positive health effects of human milk: HMOs were described to have a protective effect against gastrointestinal diseases and diarrhea (Salone et al. 2013, Lamberti et al. 2011) and against respiratory tract infections (Duijts et al. 2009, Bachrach et al. 2003, Chantry et al. 2006).

However, it has to be mentioned that because of their complex structure, oligosaccharides with a structure identical to that of HMOs are not yet available for dietary supplementation purposes; at present galactooligosaccharides (GOS), fructooligosaccharides (FOS) and oligofrucose are considered as the most relevant prebiotic oligosaccharides used in supplementation studies. In this review we could not confirm all the preventive health effects ascribed to HMOs also for non-human milk oligosaccharides.

4. Novel findings and practical applications

1. Our systematic review based on 51 publications showed significantly lower contribution of both arachidonic and docosahexaenoic acid to plasma total lipids and plasma phospholipids in men than in women; in erythrocyte membrane total lipids DHA values were significantly lower in men than in women.

2. In supplementation studies reporting fatty acid composition in serum PL, serum total lipids or erythrocyte membrane lipids, gender distribution should be regarded as significant potential confounding variable.

3. The data systematically reviewed here indicate that in patients with PKU n-3 LCPUFA supply is insufficient. However, data from RCTs suggest that DHA status in patients with PKU may be reflectively improved by dietary supplementation.

4. There is only limited evidence about the optimal LCPUFA supplementation dosage in the different age groups. It is of clinical importance to investigate whether LCPUFA deficiency is present in all age groups, especially in adolescents and adults who usually follow more relaxed diets.

5. Currently available evidence suggests that the preventive use of prebiotics decreases the rate number of infections requiring antibiotic therapy in infants and children aged 0-24 months. Data from only one randomized controlled study indicate that prebiotics may also be effective in decreasing the rate of overall infections.

6. Further studies should be carried out in the 3-18 years old age group to answer the question whether prebiotics can be considered for the prevention of acute infectious diseases also in the older pediatric age group.

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8. List of publications

Publications on which the thesis was based

- ***Lohner S***, Fekete K, Marosvölgyi T, Decsi T: *Gender differences in long-chain polyunsaturated fatty acid status: systematic review of 51 publications*. Ann Nutr Metab. 2013;62(2):98-112 (IF_{2012}: **1.661**)

- ***Lohner S***, Fekete K, Decsi T. *Lower n-3 long-chain polyunsaturated fatty acid values in patients with phenylketonuria: a systematic review and meta-analysis*. Nutr Res. 2013 Jul;33(7):513-20. (IF_{2012}: **2.142**)

- ***Lohner S***, Küllenberg D, Antes G, Decsi T, Meerpohl JJ. *Prebiotics in healthy infants and children for prevention of acute infectious diseases: a systematic review and meta-analysis.* Nutr Rev. 2014. *Accepted for publication* (IF_{2012}: **4.597**)

Other publications

- ***Lohner S***, Vágási J, Marosvölgyi T, Tényi T, Decsi T. *Inverse association between 18-carbon trans fatty acids and intelligence quotients in smoking schizophrenia patients.* Psychiatry Res. 2014 Jan 30;215(1):9-13. (IF_{2012}: **2.456**)

- ***Lohner Sz***, Vágási J, Péterfia Cs, Decsi T. *Halolajat tartalmazó étrend-kiegészítők szerepe az atópiás betegségek kezelésében.* Gyermekorvos Továbbképzés 2013; XII (5):221-223

- Płudowski P, Karczmarewicz E, Bayer M, Carter G, Chlebna-Sokół D, Czech-Kowalska J, Dębski R, Decsi T, Dobrzańska A, Franek E, Głuszko P, Grant WB, Holick MF, Yankovskaya L, Konstantynowicz J, Książyk JB, Księżopolska-Orłowska

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- ***Lohner S***, Fekete K, Berti C, Hermoso M, Cetin I, Koletzko B, Decsi T. Effect of folate supplementation on folate status and health outcomes in infants, children and adolescents: A systematic review. Int J Food Sci Nutr. 2012 Dec; 63(8):1014-1020 (IF_{2012}: **1.257**)

- ***Lohner Sz***, Marosvölgyi T, Burus I, Schmidt J, Molnár D, Decsi T. *Elhízott gyermekek étrendjének kiegészítése napi 1000 mg alfa-linolénsavval. Placebóval kontrollált, kettősen vak vizsgálat (Dietary supplementation of obese children with 1000 mg alpha-linolenic acid per day: a placebo-controlled double blind study, in Hungarian with English summary)*; Orvosi Hetilap 148(32):1499-1513, 2007.

- ***Lohner Sz***, Marosvölgyi T, Schmidt J, Molnár D, Decsi T. *Az alfa-linolénsav jelentősége a gyermekkori elhízáshoz társuló fokozott kardiovaszkuláris kockázat csökkentésében (Role of alpha-linolenic acid in amelioration of the*

cardiovascular risk related to obesity, in Hungarian with English summary); Gyermekgyógyászat 57(3): 345-350, 2006.

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- Szabó É, ***Lohner Sz***, Molnár D, Decsi T. *A transz izomér telítetlen zsírsavak kedvezőtlen hatásai a perinatális időszakban (Unfavorable effects of trans isomeric fatty acids in the perinatal period, in Hungarian)*; Gyermekorvos Továbbképzés IV(4): 48-51, 2006.

Cumulative impact factor of publications: **15.831**

Cumulative impact factor of publications as first author: **12.113**

Book chapters

- ***Lohner S,*** Decsi T. *Role of Long-Chain Polyunsaturated Fatty Acids in the Prevention and Treatment of Atopic Diseases.* In: Polyunsaturated Fatty Acids: Sources, Antioxidant Properties and Health Benefits (edited by: Angel Catalá). NOVA Publishers. 2013. Chapter 11, pp. 1-24. (ISBN 978-1-62948-151-7).

- ***Lohner Sz***, Vágási J, Decsi T. *Long-chain polyunsaturated fatty acid supplementation in the treatment of children with atopic dermatitis.* In: 10. Országos Interdiszciplináris Grastyán Konferencia előadásai (edited by: Szamonek V). 2012, pp. 207-212. (ISBN 978-963-642-471-8).

- Vágási J, ***Lohner Sz***, Marosvölgyi T, Tényi T, Decsi T. *A többszörösen telítetlen zsírsavakkal való ellátottság vizsgálata szkizofrén betegekben (Long-chain polyunsaturated fatty acid status of patients with schizophrenia, in Hungarian)*. In: 10. Országos Interdiszciplináris Grastyán Konferencia előadásai (edited by: Szamonek V). 2012, pp. 382-389. (ISBN 978-963-642-471-8).

- ***Lohner Sz***, Fekete K, Decsi T. *A folsav szupplementáció gyermekgyógyászati vonatkozásai: eredmények és további lehetőségek (Paediatric aspects of folic acid supplementation: results and further possibilities, in Hungarian with English summary). In:* Interdiszciplináris Doktorandusz Konferencia 2012 Konferenciakötet (edited by: Sipos N, Gunszt D). pp. 261-269. (ISBN 978-963-642-484-8).

Oral presentations

- ***Lohner Szimonetta:*** *Veleszületett anyagcsere-betegségben szenvedő gyermekek étrendjének kiegészítése*. Magyar Gyermekorvosok Társasága 2014. évi Jubileumi Nagygyűlése, 2014 május, Budapest

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- Krisztina Mihályi, Eszter Györei, Éva Szabó, ***Szimonetta Lohner,*** Tamás Marosvölgyi, Tamás Decsi: *Changes of the fatty acid composition of breast milk during the lactation* 47th Annual Meeting of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition, 2013 May, London

- ***Lohner Szimonetta,*** Fekete Katalin, Decsi Tamás: *A fehérjeszegény diétát tartó fenilketonúriás betegek n-3 többszörösen telítetlen zsírsavakkal való ellátottsága nem*

megfelelő. XI. Országos Interdiszciplináris Grastyán Konferencia, 2013. április, Pécs (***A legközérthetőbb tudományos előadás díja)***

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