Aus der Kinderklinik und Kinderpoliklinik im
Dr. von Haunerschen Kinderspital
der Ludwig-Maximilians-Universität München
Direktor: Professor Dr. Dr. Christoph Klein

Bestimmung von Glycerophospholipid gebundenen Fettsäuren in Erythrozyten und Vergleich des Einbaus von Docosahexaensäure in Glycerophospholipide aus Plasma, Erythrozyten und Wangenschleimhautzellen

Dissertation

Zum Erwerb des Doktorgrades der Humanmedizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Sabrina Klem
aus
Freiburg im Breisgau

2014

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Professor Dr. Berthold Koletzko
Mitberichterstatter:	Professor Dr. Clemens von Schacky,
	Priv. Doz. Dr. Uli C. Brödl
Mitbetreuung durch den	
promovierten Mitarbeiter:	Dr. Johann Demmelmair

Dekan: Professor Dr. med. Dr. h. c. M. Reiser, FACR, FRCR

Tag der mündlichen Prüfung : 22.05.2014

Einleitende Zusammenfassung der schriftlichen, kumulativen Promotion

gemäß § 4a der Promotionsordnung der LMU vom 1.Juni 1983 in der achten Fassung der Änderungssatzung vom 1.April 2009

Inhaltsverzeichnis

Abkürzungen

CC	cheek cells: Wangenschleimhautzellen
BHT	Butylhydroxytoluol
CE	Cholesterolester
DHA	Docosahexaensäure
EPA	Eicosapentaensäure
FAME	Fettsäuremethylester
GPL	Glycerophospholipiden
LC-PUFA	long chain polyunsaturated fatty acid: langkettige mehrfach ungesättigte Fettsäuren
PC	Phosphatidylcholin
PE	Phosphatidyletholamin
PI	Phosphatidylinositol
PL	Phospholipide
PS	Phosphatidylserin
RBC	red blood cells: Erythrozyten
SM	Sphingomyeline
TAG	Triglyzeride

1 Einleitung

In den letzten Jahrzehnten sind die langkettigen ungesättigten RBC Fettsäuren (LC-PUFA) in das Interesse wissenschaftlicher Forschungen gerückt, insbesondere die Docosahexaensäure (DHA) und die Eicosapentaensäure (EPA). Im Rahmen von zahlreichen, aufwändigen klinischen Studien und laborchemischen Untersuchungen konnte eine Assoziation zwischen der ausreichenden Versorgung mit LC-PUFA und der menschlichen Gesundheit (kardiovaskuläre Funktion, Herzkreislauferkrankungen [1, 2], verbesserte frühkindliche Entwicklung und der Vermeidung von Depression und anderen psychischen Krankheiten festgestellt werden [3].

All diese Studien beziehen die analysierten Fettsäuren auf unterschiedlich definierte klinische Outcomeparameter, da es derzeit in der Fettsäureanalytik kein Standard-prozedere gibt. Es werden verschiedene biologische Kompartimente, wie Gesamtblut, Erythrozyten (Red Blood Cells = RBC), Plasma und Fettgewebe als Biomarker genutzt, um den individuellen Status an Omega-3 Fettsäuren zu bestimmen.
Phospholipide (PL) bestehen aus Glycerophospholipiden (GPL) und Sphingomyelinen (SM) [4]. Da Sphingomyeline nur Spuren von Omega-3 Fettsäuren enthalten, sind sie in der LC-PUFA Analytik nicht von erheblichem Interesse [5]. Die Hauptbestandteile der GPL sind Phosphatidylcholin (PC), Phosphatidyletholamin (PE), Phosphatidylinositol (PI) und Phosphatidylserin (PS). Die am häufigsten genutzten Biomarker sind Plasma bzw. Serum PL, Triglyzeride (TAG), Cholesterolester (CE), PC und Erythrozyten PL, PC und PE [6, 7].

Die Analytik der individuellen Lipiduntergruppen (PC, PE, PS, PI) benötigt Separation mittels Dünnschichtchromatographie oder Festphasenextraktion und Derivatisierung der Fettsäuren. Diese Methoden sind meist zeitaufwändig und kostspielig. In den letzten Jahren wurden daher neue Methoden entwickelt, mit welchen die Analytik vereinfacht werden konnte, um den Arbeitsaufwand und dadurch die Kosten zu minimieren und den Probendurchsatz zu erhöhen [8]. Für gewöhnlich werden dafür Gesamtblut, Plasma/ Serum oder RBC Gesamtlipide verwendet.

Unsere Forschungsgruppe hat eine neue, sensitive und robuste Methode entwickelt, um selektiv die Fettsäuren der Glycerophospholipidfraktion im Plasma zu bestimmen, welche nur wenig vom postprandialen Status des Probanden beeinflusst werden [9]. Bei diesem neuen Verfahren wird die konventionelle Lipidextraktion und deren Auftrennung durch eine methanolische Ausfällung der Proteine mit zusätzlicher Ausfällung von TAG und CE ersetzt. In Kombination mit basenkatalysierter Synthese von Methylestern bei Raumtemperatur wird die ausschließliche Umesterung der GPL Fettsäuren gesichert [9]. In der Vergangenheit hat sich gezeigt, dass sich dieses Verfahren nicht einfach von Plasma auf RBC übertragen lassen, sodass es notwendig war die Anwendbarkeit der Plasma-GPL Methode auf RBC zu überprüfen, zu optimieren und zu validieren, bevor sie in klinischen Studien Anwendung finden kann.

Im Rahmen dieser Untersuchung bestimmten wir auch den Omega-3-Index. Er berechnet sich aus dem Anteil von DHA plus EPA in den RBC, und wird als Prozentanteil an den gesamten Fettsäuren der RBC ausgedrückt und stellt einen Risikofaktor für koronare Herzerkrankung dar [10, 11]. Diese Untersuchungen beschreibt die Publikation 1.

Des Weiteren beschäftigte sich unsere Forschungsgruppe mit der Bestimmung des Fettsäurestatus aus Wangenschleimhautzellen (cheek cells = CC). Die bisher verwendeten Verfahren benutzen invasive Techniken zur Gewinnung von zu analysierenden Proben (Blutproben durch Blutentnahmen, Fettgewebe durch Gewebebiopsie), was bei Studien mit Säuglingen und Kleinkindern ein limitierender Faktor ist. Wangenschleimhaut-PL als biologische Marker für die Fettsäureaufnahme wurden zwar bisher empfohlen [12], doch kaum in klinischen Studien genutzt. Die Gründe hierfür könnten in der unsicheren Probenqualität und -quantität und zusätzlich der Unsicherheit in der Probenaufarbeitung liegen [13].

Wir haben eine robuste Methode für die Analyse von CC GPL Fettsäuren entwickelt, welche nur wenig Probenmaterial benötigt und einfach anzuwenden ist [14].

Die genannten Methoden zur Extraktion von GPL wurden im Rahmen einer klinischen Interventionsstudie evaluiert. Dabei wurden vor allem der DHA-Anstieg im zeitlichen Verlauf der Studie zwischen den verschiedenen Kompartimenten Plasma, RBC und Wangenschleimhautzellen verglichen und Korrelationen berechnet, um eine

Aussage darüber treffen zu können, welcher Biomarker, welche Änderungen widerspiegelt und wie geeignet die Analyse von GPL der Fettsäuren im Vergleich zu langjährig etablierten Markern, wie PL ist (siehe Publikation 2).

2 Material und Methoden

Die vorliegende Dissertation basiert auf der Durchführung einer klinischen Supplementationsstudie mit DHA (510 mg/d), für welche 13 freiwillige, gesunde Probanden rekrutiert wurden. Die Studie wurde nach den Richtlinien der Deklaration von Helsinki durchgeführt, von dem Ethischen Komitee der Universität München (Medical Center 034-10) geprüft und unter ClinicalTrials.gov NCT01192269 registriert.

Sieben Frauen und sechs Männer im Alter zwischen 20 und 40 Jahren und einem BMI von 20-25 kg/m^2 wurden eingeschlossen. Ausschlusskriterien waren die Einnahme von Omega-3 Fettsäuren oder von Medikamenten, die den Lipidmetabolismus beeinflussen bis zu drei Monate vor Studienbeginn, genauso wie Schwangerschaft, kürzlich durchgeführte Reduktionsdiäten, Alkohol- oder Drogenabusus und der Genuss von fettem Fisch häufiger als einmal pro Woche. Die Probanden wurden aufgefordert vor Interventionsbeginn über drei Tage ein Ernährungsprotokoll auszufüllen. Aufgenommene Nährstoffe wurde mittels PRODI (version 4.5.LE, Nutri-Science) berechnet.

Die Studie wurde in eine 2-wöchige baseline-Periode und eine 29-tägige Interventionsperiode geteilt. Während diesem Zeitraum wurden an 11 Messtagen Blutproben und Wangenschleimhautabstriche morgens nüchtern gewonnen: Tag 0 (14 Tage vor Interventionsbeginn), 1 (Interventionsbeginn), 2, 3, 4, 9, 14, 18, 24 und 29 (Interventionsende). Der Wangenschleimhautabstrich wurde mit Hilfe einer endozervikalen Bürste gewonnen, indem der Proband nach vorherigem Ausspülen des Mundes die Wangeninnenseite ca. 20 Mal abbürstete und das erhaltene Sputum mit den Wangenschleimhautzellen in ein Reagenzglas überführte.
Klinische Untersuchungen (Anthropometrie, Körperfettanteil) und laborchemische Untersuchungen (Lipidprofil) wurden an Tag 0 und Tag 29 durchgeführt.
Das Studiensupplement bestand aus 950 µl DHASCO ©-S Mikroalgenöl, welches 510 mg DHA pro Kapsel enthielt. Der Anteil anderer Omega-3 Fettsäuren und EPA war vernachlässigbar gering (<0.4%). An den ersten 5 Tagen wurde die Kapsel direkt nach Blutentnahme und Wangenschleimhautabstrich eingenommen. Im

weiteren Verlauf der Studie wurde die Kapsel dem Probanden ausgehändigt, zu Hause nach dem Frühstück eingenommen, und die Uhrzeit der Einnahme notiert.

Die gewonnenen Proben wurden umgehend auf Eis gestellt und maximal 2 Stunden nach der Gewinnung aufgearbeitet. Die einzelnen Methoden zur Aufarbeitung von Wangenschleimhautzellen und RBC sind in den folgenden Publikationen detailliert nachzulesen. Die Analytik der Plasma GPL wurde nach Glaser et al. durchgeführt [9].

Im Rahmen der Validierung der Methode für GPL in RBC wurden die Proben mit BHT (0,2 %) bei -80° C gelagert und im Zeitraum von 8 Monaten aufgearbeitet. Die Vergleichsmethoden zur Analytik von RBC PL und PC und PE sind ebenfalls kurz in Publikation 1 beschrieben und basieren auf Folch [15] und Geppert [16].

Die, durch oben genannte Methoden erhaltene Fettsäuremethylester (FAME) wurden mit Hilfe der Gaschromatographie quantifiziert (Agilent 5890 series II, Waldbronn, Germany) [9] und die erhaltenen Peaks mittels EZChrom Elite (Version 3.1.7, Agilent) ermittelt und integriert. Der relative Anteil der Fettsäuren (%wt/wt und mol%) wurde auf der Grundlage von 20 cis-Fettsäuren berechnet und als Mittelwert und Standardabweichung dargestellt.

Die statistischen Analysen wurden mit IBM SPSS Statistics for Windows, Ver. 19.0.0.1 durchgeführt werden.

3 Zielsetzung der Untersuchungen

3.1 Fragestellungen der Publikationen

- Validierung und Optimierung einer neuen Methode zur Analyse von GPL Fettsäuren in Erythrozytenmembranen ohne Liquid-Liquid Extraktion und ohne Dünnschichtchromatographie
    - Vermeidung der Verklottung von RBC während der Aufarbeitung
    - Bestimmung der Haltbarkeit von RBC Proben

- Überprüfung der Anwendbarkeit dieser neuen Methode in einer DHA Interventionsstudie und Vergleich der Ergebnisse zur etablierten Markern (z.B. Omega-3 Index)

- Untersuchung der Validität der GPL aus RBC als Fettsäure Statusmarker

- Vergleich des Zeitverlaufs der DHA-Aufnahme in die unterschiedlichen Kompartimente (Wangenschleimhautzellen, Plasma und RBC) in einer DHA Supplementationsstudie

- Bestimmung der Korrelationen des prozentualen DHA-Anstieges in den unterschiedlichen Geweben Plasma, RBC und Wangenschleimhautzellen

- Validierung der GPL-Methode in Wangenschleimhautzellen und Erfassung der zeitlichen Änderung des Omega-3 Gehaltes in CC im Rahmen einer diätetischen Supplementation

- Spiegeln Wangenschleimhaut-GPL kurz- oder längerfristige Änderungen der Fettsäureaufnahme wider?

Im letzten Kapitel dieser kumulativen Dissertation sind die Ergebnisse der beiden Publikationen kurz in deutscher und englischer Sprache zusammengefasst und allgemeine Schlussforderungen formuliert.

4 Publikationen

4.1 Publikation 1 - PloS ONE

„Efficient and Specific Analysis of Red Blood Cell Glycerophospholipid Fatty Acid Composition.“

Submitted: 19. August 2011
Accepted: 21. Februar 2012
Published: 30.März 2012

OPEN ACCESS Freely available online PLoS one

Efficient and Specific Analysis of Red Blood Cell Glycerophospholipid Fatty Acid Composition

Sabrina Klem, Mario Klingler, Hans Demmelmair, Berthold Koletzko*

Division of Metabolic and Nutritional Medicine, Dr. von Hauner Children's Hospital, University of Munich Medical Center, Munich, Germany

Abstract

Background: Red blood cell (RBC) n-3 fatty acid status is related to various health outcomes. Accepted biological markers for the fatty acid status determination are RBC phospholipids, phosphatidylcholine, and phosphatidyletholamine. The analysis of these lipid fractions is demanding and time consuming and total phospholipid n-3 fatty acid levels might be affected by changes of sphingomyelin contents in the RBC membrane during n-3 supplementation.

Aim: We developed a method for the specific analysis of RBC glycerophospholipids. The application of the new method in a DHA supplementation trial and the comparison to established markers will determine the relevance of RBC GPL as a valid fatty acid status marker in humans.

Methods: Methyl esters of glycerophospholipid fatty acids are selectively generated by a two step procedure involving methanolic protein precipitation and base-catalysed methyl ester synthesis. RBC GPL solubilisation is facilitated by ultrasound treatment. Fatty acid status in RBC glycerophospholipids and other established markers were evaluated in thirteen subjects participating in a 30 days supplementation trial (510 mg DHA/d).

Outcome: The intra-assay CV for GPL fatty acids ranged from 1.0 to 10.5% and the inter-assay CV from 1.3 to 10.9%. Docosahexaenoic acid supplementation significantly increased the docosahexaenoic acid contents in all analysed lipid fractions. High correlations were observed for most of the mono- and polyunsaturated fatty acids, and for the omega-3 index ($r = 0.924$) between RBC phospholipids and glycerophospholipids. The analysis of RBC glycerophospholipid fatty acids yields faster, easier and less costly results equivalent to the conventional analysis of RBC total phospholipids.

Citation: Klem S, Klingler M, Demmelmair H, Koletzko B (2012) Efficient and Specific Analysis of Red Blood Cell Glycerophospholipid Fatty Acid Composition. PLoS ONE 7(3): e33874. doi:10.1371/journal.pone.0033874

Editor: Matej Oresic, Governmental Technical Research Centre of Finland, Finland

Received August 19, 2011; **Accepted** February 21, 2012; **Published** March 30, 2012

Funding: This study was financially supported by the Federal Ministry of Education and Research (project number: 0315680B). B. Koletzko is the recipient of a Freedom to Discover Award of the Bristol-Myers Squibb Foundation, New York, NY. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: office.koletzko@med.uni-muenchen.de

Introduction

The supply of n-3 long chain polyunsaturated fatty acids (LC-PUFA) is related to cardiovascular function, heart disease, morbidity and mortality, and in the perinatal period to child development [1,2]. Commonly used biological markers for the dietary n-3 fatty acid intake are fatty acid composition of plasma or serum phospholipids (PL), triacylglycerides (TAG), cholesterol esters (CE), phosphatidylcholine (PC) and of red blood cell (RBC) PL, PC and phosphatidylethanolamine (PE) [3,4]. The analysis of individual lipid species requires lipid separation usually by thin layer chromatography or solid phase extraction and the acid-catalysed derivatisation of fatty acids. In recent years, novel methods were developed avoiding these steps to reduce processing time and costs, and to increase sample throughput [5]. The application of these methods usually requires whole blood, plasma/serum, or RBC total lipids for the evaluation of the fatty acid status. Good correlations exist between fatty acid contents of these biological matrices and the diet. This is similar to correlations found between dietary fat intake and individual lipid classes [4].

Recently, our group developed a new, sensitive and robust method for the selective determination of plasma glycerophospholipids (GPL) fatty acids, which is independent of the postprandial state of a subject [6]. The conventional lipid extraction and separation has been replaced by the methanolic precipitation of proteins with co-precipitation of TAG and CE. In combination with a base catalysed synthesis of methyl esters at room temperature, this ensures the specific transesterification of GPL fatty acids [6]. In the past, it has been shown that extraction procedures cannot easily be transferred from plasma to RBC [7]. Therefore, it is essential to test the applicability of the plasma GPL method on RBC before its use in clinical studies.

The aim of the present study is to optimise and validate a new method for the analysis of GPL in RBC membranes, which avoids liquid-liquid extractions steps and chromatographic lipid class isolation. The application of the new method in a DHA supplementation trial and the comparison to established markers such as RBC PL, PC and PE will determine the relevance of RBC GPL as a valid fatty acid status marker in humans.

Materials and Methods

Subjects

Thirteen healthy subjects (6 males, 7 females) from the Munich area with an average age of 25.8±2.7 years (mean ± SD) and a BMI of 21.9±1.6 kg/m^2 participated in an open-label, single-group assignment supplementation study with DHA. Taking supplements containing n-3 LC-PUFA or drugs interfering with the lipid metabolism were exclusion criteria as well as fatty fish consumption of more than once per week. The study was registered at ClinicalTrials.gov (NC T01192269). The Ethical Committee of the University of Munich Medical Center approved the study (034-10) and participants signed informed consent forms before they entered the study.

Study design and supplements

Participants were asked to take a 950 µl DHASCO®-S microalgae oil capsule (Martek Biosciences, Columbia, MD) during breakfast daily over a period of 30 days. The capsule contained 520 mg DHA, but no other n-3 fatty acids. Participants were asked to record the intake of the capsules including time and date of consumption. Blood samples collected before and after the intervention period were analysed. This study was part of a larger project evaluating the DHA incorporation into plasma, RBC and buccal cell GPL.

Sample preparation

After an overnight fasting period antecubital venous blood was collected into 7.5 ml EDTA-containing monovettes (Sarstedt, Nümbrecht, Germany). Samples were directly placed on ice and processed within 2 hours after collection. Plasma and RBC were separated by centrifugation (1000×g, 10 min, 4°C). RBC were washed 3 times with saline solution (0.9% NaCl). For the determination of PC and PE fatty acids aliquots of 500 µl RBC were haemolysed with 250 µl distilled water and suspended in 8 ml isopropanol containing BHT (0.05%). For the analysis of PL and GPL 100 µl aliquots of RBC were haemolysed with 100 µl distilled water and suspended in 260 µl methanol containing BHT (0.2%). All samples were stored at −80°C until analysis.

GPL fatty acid analysis

The method of Glaser et al. for the analysis of plasma GPL was adapted for the determination of RBC GPL fatty acids [6,8]. Intra- and inter-assay analyses were performed to validate the method before study commencement. In total 1.3 ml methanol and 100 µl of internal standard solution (14.6 mg PC15:0 in 100 ml methanol; Sigma Aldrich, Taufkirchen, Germany) were added to 200 µl haemolysed RBC. After continuous shaking on a Vibrax shaker (IKA, Stauffen, Germany) at 1000 rpm for 5 min samples were treated for 5 min in an ultrasound water bath (40 kHz, 120 W). The RBC suspension was centrifuged at 3030×g for 10 min at 4°C to separate the methanolic supernatant from cell fragments and precipitated proteins. After the transfer of the supernatant into a 4 ml brown glass vial synthesis of fatty acid methyl ester (FAME) was initiated by adding 50 µl sodium methoxide solution (25 wt% in methanol; Sigma Aldrich). The reaction was performed at room temperature and stopped after 4 min by adding 150 µl 3 M methanolic HCl (Sigma Aldrich). FAME were extracted twice with 600 µl hexane and the extracts were combined. Solvents were evaporated under a nitrogen flow and FAME redissolved in 50 µl hexane containing BHT (0.2%). Extracts were stored at −20°C until gas chromatographic (GC) analysis.

Analysis of RBC PL fatty acids

1.8 ml chloroform, 540 µl methanol and 100 µl internal standard solution (PC15:0 in methanol) were added to a thawed RBC sample to obtain a chloroform-methanol ratio of 2:1 v/v for lipid extraction [9]. A sodium chloride solution (2%) was added to the solvent mixture to obtain phase separation after subsequent centrifugation for 10 min at 3030×g and 4°C. The organic phase containing the lipids was dried under reduced pressure. The dried extract was redissolved in 400 µl chloroform/methanol (1:1 v/v), applied on a 20×20 cm silica gel plate (Merck, Darmstadt, Germany) and lipid classes were separated using heptane, diisopropyl ether and acetic acid (60:40:3) as mobile phase [10]. Individual lipid bands were visualised with 2,7 di-chlor-fluorescein. The PL band was scraped off and transferred into a brown glass vial. FAME were synthesised in a closed vial with 3 N methanolic HCl at 85°C for 45 minutes. Samples were neutralised with a mixture of sodium carbonate, sodium hydrogen carbonate and sodium sulphate (1:2:2, Merck, KGaA). FAME were extracted twice with 1 ml hexane and redissolved in 50 µl hexane containing BHT (0.2%). Samples were stored at −20°C until GC analysis.

Analysis of RBC PC and PE

The analysis of PC and PE in RBC membranes was performed as previously described by Geppert et al. [11]. Briefly, after extracting total lipids twice with 7 ml isopropanol/chloroform (3:2 v/v) and 3 ml chloroform, the solvents were evaporated under reduced pressure. The separation of individual lipid fractions was achieved by thin layer chromatography using chloroform/ methanol/ ammonia solution(25%)/distilled water (73:27:2,2:2,8 by vol) as mobile phase. Corresponding PC and PE bands were scraped of the plate and transferred into 4 ml brown glass vials. FAME for GC analysis were obtained as describe above.

Gas chromatography

FAME were quantified by GC with flame ionisation detection (Agilent 5890 series II, Waldbronn, Germany). The applied settings have previously been published by Glaser et al [6]. Peak integration was performed with EZChrom Elite (Version 3.1.7, Agilent).

Statistical analysis

Relative fatty acid contents (% wt/wt) were calculated based on 20 cis-fatty acids and presented as mean and standard deviation. Precision analyses were performed by analyzing 8 aliquots of one RBC sample at the same day (intra-assay) or 26 aliquots over a period of 2 months (inter-assay) and calculated as coefficient of variation (CV). Intra-laboratory method performance were tested by comparing intra-assays (n = 8) of different staff members. Statistics for evaluating the effect of storage was performed with ANOVA repeated measures. The effect of DHA supplementation on fatty acid contents of different lipid fractions was assessed using paired t-tests. Relative DHA changes from baseline between PC, PE, GPL and PL were assessed with one-way ANOVA and Bonferroni post-hoc test. Correlations between fatty acid contents of different RBC compartments were evaluated according to Pearson. P-values <0.05 were considered to be statistically significant. All statistical analyses were computed using IBM SPSS Statistics for Windows, Version 19.0.0.1.

Results

Table 1 shows the intra- and inter-assay data of RBC samples donated by different volunteers. The CV of the intra-assay evaluation (n = 8) ranged from 1.0 to 10.5% for all fatty acids and

Table 1. Intra- and inter-assay reproducibility of the GPL fatty acid analysis.

	Intra-assay (n = 8)			Inter-assay (n = 26)		
	Mean	**SD**	**CV [%]**	**Mean**	**SD**	**CV [%]**
Saturated Fatty Acids						
C14:0	0.35	0.01	3.7	0.32	0.04	12.6
C16:0	22.99	1.01	4.4	23.31	0.80	3.4
C17:0	0.34	0.02	4.6	0.32	0.04	11.3
C18:0	18.34	0.27	1.5	17.92	0.40	2.3
Monounsaturated Fatty Acids						
C16:1n-7	0.29	0.02	7.6	0.35	0.03	8.6
C18:1n-7	1.45	0.03	2.0	1.42	0.15	10.9
C18:1n-9	14.07	0.22	1.5	13.38	0.22	1.6
C20:1n-9	0.29	0.00	3.3	0.27	0.01	4.9
n-9 Polyunsaturated Fatty Acids						
C20:3n-9	0.11	0.00	4.2	0.11	0.01	9.5
n-6 Polyunsaturated Fatty Acids						
C18:2n-6	11.84	0.50	4.3	12.34	0.16	1.3
C18:3n-6	0.06	0.00	3.7	0.08	0.01	10.1
C20:2n-6	0.22	0.01	6.4	0.25	0.01	5.3
C20:3n-6	2.37	0.02	1.0	2.21	0.04	1.7
C20:4n-6	15.39	0.84	5.5	15.57	0.55	3.5
C22:4n-6	2.37	0.25	10.5	3.20	0.20	6.3
C22:5n-6	0.67	0.02	3.0	0.64	0.03	5.0
n-3 Polyunsaturated Fatty Acids						
C18:3n-3	0.15	0.01	4.6	0.10	0.01	8.7
C20:5n-3	0.75	0.04	5.5	0.69	0.05	6.6
C22:5n-3	2.35	0.22	9.3	2.31	0.14	6.2
C22:6n-3	5.68	0.17	2.9	5.16	0.26	5.0

Mean and SD are expressed as %wt/wt.
doi:10.1371/journal.pone.0033874.t001

was <5% in most fatty acids. The inter-assay reproducibility (n = 26) was comparable to that of the intra-assay for all fatty acids (CV 1.3–10.9%), which contributed more than 0.5% to total fatty acids. Moreover, the inter-observer variability was tested by three different laboratory members, which achieved constantly a CV <10% for the 20 analysed RBC GPL fatty acids.

The extraction efficiency for of GPL was tested by applying 4 different extraction procedures (Figure 1). In total 16 aliquots (4×4) of an RBC sample were tested. Continuous shaking of RBC dissolved in methanol for 5 min yielded in 185±120 µg total GPL fatty acids per 100 µl RBC, which was similar to prolonged shaking for 10 min (241±63 µg) or additional ultrasound treatment for another 5 min (301±46 µg). Adding methanol to RBC without shaking caused clotting of RBC and lower recovery (37±24 µg). Partial clotting was also observed after shaking the samples for 5 or 10 min, but not when samples were treated with ultrasound. The fatty acid pattern of the 16 RBC aliquots did not differ to any appreciable extent (data not shown).

Contamination of the GPL containing supernatant with TAG and CE fatty acids was evaluated by separating lipid fractions in the methanolic supernatant via thin layer chromatography prior to the base-catalysed transesterification. Based on GPL total fatty acids the contamination originating from TAG fatty acids was 0.9% and from CE 0.4%. Only palmitic-, stearic-, oleic-, and linoleic acids derived from TAG or CE were detected.

Changes in fatty acid compositions caused by long-term storage are shown in Table 2. Washed RBC were kept in methanol containing BHT (0.2%) for 8 months at −80°C. Differences in fatty acid compositions were calculated based on changes relative to baseline and expressed in percent. Significant differences were found for some fatty acids, but values determined without storage were less than 10%, except for C17:0 (+29.5%), C18:3n-6 (+32.0%) and C22:4n-6 (+15.8%). The contribution of the first two fatty acids to total fatty acids was less than 0.5%. Palmitic acid was not affected under these storage conditions, whereas stearic acid decreased slightly (−2.8%). Fatty acids with a high potential for oxidative damage, such as n-3 and n-6 PUFA were affected differently. During storage the DHA percentage changed by 6.3%, (p = 0.061), whereas arachidonic acid (ARA) contents increased by 3.7% (p = 0.160), but differences were not statistically significant.

Table 3 shows the fatty acid composition of individual PL classes and total PL before and after the supplementation period. The micro algae oil supplementation increased DHA contents relative to baseline in PC by 92.3%±52.1, which was higher then in PE (33.2%±16.0), GPL (27.4%±16.5) and PL (13.3%±16.0) (ANOVA; $P<0.001$). ARA levels decreased during the supplementation period, which was significant in PL (−9.5%) and GPL (−3.9%), but not in PE (−0.6%) or PC (−6.9%). Similar results were shown for n-3 and n-6 docosapentaenoic acid (DPA). EPA was not affected by the supplementation, although by trend a slight increase could be observed in all fractions. At the beginning of the study contents of stearic acid in PC (10.7%±1.1) and PE (8.4%±1.1) were lower then in GPL (17.3%±0.5) or PL (18.5%±0.7), whereas oleic acid was more abundant in PC (17.2%±1.1) and PE (17.3%±0.9) then in GPL (14.6%±0.8) or PL (13.3%±0.8). All other GPL and PL fatty acid contents ranged between the levels of the respective fatty acids of PE and PC. The DHA supplementation had no effect on these observations.

Relationships of individual fatty acids between GPL and PC, PE or PL before and after the supplementation period are shown in Table 4. In general, SFA did not correlate between the different lipid fractions. At the beginning of the study high correlations were found for DHA between GPL/PE (r = 0.818) and GPL/PL (r = 0.940), and a good correlation between GPL/PC (r = 0.555). Similar correlations were shown for other n-3 and n-6 fatty acids, such as EPA, n-6 DPA and di-homo-γ-linolenic acid. GPL ARA contents were only correlated with PC ARA levels (r = 0.625), but not with ARA contents of the other fractions. The supplementation of micro algae oil influenced the correlations of individual fatty acids. The correlation of DHA between GPL/PE (r = 0.725) and GPL/PL (r = 0.729) was lower than at the beginning of the study. No correlations were found for DHA between GPL and PC. ARA, which did not correlate between GPL and PE showed a significant r-value of 0.657 after supplementation. The changes were most significant between GPL and PL as for most of the n-3 and n-6 fatty acid no longer correlations were found, except for DHA (r = 0.729), α-linolenic acid (r = 0.853) and di-homo-γ-linolenic acid (r = 0.874).

Omega-3-indices based on GPL and PL were calculated from data determined at study start (Figure 2). The sum of EPA and DHA percentages was highly correlated between both lipid fractions (r = 0.924; $P<0.001$). At the end of the study a correlation of r = 0.780 ($P = 0.002$) was found.

Discussion

This study shows that the analysis of RBC GPL is well suited for a fatty acid status determination in human. The base-catalyzed

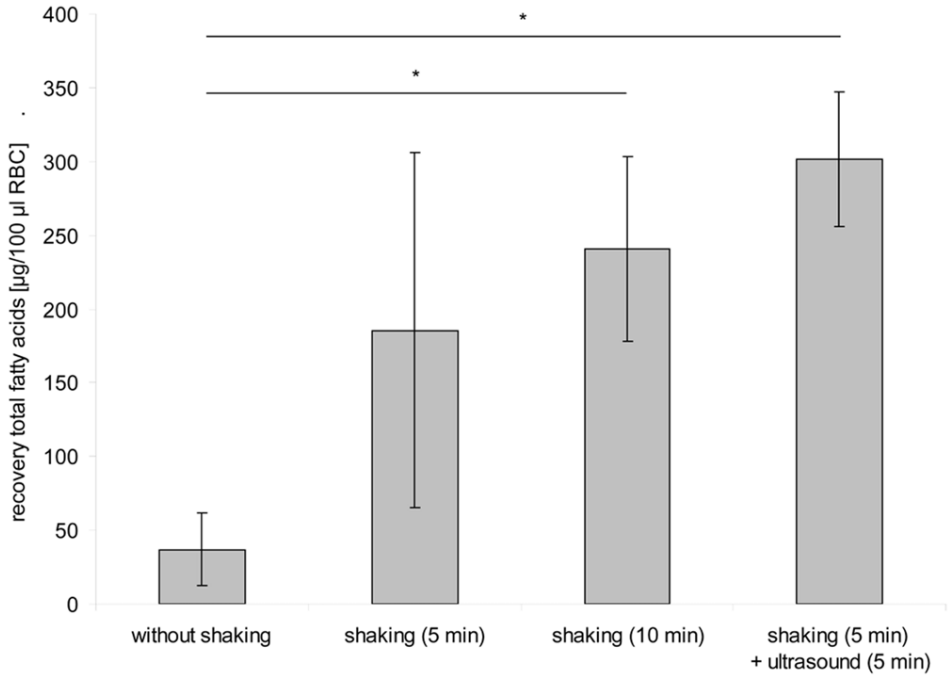

Figure 1. Recovery of total RBC fatty acids using different extraction procedures ($P<0.05$).
doi:10.1371/journal.pone.0033874.g001

Table 2. Changes in fatty acids (%wt/wt) during storage of RBC samples (n = 13) in methanol over a period of 8 months at −80°C.

	Analysis without storage		Analysis after 8 months storage		Difference*	
	Mean	**SD**	**Mean**	**SD**	**[%]**	***P***
C14:0	0.28	0.07	0.29	0.08	4.09	*<0.001*
C16:0	22.62	1.21	22.81	1.00	0.85	*n.s.*
C17:0	0.33	0.04	0.42	0.08	29.50	*<0.001*
C18:0	17.75	0.55	17.26	0.58	−2.75	*<0.001*
C16:1n-7	0.35	0.13	0.37	0.14	6.90	*n.s.*
C18:1n-7	1.35	0.11	1.39	0.12	3.32	*n.s.*
C18:1n-9	15.04	0.89	14.73	0.74	−2.03	*0.040*
C20:1n-9	0.31	0.07	0.29	0.04	−7.59	*n.s.*
C20:3n-9	0.11	0.03	0.10	0.02	−3.45	*n.s.*
C18:2n-6	11.79	1.29	11.57	1.10	−1.90	*n.s.*
C18:3n-6	0.06	0.03	0.07	0.03	31.97	*0.014*
C20:2n-6	0.28	0.04	0.26	0.03	−6.41	*n.s.*
C20:3n-6	1.91	0.43	1.82	0.39	−4.42	*n.s.*
C20:4n-6	16.04	1.24	16.63	0.86	3.69	*n.s.*
C22:4n-6	2.76	0.44	3.19	0.52	15.79	*0.004*
C22:5n-6	0.78	0.15	0.70	0.15	−9.74	*0.048*
C18:3n-3	0.14	0.03	0.14	0.03	−1.15	*n.s.*
C20:5n-3	0.58	0.21	0.60	0.20	3.10	*n.s.*
C22:5n-3	2.04	0.31	2.22	0.35	8.44	*n.s.*
C22:6n-3	5.61	1.16	5.26	0.93	−6.30	*n.s.*

*Differences in fatty acid contents caused through sample storage were related to fatty acid contents of samples without storage. Mean and SD are expressed as %wt/wt. n.s.: not significant.
doi:10.1371/journal.pone.0033874.t002

Table 3. Effects of DHA supplementation on fatty acid composition (%wt/wt) of individual RBC PL fractions.

	PC					PE					GPL					PL				
	study start		study end			study start		study end			study start		study end			study start		study end		
	mean	SD	mean	SD	% diff	mean	SD	mean	SD	% diff	mean	SD	mean	SD	% diff	mean	SD	mean	SD	% diff
C16:0	36.2	1.3	36.0	1.4	−0.5	17.7	1.9	17.3	1.5	−2.6	22.4	1.1	22.8	0.8*	1.9	25.6	1.1	27.6	1.5*	8.0
C18:0	10.6	1.1	10.4	1.1	−1.6	8.4	1.1	8.1	0.5	−3.6	17.3	0.5	16.8	0.6**	−3.0	18.5	0.7	19.1	1.4	4.7
C16: 1n-7	0.6	0.2	0.6	0.2	15.6	0.3	0.1	0.2	0.1	−6.7	0.4	0.1	0.4	0.1	−5.7	0.3	0.1	0.4	0.1	46.4
C18: 1n-7	2.0	0.1	2.1	0.1	1.0	1.4	0.3	1.3	0.1	−9.0	1.4	0.1	1.3	0.1*	−5.3	1.2	0.2	1.1	0.1	−3.9
C18: 1n-9	17.2	1.1	17.0	1.1	−1.4	17.3	0.9	17.0	1.2	−8.8	14.6	0.8	14.5	0.7	−0.7	13.3	0.8	13.1	0.7*	−1.7
C20: 3n-9	0.1	0.0	0.1	0.0	−3.3	0.1	0.0	0.1	0.0	8.6	0.1	0.0	0.1	0.0	−5.8	0.1	0.0	0.1	0.0	31.1
C18: 2n-6	19.5	1.6	19.7	1.6	1.2	5.6	0.9	5.4	0.9	−4.0	11.6	1.2	11.3	1.0	−2.1	10.2	1.0	9.9	0.8	−2.1
C18: 3n-6	0.1	0.0	0.1	0.0	−6.4	0.1	0.0	0.1	0.0	0.4	0.1	0.0	0.1	0.0	−5.0	0.1	0.0	0.1	0.0	20.5
C20: 3n-6	2.1	0.6	2.1	0.5	−2.9	1.3	0.3	1.2	0.2	−3.4	1.9	0.4	1.7	0.4***	−7.7	1.6	0.5	1.4	0.4***	−14.6
C20: 4n-6	6.8	1.0	6.4	0.9	−5.8	25.4	1.9	25.5	1.4	−0.6	16.8	0.9	16.1	0.7*	−3.9	16.3	1.1	14.7	0.9***	−9.5
C22: 4n-6	0.4	0.1	0.3	0.0	−6.9	8.2	1.1	7.8	1.0*	−6.0	3.1	0.6	3.2	0.5	2.2	3.7	0.4	3.1	0.4***	−16.2
C22: 5n-6	0.2	0.1	0.1	0.0	−3.0	1.0	0.2	0.9	0.2**	−13.3	0.7	0.2	0.7	0.1***	−11.0	0.6	0.2	0.4	0.2***	−36.7
C18: 3n-3	0.2	0.1	0.2	0.1	19.3	0.1	0.0	0.1	0.0	15.1	0.1	0.0	0.1	0.0	2.0	0.1	0.0	0.1	0.0	−9.7
C20: 5n-3	0.5	0.4	0.5	0.1	27.5	1.0	0.3	1.0	0.3	4.3	0.6	0.3	0.6	0.1	9.3	0.6	0.2	0.6	0.2	9.5
C22: 5n-3	0.5	0.1	0.4	0.1*	−12.5	4.5	0.6	4.2	0.6***	−7.9	2.3	0.4	2.1	0.3	−4.4	2.3	0.3	1.9	0.4**	−17.4
C22: 6n-3	1.4	0.2	2.6	0.5***	92.3	6.2	0.9	8.3	0.9***	33.2	4.6	0.8	5.8	0.6***	27.3	4.3	0.8	4.9	0.9**	13.3

Differences between study start and end were based on baseline values. Paired t-test: *p<0.05, **p<0.01, *p<0.001.

doi:10.1371/journal.pone.0033874.t003

transesterification of RBC GPL fatty acids presented here has been applied for the analysis of plasma and cheek cell GPL before [6,8,12]. A good precision and robustness, a high sample throughput and a low-sample volume distinguish this method from fatty acid analyses using chromatographic separation of lipid fractions [13]. RBC GPL fatty acids before and after DHA supplementation were very similar to the fatty acid composition of total RBC PL analysed by the much more cumbersome conventional methodology.

A major challenge was clotting of RBC in methanol, which affected the total GPL fatty acid recovery. Extending the shaking time of RBC in methanol to 10 min had little effect on clot formation, but variations between measurements decreased compared to shorter shaking periods of 5 min. The application of ultrasound (indirect application in a water bath) after shaking resulted in a fine grained RBC suspension. The ultrasound treatment increased the recovery rate of the total fatty acid concentration and further decreased the variation of results between measurements. However, the PC standard did not compensate for the differences in extraction efficacy. We assume that the inclusion of PL into clots causes the loss of PL and not the partitioning of PL between solid and liquid phase during extraction, in line with the observation that fatty acid compositions were not affected. The ultrasound treatment is an integral part of the procedure to optimise recovery. Ultrasound treatment of 5 min seems to be sufficient to totally disperse the RBC clots in methanol.

For the analysis of GPL fatty acids in plasma an intra-assay CV of <3.7% and inter-assay CV of <10.7% was achieved for all studied fatty acids [6]. The precision of the fatty acid analysis in cheek cells was comparable, with CV ranging from 0.7% to 14.1% [12]. The precision data determined in this study for RBC were similar. This shows that the two step procedure, methanolic protein precipitation and base catalysed transesterification, is reliable for the GPL fatty acid determination in plasma, RBC, and cheek cells.

Storage of RBC samples over a longer period may be necessary in trials with large subject numbers [14]. Fatty acids of RBC samples are stable at temperatures below −50°C with or without free radical scavenging or iron binding agents [15]. Treating the washed RBC samples prior to freezing seems unnecessary, but when adding BHT a solvent is required as this antioxidant is insoluble in water. When our samples were stored in methanol containing BHT (0.05 mg/ml of RBC) for 8 months at −80°C,

Table 4. Correlations between RBC GPL fatty acids and other RBC PL fractions before and after n-3 supplementation.

Correlation of individual GPL fatty acids *before study:*

	PC		*PE*		*PL*	
	R	*P*	**R**	*P*	**R**	*P*
C16:0	0.525	n.s.	0.081	n.s.	0.343	n.s.
C18:0	0.529	n.s.	0.291	n.s.	0.162	n.s.
C16:1n-7	0.801	0.001	0.893	<0.001	0.874	<0.001
C18:1n-7	0.509	n.s.	0.725	0.005	0.409	n.s.
C18:1n-9	0.919	<0.001	0.441	n.s.	0.898	<0.001
C20:3n-9	0.352	n.s.	0.142	n.s.	0.743	0.006
C18:2n-6	0.797	0.001	0.446	n.s.	0.970	<0.001
C18:3n-6	0.436	n.s.	0.104	n.s.	0.427	n.s.
C20:3n-6	0.766	0.002	0.905	<0.001	0.967	<0.001
C20:4n-6	0.625	0.022	0.497	n.s.	0.473	n.s.
C22:5n-6	0.746	0.003	0.909	<0.001	0.820	0.001
C18:3n-3	0.930	<0.001	0.723	0.005	0.566	n.s.
C20:5n-3	0.899	<0.001	0.779	0.002	0.966	<0.001
C22:5n-3	0.739	0.004	0.704	0.007	0.594	0.042
C22:6n-3	0.555	0.049	0.818	<0.001	0.940	<0.001

Correlation of individual GPL fatty acids *after study:*

	PC		*PE*		*PL*	
	R	*P*	**R**	*P*	**R**	*P*
C16:0	0.420	n.s.	0.281	n.s.	−0.073	n.s.
C18:0	0.529	n.s.	−0.363	n.s.	0.163	n.s.
C16:1n-7	0.943	<0.001	0.952	<0.001	0.313	n.s.
C18:1n-7	0.470	n.s.	0.547	n.s.	0.446	n.s.
C18:1n-9	0.878	<0.001	0.739	0.004	0.807	<0.001
C20:3n-9	0.341	n.s.	0.376	n.s.	−0.034	n.s.
C18:2n-6	0.524	n.s.	0.833	<0.001	0.498	n.s.
C18:3n-6	0.529	n.s.	0.489	n.s.	−0.141	n.s.
C20:3n-6	0.816	<0.001	0.913	<0.001	0.874	<0.001
C20:4n-6	0.681	0.010	0.657	0.015	0.427	n.s.
C22:5n-6	0.575	0.040	0.948	<0.001	0.226	n.s.
C18:3n-3	0.875	<0.001	0.761	0.003	0.853	<0.001
C20:5n-3	0.255	n.s.	0.946	<0.001	0.291	n.s.
C22:5n-3	0.577	0.039	0.885	<0.001	0.016	n.s.
C22:6n-3	0.454	n.s.	0.725	0.005	0.729	0.005

n.s. not significant.

doi:10.1371/journal.pone.0033874.t004

most of the fatty acid proportions did not change to an appreciable extent from pre-storage values. This is comparable with other published RBC conservation methods, which stored samples for 12 months or longer [14,15,16,17]. However, a non significant trend towards a selective degradation of DHA was observed, and losses might become significant after 12 months of storage. Increasing the BHT concentration in the sample [15] or excluding the hemolysis of RBC with distilled water prior to freeze storage [14,17] might improve the DHA stability, but this needs to be determined for GPL bound DHA.

The supplementation of micro algae oil, rich in DHA, significantly increased the DHA contents of PC and PE in the study subjects. The relative increase in PC was higher then in PE, which has also been described by other authors [11,18,19]. The non-uniform distribution of PC and PE in the RBC membrane and the different mechanisms for the fatty acid exchange of PC and PE with plasma may explain these observations.

SM in the RBC membrane behave differently during n-3 intervention and may affect the n-3 fatty acid status determination, as PC and PE proportions decrease and the SM proportion increases slightly with n-3 LC-PUFA supplementation [20]. This might be explained by the fact that the RBC membrane homeostasis is not only maintained by the exchange of other highly unsaturated fatty acids with DHA, i.e. ARA, but also by an increase of SM, which counteracts effects of high n-3 levels in PC or PE on membrane fluidity [21]. Our findings of increased SFA

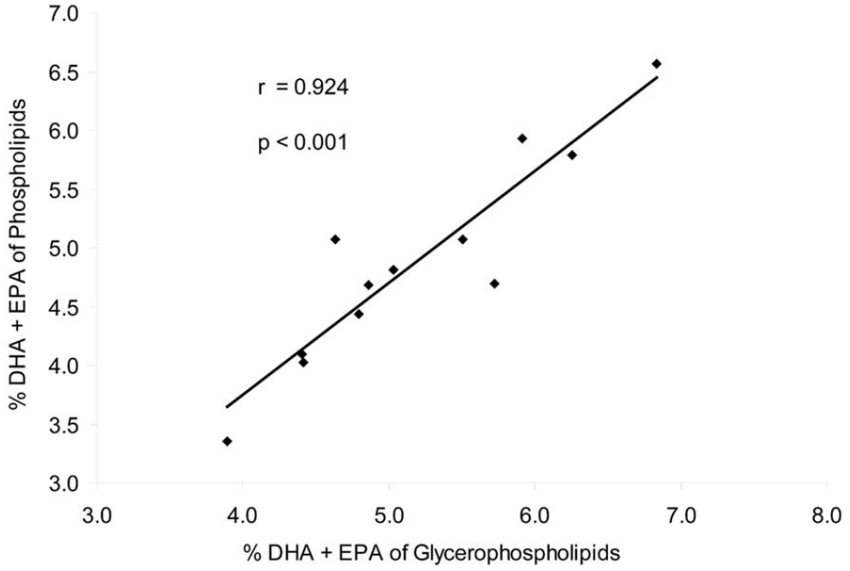

Figure 2. Correlation of RBC total PL and RBC GPL omega-3 index (n = 13).
doi:10.1371/journal.pone.0033874.g002

and decreased ARA contents in PL at the end of the study agree with this hypothesis.

Our data show a trend towards increased EPA levels after the supplementation of DHA in all studied fractions. This might be related to the retroconversion of DHA to EPA. In humans a retro conversation rate of at least 5% is observed [22]. This needs to be considered when DHA is given as the only n-3 fatty acid source.

The omega-3 index, based on the relative EPA+DHA content, is described as risk factor for coronary heart diseases [23]. We found a high correlation between the omega-3 index in RBC PL and GPL. It has to be determined, whether the omega-3 index proposed by Harris and von Schacky is comparable to our results, as different methods are applied and calculations of EPA+DHA might be based on different definitions of total fatty acids [23]. However, our results show a strong correlation between PL and GPL based omega-3 indices, therefore DHA and EPA proportions of GPL analysed with our method may be applicable for an omega-3 index determination.

In conclusion, the different responses of individual PL fractions to an n-3 supplementation have to be considered for the interpretation of changes in n-3 fatty acid status. Absolute and relative changes in RBC PC seem to be higher then in PE or GPL. The PL fatty acid composition is affected by different regulatory mechanisms in the RBC membrane, which affect the contribution of SM to total PL. Thus, we propose that our method for the analysis of RBC GPL fatty acids is advantageous over the RBC PL analysis, as changes of SM are excluded. Moreover, if the focus is not on a specific GPL subfraction, our method is an excellent alternative to monitor n-3 supplementation on fatty acid composition as it avoids the labour intensive, time-consuming and expensive separation of individual PL fractions.

Author Contributions

Conceived and designed the experiments: MK HD BK. Performed the experiments: SK MK. Analyzed the data: SK MK. Contributed reagents/materials/analysis tools: BK. Wrote the paper: SK MK HD BK.

References

1. Bucher HC, Hengstler P, Schindler C, Meier G (2002) N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. Am J Med 112: 298–304.
2. Koletzko B, Lien E, Agostoni C, Bohles H, Campoy C, et al. (2008) The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. J Perinat Med 36: 5–14.
3. Fekete K, Marosvolgyi T, Jakobik V, Decsi T (2009) Methods of assessment of n-3 long-chain polyunsaturated fatty acid status in humans: a systematic review. Am J Clin Nutr 89: 2070S–2084S.
4. Hodson L, Skeaff CM, Fielding BA (2008) Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. Prog Lipid Res 47: 348–380.
5. Klingler M, Koletzko B (2012) Novel methodologies for assessing omega-3 fatty acid status - A systematic review. Br J Nutr, Accepted for publication.
6. Glaser C, Demmelmair H, Koletzko B (2010) High-throughput analysis of fatty acid composition of plasma glycerophospholipids. J Lipid Res 51: 216–221.
7. Rose HG, Oklander M (1965) Improved Procedure for the Extraction of Lipids from Human Erythrocytes. J Lipid Res 6: 428–431.
8. Glaser C, Demmelmair H, Koletzko B (2009) High Throughput method for analyzing the fatty acid composition in palsma phosphoglycerides. In:, , Ludiwg-Maximilians-Universität, editor. Germany.
9. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226: 497–509.
10. Carnielli VP, Pederzini F, Vittorangeli R, Luijendijk IHT, Boomaars WEM, et al. (1996) Plasma and Red Blood Cell Fatty Acid of Very Low Birth Weight Infants Fed Exclusively with Expressed Preterm Human Milk. Pediatric Research 39: 671–679.
11. Geppert J, Kraft V, Demmelmair H, Koletzko B (2005) Docosahexaenoic acid supplementation in vegetarians effectively increases omega-3 index: a randomized trial. Lipids 40: 807–814.
12. Klingler M, Demmelmair H, Koletzko B, Glaser C (2011) Fatty Acid Status Determination by Cheek Cell Sampling Combined with Methanol-Based Ultrasound Extraction of Glycerophospholipids. Lipids.
13. Burdge GC, Wright P, Jones AE, Wootton SA (2000) A method for separation of phosphatidylcholine, triacylglycerol, non-esterified fatty acids and cholesterol esters from plasma by solid-phase extraction. Br J Nutr 84: 781–787.
14. Hodson L, Skeaff CM, Wallace AJ, Arribas GL (2002) Stability of plasma and erythrocyte fatty acid composition during cold storage. Clin Chim Acta 321: 63–67.
15. Di Marino L, Maffettone A, Cipriano P, Celentano E, Galasso R, et al. (2000) Assay of erythrocyte membrane fatty acids. Effects of storage time at low temperature. Int J Clin Lab Res 30: 197–202.
16. Otto SJ, Foreman-von Drongelen MM, von Houwelingen AC, Hornstra G (1997) Effects of storage on venous and capillary blood samples: the influence of deferoxamine and butylated hydroxytoluene on the fatty acid alterations in red blood cell phospholipids. Eur J Clin Chem Clin Biochem 35: 907–913.
17. Stanford JL, King I, Kristal AR (1991) Long-term storage of red blood cells and correlations between red cell and dietary fatty acids: results from a pilot study. Nutr Cancer 16: 183–188.

18. Innis SM, Elias SL (2003) Intakes of essential n-6 and n-3 polyunsaturated fatty acids among pregnant Canadian women. American Journal of Clinical Nutrition 77: 473–478.
19. Popp-Snijders C, Schouten JA, de Jong AP, van der Veen EA (1984) Effect of dietary cod-liver oil on the lipid composition of human erythrocyte membranes. Scand J Clin Lab Invest 44: 39–46.
20. Popp-Snijders C, Schouten JA, van Blitterswijk WJ, van der Veen EA (1986) Changes in membrane lipid composition of human erythrocytes after dietary supplementation of (n - 3) polyunsaturated fatty acids. Maintenance of membrane fluidity. Biochimica et Biophysica Acta (BBA) - Biomembranes 854: 31–37.
21. Hermansson M, Hokynar K, Somerharju P (2011) Mechanisms of glycerophospholipid homeostasis in mammalian cells. Prog Lipid Res 50: 240–257.
22. Plourde M, Chouinard-Watkins R, Vandal M, Zhang Y, Lawrence P, et al. (2011) Plasma incorporation, apparent retroconversion and beta-oxidation of 13C-docosahexaenoic acid in the elderly. Nutr Metab (Lond) 8: 5.
23. Harris WS, Von Schacky C (2004) The Omega-3 Index: a new risk factor for death from coronary heart disease? Prev Med 39: 212–220.

4.2 Publikation 2 - British Journal of Nutrition

„Comparison of the incorporation of orally administered DHA into plasma, erythrocyte and cheek cell glycerophospholipids.“

Submitted: 23. Januar 2012
Accepted: 29. April 2012
Published: September 2012

British Journal of Nutrition, page 1 of 7
doi:10.1017/S000711451200222X

Comparison of the incorporation of orally administered DHA into plasma, erythrocyte and cheek cell glycerophospholipids

Mario Klingler, Sabrina Klem, Hans Demmelmair and Berthold Koletzko*

Division of Metabolic and Nutritional Medicine, University of Munich Medical Center, Dr. von Hauner Children's Hospital, Lindwurmstrasse 4, 80337 Munich, Germany

(Submitted 23 January 2012 – Final revision received 25 April 2012 – Accepted 29 April 2012)

Abstract

Adequate intake of *n*-3 fatty acids plays an important role in human health. The analysis of various blood lipids is used as a measure of fatty acid status in humans. Cheek cell phospholipids (PL) have also been proposed as biological markers, but are rarely used in clinical studies due to limitations in sample quality and quantity. An improved method for the analysis of cheek cell glycerophospholipid fatty acids is applied in a 29 d supplementation trial with 510 mg DHA daily. The DHA increases in cheek cell, plasma and erythrocyte glycerophospholipids are compared. High correlations are shown for glycerophospholipid DHA between cheek cells and plasma (r 0·88) and erythrocytes (r 0·76) before study commencement. After the daily supplementation of DHA, the half-maximal glycerophospholipid DHA level is reached after about 4 d in plasma, 6 d in erythrocytes and 10 d in cheek cells. The mean DHA increase (mol%) relative to baseline was most prominent in plasma (186 %), followed by cheek cells (180 %) and erythrocytes (130 %). Considering a lag phase of about 5 d, cheek cells reflect short-term changes in dietary fat uptake. Based on the data of the present study, they can be used alternatively to plasma and erythrocyte PL as non-invasive *n*-3 fatty acid status markers.

Key words: Cheek cells: DHA: Glycerophospholipids: Fatty acids

Adequate intake of *n*-3 long-chain PUFA, such as DHA and EPA, plays an important role in human health[1]. High DHA and EPA levels in blood lipids have been associated with improved infantile development, lower risk of CHD, lower incidence of cancer and avoidance of mental diseases[1].

A modest increase in DHA and EPA uptake (< 300 mg/d) can rapidly alter the fatty acid composition of blood lipids[2]. The incorporation of EPA and DHA into individual blood lipid fractions is time- and dose-dependent and differs between *n*-3 fatty acids[3]. The quantity of administered *n*-3 fatty acids determines total changes in tissues[4–6]. Plasma phospholipids (PL) or cholesteryl esters reach *n*-3 fatty acid equilibrium within 2 weeks, erythrocytes after approximately 120 d and adipose tissues after 1–2 years[6,7]. In most biological compartments, changes of EPA levels occur earlier and are more pronounced than changes of DHA[4–6,8]. This might be related to different affinities of EPA and DHA to lecithin-cholesterol acyltransferase[9], different clearance rates of both *n*-3 fatty acids from plasma to adipose tissue[8] or the displacement of DHA by EPA in plasma PL[7]. Moreover, the conversion of EPA to DHA is very limited[10], whereas retroconversion of DHA to EPA was observed after DHA supplementation[11].

Strong correlations exist for EPA and DHA percentages between plasma and erythrocyte lipids[7,12] and other tissues, such as cardiac tissue[13], brain cortex[14] and cheek cell glycerophospholipids (GPL)[15]. Correlations of *n*-3 long-chain-PUFA contents between adipose tissue and blood lipids are low or absent[16,17]. While the fatty acid analysis of blood lipids offers a measure for the fatty acid intake over the last few weeks, the analysis of subcutaneous fat reflects long-term fat intake[18]. Plasma PL or cholesteryl esters, erythrocyte PL, whole blood or plasma total lipids and adipose tissue are the preferred markers for *n*-3 fatty acid status in humans since *n*-3 long-chain-PUFA contents of these tissues are strongly correlated with dietary fat intake[19].

Cheek cell PL have also been recommended as a biological marker for dietary fatty acid intake[20], but they have rarely been used in clinical studies. This might be related to insecure sample quality and quantity and additionally required sample handling procedures[13]. On the other hand, sampling of cheek cells is less invasive than blood or adipose tissue sampling and therefore better accepted, particularly when applied in infants or children. Recently, we developed a robust method for the analysis of cheek cell GPL fatty acids, which requires only minimal sample amounts[15].

Abbreviations: ARA, arachidonic acid; FAME, fatty acid methyl ester; GPL, glycerophospholipids; PL, phospholipids.

* **Corresponding author:** Professor B. Koletzko, fax +49 89 5160 7742, email office.koletzko@med.uni-muenchen.de

British Journal of Nutrition

This method has been applied in a 29 d DHA supplementation trial. The supplement did not provide appreciable amounts of *n*-3 fatty acids other than DHA to avoid influences of these fatty acids on DHA incorporation into the studied compartments. The aims of the present study were the comparison of the time course of DHA incorporation into cheek cell, plasma and erythrocyte GPL, and the determination of the correlation of DHA between these tissues. The results of this study will show whether cheek cells reflect short-term or long-term changes in dietary fat intake and may underpin the suitability of cheek cells as a fatty acid status marker.

Materials and methods

Subjects

A total of thirteen volunteers were recruited for a supplementation study with DHA. Towards this, seven healthy females and six males between 20 and 40 years of age with a BMI of 20–25 kg/m^2 were invited. Participants ought not to have taken *n*-3 long-chain-PUFA supplements or medication assumed to interfere with the lipid metabolism 3 months before the start of the study. Further exclusion criteria were pregnancy, fatty fish consumption more than once per week, a weight reduction diet 4 weeks before study commencement and the abuse of alcohol or drugs.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethical Committee of the University of Munich Medical Center (034-10). Written informed consent was obtained from all subjects before study commencement. The trial was registered at ClincialTrials.gov (NCT01192269).

Experimental design and supplements

The study consisted of a 2-week baseline period followed by a 29 d intervention period and included clinical examinations at the beginning and the end of the study. Blood and cheek cells were sampled eleven times during the trial, on days −14, 0 (start of intervention), 1, 2, 3, 4, 9, 14, 18, 24 and 29 (end of intervention). The study supplement consisted of a 950 μl DHASCO®-S microalgae oil capsule (Martek Biosciences) containing 510 mg DHA (Table 1). The content of EPA and other *n*-3 fatty acids was negligible (<0·4 %). Over the first 5 d, capsules were administered directly after blood and cheek cell sampling. The capsules for the remaining intervention period were handed out at day 5, and the participants were asked to take one capsule daily with breakfast and to record the time of consumption. Capsule counts were conducted at the end of the study.

Glycerophospholipid fatty acid analysis of erythrocyte, cheek cell and plasma lipids

The analysis of erythrocytes was conducted with a modified method for plasma GPL analysis[21]. Briefly, after an overnight fast, venous blood was collected into 7·5 ml EDTA Monovettes (Sarstedt) and directly placed on ice. Cooled samples were centrifuged (1000 ***g***, 10 min, 4°C) within 2 h after sampling. Plasma was separated, the buffy-coat was discarded, and remaining blood cells were washed three times with saline (0·9 % NaCl).

Table 1. Selected fatty acids of the study supplement (950 μl capsule) according to the manufacturer

Fatty acid	% w/w
C14 : 0	11·3
C16 : 0	6·0
C18 : 0	0·2
C18 : 1	9·6
C18 : 2*n*-6	0·2
C20 : 4*n*-6	ND
C22 : 5*n*-6	<0·1
C18 : 3*n*-3	0·3
C20 : 5*n*-3	<0·1
C22 : 6*n*-3	59·8

ND, not detected.

A volume of 100 μl erythrocytes was haemolysed by the addition of 100 μl water; thereafter, 1300 μl methanol plus 100 μl internal standard (14·6 mg dipentadecanolyl-sn-glycero-phosphocholine, phosphatidylcholine 15:0, in 100 ml methanol; Sigma Aldrich) were added during continuous shaking. The suspension was kept in an ultrasound water bath (40 kHz, 120 W) for 5 min. Precipitated proteins were separated by centrifugation (3030 ***g***, 10 min, 4°C), and the methanolic supernatant containing polar lipids was transferred into a small brown glass. Then, 50 μl of sodium methoxide solution (25 wt% in methanol; Sigma Aldrich) were added to synthesise fatty acid methyl esters (FAME) from erythrocyte GPL at room temperature. After 4 min, the reaction was stopped with 150 μl 3 M-methanolic HCl (Sigma Aldrich). FAME were extracted twice into 600 μl hexane, the extracts were combined, hexane was evaporated under a continuous flow of N_2, and the FAME were re-dissolved in 40 μl hexane (containing 2 g/l butylated hydroxytoluene). Samples were stored at −20°C until GC analysis.

The analysis of GPL fatty acids from cheek cells and plasma required a slightly different sample preparation and was performed as recently described[15,21]. Briefly, cheek cells were collected with an endocervical brush and additional mouth rinse. Cheek cells were isolated by centrifugation before they were suspended in 1400 μl methanol including phosphatidylcholine 15:0 as internal standard. The methanolic cell suspension was treated with ultrasound for 20 min and the precipitated proteins were removed by centrifugation. FAME synthesis and extraction were performed as described previously. The analysis of plasma GPL did not require sample pre-treatment. Methanol and internal standard were added directly to plasma for protein precipitation.

FAME were quantified by GC with flame ionisation detection (Agilent 5890 series II), using a 25 m × 0·22 mm (inner diameter) BPX70 column (SGE). Injection temperature was set to 250°C, the split ratio was 1/30 and He was used as the carrier gas. The oven temperature was programmed to rise from 150 to 180°C at 2·5°C/min, followed by 1·5°C/min to

British Journal of Nutrition

British Journal of Nutrition

a final temperature of 200°C, which was held for 1 min. The pressure program started at 0·9 bar, and pressure increased by 0·02 bar/min to 1·2 bar, 0·05 bar/min to 1·5 bar, and 0·1 bar/min to a final pressure of 2·0 bar. This pressure was held until the temperature program was completed[21].

FAME were identified by comparison with a FAME standard mixture (GLC-569B, Nu-Check Prep, Inc.). All FAME response relative to pentadecanoic acid methyl ester (internal standard) was determined using GLC-85 (Nu-Check Prep, Inc.) as external standard. EZChrom Elite (version 3.1.7, Agilent) was used for peak integration.

Dietary records

Participants recorded their total food and beverage consumption on three consecutive days including one weekend day a week before the start of the intervention period. Nutrient intakes were calculated using PRODI (version 4.5 LE, Nutri-Science), which is based on the nutrient data bank of Souci-Fachmann-Kraut (version 2000) and the 'Bundeslebensmittelschlüssel' (version 2.3).

Mathematical modelling and statistical analysis

Curves of averaged DHA percentage increases (y) of plasma, erythrocyte and cheek cell GPL were fitted according to the least square using OriginPro, version 8.5 software (originLab), by varying the parameters a, b and c of the equation

$$y = a \times (1 - e^{-bx})^c$$

where x is the time in d since the onset of supplementation, and a, b and c are constants. The parameter a represents the upper limit of the DHA percentage increase, which is approached with infinitive time (x), while parameters b and c define the shape of the exponential increase. With $c = 1$, this equation was used by Katan *et al.*[6] to model changes of EPA and DHA in cholesterol esters, erythrocytes and adipose tissue during fish oil supplementation. The time of the half-maximal DHA incorporation $t_{1/2}$ can be calculated as $\mathrm{DHA}t_{1/2} = -1/b \times \ln(1 - 2^{-1/c})$.

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 19.0.0.1 (IBM). Relative fatty acid contents (mol%) are given as mean and standard deviation based on twenty detected *cis*-fatty acids with chain lengths between 14 and 24 carbon atoms[21]. Changes from baseline to day 29 were expressed as mean difference and 95 % CI, significance of differences was evaluated using paired t tests. Correlation coefficients between compartments at baseline were evaluated according to Pearson. P values $<0{\cdot}05$ were considered as statistically significant.

Results

Baseline characteristics and nutrient intake

The compliance of the subjects was very good, and twelve of the thirteen participants followed exactly the study protocol. However, one participant consumed twenty-eight instead of twenty-nine capsules. This resulted in a DHA intake of about 96 % of the planned dose; therefore this subject was not excluded from the study.

Baseline characteristics of the study subjects and their average nutrient intake are presented in Table 2. The characteristics described did not change during the study (data not shown). No adverse effects were reported during the intervention period.

Plasma, erythrocyte and cheek cell glycerophospholipid fatty acid compositions

Table 3 shows the GPL fatty acid compositions of plasma, erythrocytes and cheek cells, determined before (averaged fatty acid baseline values of day −14 and day 0) and after the supplementation period (day 29). The majority of individual GPL fatty acid proportions differed significantly between the three compartments. Palmitic-, stearic-, oleic-, linoleic- and arachidonic acids (ARA) were the predominant fatty acids in plasma and erythrocytes, averaging 88·0 (SD 1·4) and 85·0 (SD 1·2) mol%, respectively. In cheek cells, palmitic-, stearic-, oleic-, linoleic- and palmitoleic acids presented the major fatty acids comprising 86·3 (SD 1·0) mol%. Erythrocytes contained the highest levels of ARA and DHA averaging 15·2 (SD 1·6) and 4·3 (SD 0·8) mol%, followed by plasma with 10·1 (SD 1·5) and 2·7 (SD 0·5) mol% and cheek cells with 3·2 (SD 0·6) and 0·7 (SD 0·1) mol%, respectively.

Table 2. Characteristics of the study participants (n 13) and their nutrient intake at baseline

(Mean values and standard deviations)

	Mean	SD
Characteristics		
Age (years)	25·8	2·7
BMI (kg/m^2)	21·9	1·6
Body fat (%)	20·9	8·2
Waist circumferrence (cm)	81·7	5·7
Blood pressure		
Systolic (mmHg)	128	15
Diastolic (mmHg)	70	6
Heart rate (beats/min)	69	7
GT (U/l)	16	5
GPT (U/l)	16	5
GOT (U/l)	20	4
Cholesterol (mg/l)	1640	190
TAG (mg/l)	830	260
CRP (high sensitivity) (mg/l)	2	4
LDL (mg/l)	800	200
HDL (mg/l)	670	90
LDL:HDL ratio	1·2	0·3
Nutrient intake		
Energy (MJ/d)	9·1	1·4
Protein (percentage of energy)	14·8	2·9
Carbohydrates (percentage of energy)	49·6	9·7
Total fat (percentage of energy)	33·8	11·3
SFA (percentage of energy)	15·1	4·9
MUFA (percentage of energy)	11·8	3·3
PUFA (percentage of energy)	4·9	1·8
DHA (mg/d)	79	51
EPA (mg/d)	43	28

GT, glutamyl transpeptidase; GPT, glutamic pyruvic transaminase; GOT, glutanic oxaloacetic transaminase; CRP, C-reactive protein.

Table 3. Fatty acid compositions (mol%) of plasma, erythrocytes† and cheek cells at baseline
(Mean values, standard deviations, mean difference and 95 % confidence intervals; *n* 13)

	Plasma				Erythrocytes				Cheek Cells			
	Baseline				Baseline				Baseline			
	Mean	SD	Change at day 29	95 % CI	Mean	SD	Change at day 29	95 % CI	Mean	SD	Change at day 29	95 % CI
C16 : 0	31·16	1·54	0·90	0·45, 1·35**	24·99	2·15	0·59	−0·80, 1·99	16·55	1·81	−0·09	−0·85, 0·68
C18 : 0	12·29	1·00	−0·15	−0·53, 0·23	17·84	0·55	−0·32	−0·63, −0·01*	15·31	1·43	−0·30	−1·05, 0·46
C16 : 1*n*-7	0·94	0·46	−0·06	−0·16, 0·04	0·41	0·19	−0·02	−0·07, 0·02	7·06	1·13	−0·31	−0·57, −0·05*
C18 : 1*n*-7	1·52	0·17	0·11	0·03, 0·20*	1·35	0·13	−0·01	−0·05, 0·03	4·68	0·61	0·14	−0·14, 0·42
C18 : 1*n*-9	11·68	1·30	−0·22	−0·75, 0·31	15·02	0·78	0·09	−0·26, 0·44	30·06	2·04	−0·23	−1·10, 0·65
C18 : 2*n*-6	22·80	2·70	−1·35	−2·47, −0·23*	12·02	1·22	−0·08	−0·58, 0·42	17·32	1·77	0·56	−0·28, 1·39
C18 : 3*n*-6	0·16	0·07	−0·06	−0·10, −0·03**	0·08	0·03	−0·04	−0·06, −0·02***	0·20	0·08	0·02	−0·04, 0·07
C20 : 3*n*-6	2·93	0·75	−0·23	−0·47, 0·01	1·82	0·36	−0·12	−0·18, −0·07***	1·42	0·35	−0·05	−0·15, 0·05
C20 : 4*n*-6	10·07	1·49	−1·01	−1·52, −0·49**	15·16	1·59	−0·76	−1·78, 0·25	3·20	0·64	−0·31	−0·67, 0·04
C22 : 5*n*-6	0·28	0·10	−0·10	−0·13, −0·06***	0·71	0·15	−0·09	−0·12, −0·05***	0·07	0·03	−0·01	−0·02, 0·00
C18 : 3*n*-3	0·28	0·13	−0·02	−0·07, 0·03	0·15	0·04	0·00	−0·01, 0·01	0·24	0·12	0·06	−0·09, 0·20
C20 : 5*n*-3	0·76	0·53	−0·01	−0·22, 0·21	0·55	0·23	0·01	−0·05, 0·06	0·21	0·11	0·04	−0·02, 0·10
C22 : 5*n*-3	0·73	0·22	−0·21	−0·27, −0·14***	1·85	0·36	−0·16	−0·36, 0·03	0·23	0·08	−0·04	−0·08, 0·00
C22 : 6*n*-3	2·69	0·52	2·20	1·66, 2·73***	4·28	0·76	1·18	0·89, 1·46***	0·69	0·14	0·54	0·43, 0·66***

Significant changes of individual fatty acid contents during intervention are indicated as * $P<0·05$, ** $P<0·01$ or *** $P<0·001$; one-sample *t* test.
† Erythrocyte values for samples stored for 8 months have been reported elsewhere[34].

The additional DHA intake of 510 mg/d significantly increased the DHA content in all three compartments, which was by 2·20 mol% (95 % CI 1·66, 2·73; $P<0·001$) in plasma, 1·18 mol% (95 % CI 0·89, 1·46; $P<0·001$) in erythrocytes and 0·54 mol% (95 % CI0·43, 0·66; $P<0·001$) in cheek cells at the end of the study. ARA proportions decreased during the same period, but differences were only in plasma statistically significant (−1·01 mol%; 95 % CI −1·52, −0·49; $P < 0·002$). Proportions of plasma linoleic acid decreased during the intervention period (−1·35 mol%; 95 % CI −2·47, −0·23; $P=0·022$), but this change was not observed in erythrocytes or cheek cells. EPA contents were not significantly affected by DHA supplementation. The study was not adequately powered to determine reliably changes in fatty acids other than DHA; thus the changes and correlations between percentages in different compartments were analysed on an explorative basis only.

Correlation coefficients were computed between individual fatty acids of all three compartments at baseline (Table 4). Major cheek cell fatty acids, such as oleic- and linoleic acid did not correlate with erythrocytes and plasma, while significant correlations were found for palmitic (*r* 0·64) and stearic acids (*r* 0·70). High correlations were found for DHA contents between cheek cells and erythrocytes as well as cheek cells and plasma (*r* 0·88 and 0·76, respectively), and for EPA between the same compartments (*r* 0·79 and *r* 0·66, respectively). The sum of both *n*-3 fatty acids DHA and EPA was also highly correlated (*r* 0·87 and *r* 0·72, respectively). Correlations for ARA were only found between cheek cells and plasma (*r* 0·65), but not between other compartments.

Table 4. Correlation coefficients of individual glycerophospholipid fatty acids between cheek cells, erythrocytes and plasma before DHA supplementation

Fatty acid	*R*	*P*	*R*	*P*	*R*	*P*
C16 : 0	0·32	NS	0·64	0·026	0·33	NS
C18 : 0	0·33	NS	0·70	0·010	0·59	0·04
C16 : 1*n*-7	−0·47	NS	−0·37	NS	0·84	<0·001
C18 : 1*n*-7	0·14	NS	0·15	NS	0·23	NS
C18 : 1*n*-9	0·10	NS	0·40	NS	0·26	NS
C18 : 2*n*-6	−0·05	NS	−0·04	NS	0·71	<0·01
C18 : 3*n*-6	0·57	NS (0·05)	0·50	<0·001	0·86	<0·001
C20 : 3*n*-6	0·65	0·020	0·74	<0·001	0·78	<0·01
C20 : 4*n*-6	0·17	NS	0·65	0·01	0·49	NS
C22 : 5*n*-6	0·70	0·010	0·78	<0·001	0·94	<0·001
C18 : 3*n*-3	0·13	NS	0·11	NS	0·72	<0·01
C20 : 5*n*-3	0·79	<0·01	0·66	NS	0·88	<0·001
C22 : 5*n*-3	0·39	NS	0·85	NS	0·59	0·04
C22 : 6*n*-3	0·88	<0·001	0·76	<0·001	0·89	<0·001
EPA + DHA	0·87	<0·001	0·72	<0·01	0·88	<0·001

NS, $P>0·05$, Pearson's correlation coefficient.

British Journal of Nutrition

Most of the fatty acids in plasma and erythrocytes were highly correlated, except for palmitic acid, oleic acid, vaccenic acid, and ARA. Correlations calculated for EPA, DHA and EPA + DHA were similar to those of cheek cells and erythrocytes.

At the end of the study, correlations for DHA levels between cheek cells and plasma (r 0·60, P=0·03) or erythrocytes (r 0·77, P = 0·002) tended to be lower, whereas DHA levels did not correlate any more between plasma and erythrocytes (r 0·353, NS).

Incorporation of DHA in plasma, erythrocytes and cheek cells

Fig. 1 shows changes of DHA mol% in plasma, erythrocyte and cheek cell GPL over the course of supplementation. The mean DHA increase (mol%) relative to the baseline level was most prominent in plasma (186 %), followed by cheek cells (180 %) and erythrocytes (130 %).

The parameter a, representing the estimated maximal increase of DHA_{∞}, was 2·25, 1·09 and 0·68 for plasma, erythrocytes and cheek cells. The parameters b and c describe the course of the increase over time with 0·07 and 0·52 for plasma, 0·10 and 0·97 for erythrocytes, and 0·10 and 1·68 for cheek cells. The parameters are based on the collected data points until day 29 and thus may not reflect processes mostly effective during longer intervention periods. Solving the equation used for time until half of the expected maximal increase is reached ($DHAt_{1/2}$), yielded 4·4 d for plasma, 6·4 d for erythrocytes and 10·4 d for cheek cells.

Discussion

This is the first study evaluating the incorporation rate of DHA into cheek cell GPL in comparison to plasma and erythrocyte GPL. High correlations are found for DHA between all three compartments. In our 29 d supplementation trial (510 mg DHA/d), a half-maximal GPL DHA level is reached after about 4 d in plasma, 6 d in erythrocytes and 10 d in cheek cells. The relative response to DHA supplementation is highest in plasma and cheek cells. Our findings support the use of cheek cells as a n-3 fatty acid status marker.

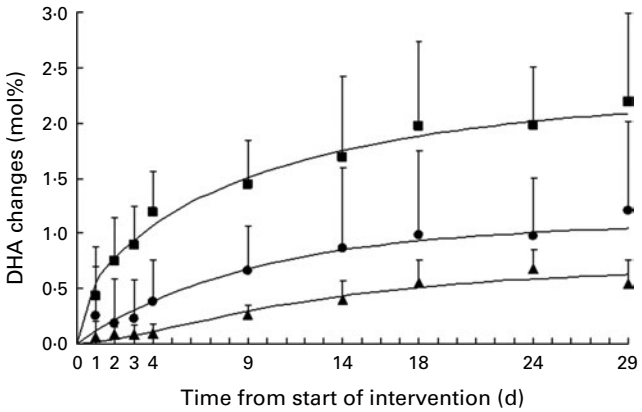

Fig. 1. DHA changes from baseline in different compartments of subjects supplemented with 510 mg DHA daily over 29 d. Values are means with standard deviations represented by vertical bars. Curves were fitted to $y = a \times (1 - e^{-bx})^c$ resulting in the following parameters for plasma (■): a = 2·25, b = 0·07, c = 0·52; erythrocytes (●): a = 1·09, b = 0·10, c = 0·97 and cheek cells (▲): a = 0·68, b = 0·10, c = 1·68.

The distribution of total GPL fatty acids in cheek cell and plasma determined in our study cohort is comparable to other studies[15,21,22]. Data for fatty acid contents of total GPL in erythrocytes are not available. However, our results can be compared to those reported for erythrocyte total fatty acids[12], although differences for some individual fatty acids are indicated. This might be related to the contribution of sphingomyelin fatty acids to erythrocyte total fatty acids. Sphingomyelin contains high amounts of palmitic acid and only traces of n-3 fatty acids[23]. This is reflected in the respective patterns of erythrocyte total and GPL fatty acids.

At the start of the study, GPL DHA proportions of cheek cells in our subjects averaged 0·7 mol% (0·8 wt%), which is comparable to DHA levels in cheek cell PL reported for breastfed infants[24,25], elderly people[13] and cheek cell GPL in adults[15]. In comparison to plasma and erythrocytes, the DHA content of cheek cells is approximately one-third. This may limit the validity of cheek cell GPL as a fatty acid status marker, but it has been shown that changes of n-3 and n-6 fatty acid uptakes are reflected in cheek cell lipids similarly to erythrocytes or plasma[13,24–26]. Moreover, the outcome of our supplementation study shows that the relative DHA increase in cheek cells is comparable to that in plasma, which is in agreement with DHA changes reported for plasma (104 wt%) and cheek cell PL (95 wt%) in patients receiving 400 mg DHA per d over a period of 6 months[13].

Little is known about DHA incorporation into cheek cells. The oral mucosa is an avascular stratified squamous epithelium[27]. Cells of the base membrane are continuously renewed by mitosis, and migrate through the epithelium to the surface[28]. The nutrient and metabolite content of the outer epithelium layer is determined by cell migration and to a smaller extent by diffusion[27]. The estimated renewal time of buccal cheek cells is 5–8 d[29,30]. These characteristics of the oral mucosa suggest that DHA changes in the analysed outer epithelial layer can be expected not earlier than 5 d after the onset of supplementation. Such a delay is observed in our study, although an increase is indicated after 1 d, which might be explained by passive transport mechanisms. However, we have no information about the exact time when the increase took place, as samples between day 5 and day 8 were not collected. Considering the lag-phase of at least 5 d, half-maximal DHA levels are reached quickly, which is comparable to plasma. DHA contents in cheek cells do not further increase after 24 d, suggesting that DHA equilibrium is reached at about this time. These data indicate that cheek cells reflect short-term changes of the dietary n-3 fatty acid pattern; however, a delayed increase at the start of the intervention has to be considered.

Plasma and erythrocyte lipids are used as biological markers for dietary fat intake. Their n-3 and n-6 fatty acid contents are highly correlated[12]. Correlations described for cheek cells with other biological markers are mainly related to DHA, EPA and ARA. Strong correlations have been shown for DHA between cheek cell PL and plasma PL (r 0·83), erythrocyte

British Journal of Nutrition

total lipids (r 0·72)[25], plasma total lipids (r 0·61)[24] and serum PL (r 0·72)[31]. In our study, correlation coefficients of $r > 0{\cdot}75$ have been determined between DHA in cheek cell, plasma and erythrocyte GPL. Correlations between cheek cell and plasma EPA have been only reported in a single study[31], in which the r-value of 0·56 is similar to that in our study. Correlations of ARA levels between cheek cells and blood compartments have also already been determined, but results are inconsistent. Whereas ARA contents correlated between cheek cell and serum PL[31], none or weak relationships were reported between cheek cell PL and plasma PL or erythrocyte total lipids[24,25]. No correlations have been found between ARA levels in plasma and erythrocyte total fatty acids[32]. Our results confirm previous findings, where correlations were only indicated between cheek cells and plasma, but not between the other compartments.

The supplementation of DHA as an individual *n*-3 fatty acid was chosen to exclude the effects of other fatty acids on the incorporation of DHA into GPL. Consuming fish or fish oil capsules may result in different DHA levels than those observed in our study due to the competition of EPA and DHA for the sn-2 position of GPL. There was no control group without DHA supplementation included, and hence we cannot compare the intervention effects to a reference group. Systematic changes of fatty acid compositions during the study period cannot be excluded, but such changes are not expected during a 4-week period. An estimate for random variation was obtained by duplicate baseline measurements within 2 weeks before study start. In all compartments, differences for DHA percentages were small compared to those observed after supplementation (data not shown). Also, providing DHA only allows detecting changes in EPA related to retroconversion. Based on the EPA results, retroconversion did not take place during the supplementation period of 29 d. We cannot exclude that with a prolonged intervention time a further increase in DHA proportions would have occurred in the three compartments. However, this seems unlikely as DHA in cheek cells derives from plasma lipids, and plasma DHA levels reach equilibrium within 1 month.

While the studied daily supplementation with 510 mg DHA is clearly above the average habitual DHA intake in most countries[33], this dosage has frequently been applied in interventional studies to test DHA effects[2]. We tested only the kinetics of DHA incorporation following a change in intake from about 80 mg DHA to 590 mg per d. Nevertheless, we assume that with lower DHA intakes similar curves, with lower maximal changes, would be observed as for DHA supplementations up to 1 g/d increases in plasma PL DHA percentages have been found to be proportional to intake increases[7]. On the other hand, a further increase of the supplementation dose leads to disproportional increases of DHA in plasma[7] and kinetics will probably differ. In case of very low basal DHA levels and/or minute changes of DHA intakes, cheek cell GPL analysis might be disadvantageous compared to plasma or erythrocytes, as cheek cells contain less GPL DHA which may influence the relative error of measurements unfavourably.

In summary, after a lag-phase of a few days, cheek cells respond quickly to DHA supplementation. The relative increase over 4 weeks is comparable to plasma, although the proportion of DHA in cheek cells is small compared to plasma and erythrocytes. This indicates that cheek cells reflect short-term changes in dietary fatty acid composition. Furthermore, sampling of cheek cells is simple and applicable in a non-clinical environment. Based on the results of this study, cheek cell GPL are an alternative to plasma and erythrocyte PL as biological markers for *n*-3 fatty acid status, especially in *n*-3 fatty acid supplementation trials and studies, where blood sampling is difficult or not applicable.

Acknowledgements

The authors gratefully acknowledge the provision of the DHASCO®-S microalgae oil by Martek Biosciences. Furthermore, the authors thank Dr Claudia Matthies for her valuable support during the intervention trial and subsequent sample analysis. The present study was financially supported by the Federal Ministry of Education and Research (0315680B). B. K. is receiving a Freedom to Discover Award of the Bristol-Myers Squibb Foundation, New York, NY. The methods for the GPL FA analysis presented in this article are in patent pending status. M. K., H. D. and B. K. conceived and designed the experiments of the clinical study; M. K. and S. K. performed the experiments; M. K. and H. D. analysed the data. M. K., S. K., H. D. and B. K. wrote the paper and were responsible for the review and approval of the manuscript. The authors have no conflicts of interest to declare.

References

1. Riediger ND, Othman RA, Suh M, *et al.* (2009) A systemic review of the roles of *n*-3 fatty acids in health and disease. *J Am Diet Assoc* **109**, 668–679.
2. Fekete K, Marosvolgyi T, Jakobik V, *et al.* (2009) Methods of assessment of *n*-3 long-chain polyunsaturated fatty acid status in humans: a systematic review. *Am J Clin Nutr* **89**, 2070S–2084S.
3. Masson S, Latini R, Tacconi M, *et al.* (2007) Incorporation and washout of *n*-3 polyunsaturated fatty acids after diet supplementation in clinical studies. *J Cardiovasc Med (Hagerstown)* **8**, Suppl. 1, S4–10.
4. Brown AJ, Pang E & Roberts DC (1991) Persistent changes in the fatty acid composition of erythrocyte membranes after moderate intake of *n*-3 polyunsaturated fatty acids: study design implications. *Am J Clin Nutr* **54**, 668–673.
5. Di Stasi D, Bernasconi R, Marchioli R, *et al.* (2004) Early modifications of fatty acid composition in plasma phospholipids, platelets and mononucleates of healthy volunteers after low doses of *n*-3 polyunsaturated fatty acids. *Eur J Clin Pharmacol* **60**, 183–190.
6. Katan MB, Deslypere JP, van Birgelen AP, *et al.* (1997) Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: an 18-month controlled study. *J Lipid Res* **38**, 2012–2022.
7. Arterburn LM, Hall EB & Oken H (2006) Distribution, interconversion, and dose response of *n*-3 fatty acids in humans. *Am J Clin Nutr* **83**, 1467S–1476S.

British Journal of Nutrition

8. Zuijdgeest-van Leeuwen SD, Dagnelie PC, Rietveld T, *et al.* (1999) Incorporation and washout of orally administered *n*-3 fatty acid ethyl esters in different plasma lipid fractions. *Br J Nutr* **82**, 481–488.
9. Subbaiah PV, Kaufman D & Bagdade JD (1993) Incorporation of dietary *n*-3 fatty acids into molecular species of phosphatidyl choline and cholesteryl ester in normal human plasma. *Am J Clin Nutr* **58**, 360–368.
10. Brenna JT, Salem N Jr, Sinclair AJ, *et al.* (2009) α-Linolenic acid supplementation and conversion to *n*-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins Leukot Essent Fatty Acids* **80**, 85–91.
11. Plourde M, Chouinard-Watkins R, Vandal M, *et al.* (2011) Plasma incorporation, apparent retroconversion and beta-oxidation of 13C-docosahexaenoic acid in the elderly. *Nutr Metab (Lond)* **8**, 5.
12. Geppert J, Kraft V, Demmelmair H, *et al.* (2005) Docosahexaenoic acid supplementation in vegetarians effectively increases omega-3 index: a randomized trial. *Lipids* **40**, 807–814.
13. Harris WS, Sands SA, Windsor SL, *et al.* (2004) Omega-3 fatty acids in cardiac biopsies from heart transplantation patients: correlation with erythrocytes and response to supplementation. *Circulation* **110**, 1645–1649.
14. Makrides M, Neumann M, Byard R, *et al.* (1994) Fatty acid composition of brain, retina, and erythrocytes in breast- and formula-fed infants. *Am J Clin Nutr* **60**, 189–194.
15. Klingler M, Demmelmair H, Koletzko B, *et al.* (2011) Fatty acid status determination by cheek cell sampling combined with methanol-based ultrasound extraction of glycerophospholipids. *Lipids* **46**, 981–990.
16. Baylin A, Kim MK, Donovan-Palmer A, *et al.* (2005) Fasting whole blood as a biomarker of essential fatty acid intake in epidemiologic studies: comparison with adipose tissue and plasma. *Am J Epidemiol* **162**, 373–381.
17. Ogura T, Takada H, Okuno M, *et al.* (2010) Fatty acid composition of plasma, erythrocytes and adipose: their correlations and effects of age and sex. *Lipids* **45**, 137–144.
18. Arab L (2003) Biomarkers of fat and fatty acid intake. *J Nutr* **133**, Suppl. 3, 925S–932S.
19. Hodson L, Skeaff CM & Fielding BA (2008) Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Prog Lipid Res* **47**, 348–380.
20. McMurchie EJ, Margetts BM, Beilin LJ, *et al.* (1984) Dietary-induced changes in the fatty acid composition of human cheek cell phospholipids: correlation with changes in the dietary polyunsaturated/saturated fat ratio. *Am J Clin Nutr* **39**, 975–980.
21. Glaser C, Demmelmair H & Koletzko B (2010) High-throughput analysis of fatty acid composition of plasma glycerophospholipids. *J Lipid Res* **51**, 216–221.
22. Glaser C, Demmelmair H, Sausenthaler S, *et al.* (2010) Fatty acid composition of serum glycerophospholipids in children. *J Pediatr* **157**, 826–831, (e821).
23. Kornsteiner M, Singer I & Elmadfa I (2008) Very low *n*-3 long-chain polyunsaturated fatty acid status in Austrian vegetarians and vegans. *Ann Nutr Metab* **52**, 37–47.
24. Connor SL, Zhu N, Anderson GJ, *et al.* (2000) Cheek cell phospholipids in human infants: a marker of docosahexaenoic and arachidonic acids in the diet, plasma, and red blood cells. *Am J Clin Nutr* **71**, 21–27.
25. Hoffman DR, Birch EE, Birch DG, *et al.* (1999) Fatty acid profile of buccal cheek cell phospholipids as an index for dietary intake of docosahexaenoic acid in preterm infants. *Lipids* **34**, 337–342.
26. Hoffman DR & Uauy R (1992) Essentiality of dietary omega 3 fatty acids for premature infants: plasma and red blood cell fatty acid composition. *Lipids* **27**, 886–895.
27. Salamat-Miller N, Chittchang M & Johnston TP (2005) The use of mucoadhesive polymers in buccal drug delivery. *Adv Drug Deliv Rev* **57**, 1666–1691.
28. Hill MW (1984) Cell renewal in oral epithelia. In *The Structure and Function of Oral Mucosa*, pp. 53–81 [J Meyer, CA Squier and SJ Gerson, editors]. Oxford: Pergamon Press Ltd.
29. Gillespie GM (1969) Renewal of buccal epithelium. *Oral Surg Oral Med Oral Pathol* **27**, 83–89.
30. Kaidbey KH & Kurban AK (1971) Mitotic behaviour of the buccal mucosal epithelium in psoriasis. *Br J Dermatol* **85**, 162–166.
31. Laitinen K, Sallinen J, Linderborg K, *et al.* (2006) Serum, cheek cell and breast milk fatty acid compositions in infants with atopic and non-atopic eczema. *Clin Exp Allergy* **36**, 166–173.
32. Bailey-Hall E, Nelson EB & Ryan AS (2008) Validation of a rapid measure of blood PUFA levels in humans. *Lipids* **43**, 181–186.
33. Ian Givens D & Gibbs RA (2008) Current intakes of EPA and DHA in European populations and the potential of animal-derived foods to increase them. *Proc Nutr Soc* **67**, 273–280.
34. Klem S, Klingler M, Demmelmair H, *et al.* (2012) Efficient and specific analysis of red blood cell glycerophospholipid fatty acid composition. *PLoS One* **7**, e33874.

British Journal of Nutrition

5 Zusammenfassung

5.1 Zusammenfassung (deutsch)

Ziele: Klinische und epidemiologische Studien zeigen die Wichtigkeit von Omega-3 Fettsäuren in der menschlichen Ernährung und deren Zusammenhang mit Morbidität und Mortalität bei Erkrankungen des Herz-Kreislaufsystems, psychischen Erkrankungen und ihre Bedeutung in Schwangerschaft und Säuglingsalter. Detaillierte Untersuchungen der Zusammenhänge zwischen der individuellen Fettsäurezusammensetzung und der Gesundheit erfordern die Analytik des Fettsäurestatus bei großen Probenzahlen. Es gibt verschiedene Verfahren in der Fettsäureanalytik, welche oft durch aufwändige Probenaufarbeitungsprozeduren begrenzt, kostspielig oder unangemessen invasiv sind.

Ziel dieser Arbeit war die Optimierung und Validierung zweier Biomarker für den Fettsäurestatus im Menschen: Wangenschleimhautzellen-Glycerophospholipide und RBC-Glycerophospholipide basierend auf einer klinischen Supplementationsstudie mit DHA. Des Weiteren wurden beide neuen Verfahren mit etablierten Methoden verglichen und Korrelationen berechnet, um eine Aussage über deren Nutzen und Anwendbarkeit treffen zu können und zu bestimmen, ob Wangenschleimhaut-Glycerophospholipide und RBC-Glycerophospholipide kurz- oder langfristige Biomarker des Fettsäurestatus sind.

Methoden: Die Methylester der Glycerophospholipid-Fettsäuren sowohl in den RBC, als auch in den Wangenschleimhautzellen werden durch Fällung der Proteine und die anschließende basenkatalysierte Methylestersynthese selektiv herausgelöst. Die Lösung der GPL innerhalb der Probe wird durch die Behandlung mit Ultraschall verbessert.

Der Fettsäurestatus in RBC GPL und Wangenschleimhaut-GPL wurde in einer 29-tägigen Supplementationsstudie mit DHA (510mg/d) ausgewertet. Zum Vergleich wurde der DHA-Anstieg in den unterschiedlichen Kompartimenten und deren Korrelationen mit SPSS bestimmt.

Ergebnisse: Bei der Validierung der RBC GPL Methode fanden sich im Intra-Assay Variationskoeffizienten zwischen 1,0 und 10,5 %, im Inter-Assay Werte zwischen 1,3 und 10,9 %. In allen analysierten Lipidfraktionen (PE, PC, GPL und Gesamt-PL) stieg der DHA-Anteil während der Supplementation signifikant an. Außerdem wurden hohe

Korrelationen für die meisten einfach- und mehrfach ungesättigten Fettsäuren und für den Omega-3-Index zwischen RBC GPL und RBC PL (r=0.924) gefunden.
Ebenso ergaben sich hohe Korrelationen zwischen dem DHA-Gehalt in Wangenschleimhaut-GPL und Plasma-GPL (r=0,88) und RBC-GPL (r=0,76) vor Studienbeginn.
Das „half-maximal-Level“ von DHA in den GPL fand sich nach 4 Tagen in Plasma, nach 6 Tagen in RBC und nach 10 Tagen in Wangenschleimhautzellen nach täglicher DHA-Supplementation. Der mittlere DHA-Anstieg (mol%) im Bezug zum Ausgangswert war am deutlichsten im Plasma (186%) zu erkennen, gefolgt von Wangenschleimhautzellen (180%) und RBC (130%).
Diskussion und Schlussfolgerungen: Im Rahmen der Studie der RBC GPL Methode zeigte sich eine große Herausforderung in der Vermeidung der Verklottung der RBC in Methanol, was die Ausbeute an Methylestern deutlich beeinflusste. Das Verfahren wurde durch die Behandlung der Proben mit Ultraschall im Wasserbad optimiert, nachdem die Verlängerung der Schüttelzeit kaum Erfolg zeigte. Deshalb sehen wir die Ultraschallbehandlung als essentiellen Bestandteil des Aufarbeitungsprozesses in der Analyse der RBC GPL an um die Ertragsrate zu erhöhen. Die Überprüfung der Präzision mit Intra- und Inter-Assay war vergleichbar mit den bereits etablierten Verfahren der GPL in Wangenschleimhautzellen und Plasma, sodass die Prozedur der Proteinfällung mit anschließender basenkatalysierter Umesterung für alle 3 Kompartimente angewandt werden kann.
Für die Lagerzeit können folgende Empfehlungen ausgesprochen werden: eine Lagerung in Methanol mit BHT (0,05 mg/ml) für 8 bis 12 Monate bei -80° C beeinflusst die meisten Fettsäuren kaum, was vergleichbar ist zu anderen Lagerungsmethoden der RBC. Jedoch konnte eine nicht signifikante Abnahme des DHA-Gehaltes nachgewiesen werden, was im Rahmen einer Lagerungszeit von über 12 Monaten kritisch werden könnte.
Der Omega-3-Index in RBC GPL zeigte eine hohe Korrelation zu dem Omega-3-Index der RBC Gesamt-PL, sodass mit der Methode auch die Bestimmung des Omega-3-Indexes möglich scheint.
Zusammenfassend lässt sich festhalten, dass die Analytik der Fettsäuren in GPL RBC eine exzellente Alternative zum Monitoring der Fettsäuren während einer Omega-3-Supplementation ist und zusätzlich eine präzise und robuste Methode darstellt, die es erlaubt schnell und kostengünstig eine große Zahl von kleinen

Probenvolumina zu untersuchen, indem sie auf zeitraubende Aufarbeitungsschritte, wie Dünnschichtchromatographie, verzichtet.

In dieser Studie wurde erstmals gleichzeitig die Aufnahme von DHA in GPL der Kompartimente Wangenschleimhautzellen, RBC und Plasma untersucht. Es fanden sich hohe Korrelationen. Der DHA-Anteil in CC betrug jedoch im Vergleich zu Plasma und RBC nur ein Drittel, aber es wurde gezeigt, dass die Änderung der Omega-3 und Omega-6-Fettsäureaufnahme sich in allen Kompartimenten ähnlich äußert.

Unter Beachtung einer Verzögerungsphase von 5 Tagen (durch Erneuerung der Mundschleimhautzellen) spiegeln die Wangenschleimhautzellen kurzzeitige Änderungen der diätetischen Fettsäureaufnahme wider und reagieren schnell auf DHA-Supplementation. Nicht außer Acht zu lassen sind die Vorteile der Probengewinnung der Wangenschleimhautzellen, welche durch Abbürsten einfach, schnell, nicht invasiv und in einer nicht klinischen Umgebung durchzuführen ist.

Somit lässt sich schlussfolgern, dass Wangenschleimhaut GPL als Biomarker des Omega-3-Fettsäurestatus herangezogen werden können, vor allem in klinischen Studien, in denen Blutentnahmen schwierig oder nicht zumutbar/ anwendbar sind.

5.2 Zusammenfassung (englisch)

Objectives: Clinical and epidemiological studies demonstrate the importance of long chain omega-3 fatty acids in human nutrition and their relationship to morbidity and mortality in diseases of the cardiovascular system, mental illness and their importance in pregnancy and infancy. Detailed studies of the relationships between fatty acid composition and health require the analysis of the fatty acid status of large numbers of samples. There is a variety of methods for fatty acid analysis available, but they are often limited by time-consuming sample preparation procedures or invasive sample collection is required.
The aim of this thesis was to optimize and validate two biomarkers for fatty acid status in humans: cheek cell glycerophospholipids (GPL) and red blood cell (RBC) glycerophospholipids based on a clinical supplementation trail with docosahexaenoic acid (DHA). The new method was compared with established methods and correlations were calculated to evaluate their usefulness and applicability and to determine, whether cheek cell GPL and RBC GPL are short- or longterm biomarkers of fatty acid status in human.
Methods: The methylesters of the GPL fatty acids, in both the RBC and in cheek cells are selectively transferred into methylesters by a combination of methanolic precipitation of proteins and base-catalyzed transesterification. The solubility of GPL in the sample is improved by ultrasound treatment.
The fatty acid status in RBC GPL and CC GPL was evaluated in a 29-day supplementation trial with DHA (510mg/d). In order to compare the increase of DHA, the different compartments were measured and their correlations were determined.
Results: In the validation of the RBC GPL method intra-assay coefficients of variation were found between 1.0 and 10.5% in the inter-assay values from 1.3 to 10.9% were obtained. In all analyzed lipid fractions (PE, PC, GPL and total PL) DHA-content increased significantly during supplementation. In addition, high correlations for most mono- and polyunsaturated fatty acids and the omega-3-index between RBC GPL and RBC total phospholipids ($r = 0.924$) were found.
Similarly, high correlations between the DHA-content in cheek cell GPL and plasma GPL ($r = 0.88$) and RBC GPL ($r = 0.76$) were found at baseline.
The half-maximal GPL DHA-level was detected after 4 days in plasma, after 6 days in RBC and after 10 days in CC, after daily DHA-supplementation. The mean increase in DHA (mol%) in relation to baseline, was seen most clearly in the plasma (186%), followed by CC (180%) and RBC (130%).

Discussion and Conclusions: In the study of the RBC GPL method a major challenge was to avoid the clotting of RBC in methanol, which significantly influenced the yield of methylesters. The process has been optimized by treating the sample with ultrasound in a water bath, after extension of the shaking time was hardly successful. Therefore, we see the ultrasound treatment as an essential part of the analysis of RBC GPL. The precision indicated by intra- and interassays was similar in CC and plasma. Thus, the procedure of protein precipitation followed by base-catalyzed transesterification can be applied for all 3 matrices with slight adaptations.

Concerning the storage stability of samples the following recommendations can be made: a storage in methanol with BHT (0.05 mg/ml) for 8 to 12 months at -80° C hardly affects most fatty acids, which is comparable to other conservation procedures for RBC. However, a non-significant decrease in DHA content, which could be critical for a storage time of about 12 months was observed.

The omega-3-index in RBC GPL showed a high correlation with the omega-3 index of RBC total PL, which indicates the possibility to determine an omega-3 index with this method.

In summary, it can be stated that the analysis of fatty acids in RBC GPL is suitable to monitor the fatty acid status during an omega-3 supplementation and is an accurate and robust method that quickly allows to investigate a large number of samples from small sample amounts and with relatively low costs by avoiding time-consuming purification steps, such as thin layer chromatography.

In this study the incorporation of DHA into GPL of the compartments cheek cells, RBC and plasma was investigated simultaneously for the first time. High correlations were detected. Although the DHA-content in CC was only one-third compared to plasma and RBC, it was shown that the relative change of the omega-3 and omega-6 fatty acids is similar in the compartments.

Taking into account a lag phase of 5 days for renewal of oral mucosal cells, the CC reflect short-term changes and respond quickly to DHA-supplementation. Besides, the collecting samples of cheek cells by brushing is simple, fast, non-invasive and easy to apply in a non-clinical setting.

Thus, it can be concluded that CC GPL seem to be valuable biomarkers of omega-3 fatty acid status, especially in clinical studies in which blood sampling is not adequate or applicable.

6 Literaturverzeichnis

1. Harper, C.R. and T.A. Jacobson, *Usefulness of omega-3 fatty acids and the prevention of coronary heart disease.* Am J Cardiol, 2005. **96**(11): p. 1521-9.
2. Kris-Etherton, P.M., W.S. Harris, and L.J. Appel, *Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease.* Arterioscler Thromb Vasc Biol, 2003. **23**(2): p. e20-30.
3. Riediger, N.D., et al., *A systemic review of the roles of n-3 fatty acids in health and disease.* J Am Diet Assoc, 2009. **109**(4): p. 668-79.
4. *Thieme Chemistry (Hrsg.): Römpp Online. Version 3.1. Georg Thieme Verlag, Stuttgart 2007.*
5. Kornsteiner, M., I. Singer, and I. Elmadfa, *Very Low n–3 Long-Chain Polyunsaturated Fatty Acid Status in Austrian Vegetarians and Vegans.* Annals of Nutrition and Metabolism, 2008. **52**(1): p. 37-47.
6. Hodson, L., C.M. Skeaff, and B.A. Fielding, *Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake.* Progress in Lipid Research, 2008. **47**(5): p. 348-380.
7. Fekete, K., et al., *Methods of assessment of n-3 long-chain polyunsaturated fatty acid status in humans: a systematic review.* Am J Clin Nutr, 2009. **89**(6): p. 2070S-2084S.
8. Klingler, M. and B. Koletzko, *Novel methodologies for assessing omega-3 fatty acid status - a systematic review.* Br J Nutr, 2012. **107 Suppl 2**: p. S53-63.
9. Glaser, C., H. Demmelmair, and B. Koletzko, *High-throughput analysis of fatty acid composition of plasma glycerophospholipids.* J Lipid Res, 2010. **51**(1): p. 216-21.
10. Harris, W.S., *The omega-3 index as a risk factor for coronary heart disease.* Am J Clin Nutr, 2008. **87**(6): p. 1997S-2002S.
11. Harris, W.S., *The omega-3 index: from biomarker to risk marker to risk factor.* Curr Atheroscler Rep, 2009. **11**(6): p. 411-7.
12. McMurchie, E.J., et al., *Dietary-induced changes in the fatty acid composition of human cheek cell phospholipids: correlation with changes in the dietary polyunsaturated/saturated fat ratio.* Am J Clin Nutr, 1984. **39**(6): p. 975-80.

13. Harris, W.S., et al., *Omega-3 fatty acids in cardiac biopsies from heart transplantation patients: correlation with erythrocytes and response to supplementation.* Circulation, 2004. **110**(12): p. 1645-9.

14. Klingler, M., et al., *Fatty Acid Status Determination by Cheek Cell Sampling Combined with Methanol-Based Ultrasound Extraction of Glycerophospholipids.* Lipids, 2011.

15. Folch, J., M. Lees, and G.H. Sloane Stanley, *A simple method for the isolation and purification of total lipides from animal tissues.* J Biol Chem, 1957. **226**(1): p. 497-509.

16. Geppert, J., et al., *Docosahexaenoic acid supplementation in vegetarians effectively increases omega-3 index: a randomized trial.* Lipids, 2005. **40**(8): p. 807-14.

17. Klingler, M., et al., *Analysis of FA contents in individual lipid fractions from human placental tissue.* Lipids, 2003. **38**(5): p. 561-6.

7 Eigenanteil an den vorgelegten Arbeiten

Die Doktorandin
Sabrina Klem, geb. am 25.10.1984 in Freiburg im Breisgau,
die in einer Publikation als Erstautor und der zweiten Publikation als Zweitautor auftritt, hat das Thema der Dissertation gemeinsam mit Herrn Prof. Dr. med. Berthold Koletzko, Herrn Dr. rer. nat. Johann Demmelmair und Herrn Dr. hum. biol. Mario Klingler entwickelt und vorbereitet.

Die Durchführung der klinischen Studie mit Probandengespräch, körperlicher und biometrischer Untersuchung, Probengewinnung und Lagerung erfolgte eigenständig. Die Aufarbeitung der Proben, die Analyse der Daten und Auswertung der Ergebnisse gelang mit Hilfe von Dr. Mario Klingler.

Beide Publikationen wurden zunächst durch den/die Autor/in mit Hilfe des(r) Zweitautoren/in verfasst und anschließend durch Herrn Dr. Johann Demmelmair revidiert und in gemeinsamer Arbeit in ihre endgültige Fassung gebracht.

Alle Koautoren standen stets unterstützend bei speziellen Fragestellungen zum Manuskript, zur statistischen Auswertung und der graphischen Darstellung von Studienergebnissen zur Verfügung.

München, den 25.05.2014

_____

Sabrina Klem

8 Danksagung

Herrn Professor Dr. med. B. Koletzko danke ich für die Überlassung des Themas und für die Mühe und angenehme Betreuung während der Anfertigung meiner Dissertation.

Herzlich danken möchte ich allen Mitarbeitern des Stoffwechsellabors der Dr. von Haunerschen Kinderklinik für ihre Kollegialität und warmherzige Unterstützung. Ihr seid zu Freunden für mich geworden.

Besonderer Dank gebührt Herrn Dr. hum. biol. Mario Klingler für seine Rolle als Betreuer, Mentor und Motivator und ebenso Herrn Dr. rer. nat. Hans Demmelmair für die freundliche Begleitung, sowie für die sorgfältige Durchsicht und Verbesserung der Texte. Durch ihre fachlichen Anregungen sowie konstruktive Kritik waren sie maßgeblich am Gelingen meiner Arbeit beteiligt.

Weiterhin möchte ich mich an dieser Stelle bei allen teilnehmenden Probanden bedanken, mit deren zuverlässiger Hilfe und engagierter Mitarbeit die Durchführung des klinischen Teils der Studie erst möglich wurde.

Mein ganz besonderer Dank gilt Alexander für seine sprachliche Hilfe beim Anfertigen dieser Arbeit, aber insbesondere für eine Schulter zum Anlehnen, Worte, die berühren, viel Lachen und seine unendliche Liebe.

Ich danke meiner besten Freundin Julia für das feste unsichtbare Freundschaftsband, das wir seit unserer Kindheit halten und die Kraft, Energie und den Mut, den sie mir dadurch gibt.

Zuletzt danke ich von ganzem Herzen meinen Eltern und meiner liebsten Schwester Verena, die immer an mich glauben und mich in allen Lebenslagen und Situationen unterstützen und motivieren. Ohne Euch wäre das alles nicht möglich gewesen.