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In vitro dissolution behaviour and absorption in humans of a novel mixed L-lysine salt formulation of EPA and DHA

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ABSTRACT

Introduction: Supplements with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are generally oil-based formulations containing their triacylglycerols, phospholipids or ethyl-esters (EE). Recently, a L-lysine salt of carboxylic EPA and DHA became available (Lys-FFA), which necessitated to study its oral absorption and plasma kinetics in humans.

Objectives: The in vitro dissolution characteristics, oral bioavailability and 48 h plasma profiles of EPA and DHA (as triacylglycerides) of Lys-FFA, relative to a commercially available oil-based EE supplement.

Methods: Dissociation of the lysine from the FFAs was studied in vitro applying simulated gastric (12 h) and intestinal (3 h) conditions. In an open label, randomized, two-way cross-over design, oral administration of Lys-FFA (500 mg EPA plus 302 mg DHA) versus EE (504 mg EPA plus 378 mg DHA) was studied over 48 h, in eight female volunteers. Plasma profiles of EPA and DHA were described by Area Under the Curve (AUC; 0–12 h), C_{max} and T_{max}.

Results: Dissolution studies with Lys-FFA showed complete dissociation under both conditions. In volunteers Lys-FFA showed rapid absorption and high bioavailability indicated by significant differences in both the AUC_{0-12hr} and C_{max} when compared to the EE comparator ($p<0.001$), with AUC_{0-12hr} which was for EPA 5 times higher with Lys-FFA than with the EE formulation.

Conclusion: This first-in-man study of Lys-FFA demonstrated rapid absorption of EPA and DHA and a considerably higher bioavailability compared to an EE supplement under fasting conditions. The release and absorption characteristics from this solid form offer several new options in terms of formulation technology and dosing.

Abbreviations

DHA: Docosahexaenoic acid;
EPA: Eicosapentaenoic acid;
PK: pharmacokinetic;
TAG: triacylglyceride;
n-3 LC PUFA: n-3 Long chain poly-unsaturated fatty acids,
EE: ethyl ester;
Lys-FFA: L-lysine salt formulation of EPA and DHA as carboxylic acids

1. Introduction

n-3 Long Chain Poly-Unsaturated Fatty Acids (*n-3* LC PUFAs) play vital roles as components of biological membranes and precursors of several important signalling molecules [1]. Because humans do not possess enzymes to insert a double bond in the n-3 position, n-3 (and also n-6) fatty acids are essential dietary components [2]; with the main n-3 LC PUFAs in the human diet being α-linolenic acid (ALA; 18:3n-3), predominantly obtained from plant sources, and the 'marine' forms eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) [3]. Typically, rich sources of EPA and DHA are fatty fish (e.g. herring, salmon, mackerel), as well as in certain algae and krill [3]. As endogenous elongation from ALA is limited, in particular in adults, their adequate intake is important for normal development and prevention of disease [3-5]. However, in many populations and individuals, supply from the diet remains below recommendations [3]. Furthermore,

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several lines of evidence support specific clinical use of EPA and (or) DHA at doses higher than those obtained from a normal diet [6]. Together, this generates an increasing demand to supply n-3 LC PUFAs via food supplements, clinical nutrition products, or as pharmaceutical products.

Attaining adequate *n-3* LC PUFA via supplementation can be affected by the chemical form of the EPA and DHA, the required steps in digestion and thus the implications for bioavailability (in this sense, the speed to which the *n-3* LC PUFAs appear in the blood post-absorption). A very common form of EPA/DHA supplement on the market is an ethyl ester, where the natural triglyceride form of EPA and DHA is trans-esterified to an ethyl ester in place of the glycerol backbone [7]; invariably this is to allow the EPA and DHA to be more easily purified and concentrated (triglyceride and phospholipid forms are also available). To enable absorption in the gut, the ethyl ester form requires emulsification via bile salts and hydrolysis by pancreatic lipases. In comparison, the free fatty acid form of EPA/DHA requires no emulsification or enzymatic hydrolysis in order to allow incorporation into the mixed micelle and thus progress to absorption in the gut [8]. Although it is already known for several years that EPA and DHA given as free fatty acids are well absorbed [9,10], their use in oral preparations is hampered by a pungent taste, GI irritating effects and sensitivity to oxidation [11,12]. To overcome this, a mixed L-lysine salt of the carboxylic form of EPA and DHA has been developed. The underlying principle is that the salt will dissociate in the acidic environment of the stomach, allowing the free fatty acids to be absorbed from the small intestinal tract. To test this concept, studies were carried out first to investigate the in vitro dissociation behaviour of the salt. This was followed by the first study in human volunteers to determine the plasma profile and bioavailability of both fatty acids.

2. Materials and methods

2.1. Omega-3 lysine salt

The mixture of EPA L-lysine salt (EPA-Lys; CAS 171,228–62–9) and DHA L-lysine salt (DHA-Lys; CAS 171,228–62–9) is prepared by mixing equimolar amounts of the fatty acids dissolved in ethanol with an aqueous solution of L-lysine, followed by spray-drying. Fatty acids were obtained via alkaline hydrolysis from a commercially available mixture of EPA/DHA ethyl esters containing at least 50% EPA-OEt and 20% DHA-OEt. This results in a salt with a yellowish to brownish colour.

2.2. Study 1: in vitro dissolution testing

First, a pilot study was performed to study the dissolution / dissociation behaviour of the fatty acid-lysine salts under stomach-like (low pH) conditions. To this end, the L-lysine salt preparation, in amounts of 1.25 and 5.0 g, respectively, was added to 250 ml of an aqueous 0.1 N hydrochloric acid solution in a beaker. Using a magnetic stirrer, resulting mixtures were stirred overnight at ambient temperature. For reference, similar tests were carried out with a mixture of DHA and EPA as free fatty acids, prepared from TAG- or ethyl-esters, respectively, with L-lysine monohydrate, and with a 'synthetic' mixture of above free fatty acids and L-lysine in solution. Quantification of DHA and EPA was carried out using reversed-phase HPLC with UV detection, whereas L-lysine was analysed by acidimetric titration using 0.1 N perchloric acid as the titrant and formic acid/glacial acetic acid as solvent. The equivalence point was determined potentiometrically. The lysine recovery in the aqueous phase was close to 100% for all sample solutions tested, with no notable difference between the reference and test sample solutions. Data from method validation confirmed lysine assay repeatability of RSD $<$ 0.1%. Next to this, ^{1}H NMR spectroscopy was utilized to determine the lysine profile of the aqueous phases for the reference (i.e., L-lysine monohydrate) and the test sample solutions. It was found that the lysine generated from the salt formulation existed mainly in the form of the hydrochloride. The addition of the fatty acid lysine salts, the free fatty acids and the esters resulted in turbid aqueous phases and a small volume of an oily/waxy upper phase. Because of this, both phases were separately sampled for analysis after stirring had stopped. The lysine hydrate reference dissolved completely in the aqueous phase.

Subsequently, the dissolution behaviour of the salt was investigated using a standardized United States Pharmacopoeia (USP) in vitro set-up. To this end, a USP Apparatus 2 (paddle method, www.usp.org) was used according to the corresponding monograph, with fed state simulated small intestinal fluid (FeSSIF) at pH 5, considered representative for duodenum and jejunum regions of the small intestine. The fed state buffer was chosen because it is generally recommended to consume n-3 fatty acids along with meals. Appropriate dissolution mixtures were prepared using a commercially available product (Biorelevant.com Ltd, UK) [13] adjusted to pH 5.0 according to manufacturer's instructions. Test volume was 250 ml. Amounts tested were equivalent to concentrations of approximately 100 mg/100 ml EPA + DHA and 600 mg / 100 ml EPA + DHA, to simulate a low dose (500 mg EPA+DHA) and a high dose, respectively. Tests were performed in triplicate at 37 °C applying an incubation time of 3 h. Samples were taken after 3 h for analysis by HPLC.

2.3. Study 2: bioavailability and plasma kinetics in human volunteers

2.3.1. Ethical approval

This study was approved by the medical ethical committee of Wageningen University (METC-WU; NL 63,619.081.18) and conducted in accordance with the principles of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and with the Medical Research Involving Human Subjects Act (WMO). All subjects gave their written informed consent.

2.3.2. Subjects

Healthy adult (aged 18–28yrs) female volunteers with a body mass index (BMI) of 18.5–25 kg/m^2 were recruited from the area local to Wageningen University, The Netherlands.

Exclusion criteria included: evidence of current illness, any gastrointestinal conditions/diseases within the 3 months prior to the intervention, use of medication two months before and during the intervention (except for oral contraceptives and occasional use of painkillers), reported weight loss or weight gain of $>$ 2 kg in the month prior to the intervention, use of omega-3 or fish oil supplements up to three weeks before- or during the intervention, allergies to test products ((shell)fish or soy products), drug abuse, smoking, alcohol consumption of $>$10 glasses per week, recent or planned blood donation ($<$4 month prior to first study day or during intervention), haemoglobin (Hb) level $<$ 7.5 mmol/L, been pregnant or breastfeeding in the last 6 months, or plan to become pregnant or breastfeed during the study and (planned or recent) participation in other research.

A total of eight healthy women were recruited and after providing written informed consent, were randomly allocated to their respective trial sequence. The study was carried out in one period (June to July 2018) and participants were recruited between May and June 2018.

2.3.3. Study design and randomisation

To allow a direct comparison of kinetic profiles between Lys-FFA and EE, the study was a prospective, open-label, randomized, cross-over design with two single-dose treatment periods. The study was not blinded due to the dose of the experimental formulation (powder) being contained within hard capsules, whereas the EE comparator was a softgel capsule. Participants were randomly allocated to a test sequence.

The two experimental periods lasted 48 h each and were separated by a wash-out period of two weeks, calculated from the first dosing. To reduce upfront the possible effect of disparities between groups when drop-out might occur, both preparations were tested in 4 subjects during each session.

During the first 24 h of a test session, subjects were required to stay within the Wageningen University Human Research Facilities, until the 24 h blood sample had been collected. For the samples taken at 32 h and 48 h, subjects returned to the university facilities. During a session, subjects were provided standardized meals, supplied by the university and composed by a research dietician, at $T = 2$, 6, 11, 24, 28 and 34 h after ingestion of the study products. Meal composition data are provided in the supplemental materials. The meals provided were individually standardized based on the estimated energy requirement of each subject, taking weight, height and approximate physical activity level into account. The standardized meals at 28 and 34 h were eaten outside the facility. A standardized snack was provided at $T = 9$. The subjects did not consume any other food product besides the meals provided, which were free of 'marine' sources of fatty acids. Drinks such as water, coffee or tea without milk and sugar were allowed. After the sampling period, the second morning (48 h), subjects were provided with a breakfast.

On each first test day, participants arrived at the research facility in the morning in a fasted state. The evening before they were requested to consume a standardized meal provided by the university. Before the start, subjects were requested to fill out a well-being questionnaire. After placement of a venous catheter and donating a baseline blood sample ($T = 0$), subjects received either the test ingredient of the L-lysine salt formulation of EPA and DHA in the free fatty acid form (Lys-FFA) or the comparator of EPA and DHA in the ethyl ester form (EE), both in the form of capsules, with 150 mL of tap water. Subsequently, blood samples (11 mL until $T = 4$ h, 6 mL at the remaining timepoints) were taken at $T = 0.5$, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 20, 24, 32, 48 h after ingestion using tubes containing EDTA + aprotinin and centrifuged at 4 °C within minutes after centrifugation. Plasma samples were stored at −80 °C until analysis. All adverse events were recorded, either spontaneously reported by the research subject or observed by the research staff, regardless of being or not considered related to the investigational products or the study.

2.3.4. Study products and dosing

On each occasion, research subjects received either a preparation of L-lysine salts of EPA and DHA in their free fatty acid form (Lys-FFA) known commercially as AvailOm® (EP patent EP3236782B1), or a conventional fish oil food supplement containing EPA and DHA in the ethyl ester form (EE). The Lys-FFA was provided by Evonik Operations GmbH (Hanau, Germany). The fish oil supplement (EE) was purchased from a local store (Lucovitaal Puur Koudwater Omega 3 Visolie). Its relative fatty acid composition was checked by GC after saponification. In addition, ^{13}C NMR was used to determine the molecular structure of the preparation. EPA and DHA were found to be entirely present as their ethyl-esters. No NMR signals indicative for triacylglycerides or free fatty acids were detected. Analytical data for the reference product are provided in the supplementary material.

The Lys-FFA preparation was administered in a dose of 1400 mg, given in hard capsules; the EE supplement was administered in a dose of 1400 mg, given in a soft gel capsule as supplied. The Lys-FFA preparation contained per 1400 mg dose: 499.8 mg EPA and 302.4 mg DHA (EPA/DHA molar ration 1.79). The preparation of this product was performed according to HACCP food safety guideline and food cGMP requirements as defined in 21CFR§110 at an ISO22000 certified production site. The EE supplement specifications were per dose of 1400 mg: 504 mg EPA and 378 mg DHA (EPA/DHA molar ration 1.44). This relative composition was confirmed both by NMR and GC analysis.

2.3.5. Plasma fatty acid analysis

Following centrifugation (2418 rpm, 1190 *G*, t = 4 °C), plasma (EDTA-aprotinin) was separated and stored at −80 °C until further analysis. DHA and EPA values were quantified in the triglyceride fraction of EDTA plasma using a modified version of a previously described protocol [14]. In short, 650 μL EDTA plasma was extracted using hexane in the presence of a C19:0 TAG internal standard, and lipids were purified by solid phase extraction using silica columns. Subsequently, fatty acid methylesters are formed, which are analysed using gas chromatography coupled to flame ionization detection (GC-FID) as described before [14]. Concentrations were calculated via single-point calibration, using the C19-TAG as standard. Per batch of 48 samples, 3 QC samples were co-analysed in duplicate. The intra-batch coefficient of variability (CV) for DHA-TAG was 13%, for EPA-TAG 17%.

2.3.6. Sample size calculation

No kinetic data had been available for Lys-FFA prior to the study, however, based on data by El Boustani et al. [15], the pharmacokinetics of an EPA-arginine salt has been described which was the nearest possible resource to utilise for sample size calculation.

A total of eight subjects was calculated to be adequate based on: 80% power (β), 0.05 level of significance (α) with expected means (±SD) of 1) control (1 g EPA ethyl-ester): 28 ± 4.7 (SEM with $n = 4$), EPA to amino acid plasma ratio at $t = 5$ h and 2) treatment (1 g EPA arginine):89 ± 26 (SEM with $n = 4$) EPA to amino acid plasma ratio at $t = 5$ h, results in a sample size of 7.25 (via a t-test due to $n<30$).

2.3.7. Data handling and data analysis

Data for the plasma fatty acid concentrations were analysed using the 'R' statistical package, version 3.4.4 [16]. All data are presented as median and interquartile range, except where specified otherwise and all P values were considered significant if $p \leq 0.05$.

For the primary endpoints (Table 1), the descriptive kinetic values of maximum peak height (C_{max}) and time-to-peak (T_{max}) were directly estimated from the individual plasma curves. Baseline-adjusted Area Under the Curve from $T = 0$ until $T = 12$ h ($AUC_{0–12\ h}$) were calculated for each participant separately by the trapezoid rule. A baseline adjustment of $AUC_{0–12\ h}$ and C_{max} was completed to account for the endogenous levels of EPA and DHA that were already present in the circulating plasma of the participants.

In Table 2, for each pharmacokinetic parameter in the first column, we considered the sample statistics (median, arithmetic mean and geometric mean) for the paired ratios between the Lys-FFA and EE values. The pairing was done by participant, that is for each participant j (1 to 8) we defined the ratio x_j/y_j, where x_j is the value of the Lys-FFA-parameter and y_j is the corresponding EE-parameter. The last column presents the p-value of the applied tests. Namely the variables with the p-values 0.004 are referred to a one sample Sign test (binomial) with

Table 1
Summary of the baseline-adjusted pharmacokinetic parameters of total EPA + DHA, total EPA, and total DHA in plasma.

Pharmacokinetics parameter	L-FFA	EE
TOTAL EPA:		
Cmax (μg/ml)	42.56 [24.54, 52.31]	4.99 [3.19, 6.98]
tmax (hour)	3 [3, 3]	5 [4,12]
AUC(0,12) (μg hour/ml)	140.99 [104.71, 173.94] gm:127.16	26.17 [13.23, 30.94] gm:22.18
TOTAL DHA:		
Cmax (μg/ml)	24.54 [13.13, 31.63]	6.22 [2.65, 8.86]
tmax (hour)	3 [3, 3]	4 [3, 8]
AUC(0,12) (μg hour/ml)	71.89 [57.74, 94.93] gm:77.53	24.68 [12.26, 31.61] gm:13.3
TOTAL EPA+DHA:		
Cmax (μg/ml)	67.11 [37.61, 83.94]	9.54 [8.31, 14.86]
tmax (hour)	3 [3, 3]	5 [4,12]
AUC(0,12) (μg hour/ml)	207.13 [159.6, 268.56] gm:200.39	44.88 [31.01, 50.8] gm:36.2

NOTES: Each cell presents the median and the IQR interval [25th, 75th percentile]. Just for the AUC parameters, the geometric mean is also reported as 'gm'. ABBREVIATIONS: AUC, area under the concentration-time curve; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. AUC(0,12), area under the plasma concentration versus time curve from time 0 to 12 h postdose; Cmax, maximum measured plasma concentration.

Table 2
Summary of the statistical comparisons of the pharmacokinetic parameters of baseline-adjusted total EPA+DHA, total EPA, and total DHA in plasma.

Pharmacokinetics parameter	Median of ratios	Arithmetic mean of ratios	Geometric mean ratio	P-value
TOTAL EPA:				
AUC(0,12) (μg hour/ml)	5.45	6.73	5.73 (90% CI: 3.9 - 8.42)	< 0.00006
Cmax (μg/ml)	6.75	9.71	7.84	< 0.004
TOTAL DHA:				
AUC(0,12) (μg hour/ml)	2.92	14.65	5.83	< 0.004
Cmax (μg/ml)	4.1	6.91	5.22	< 0.004
TOTAL EPA+DHA:				
AUC(0,12) (μg hour/ml)	5.04	6.59	5.54	< 0.004
Cmax (μg/ml)	6.73	6.72	6.07	< 0.004

ABBREVIATIONS: AUC, area under the concentration-time curve; CI, confidence interval; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. AUC(0,12), area under the plasma concentration versus time curve from time 0 to 12 h postdose; Cmax, maximum measured plasma concentration. The p-value and CI for EPA AUC(0,12) refer to a *t*-test for the geometric mean, while the p-values for the other quantities refer to a sign test for the median.

null hypothesis H0: the median of the ratios x_j/y_j is equal 1. H0 can always be rejected for the alternative HA: the median of the ratios x_j/y_j is greater than 1. The first p-value < 0.00006 is instead related to the test on the geometric mean ratio for the parameter EPA-AUC($_{0–12}$). Here the null hypothesis was H0: the mean of the paired differences log(x_j)-log (y_j) is 0. By a one-sample Student's *t*-test and the antilog transformation we obtain the 90% CI for the geometric mean ratio. For EPA-AUC($_{0–12}$) also the median of the ratios passes the related test with p-value < 0.004. For DHA, due to its irregular behaviour, we provide just a test for the median.

3. Results

3.1. In vitro release characteristics

The first pilot study (Study 1), using aqueous 0.1 N hydrochloric acid to simulate gastric conditions showed that the lysine salts dissociate into free fatty acids (primarily composed of EPA and DHA) and L-lysine. While the L-lysine dissolved completely in the aqueous solution, the majority of the free omega-3-fatty acids were identified in the upper oily/waxy phase. The same behaviour was observed for samples prepared solely from the corresponding free fatty acids and lysine references or 'synthetic' mixtures thereof. In all cases, an emulsion was initially formed by stirring, which then separated into two phases when stirring was stopped. These observations led to the conclusion the n-3 LC PUFA L-lysine salts dissociate in simulated gastric fluid and behave very similar to free fatty acids and L-lysine.

Results with the USP paddle method and fed state simulated small intestinal fluid (FeSSIF) at pH 5 confirmed that also under these conditions, the L-lysine salt completely dissociated into free fatty acids and L-lysine. The dissociation behaviour was shown to be consistent in triplicate runs, No new or unknown compounds were observed.

3.2. In vivo results

3.2.1. Participant characteristics

All 8 women completed the study and compliance to the intervention products was 100%.

At the start of the first study period, the baseline anthropometric characteristics for the participants (mean ±SD) were: age 23.4 ± 1.5 years; height 1.69 ± 7 cm; weight 62.0 ± 5.8 kg; BMI 21.7 ± 2.0 kg/m^2.

3.2.2. Tolerability and adverse effects

All volunteers involved successfully completed the study and both preparations were reported to be well tolerated by the subjects. No burping (as sometimes reported by users of n-3 LC PUFA preparations) was reported. Mild nausea was reported once, but its relation to the study product was unclear. On two occasions some transient venous irritation resulting from the in-dwelling cannula was noted. No serious adverse effects were reported.

3.2.3. Plasma TAG-derived EPA and DHA concentrations

The plasma TAG-derived concentrations for EPA and DHA, after a single dose of Lys-FFA versus EE in the fasted state are presented in Fig. 1 and Tables 1 and 2.

Fig. 1A displays the boxplots of the baseline-adjusted plasma EPA concentrations of the 8 participants for all the timepoints (48-hour blood collection window), both for the EE and the Lys-FFA treatment. In this side-by-side boxplot the black horizontal line denotes the median, the coloured region delimited by the lower and upper hinges denotes the inter-quantile range [25th to 75th percentiles]. The maxima and minima detected are at the top and bottom respectively; outliers are denoted as dots. At the T_{max} of 3 h for Lys-FFA, the median peak concentration of EPA for EE is 2.1 μg/ml and lies in the IQR interval [1.738, 3.558]. At the same time point, the median peak concentration of EPA for the Lys-FFA intervention is 42.56 μg/ml and lies in the IQR interval [20.96, 52.31].

Fig. 1B presents the analogous boxplots for the baseline-adjusted plasma DHA concentrations. Both figures show that the Lys-FFA concentrations are much higher than the respective EE comparators, with a peak after 3 h and a return to the baseline at approximately 12 h. Fig. 1C displays the time evolution plot of the mean values for EPA and DHA corresponding to the two products Lys-FFA and EE. Again, at T_{max} of 3 h, the mean concentration of Lys-FFA EPA is 37.17 μg/ml while the mean of EE EPA is 2.6 μg/ml.

Table 1 further summarises the main pharmacokinetic parameters considered in the statistical analysis: AUC$_{(0–12),}$ Area under the plasma concentration versus time curve from time 0 to 12 h post-dose; C_{max}, maximum measured plasma concentration, from time 0 to 12 h post-dose; T_{max}, time (hour) of maximum measured plasma concentration, from time 0 to 12 h post-dose.

For EPA, the median ratio between Lys-FFA-AUC($_{0–12}$) and EE-AUC ($_{0–12}$) is 5.45, while the median ratio between Lys-FFA-C_{max} and EE-C_{max} is 6.75. For DHA, the median ratio between Lys-FFA-AUC($_{0,12}$) and EE-AUC($_{0,12}$) is 2.92, while the median ratio between Lys-FFA-C_{max} and EE-C_{max} is 4.1. For EPA+DHA: the median ratio between Lys-FFA-AUC($_{0–12}$) and EE-AUC($_{0–12}$) is 5.04, while the median ratio between Lys-FFA-C_{max} and EE-C_{max} is 6.73. In terms of relative bioavailability (as measured by the geometric mean for AUC($_{0–12}$)), Lys-FFA resulted in approximately 5.73 times the value for total EPA compared with EE (Table 2). The magnitude of the spread can be better appreciated by considering the 90% confidence interval. All values of Tables 1 and 2 are referred to the original measurements. By applying a proper dose correction, the values increase: namely for AUC: the value of the median of ratios becomes 5.49 for EPA and 3.65 for DHA. Moreover the geometric mean ratio for EPA-AUC($_{0–12}$) becomes 5.78 (with CI: 3.93–8.5).

4. Discussion

To the best of our knowledge, this is the first published study investigating the oral absorption characteristics of EPA and DHA in humans administered in the form of their mixed L-lysine salts. Results show that both n-3 LC PUFAs are rapidly and well absorbed from this new formulation when taken on an empty stomach. Administration of the Lys-FFA resulted in significantly higher EPA and DHA plasma levels than those from the EE comparator, particularly within the first 12 h post-dose. The sharp peak in plasma EPA and DHA triacylglyceride (TAG) levels occurring 3 h after ingestion of the products, and the results obtained with the in vitro dissolution tests both underline our

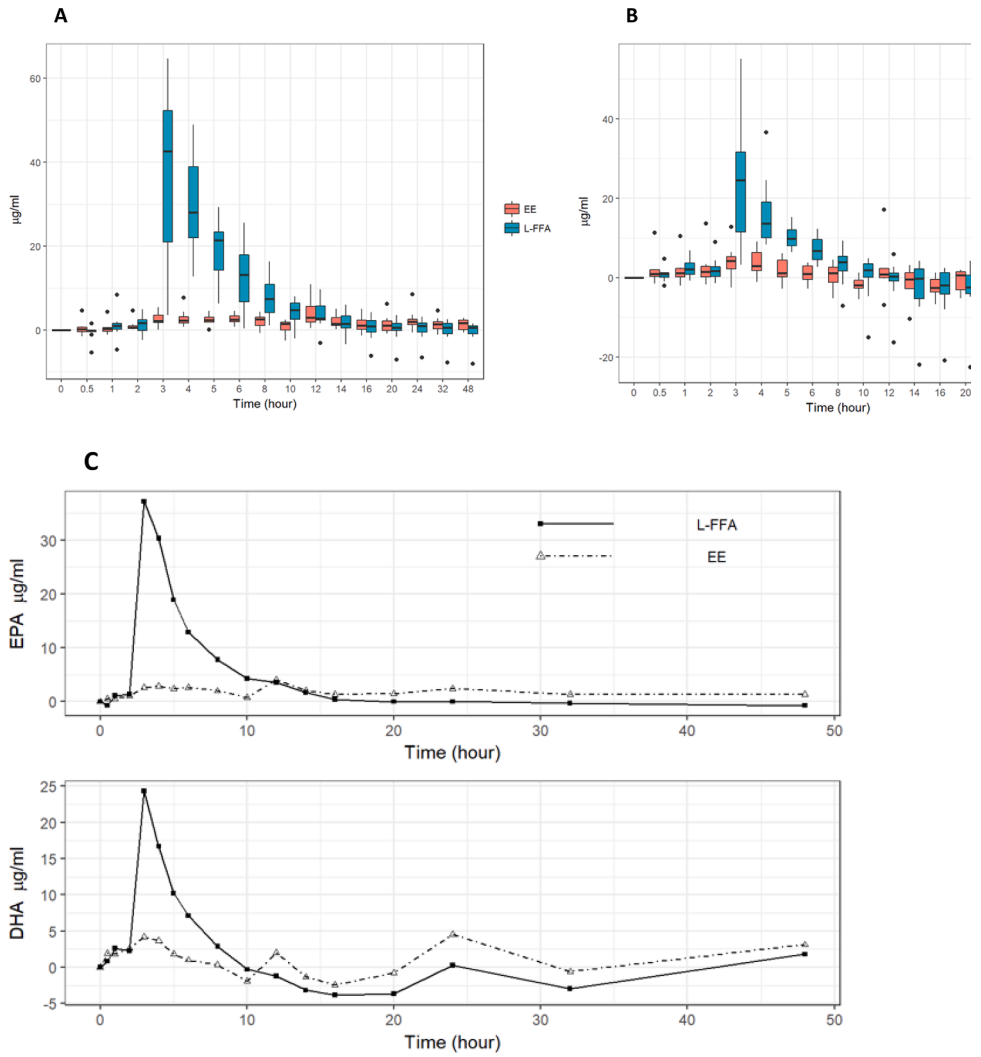

Fig. 1. Baseline-adjusted EPA and DHA concentration in the plasma TAG fraction versus the 48-hour time curve obtained after the administration of the single bolus dose of Lys-FFA versus the Ethyl Ester (EE) comparator ($n = 8$) - **A**: EPA (Median (IQR)); **B**: DHA (Median (IQR)); **C**: Arithmetic Mean EPA and DHA. Baseline concentrations for DHA and EPA were 10.44 ± 3.12 µg/ml and 4.56 ± 0.56 µg/ml, respectively.

hypothesized mechanisms of release and absorption of free fatty acids from the first parts of the small intestine.

Salt formation, including by using amino acids, is a common and effective method of increasing solubility and dissolution rates of acidic and basic drugs [17]. Although the idea of preparing salts of n-3 LC PUFA with basic amino acids like arginine or lysine is not entirely new [18], this principle had not resulted in a marketed preparation so far. Long chain fatty acids like EPA and DHA are known to be well absorbed from the GI tract in their free form [9,10,15,19]. Independent of their original molecular form, they will be incorporated into micelles and taken up by enterocytes via diffusion or transport mechanisms involving CD36/ FABP or FATP4 [20-22]. There, fatty acids are again incorporated into TAGs. These TAGs are packaged with cholesterol, lipoproteins and other lipids into chylomicrons, which are transported through the lymphatic system and delivered into the blood circulation via the thoracic duct. In the circulation and the rest of the body, fatty acids are bound to- and incorporated in different pools in a time-dependant manner; as free fatty acids (non-covalently bound to albumin), as TAGs and cholesteryl-esters in circulating triglyceride-rich lipoproteins, chylomicrons, very low-density lipoproteins, in erythrocytes in adipose tissue etc. [23-28]. In the present study, the plasma TAG fraction was used to monitor EPA and DHA absorption. Previous studies, including those with isotope labelling have confirmed that most of the newly absorbed DHA and EPA will be incorporated in this fraction during the first 24 h following absorption [29]. Enzymatic hydrolysis of fatty acid esters by pancreatic lipases prior to fatty acid absorption represents a potentially rate-limiting process. It has been demonstrated that the triglyceride forms of EPA and DHA are more resistant to pancreatic lipase hydrolysis compared with other polyunsaturated fatty acids [15,30,31]. Moreover, their semi-synthetic ethyl-esters appear up to 50 times more resistant to pancreatic lipases compared to the triglyceride forms [10, 30, 32]. Previous studies have shown that administration of DHA and (or) EPA as free fatty acids leads to a several fold higher plasma peak and bio-availability during the first 12 −24 h following administration compared to that after administration in the form of EEs or TAGs [8,19, 33]. This was confirmed in the present study. Although the present design does not allow determination of the absolute bioavailability, i.e. relative to that after i.v administration, the relative bio-availability during the first 12 h following administration compared to that of the comparator preparation was on average 5.7 times higher for EPA. Notwithstanding the kinetic advantages of EPA and DHA in the form of free fatty acids, their practical use is hampered by unfavourable organoleptic properties and issues with stability. There is one formulation on the market (Epanova®), for medical prescription that contains EPA and DHA as free carboxylic acids in a coated formulation. Interestingly, this product was found to produce a plasma profile very similar

to that of our study, despite a somewhat delayed peak likely due to the controlled-release formulation [8]. Compared to the EE product used as comparator in that study, oral bioavailability of the free fatty acid product was also higher, again quite similar to our results. This confirms our hypothesis that free fatty acids can be absorbed easily and rapidly from the upper duodenum, before ester hydrolysis normally takes place.

In the present study, the Lys-FFA was given in gelatine capsules and did not produce any organoleptic side-effects, including burping or an unpleasant after-taste. Apparently, and in line with our in vitro findings, free fatty acid release occurs rather gradually on an empty stomach. Therefore, next to its interesting absorption characteristics, the solid formulation shows favourable stability, formulation and technical properties [34]. Together with its plasma-time profile, this offers opportunities to develop novel dosing forms and possibly also allows lower dosing and hence a reduced demand for natural resources. This is particularly pertinent as the Lys-FFA salt comes in the form of a compressible powder, thus allowing a higher loading of EPA and DHA (45%+) than typically found in solid omega 3 dietary supplementation. Creating a powdered form of EPA and DHA also opens opportunities to combine with additional vitamins, minerals or other ingredients.

A power calculation was completed for the trial; however, it was found that whilst the EPA concentration curve was relatively uniform within the study participants, there was greater variability for the DHA time curve. Whilst there was a clear return to baseline for both EPA and DHA at 12 h post-dose for the group (thus justifying the use of calculating AUC as 0–12 h), as can be seen visually in Fig. 1B&C, the DHA concentrations continued to flux to a small degree over the remaining 36 h. Due to the nature of the methodology of measuring the fatty acid concentration within the circulating TAG (i.e. it appears to capture a snapshot of the post-prandial and post-absorptive phase) and the lack of any further EPA or DHA consumed post-administration of the investigational products, it is conceivable that the fluctuations within the DHA time curve after 12 h are no longer representative of the absorption of the Lys-FFA or EE, but instead are indicating a separate biological process involving DHA.

Carry-over effects were not deemed to be an issue in this study, due to the large wash-out period (two weeks) relative to the size of the single bolus dose of n-3 LC PUFAs that the participants received.

Future research with Lys-FFA should include further studies on the relationships between single or multiple dosing and patterns of different plasma-lipids, including the incorporation of EPA and DHA in erythrocytes, possible adipose tissue and effects on n-3/n-6 ratio's. Within the body, EPA and DHA are incorporated into different pools, including plasma TAGs, phospholipids and cholesteryl esters, and gradually in platelets, erythrocytes and other cells, in particular those in fat-, immune- and neuronal tissues [3, 5, 29, 35]. This occurs in a time-dependant manner which makes it of further interest to perform studies involving different dosing schedules and studying the effects of the relative fatty acid composition in these compartments. Given the fact that Lys-FFA is a solid, new formulations can be developed, which need to be evaluated as well and compared with existing conventional food supplements like those based on fish oil, krill oil or EE. A further consideration is that absorption of the EPA and DHA mainly takes place from the small intestine, in particular the duodenum, and so gastric emptying is likely to become rate-limiting under certain conditions, for example when consumed with a high fat meal. The present study was carried out in a group of young females, which limits to some extent its extrapolability to other populations. This also includes sex-dependency, as differences in gastric emptying (also to some extent depending on the menstrual cycle), peristalsis and absorption processes as well as plasma transport and deposition may show sex-differences. Another interesting question is whether certain drugs like proton-pump inhibitors will interfere, as these medicines cause a considerable and sustained increase of the stomach pH.

5. Conclusion

Results of the first in man study demonstrate that administration of EPA and DHA in the form of its lysine salt provides a novel and effective way to increase plasma levels of these n-3 LC-PUFAs. The stability and technical properties of Lys-FFA provide additional advantages allowing the development of novel product formulations possibly also necessitating lower doses.

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Evonik Operations GmbH provided financial support for the practical conduct of the study; as such, Evonik took no part in the study design and data collection. Data analysis and interpretation, and latterly the manuscript preparation was jointly conducted by both partners. The final draft and decision to submit was approved by all authors.

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Declaration of Competing Interest

LH, RB, and MS declare competing interests as employees of Evonik Operations GmbH. No conflicts of interest to declare for the remaining authors.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2020.102232.

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