final report

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DPA pilot

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Study 1 - the Acute Study:

Main Objective: To investigate the partitioning of DPA into chylomicron lipids in the post-prandial phase of fat absorption (first 5 hours) using lipidomic analysis of all chylomicron lipids following a single dose of DPA after an overnight fast.

Secondary Objective: To investigate the metabolism of DPA to EPA and to DHA in the post-prandial phase using fatty acid analysis of the chylomicron lipids and plasma lipids by gas chromatography

Design: To use EPA and oleic acid as control and placebo fats, respectively, in this acute study

This phase of the project has been completed and a draft manuscript has been prepared in anticipation of submission within the next 4 months to the European Journal of Nutrition (July 2012).

A copy of the ***draft manuscript*** is attached in

Appendix 1. A **summary** of this study follows:

1. The group fed DPA over the 5 hours showed a significantly reduced chylomicronemia compared with the EPA and placebo groups.
2. The group fed DPA over the 5 hours showed a significantly reduced plasma triglyceride levels compared with the EPA and placebo groups.
3. Both EPA and DPA were incorporated into the chylomicron triglyceride fraction, based on the analysis of the triglyceride fatty acid composition (gas chromatography analysis conducted in Finland). At all time points, approximately twice as much EPA was incorporated into these triglycerides as for DPA. This is consistent with the data in point 1.
4. There was no evidence of EPA and DPA being incorporated into the chylomicron phospholipid fatty acids (gas chromatography analysis conducted in Finland),
5. Lipidomic analysis of the chylomicron triglycerides revealed the identity of the molecular species of TAG into which the EPA or DPA were incorporated (analyses conducted by Dr Kaisa Linderborg at the Baker Institute).
6. The main species that EPA and DPA were incorporated into were triglycerides with the following molecular structures, respectively:

EPA:18:1:18:1 and DPA:18:1:18:1. This is consistent with the fact that the DPA and EPA were mixed into olive oil which is rich in 18:1.

7. There does not appear to be any evidence in this acute study for either DPA or EPA being metabolised to DHA (over the 5 hour period).

The **key message** here is that DPA is not 'absorbed and incorporated' into chylomicrons **as efficiently** as EPA. The reasons for this reduced efficiency are not known at this stage.

Once absorbed, both omega 3 PUFA are incorporated into the TAGs of the chylomicrons and deposited in tissues such as liver.

There is no evidence of metabolism of DPA or EPA to DHA in this 5 hour acute study.

Study 2 - the Chronic Study:

Main Objective: To investigate the partitioning of DPA into plasma lipid species at day 4 and day 7 following daily doses of DPA, using lipidomic analysis of all plasma lipids and fatty acids analysis of the plasma by gas chromatography

Secondary Objective: To investigate the metabolism of DPA to EPA and to DHA at day 4 and day 7 using fatty acid analysis of the plasma lipids by gas chromatography

Design: To use EPA and oleic acid as control and placebo fats, respectively in this chronic study

This phase of the project has been completed and a manuscript has been submitted to the European Journal of Nutrition (March 2012).

A copy of the manuscript is attached in **Appendix 2**.

A **summary** of this study follows:

Purpose Despite the detailed knowledge of the absorption and incorporation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into plasma lipids and red blood cells (RBC) in humans, very little is known about docosapentaenoic acid (DPA, 22:5 n-3). The aim of this study was to investigate the uptake and incorporation of pure DPA and EPA into human plasma lipid RBC lipids.

Methods Ten female participants received 8 g of pure DPA or pure EPA in

randomised crossover double blinded manner over a 7-day period. The placebo treatment was olive oil. Blood samples were collected at days zero, four and seven, following which the plasma and RBC were separated and used for the analysis of fatty acids.

Results Supplementation with DPA significantly increased proportions of DPA in the plasma phospholipids (PL) (by 2-fold) and triacylglycerol (TAG) fractions (by 2.3-fold, day 4). DPA supplementation also significantly increased proportions of EPA in TAG (by 3.1-fold, day 4) and cholesterol ester (CE) fractions (by 2.0-fold, day 7) and of DHA in TAG fraction (by 3.1-fold, day 4). DPA proportions in RBC PL did not change following supplementation. Supplementation with EPA significantly increased the proportion of EPA in the plasma CE and PL fractions, (both by 2.7-fold, day 4 and day 7) and in the RBC PL (by 1.9-fold, day 4 and day 7). EPA supplementation did not alter the proportions of DPA or DHA in any lipid fraction. These results showed that within day 4 of supplementation DPA and EPA were both incorporated into plasma and RBC lipids, but each FA demonstrated different and specific incorporation patterns.

Conclusions The results of this short-term study suggest that DPA may act as a reservoir the major long chain n-3 fatty acids (LC n-3 PUFA) in humans.

Overall outcomes of the project

The investigators are very pleased with the overall outcomes of the project. We are delighted that we have been able to marshall resources from around the world (Finland and Malaysia) and within Victoria (Deakin University, Burwood, Waurn Ponds & Warrnambool; and The Baker Institute in Melbourne).

The main conclusions are:

Somewhat surprisingly, DPA (omega 3 DPA) and EPA are not handled in an identical manner in humans.

There was a reduced efficiency of DPA absorption compared with EPA.

DPA was apparently metabolised to both EPA and DHA in the Chronic Study. In contrast, EPA was NOT metabolised to DPA or DHA.

DPA may be a reservoir of EPA and or DHA in the body.

APPENDIX-1

Draft Manuscript

Study 1 – Acute Study

Postprandial metabolism of docosapentaenoic acid (DPA, 22:5n-3) and eicosapentaenoic acid (EPA, 20:5n-3) in humans

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Kew words

n-3 polyunsaturated fatty acids (PUFA), docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), chylomicron, human

Abstract

Study of the molecular level metabolism of DPA in humans has thus far been limited by the fact that docosapentaenoic acid (DPA, 22:5n-3) is found side by side with eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) in natural products. In this cross-over study, pure DPA and EPA were incorporated in meals served to healthy female volunteers. Special interest was laid in the subsequent metabolism of the n-3 PUFA in chylomicron triaclyglycerol (TAG) and phospholipids (PL). The meals served contained 18g of olive oil together with 2g of EPA or of DPA. The control meal contained 18g of olive oil together with an additional 2g of olive oil. Plasma lipemia as well as chylomicronemia were significantly reduced after the breakfast containing DPA compared to the breakfasts containing EPA or olive oil only. This indicates that the DPA hindered the digestion or ingestion of also the olive oil

present in the meal or that the lipids were transported in plasma as free fatty acids after the DPA meal. Both gas chromatography and tandem mass spectrometry were used to study the fate of the lipids. (incomplete).

Introduction

The essential fatty acid alpha-linoleic acid (ALA, 18:3n-3) can be metabolized *in vivo* by elongation and desaturation enzymes to form a series of polyunsaturated fatty acids (PUFA) of the n-3 series. In addition to potentially being metabolized from ALA, eicosapentaenoic acid (EPA, 20:5n-3), docoxahexaenoic acid (DHA, 22:6n-3) and docosapentaenoic acid (DPA, 22:5n-3) are provided from diet, mainly from fish and fish oil products. Currently a large amount of information exists on the metabolism of both EPA and DHA, while less is known about the metabolism of their intermediate product DPA. *In vivo*, DPA is formed by chain elongation of EPA by the action of fatty acid elongases 2 and 5, while the conversion of DPA to DHA requires an elongation to 24:5n-3 and desaturation to 24:6n-3 before oxidation to yield DHA. As recently reviewed, ALA supplementation generally leads to an increase in plasma EPA and DPA, but has little or no effect on DHA levels (Brenna et al 2009).

To date, research of DPA metabolism in humans has been limited by the availability of pure DPA for human trials. The supplements used in the study of Meyer et al (2009) contained a significant amount of DPA, but together with EPA and DHA. In rats, short term DPA supplementation significantly increased the concentration of DHA in liver and the concentration of EPA in the liver, heart and skeletal muscle, presumably by the process of retroconversion (Kaur et al 2010; Holub's article in Lipids 2011 will be also be discussed).

The aim of this cross over study was to study and compare the postprandial metabolism of DPA and EPA in humans after a meal containing purified EPA-triacylglycerols or purified DPA-triacylglycerols added to high-oleic acid olive oil. Special interest was laid in metabolism of the n-3 PUFA in chylomicron triacylglycerols and phospholipids.

Materials and Methods

Study design

Ten healthy normal weight females between the age 20 to 30 of took part in the randomized cross over study with three different breakfast meals. The subjects had a BMI between 20 to 25 kg/m^2 and their habitual consumption of omega-3 PUFA per day was not more than 500mg (per day) as assessed from a PUFA food frequency questionnaire. Subjects with a high risk of any form of CVD based on self reported medical status and family history were excluded from the study. All subjects provided written informed consent. Ethics approval was obtained from the Deakin University Human Research Ethics Committee (EC2011-023).

The study breakfast consisted of 180 grams of instant mashed potato (Continental Deb™) mixed with 70 ml boiled water and 20 grams of oil. In each of the tree meals 18 grams of lipids consisted of olive oil (La Espanola Pure Olive Oil, Seville, Spain). Additionally, the DPA breakfast included 2 g of DPA TAGs (Equateq Ltd, Breasclete, Callanish, Scotland), the EPA breakfast 2 g of EPA TAGs (Equateq Ltd, Breasclete, Callanish, Scotland) and the control (olive oil) meal an additional 2 g of olive oil. The subjects could use salt, pepper or chicken flavoured salt with them meal and were provided with water throughout the study ad libitum.

Subjects attended the clinic after a 10 hour fast, and consumed the study meal within 15 minutes.

During the three week period of the cross-over trial subjects were requested to refrain from consuming high long chain omega-3 PUFA products including fish, red meat and omega-3 fortified products (<2 marine and/or 2 red meat meals/week and <2 omega-3 fortified products/week), in order to prevent false increases in plasma circulating long chain omega-3 PUFA.

Isolation of plasma, chylomicrons and chylomicron lipids

Venous blood was drawn at the fasting state and hourly for one to five hours postprandially. EDTA blood samples were immediately centrifuged for fifteen minutes at 591 x g for 15 min to remove the plasma.

A chylomicron-rich fraction (Svedberg flotation unit (Sf) > 400) was isolated from plasma by ultracentrifugation using a Beckman ultra centrifuge and TLA 100.4 rotor (Beckman instruments, Palo Alto, CA) as previously described (Ågren et al). Briefly 1.8 ml of EDTA plasma was overlaid with saline solution (density = 1.006 kg/l) in ultracentrifuge tubes and centrifuged at 35,000 x g for 30 min at 23°C. The top 1 millimetre was aspirated to remove the chylomicron-rich fraction. All samples were frozen at -80°C prior analysis.

Fatty acid analysis

For fatty acid anlaysis, an internal standard mixture of triheptadecanoin (Sigma-Aldrich, St.Louis, MO, USA), dinonadecanoylphosphatidylcholine (Sigma-Aldrich, St.Louis, MO, USA) and cholesterylpentadecanoate (Nu-Chek Prep. Inc., Elysian, MN, USA) was added to the isolated chylomicrons. 1.5 ml methanol, 3 ml chloroform and 0.8 ml 0.88 % KCl in water were added and the blend was troughly vortexed after each addition. Vials are centrifuged 2000 x g for 3 minutes to separate the layers, and the chloroform rich layer was removed and evaporated to dryness (Folch et al. 1957). Triacylglycerols, phospholipids and cholesteryl esters were isolated from the extracted lipid mixture with solid phase extraction based on silica columns (Hamilton and Comai, 1988).

Fatty acid methyl esters (FAME) were prepared with sodium methoxide method. In short, triacylglycerols were suspended to 1 ml dry diethylether. 25 µl methylacetate and 25 µl sodium methoxide were added and the blend was incubated for 5 minutes while shaken at times. The reaction is stopped with 6 µl acetic acid. Vials were centrifuged 2000 x g for 5 minutes, after which the supernatant was removed and gently evaporated to dryness. The resulting fatty acids were transferred to 100 µl inserts in hexane (Christie 1982). The FAME were analysed with gas chromatography (Shimadzu GC-2010 equipped with AOC-20i auto injector, flame ionization detector (Shimadzu corporation, Kyoto, Japan) and wall coated open tubular column DB-23 (60 m x 0.25 mm i.d., liquid film 0.25 µm, Agilent technologies, J.W. Scientific, Santa

Clara, CA, USA). Splitless/split injection was used and the split was opened after 1 min. Supelco 37 Component FAME Mix (Supelco, St. Louis, MO, USA), 68D (Nu-Check-Prep, Elysian, MN, USA) and GLC-490 (Nu-Check-Prep, Elysian, MN, USA) were used as external standards.

Analysis of triacylglycerol species

Extraction method of lipids will be added.

TAGs analysis of the one, three and five hour samples was performed by liquid chromatography, electrospray ionisation-tandem mass spectrometry using an Applied Biosystems 4000 QTRAP mass spectrometer running Analyst 1.5 software. Liquid chromatography was performed on a Zorbax C18, 1.8 µm, 50 x 2.1 mm column (Agilent technologies, Santa Clara CA, USA). Samples were resuspended in 50 µl saturated butanol and 50µl MeOH containing 10mM ammonium formate (NH_4COOH). The mobile phase was tetrahydrofuran:methanol:water in a 30:20:50 ratio (A) and 75:20:5 (B) both containing 10 mM NH_4COOH. TAG were separated with an isocratic flow (100 mL/min) of 85% mobile phase B. Quantification of individual TAG species was performed using scheduled multiple-reaction monitoring (MRM) in positive ion mode (Murphy et al 2007). Lipid concentrations were calculated by relating the peak area of each species to the peak area of the internal standard (using Multiquant 1.2 software). Total TAG was calculated by summing the individual species. This analysis resulted in TAG FA combinations where the location of the FA in different sn position was not determined. As there were no standards available for each TAG species, no adjustment was made for different response factors and the relative proportions of different species should be taken as semi-quantitative.

TAGs that were likely to contain arachidonic acid, EPA, DPA or DHA were selected for further neutral loss experiments. Each molecular species selected where screened for the neutral loss of 16:0, 16:1, 18:1, 18:2, 18:3, 20:4, 20:5, 22:5 and 22:6. Most likely TAG FA combinations were calculated from the results.

Statistical analysis

Normal distribution of the data was tested with the Shapiro-Wilk test. Paired samples t-test or Wilcoxon matched-pairs signed ranks test, depending on the normality of the

data, was used to compare the measured responses to control. ANOVA for repeated measurements (GLM) was used to compare the timepoints within treatments. Paired samples t/test or Wilcoxon matched-pairs signed ranks test with Bonferroni correction was used for post hoc comparisons. Statistical significance was indicated by $P < 0.05$. Statistical analyses were performed with SPSS 18.0 software (SPSS Inc, Chicago, IL, USA).

Results

Plasma TAGs remained at almost an fasting level after the DPA breakfast indicating that the oil was not ingested efficiently. The chylomicronemia after the DPA breakfast was very modest. Figure 1 (Statistical tests need to be done for areas under the curves).

The fatty acid composition of chylomicron triacylglyserols is presented in figure 2. At all of the time points EPA was significantly higher in the chylomicron TAGs after the breakfast containing EPA than after the breakfasts containing olive oil only or DPA. There was much more between subject deviation in the proportion of DPA in chylomicron TAGs after the DPA breakfasts and even though DPA was higher after the DPA breakfast compared to the olive oil or EPA breakfasts at all time points, only the time points of 2h, 4h and 5h were significant. DPA did not significantly raise the proportion of EPA in chylomicron TAGs. At 5h, there was more DPA after the EPA breakfast than after the olive oil breakfast. DHA was significanlty increased after the DPA breakfast compared to the olive oil breakfast at 2h and 3h.

The fatty acid composition of chylomicron phospholipids is presented in figure 3. Lipids of the meal affected chylomicron phospholipids especially at 2h, where the proportion of EPA was increased after the EPA breakfast compared to the two other breakfasts. At 2h, the EPA breakfast also increased the amount DPA in phospoholipids compared to the olive oil breakfast.

The chylomicron TAGs (1, 3 and 5hours) detected by HPLC-MS/MS are presented in figure 4 as molar percentages. Significant differences between breakfasts were detected in the TAGs potentially containing PUFA. Significant (statistical analysis yet

to be done) differences were detected between breakfasts. Major species that contained EPA after the EPA breakfast included 20:5/18:1/16:0 and 20:5/18:1/18:1. DPA was spread to a larger variety of molecules and its overall presence was lower as seen also from the TAG concentration and fatty acid composition data. The major TAGs containing PUFA after the DPA breakfast were 20:5/18:1/18:0 which is an indication of retroconversion (or an analysis mistake – could be a mix up with 20:4/18:1/18:1, maybe not because there are an equal amount of it after all three breakfasts. Needs to be checked from the neutral loss experiments or from spectra), 22:5/18:1/18:1, 22:5/16:0/18:2 and 22:5/16:0/18:1 (TAGs with two 20 carbon FA were not looked for).

TAGs potentially containing long chain PUFA were further selected for intensive neutral loss experiments which revealed the most prevalent FA combinations in each molecular weight species. This data is presented in figures 6,7 and 8 as molar proportions of the TAGs shown. It can be seen from these pictures that while 18:2/18:2/18:1, 18:3/18:1/18:1, 20:4/18:1/16:0 and 20:4/18:1/18:1 were the most prevalent potentially PUFA containing TAGs after the olive oil breakfast, 20:5/18:1/18:1 and 20:5/18:1/16:0 and 20:5/18:2/18:1 were abundant TAGs in addition to 18:2/18:2/18:1 after the EPA breakfast. After the DPA breakfast the most prevalent respective combinations were 22:5/18:1/18:1, 22:5/18:1/16:0 in addition to the 18:2/18:2/18:1 which was actually the most prevalent TAG in this group indicating of inferior corporation of DPA to chylomicron TAGs compared to EPA (or also the fact that there was less DPA in the meal).

The neutral loss experiments also revealed that there were most counts for the loss of LC PUFA after the EPA breakfast indicating of less LC PUFA being transported in the chylomicrons after the DPA breakfast compared to the EPA breakfast.

HPLC-MS/MS analysis revealed that phosphatidyl cholines were abundant phospolipid species in chylomicrons followed by inositols and ethanolamines. Phosphatidyl serines were not abundant enough to be quantified. There were no between breakfast differences in individual phospholipids between the breakfasts. No differences were found in chylomicron cholesteryl esters species between breakfasts either (data not

shown). Three charts of phospholipids and one of cholesteryl esters are ready if needs to be added.

Discussion

(Discussion is still just random thoughts - incomplete at this stage).

The current knowledge on the metabolism of DPA in humans is very limited due to the difficulties in the availability of adequate amounts of pure DPA for human studies. Previous studies demonstrated a significant elevation in the level of DPA in the circulating lipid fractions when human subjects have received seal oil (Conquer et al 1999, Myer et al 2009) as well as a significant rise in DPA concentrations in tissue lipids when animals have received seal oil (Murphy et al 1997). However, such effects cannot be directly attributed to the consumption of DPA since it represents approximately 5% of the fatty acids in seal oil with a higher level of EPA which has a capacity to generate considerable amounts of DPA via chain elongation.

In population studies a higher level of DPA in the circulation has been associated with a lower risk of coronary heart disease (Simon et al 1995), and a higher intake of DPA and a higher level of DPA in the circulation have been associated with the protection against carotid atherosclerosis (Hino et al 2004). DPA has also shown the ability to inhibit human platelet aggregation in vitro and to suppress thrombozane formation (Cheryk et al 1999).

Studies on purified DPA are scarce. Kaur et al (2010) evaluated the effects of purified dietary DPA on the fatty acid composition of liver, adipose, heart, skeletal muscle and brain in rats. The further study of Holub et al (2011) measured changes in plasma and tissue lipid levels and fatty acid compositions resulting from DPA ingestion including an extensive analysis of the resulting fatty acid alterations in the liver, heart, and the kidney and individual phospholipid types. In the circulation, serum phospholipids were the single most predominant reservoir of DPA whereas the triacylglycerol fraction showed a moderately greater enrichment in DPA compared to phospholipids. The current knowledge available on the metabolism and the biological effects of DPA has been recently reviewed (Kaur et al 2011).

Much of our current understanding of the role of TAGs comes from measurement either of the total quantity of TAG present or of fatty acid that can be released from TAGs after isolation of TAGs and saponification rather than from knowledge of changes in unique TAG molecular species.

As increased postprandial plasma triacylglycerol (TAG) level a risk factor for cardiovascular diseases (Sanders 2003, O'Keefe and Bell 2007), and most of the day in spent in the postprandial state, it is important to characterize how different fatty acids behave in the postprandial state and influence the magnitude of postprandial lipemia. However, the effects of DPA on plasma lipids should also be studied in a long term human trial.

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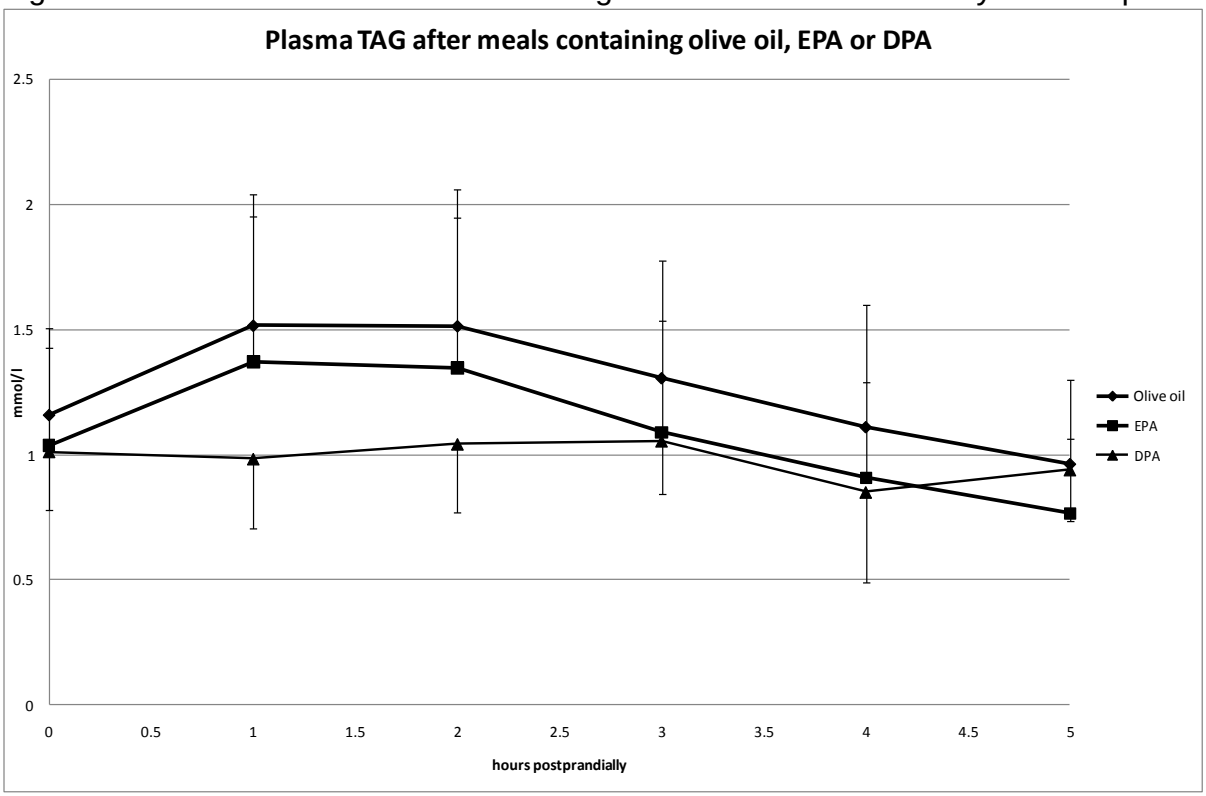
Figure 1. These need to be made matching. Error bars needed for chylomicron picture.



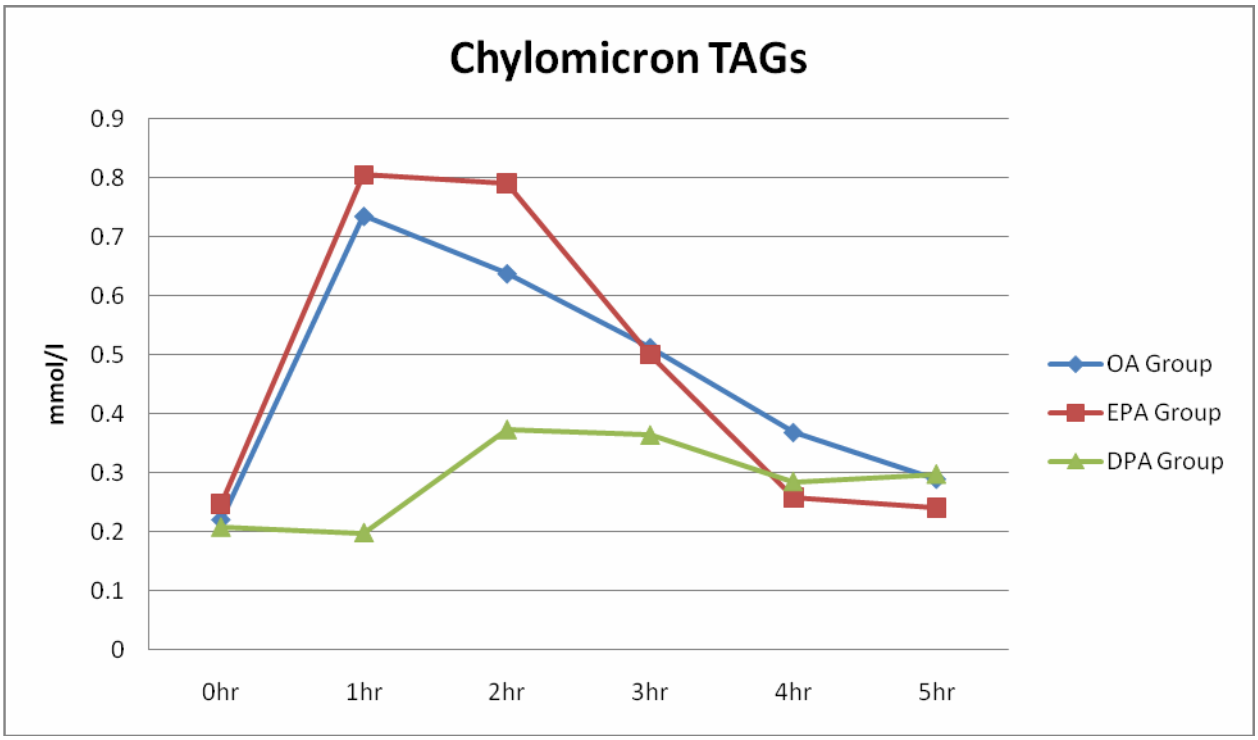
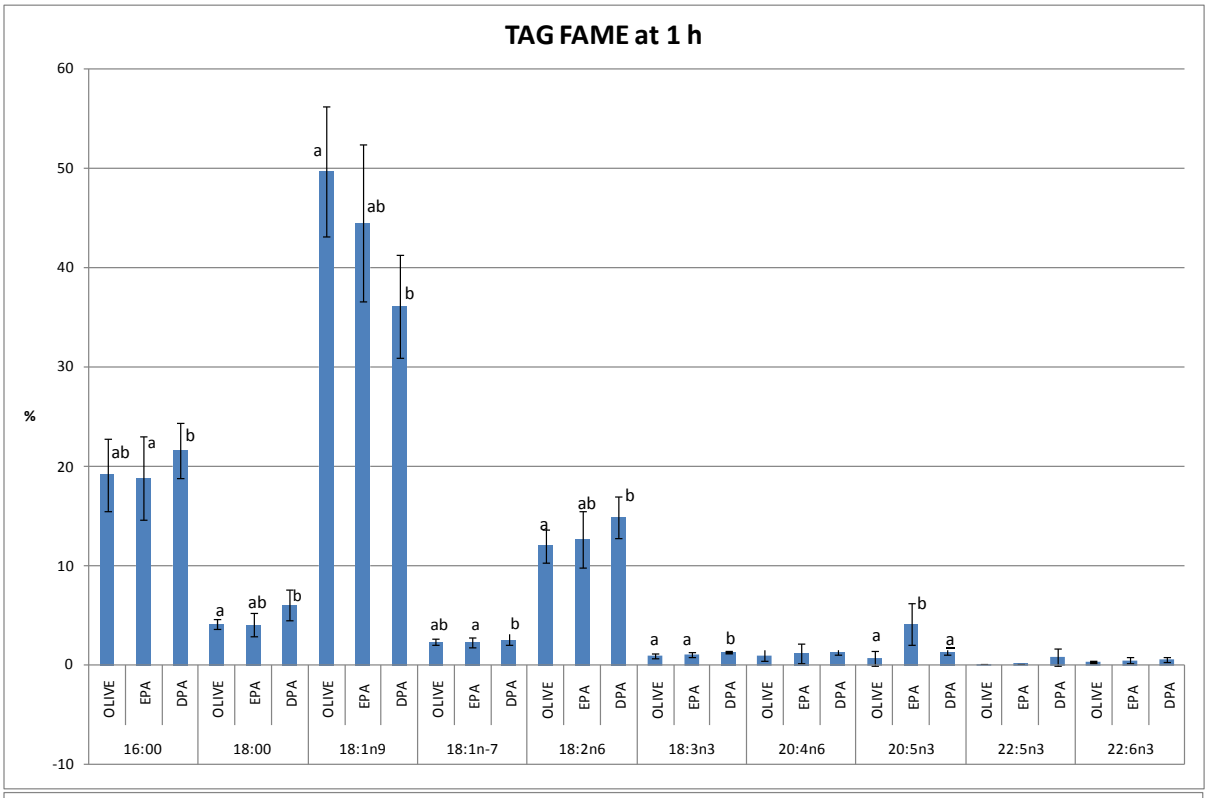
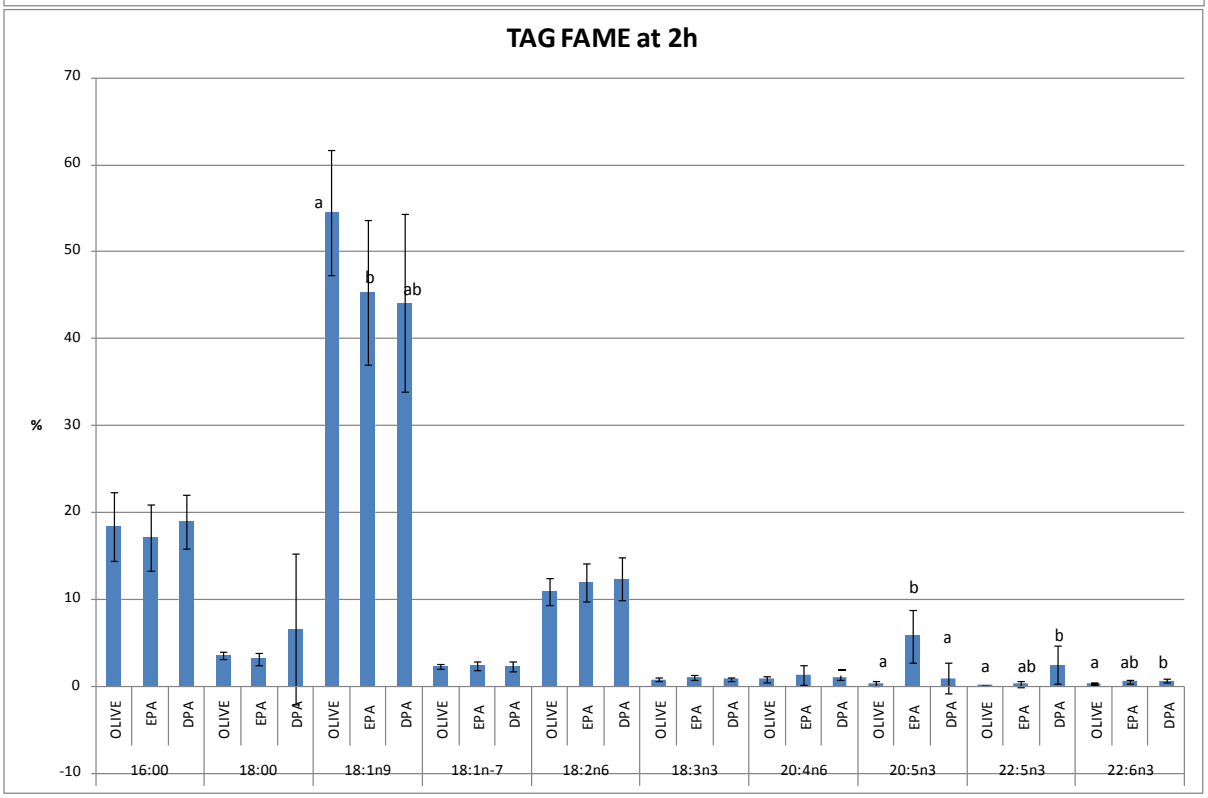
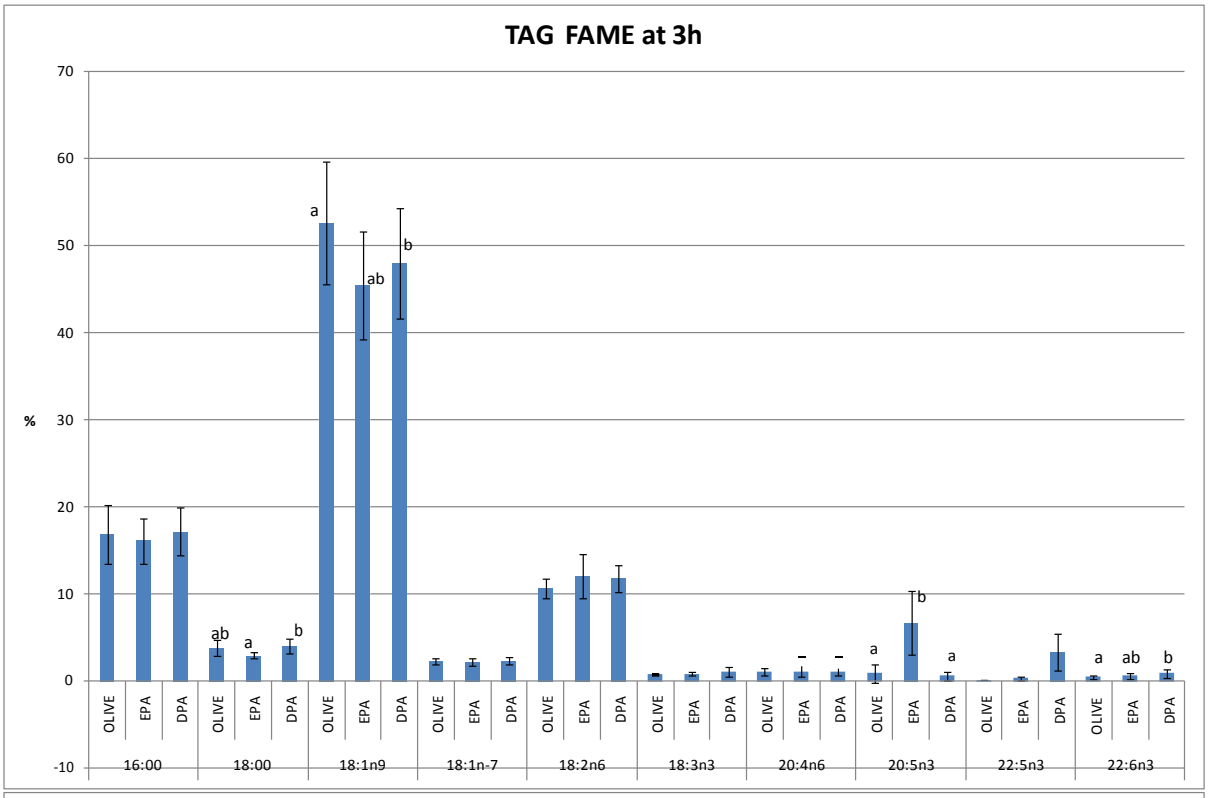


Figure 2. Chylomicron TAG FA composition at one to five hours postprandially. The five figures below will be combined into a single figure if significances can still be shown. Some error in 18:3n-3 => will be corrected. Y axis needs to start from 0.


TAG FAME at 1 h
%
60
50
40
30
20
10
0
-10
OLIVE
EPA
DPA
16:00
18:00
18:1n9
18:1n-7
18:2n6
18:3n3
20:4n6
20:5n3
22:5n3
22:6n3


TAG FAME at 2h
%
70
60
50
40
30
20
10
0
-10
OLIVE
EPA
DPA
16:00
18:00
18:1n9
18:1n-7
18:2n6
18:3n3
20:4n6
20:5n3
22:5n3
22:6n3


TAG FAME at 3h
%
70
60
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30
20
10
0
-10
a
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ab
b
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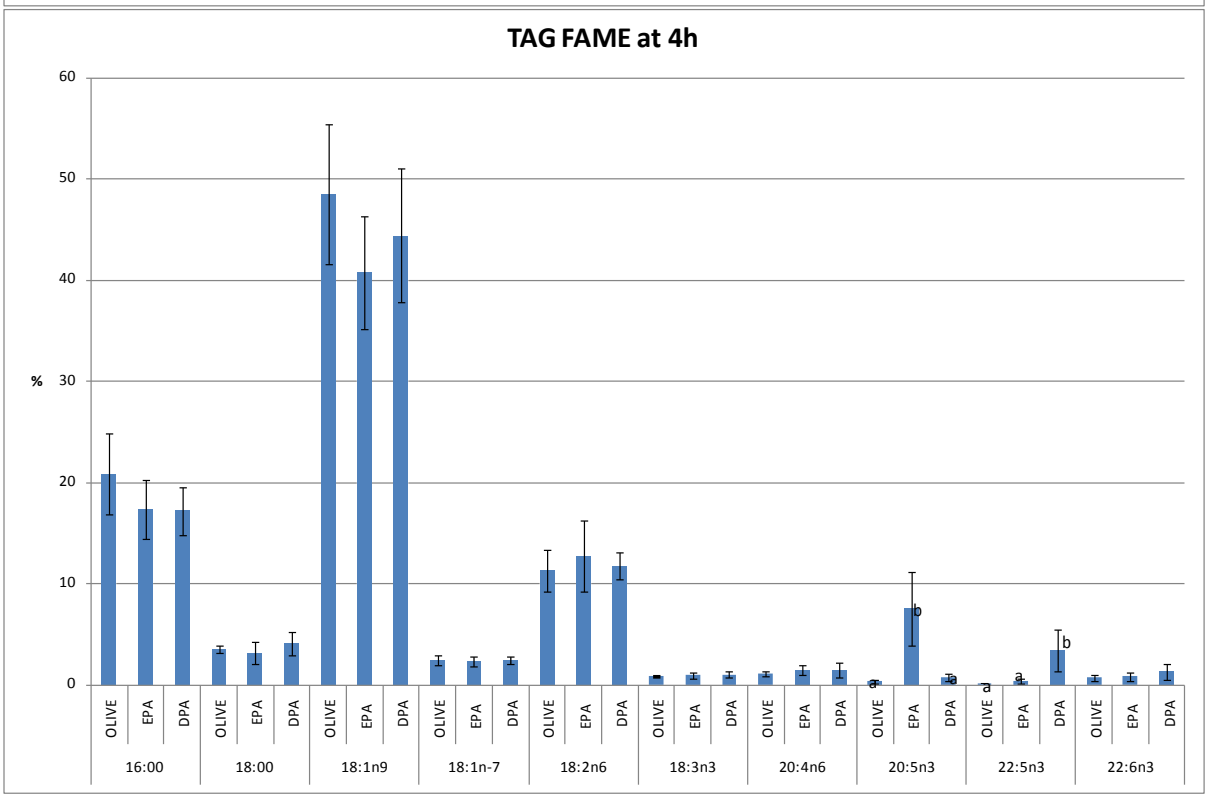
a
a
a
ab
b
OLIVE
EPA
DPA
16:00
18:00
18:1n9
18:1n-7
18:2n6
18:3n3
20:4n6
20:5n3
22:5n3
22:6n3


TAG FAME at 4h
%
60
50
40
30
20
10
0
a
b
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a
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b
OLIVE
EPA
DPA
16:00
18:00
18:1n9
18:1n-7
18:2n6
18:3n3
20:4n6
20:5n3
22:5n3
22:6n3

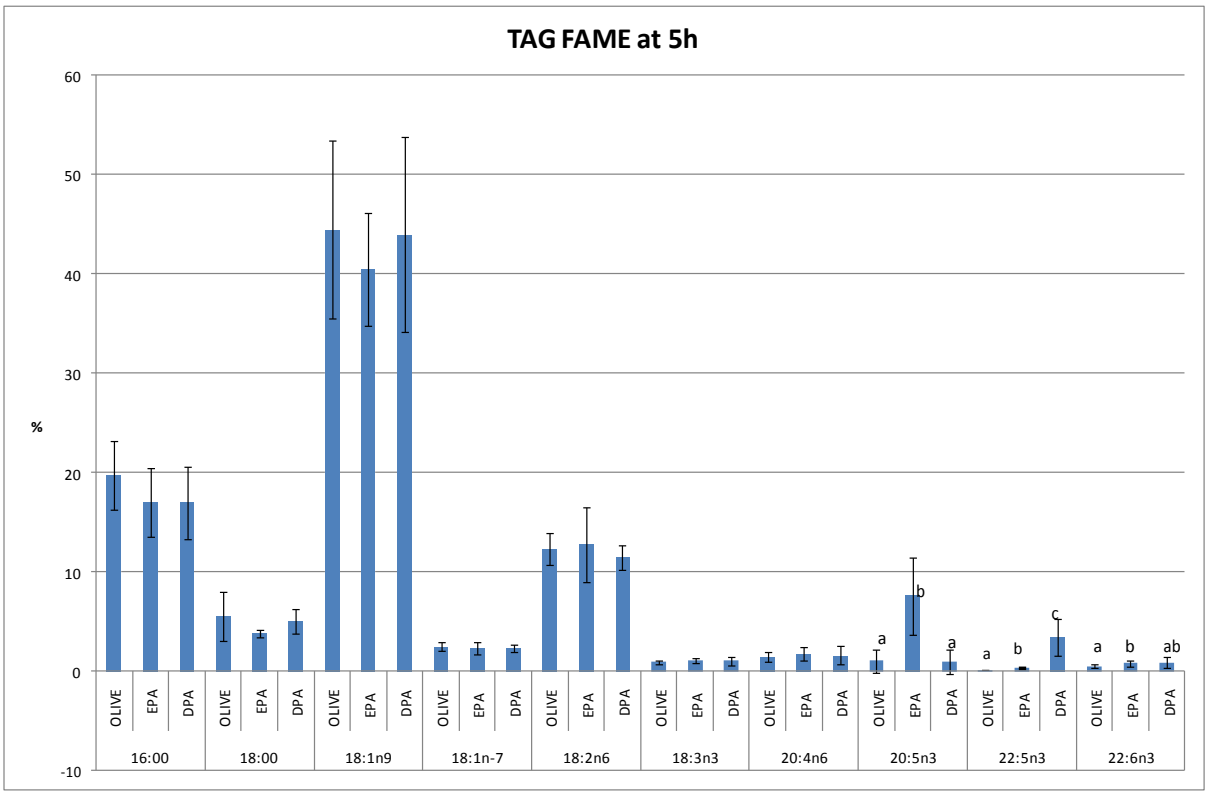
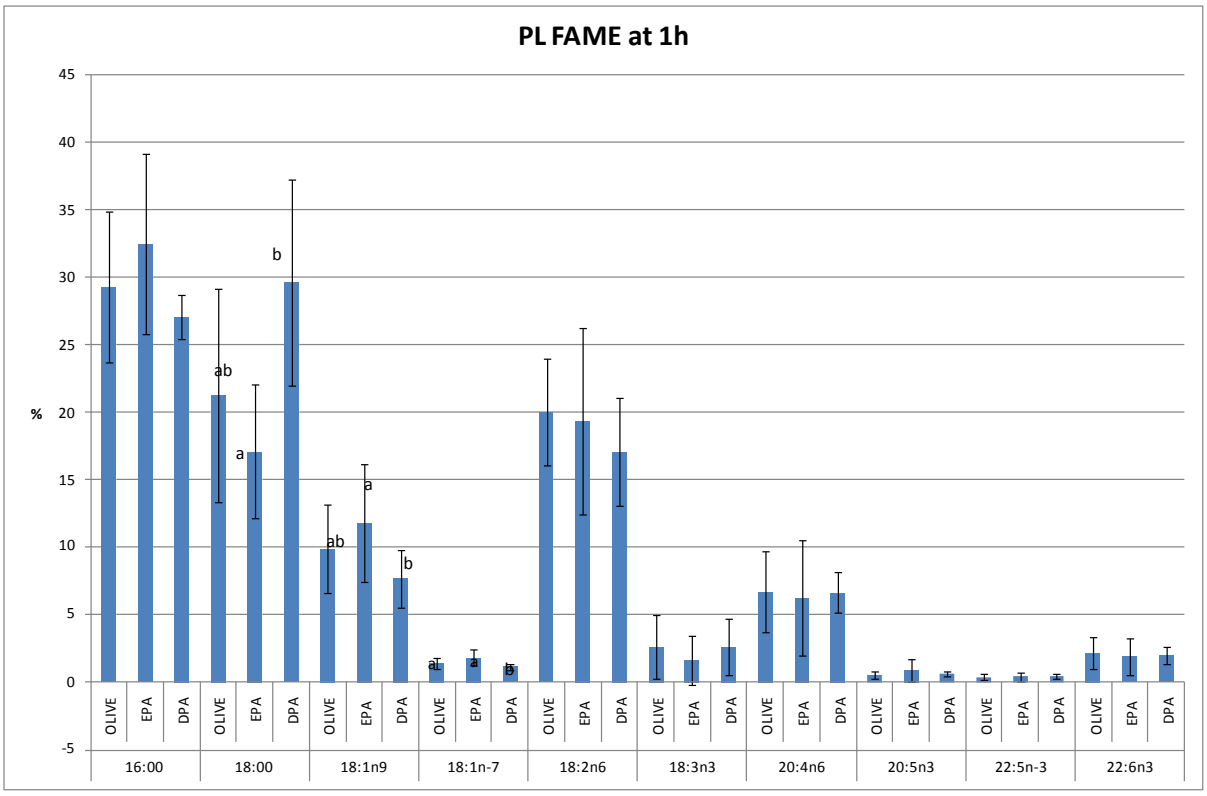
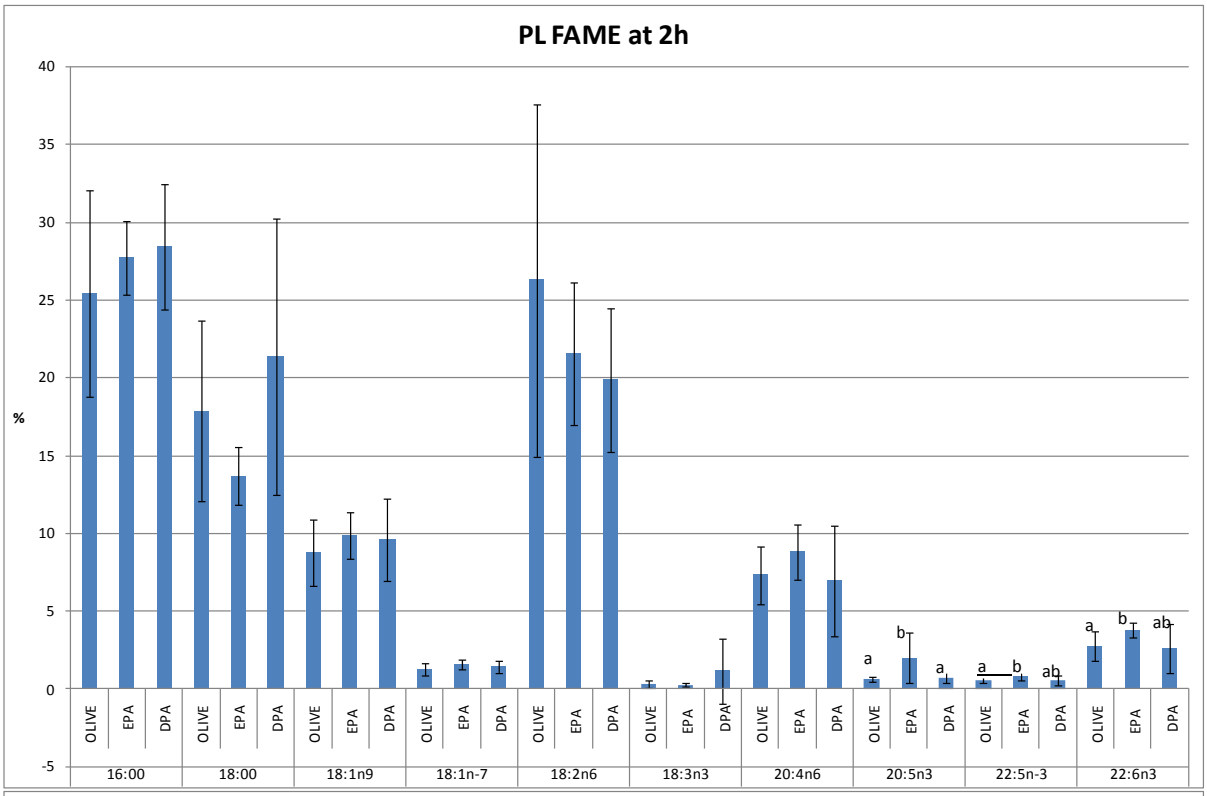
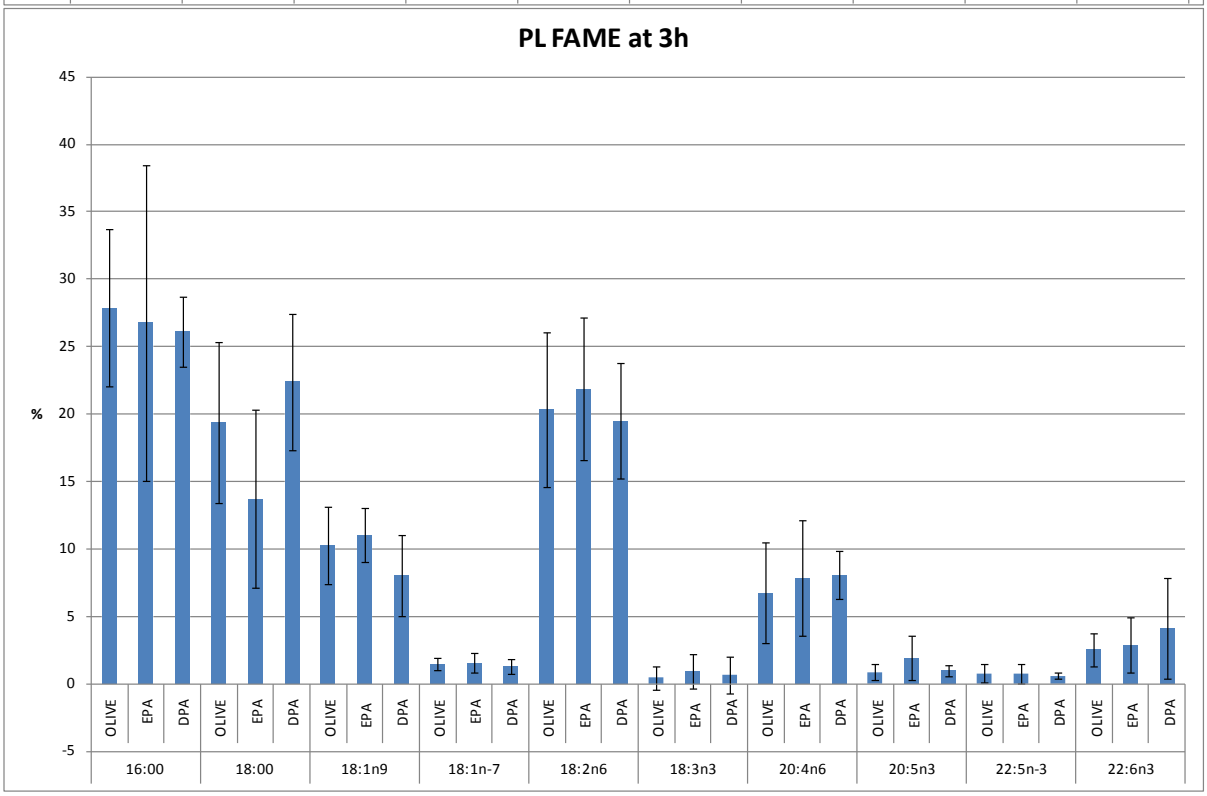


Figure 3. Phospoholipid FA composition at one to five hours postprandially. The five figures below will be combined into a single figure if significances can still be shown. Some error in 18:3n-3 => will be corrected. Y axis needs to start from 0.




PL FAME at 2h
%
40
35
30
25
20
15
10
5
0
-5
OLIVE
EPA
DPA
16:00
18:00
18:1n9
18:1n-7
18:2n6
18:3n3
20:4n6
20:5n3
22:5n-3
22:6n3
a
b
ab


PL FAME at 3h
%
45
40
35
30
25
20
15
10
5
0
-5
OLIVE
EPA
DPA
16:00
18:00
18:1n9
18:1n-7
18:2n6
18:3n3
20:4n6
20:5n3
22:5n-3
22:6n3

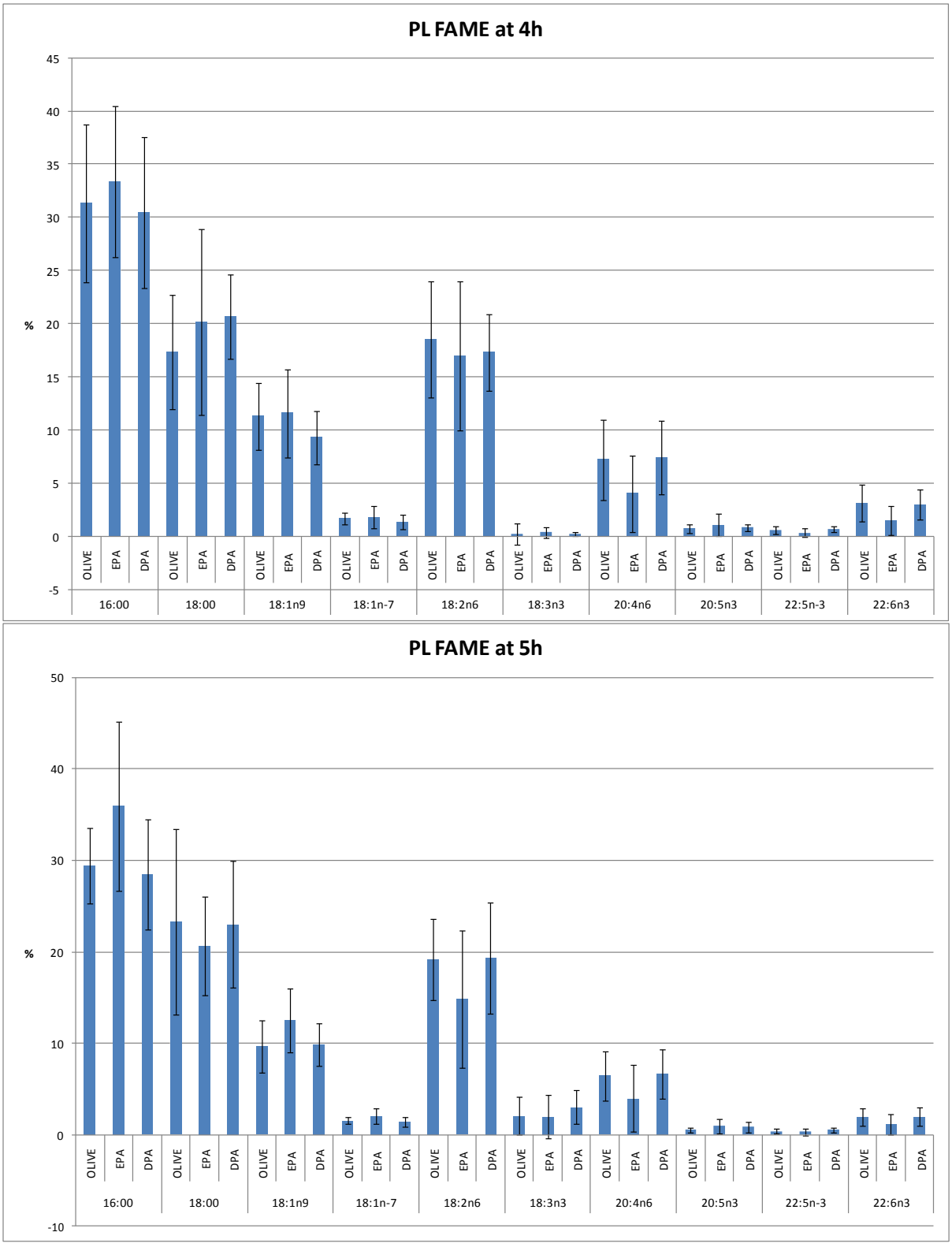


Figure 4. Chylomicron TAGs (1,3 and 5h) after breakfasts containing olive oil, EPA or DPA. Some TAGs need to be combined???

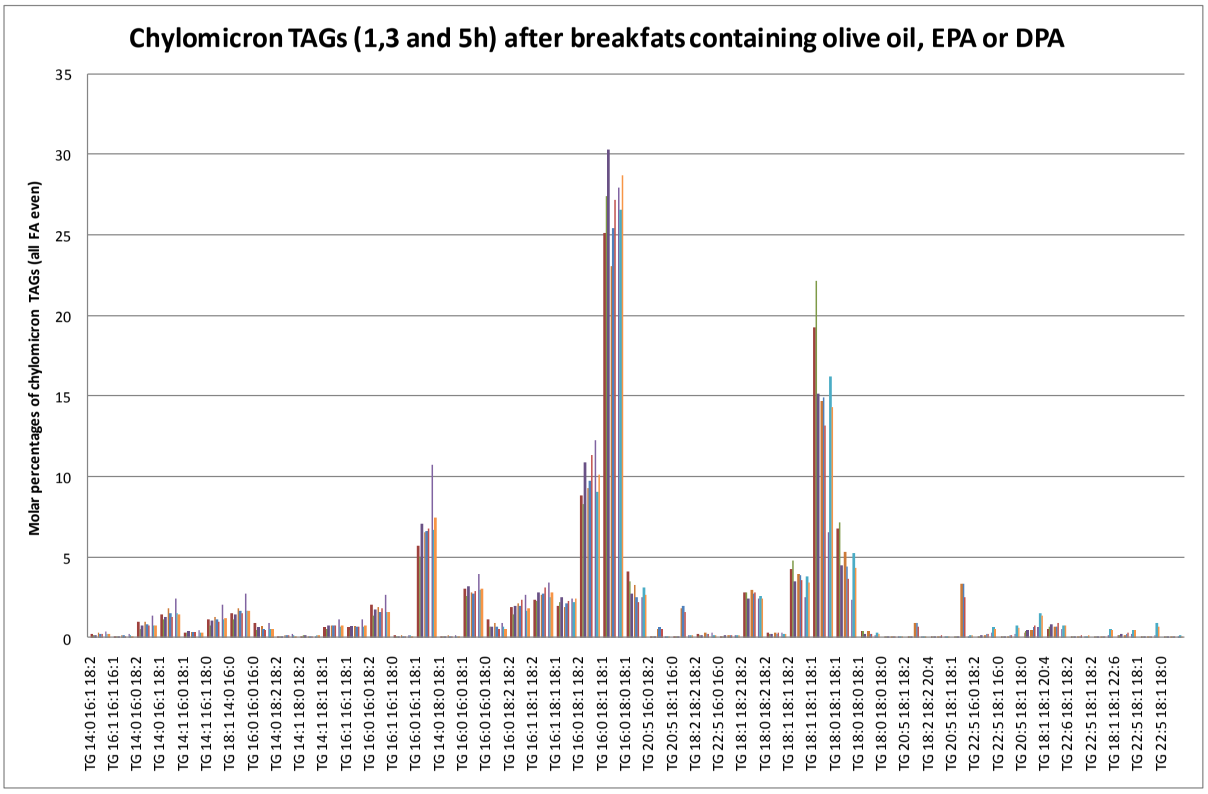


Figure 5. Chylomicron TAGs containing long chain polyunsaturated TAGs at 1,3 and 5h after the breakfasts containing olive oil, EPA or DPA. Statistical tests have not been done yet. TAG 20:5/18:1/18:0 is strange. Needs to be verified with neutral loss experiments. Might be safest to change this chart into a ACN:DB chart and discuss the FA combinations in the next picture.

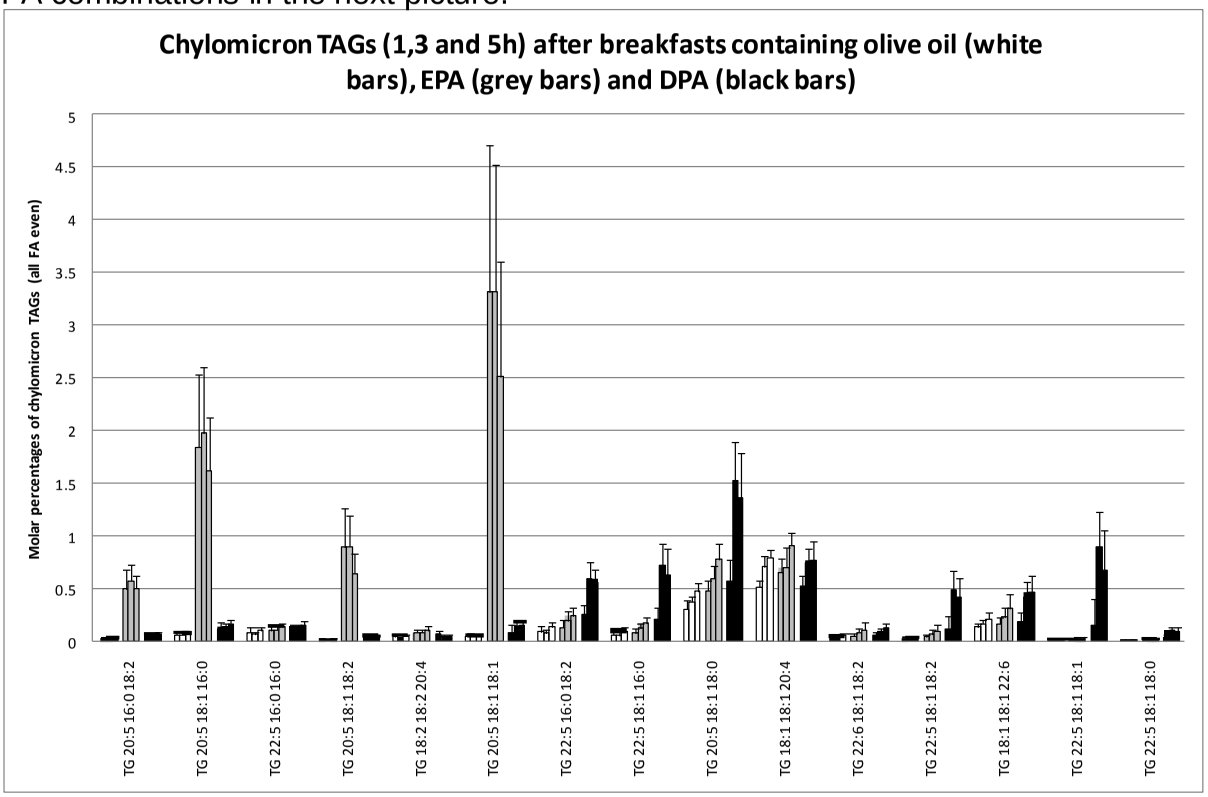


Figure 6.

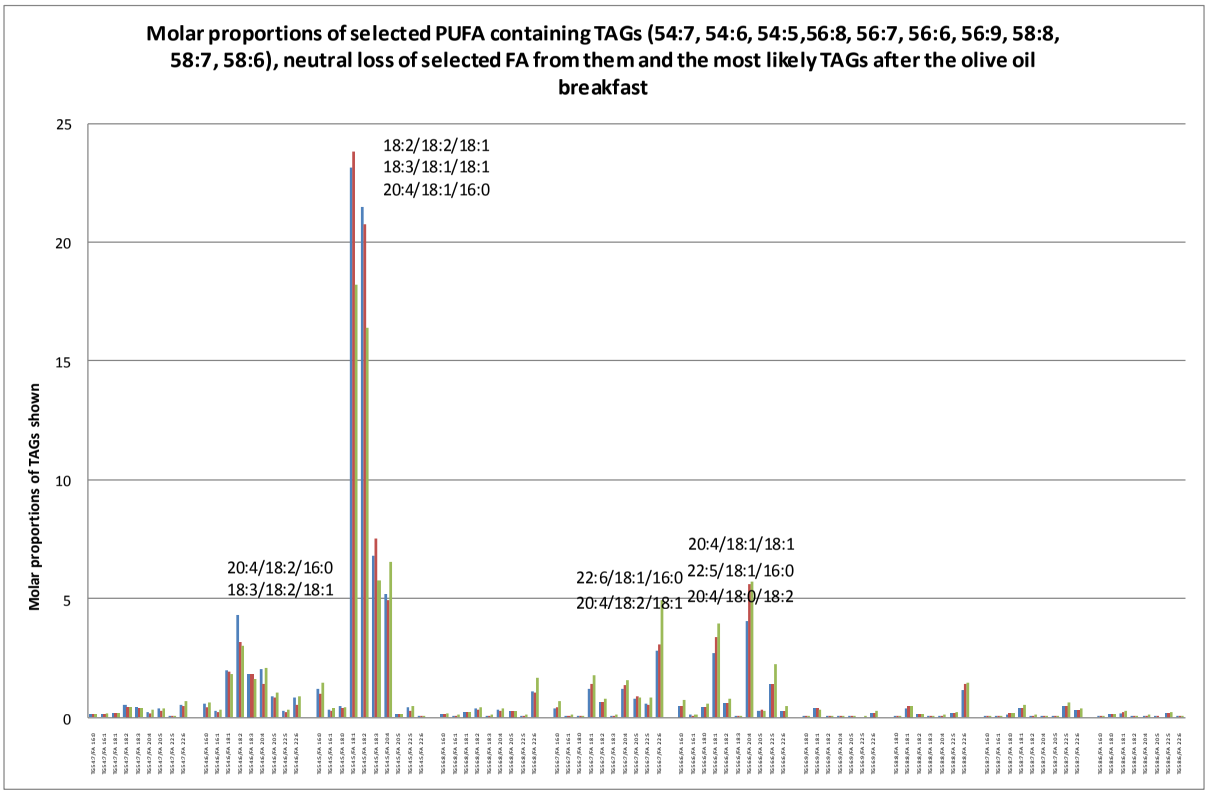


Figure 7

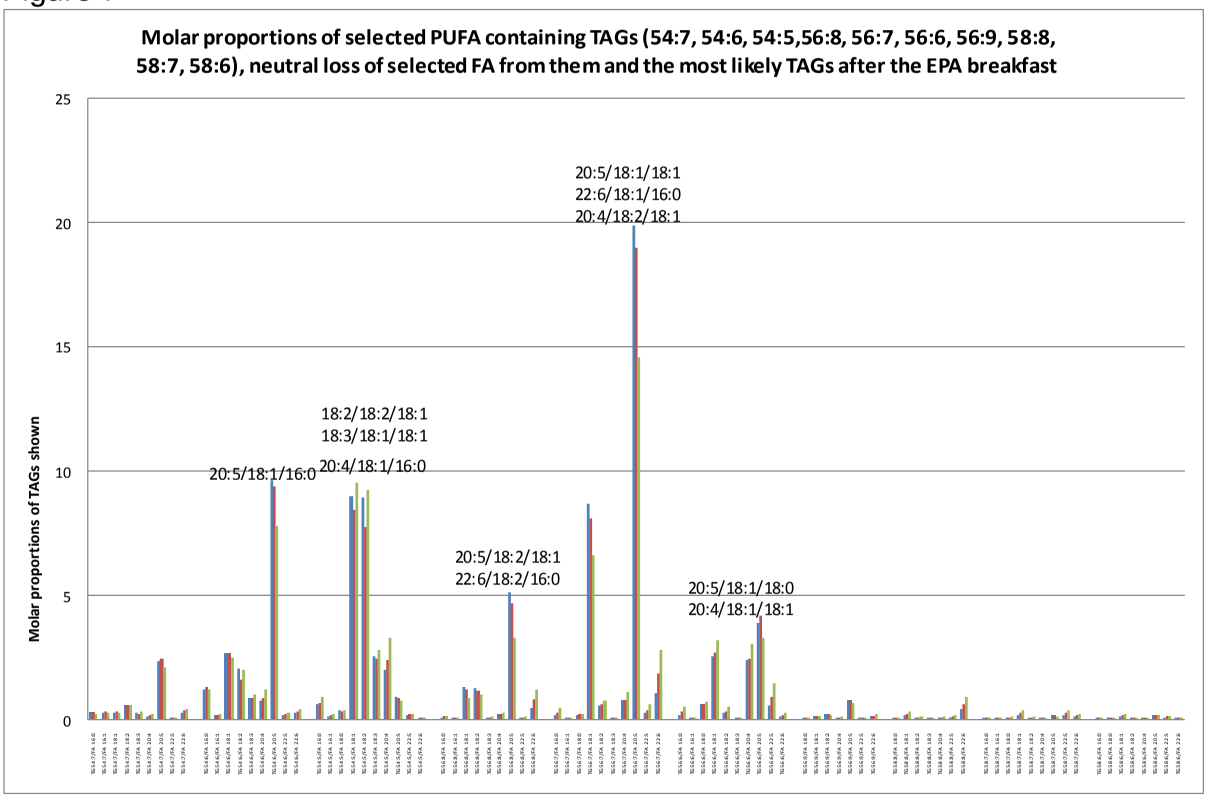
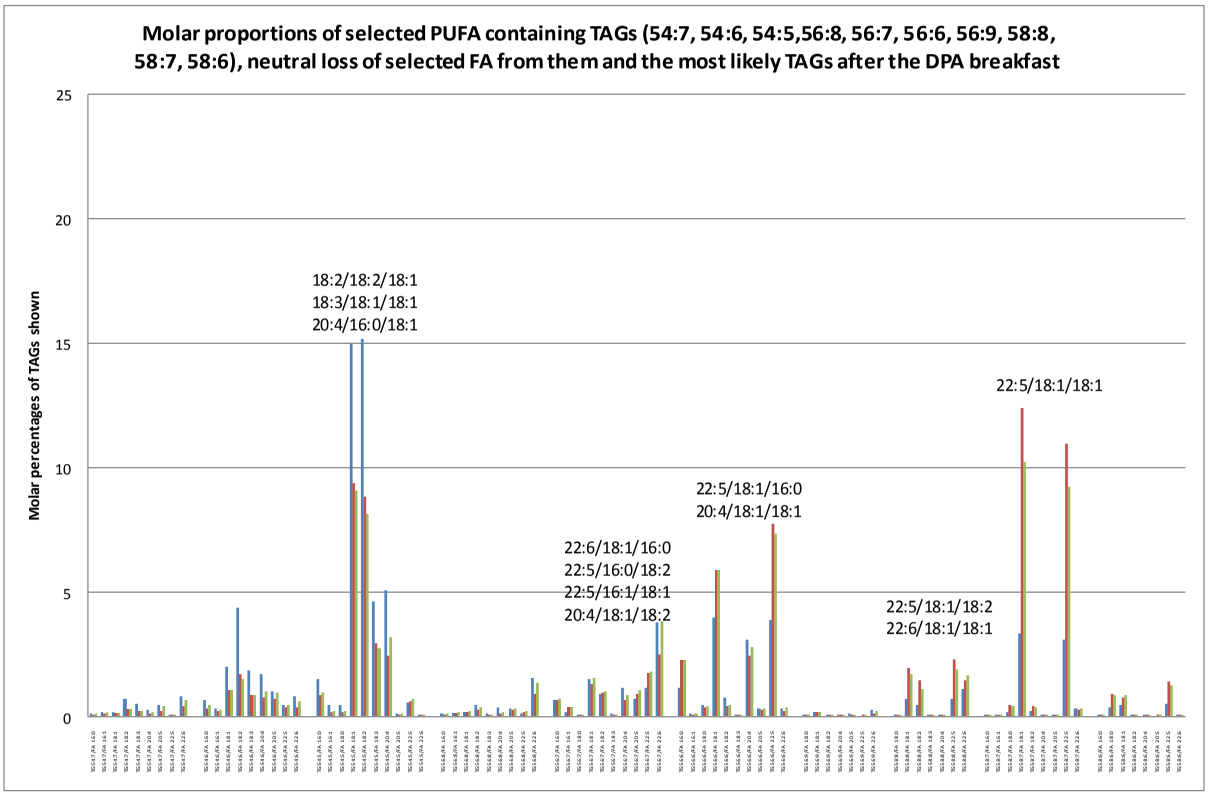


Figure 8


Molar proportions of selected PUFA containing TAGs (54:7, 54:6, 54:5,56:8, 56:7, 56:6, 56:9, 58:8, 58:7, 58:6), neutral loss of selected FA from them and the most likely TAGs after the DPA breakfast
Molar percentages of TAGs shown
25
20
15
10
5
0
18:2/18:2/18:1
18:3/18:1/18:1
20:4/16:0/18:1
22:6/18:1/16:0
22:5/16:0/18:2
22:5/16:1/18:1
20:4/18:1/18:2
22:5/18:1/16:0
20:4/18:1/18:1
22:5/18:1/18:2
22:6/18:1/18:1
22:5/18:1/18:1

APPENDIX-2

Submitted Manuscript

Study 2 – Chronic Study

A Short-Term n-3 DPA Supplementation Study in Humans

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Running Title: *Metabolism of n-3 DPA in humans*

Key Words:

n-3 polyunsaturated fatty acids (PUFA), docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), fatty acid metabolism

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Abstract

Purpose Despite the detailed knowledge of the absorption and incorporation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into plasma lipids and red blood cells (RBC) in humans, very little is known about docosapentaenoic acid (DPA, 22:5 n-3). The aim of this study was to investigate the uptake and incorporation of pure DPA and EPA into human plasma lipid RBC lipids.

Methods Ten female participants received 8 g of pure DPA or pure EPA in randomised crossover double blinded manner over a 7-day period. The placebo treatment was olive oil. Blood samples were collected at days zero, four and seven, following which the plasma and RBC were separated and used for the analysis of fatty acids.

Results Supplementation with DPA significantly increased proportions of DPA in the plasma phospholipids (PL) (by 2-fold) and triacylglycerol (TAG) fractions (by 2.3-fold, day 4). DPA supplementation also significantly increased proportions of EPA in TAG (by 3.1-fold, day 4) and cholesterol ester (CE) fractions (by 2.0-fold, day 7) and of DHA in TAG fraction (by 3.1-fold, day 4). DPA proportions in RBC PL did not change following supplementation. Supplementation with EPA significantly increased the proportion of EPA in the plasma CE and PL fractions, (both by 2.7-fold, day 4 and day 7) and in the RBC PL (by 1.9-fold, day 4 and day 7). EPA supplementation did not alter the proportions of DPA or DHA in any lipid fraction. These results showed that within day 4 of supplementation DPA and EPA were both incorporated into plasma and RBC lipids, but each FA demonstrated different and specific incorporation patterns.

Conclusions The results of this short-term study suggest that DPA may act as a reservoir the major long chain n-3 fatty acids (LC n-3 PUFA) in humans.

Introduction

A vast amount of information exists in relation to the beneficial cardiovascular [CV] health actions of long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA), namely EPA and DHA [1-2]. In contrast, very little is known about the regularly consumed intermediary product DPA (22:5 n-3). DPA is found in most fish and marine foods, and is also present in lean red meat from ruminant animals [3-4]. On average, the intake of DPA in adult Australian population is 71 mg/day that represents approximately 29 % of total LC n-3 PUFA intake [5].

The available literature, based on *in vitro*, *ex vivo* and animal studies, suggests that n-3 DPA may exert beneficial CV health effects [6-8]. DPA has been shown to be the most potent inhibitor of platelet aggregation in rabbit platelets, compared with either EPA or DHA. Platelet aggregation is an early event in the development of thrombosis or blood clot formation and is initiated by thromboxin A_2 (TXA_2) [9]. DPA inhibits cyclooxygenase-1 which is required for the synthesis of TXA_2 thereby inhibiting platelet aggregation. In human subjects, DPA is equally effective as EPA and DHA in inhibiting platelet aggregation (ex vivo) in female subjects, however, in male subjects only EPA inhibited platelet aggregation [10]. Furthermore, DPA exhibits additional physiological actions, including the ability to suppress the expression of lipogenic genes in cultured liver cells and in mice receiving DPA supplementation [7, 11]. Mechanistically, these actions may be due, in part, to the ability of DPA to also induce the expression of peroxisome proliferator activated receptor (PPARα), which negatively regulates lipolysis in favour of increased fat oxidation [12]. In addition, DPA is involved in the reduction of the expression of inflammatory genes such as tumor necrosis factor (TNF-α) in cell culture models [13]. The beneficial role of DPA in CV health is also supported by the studies investigating the metabolism of n-3 DPA, which have shown that DPA is highly incorporated in heart phospholipids (PL) compared with EPA, [14-15]. In addition, there is evidence from *in vitro* [7] and *in vivo* [14-15] studies that DPA can be metabolised into DHA and retro-converted to EPA. Collectively these studies suggest that DPA may provide an additional source of beneficial LC n-3 PUFA.

To date, there has only been one clinical study using a supplement rich in DPA, namely seal oil. However, this supplement also contained EPA and DHA in higher proportions than DPA [16]. Therefore, the benefits observed cannot be attributed

purely to DPA rather than the EPA or DHA present. The present study was conducted to investigate the actions of a highly purified DPA source, relative to pure EPA, on the incorporation into plasma and RBC lipids, following a 7-day supplementation period. It was hypothesised that the pattern of incorporation of DPA into human blood lipids would be similar to EPA.

Material and methods

Study population

Ten healthy lean females with a body mass index (BMI) of 20-25 kg/m^2, aged between 21-30 years were recruited for the study. Participants provided a written informed consent and completed a medical questionnaire and PUFA food frequency questionnaire (FFQ). Participants were excluded if they consumed more than 500 mg of LC n-3 PUFA per day (based on results of PUFA FFQ [18-20]), were at high risk of any form of CVD (based upon family history information obtained from medical questionnaire) or were overweight as indicated by their BMI (>26 kg/m^2). Ethics approval was obtained from the Deakin University Human Research Ethics Committee (EC2011-023).

Daily supplements

Purified EPA (99.8%; w/w) and DPA (99.8%; w/w) were sourced from Equateq Ltd, Breasclete, Callanish, Scotland. The participants consumed the supplements for seven consecutive days. The participants consumed 2 g of the supplement in question on the first day of the study and 1 g daily for the subsequent 6 days. The total DPA or EPA that was consumed per supplementation period per subject was 8 g. As the supplements were diluted in olive oil, the participants received a total of 8 g of olive oil during the EPA and DPA study periods. The placebo supplements contained a total of 16 g of olive oil. The initial dose was double that of the remaining doses in an attempt to boost the DPA or EPA levels from the beginning of the study.

Study design

Following screening, the participants were randomised to receive the olive oil, DPA, EPA supplements in a random order. The participants consumed a standardized dinner meal (containing pasta (dry 200g), tomato stir through sauce (70g) and a packet pudding the night before the start of the study and were given instructions to

fast overnight for 10 hours. On the first study day, a fasted blood sample (day 0) was drawn and following this the participants were asked to consume a meal of 180 g of instant mashed potato (Continental, Deb™, Unilever Australasia) which contained the 2 g of DPA or EPA mixed with 18 ml of olive oil. The placebo group consumed 20 ml of olive oil in the 180 g of mashed potato. The participants consumed this 'breakfast' in 15 minutes.

For the next 6 days, participants were provided with six, 2 g aliquots of a 1:1 mixture of DPA or EPA in olive oil in 2 ml cryovials in a box (protecting the oils from exposure to light). Participants were provided with instructions to keep the supplements in the fridge and to consume them each morning. During the placebo period the participants received six 2 g aliquots of olive oil. For days 1 to 6, the participants were asked to pour the contents of the cryovial into 200 ml of standard commercial orange juice to aid in palatability. On the mornings of day 4 and 7 of their supplementation week, participants attended the clinical facility to provide a fasting blood sample.

During the three supplementation week's, participants were requested to refrain from consuming high LC n-3 PUFA products including fish, red meat and LC n-3 PUFA fortified products (<2 marine and/or 2 red meat meals/week and <2 LC n-3 PUFA fortified products/week), in order to prevent false increases in plasma circulating LC n-3 PUFA. The participants were asked to give a recall of their diet 24 hours before they came in to provide blood samples at day 0, 4 and 7. It was found that the participants did not consume any fish during the study period and consumption of red meat was <2 serves per week. Each supplementation period lasted seven days with a two-week washout period prior to crossover.

Plasma and red blood cell lipid analysis

Venous blood was collected into two 8 ml EDTA vacutainers. Samples were immediately centrifuged for fifteen minutes at 591 x g and 15°C. Plasma at each time point was aliquoted and stored at -80°C until further analysis. The RBC remaining after the removal of the plasma were washed twice with 0.9% sodium chloride, centrifuged each time and after removal of the upper saline solution, the washed red cells were aliquoted into storage vials and stored as the plasma (above).

Total plasma lipids were extracted from plasma as described by Sinclair et al [20]. In brief, 850 µl of plasma was extracted using 15 ml of dichloromethane: methanol 2:1 (v/v) containing 0.01 mg butylated hydroxytoluene (BHT) and reference internal lipid standards, specifically TAG-17:0, CE-17:0 (NuCheck Prep, Minnesota, USA) and phosphatidyl choline (PC)-17:0 (Avant Polar Lipids, USA). The major neutral lipid classes were separated by thin layer chromatography (TLC) and the CE, TAG & PL fractions were scraped from the TLC plates and transmethylated with 5% H_2SO_4 in methanol prior to GC. The resulting fatty acid methyl esters (FAME) were isolated and identified using an Agilent Technologies GC 7890A (Agilent Technologies, Santa Clara, California, USA) equipped with an Omegawax 250 capillary column (30 m x 0.25 µm internal diameter, 0.25 µm film thickness, Supelco, Bellefonte, PA, USA), a flame ionisation detector (FID), and an Agilent Technologies 7693 auto-sampler. Each of the FAME peak was identified relative to known external standards; a FAME mix of three PUFA, these being a marine source, animal source and a menhaden oil (Supleco, Bellefonte, PA, USA). The resulting peaks were then corrected by the theoretical relative FID response factors and quantified relative to the internal standard used at the lipid extraction stage [21].

Total RBC lipids were extracted from 200 µl of RBC with dichloromethane: methanol (1:1 v/v, containing a known amount of PC-17:0 standard), similar to the Folch method [22], with modifications by Armstrong et al [23]. The RBC lipids were separated by TLC and the PL fraction was scraped from the TLC plates and transmethylated and the FAME were analysed as described above.

Plasma TAG concentration

Plasma TAG concentrations were measured on a Roche Cobas Integra 400 plus autoanalyser an enzymatic colorimetric method using a commercially available kit (TRIGL) as per the manufacturer's instructions (Roche, Lavel, Quebec, Canada).

Statistical analysis

Data calculations and statistical analysis were performed using the Minitab Statistical Software (Minitab Version 15; Minitab Inc., USA). Data was analysed using two way ANOVA repeated measures and pair wise comparisons were made using Tukey. A value of $p<0.05$ was taken as significant.

Results

Subject characteristics

The 10 healthy female participants had a mean age of 25.5±3.3 years, with a BMI of 22.3±1.6 kg/m2; they were non-diabetic, not taking CVD medication and did not regularly consume fish oil capsules. All participants completed a PUFA FFQ [17-19] and were all found to consume 102±66 mg LC n-3 PUFA/day.

Acceptability of the supplements

Participants who experienced any adverse reaction were requested to inform investigators immediately. Three cases of mild diarrhoea were reported by participants during the DPA and EPA supplementation periods, respectively, with severity ranging from very mild to moderate. These events were found to occur only during first four days of supplementation and were most commonly reported to occur within the first hour following supplement consumption. Participants received daily reminders in person or by e-mail to consume their supplement and upon returning for the final blood collection, participants were requested to return the box containing all the vials the supplements were provided in as a way to ascertain compliance with consumption of the supplement. It was found that all returned vials were empty.

Plasma and red blood cell fatty acid composition

Plasma PL

The average baseline levels of LC n-3 PUFA in plasma PL were 0.8% for EPA and DPA and 2.3% for DHA (Fig 1). For those consuming the DPA supplement, there was a significant increase in the proportion of DPA at day 4 (from 0.7% to 1.4%), compared with day 0 value ($p=0.006$). Although, there was a trend for an increase in day 7 DPA levels this was not statistically significant ($p= 0.076$). In the EPA group, there was a significant increase in the proportion of EPA (from 1% to 2.7%) relative to baseline at both days 4 and 7 ($p<0.01$). There were no significant changes in the proportion of LC n-3 PUFA for participants consuming the olive oil placebo.

Plasma TAG

The average baseline levels of LC n-3 PUFA in plasma TAG were 0.3% for EPA and DPA, and 0.6% for DHA (Fig 2). After DPA supplementation, there was a significant

rise in the proportion of DPA (from 0.5% to 1.2%) at day 4 (p=0.027), in the proportion of EPA (from 0.3% to 0.9%) at day 4 (p=0.05), as well as in the proportions of DHA (from 0.7% to 2.2%) at day 4 (p=0.004). There were no significant changes in the proportions of EPA, DPA or DHA in plasma TAG for participants consuming the EPA supplements or the olive oil placebo, compared with day 0.

Plasma CE

The average baseline levels of LC n-3 PUFA in plasma CE were 0.9% for EPA, 0.8% for DPA and 0.7% for DHA (Fig 3). As shown in Fig 3, there were no significant changes in the proportion of LC n-3 PUFA caused by the olive oil supplementation. After DPA supplementation, there was a significant rise in EPA proportions at day 7 (from 0.9 to 1.7%, p=0.027) compared to day 0. After supplementation with EPA, there was a significant rise in the proportion of EPA at day 4 (from 0.9% to 2.4%, p<0.01) and day 7 (from 0.9% to 2%, p<0.01), compared with day 0.

RBC PL

The average baseline levels of EPA, DPA and DHA in RBC PL were 1.0%, 2.2% and 6.7%, respectively (Fig 4). After the DPA supplementation, there was no significant change in the proportion of DPA in RBC PL. After the EPA supplementation, there was a significant increase in the proportion of EPA at both day 4 (from 1.1% to 2.0%) and 8 (from 1.1% to 1.9%), compared with the baseline value (p<0.01). There were no significant changes in the proportions of DPA or DHA in any of the treatment groups.

Plasma TAG concentrations

There were no significant changes in the concentration of TAG in plasma samples between the control and LC n-3 PUFA supplement periods, or with time in any of the three groups (data not shown).

Discussion

The ingestion of LC n-3 PUFA rich marine oils, either as fish or in purified oil supplements, is a widely accepted strategy for the reduction of plasma TAG levels [24-27]. This is supported by a considerable quantity of data on the effect of LC n-3 PUFA in cell models [28-29], experimental animals [30-31] and intervention clinical studies [32-33]. DPA is one of the three major LC n-3 PUFA in marine oils, yet there is no

available data on the plasma lipid or RBC phospholipid distribution of DPA following supplementation with pure DPA in humans. Therefore, the aim of this study was to investigate the partitioning of pure DPA into human plasma and red blood cell lipid fractions following a 7-day dietary supplementation period.

The most striking finding, contrary to expectations, was that DPA and EPA partitioned into different lipid fractions in both plasma and RBC phospholipids. In the steady state (baseline values), DPA and EPA were both present in RBC PL, plasma CE and plasma PL in higher proportions than in the plasma TAG fraction. With DPA supplementation there was a significant increase in proportions of DPA in the plasma TAG and PL fraction, but not in RBC PL or plasma CE fractions. Consistent with the steady state, EPA supplementation significantly increased the proportion of EPA in plasma CE and PL fractions and RBC PL, but not in the plasma TAG fraction. The failure of DPA to be incorporated into the plasma CE fraction and RBC PL fraction was unexpected and reveals in the time-frame of this study a highly interesting difference between how DPA and EPA are processed in the body. This differential processing might occur perhaps at the level of incorporation of the PUFA into chylomicron TAG and/or at the level of the liver following the uptake of the PUFA from chylomicron remnants, subsequent processing into VLDL lipids and exchange between lipoproteins and red blood cell lipids.

The average baseline levels of LC n-3 PUFA in the plasma PL in our study were 0.8% for both EPA and DPA; and 2.3% for DHA, which is consistent with previously reported data for Australian subjects [34-35]. In our study, supplementation with DPA led to a significant increase in DPA levels (by 2 fold) in plasma PL which peaked by 4 days. Seal oil supplementation (which contains a higher proportion of DPA than other marine oils) led to a a sigificant increase in DPA proportions in plasma PL [36]. No changes were observed in EPA levels in plasma PL as a result of DPA supplementation, which means any DPA that was retro-converted to EPA, say in the liver, was not incorporated into the plasma PL in the timeframe of this study. In our study, supplementation with EPA significantly increased the EPA levels by approximately 2.7 fold in plasma PL. Similar findings have also been reported by Mori et al [37] who fed 4 g/day of pure EPA to human subjects for 6 weeks and showed increases in EPA levels in plasma PL. In addition, Mori et al showed an increase in the DPA level. In our study,

although, DPA levels in the EPA group were higher than baseline levels, this increase did not achieve statistical significance. It should be noted that the Mori et al study was over an extended period (6 week vs. 1 week). Studies utilising fish oil supplements have also shown increased levels of all three LC n-3 PUFA in plasma PL after 6 weeks of supplementation [38].

The mean baseline values of LC n-3 PUFA in plasma TAG in our study were 0.3% for EPA and DPA and 0.6% for DHA, comparable to previous reports [35]. In the present study, DPA supplementation significantly increased plasma TAG DPA, EPA and DHA levels at day 4. The increases in all 3 major n-3 PUFA species indicates that DPA is both being retro-converted back to EPA and further elongated onto DHA. Retro-conversion involves both peroxisomal acyl-CoA oxidase and β-oxidation [39-40]. Retro-conversion has been demonstrated in hepatocytes [12], yet may also be present in endothelial cells [41] and fibroblasts [39,42]. Recently, evidence of retro-conversion of DPA to EPA was found to be present in a wide variety of tissues including liver, heart and skeletal muscle [14-15]. The current study demonstrated that in healthy female volunteers 7 days of supplementation of DPA increased plasma TAG EPA demonstrating retro-conversion and augmentation of this EPA pool. The increase in DHA levels in plasma TAG fractions following DPA supplementation demonstrates DPA can act as a source of DHA in the body. This increase in DHA levels is supported by animal studies with pure DPA supplementation by Kaur et al [15], Holub et al [14] and Gotoh et al [11] who all reported increased DHA levels in liver tissue.

The mean baseline values for LC n-3 PUFA in plasma CE in our study were 0.9% for EPA, 0.8% for DPA and 0.7% for DHA, consistent with previously published data [43]. In the current study, DPA supplementation trended towards an increased DPA and DHA levels in plasma CE, however statistical significance was not achieved. It is possible that long term supplementation might result in increased DPA and DHA levels in plasma CE. There was further evidence of DPA retro-conversion to EPA, since there was a significant increase in EPA in the plasma CE fraction (by-2 fold at day 8). This suggests that DPA can act as a source of EPA in the body. Our data shows that EPA supplementation significantly increased plasma CE EPA levels by approximately 2.7 and 2.3 fold at day 4 and day 8, but did not impact on DPA levels. Previous long-term studies [43] have shown that an EPA-rich fish oil lead to significant increases in

plasma CE proportions of EPA within 30 days of commencing the study (12 month study). The present study showed that EPA can be incorporated into plasma CE within 4 days of commencing the supplement.

The mean baseline values of LC n-3 PUFA in RBC PL in our study were 1.0% for EPA, 2.2% for DPA and 6.7% for DHA, consistent with data published previously [38, 44]. In our study there was no increase in DPA levels in RBC PL in any group. A seal oil supplementation study showed a considerable increase in EPA (0.8-fold) and DHA (1-fold) levels in erythrocytes and an only modest increase in DPA (0.2-fold) [45]. Since, seal oil also contains EPA along with DPA, the modest increases in DPA levels could be from conversion of EPA into DPA, rather than direct incorporation of DPA itself.

Pure EPA supplementation for 6 weeks has been reported to increased EPA levels in RBC membrane [44]. Similarly, our data shows that EPA supplementation for 1 week significantly increased RBC PL EPA levels at day 4. As RBC lifespan is approximately 120 days [43], incorporation of EPA into RBC PL is unlikely to be achieved through a process involving RBC turnover. Incorporation of EPA into the RBC is more likely achieved through exchange between plasma and RBC PL, as speculated previously [46-48].

The metabolism of DPA has not been studied previously in humans. This short term supplementation study in healthy volunteers demonstrated that DPA, along with EPA were incorporated into plasma and RBC lipid fractions, yet notable differences are evident. Future studies should examine the incorporation of DPA into chylomicrons, chylomicron remnants and VLD lipoproteins to help explain the differential metabolism of DPA and EPA. The most novel finding is that in the context of this short-term study, DPA showed evidence of metabolism to both EPA and DHA. This suggests that DPA could function as a reservoir or buffer of the other LC n-3 PUFA.

Acknowledgments

Research support from Meat & Livestock Australia for financial support, Equateq Ltd (UK) for the generous provision of the pure supplements and Deakin University Strategic Research Centre for Molecular Medicine is gratefully acknowledged. EM, AL, DCS and AJS planned and designed the study; EM and AL recruited the participants

and collected samples and dietary data; GK, EM and GT conducted the plasma analyses; GK, SPL and GT conducted the RBC analyses; GK conducted the statistical analysis; GK, AJS, and DCS wrote the manuscript; GK, AJS, DCS, KL and HSW made significant contributions to the discussion.

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Figure captions

Fig 1 Fatty acid composition of plasma phospholipids (PL) from human participants supplemented with olive oil, EPA or DPA with a dose of 2 g for the first day and 1 g for the subsequent 6 days.

Results are expressed as percentage mean ± SEM (n=10). Data was analysed using two way ANOVA repeated measures and pair wise comparisons were made using tukey. The superscripts with capital alphabets represent a combined supplementation effect and different superscripts represents values which are significantly different ($p<0.05$). The superscripts with small alphabets represent time effect within each supplementation group and different superscripts represents values which are significantly different ($p<0.05$). The values with no superscripts show no significant differences. OO, olive oil; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Fig 2 Fatty acid composition of plasma triacylglycerides (TAG) from human participants supplemented with a dose of 2 g for the first day and 1 g for the subsequent 6 days.

Results are expressed as percentage mean ± SEM (n=10). Data was analysed using two way ANOVA repeated measures and pair wise comparisons were made using tukey. The superscripts with capital alphabets represent over all supplementation effect and different superscripts represents values which are significantly different ($p<0.05$). The superscripts with small alphabets represent time effect within each supplementation group and different superscripts represents values which are significantly different ($p<0.05$). The values with no superscripts show no significant differences. OO, olive oil; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

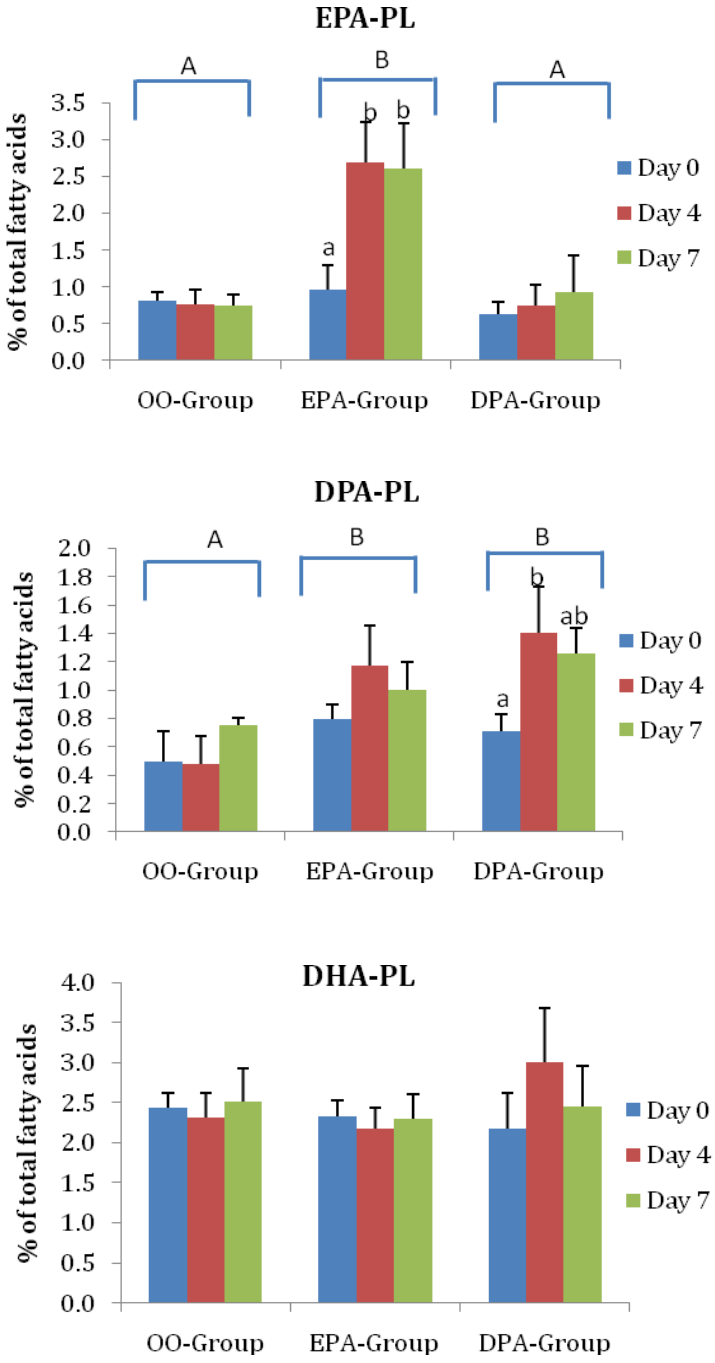
Fig 3 Fatty acid composition of plasma cholesterol ester (CE) from human participants supplemented with olive oil, EPA or DPA with a dose of 2 g for the first day and 1 g for the subsequent 6 days.

Results are expressed as percentage mean ± SEM (n=10). Data was analysed using two way ANOVA repeated measures and pair wise comparisons were made using tukey. The superscripts with capital alphabets represent over all supplementation effect and different superscripts represents values which are significantly different ($p<0.05$). The superscripts with small alphabets represent time effect within each supplementation group and different superscripts represents values which are significantly different ($p<0.05$). The values with no superscripts show no significant differences. OO, olive oil; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Fig 4 Fatty acid composition of red blood cell phospholipids (PL) from human participants supplemented with olive oil, EPA or DPA with a dose of 2 g for the first day and 1 g for the subsequent 6 days.

Results are expressed as percentage mean ± SEM (n=10). Data was analysed using two way ANOVA repeated measures and pair wise comparisons were made using tukey. The superscripts with capital alphabets represent over all supplementation effect and different superscripts represents values which are significantly different ($p<0.05$). The superscripts with small alphabets represent time effect within each supplementation group and different superscripts represents values which are significantly different ($p<0.05$). The values with no superscripts show no significant differences. OO, olive oil; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

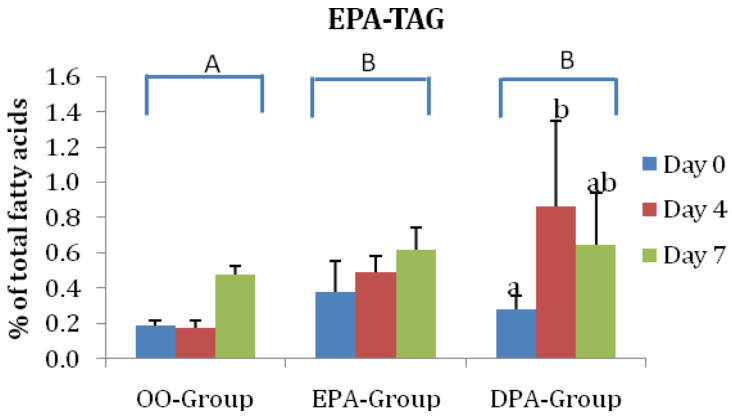
Fig 1



Overall Significance(ANOVA)
Combined supplementation effect; p<0.01
Day effect; p<0.01
Breakfast*Day interaction;

Overall Significance(ANOVA)
Combined supplementation effect; p=0.393
Day effect; p=0.654
Breakfast*Day interaction; p=0.257

Fig 2



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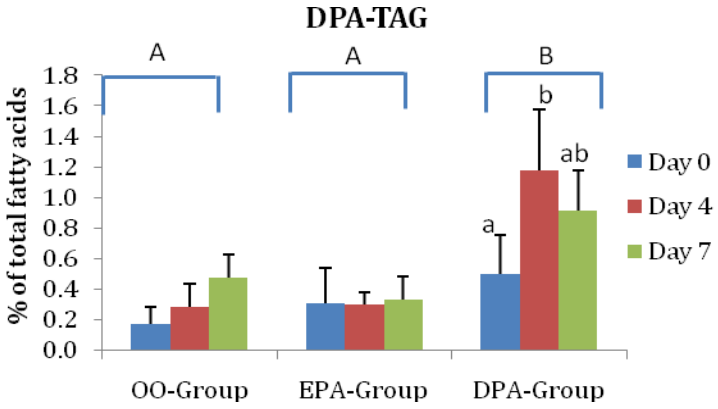

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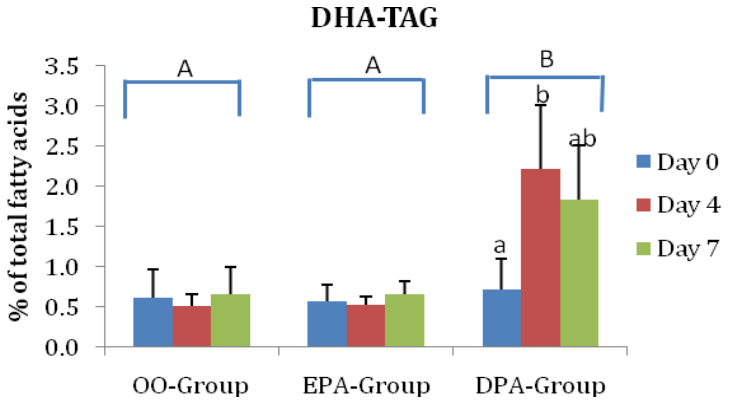

Overall Significance(ANOVA)
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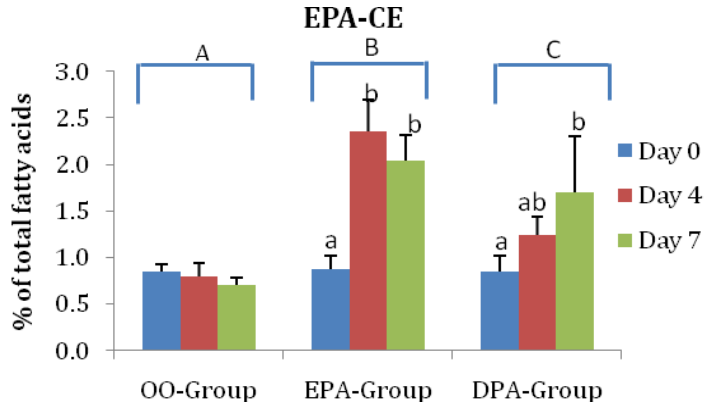
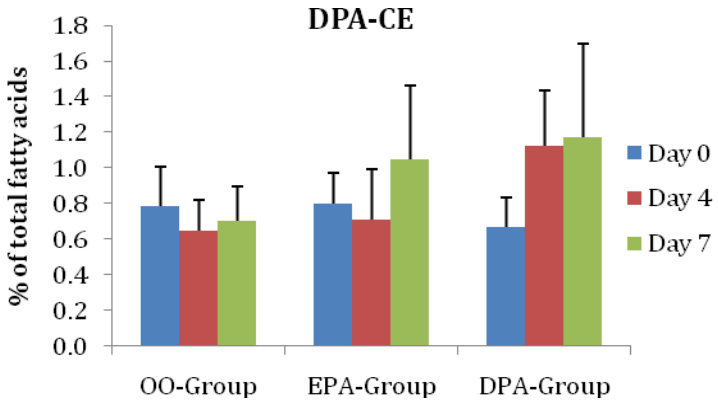
Fig 3



Overall Significance(ANOVA)
Combined supplementation effect; p<0.01
Day effect; p<0.01
Breakfast*Day interaction;



Overall Significance(ANOVA)
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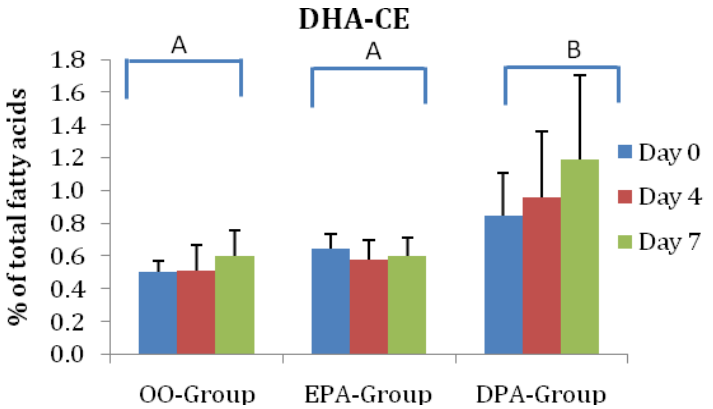
Day effect; p=0.348
Breakfast*Day interaction;



Overall Significance(ANOVA)
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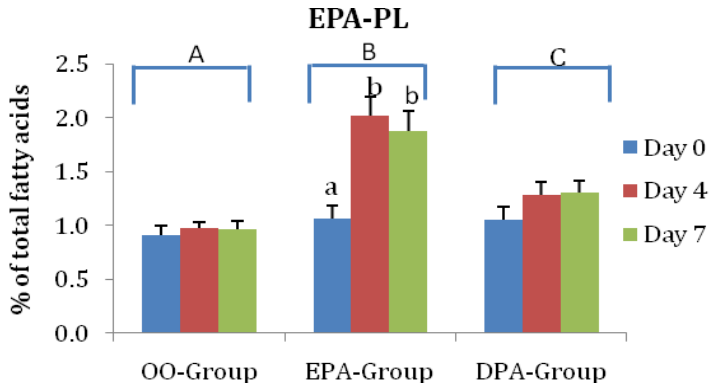
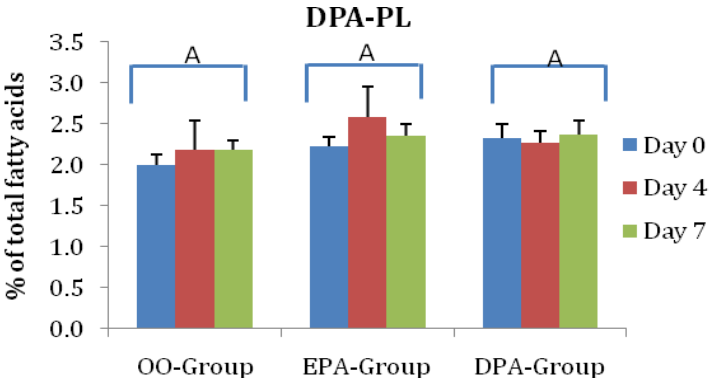
Day effect; p=0.558
Breakfast*Day interaction;

Fig 4



Overall Significance(ANOVA)
Combined supplementation effect;p<0.01
Day effect; p<0.01
Breakfast*Day interaction;



Overall Significance(ANOVA)
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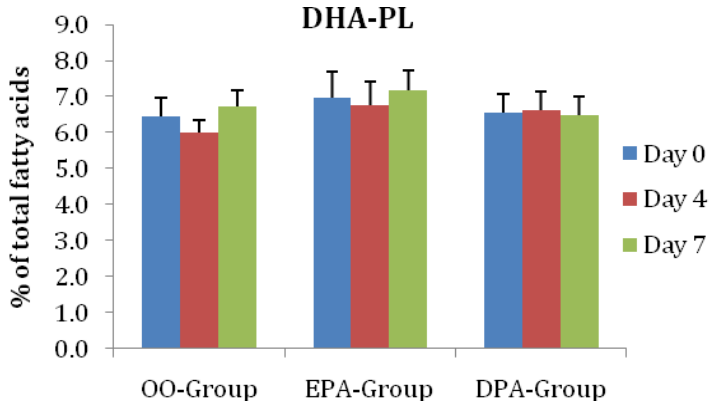
Day effect; p=0.328
Breakfast*Day interaction;



Overall Significance(ANOVA)
Combined supplementation effect;p=0.140
Day effect; p=0.538
Breakfast*Day interaction;