Prostaglandins, Leukotrienes and Essential Fatty Acids 115 (2016) 60–66

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Measurement of red blood cell eicosapentaenoic acid (EPA) levels in a randomised trial of EPA in patients with colorectal cancer liver metastases☆

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ARTICLE INFO

Keywords:
Colorectal cancer
Eicosapentaenoic acid
Omega-3 polyunsaturated fatty acid

ABSTRACT

We investigated red blood cell (RBC) PUFA profiles, and the predictive value of RBC EPA content for tumour EPA exposure and clinical outcomes, in the EMT study, a randomised trial of EPA in patients awaiting colorectal cancer (CRC) liver metastasis surgery (Cockbain et al., 2014) [8]. There was a significant increase in RBC EPA in the EPA group (n=43; median intervention 30 days; mean absolute 1.26[± 0.14]% increase; $P < 0.001$), but not in the placebo arm (n=45). EPA incorporation varied widely in EPA users and was not explained by treatment duration or compliance. There was little evidence of 'contamination' in the placebo group. The EPA level predicted tumour EPA content (r=0.36; P=0.03). Participants with post-treatment EPA≥1.22% (n=49) had improved OS compared with EPA < 1.22% (n=29; HR 0.42[95%CI 0.16–0.95]). RBC EPA content should be evaluated as a biomarker of tumour exposure and clinical outcomes in future EPA trials in CRC patients.

1. Introduction

The clinical efficacy of omega-3 polyunsaturated fatty acids (PUFAs) has been tested in randomised, double-blind, placebo-controlled trials in multiple adult healthcare settings including studies of cardiovascular disease [1], inflammatory bowel disease [2], non-alcoholic fatty liver disease [3] and cancer [4,5]. However, despite the widespread availability of mass spectrometric techniques for measuring PUFAs, only a minority of clinical omega-3 PUFA studies with a primary or secondary clinical endpoint have reported target organ PUFA incorporation and/or individual blood PUFA profiles [3,5,6], with which to interpret PUFA 'bioavailability' (a term usually used in omega-3 PUFA studies to indicate the tissue or blood level of omega-3 PUFAs), compliance and 'contamination' by excess 'own use' of the intervention or dietary omega-3 PUFA intake, the latter being a particular threat to correct interpretation of trial data and the statistical power of a trial for any substance that is easily available 'over the counter' or as a dietary constituent [7].

We have previously reported the results of a Phase II randomised, double-blind, placebo-controlled trial of 99% pure eicosapentaenoic acid (EPA) in the free fatty acid (FFA) form in patients undergoing liver resection surgery for colorectal cancer liver metastasis (CRCLM), called the EMT study [8]. In this 'window of opportunity' trial, gastro-resistant EPA-FFA or identical placebo capsules were taken for a variable amount of time between the decision to undergo surgery and liver resection, at which time the trial intervention was stopped. We demonstrated that EPA-FFA 2 g daily before liver surgery was safe and well-tolerated. Analysis of resected CRCLM tissue confirmed that orally administered EPA was incorporated into target tumour tissue and was associated with a reduction in tumour vascularity as measured by CD31-positive microvessel density [8]. An intriguing preliminary observation was that EPA may provide prolonged overall survival (OS) and disease-free survival (DFS) benefit after treatment cessation following liver surgery [8].

A methodological limitation of the trial was that we were only able to obtain CRCLM tissue at surgery after EPA treatment, thus excluding any longitudinal analysis of PUFA incorporation and 'washout' in target CRCLM tissue. However, we obtained blood from participants at randomisation (baseline), the day prior to liver surgery (post-treatment), and several weeks after surgery ('washout'). Red blood cell

Abbreviations: AA, arachidonic acid; CRC, colorectal cancer; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LM, liver metastasis; OS, overall survival; PUFA, polyunsaturated fatty acid; RBC, red blood cell

☆ This work was funded by Leeds Teaching Hospitals Charitable Trustees, Yorkshire Cancer Research and Department of Health/Cancer Research UK funding of the Yorkshire Experimental Cancer Medicine Centre.

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http://dx.doi.org/10.1016/j.plefa.2016.10.003
Received 17 August 2016; Received in revised form 5 October 2016; Accepted 6 October 2016

(RBC) membrane omega-3 PUFA content is widely accepted as the best surrogate biomarker of omega-3 PUFA content in other tissues such as the heart and liver [9,10].

Therefore, we analysed the RBC membrane omega-3 PUFA content at baseline, post-treatment and at follow-up after surgery in the EMT study, allowing us to interpret individual PUFA content profiles in both active (EPA) and placebo groups, correlate RBC PUFA content with CRCLM levels, and explore the use of RBC EPA content as a predictor of survival in patients who have undergone liver surgery for CRCLM.

2. Methods

2.1. The EMT study

This Phase II randomised, double-blind, placebo-controlled trial (ClinicalTrials.gov NCT01070355) has been described in detail previously [8]. Participants were randomised to EPA-FFA 2 g taken as two gastro-resistant capsules twice daily with food for a median (range) duration of 30 (12–65) days (n=43) or placebo (capric and caprylic acid triglycerides) capsules for a median 26 (15–73) days (n=45). Blood samples were obtained at randomisation (baseline; n=87), the day prior to liver surgery (post-treatment; n=79), and approximately six weeks (EPA median [range] 47 [16–110] days; placebo 44 [21–68]) after surgery (washout; n=70). Participants were stratified by prior fish oil supplement use and/or high dietary (>2 oily fish portions per week) omega-3 PUFA intake. We have previously reported that dietary omega-3 PUFA intake did not change significantly in either treatment group during the trial as measured by a modified validated food frequency questionnaire [8]. Percentage compliance with capsules was calculated from the difference in actual and expected capsules use based on a capsule count at the trial visit immediately prior to surgery. Participants were all given capsules in slight excess of requirements and were admitted for surgery at different times after randomisation. A lower than expected capsule count before surgery is expressed as 'compliance' >100%.

2.2. PUFA measurement

Blood was collected in two EDTA-coated VACUETTE® tubes on ice and the RBC slurry was obtained after centrifugation at 700*g* for 10 min at 4 °C within three hours of venipuncture. Isolated RBCs were stored at −80 °C until fatty acid extraction.

Fatty acids were extracted from erythrocytes using the following method adapted from the protocol first published by Rose et al. [11]. Isolated erythrocytes were washed three times with 5 volumes of 0.89% Sodium chloride (NaCl), before transferring 50 μL into a 1.7 mL Eppendorf® tube. Erythrocytes were mixed with 50 μL distilled water and allowed to stand for 15 min. 550 μL isopropanol was added slowly with mixing, and following incubation for 1 h at room temperature, 350 μL chloroform was added. After a further 1 h incubation, samples were centrifuged at 10 000*g* for 5 min. The supernatant was then evaporated in a rotary evaporator and reconstituted in 500 μL acetonitrile. Hydrolysis was performed with the addition of 50 μL 5 M HCl followed by incubation at 80 °C for 1 h. 50 μL of 5 M NaOH was then added, and the sample mixed, before addition of 350 μL chloroform. The sample was left to stand for 5 min to allow the solvent layers to separate, before taking the top 800 μL and evaporating in a rotary evaporator. Extracted fatty acids were then reconstituted in 50 μL methanol prior to derivatization.

We measured FAs by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [12]. The following FAs were measured with the relative level of each PUFA expressed as the % of the total FA content: alpha-linolenic acid (C18:3ω3), EPA (C20:5ω3), docosapentaenoic acid (DPA; C22:5ω3), docosahexaenoic acid (DHA; C22:6ω3), linoleic acid (C18:2ω6), arachidonic acid (AA;C20:4ω6), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1ω9). Tumour tissue PUFA content was measured previously and has been reported in the EMT study publication [8].

2.3. Statistical analysis

PUFA data are quoted as the mean (±standard error of the mean [SEM]) relative (%) amount of each PUFA compared with the total fatty acid content measured by LC-MS/MS. Individual absolute and fold changes in PUFA content over time were compared using the one-sample *T* test. Pairwise differences in relative PUFA content over time were analysed by the paired-sample T test. Data that were not normally distributed were analysed by the Mann Whitney U test. Pearson's correlation test was used to explore the relationship between continuous variables including RBC and tumour % PUFA content. Comparison of OS in trial participants with a % RBC EPA level or 1.22 (the lowest post-treatment % EPA level in the EPA group) was performed using Kaplan-Meier survival analysis and log rank test.

3. Results

3.1. Time course of RBC EPA incorporation and 'washout' in CRCLM patients

Baseline % RBC PUFA levels in patients with CRCLM are displayed in Fig. 1. There was a rise in RBC EPA content in patients randomised to EPA-FFA treatment with a mean absolute 1.26 (±0.14 [SEM])% increase during treatment ($P < 0.001$, one-sample T test; Figs. 1A and 2A), which was not observed in the placebo group (−0.03 ± 0.04%; Figs. 1A and 2B). This equated to a mean 2.40 (±0.16)-fold increase in RBC EPA content during EPA treatment (placebo 1.02 ± 0.04 fold change). There was also a smaller increase in RBC DPA content (mean 0.60 ± 0.18[SEM] absolute increase; $P < 0.05$; Figs. 1B and 2C). A small, but statistically significant, reduction in relative DHA content in RBC membranes (mean absolute 0.31 ± 0.24% reduction) was also seen following EPA treatment (Fig. 1C). There was a concurrent, statistically significant decrease in omega-6 PUFA AA content (mean absolute 1.31 ± 0.57% reduction) in those individuals receiving EPA ($P < 0.05$; Figs. 1D and 2G). The EPA/AA ratio is widely quoted as a predictive biomarker of omega-3 PUFA activity, particularly in cardiology studies [13]. There was a mean absolute increase in EPA/AA ratio of 0.12 (SEM 0.01) at the end of the intervention period in those allocated to EPA with RBC EPA/AA ratio values at the end of the intervention period ranging between 0.09 and 0.37 (mean 0.21).

There was no overall change in % RBC content of any PUFA in the placebo group (Figs. 1 and 2B, D, F and H). For each PUFA, there was significant, but incomplete, reversal of the changes in PUFA content related to EPA treatment during the post-intervention 'washout' period (Figs. 1 and 2A, C, E and G). A persistent, small elevation in RBC EPA and DPA content was observed at six weeks after cessation of EPA treatment (Fig. 1A and B), along with reduced AA content (Fig. 1D).

Individual RBC PUFA content profiles varied significantly between individuals in both EPA and placebo groups (Fig. 2A–H). Previous fish oil supplement users (who were required to stop this for the duration of the trial) and those with high dietary omega-3 PUFA intake (n=23) had a significantly higher baseline % RBC EPA content (1.39 ± 0.15%) than participants 'naïve' to both (0.97 ± 0.04%; n=64; P=0.003). The four participants in the placebo arm with the highest baseline % EPA content (all >2%) were all fish oil supplement users or those classified as having a high dietary omega-3 PUFA intake (Fig. 2B). All these individuals that supplied blood samples after baseline displayed a reduction in RBC EPA content during the intervention period consistent with adherence to the protocol, which demanded cessation of existing omega-3 PUFA supplement use (Fig. 2B). Thirty-three out of 36 (92%) participants, who received EPA and for whom data from all three time-points were available, demonstrated an increase in RBC EPA content during the intervention period, which then reduced after

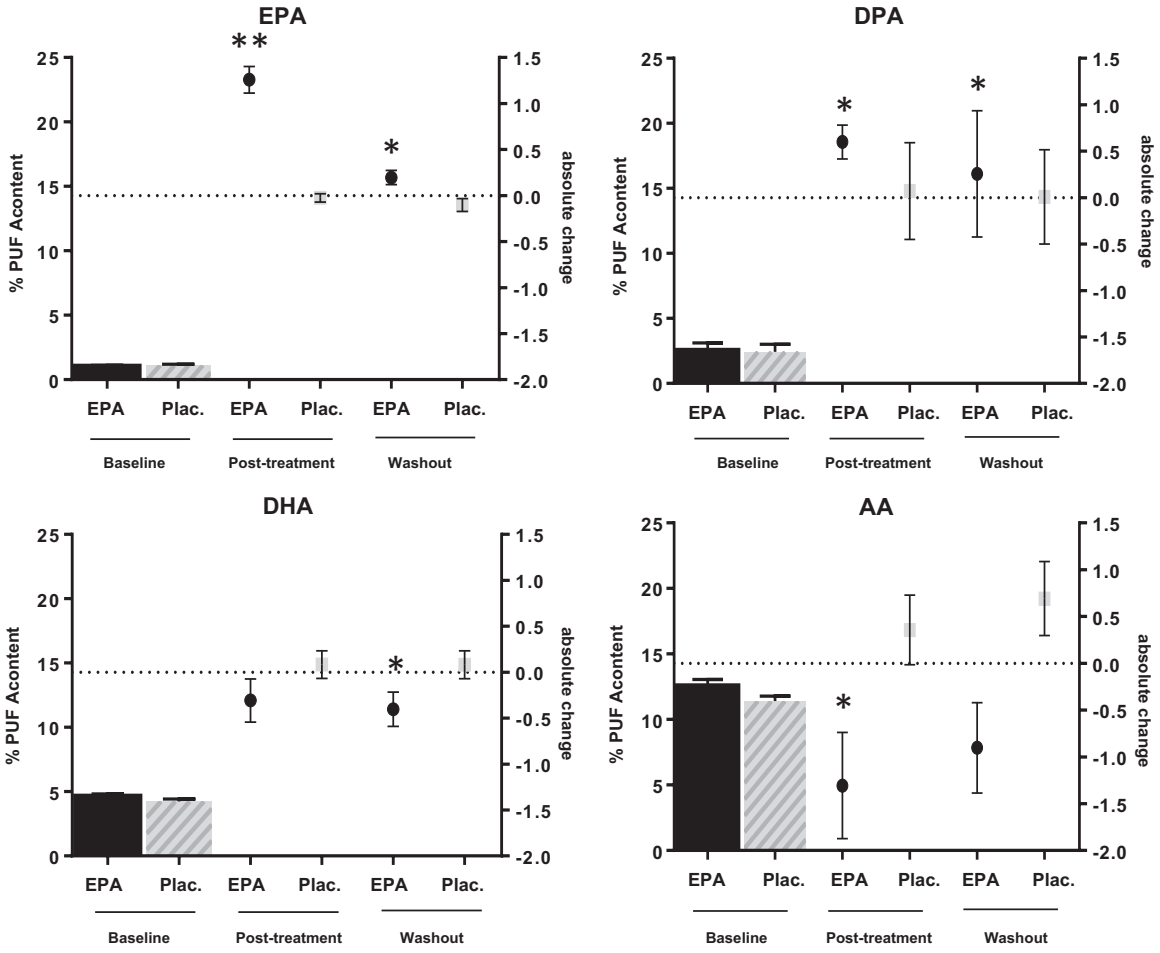

Fig. 1. Baseline % RBC PUFA level and absolute difference in % RBC PUFA level between baseline and post-treatment or after surgery (washout). In each case (A–D), the left y axis is the baseline % RBC PUFA value and the right y axis is the absolute difference between the post-treatment value or 'washout' post-operative value and baseline % level. Columns (baseline % values) and symbols (absolute difference in % value from baseline) denote the mean for EPA and placebo (Plac.) groups. Error bars denote the standard error of the mean.*P < 0.05, **P < 0.001; one-sample *t* test.

treatment cessation (Fig. 2A). This suggests excellent compliance with trial treatment. However, the magnitude of the EPA 'response' varied widely with only 20 individuals displaying a %EPA RBC content of greater than 2% (Fig. 2A).

There was no statistically significant correlation between the % RBC EPA content at the end of the intervention period and the duration of EPA treatment (r=0.25; P=0.12; Fig. 3A). The post-treatment absolute EPA level attained in females (mean 2.68 ± 0.23%; n=16) was greater than in male participants (mean absolute 2.07 ± 0.0.14%; n=23) in the EPA group (P=0.02). There was no difference in baseline or post-intervention RBC PUFA levels in concurrent aspirin users (n=9) compared with non-users in the EPA group (data not shown). 'Compliance' with capsules measured by 'pill counting' in both active and placebo groups was good and has been reported previously [8]. Mean compliance in the EPA group was 89.5 ± 2.9% (range 40.9–116.7). There was no significant correlation between % compliance and post-treatment % RBC EPA level (r=0.09; P=0.59) in EPA users. Multivariate linear regression inputting gender, treatment duration and compliance confirmed that gender alone (beta −0.35, P=0.039) remained the only significant predictor of post-treatment RBC EPA level in the EPA treatment group.

Of note, the individual with the largest increase in RBC EPA content (gold symbols and line) also demonstrated a marked increase in DPA and DHA, but also AA (Fig. 2A, C, E and G). The most likely explanation is 'contamination' by concurrent use of a mixed omega-3 and omega-6 PUFA supplement, many of which are available commercially. Only one individual allocated to placebo demonstrated an increase in RBC EPA content over time (yellow crosses and line), also suggesting possible 'contamination' by 'own' omega-3 PUFA use.

3.2. Relative RBC EPA content predicts the CRCLM tissue EPA level

Measurement of the % RBC EPA content the day before surgery allowed a pairwise comparison of RBC EPA content and the corresponding tumour EPA level at CRCLM resection (Fig. 3B). There was a significant correlation between the % RBC EPA level just prior to surgery and the CRCLM EPA content in those participants who received EPA (r=0.35, P=0.03) and placebo (r=0.72; P < 0.001: Fig. 3B). Interestingly, those individuals in the placebo group with relatively high % RBC EPA content, as a consequence of prior omega-3 PUFA use or high dietary intake, had a correspondingly high tumour EPA content (Fig. 3B).

3.3. RBC EPA incorporation and long-term clinical outcomes

Long-term clinical outcomes were an exploratory end-point in the Phase II EMT study [8]. A preliminary finding was that those patients who were randomised to EPA treatment had OS benefit compared with placebo, although the difference was not statistically significant in this small randomised trial [8]. On the basis that the lowest post-treatment % RBC EPA level attained in the EPA group was 1.22%, which is higher than all the respective placebo group values except for 10 individuals (6 of whom had high baseline levels due to significant prior dietary or supplement omega-3 PUFA exposure), we compared OS in individuals

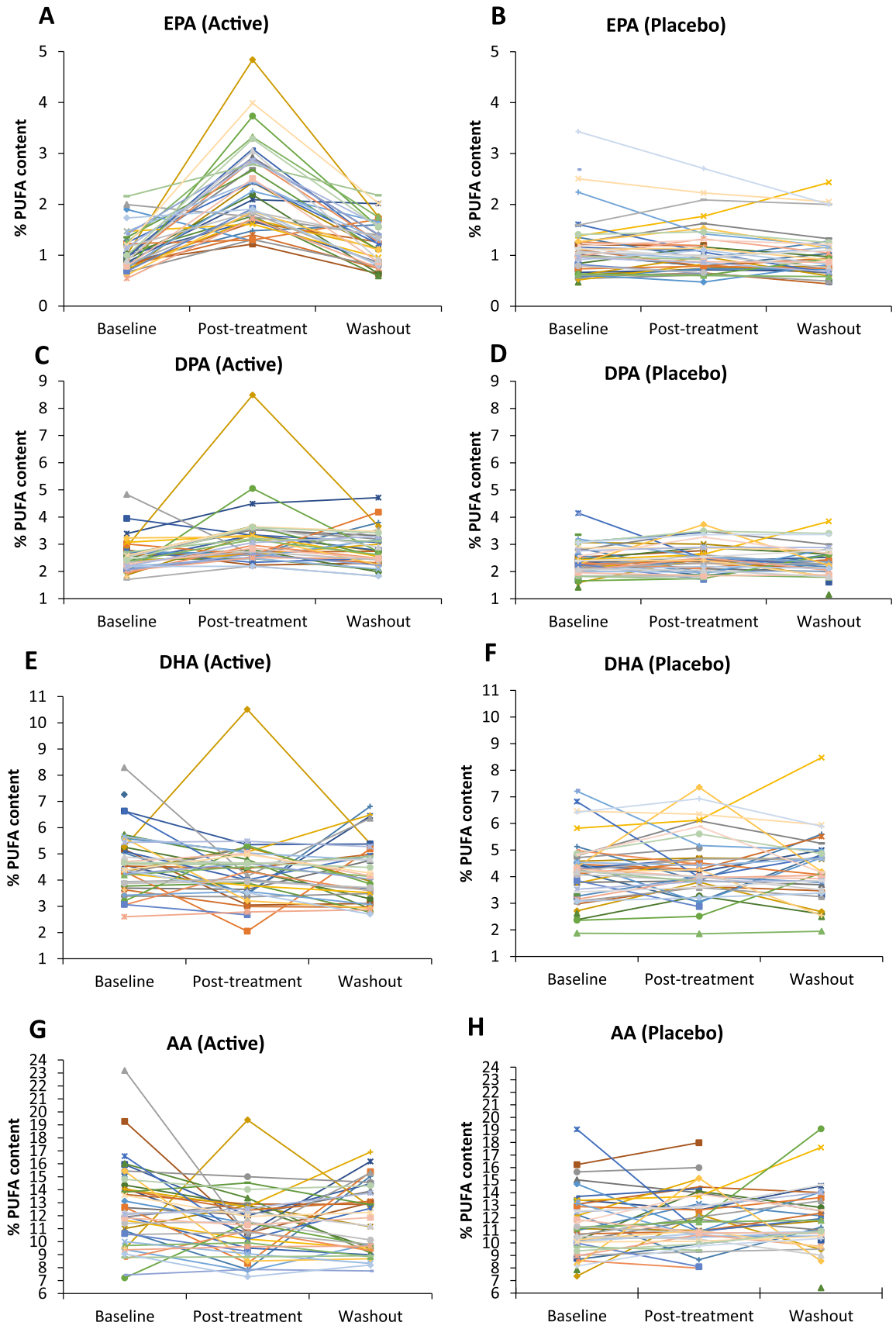

Fig. 2. Individual % RBC PUFA profiles for individuals randomised to either EPA (A, C, E, G) or placebo (B, D, F, H). Different coloured lines join data points for all individuals.

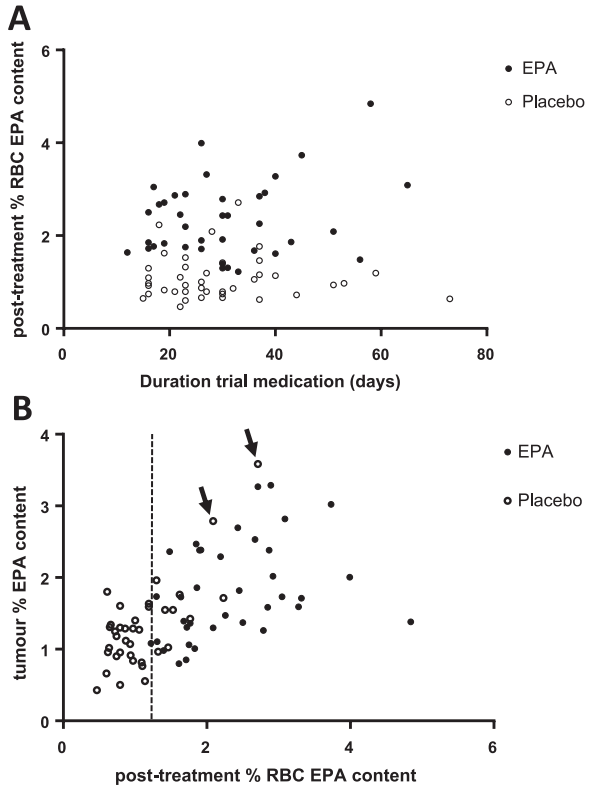

Fig. 3. Relationship between the % RBC EPA level at the end of the trial intervention and either treatment duration (A) or tumour EPA content at surgery (B). Open symbols denote individual data from the placebo group and filled symbols denote EPA group data. B) The dashed line denotes the RBC % EPA 'cut-off' (1.22 for survival analysis). Arrows denote examples of individual patient data in prior omega-3 PUFA supplement users allocated to placebo, who had high tumour % EPA content.

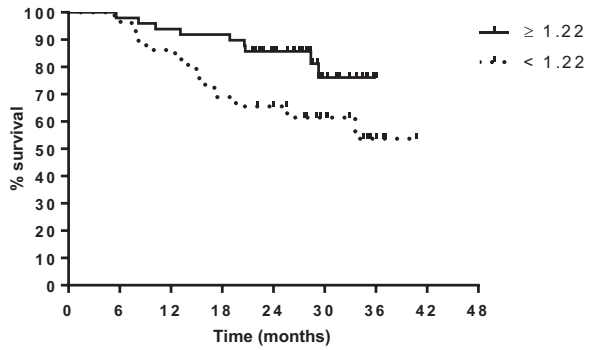

Fig. 4. Overall survival analysis of CRCLM patients stratified on the basis of post-treatment % RBC EPA level, not treatment allocation. The cut-off of 1.22% was based on the lowest value indicative of an EPA 'response' (evidenced by an increase in EPA level from baseline to post-treatment measurement and then 'washout') in the EPA group. Vertical ticks denote censored cases. Solid line denotes events in EPA 'incorporators' (post-treatment RBC % EPA level≥1.22%. Dashed line denotes events in EPA 'non-incorporators' (post-treatment RBC % EPA level < 1.22%. Log rank P=0.04.

with a post-treatment % RBC EPA content of ≥1.22% with those with a post-treatment RBC EPA content of < 1.22%, regardless of treatment allocation in the trial. Participants with a post-treatment % RBC EPA content of ≥1.22% (n=49) demonstrated improved OS compared with those with a RBC EPA level < 1.22% (n=29; Fig. 4). The hazard ratio was 0.42 (95% confidence interval 0.16–0.95) with a log rank P value of 0.04.

4. Discussion

This is the first time that individual RBC and tumour PUFA content values have been analysed together in a randomised, placebo-controlled trial of an omega-3 PUFA in cancer patients. We measured % RBC omega-3 PUFA content as the biomarker of choice, with least within-subject variability, for assessment of omega-3 PUFA 'bioavailability' in a clinical study lasting several weeks [9,10].

We report that RBC EPA incorporation predicts CRCLM EPA content and therefore has potential as a surrogate biomarker of short-term target organ EPA exposure, at least in CRCLM. Consistent with this finding, RBC and cardiac/breast tissue EPA content correlate strongly after omega-3 PUFA supplementation in humans [14–16], and RBC omega-3 PUFA levels correlate with omega-3 PUFA content in multiple mouse and rat tissues after dietary supplementation [17,18].

Baseline and post-treatment RBC PUFA levels in EMT study participants were similar to those reported previously in studies of omega-3 PUFA supplementation in healthy volunteers [13,19], women with breast hyperplasia [20,21] and in patients with lung cancer [22]. In particular, post-treatment % RBC EPA values were similar to those observed in an eight-week study of exactly the same EPA-FFA formulation and dose in healthy volunteers and inflammatory bowel disease patients [23]. In keeping with previous studies of pure EPA treatment, there was some evidence of elongation of EPA to DPA leading to a small, statistically insignificant increase in RBC DPA incorporation [8,23,24]. However, there was no evidence of DHA incorporation in RBC membranes, with an actual small reduction in % DHA content in EPA users, unlike the previous study with EPA-FFA [23]. This may be explained by the shorter duration of EPA supplementation in the EMT study, which may lead to displacement of membrane DHA by excess EPA-FFA, prior to *de novo* synthesis of DHA from EPA. Alternatively, it is understood that excess EPA may saturate the second *ELOVL2* elongase reaction necessary for DPA to DHA conversion, which could lead to a reduced relative DHA content [24].

The literature on omega-3 PUFA supplementation in healthy volunteers and patient groups would suggest that omega-3 PUFA incorporation is associated with a reciprocal reduction in membrane content of the omega-6 PUFA counterpart to EPA, AA [19–21,23,25,26]. We also observed this phenomenon in the EMT study, despite the relatively short duration of EPA supplementation.

Overall, there was consistent and uniform, but incomplete, EPA 'washout' over the relatively short time period between surgery and post-operative follow-up mandated in the trial. Longer-term follow-up would have been required in order to confirm the return of the PUFA profile in RBCs to that observed pre-intervention. 'Washout' kinetics were not dependent on the prior degree of RBC incorporation or duration of EPA treatment and are similar (in these CRC patients) to the decay in RBC EPA content observed in at least three healthy volunteer studies, in which peak RBC EPA content was similar to that seen in the EMT study [13,19,27], as well as two more recent studies of pre- and post-menopausal women [20,21].

Individual omega-3 PUFA profiles allowed us to investigate inter-patient variability in EPA incorporation in CRC patients. There was significant heterogeneity in the increase in EPA content of RBC membranes in response to EPA treatment, which has previously been noted in intervention studies of daily supplementation with combined EPA and DHA formulations (measured as the omega-3 index) in atherosclerotic patients [28,29], but not cancer patients. This is in contrast to the more uniform incorporation of EPA into RBC membranes observed in the healthy volunteer study of EPA-FFA by Scaioli et al. [23]. A similar degree of inter-individual variability in plasma phospholipid DHA level was observed in a DHA intervention study in breast cancer patients [30].

There was a small but statistically significant difference in RBC EPA incorporation in women compared with men in the EPA group, which remained even after adjustment for treatment duration and compliance. A meta-analysis of gender differences in omega-3 PUFA levels in RBC membranes has previously reported a significantly higher DHA,

but not EPA, content in RBCs in women compared with men [29]. However, a previous supplementation study, which undertook a gender-specific analysis, reported on gender difference in omega-3 index [31].

Individual omega-3 PUFA profiles also highlighted possible cases of 'contamination' by prior or concurrent omega-3 PUFA intake separate from the Investigational Medicinal Product. This was most easily observed in several instances in the placebo arm, in which a concurrent increase in DHA (but not DPA) suggested use of a combined EPA/DHA omega-3 PUFA formulation in addition to placebo. 'Contamination' by extra omega-3 PUFA use may also explain the high EPA and DHA 'outlier' in the EPA arm of the trial although the concurrent high RBC DPA content may signal that this individual was, in fact, capable of extremely efficient EPA-DPA-DHA conversion. There is increasing understanding of the genetic basis of inter-individual variability in omega-3 PUFA interconversion, underlying which genetic polymorphisms in the *FADS1-FADS2* cluster and *ELOVL2/5* genes modulate desaturase and elongase activities [32].

Placebo group contamination is an ever-present risk in a randomised trial of any intervention available 'over the counter' [33], particularly when fish oil use is so widespread (7.8% of US adults in 2012 [34]). However, contamination by omega-3 PUFA use in the placebo arm (suggested by an increase in omega-3 PUFA RBC content during the intervention period) appeared relatively low, occurring in perhaps 4 (9%) of 45 individuals allocated to placebo, and possibly in a single case (2%) in the EPA group. Therefore, contamination by additional omega-3 PUFA use is unlikely to have affected significantly the laboratory and clinical endpoints of the EMT study.

There has been only one previous randomised omega-3 PUFA intervention trial in cancer patients that has reported individual omega-3 PUFA levels during treatment [6] and none that have included an analysis of omega-3 PUFA 'washout' after cessation of omega-3 PUFA supplementation. Van der Meij et al. described variability in plasma phospholipid EPA levels after double-blind intervention for 5 weeks with a daily supplement containing 2 g EPA and 0.9 g DHA or isocaloric control in patients with non-small cell lung cancer [6]. These authors obtained similar individual EPA profiles to the EMT study with a number of 'non-responders' in the intervention arm and, conversely, some individuals with high baseline EPA values or an 'EPA response' in the control arm of the study [6]. We suggest that contamination is likely to occur in all trials of omega-3 PUFA supplements with individual randomisation and can be, at least partly, adjusted for by analysis by absolute or relative change in RBC omega-3 PUFA levels.

To the best of our knowledge, survival analysis based on EPA incorporation in RBCs, as a surrogate biomarker of tumour EPA exposure, has not been reported previously. Despite the relatively small sample size of the EMT trial, there was a statistically significant difference between individuals dichotomised on the basis of the RBC EPA content at the end of the intervention period (irrespective of the trial treatment allocation). Effectively, this moved those individuals in the placebo group with relatively high RBC EPA levels (explained mainly by high dietary marine omega-3 PUFA intake or prior supplement use) into the active (EPA) group. A methodological weakness of this approach is the rather arbitrary cut-off value, which, in this case, was selected as the lowest relative RBC EPA level in an 'EPA-responder' (increase in EPA content during intervention and then reduction during 'washout'). However, the EPA value used (1.22%) was similar to equivalent values used in previous cohort and case-control studies of the association between omega-3 PUFA levels and CRC risk [35], in particular the study of Cottet et al. which measured RBC EPA content [36]. Moreover, in the absence of a widely recognised, standardized method for measurement of RBC fatty acid content, we used a LC-MS/MS method, thus restricting future comparison with other studies.

We suggest that a so-called 'contamination-adjusted' secondary analysis [7] should be undertaken in future randomised, double-blind trials of omega-3 PUFAs in which PUFA levels are measured. Such an approach was recently taken in the WELCOME study of non-alcoholic fatty liver disease patients, in which placebo- and active-group contamination occurred to a similar extent to the EMT study [3].

In conclusion, we report significant variability in RBC omega-3 PUFA profiles during a randomised trial of EPA in patients with CRCLM awaiting liver resection. There was evidence of contamination by 'own use' omega-3 PUFA in only a small proportion of participants. There was a significant correlation between the RBC EPA content and CRC liver metastasis EPA level measured within 24 h of each other in this EPA intervention trial, implying that the RBC omega-3 PUFA content may reflect tumour omega-3 PUFA exposure, at least in CRCLMs.

A fascinating preliminary observation is that OS analysis based on post-intervention RBC EPA content, rather than treatment allocation, suggested OS benefit in those individuals with a high relative EPA level. This finding adds further to the rationale for a phase III omega-3 PUFA intervention trial in CRCLM patients. Consistent with the hypothesis that EPA therapy may provide survival benefit in this group of patients, Song et al. have recently reported that higher marine omega-3 PUFA intake is associated with reduced post-diagnosis CRC mortality in two US cohort studies [37].

Conflict of interest statement

Mark Hull has received a travel grant and unrestricted scientific grant from SLA Pharma AG. Mark Hull has received an unrestricted scientific grant from Smartfish®. Mark Hull is a member of the Scientific Advisory Board for Thetis Pharmaceuticals LLC. None of the other Authors declares a potential Conflict of Interest.

Author contributions

AJC, GJT and MAH designed the EMT study and PUFA analysis; HW, JS, AR, MV and PML conducted the research; HW and MAH analysed data; HW and MAH wrote the manuscript; MAH has primary responsibility for final content. All Authors read and approved the final manuscript.

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