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Assessment of Plasma Antioxidants, Oxidative Stress and Polyunsaturated Fatty Acids in Paediatric Cancer Patients: A Prospective Cohort Pilot Study

Revuelta Iniesta R[1,2], Wilson DC[2,3], Brougham MFH[4], Smail NF[1], Davidson I[1] and McKenzie J[1]

[1]*Dietetics, Nutrition and Biological Health Sciences, Queen Margaret University, Edinburgh, United Kingdom*

[2]*Child Life and Health, University of Edinburgh, Edinburgh, United Kingdom*

[3]*Department of Paediatric Gastroenterology and Nutrition, Royal Hospital for Sick Children, Edinburgh, United Kingdom*

[4]*Department of Haematology and Oncology, Royal Hospital for Sick Children, Edinburgh, United Kingdom*

***Corresponding Author:** Raquel Revuelta Iniesta, Department Dietetics, Nutrition and Biological Science Queen Margaret University, Queen Margaret University Drive, Edinburgh EH21 6UU, United Kingdom.

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Abstract

Background: Paediatric cancer patients may have a limited dietary intake, particularly nutrients high in antioxidants, docosahexanoic acid (DHA) and eicosapentanoic acid (EPA).

Objective: To investigate the antioxidant status (TAS), antioxidant capacity (TAC), oxidative stress, DHA and EPA of paediatric cancer patients during treatment.

Methods: A prospective cohort study of Scottish children aged <18 years, diagnosed with and treated for cancer between April-2013 to Jan-2014 was performed. Clinical data and blood samples were collected at baseline and 6 months. Data were stratified by treatment risk (low, medium and high) and nutritional support. We used Oxygen Radical Absorbance Capacity (ORAC) Antioxidant Assay to measure TAC, thiobarbituric acid reactive substances (TBARS) for lipid peroxidation and high performance liquid chromatography and Inductively Coupled Plasma Mass Spectrometry for TAS. The analyses of DHA and EPA were performed by analysing fatty acid methyl esters (FAME) using gas-liquid chromatography. The reference ranges used were: Yagi 1998 (1.86-3.94) µmol for lipid peroxidation and Damsgaard., *et al*. 2014 for EPA (0.45-0.77) % and DHA (2.22-3.76) %.

Results: 20 patients (median (IQR) age 4.2 (1.5-8.5) years; 50% males) were recruited. There were no significant changes in plasma TAS, TAC and EPA, but lipid peroxidation significantly decreased from 7.4 (6.2-9.0) at baseline to 5.3 (4.5-6.4) µmol/MDA at 6 months ($p = 0.003$). The median (IQR) blood percentage of DHA significantly increased from 1.3 (0.9-1.9) to 1.8 (1.3-2.1) ($p = 0.001$). Lipid peroxidation was high in 95% (19/20) of patients at baseline and 94% (15/16) at 6 months; whilst DHA and EPA were low in 95% (19/20) and 70% (14/20) at baseline and 87.5% (14/16) and 60% (12/16) at 6 months. Children on high-treatment risk exhibited the highest oxidative stress levels. No statitically significant differences were found between non-supplemented and supplemented children in any of the following parameters (TAS, TAC, oxidative stress, EPA and DHA).

Conclusion: There was a high prevalence of oxidative stress, especially in children treated with high-risk protocols and during the initial phases of treatment. Nutritional support does not appear to provide enough TAS, EPA and DHA in this cohort; however, larger high-quality population based studies are warranted to confirm these findings.

Keywords: *Paediatric cancer; Antioxidants; Oxidative stress; Docosahexanoic acid; Eicosapentanoic acid*

Abbreviations: AA: Arachidonic Acid; ALL: Acute Lymphoblastic Leukaemia; BMI: Body mass index; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; FAME: Fatty acid methyl esters; FR: Free radicals; HPLC: High performance liquid chromatography; MDA: Malondialdehyde; ORAC: Oxygen Radical Absorbance Capacity; PUFA: Polyunsaturated fatty acids; ss-CRP: Standard sensitivity C-Reactive Protein; TAC: Total antioxidant capacity; TAS: Total antioxidant status; TBARS: Thiobasbituric Acid Reactive Substances; TE: Trolox equivalents

Introduction

Survival rates of paediatric cancer patients have improved considerably in the last 40 years with the implementation of more intensive and progressive treatments. Consequently, attention is shifting to the reduction of treatment related side-effects during and after completion of therapy [1]. At present, there is a great deal of interest in the benefits of antioxidants and polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), on health. Both have been demonstrated to possess anti-inflammatory properties and may therefore protect against endothelial cell senescence (aging) [2], early cardiovascular disease (CVD), neurodegenerative disorders and cancer [3,4], of which survivors of paediatric cancer are at increased risk compared to the general population [1,5].

Free radicals (FR) at low/moderate concentrations are essential for normal body functions [6]. FR are involved in cell signaling, are bactericidal in response to infection and are also secreted as by-products in mitochondrial respiration playing an essential role in homeostasis [6]. Cancer generates high levels of FR induced by an inflammatory response. In addition some cancer treatments including chemotherapy, such as alkylating agents and anti metabolites, and radiotherapy generate FR to induce cancer-cell apoptosis [3]. This in turn may lead to oxidative stress [7] by promoting lipid peroxidation, oxidative mediation of proteins and by damaging nuclear and mitochondrial DNA [8,9]. Excess production of FR damages both cancer cells and healthy tissues [10]. Some authors [10-13] have reported that higher levels of total antioxidant status (TAS) and total antioxidant capacity (TAC), which is the cumulative action of the antioxidants present in plasma [11], may be beneficial and counteract some of the toxic effects of FR, thus reducing treatment related side-effects and improving clinical outcome in children with ALL.

The polyunsaturated fatty acids omega-3 (ω-3 PUFA) and omega-6 (ω-6 PUFA) are essential as they need to be supplied from the diet [14]. Linoleic acid, the most abundant ω-6 PUFA in the western diet, is a precursor of arachidonic acid (AA). AA is a potent precursor of inflammatory markers [14] whereas, the main ω-3 PUFAs are DHA, EPA and α-linolenic acid (14). Both DHA and EPA are anti-inflammatory fatty acids [14,15]. A balance between ω-3 PUFA and ω-6 PUFA is necessary for homeostasis of the immune system and optimal body and brain development. This is particularly important in younger children [16]. Supplementation of ω-3 PUFA, particularly DHA, has been shown to induce neuroblastoma cell death in vitro [17] and in vivo [16]; whilst, excessive AA may promote tumour promotion and progression [14,15].

Despite the potential protective role of antioxidants and ω-3 PUFA against the development of CVD, neurodegenerative disorders and cancers, until now no studies have investigated the profile of TAS, TAC and markers of oxidative stress as well as plasma PUFA levels in paediatric cancer patients. In addition, nutritional profile in current clinical practice usually only consists of growth assessment, routine biochemical and haematological blood tests. However emerging evidence suggest that plasma TAS, TAC, markers of oxidative stress and lipid levels may provide a further indicator of nutrient adequacy and nutritional functional markers [3,16,18,19]. Therefore, the aims of this study were: (i) to assess the TAS, TAC and oxidative stress as well as the lipid profile in paediatric cancer patients at diagnosis and 6 months into treatment; (ii) to establish whether there were any changes in these parameters following 6 months of treatment (iii) and whether there were any differences between children who were on nutritional support compared to those who were not.

Methods

A prospective cohort pilot study was performed. The eligibility criteria were: (i) children aged < 18 years; (ii) diagnosed with cancer (ICCC-3) [20] or Langerhans Cell Histiocytosis between April-2013 to Jan-2014; (iii) attending the South East Scotland regional centre for Haematology and Oncology at the Royal Hospital for Sick Children (RHSC), Edinburgh for treatment. We excluded children who were

treated with palliative intent from diagnosis and discontinued monitoring those whose care was re-orientated to palliation during the study period, out of respect for the patients and their families. Two measurements were taken at different time points; one at initial diagnosis and again at 6 months into treatment (± 3 months).

The following clinical data was collected: diagnosis, treatment intensity stratified according to Kazka., *et al*. [21] into low, medium and high risk (intensity) [21] and length of treatment. Biochemical blood parameters (standard sensitivity C-Reactive protein (ss-CRP)) and demographic data (age, gender, ethnicity and socio-economic deprivation) were also collected from the medical notes. As a proxy marker for socioeconomic deprivation, we used individual residence postcodes to assess deprivation level of areas of residence using the Standard Index of Multiple Deprivation (SIMD) [22].

The paediatric cancer cohort was grouped according to the wider definition of solid tumours, haematological cancers, brain tumours and other associated diagnoses. Height (or length) and weight were measured using standard procedures (RRI). BMI centile was calculated and UK BMI growth centiles were used. Nutritional status was classified as underweight (BMI ≤ 2.3th centile) and overweight (BMI ≥ 85th) [23].

Blood collection, procedure and analysis of samples

TAS was assessed by measuring vitamin A, vitamin E/Cholesterol (E/Ch) and the trace metals zinc, copper and selenium. Blood samples were collected by trained nurses. High performance liquid chromatography (HPLC) with UV detection was used to measure vitamin A and E/Ch and Inductively Coupled Plasma Mass Spectrometry was used for trace metals. All blood samples were measured in the Royal Infirmary Laboratory, Glasgow by their own standard operating procedures.

To analyze TAC levels and oxidative stress blood samples were collected in 1.3 ml HEPARIN tubes, centrifuged at 1600 ppm for 15 minutes at 4°C. The plasma was then stored at -80°C until analyses were performed. TAC was analyzed using the Oxygen Radical Absorbance Capacity (ORAC) Antioxidant Assay [8,9] and the results were expressed in µmol of Trolox equivalents (TE) per gram (µmol TE/g) [8,9]. Oxidative stress was analyzed measuring Thiobarbituric Acid Reactive Substances (TBARS) with a TBARS Assay Kit (Cayman, USA). The results are an indicator of lipid peroxidation and thus oxidative stress [24]. The total lipid peroxidation is quantified in terms of malondialdehyde (MDA) per µmol and the within individual runs coefficient of variation were 0.9-1.7% for ORAC and 1.8-3.3% for TBARS [24,25].

To measure DHA and EPA levels, 50 µl of blood was dropped onto a Guthrie card. This was then placed in individual foil pockets and stored at -20°C. The analyses of DHA, EPA and AA were then performed by analysing fatty acid methyl esters (FAME) using gas-liquid chromatography at the University of Stirling laboratory facilities [26]. Results were expressed as a percentage of the total plasma lipid profile and the ratio between AA:EPA was calculated.

The reference ranges for lipid peroxidation was 1.86-3.94 µmol [25,27] and for EPA (0.45-0.77), DHA (2.22-3.76), AA (7.91-10.46) and AA:EPA (> 14.59), which were adjusted for both girls and boys and expressed as a percentage of whole blood total fatty acids [28]. The reference ranges published by NHS Scotland Laboratory Handbook were used for TAS and no reference ranges exist for antioxidant capacity (ORAC).

Dietary intake and nutritional support

TAS from dietary intake was assessed by using a 24-hour multi-pass recall method and analyzed using WinDiets® (Univation Ltd 2005) [29]. Information on nutritional treatment was recorded and this was prescribed according to Subjective Global assessment and consisted of enteral +/- parenteral nutrition (macronutrient), micronutrient supplementation, and a combination of macronutrients and micronutrients.

Statistical analyses

The Statistical Package for Social Science (IBM-SPSS for Windows Statistics, version 19) was employed to analyze all data. Descriptive statistics were used to evaluate TAS, TAC, oxidative stress (lipid peroxidation) and lipid profiles (DHA, EPA and) at baseline and at 6 months. Wilcoxon-Signed Rank Test was used to establish changes from baseline to 6 months in these variables (aim i). Associations between TAS (vitamin A, vitamin E/Ch, Zinc, Copper and Selenium), TAC (ORAC) and oxidative stress and inflammation (TBARS, ss-CRP) were performed using Spearman's correlation (aim ii); and univariate associations between treatment risk and whether the patients were on nutritional support were established by $\chi 2$-test (aims ii and iii). Mann-Whitney test was used to establish differences in TAS, TAC and oxidative stress, and also in fatty acids profiles between children who were on nutritional support and those who were not (aim iv). Finally Spearman's correlation were used to established correlations between antioxidant intakes (vitamin A, vitamin E, Zinc, Copper and Selenium) and TAS, TAC and oxidative stress (aim iv). $P < 0.05$ was considered statistically significant. We followed the STROBE guidelines for the presentation of the data (www.strobe-statement.org).

This study was granted ethical approval from NHS Scotland (NHS REC 06-51104-52) and all patients' data were anonymised and kept confidential.

Results

Demographic and clinical characteristics

Of the 25 eligible patients, 2 refused to participate and 3 were excluded due to insufficient samples. Thus 20 paediatric cancer patients had TAS, TAC (ORAC), oxidative stress (TBARS) and PUFA (DHA, EPA and AA) measured at baseline. Of these, 16 were also collected at six months as 4 were unavailable due to the following reasons: treatment given outside reach (Birmingham) (n = 2) and no-routine bloods performed at the time of measurements (n = 2), thus precluding study bloods being taken as per ethical approval. The baseline demographic and clinical characteristics of the population are presented in table 1. Twelve different treatment protocols were used to treat this cohort, the median (IQR) time from the time of diagnosis until baseline measurements was 15.5 (11.0-21.5) days and from baseline to the second measurement at 6 months (+/- 3 months) was 101 (72.2-160) days. BMI centile expressed as median (IQR) increased from 47.5 (18.5-71.2) at baseline to 54.0 (23.5-73.2) at 6 months; however this was not significant (p = 0.7).

Paediatric cancer cohort	Median	IQR
Age at diagnosis (years)	4.2	1.5-8.5
	n	%
Gender		
Male	10	50
Female	10	50
Ethnicity		
White	18	90
Non-white	2	10
SIMD		
I	4	20
II	1	5
III	4	20
IV	8	40
V	3	15
Diagnostic criteria		

Solid tumours	6	30
Haematological malignancies	12	60
Brain tumours		
(low grade Glioma)	1	5
OAD (LCH)	1	5
Treatment Risk		
Low risk	7	35
Medium risk	5	25
High risk	8	40

Table 1: *Demographic and clinical characteristics of the paediatric cancer cohort (n = 20) at baseline.*
LCH: Langerham's cell Histiocytosis; SIMD: Standard Index of Multiple Deprivation presented as a quintile where "I" denotes the most deprived and "V" the least deprived.

Plasma antioxidants, oxidative stress and PUFA levels of paediatric cancer patients

Changes from baseline to 6 months in plasma TAS, TAC and PUFA levels are presented in table 2. Vitamin A levels were within the normal range in 76.5% (13/17) of patients at baseline and 94% (15/16) at 6 months, whilst 12% (2/17) had hypovitaminosis A at baseline and 6% (1/16) at 6 months. 12% (2/17) of patients had hypervitaminosis A at baseline and none at 6 months. Vitamin E/Ch levels were within the normal range in 94% (16/17) of patients at baseline and in 100% (16/16) at 6 months, whilst 6% (1/16) had hypervitaminosis E/Ch at baseline. The prevalence of high lipid peroxidation was 95% (19/20) at baseline and 94% (15/16) at 6 months (normal range: 1.86-3.94 µmol/MDA) [25,27].

Plasma levels	Baseline		6 months		P value[1]
	Median	IQR	Median	IQR	
Vitamin A (µmol/L)	0.85	0.7-1.3	1.2	0.87-1.42	0.2
Vitamin E/Ch (µmol/L)	6.05	4.6-7.0	5.6	4.9-6.8	0.9
Copper (µmol/L)	16.6	10.9-21.0	16.8	14.1-18.3	1
Selenium (µmol/L)	0.97	0.8-1.4	0.9	0.8-1.1	0.3
Zinc (µmol/L)	10.4	8.9-20.2	10.7	9.1-13.0	0.4
ORAC (µmol TE/g)	83.0	71.5-89.0	90.2	74.4-97.3	0.3
TBARS (MDA µmol)[2]	7.40	6.20-9.00	5.30	4.50-6.40	0.003
CRP mg/L	4.0	1.0-8.0	1.0	1.0-4.0	0.1
EPA (20 5n-3) %	0.4	0.3-0.5	0.4	0.3-0.6	0.6
DHA (22 6n-3) %	1.3	0.9-1.9	1.8	1.3-2.1	0.001
AA (20 4n-6) %	6.0	5.4-6.8	7.3	5.5-8.1	0.05
AA/EPA ratio (%)	17.1	10.5-22.2	18.7	13.3-25.0	0.3

Table 2: *Plasma antioxidants, oxidative stress and PUFA of paediatric cancer patients at baseline and at 6 months.*
AA: Arachidonic acid; CRP: C-reactive protein; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; ORAC: Oxygen Radical Absorbance Capacity; TBARS: Thiobarbituric Acid Reactive Substances;
[1]Wilcoxon-Signed Rank Test;
[2]TBARS reference range for human plasma lipid peroxidation level is 1.86 -3.94 uM in terms of MDA.

Most paediatric cancer patients had plasma PUFA levels below the reference ranges [28]. The prevalence of patients with low EPA (< 0.45% of total whole blood weight) was 70% (14/20) at baseline and 60% (12/16) at 6 months. DHA was low (< 2.22%) in 95% (19/20) at baseline and in 87.5% (14/16) at 6 months. The ratio AA/EPA was high (> 14.59%) in 100% (20/20) of patients at baseline and in 75% (12/16) at 6 months. Interestingly, AA was also low (< 7.91%) in 60% (12/20) and 50% (8/16) of patients at baseline and 6 months respectively.

Stratification of the data by treatment risk (Figure 1 and Figure 2) showed that plasma vitamin A levels were highest in the high-risk treatment group in comparison with both low and medium-risk treatment groups (Figure 1a), whilst plasma vitamin E/Ch did not differ (Figure 1b) at either baseline or 6 months. Also, plasma lipid peroxidation levels (TBARS) of paediatric cancer patients on a high-risk treatment protocol were higher than those on low and medium-risk treatment protocols at both time points (Figure 1c). In contrast, antioxidant capacity (ORAC) did not differ in any of the treatment risk groups (Figure 1d).

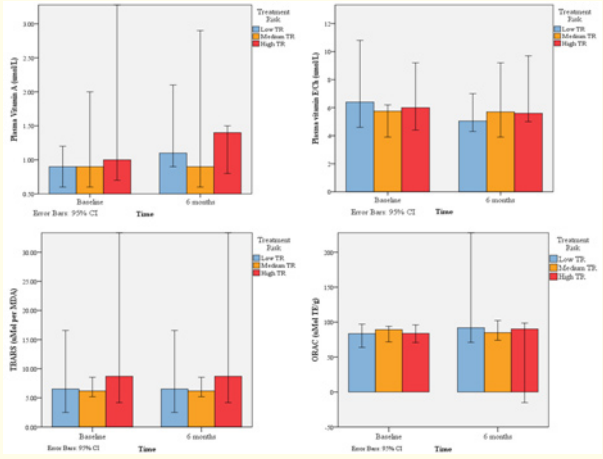

Figure 1: *Plasma antioxidant levels, antioxidant capacity and oxidative stress in paediatric cancer patients with data stratified by treatment risk.*

Figure 1a (top left) Plasma vitamin A levels; figure 1b (top right) Plasma vitamin E/Ch levels; figure 1c (bottom left) Plasma lipid peroxidation levels (TBARS); 1d (bottom right) Plasma antioxidant status (ORAC).

DHA and EPA levels were highest in children treated with high-risk treatment groups followed by medium and low-risk treatment (Figures 2s and 2b). Additionally, AA levels did not differ between any of the groups at baseline and were highest in the low-risk treatment group at 6 months. Finally, AA/EPA ratio was highest in those treated with low-risk treatment in comparison with high and medium-risk treatment (Figure 2d).

Associations between plasma antioxidant status and capacity, oxidative stress and lipid levels

The antioxidants plasma vitamin A and vitamin E/Ch were not significantly associated with antioxidant capacity. Likewise, no significant correlation was found between lipid peroxidation and antioxidant capacity at baseline or 6 months. However, lipid peroxidation correlated with copper (r = -0.9 (very strong); p < 0.001) and zinc (r = -0.4 (moderate); p = 0.01) at baseline.

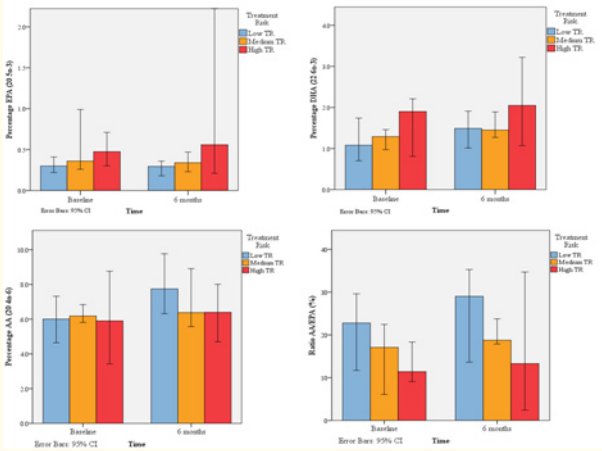

Figure 2: *Plasma PUFA levels in paediatric cancer patients with data stratified by treatment risk . Figure 2a (top left) Plasma eicosahexanoic acid (EPA) expressed as a percentage; figure 1b (top right) Plasma decosahexanoic acid (DHA) expressed as a percentage; figure 1c (bottom left) Plasma arachidonic acid (AA) expressed as a percentage; 1d (bottom right) Plasma AA/EPA ratio expressed as a percentage.*

Lipid peroxidation (TBARS) did not correlate with any of the following parameters at baseline or 6 months: plasma DHA and EPA did positively correlate with AA levels (r = 0.8 (very strong); p < 0.001) and negatively correlate with BMI centile at (r = -0.5 (moderate); p = 0.04) at baseline. Finally, only DHA positively correlated with BMI centile (r = 0.9 (very strong); p < 0.001) at baseline.

Dietary antioxidants and nutritional support

The median (IQR) antioxidant intakes of paediatric cancer patients at baseline and 6 months are presented in table 3 and Spearman's correlation between dietary antioxidants and corresponding plasma TAS, TAC (ORAC) and oxidative stress (TBARS) are presented in table 4.

Antioxidant intakes	Baseline n = 20		6 months n = 18		P value1
	Median	IQR	Median	IQR	
Vitamin A (µd/day)	971	971-1732	264	264-820	< 0.001
Vitamin E (mg/day)	8.6	4.0-9.2	14	5.0-13.0	0.7
Copper (mg/day)	0.7	0.5-0.75	1.7	1.7-1.8	< 0.001
Selenium (µg/day)	41	15-41	28	21-36.5	0.6
Zinc (mg/day)	6.1	6.1-7.7	8.0	7.5-15.6	<0.001

Table 3: *Antioxidant intakes of paediatric cancer patients at baseline and 6 months*

[1]Wilcoxon-Signed Rank Test.

Dietary antioxidants	Corresponding plasma antioxidant levels		Antioxidant capacity (ORAC)		Oxidative stress (TBARS)	
	Baseline	6 months	Baseline	6 months	Baseline	6 months
Vitamin A (µg/day)	r = 0.9; p < 0.001	r = 0.7; p = 0.001	r = 0.5 p = 0.03	r = 0.9; p < 0.001	r = 0.7; p < 0.001	r = 0.5; p = 0.01
Vitamin E (mg/day)	r = 0.9; p < 0.001	r = 0.2; P = 0.6	r = -0.3; p = 0.2	r = 0.7; p = 0.001	r = -0.7; p = 0.001	r = -0.4 p = 0.1
Copper (mg/day)	r = 0.7; p = 0.01	r = 0.5; P = 0.02	r = 0.7; p= 0.008	r = 0.5 p = 0.03	r = -0.8; p< 0.001	r = -0.1; p = 0.6
Selenium (µg/day)	r = -0.5; p = 0.03	r = 0.9; p < 0.001	r = -0.3; p = 0.1	r = 0.8; p < 0.001	r = -0.8; p< 0.001	r = -0.9; p < 0.001
Zinc (mg/day)	r = 0.7; p < 0.001	r = 0.9; p < 0.001	r = 0.7; p < 0.001	r = 0.9; p = 0.001	r = 0.6; p= 0.003	r = 0.9; p < 0.001

Table 4: *Correlations between dietary antioxidants and plasma antioxidant levels, antioxidant capacity and oxidative stress.*

At baseline, there were 8/20 (40%) patients on nutritional support. Of these, 100% were on macronutrient supplementation; 3/8 (37.5%) were on oral nutrition support (ONS), 3/8 (37.5%) on Nasogastric (NG) feeding and 2/8 (25%) on total parenteral nutrition (TPN). At 6 months, 12/16 (75%) patients were on nutritional support. Of these 6/12 (50%) were on micronutrient, 4/12 (33%) were on macronutrient supplementation only; of which 3/4 (75%) were on either NG or feeding via Percutaneous Endoscopic Gastrostomy (PEG) and 1/4 (25%) on ONS, and 2/5 (40%) were on complex nutritional support (NG-feed and TPN). Finally, one patient was on both micronutrient and macronutrient supplementation. Three out of 20 (15%) patients were on nutritional support at both baseline and 6 months. Of these, 2/3 patients were on macronutrient supplementation and 1/3 was on both macronutrient (NG-feeding) and micronutrient supplementation. The median (IQR) time the patients were on nutritional support was 11.5 (2.2-17.8) days at baseline and 82.5 (55.7-100) days at 6 months.

Table 5 shows that there were no statistical significant differences between paediatric cancer patients who were on nutritional support compared to those who were not at both baseline and 6 months in the following parameters measured: TAC (ORAC), TAS (vitamins: vitamin A and vitamin E/Ch; minerals: zinc, selenium and copper) and oxidative stress (TBARS). Likewise, EPA, DHA and AA/EPA ratio did not statistically differ between these two groups at baseline and 6 months.

Discussion

This is the first prospective cohort pilot study investigating plasma TAS, TAC and lipid peroxidation as well as the PUFA's profile of paediatric cancer patients. We have shown that 95% and 94% of paediatric cancer patients had lipid peroxidation levels above the reference range at baseline and 6 months respectively, and that this cohort had higher levels of lipid peroxidation at baseline despite antioxidant status remaining at similar levels. Interestingly, TAC increased slightly at 6 months. Moreover, there was a high percentage of patients with low levels of EPA% (70% at baseline and 60% at 6 months) and DHA% (95% at baseline and 87.5% at 6 months), and high levels of AA/EPA ratio (100% at baseline and 60% at 6 months). Although, DHA levels improved following 6 months of treatment, AA increased and EPA remained the same. Consequently, AA/EPA ratio also increased.

Children on high-risk treatments exhibited the highest lipid peroxidation and plasma vitamin A levels during the study period, whilst antioxidant capacity and the remaining antioxidants measured (Vitamin E/Ch, copper, zinc and selenium) were similar in all treatment risk groups. Also, nutritional support did not make any difference to plasma TAS, TAC and oxidative stress (lipid peroxidation) in this cohort; however vitamin E, selenium and copper intakes significantly contributed to higher plasma TAS, higher TAC and lower lipid peroxidation. Finally, we reject the hypothesis that the assessment of vitamins and minerals with antioxidant function is a reliable marker of antioxidant capacity due to the lack of correlation found between plasma TAS, TAC and oxidative stress.

	Baseline					6 months				
	NS		No NS		P value[1]	NS		No NS		P value[1]
	Median	IQR	Median	IQR		Median	IQR	Median	IQR	
Vitamin A (µmol/L)	1.2	1.0-2.6	0.9	0.7-1.2	0.3	1.0	0.8-1.4	1.4	1.4-1.9	0.2
Vitamin E/Ch (µmol/L)	8.5	4.4-10.0	6.2	5.6-6.4	1	5.8	5.0-6.8	5.5	4.9-5.6	0.1
Copper (µmol/L)	16.8	12.9-23.4	14.5	9.4-18.6	0.4	16.8	13.7-19.5	16.3	14.5-17.7	0.9
Selenium (µmol/L)	0.8	0.6-1.2	12	0.8-1.4	0.2	0.9	0.7-0.9	1.2	0.9-1.5	0.05
Zinc (µmol/L)	16.6	11.1-26.1	9.9	9.1-18.4	0.3	10.6	9.4-12.5	15.6	7.7-25.4	0.2
ORAC (µmol TE/g)	82.6	78.8-85.3	87.2	71.3-91.9	0.4	84.9	75.5-97.7	90.2	81.1-90.9	0.6
TBARS (MDA µmol)[2]	8.5	6.5-11.2	6.2	5.2-8.0	0.2	5.3	4.5-6.4	5.5	4.3-6.4	1
CRP mg/L	1.0	1.0-6.0	3	1.0-9.0	0.5	1.0	1.0-3.5	1.0	1.0-4.0	0.9
EPA (20 5n-3) %	0.4	0.3-0.6	0.4	0.3-0.5	0.9	0.4	0.3-0.6	0.3	0.2-0.5	0.3
DHA (22 6n-3) %	1.4	1.1-1.9	1.3	0.9-1.8	0.8	1.9	1.4-2.2	1.7	1.0-1.9	0.3
AA (20 4n-6) %	5.8	5.3-6.7	6.2	5.2-7.1	0.5	6.9	5.5-7.9	7.5	5.5-8.9	0.6
AA/EPA ratio (%)	12.1	9.4-22.5	17.7	12.1-22.7	0.4	18.3	9.0-22.6	19.8	15.3-33.6	0.2

Table 5: *Plasma antioxidants, oxidative stress and PUFA levels of paediatric cancer patients at baseline and 6 months with data stratified by nutritional support. AA: Arachidonic acid; CRP: C-reactive protein; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; ORAC: Oxygen Radical Absorbance Capacity; NS: Nutrition support; TBARS: Thiobarbituric Acid Reactive Substances; [1]Mann-Whitney test.*

Plasma antioxidant levels, antioxidant capacity and oxidative stress

None of the plasma antioxidant levels (vitamin A, vitamin E/Ch, zinc, copper and selenium) significantly changed over 6 months. This finding is supported by a study from Brazil, in which 45 children diagnosed with and treated for ALL reported that neither zinc nor copper significantly changed during treatment [30]. Likewise, no changes in selenium levels were reported in a French study, in which 170 children diagnosed with and treated for paediatric cancer were included [31]. However, by contrast, a third study demonstrated a significant decrease in zinc levels [31] and a reduction in selenium levels [32] in children with ALL receiving treatment [31,32]. It is well established that zinc, copper and selenium are acute phase reactants and their plasma concentration may change during inflammation [33]. Plasma zinc and selenium may decrease during inflammation [34]. In the case of zinc an increased uptake by the liver allows zinc to bind to the protein matallothionein, which acts as an antioxidant; whilst with selenium an increase in capillary escape of selenoprotein-P, the main selenium containing protein in plasma, is seen [34]. An explanation for this discrepancy in our findings and those published elsewhere [30-32], could lie in the timing of measurements as all our samples were collected during treatment rather than before.

Contrary to all studies published to date, which have investigated antioxidant capacity and oxidative stress using a variety of techniques (antioxidant capacity using ORAC [13] or different techniques [10,11,35-40], and oxidative stress using TBARS [10,11] or different techniques [37,39,41-44]), we found that paediatric cancer patients were more stressed and had lower plasma TAC levels at baseline than 6 months into treatment. This may reflect that treatment protocols are most intensive during the first 3 months of treatment, especially in the ALL group, which represented 60% of the total cohort. Furthermore, children in the high-risk treatment group had higher levels of lipid peroxidation, which further confirms that more intensive treatments are associated with higher oxidative stress.

We have shown that antioxidant intakes contribute to higher plasma TAS and TAC and help reduce oxidative stress. Also, lower BMI centiles were associated with higher oxidative stress, suggesting that both Undernutrition and reduced antioxidant intake may lead to more cell damage and may explain the higher toxicity rates seen in undernourished children as reported elsewhere [45,46]. Unfortunately, due to the small sample size and short follow up period, the current study was unable to establish any associations between TAC, oxidative stress and clinical outcomes, and the short and long term implications of reduced TAC and high oxidative stress during the treatment phase remain under debate. TAS and TAC have been associated with reduced chemotherapy toxicity, less delays in treatment and thus fewer days of hospital stay in children with ALL [13,47] and in children with soft tissue sarcoma and neuroblastoma [44]. However, plasma zinc and copper did not correlate with prognosis in children with ALL [30]. As most evidence suggests that higher oxidative stress is associated with poorer clinical outcomes [11,41,43], it is fair to assume that a higher antioxidant intake reduces oxidative stress and would in turn be beneficial for this cohort; however it is first essential to establish optimal TAS levels in this population.

Plasma PUFA levels

Our cohort had low levels of EPA and DHA and high levels of AA/EPA ratio. Similar findings have been reported in a small cohort of children diagnosed with CNS tumours [18] and in adults diagnosed and treated for non-Hodgkin's Lymphoma [48]. Moreover, we showed that children with higher AA levels also had higher lipid peroxide levels. AA is not only a precursor of inflammatory markers [14], but it is easily oxidised and the main precursor of MDA; therefore contributing further to oxidative stress [49]. Interestingly, plasma copper and zinc concentration negatively correlated with lipid peroxidation, and although only plasma copper was statistically significant, this study suggests that copper and zinc may have an essential role at reducing oxidative stress caused by MDA in this patient cohort during cancer therapy. Copper and zinc are co-factors for the metalloenzyme superoxide dismutase, which de-oxidises the extremely toxic MDA into the less toxic hydrogen peroxide [49].

Unlike healthy individuals [14,49-51], this study did not establish that higher levels of EPA% and DHA% were associated with lower plasma lipid peroxide, but they were negatively associated with ss-CRP. No studies including paediatric cancer patients have investigated this relationship; however such findings have been established in adults with chronic conditions such as cancer [15,52] and cardiovascular disease [14,53]. In line with our findings, others have also reported a positive association between PUFA levels, particularly DHA, and nutritional status [54,55].

Nutritional Support

The TAS, TAC and oxidative stress as well as the PUFA profile (DHA and EPA) of paediatric cancer patients on nutritional support did not statistically differ from those who were not. Furthermore, children on high-risk treatment tended to receive more nutritional supplementation than those in low or medium-risk treatment. Given that we demonstrated a positive relationship between antioxidant intakes and plasma TAS, it can be hypothesized that current nutritional treatment formulas may not support the needs of paediatric cancer patients, especially those receiving intensive treatments. Due to the lack of studies performed in the same population, comparison of these results is difficult. However a randomized-placebo control trial (RCT) in which 15 children with active Crohn's disease were treated with exclusively polymeric diet or antioxidant (glutamine) enriched polymeric diet found no differences in plasma antioxidant concentration [56], which contrasts with our results.

Limitations of the study and future research

We have identified several limitations in this study, which may have affected our results. An underestimation of the percentage of EPA and DHA as well as an overestimation of AA/EPA ratio may have occurred as samples were stored for a period of 3 months to 1 year. A reduction of 9% and 4% in EPA and DHA respectively has been reported following 1 month storage at -20°C; whilst AA is a more stable PUFA with no reduction of stability during this period of time when stored also at -20°C [26]. The reduced sample size precluded stratification of the data by diagnostic criteria and therefore the establishment of associations between TAS, TAC, oxidative

stress and lipid profile, and clinical outcomes; including survival and events. Owing to the nature of this population, the collection of fasted samples was impossible, which could potentially have affected both TAS and TAC. Antioxidant intake from children who were not receiving nutritional support was assessed using a 24-hour dietary recall, which may have led to some inaccuracies.

Future research should ideally include: (i) larger population-based epidemiological studies in which the TAS, TAC and oxidative stress of paediatric cancer patients are followed up for a longer period of time. Studies should take into consideration treatment related side-effects, response to therapy and clinical outcome, such as relapse, death and survival; (ii) well-designed RCT investigating the effectiveness of different forms of supplementation, in which different doses of antioxidants are considered is warranted; (iii) Owing to the paucity of evidence and the fact that at present no specific micronutrient (including those with antioxidant function) and PUFA requirements (including EPA and DHA) have been established for paediatric cancer patients or for critically ill individuals [57], more research is needed to set optimal intakes for this population; (iv) Finally, more *in vivo* (and *in vitro*) studies should investigate whether different antioxidants, DHA and EPA have a protective role against the onset of endothelial cell senescence in paediatric cancer patients during treatment and whether their use would reduce their risk of chronic conditions.

Conclusion

In conclusion, we have highlighted that our cohort of paediatric cancer patients had a high prevalence of oxidative stress, which was highest in children treated with high-risk treatment protocols and soon after the initiation of treatment. Importantly, nutritional support did not contribute to higher TAS, EPA and DHA in this cohort. Nonetheless, larger high-quality population based studies and clinical trials are now justified. This should investigate the effects of nutritional support, in which different concentrations of PUFA and antioxidants are included to establish optimal levels for this population and whether they might play a protective role against early development of chronic conditions in this population.

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Conflicts of Interest

No conflicts of interest to declare.

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