**Author’s response to reviews**

**Title:** The influence of prenatal exposure to trans-fatty acids for development of childhood haematopoietic neoplasms (EnTrance): a natural societal experiment and a case-control study

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**Author’s response to reviews:**

Reviewer reports:

Editor: Can you please ensure that you have the protocol considered by an expert in cancer diagnosis and incorporate their advice.

Answer: Three of the co-authors are cancer experts working at the International Agency for Research on Cancer (IARC). ESF has an insight into childhood cancer epidemiology, IH is a specialist in nutrients and cancer and VC is a world expert in fatty acid analysis and its relationship with different cancer sites. VC is doing the blood analysis of fatty acids, with many years of experience doing this in relation to cancer.

Reviewer #1: In this paper, the authors reported the study protocol including background, objective, design, and methods of the EnTrance study. The study objectives are two-fold: 1) to investigate whether the Danish iTFA legislation ban influenced the risk of childhood haematopoietic neoplasms in children born either before or after the change in legislation in a natural societal experiment; and 2) to examine the associations between trans-fatty acid concentrations measured from stored dried blood spots and childhood haematopoietic neoplasms in a nested case-control study. The research question is of importance to the field and is of interest to the readership of the journal. However, the manuscript may be improved by addressing several concerns.

1. Indeed, trans-fat is a type of unsaturated fat common in industrially produced foods (iTFA). However, industrially produced foods are not the exclusive source of trans-fat. It
is important to acknowledge that two dietary sources exist for the TFAs present in the food supply: industrial production and natural sources, in which TFAs are found in smaller amounts in ruminant-derived food products. Emerging evidence suggests that TFAs from industrially produced and from natural sources have different effects on cardiometabolic outcomes. Given that the reported methodology of fatty acid measurement using gas chromatography "allows a complete separation, identification and quantification of sixty fatty acids including 15 individual isomers of industrial trans fatty acids and natural trans fatty acids (CLA, vaccenic acid)." The authors may need to clarify whether Aim 2 focuses on the former (industrial source) or the latter (natural source). If the former, it would be important to assess both the independent and mutually adjusted associations with childhood haematopoietic neoplasms.

Answer: Thank you for this very relevant comment. In lines 65-68 we have described the two types of TFA. We have included a sentence with information on where rTFA can be found (line 71) and a sentence about different effects of the two TFA types (line 73). Yes, we will investigate both TFAs. This has been specified now in lines 238-239.

2. The authors reported that identification and quantification of sixty fatty acids will be conducted by the International Agency of Research of Cancer; however, more information is needed regarding the methodology for fatty acids measurement. First, how will the fatty acid concentrations be reported and analyzed, in absolute concentrations or relative % weight of the total fatty acids?

Answer: Plasma phospholipid fatty acid concentrations will be determined at IARC. Samples from cases and their matched controls will be analysed within the same daily batch, with two quality controls in each batch. The technical staff will not have any information on the case/control status. Total lipids will be extracted from plasma samples with chloroform-methanol containing antioxidant butylated hydroxytoluene and L-A-phosphatidylcholine-dimyristoyl-d as an internal standard. Phospholipids will be purified by adsorption chromatography; fatty acid methyl esters will be separated through gas chromatography. The relative amount of each fatty acid, expressed as percent of total fatty acids, will be quantified by integrating the area under the peak and dividing the result by the total area. Fatty acids will be also expressed as absolute concentrations in plasma (µmol/liter) based on the quantity of the methyl deuterated internal standard. This now appears in the manuscript.

Second, for certain trans-fatty acids, particularly for measurements using up to 20-year-old dried blood spots, it is likely that a considerable amount of measurements may have concentrations below the LODs. If levels are below the LODs, what are the corresponding data preprocessing procedures?

Answer: A study investigated the effect of long-term storage on plasma phospholipid fatty acid composition and reported no significant effect of up to 4-year storage at -80°C on plasma fatty acid concentrations (Hodson et al, 2002). Moreover, in our study, case and control subjects will be matched on date at blood collection; thus, the possibility that long-term storage may have differential influence on plasma fatty acids from subjects who developed leukemia and those who did not is unlikely.
Further, in a previous study we did not have more observations below LOD when measuring vitamin D in up to 35 years old DBS (Eyles et al. A sensitive LC/MS/MS assay of 25OH vitamin D3 and 25OH vitamin D2 in dried blood spots. Clin Chim Acta 2009;403:145–51.), however we have not investigated fatty acids before in these DBS so it might not be the case here. Levels below LOD will thus be imputed by the maximum likelihood single imputation method.

Third, report the laboratory CVs for fatty acids, particularly trans-fatty acids measurement.

Answer: Based on this methodology, coefficients of variation (CVs) for fatty acids have been calculated for 60 samples. CVs ranged from 0.013% for large peaks to 7.75% for the smallest peaks (less than 0.10% of total fatty acids). Overall CVs (within-batch and between batch CVs) are very good for industrial trans fatty acid isomers, mainly for elaidic acid (CV=0.137%), whose mean level in plasma phospholipids in the EPIC cohort is 0.36 (SD = 0.24). Overall CV is 0.122% for total industrial trans fatty acids. CVs for natural trans fatty acids are also good, mainly for vaccenic acid, the main natural trans fatty acid isomer (CV=0.282%), with a mean level in the EPIC cohort of 0.30 (SD = 0.15). CV for total natural trans fatty acids is 0.123%. The comments above as well as a revision of the laboratory analysis have been included in the manuscript (page 10).

3. For Aim 2, it is not entirely clear how the control will be selected. Specifically, which control will be selected if more than one participant meets the matching criteria? On the other hand, what if there are no qualified controls meeting the matching and selection criteria - would the matching criteria be modified and how?

Answer: Thank you for this very relevant comment. We have included the following (lines 191-194): “If more than one participant meets the matching criteria a random control from the eligible participants will be selected. If no participant meets the matching criteria we will relax the date of birth or place of birth criteria as needed to identify controls and report the relevant adjustment.”

4. Also for Aim 2, it is important to consider whether trans-fatty acids of interest are truly bioactive, or are related to food products rich in trans-fat, such as baked foods, fried foods, and dairy products. Is diet data available in the study via dietary assessment such as food frequency questionnaire and dietary recalls or records? The residual confounding due to total energy, afore-mentioned foods, and overall dietary pattern would be a major concern.

Answer: Unfortunately dietary information is not available which is indeed a limitation of our study. In line 338-341 we have added: “However, since we do not know the exact mechanism between TFA and childhood neoplasms, we cannot account for a residual confounding which might affect observed associations. Residual confounding could be caused by additional components in food products with high contents of TFA.”

5. Further, the authors would like to examine whether levels of trans-fats in dried blood spots from newborns are associated with the risk of haematopoietic neoplasms by age of
7 years. A potential issue of residual confounding may arise due to the lack of data (or description if such data are available?) on trans-fat levels in childhood and other important factors such as breastfeeding.

Answer: True we do not know TFA levels during childhood but since this study is investigating fetal exposure, this is not relevant. Breastfeeding is very prevalent in Denmark with approximately 97% breastfeeding, so we do not believe this to be a problem in this study, considering that maternal TFA intake remains stable during pregnancy and breast-feeding.

Reviewer #2:

Thank you for giving me an opportunity to review the manuscript "The influence of prenatal exposure to trans-fatty acids for development of childhood haematopoietic neoplasms (EnTrance): a natural societal experiment and a case control study" (NUTJ-D-17-00185).

The write-up submitted by the authors is a grant proposal complete with hypothesis, objectives, detailed methodology along with a checklist of recommended items to address in a clinical trial protocol and related documents. And there is no results.

Answer: This is a protocol paper, normally considered by Nutrition Journal, thus the missing results, we simply followed the instructions for authors pertinent to a protocol paper.

Reviewer #3: Dear Authors,

This study aims to bring new knowledge as to whether trans-fat and other fatty acids may also increase the risk of developing haematopoietic neoplasms during childhood. The manuscript addresses an interesting topic and represents a well done conducted study protocol. The authors intend to improve the quality, integrity and transparency of the study.

Background)

In general, background should be synthesized. Some non-essential information may be removed.

Page 3, Lines 5-7: Please, consider to include some prevalence of childhood cancer (How rare is it?).

Answer: We have now removed what we believe might be non-essential information in the background.

Information on the incidence of leukaemia is given in the first paragraph of the Background.

Page 3, Lines 15-51: Second paragraph should be shorter. The sentence "Alarmingly, childhood cancer incidence rates in Europe are rising with an overall average annual incidence increase of around 1% [6" could be located in the previous paragraph.

Answer: Thank you, we have rearranged and shortened the paragraph.
Page 4: The paragraph "Evidence of adverse…" should be shorter.

Answer: the paragraph has been shortened.

Page 4, Lines 18-19: "Guidance to minimize… …infarction of 23%" is an important sentence. Please, include a reference to this information.

Answer: Done.

Page 4, Line 35: I recommend to start a new paragraph "A case-control…" to improve the scientific writing.

Answer: Done.

Hypotheses and objectives)

This section should be rewrite. Please, write more clearly the hypotheses and the two objectives of your study protocol. Several information may be removed and/or replaced to the end of the Background.

Answer: Thank you, this has been done.

Methods)

Page 9, Power Calculation: Please, include the formula used and/or the reference.

Answer: The power analysis for the cohort study was performed in Stata 14 using the stpower command for the log-rank test for comparison of two survival functions: Freedman, L. S. 1982. Tables of the number of patients required in clinical trials using the logrank test. Statistics in Medicine 1: 121-129.

The power analysis for the matched 1:1 case-control study was performed using the power mcc command in Stata 14.


This has now been added to the manuscript.

These have now been included in the manuscript line 243-251.

Discussion)
Paragraphs with only one sentence are not indicated in good scientific writing. Please, rewrite these paragraphs.

Answer: We have rearranged so that no paragraphs with only one sentence occur.