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Genome-wide identification of mononuclear cell DNA methylation sites potentially affected by fish oil supplementation in young infants: A pilot study

M.V. Lind [a,1], D. Martino [b,1], L.B.S. Harsløf [a], Z.O. Kyjovska [c], M. Kristensen [a], L. Lauritzen [a,*]

[a] Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, Frederiksberg, Denmark
[b] Murdoch Childrens Research Institute, Royal Children's Hospital, University of Melbourne, Australia
[c] National Research Centre for the Working Environment, Copenhagen, Denmark

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ABSTRACT

Recent evidence suggests that the effects of n-3LCPUFA might be mediated through epigenetic mechanisms, especially DNA-methylation, during pregnancy and early life. A randomized trial was conducted in 133 9-mo-old, infants who received 3.8 g/day of fish oil (FO) or sunflower oil (SO) for 9 mo. In a subset of 12 children, buffy-coat DNA was extracted before and after intervention and analyzed on Illumina-Human-Methylation 450-arrays to explore genome-wide differences between the FO and SO groups. Genome-wide-methylation analysis did not reveal significant differences between groups after adjustment for multiple testing. However, analysis of the top-ranked CpG-sites revealed 43 CpG's that appear modified with an absolute difference in methylation of $\geq 10\%$. Methylation levels at these sites were associated with phenotypic changes mainly in blood pressure. In conclusion, our analyses suggest potential epigenome effects that might be associated with functional outcomes, yet the effect sizes were small and should be verified by additional investigation.

1. Introduction

Consumption of fish and fish oil (FO) rich in long-chain n-3 fatty acids (n-3 LCPUFA) including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) has been shown to be associated with protection against atopic disease [1,2] and low grade inflammation [3], and affect several features of the metabolic syndrome [3–5]. Recent evidence suggests that DHA supplementation affects epigenetic regulation through DNA methylation [6]. This could be one of the mechanisms by which FO affects metabolic disease and immune regulation as some immunological markers [7] and metabolic syndrome [8] has been shown to be associated with DNA methylation. Epigenetic modification such as DNA methylation is of particular interest as a potential mechanism for the developmental origins of many diseases [9,10]. This emphasizes the importance for exploring the relationship between FO supplementation, DNA methylation and metabolic and phenotypic outcomes.

n-3 LCPUFA might affect epigenetics through an altered one-carbon metabolism. A meta-analysis found that high consumption of n-3 LCPUFA was associated with a significant decrease in plasma homocysteine [11]. Furthermore, n-3 LCPUFA has been shown to upregulate the expression of genes involved in the one-carbon metabolism pathway potentially increasing the remethylation of homocysteine to methionine [12,13]. Recycling of homocysteine to methionine could possibly lead to an increase in the ratio of *s*-adenosylmethionine to *s*-adenosylhomocysteine which is used as an indicator of cellular methylation capacity [14,15].

DHA has been shown to modify DNA methylation in rats fed on a vitamin B_{12} deficient diet indicating that DHA plays a role in one-carbon metabolism [16]. Hirabara et al. found that FO supplementation for two generations lowered hepatic DNA methylation [17]. Further, a recent study found that maternal DHA supplementation

Abbreviations: BP, blood pressure; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; HOMA-IR, homeostatic model assessment of insulin resistance; IL, interleukin; LCPUFA, long-chain polyunsaturated fatty acids; LPS, lipopolysaccharide; MAP, mean arterial pressure; PCA, principal component analysis; RBC, erythrocytes; SBP, systolic blood pressure; SNP, single nucleotide polymorphism; SO, sunflower oil; TAG, triacylglycerol

* Correspondence to: Department of Nutrition, Exercise and Sports, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark.
Tel.: +45 3533 2508; fax: +45 3533 2483.
E-mail address: ll@nexs.ku.dk (L. Lauritzen).
[1] Shared first authorship.

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during pregnancy increases DNA methylation of an overall marker of DNA methylation long-interspersed element 1, in infants of mothers who smoked [6]. We have performed a FO supplementation trial in late infancy, with the primary aim of examining effects on growth, metabolic markers and immune function. The main results of this trial was a reduction in triglycerides, blood pressure, and ex-vivo stimulated interleukin (IL)-6 [18–20]. In this pilot substudy, the aim was to identify differently methylated regions between the FO supplemented infants and controls and explore if differences in methylation might be linked to functional outcomes. To our knowledge this is the first paper, examining epigenome-wide effects of supplementing infants with fish oil, and may serve as an initial "proof of concept" of a mechanism for potential programming effects of fish oil.

2. Methods

2.1. Subjects and study design

The study design has been described in detail elsewhere [18]. In brief, this study was a double-blind controlled parallel intervention study in which 154 9-mo-old infants were randomly allocated to receive either a teaspoon-full of FO or sunflower oil (SO) for a period of 9 mo. The participants were examined at the beginning and end of the intervention at Department of Nutrition, Exercise, and Sports, University of Copenhagen from January 2008 to March 2009. The infants were all term normal-weight healthy singletons with no previous FO supplementation. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures all procedures were approved by the Ethical Committees of the Capital Region of Copenhagen, Denmark (H-A-2007-0088) and registered in www.clinical-trials.gov (NCT 00631046). Written informed consent was obtained from all parents of the participants in the study.

2.2. Background diet, dietary intervention and adherence

The investigators were at all times (at allocation, during intervention, lab analysis and statistics) blinded to the received treatment. The FO supplement was cod-liver oil rich in EPA and DHA but without vitamins. The intended dose was 1.6 g/d n-3 LCPUFA. We chose SO which provided 3.1 g/d linoleic acid as the control because we wanted to investigate the specific effects of n-3 LCPUFA. For a detailed description of the estimated daily intake of specific fatty acids from the supplements, see Supplementary Table 1. At baseline, the parents were provided with intervention oil for the entire intervention period and requested to return all leftovers at the 18-mo examination and to report spilling etc. Compliance was estimated from the original weight of the bottles and the returned bottles including leftovers plus the exact number of days a given participant had been in the study. Compliance was furthermore supported by the erythrocyte (RBC) fatty acid composition, before and after the intervention.

2.3. Examinations

Detailed questionnaires were completed at baseline (e.g. birth outcome, breastfeeding and maternal fish intake, etc.) and when infants were 18 mo of age, and parents and caretakers filled in precoded food diaries for the infants during seven consecutive days before both examinations. The anthropometric measurements were performed by trained investigators according to Gibson [21]. All anthropometric measures, except weight, were obtained in triplicates, and mean values were used in analyses. The recumbent length of the infants was measured to the nearest millimeter on a measuring board (Force Technology, Broendby, Denmark). Naked weight was measured sitting or lying down on a pediatric infant scale (Sartorius IP65; Bie & Berntsen, Rødovre), which took 40 consecutive weights in 10 s and displayed the mean, or on an adult scale (Lindeltronic 8000; Samhall Lavi AB, Kristianstad, Sweden) if the infant weighed >12 kg. Blood pressure (BP) measurement has been described in detail elsewhere [19]. We aimed at measuring BP 3 times during 15 min, while infants sat on the parent's lap and were distracted with a toy to ensure minimum movement. We used two different automated oscillometric devices (model 506n, Criticare Systems Inc., Waukesha, WI, USA and Spot Vital Signs LXi, Welch Allyn, Skaneateles Falls, NY, USA) and the mean of the BP measurements was used in analyses.

At the end of both examinations, 6 mL blood samples were taken by venipuncture, kept on ice and separated into plasma, buffy coat, and RBC by centrifugation at 2300*g* for 10 min at 4 °C. Samples were kept at −80 °C until analysis. The fatty acid composition of RBC from heparin-coated tubes was determined as previously described [22]. Values are expressed as area% of the specific fatty acid relative to the total chromatogram area (FA%). Triglycerides (TAG) were determined in heparinized plasma using an automated, enzymatic, colorimetric assay on ABX Pentra 400 (HORIBA ABX, Montpellier, France). Glucose concentration was measured in EDTA whole blood immediately after blood sampling on a HemoCue analyzer (HemoCue Danmark, Vedbaek, Denmark) and the equivalent plasma values were obtained by multiplying with 1.11 [23]. Plasma insulin was determined in heparinized plasma by automated assays on an Immulite 1000 (Siemens Healthcare Diagnostics, Ballerup, Denmark). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as plasma glucose (mmol/L) × plasma insulin (mIU/L)/22.5 [24].

2.4. Functional immune assessment

The functional immune assessment was measured as ex vivo cytokine production in whole blood cultures stimulated with lipopolysaccharide (LPS) (20 ug/mL from *Escherichia Coli* O26:B6, Sigma Aldrich, Mannheim, Germany) and UV-killed *Lactobacillus paracasei* (20 ug/mL CRL431 from Chr. Hansen A/S, Hoersholm, Denmark) as described in detail elsewhere[20]. In brief, cultures were made in triplicates for each of the two conditions and controls (no LPS or bacteria) with medium (RPMI-1640 with antibiotics and L-glutamine, Cambrex, East Rutherford, NJ) and incubated at 37 °C, 5% CO_2 for 24 h (range 23–25 h). Interleukin (IL)-6 in supernatant was measured by commercial enzyme-linked immune-sorbent assay (Duoset, R&D Systems, Abingdon, UK). Median intra-assay CV% for the triple determinations was 14%. Limits of detection in the assay were 184 pg/mL. Measurements below the detection limit were defined as 0.5 × the detection limit.

2.5. Nucleic acid extraction for DNA methylation analysis

The genome wide methylation analysis was performed in 12 out of 133 completers from the study. The 12 children were selected in order to be representative of the group changes according to the following criteria: three girls and three boys from each group. For the FO intervention group the children were selected if they had: 1) a large increase in DHA from 9 mo to 18 mo 2) lowered systolic blood pressure (SBP) at 18 mo and 3) lowered triceps/subscapular skinfold measurement. For the SO group had: 1) a large decrease in DHA from 9 mo to 18 mo 2) higher or unchanged SBP and 3) higher or unchanged triceps/subscapular skinfold measurement. Genomic DNA was isolated from buffy coat using the salting-out method [25]. A total of 500 ng of purified genomic DNA was bisulphite converted using the Methyl Xceed kit from Human Genomic Signatures (North Ryde, Australia). Successful bisulphite conversion was evaluated for all samples using an in-house bisulphite-specific PCR.