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Brief Title:	Omega-3 Supplementation to Both Parent and Adolescent
Official Title:	Omega-3 Supplementation to Both Parent and Adolescent to Reduce Behavior Problems

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Experimental Design and Methods

This section provides the design and method of the overarching study within which the three aims will be tested. We also briefly specify further programmatic goals of this line of study, and also how the specific expertise of the research team will help ensure feasibility. As with the pilot study, methodology and ultimately reporting of results will follow CONSORT 2010 guidelines (Moher et al., 2010).

Design Overview

The study design will consist of a stratified, double-blind, placebo-controlled, factorial, randomized controlled trial of children and their caregivers in a community sample in Mauritius. This closely follows the design of the pilot study (Raine et al. 2015). Unlike the pilot study however, it will utilize a 2x2 factorial design, in which children *and* their parents will be randomly assigned to either omega-3 supplementation (treatment condition) or a placebo (control condition), and will have an extended follow-up at 18 months. This results in 4 groups: (1) child and caregiver both receiving omega-3, (2) child receives omega-3, caregiver receives placebo, (3) caregiver receives omega-3, child receives placebo, (4) both parent and child receive placebo. Assessments will take place at 0 months (baseline), 6 months (end of supplementation), 12 months (6 months post-treatment), and 18 months (12 months post-treatment).

Background To The Sample and the Mauritius Child Health Project

Adolescents and caregivers will be drawn from the Mauritius Child Health Study, a multi-generational study involving three generations of subjects. Details of these three generations are provided in a cohort profile (Raine et al. 2010). Originally set up by Venables (Consultant), this longitudinal study covers early child health and development and the identification of early risk factors (including nutrition) for later psychopathology, as well as early primary prevention. Early factors that shape later cognitive functioning have also been a focus. Given the pilot findings and longitudinal data on the parents, it provides a unique platform on which to build.

Participants, Potential Attrition, and Strategies to Deal with Attrition

Participants will consist of 400 pairs of children and their caregivers (total N = 800). This replicates cell sizes in the pilot data and allows close comparability, with 100 children and 100 adults in each of four cells. The caregivers consist of the second generation participants (their parents are the first generation) who originally comprised the birth cohort. Their adolescent children are the third generation. All caregivers have previously participated in the Mauritius Child Health Project (Raine et al., 2010). Adolescents will be aged between 11 and 18 years old, willing to participate in an RCT, and residing in the community. Exclusion criteria consist of: (1) allergy to fish or fish products, (2) use of fish oil supplementation in the past six months, (3) intellectual disability, (4) participation in the prior RCT. There are currently 1,126 children aged 11-18 years actively participating in the Mauritius Child Health Project (51.2% female; 73.2% Indian, 24.5% Creole, 2.3% other), 124 of whom took part in the prior RCT and will be excluded. Written informed consent will be obtained from the parents with assent obtained from adolescents. The study will take place in interview rooms at the project headquarters in Quatre Bornes.

Attrition is an issue in all RCTs. In our pilot data however, attrition was only 8%, even after one year, and is likely attributable both to the long-standing intergenerational relationships between our research team in Mauritius and the participants. Attrition may nevertheless be slightly more with the longer follow-up. We have three strategies to deal with attrition. First, analyses will be intention-to-treat, including all participants regardless of drop-outs. Second, we will enhance participation using staggered incentives for completion. Third, we will code for missingness and assess whether it moderates treatment outcome.

Randomization

Participants will be randomized into four groups using urn randomization, a covariate adaptive randomization method (Wei & Lachin, 1988). Adaptive randomization is employed because it protects the study from bias while promoting study objectives, it improves power over standard designs, and it balances assignment of treatment across covariates in a way superior to traditional stratification (Rosenberger et al., 2012). We aim to achieve balanced groups on three main child covariates: age, gender, and ethnicity. An advantage of the Mauritius cohort is that we have data on the parents' externalizing behavior over the life-course (Raine et al. 2010) which we could add as a fourth covariate to further ensure that groups are balanced.

Omega-3 Intervention

Omega-3 supplementation. As in the pilot study, this consists of a 200 ml drink. The base drink in both treatment and placebo conditions consists of fruit juice containing vitamin D (0.85 micrograms) and antioxidants (ferric reducing ability of plasma value of 0.71 mmol/100g). For the treatment condition only, 1660 mg of omega-3 (550 mg of DHA, 550 mg of EPA, 560 mg of alpha-linolenic acid) is added to the base drink. Placebo drinks are matched exactly with the fish-oil drink in terms of size, appearance, and flavor. Caregiver and child drinks are differentially color-coded.

This drink is chosen because: (i) it contains a higher dosage of omega-3 than standard capsules in a small liquid quantity (60.6% of a standard can of cola) suitable for child / adolescent consumption, (ii) the fruit-flavored drink may be better tolerated and result in higher compliance with children than fish-oil capsules.

Treatment duration and administration. As in the pilot study, treatment duration will be six months. It was chosen because prior studies have usually been 2 - 4 months (Sinn et al., 2010), and a longer duration may be more effective in producing longer-term behavioral change. The drink will be administered by the parents to their children at any time each day.

Primary Outcome Measures

The pre-defined primary outcome measures are externalizing behavior problems. Secondary outcomes included internalizing behavior problems and neurocognitive / academic mediators. We include additional *secondary* measures of reactive-proactive aggressive behavior and callous-unemotional traits to replicate the pilot findings and explore antisocial subtypes (the pilot data showed stronger effects for reactive aggression).

Externalizing and internalizing behavior problems in children and caregivers will be assessed using the Multicultural Family Assessment Module (MFAM - Achenbach et al., 2015) which constitutes counterpart scales of the Child Behavior Checklist (CBCL/6-18), Youth Self-Report (YSR), Adult Self-Report (ASR), and Adult Behavior Checklist (ABCL). Reliability, validity, and cross-cultural generalizability have been documented in over 100 societies (Achenbach & Rescorla, 2001; Achenbach & Rescorla, 2001; Rescorla et al., 2012). The Externalizing scale includes Aggressive Behavior and Rule-Breaking Behavior, while the Internalizing scale includes Anxious/Depressed, Withdrawn, and Somatic Complaints. Additional syndromes include Attention Problems, Social Problems, and Thought Problems. Instruments are also scored on DSM-oriented scales comprising items judged by international clinical experts to be very consistent with DSM diagnostic criteria.

Adolescents will rate themselves on the Youth Self-Report YSR. The caregiver will rate their child on the CBCL/6-18, themselves on the ASR, and their spouse on the ABCL. We have established measurement invariance of the CBCL in this cohort in Mauritius (Yarnell et al., 2013). Teacher ratings of the child will be assessed using the Teacher Report Form of the MFAM. In consultation with Dr. Achenbach (Consultant), we will adopt the same system he has used in his national surveys to successfully obtain individual-based teacher ratings using payment to the teacher with a parental permission letter (Achenbach & Rescorla, 2001).

Other measures of antisocial behavior taken in the pilot data will be repeated here to assess replicability of original findings. These include the *Reactive and Proactive Aggression Questionnaire* (RPQ), Developed by the PI, this instrument assesses two overlapping yet distinct forms of aggression (Raine et al., 2006). *Psychopathic-like traits* will be assessed by caregivers using the Antisocial Personality Screening Device (APSD) consisting of three sub-scales: callous-unemotional traits, narcissism, and impulsivity (Frick et al., 2000). Due to the introduction of callous-unemotional traits in DSM as a specifier for conduct disorder, adolescents will also complete the Inventory of Callous-Unemotional Traits (ICU - Essau et al., 2006). In caregivers, psychopathy will be assessed using the self-report *Psychopathic Personality Inventory (PPI* - Lilienfeld & Andrews, 1996). These measures also showed significant changes in the pilot study.

Secondary Outcome Measures

Neurocognitive Risk Factors. These encompass cognition, emotion, and motor functioning and will be assessed using the CANTAB (Robbins et al., 1994). This battery is selected because it is language and culture independent, has been extensively validated on children and adolescents, and its user-friendliness to adolescents helps assure feasible implementation. Measures are selected based on the most pertinent neurocognitive domains relevant to antisocial / aggressive behavior (e.g. Ogilvie et al. 2011; Raine et al., 2005; Cauffman et al., 2005), including attention (Attention Switching Task), cold executive functioning (Stockings of Cambridge), hot executive functioning (Cambridge Gambling Task), emotional decision-making (Affective Go/NoGo), social / emotional cognition (Emotion Recognition Task), and impulse control (Stop Signal Task). Minimal reading (2 tasks) or no reading (4 tasks) is needed.

Academic Skills. As per C2's recommendation for "expanded assessment" to cover other domains, academic skills will be assessed using the Wide Range of Achievement Test (WRAT-4 – Wilkinson & Robertson, 2006) which provides measures of mathematics, spelling, sentence completion, and word reading. Mauritian children today are fluent in English which is the oral and written language of instruction from age 5.

The parent (CBCL) and youth (YSR) reports also include School Competence scales, while the teacher (TRF) form includes Academic Performance and Adaptive Functioning (Achenbach et al., 2015). These will extend the scope on mechanistic processes beyond neurocognitive functioning.

As in the pilot study, parental belief in treatment allocation to the omega-3 group (yes, no) will be measured at the end of treatment to assess influence on behavioral change. Demographics will again be collected on entry into the study, together with an adverse events questionnaire at the end of treatment.

Adherence to protocol and blood assays.

Adherence to the treatment regimen is a frequent problem in RCTs. As before, this will be assessed by assays of omega-3 from finger-prick blood taken from both caregiver and adolescent at all four time-points, with microwave irradiation assays conducted by team member Dr. Joe Hibbeln an NIAAA. For full methodological details see Lin, Loewke, Hyun, Leazer, & Hibbeln (2012). In response to C2, blood will additionally be assessed mid-way through supplementation (3 months). To help further enhance adherence, participants will be called every two weeks to remind them to use supplements, and to assess frequency of drink consumption by caregiver and adolescent in that time period (number of drinks / two weeks). To further facilitate compliance, participants will be given a bonus payment if their % blood omega-3 levels have risen higher than the bottom quartile of the population.

Statistical Methods

Overview. An intention-to-treat (ITT) design using all randomly assigned participants will be employed for all data analyses. All data will be blindly double-entered and checked for consistency.

Aims 1 and 2 (Primary Outcome). These hypotheses will be addressed using adolescent and caregiver externalizing responses gathered at baseline and at 6, 12, and 18 month follow-ups. Responses for the adolescent will be analyzed separately from responses for the caregiver. For each separate set of responses, linear mixed effects models (Molenberghs & Verbeke, 2005) will be used to describe both the pattern of response and the within-subject covariance structure across time points. Primary analyses will not include baseline covariates, as the randomization will balance the treatment groups and eliminate selection bias. To enhance power by controlling for variability in response (Kahan et al., 2014), a small set of baseline covariates (e.g. gender, age, ethnicity) will be included in supplementary analyses.

The response may be transformed (e.g. log transformation) to reduce levels of skewness in the response to acceptable levels, as assessed by examination of residuals. Models will include binary indicators for adolescent group (omega-3 vs placebo), caregiver group (omega-3 vs placebo), and time (four level factor for times 0, 6, 12, and 18), and group by time interaction terms, as fixed effects. Time is modeled as a discrete factor to accommodate the patterns observed in our prior study (see Figure under Preliminary Data) where the omega-3 adolescent groups are similar at baseline and 6 months, and separate at 12 months. Covariance structure models (e.g. random effects, autocorrelated residuals) will be compared using information criteria in models containing all main effects and interactions of interest. Once the covariance structure has been chosen, the fixed effects will be assessed for significance using likelihood ratio tests.

Parameter estimates, standard errors, and 95% confidence intervals will be reported. In the case of a multiple degree of freedom test (e.g. group by time interaction, with 3 df), single-df contrasts will be estimated and reported, to explain the nature of the interaction.

For the adolescent, Hypothesis 1a will be addressed using a child group by time interaction term (time trends for omega-3 and placebo group need not be parallel), or a child group main effect if the interaction term is non-significant (parallel time trends). Similar models will address the corresponding Hypothesis 1b for the caregiver responses. For both the adolescent and caregiver responses, Hypothesis 2a will be tested by an adolescent group by caregiver group by time interaction, or by an adolescent by caregiver interaction if the four adolescent by caregiver group trends are parallel.

Aim 3 (Secondary Outcome). Working hypothesis 3a (change in neurocognitive and academic factors) will be tested using the same mixed effects models as outlined above in aims 1 and 2. Working hypothesis 3b (mediation), whether improvement in neurocognitive variables (e.g. impulse control) or academic WRAT scores partly account for the treatment effect on child behavior will be assessed using mediation analyses. Based on the prior study (Raine et al, 2015), effects of supplementation should emerge by the 12-month time point. Analyses will test whether changes in risk factors between baseline and the 6 month point mediate the 12-

month treatment effect. These analyses will be conducted using the PROCESS SPSS macro (Hayes, 2012), which will also be used to assess moderated mediation (e.g. neurocognitive measures mediate outcomes more strongly in younger adolescents). To test significance of the indirect effects (i.e. mediation), bias-corrected confidence intervals for the indirect effects will be generated using 10,000 bootstrap samples. To assess extent of mediation, the reduction in variance explained in treatment outcome by the intervention after controlling for improved parental behavior (the mediator) will be calculated in a two-step regression. Equivalent analyses will be performed for the other neurocognitive variables.

Moderator Analyses. We will conduct exploratory analyses on age, gender, ethnicity, initial general nutritional status, and initial omega-3 levels. To test for moderators, we will extend the models of the primary hypotheses to include main effects for each moderator and their interaction effect with the group factor. Analyses will initially consider each moderator separately, and multivariate models will examine the joint effects of significant moderators. As a secondary analysis we hypothesize that better treatment outcome may be observed in children who start out with lower blood levels of omega-3, and have diets high in omega-6 (which compete with omgega-3 for uptake).