Principal Investigator: Floyd H. Chilton, Ph.D.; Roberta D. Brinton, Ph.D.

Study Title: Study of Effect of Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) on Biomarkers of Sub-concussion Injuries in American Football.

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Study Randomization

This study is designed to replicate and extend a study done at Texas Christian University (TCU), which looked at the effects of three doses of EPA+DHA supplementation on neurofilament light chain (NFL) levels in TCU football players over the course of a complete football season including summer training camps (Oliver et al. 2016). In that study, players were randomized to one of three supplement doses or placebo by player position, as different positions experience different degrees of head trauma. However, this resulted in very small sample sizes, and they found that the main change in their primary outcome (NFL) was for starters as opposed to non-starters, and they excluded non-starters in multiple analyses. In this study, we will modify the previous study design in two ways. First, we will use only one supplementation dose (3g DHA/EPA fish oil) vs placebo (high-oleic safflower oil). Second, we will use a block randomization scheme by which players will be randomized both by whether they are a starter or non-starter, and then by position group to receive supplementation or placebo. Starters are defined as players on the 1st and 2nd depth rosters. This definition results in the potential for n=44 starters and n=44 non-starters. The eight position groups are based on the literature (Baugh et al. 2015): offensive linemen (offensive center, guard, and tackle); defensive linemen (defensive end and defensive tackle); running backs & tight ends (fullback, running back, tight ends); linebackers (middle linebacker and outside linebacker); defensive backs (cornerback and safety); special teams (punter and kicker); wide receivers; and quarterbacks. Within blocks, we will use a random number generator to randomly assign players to treatment or placebo groups. In order to blind the contents of the capsule from both investigators and players, the supplements will then be allocated and re-labelled as "A" or "B" by a project team member who is not participating in data collection during the study.

Power, Sample size, and Confidence intervals

This study is both an independent replication of a previous omega-3 supplementation trial with college football players, as well as an exploratory, hypothesis-generating extension that will collect additional biological data including cardiometabolic biomarkers and untargeted lipidomics. For statistical power, our primary hypothesis-driven question is whether DHA+EPA supplementation will have a protective or restorative effect based on measured levels of markers of neuronal damage, such as NFL, and whether this will vary by starter/non-starter. In this study, with a samples size of n=44 starters, n=44 non-starters, and a significance level of α=0.05, we have 80% power to detect an effect size of d = 0.6, where $d=(\mu_1 - \mu_2)/\sigma$, is the difference in the group means divided by the pooled standard deviation. The TCU study looked at the differences between starters and non-starters at multiple time points and found effect sizes ranging from 0.88 to 2.07. The TCU study also found dramatic changes in NFL levels between starters on placebo, with levels more than doubling over the season, while starters on supplementation saw a more modest increase of 35% by the end of the season. If we look only at starters, and compare the supplement (n=22) and placebo groups (n=22), we are powered to see an effect size of 0.86, well below the reported differences at many time points in the TCU study. We anticipate some other biomarkers will also change over time, and we are

well-powered to detect small to moderate effects. For previously unstudied biomarkers, our focus will be on estimating the magnitude and direction of effects in order to discover which are the most informative as markers of brain injury/recovery or omega-3 supplementation.

Data analysis

REDCap provides easy data storage and manipulation (with audit trails for reporting, monitoring and querying patient records), and an automated export mechanism to common statistical packages. We will conduct most analyses using R and relevant R packages (e.g., ggplot2, dplyr, MetaboAnalyst).

This study will collect multiple types of data from each player at multiple time points. Many analyses will be exploratory in nature as this will be the first data of its kind.

Continuous outcome measures include:

- Biomarkers – NFL
- Cardiometabolic markers – TNF-a, IL-6
- Lipid panel – TG, HDL-C, LDL-C
- GC-FID for both RBC and Plasma – results in 23 free fatty acids concentrations

For these continuous outcomes, we will use a longitudinal mixed-effects model framework to look at both individual and group-level changes from baseline over time. Outcome measures will be transformed where appropriate (e.g., log, sqrt, Box-Cox). Covariates will include age, position, genotypes, treatment, and starter/non-starter, as well as a treatment x starter/non-starter interaction. We will also graphically explore the trajectories of the different biomarkers over time.

Untargeted lipidomics will generate data on ~800 lipids

We will analyze the lipidomics data using a custom analysis pipeline developed by the Chilton lab based around the MetaboAnalyst R package and custom scripts that is already in use in other metabolomics/lipidomics analyses. Data will be transformed and normalized as appropriate. We will model changes over time in a longitudinal mixed-effects model framework similar to the other biomarkers. We will also use several ordination strategies to determine how lipid species relate to omega-3 supplementation including principal components analysis (PCA), and partial least squares discriminant analysis (PLS-DA). Due to the compositional nature of untargeted lipidomics data, we will use additive log ratio transformed data in the linear models, and use the centered log-ratio transform before performing ordination analyses. We will also further explore the covariance structure of the data and determine which groups of lipid molecules are changing together or in opposing directions. We will then map lipid species to biochemical pathways to gain additional insight into the underlying biology of brain injury and recovery.