Statistical Analysis Plan

Official Title: Investigating the Stability, Variability and Mechanism of Incorporation of Lipid Mediators into Eccrine Sweat

ClinicalTrials.gov ID: NCT02935894

Unique Protocol ID: 929370

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Date: December 5, 2017

Introduction

The purpose of this study is to see what the differences are in sweat (amount and small molecule content) collected from different sites of the body and by different methods of sweat stimulation. Additionally, the investigators want to know whether the amount and small molecule content of the sweat is the same in an individual over time, and the same across individuals at a given time. Finally, the investigators want to know how consumption of over-the-counter anti-inflammatory drugs such as ibuprofen will affect the inflammatory mediator content of sweat and how that compares to blood. This information will help to better understand the composition and behavior of sweat and assess its potential utility as a routine clinical tool in skin research.

Subjects will participate in 4 study day visits that will be scheduled about 1- week apart. During the first study visit, the investigators will compare sweat collected following pharmacological stimulation of sweating (using the drug pilocarpine) to sweat collected following physiological stimulation of sweating (using a stationary bicycle). During the second study visit, the investigators will collect sweat following stimulation of sweating by the drug pilocarpine from the inner part of the forearm (near the wrist) and also the upper surface of the thigh (near the knee). During the third study visit, the investigators will collect sweat following stimulation of sweating by the drug pilocarpine from the inner part of both forearms (near the wrist). During the fourth study visit, the investigators will collect a sweat sample following stimulation of sweating by the drug pilocarpine from the inner part of the forearm (near the wrist) and a blood sample (about one teaspoon) from the other arm. After blood and sweat collection, participants will consume 400 mg (two tablets) of ibuprofen. The investigators will then collect blood and sweat from 30 minutes, 2 hours and 4 hours after participants consume the ibuprofen.

Analysis Objectives and Sets

Data from the first three study visits will be analyzed to assess the impact of different sweat stimulation techniques and collection sites on the sweat lipid mediator profile. Secondarily, data from pilocarpine-stimulated sweat collected from the volar

forearm collected at each study visit will be evaluated to assess to compare the temporal stability and intra- and inter-individual variability pharmacologically stimulated sweat lipid mediators. Data from the fourth study visit will be analyzed to determine the impact of systemic ibuprofen administration on sweat lipid mediators as a means to evaluate the source of these metabolites. Differences in sweat lipid mediators at each collection time and correlations between sweat and plasma lipid mediator concentrations at baseline will be assessed to evaluate these outcomes.

Data Analysis Workflow

Visits 1–3

Prior to statistical analyses, data will be curated such that analytes with >30% missing values across the data set are excluded. The remaining data will be screened for outliers using Huber's maximum likelihood type estimates to determine the center and spread of each analyte [1]. Missing data will be imputed by least squares multivariate normal imputation [2], after which data will be transformed to normal using the generalized logarithm transform [3], and normality will be verified using the Shapiro-Wilk test [4]. All data pretreatment will be performed in JMP Pro 12 (SAS Institute, Inc., Cary, NC, USA).

To prevent differential storage times, samples will be processed weekly along with a pooled sweat LRM. If batch effects are noted, data will be adjusted using the batch-to-concentration correlation for each lipid mediator detected in the LRM, by calculating the batch-specific residuals and subtracting these from the measured data. Batch-adjusted data will be then transformed to normal as described above. All batch adjustments will be performed in Microsoft Excel 2010 (Redmond, WA, USA).

Comparisons of mean lipid mediator concentrations in sweat stimulated by pilocarpine iontophoresis and exercise will be made by paired Student's t-tests. Comparisons of collection sites (anterior distal thigh, lower back and volar forearm) will be made by one-way ANOVAs, while assessments of sweat lipid mediator temporal stability will be made by repeated measures ANOVAs. Subject identification will be included as a

random effect in the one-way ANOVA models, as only two subjects produced sweat from all three collection sites. All univariate comparisons will be performed in JMP Pro 12 (SAS Institute, Inc.) and adjusted for multiple comparisons using the Benjamini-Hochberg procedure at $q = 0.05$ [5].

Multivariate analyses will be conducted by partial least squares discriminant analysis (PLS-DA), using two different algorithms in the R statistical environment (Vienna, Austria). Models will be constructed using either the orthogonal scores algorithm with univariate scaling and leave-one-out cross-validation in imDEV version 1.42, a Microsoft Excel add-in [6], or using the *pls* and *caret* packages in R version 3.4.1 [7, 8]. In both cases sweat stimulation technique, collection site or study visit will be used as classifiers, and variables will be clustered by Spearman correlation coefficients using the Minkowski distance and Ward agglomeration. Models built using imDEV version 1.42 will be evaluated using the Q2 score, with $Q2 \geq 0.4$ considered indicative of a predictive model [9]. Models built using the *pls* and *caret* packages in R version 3.4.1 will be evaluated using model accuracy, and the significance of model accuracy was evaluated using a chi-squared test.

Visit 4

Plasma and sweat data will be independently pretreated, and all data pretreatments will be performed in JMP Pro 13 (SAS Institute, Inc., Cary, NC, USA). Prior to statistical analyses, data will be curated such that analytes with >30% missing values across the data set will be excluded. The remaining data will be screened for outliers using Huber's maximum likelihood type estimates to determine the center and spread of each analyte [1]. Missing data will be imputed by least squares multivariate normal imputation with covariance shrinkage [2], after which data will be transformed to normal using the procedures of Box and Cox [10], and normality was verified using the Shapiro-Wilk test [4].

Linear mixed models evaluating the relationship between metabolite concentrations and time with a random intercept for subject, will be performed in R version 3.1.0 using the *lme4* package [11] after verification of population homoscedasticity at each timepoint in plasma and sweat by Bartlett's test using JMP Pro 13. Statistical significance of the fixed

effect of time was determined by likelihood ratio tests of the full model against a null model (i.e., without the fixed effect of time, but with random intercept for subject). Results of likelihood ratio test will be considered significant if $P < 0.05$ and the false discovery rate (q) as estimated by the method of Storey and Tibshirani using the *fdrtool* package is < 0.2 [12, 13]. Differences between metabolite concentrations at each timepoint will be assessed by Tukey's post-hoc HSD test using the *multcomp* package [14].

Comparisons of metabolite patterns in plasma and sweat will be performed in JMP Pro 13. Correlations between imputed but not transformed baseline (i.e. 0 h) lipid mediator concentrations in plasma and sweat will be estimated using Kendall rank correlation coefficients for analytes detected in both matrices. Additionally, relative abundances of all detected lipid mediators in each matrix will be estimated on both a per-class (e.g. relative abundance of total alcohols detected regardless of fatty acid precursor) and by fatty acid precursor within a lipid mediator class (e.g. individual abundances of alcohol metabolite isomers of arachidonic acid), and will be compared using the Wilcoxon rank-sum exact test.

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