Statistical Analysis Plan

NCT04175106

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1 Methodology

This methodology section covers the study design, which is a crossover longitudinal study. Measurements are taken for each subject across time and treatment. First, the study design is described in detail, common methodology for crossover and longitudinal studies is presented, and then a statistical analysis methodology blending the two is proposed.

1.1 Study design

A four-way, placebo-controlled crossover study will be conducted to compare phytonutrient bioavailability from two varieties of blueberries, blueberry-rich protein bar, and a placebo drink. Each of the 28 participants will partake in a three-month long study with four feeding visits, separated by washout periods lasting 7 days, ranging from 11 to 18 days. For each visit, participants will provide blood (baseline, 1, 3, 6, 9, 24, 48 hours) and urine (48 hours prior, 24 hours prior, 0-9 hours, 9-24 hours, 24-48 hours) samples to be analyzed for biomarkers necessary to measure bioavailability and other pharmacokinetic parameters. Figure 1 illustrates the steps a participant takes for one feeding visit. The study has been reviewed and approved by the Institutional Review Board at the North Carolina State University.

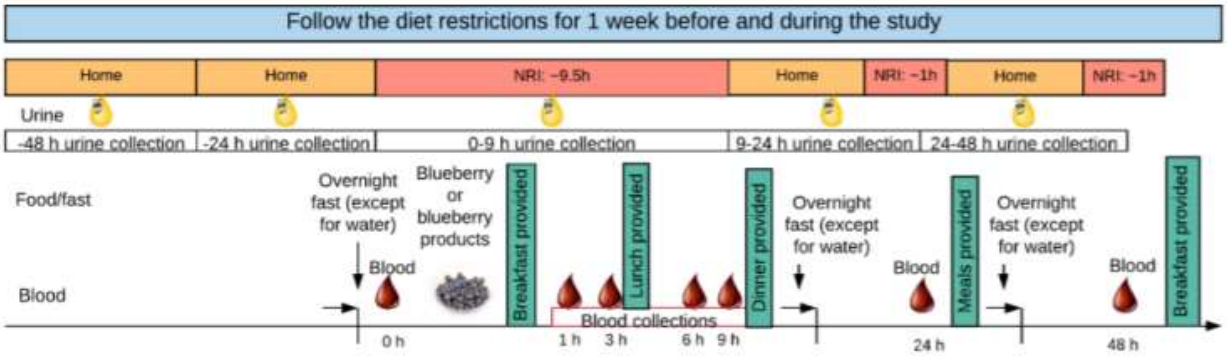


Figure 1: Guideline of Dietary Restrictions of Prior and During the study

1.2 Methodology For Longitudinal Studies

Longitudinal studies involve repeated measurements of specified variables over a period of time. Within each period *k*, each subject *i* will undergo 7 blood collections at time points of 0, 1, 3, 6, 9, 24, and 48 hours post-treatment and 5 urine collections at -48, -24 pre-intervention and 0-9, 9-24 and 24-48 hours post-treatment tracking concentrations of pre-specified metabolites reported as concentration versus time. Concentrations will be converted to corresponding pharmacokinetic parameters: maximum serum concentration (C_{max}), time at maximum concentration (Tmax), the half-life [$t_{1/2}$], the area under the curve (AUC), area under the first moment curve (AUMC), the clearance (CL), the volume of distribution (V_d), and the mean residence time (MRT) of blueberry-derived metabolites in the blood and urine using an appropriate pharmacokinetic model (PK) [BAMStudy2020]. The software Phoenix WinNonlin is planned to be used to fit the PK model. Using the calculated AUC and dosage D, the total bioavailability for each metabolite can be determined [2]. Subject specified covariates (such as gender, age, body mass index, treatment order, and sample type) may be significant in the PK

model and will be considered for potential inclusion. However, the exact nature of this model will be determined as the study is completed and is beyond the scope of this statistical analysis plan.

1.3 Methodology for Crossover Studies

In crossover studies, measurements are repeated across treatments. Each subject receives each treatment with a washout period in between. We will use a toy example where Y_{ijkl} is the outcome, i denotes the subject, j denotes the treatment, k denotes the period, and l denotes the sequence. For four treatments, A, B, C, D, there are a number of different treatment sequences (e.g. ABCD, ACDB, BACD) and four periods. We account for variability from sequences and periods in the model. An example of a crossover model is the following:

$$Y_{ijkl} = \mu + \delta_l + \beta_{i(l)} + \alpha_j + \gamma_k + \alpha_{\gamma jk} + \varepsilon_{ijkl} \quad (3.1)$$

where, μ is the overall mean, fixed sequence effect is δ_l, fixed treatment effect is α_j, fixed period effect is γ_k, and $\beta_{i(l)}$ is the random individual subject effect for individual i nested in the sequence l. The fixed interaction between treatment and period is $\alpha_{\gamma jk}$ and the random error from the realization process or measurement error is $\varepsilon ijkl$. For the random effects, we assume $\beta i(l)$ is independent of $\varepsilon ijkl$ and we have flexibility in modeling the random errors. For example, it may be reasonable for the residual error to have different variances based on treatment: $Var(\varepsilon_{i1kl}) \neq Var(\varepsilon_{i2kl})$. Naturally, one could add additional fixed effects for covariates or interactions between covariates and treatments. Mean and covariance parameters are typically estimated through maximum likelihood estimation (MLE). In SAS, PROC MIXED is a popular procedure to fit these mixed effects models. Sophisticated inference including adjustments for multiple comparisons can be carried out with PROC MIXED. In R, the nlme and lme4 [1] packages can be used to fit the models. The lmerTest package is a popular companion to lme4 for statistical inference [3].

1.4 Proposed Methodology for Crossover Longitudinal Studies

The model specified in (1.1) will serve as a framework for testing the differences of fixed effects between treatments. Using the methodology outlined in Section 1.2, the appropriate PK parameters for a given metabolite (ex. Bioavailability) will be calculated. The PK parameters will be represented as the continuous responses Y_{ijkl} in the model. Note that each measured PK parameter is specified to a given subject I receiving treatment j at a specified period k within sequence l.

It is of primary interest to test the potential differences in bioavailability of blueberry-derived metabolites across treatments. Given the number of metabolites being tested, multiple comparison adjustments will be necessary. There are a multitude of strategies for tackling such problems each with their respective advantages and disadvantages. However, given the relatively small sample size (n=28), controlling the family-wise error rate (FWER) across all metabolites

may drain requisite statistical power needed to detect any difference between treatments. It may be beneficial to set up a smaller subset of metabolites prior to analysis that are of primary interest and then investigate differences between the remaining metabolites in an exploratory nature.

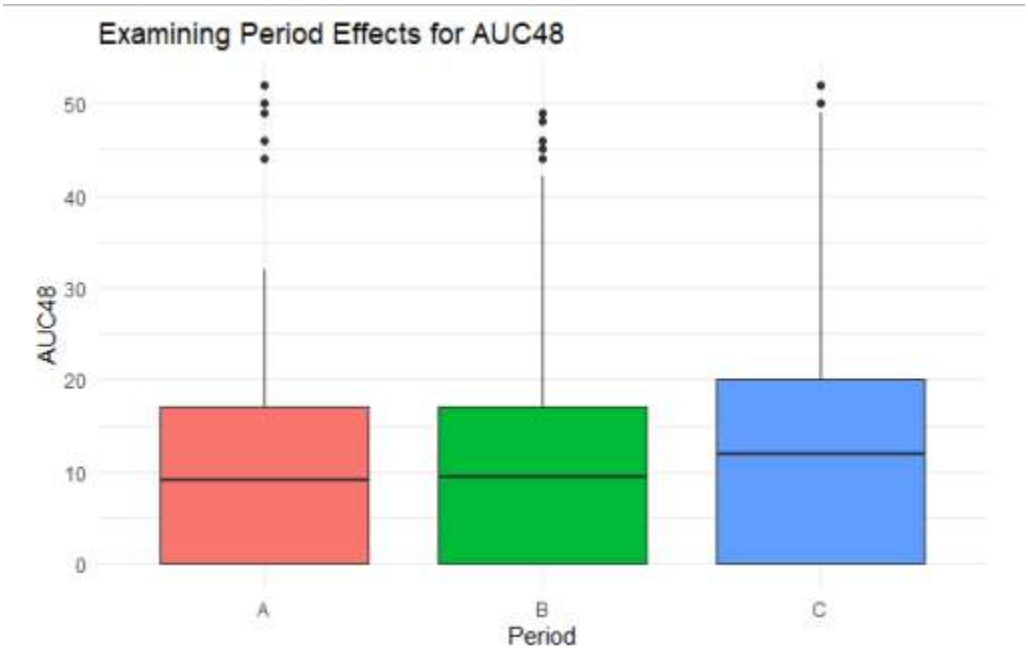
2 Recommended Analysis/Design

2.1 Exploratory Analysis

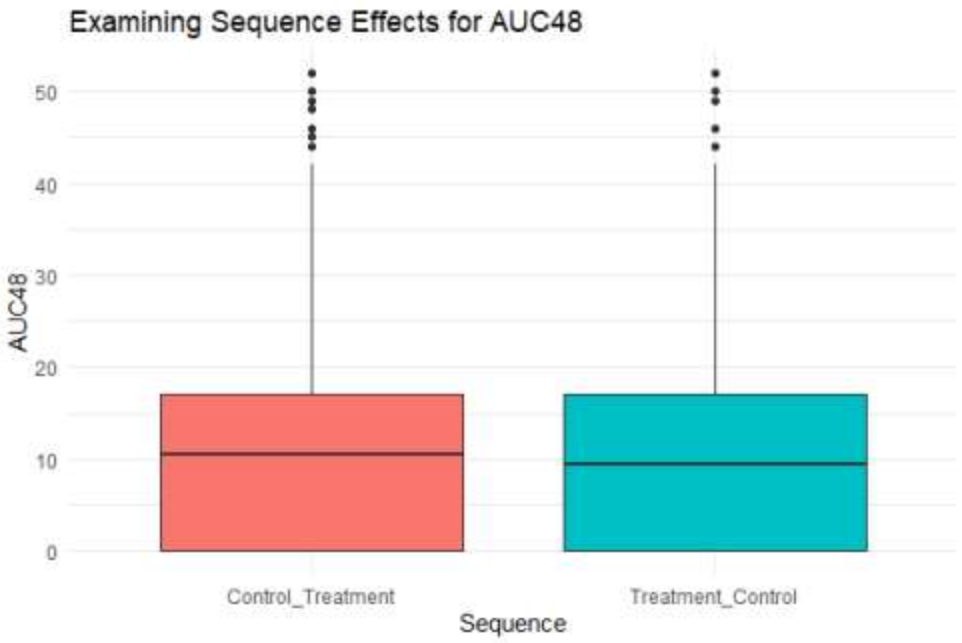
In this section, we will walk through a toy example to show how we could build a model similar to (1.1). In the subsequent section, we will build the model using the lme4 package in R and show how to conduct valid statistical inference using the lmerTest package in R.

For data manipulation, we mostly use the dplyr package and for plotting we mostly use the ggplot2 package. These are part of the "tidyverse" [4].

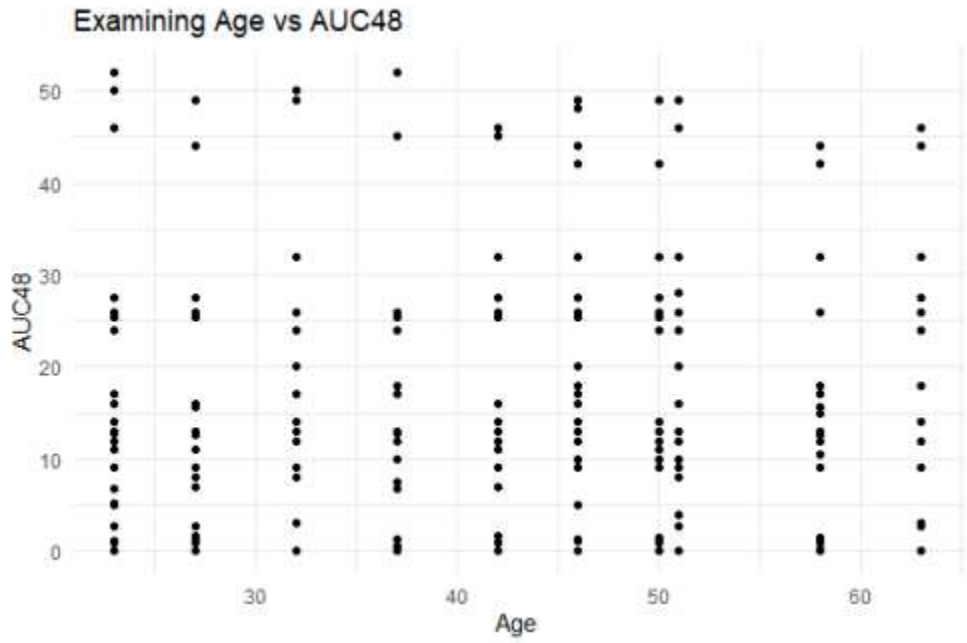
For this example, we will use AUC48 from the plasma dataset as our outcome. First, we examine the period effect using a boxplot.



Since the sequence variable is not already in the dataset, we need to create it. An example of creating the new variable is in the appendix.



Now, we are interested in which in the effect age may have on the response. We can examine this via a scatterplot.



A similar plot can easily be made for any other continuous covariate of interest. Based on these summary statistics, we can gain insight on what additional covariate we may want to include into the model.

2.2 Fitting the Model

The toy dataset provided serves to illustrate the proposed methodology of the actual analysis. The dataset contains PK parameters from 15 subjects participating in a crossover design study with three periods and two treatment groups, a blueberry treatment and a control group. At each period, subjects had concentrations of 15 different metabolites measured via blood and urine samples. Concentrations were converted to corresponding pharmacokinetic parameter using an appropriate PK model. The primary purpose of the study was to compare the bioavailability of the ten metabolites between the blueberry treatment and the control group.

We propose the following model for analysis:

$$Y^n_{ijkl} = \mu + \delta^n_l + \alpha^n_j + \gamma^n_k + (\alpha\gamma)^n_{jk} + \varepsilon_{ijkl} \tag{2.1}$$

Below are detailed descriptions of each term in the model:

- μ: Overall mean.
- δ^n_l: Sequence Effect for metabolite n.
- α^n_j: Fixed treatment effect for metabolite n.
- γ^n_k: Period effect for metabolite n.
- $\alpha\gamma^n_{jk}$: Interaction between treatment and period for metabolite.
- ε_{ijkl}: Random error from the realization process or measurement error.

The effects δ^n_l, γ^n_k, and $\alpha\gamma^n_{jk}$ can be considered fixed or random. We assume the random effects are independent of each other. Here the response Y_{ijkl}, represents the bioavailability of metabolite n. Additionally, i denotes the *i-th* subject in the dataset. If we are interested in the model for just one metabolite, we can subset the dataset to just include observations for that one metabolite and then fit a similar, smaller model. An example is:

$$Y_{ijkl} = \mu + \delta_l + \alpha_j + \gamma_k + \varepsilon_{ijkl} \tag{2.2}$$

Below are detailed descriptions of each term in the model:

- μ: Overall mean.
- δ_l: Sequence Effect.
- α_j: Fixed treatment effect.
- γ_k: Period effect.
- ε_{ijkl}: Random error from the realization process or measurement error.

Notice this is the same model as before without the interaction, but without the n superscripts, since we only have one metabolite of interest.

This model can be fit using the lmer function in the lme4 package of R. We will use the smaller model with just one metabolite for demonstration. To add the effect of one variable, X_1,

we simple include it the model statement, Y ~ X_1, the first argument for the function. To specify a random effects, we use (1|X_1) in the model statement. We can specify interactions with X_1:X_2. Below is specific example of model in 2.2, using AUC as the response, and treatment and period as fixed effects, and sequence as a random effect.

```
## fit the model
## response: AUC48
## Fixed Effects: Treatment, Period
## Random Effects: Sequence
fit <- lmer(AUC48 ~ Treatment + (1|seq) +
                    'Visit Period',
                    data=dat, REML = FALSE)
```

2.3 Interpreting Model Output

Using the lmerTest package in R, we can conduct hypothesis tests for fixed and random effects in linear mixed effects models. The anova function provides F-tests on the fixed effects, letting you evaluate if a fixed effect is useful for explaining the response. Using the above model, the anova output is as follows:

```
> anova(fit)
Type III Analysis of Variance Table with Satterthwaite's method
              Sum Sq Mean Sq NumDF  DenDF F value    Pr(>F)
Treatment     158.70  158.70     1 177.99  17.746 4.005e-05 ***
'Visit Period' 391.38  195.69     2 178.34  21.883 3.170e-09 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Based on this, we can see that both treatment and period are useful for explaining the response. Since treatment is binary (control, treatment) here, we can examine if administering the treatment increases or decreases the response, on average. The summary function is one way to do this. Below is selected output:

```
> summary(fit)
Fixed effects:
                   Estimate Std. Error        df t value Pr(>|t|)
(Intercept)         0.02752    0.84451   4.24374   0.033    0.975
TreatmentTreatment  1.91665    0.45498 177.99167   4.213 4.00e-05 ***
'Visit Period'B    -0.29409    0.55514 178.22795  -0.530    0.597
'Visit Period'C     3.24513    0.63618 178.66646   5.101 8.57e-07 ***
```

Notice the estimate for the TreatmentTreatment is 1.91665. This indicates the model the model estimates a 1.91655 increase in AUC if the Treatment is administered instead of the control. The corresponding p-value is small, so we can be confident that the treatment increases AUC, on average. To check if the random effects are useful for explaining the response. Since we are not interested in specific levels of the random effects, we just want to check if considerably variation is coming from the random effects. The ranova function runs a hypothesis testing the variance against zero. For the ANOVA model, here is the output:

```
> ranova(fit)
ANOVA-like table for random-effects: Single term deletions

Model:
AUC48 ~ Treatment + `Visit Period` + (1 | seq)
          npar  logLik    AIC    LRT Df Pr(>Chisq)
<none>       6 -454.87 921.74
(1 | seq)    5 -460.85 931.70 11.969  1   0.000541 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

This tells us that there is considerable variation between sequences. Some quick summary statistics show us the mean AUC for the control then treatment sequence is 2.80 and the treatment then control sequence is 0.50 (note this is just for this metabolite, not all metabolites like the plot in 2.1). This intuitively shows why the sequence effect has a small p-value.

3 Code Appendix

First, we present the code for the plots.

```
## load in "tidyverse"
install.packages("tidyverse")
library(tidyverse)

## boxplot based on period
ggplot(data=plasma) +
  geom_boxplot(aes(x=`Visit Period`,y=AUC48,fill = `Visit Period`)) +
  labs(x="Period",y="AUC48") +
  ggtitle("Examining Period Effects for AUC48") +
  theme_minimal() +
  theme(legend.position = "none")

## create sequence variable

## find each individual's first treatment
## create dataset with subject ID and trt1
int1 <- plasma %>%
        filter(Visit=="Day 1") %>%
        mutate(trt1=Treatment) %>%
        select(`Subject ID`,trt1) %>%
        unique()

## create dataset with subject ID and trt2
int2 <- plasma %>%
        filter(Visit=="Day 2") %>%
        mutate(trt2=Treatment) %>%
        select(`Subject ID`, Visit, trt2) %>%
        unique()

## combine datasets to make a subject ID and sequence dataset
seq_dataset <- bind_cols(int1,int2) %>%
               unite(seq,trt1,trt2) %>%
               select(`Subject ID`,seq)

## add the sequence column to the
plasma <- left_join(plasma,seq_dataset,by="Subject ID")

 ## plot
 ggplot(data=plasma) +
   geom_boxplot(aes(x=seq,y=AUC48,fill=seq)) +
   labs(x="Sequence",y="AUC48") +
   ggtitle("Examining Sequence Effects for AUC48") +
   theme_minimal() +
   theme(legend.position = "none")

 ## scatterplot with age
 ggplot(data=plasma) +
   geom_point(aes(x=Age,y=AUC48)) +
   #facet_wrap()
   labs(x="Age",y="AUC48") +
   ggtitle("Examining Age vs AUC48") +
```

```
  theme_minimal() +
  theme(legend.position = "none")
```

Now the code to fit the models and examine their output:

```
## load in packages
install.packages("lme4")
install.packages("lmerTest")
library(lme4)
library(lmerTest)
library(tidyverse)

## subset dataset
dat <- plasma %>% filter(Metabolite=="1-M-Xan_108Q")

## fit the model
## response: AUC48
## Fixed Effects: Treatment, Period
## Random Effects: Sequence
fit <- lmer(AUC48 ~ Treatment + (1|seq) +
            `Visit Period`,
            data=dat, REML = FALSE)

## examine fixed effects
anova(fit)
summary(fit)

## examine random effects
ranova(fit)

## summary stats to make sense of random effects
dat %>% group_by(seq) %>%
  summarise(mean_AUC=mean(AUC48),
            sd_AUC=sd(AUC48),
            med_AUC=median(AUC48))
```

References

[1] Douglas Bates et al. “Fitting Linear Mixed-Effects Models Using lme4”. In: Journal of Statistical Software 67.1 (2015), pp. 1–48. doi: 10.18637/jss.v067.i01.

[2] Shein Chung Chow. “Bioavailability and bioequivalence in drug development”. In: Wiley Interdisciplinary Reviews: Computational Statistics (2014). issn: 19390068. doi: 10.1002/wics.1310.

[3] Alexandra Kuznetsova, Per B. Brockhoff, and Rune H. B. Christensen. “lmerTest Package: Tests in Linear Mixed Effects Models”. In: Journal of Statistical Software 82.13 (2017), pp. 1–26. doi: 10 . 18637/jss.v082.i13.

[4] Hadley Wickham. “tidyverse: Easily Install and Load the Tidyverse”. In: (2017). R package version 1.2.1. url: https://CRAN.R-project.org/package=tidyverse.