Antibody Response Induced by HIV Vaccines and T-cell Suppression Treatments in Rhesus Macaques

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1 Introduction

The current report describes multivariate and longitudinal data analyses done on a dataset from an HIV study, in which researchers administered HIV vaccine injections and immuno-suppression treatments to 20 rhesus macaques and measured antibody characteristics. This project's main goal is to determine whether the vaccine's efficacy, reflected by the variable `Binding`, can be increased by the number of vaccine injections and the type of immuno-suppression treatments.

1.1 About the Study

A dominant vaccine development strategy is to induce neutralizing antibodies by immunizing humans with the virus' glycoproteins. However, HIV vaccines that adopted this strategy mostly failed due to the fact that HIV is an RNA virus, which mutates rapidly to escape the inhibition of neutralizing antibodies. By the time the body generates neutralizing antibodies against the glycoproteins of some HIV strains, the RNA virus has already mutated. Thus, the existing neutralizing antibody fails to recognize, bind with, and neutralize the HIV virus. One possible solution is to increase the number of potential neutralizing antibodies that will cycle in the body by releasing a variety of antibodies after glycoprotein immunization. Regulatory T (Treg) cells prevent autoimmune diseases and suppress allergic reactions by inhibiting adaptive antibody immune response in the germinal center. Theoretically, this adaptive response lowers the effectiveness of vaccines. Thus the experiment used T-cell suppression treatments, widely used in post transplant immuno-suppression treatment to prevent rejection, and to investigate whether the treatments improve the efficacy of immunization.

During the study, 20 rhesus macaques were given glycoprotein immunization and supplemental antibody doses, as well as one of three treatments (two experimental regulatory T-cell suppression

treatments and one control). The resulting dataset includes measurements of antibodies after the 20 macaques were given the same HIV vaccine at three different time points and one of three randomly selected anti-Treg treatments. Blood samples were collected two weeks after vaccine dosing, and antibodies were isolated from those samples. Limited by assay yield, the number of antibodies collected from each blood sample varied. In short, each observation contains information about the antibody isolated post the glycoprotein immunization.

A human antibody is formed heavy chains and light chains. A heavy chain has about 51 V-gene segments, 25 D-gene segments, and 6 J-gene segment. A light chain has about 71 V-gene segments and 9 J-gene segments[ref.5]. Any heavy chain V-D-J combination and light chain V-J combinations can randomly happen in germinal centers. Theoretically, there can be $51 * 25 * 6 * 71 * 9 = 4.88835 \times 10^6$ combinations of gene segments. Considering the frequent mutations and other factors, each individual can have over **10 billion** different antibodies. Thus, we followed the convention of vaccine studies and viewed each antibody as independent for multivariate data analysis. For longitudinal data analysis, since general linear and linear mixed models allow measurements from the same observational unit to correlate over time, we used different correlation structures to find a best model. This essentially implies that the antibodies from the same macaque can be correlated.

Section 7.1 shows a list of variables with a brief description from our dataset. Please note that each antibody contains two sets of heavy chain and light chain, all of which forming a Y-shape immunoglobulin. Thus many of the variables start with H or L, indicating the chain from which the information comes. Some variables have missing numbers. We chose to let R functions ignore missing values, since we wanted to keep as much data as possible.

As one of the authors for four publications that used the dataset, Kan Luo provided the dataset, which can be found here. Now we turn to our research questions, after which the methods section includes exploratory data analysis, multivariate data analysis, and longitudinal data analysis. The statistical results are included in the methods section and summarized in the results section. We discuss implications and limitations in the discussions and some final thoughts in the conclusions.

1.2 Research questions

The current project focused on understanding whether the number of vaccine injections (`Time_Point`) and the different Treg inhibitor treatments (`Drug`) caused changes in the antibody characteristics and if the changes were related to the immune responses against HIV virus. The category of antibody (`Isotype`) was not controlled by the researchers, but it could be related to the response variables and is thus included as a predictor. Our research questions are:

RQ1: Do time points and drugs have effects on the mutation frequency (`HMuFreq` and `LMuFreq`) and the amino acid count in the third complementarity determining region (`H_CDR3` and `L_CDR3`)?

RQ2: How does the binding strength of the antibodies (`Binding` or `logBinding`) develop in response to the number of vaccine dosages (`Time_Point`) and immuno-suppression treatments (`Drug`)?

2 Methods

This section first provides an overview and summaries of the dataset. It then uses multivariate and longitudinal data analyses to answer the research questions.

2.1 Data Summaries

2.1.1 An Overview of Antibodies by Time Points, Drug Types, and Isotypes

A total of 2465 antibodies, from 20 rhesus macaques, were collected at four different time points (0, 1, 2, 3) and each macaque was given one of three drugs (1 and 2 are immuno-suppressing drugs and 3 is a mock control). Figure 1 shows the histograms of antibody counts, and Table 1 and Table 2 show the antibody counts in different combinations of drugs, time points, and isoptypes.

Figure 2 shows the histograms of `Isotype`, which show that IgG and IgM occupied the biggest proportion of antibodies in all time points. Before immunization (time point 0), there were similar weight of IgG and IgM found in blood. After the first immunization (time point 1), primary immune response resulted an increase of IgM, followed by an IgG increase at later time points 2 and 3.

2.1.2 Outlier Detection

We included five response variables for the project: H_CDR3, HMuFreq, L_CDR3, LMuFreq, and Binding. As shown in Figure 3, one data point of L_CDR3 seems an outlier. The summary statistics of standardized L_CDR3 in Table 3 show that a maximum value of 30, which is quite unusual. Figure 4 shows the Mahalanobis ditances and Z scores of L_CDR3, and the same data point again appears to be an apparent outlier. The value for L_CDR3, 47, is quite unlikely. Since we were not not the original researcher and unable to reexamine the original data, we removed the data point.

2.1.3 Response Variables

While examining our responses, H_CDR3, HMuFreq, L_CDR3, LMuFreq, and Binding, we observed that H_CDR3's distributions are approximately normal with the center around 13 at different time points (Figure 5). Figure 6 shows the distributions of H_CDR3 with respect to treatments at different time points, which are again approximately normal. With L_CDR3, Figure 7 and Figure 8 show approximately normal distribution, centered around 9, and with a longer right tail. The Q-Q plots in Figure 9 show that H_CDR3 and L_CDR3 are both approximately normal.

HMuFreq and LMuFreq are calculated by dividing H_Substitution by H_VBase for heavy chains and L_Substitution by L_VBase light chains. These two variables show the degree to which the antibodies mutate. A higher mutation rate usually indicates better virus neutralization. Figure 10, Figure 11, Figure 12, and Figure 13 show that HMuFreq and LMuFreq are both approximately normal, each with a long right tail. The Q-Q plots in Figure 14 confirm the approximate normality of HMuFreq and LMuFreq.

Next, a histogram of Binding with respect to treatment at different time points and Q-Q plot are shown in Figure 15 and Figure 17. We observed that Binding is not normally distributed. Since the dataset has a large sample size (n = 2464), we can use the Central Limit Theorem and assume normality. However, since many data points have the value 0 for Binding, linear models for longitudinal analyses might lead to negative values. To avoid this problem, we transformed Binding to $log(Binding + 1)$ and called it logBinding. The Q-Q plot of logBinding is shown in Figure 16. Lastly, Figure 18 shows that none of the response variables are highly correlated.

2.2 Multivariate Data Analysis

To answer **RQ1** (Do time points and drugs have effects on the mutation frequency (`HMuFreq` and `LMuFreq`) and the amino acid count in the third complementarity determining region (`H_CDR3` and `L_CDR3`)?), we tested whether predictors `Time_Point` and `Drug` had effects on four of our five response variables: `H_CDR3`, `HMuFreq`, `L_CDR3`, and `LMuFreq`. We excluded `Binding` from this section, because it has unequal variances across time points, which violates the equal variance assumption of MANOVA. We used it as the main response variable in longitudinal analyses, since longitudinal analysis allows for unequal variances over time. We used the `manova` function in base R for the MANOVA test and the `emmeans` package for pairwise comparisons.[7].

2.2.1 MANOVA

Since we wanted to compare more than two populations, we used MANOVA to test for effects. We check that the normality assumption is met due to large sample size (n = 2464). Each antibody is assumed to be independent. However, we had some concerns about the equal variance-covariance matrices assumption, which is addressed in the Discussions section. We performed a MANOVA test with the formula $\mathbf{(H\_CDR3, HMuFreq, L\_CDR3, LMuFreq)^T \sim Time\_Point + Drug}$ and the null hypothesis the means of the different populations (of `Time_Point` and `Drug`) were equal.

The output seen at Section 7.2 shows that all of the main effects of `Time_Point` and `Drug` have very small p-values. Thus we rejected the null hypothesis and concluded that both main effects were significant to the antibodies' four traits, `H_CDR3`, `HMuFreq`, `L_CDR3`, and `LMuFreq`. To understand how exactly the effects of these three factors, we proceeded to do pairwise comparisons.

2.2.2 Pairwise Comparison

Table 4 summarizes the pairwise comparison results (see Sections 7.3, 7.4). `Time_Point` have significant pairs for `H_CDR3`, `HMuFreq`, and `LMuFreq`, and time points 0 and 1 appear to have significant differences from time points 2 and 3. `Drug` has significant pairs in `H_CDR3` and `HMuFreq`. For `H_CDR3`, drug 1 has a higher mean than drug 2. For `HMuFreq`, drug 2 has a higher mean than drug 1, which has a higher mean than drug 3 (control group). [Kan, please add/revise the interpretations here.]

2.3 Longitudinal Analysis

We used longitudinal data analyses to answer our **RQ2** (How does the binding strength of the antibodies (`Binding` or `logBinding`) develop in response to the number of vaccine dosages (`Time_Point`) and immuno-suppression treatments (`Drug`)?), including general linear models and linear mixed models. We used the `gls` and `lme` functions from the `nlme` package[8].

2.3.1 One Covariate: Time Point

We first take a look at the data over time. As seen in Figure 19 and Figure 20, the mean trend is not linear, and the different time points have different variances. This information suggests that we should use piecewise linear models and set variances as unequal over time.

We first considered a model with time point as the only covariate:

$$Y_{ij} = \beta_0 + \beta_1 Time_{ij} + e_{ij}$$

We then turned the model above into a piecewise linear model, in which each segment has different intercepts and slopes. The model includes three indicator variables: $S1, S2, S3$ as the indicator variables, where

$$S1 = \begin{cases} 1 & \text{if } 0 \leq \text{Timepoint} < 1 \\ 0 & \text{otherwise} \end{cases}$$

$$S2 = \begin{cases} 1 & \text{if } 1 \leq \text{Timepoint} < 2 \\ 0 & \text{otherwise} \end{cases}$$

$$S3 = \begin{cases} 1 & \text{if Timepoint} \geq 2 \\ 0 & \text{otherwise} \end{cases}$$

The new model is

$$Y_{ij} = S1(\beta_0 + \beta_1 Time_{ij}) + S2(\beta_2 + \beta_3 Time_{ij}) + S3(\beta_4 + \beta_5 Time_{ij}) + e_{ij}$$

We ensured that the trend is continuous at time points 1 and 2. Our first complete model

(`fit.gls`) is

$$Y_{ij} = \beta_0(S1 + 2S2 - S2Time_{ij}) + \beta_1(S1Time_{ij} + 2S2 - S2Time_{ij}) +$$

$$\beta_4(-S2 + S2Time_{ij} + S3) + \beta_5(-2S2 + 2S2Time_{ij} + S3Time_{ij}) + e_{ij}$$

where

$$\mathbf{e}_i \sim N(0, \sigma^2 I)$$

As mentioned earlier, we transformed `Binding` into `logBinding` with the formula

$$logBinding = log(Binding + 1).$$

We first used them as response variables in the above formula in two models (`fit.gls1` and `fit.gls2`, respectively) to find the better response variable. As seen in Table 5, the model using `logBinding` as the response variable has much lower AIC and BIC values. We decided to use `logBinding` as the response variable and build on this model (`gls.fit2`). Note that the plots below still use `Binding` in the y-axis, and thus we had to plug the fitted values of `logBinding` into the exponential function to find `Binding`. As seen in plots below, the fitted values of `Binding` are never negative, which was why we transformed `Binding`.

The model can also be written as

$$Y_{ij} = S1(\beta_0) + S1Time_{ij}(\beta_1) + S2(2\beta_0 + 2\beta_1 - \beta_4 - 2\beta_5) + S2Time_{ij}(-\beta_0 - \beta_1 + \beta_4 + 2\beta_5)$$

$$+ S3(\beta_4) + S3Time_{ij}(\beta_5) + e_{ij}$$

We ran the model and plugged in coefficients to find the intercepts and slopes for all three segments of the mean trend:

- S1: $exp(-0.0921720 + 0.1090234 * time)$
- S2: $exp((2*-0.0921720+2*0.1090234-0.3573358-2*-0.1070750)+(0.0921720-0.1090234+0.3573358+2*-0.1070750)*time) = exp(-0.109483 + 0.1263344 * time)$

- S3: $exp(0.3573358 - 0.1070750 * time)$

Figure 21 shows the two segments S1 and S2 have very similar slopes. So we could refit the model with only two line segments between time points 0 and 2 and between time points 2 and 3. We called them S4 and S5. The next model is therefore

$$Y_{ij} = S4(\beta_0 + \beta_1 Time_{ij}) + S5(\beta_2 + \beta_3 Time_{ij}) + e_{ij}$$

$$S4 = \begin{cases} 1 & \text{if Timepoint} < 2 \\ 0 & \text{otherwise} \end{cases}$$

$$S5 = \begin{cases} 1 & \text{if Timepoint} \geq 2 \\ 0 & \text{otherwise} \end{cases}$$

Again, we ensured that the trend is continuous at Time_Point = 2. Our second complete model (`fit.gls3`) is then

$$Y_{ij} = \beta_1(-2S4 + S4Time_{ij}) + \beta_2(S4 + S5) + \beta_3(2S4 + S5Time_{ij}) + e_{ij}$$

where

$$\mathbf{e}_i \sim N(0, \sigma^2 I)$$

The model could also be written as

$$Y_{ij} = S4(-2\beta_1 + \beta_2 + 2\beta_3) + S4Time_{ij}(\beta_1) +$$

$$S5(\beta_2) + S5Time_{ij}(\beta_3) + e_{ij}$$

After the model was constructed, we used the coefficients to find the mean trends for S4 and S5:

- S4: $exp((-2 * 0.24395790 + 0.26937508 + 2 * 0.00194581) + 0.2448519 * time) = exp(-0.2146491 + 0.2448519 * time)$
- S5: $exp(0.26937508 + 0.00194581 * time)$

As shown in Figure 22, there is a linear line between Time_Point 0 and 2 and one between Time_Point 2 and 3. The two lines are continuous at Time_Point 2. A comparison of AIC And BIC of these two models, shown in Table 6, indicates that the first model (`fit.gls2`) is still a better model.

2.3.2 Adding Random Effects

Next we checked whether adding random effects could improve our model (`fit.gls2`). We assumed that random effects existed in the intercept and slope. Our linear mixed model is then:

$$Y_{ij} = \beta_0(S1 + 2S2 - S2Time_{ij}) + \beta_1(S1Time_{ij} + 2S2 - S2Time_{ij}) +$$

$$\beta_4(-S2 + S2Time_{ij} + S3) + \beta_5(-2S2 + 2S2Time_{ij} + S3Time_{ij}) + b_{0i} + b_{1i}Time_{ij} + e_{ij}$$

where

$$\mathbf{b}_i \sim N\left(0, \mathbf{D} = \begin{bmatrix} D_{11} & D_{12} \\ & D_{22} \end{bmatrix}\right)$$

and

$$\mathbf{e}_i \sim N(0, \sigma^2 I)$$

We fit two models with random effects: `fit.a1` assumes random intercept and slope for time point, compound symmetric correlation structure, and unequal variances over time; and `fit.a2` assumes random intercept and slope for time point, AR1 correlation structure, and unequal variances over time. As shown in Table 7, the model `fit.a2` has the lowest AIC and BIC, so it is the best model. We checked residuals for three models: `fit.gls2`, `fit.a1`, `fit.a2`, as shown in Figure 23. All three Q-Q plots show approximate normality. To further investigate the effects of drugs, We built on the model `fit.a2`.

2.3.3 Inference about β

Note the meanform for our three models are:

$$E(Y_{ij}) = S1(\beta_0)+S1Time_{ij}(\beta_1)+S2(2\beta_0+2\beta_1-\beta_4-2\beta_5)+S2Time_{ij}(-\beta_0-\beta_1+\beta_4+2\beta_5)$$

$$+S3(\beta_4) + S3Time_{ij}(\beta_5)$$

We would like to know if the slopes between time points 0 and 1, 1 and 2, and 2 and 3 equal zero, which means

$$H_0 : \beta_1 = 0, -\beta_0 - \beta_1 + \beta_4 + 2\beta_5 = 0, \beta_5 = 0$$

Thus, we performed three tests:

$$\mathbf{L_1}\beta = 0$$

where $\mathbf{L_1} = (0, 1, 0, 0)$ and $= (\beta_0, \beta_1, \beta_4, \beta_5)^T$

$$\mathbf{L_2}\beta = 0$$

where $\mathbf{L_2} = (-1, -1, 1, 2)$ and $= (\beta_0, \beta_1, \beta_4, \beta_5)^T$

$$\mathbf{L_3}\beta = 0$$

where $\mathbf{L_3} = (0, 0, 0, 1)$ and $= (\beta_0, \beta_1, \beta_4, \beta_5)^T$

As shown in Table 8, all three slopes have very small p-values, which means the rates of change in all three segments are significant. As shown in Figure 21, the slopes of S1 and S2 are positive, and the slope of S3 is negative. Combining the information of very small p-values and the signs of slopes, we concluded that the binding rates increased from time points 0 to 1 and from time points 1 to 2. The binding rates decreased from time points 2 to 3. We concluded that time point 2, when the monkeys had received two vaccines, had the highest binding rates, while the third vaccine injection at time point 3 failed to increase binding rates.

2.3.4 Two Covariates: Time Point and Drug

Next we added `Drug` as a covariate to the model `gls.a2` to see if it had effects on binding rates. We used two indicator variables: `D2` and `D3`, where

$$D2 = \begin{cases} 1 & \text{if Drug = 2} \\ 0 & \text{otherwise} \end{cases}$$

$$D3 = \begin{cases} 1 & \text{if Drug = 3} \\ 0 & \text{otherwise} \end{cases}$$

Building on to the model `gls.a2` and assuming that the random effects were the same for each drug, our model (`fit.a3`) with the extra covariate `Drug` is:

$$Y_{ij} = \beta_0(S1 + 2S2 - S2Time_{ij}) + \beta_1(S1Time_{ij} + 2S2 - S2Time_{ij})+$$

$$\beta_2(-S2 + S2Time_{ij} + S3) + \beta_3(-2S2 + 2S2Time_{ij} + S3Time_{ij})+$$

$$\beta_4 D2(S1 + 2S2 - S2Time_{ij}) + \beta_5 D2(S1Time_{ij} + 2S2 - S2Time_{ij})+$$

$$\beta_6 D2(-S2 + S2Time_{ij} + S3) + \beta_7 D2(-2S2 + 2S2Time_{ij} + S3Time_{ij})+$$

$$\beta_8 D3(S1 + 2S2 - S2Time_{ij}) + \beta_9 D3(S1Time_{ij} + 2S2 - S2Time_{ij})+$$

$$\beta_{10} D3(-S2 + S2Time_{ij} + S3) + \beta_{11} D3(-2S2 + 2S2Time_{ij} + S3Time_{ij})+$$

$$b_{0i} + b_{1i}Time_{ij} + e_{ij}$$

where

$$\mathbf{b}_i \sim N\left(0, \mathbf{D} = \begin{bmatrix} D_{11} & D_{12} \\ & D_{22} \end{bmatrix}\right)$$

and

$$\mathbf{e}_i \sim N(0, \sigma^2 I)$$

Table 9 shows that the model with drug as the second covariate has the lowest AIC but not

the lowest BIC, suggesting that it is somewhat comparable to the linear mixed model without the covariate `Drug`. Considering the added complexity of the current model and the slightly improvement with respect to AIC, we considered `fit.a2` the best model. Next we made inference about β to find whether the drugs had different effects. To see whether Drug 1 and Drug 2 had different effects, we performed a hypothesis test on $H_0 : \beta_4 = \beta_5 = \beta_6 = \beta_7 = 0$ by testing

$$\mathbf{L_4}\beta = 0$$

where

$$\mathbf{L_4} = \begin{bmatrix} 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \end{bmatrix}$$

and $\beta = (\beta_0, \beta_1, \beta_2, \beta_3, \beta_4, \beta_5, \beta_6, \beta_7, \beta_8, \beta_9, \beta_{10}, \beta_{11})^T$

To see whether Drug 1 and Drug 3 had different effects, we performed a hypothesis test on $H_0 : \beta_8 = \beta_9 = \beta_1 0 = \beta_1 1 = 0$ by testing

$$\mathbf{L_5}\beta = 0$$

where

$$\mathbf{L_5} = \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix}$$

and $\beta = (\beta_0, \beta_1, \beta_2, \beta_3, \beta_4, \beta_5, \beta_6, \beta_7, \beta_8, \beta_9, \beta_{10}, \beta_{11})^T$

To see whether Drug 2 and Drug 3 had different effects, we performed a hypothesis test on $H_0 : \beta_4 = \beta_8, \beta_5 = \beta_9, \beta_6 = \beta_{10}, \beta_7 = \beta_{11}$ by testing

$$\mathbf{L_6}\beta = 0$$

where

$$\mathbf{L_6} = \begin{bmatrix} 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & -1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & -1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & -1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & -1 \end{bmatrix}$$

and $\beta = (\beta_0, \beta_1, \beta_2, \beta_3, \beta_4, \beta_5, \beta_6, \beta_7, \beta_8, \beta_9, \beta_{10}, \beta_{11})^T$

As shown in Table 10 and Table 12, the p-values are smaller than 0.05. We rejected the null hypothesis and concluded that drugs 1 and 2 and drugs 2 and 3 had different effects on binding rates. Table 11, on the other hand, shows a p-value greater than 0.05 (although not too much greater), suggesting that drugs 1 and 3 did not have different effects on binding rates. Drug 3 was in fact a mock control, which should not have any effects. From the results, we concluded that drug 2 acted differently from drug 1 and the control, and drug 2 could be used in future research for more investigation.

3 Results

For multivariate analyses, we performed a MANOVA test on the main effects of `Time_Point` and `Drug` on the response variable vector (`H_CDR3`, `HMuFreq`, `L_CDR3`, `LMuFreq`)$^{\mathrm{T}}$. We found that both main effects had very small p-values. We also performed pairwise comparison to see where the effects were, as shown in Table 4. [Kan, do you have anything to add?]

For longitudinal analyses, we found that the transformed variable `logBinding` was a better option and the linear mixed model `fit.a2` had the lowest BIC:

$$Y_{ij} = \beta_0(S1 + 2S2 - S2Time_{ij}) + \beta_1(S1Time_{ij} + 2S2 - S2Time_{ij}) +$$

$$\beta_4(-S2 + S2Time_{ij} + S3) + \beta_5(-2S2 + 2S2Time_{ij} + S3Time_{ij}) + b_{0i} + b_{1i}Time_{ij} + e_{ij}$$

where

$$\mathbf{b}_i \sim N\left(0, \mathbf{D} = \begin{bmatrix} D_{11} & D_{12} \\ & D_{22} \end{bmatrix}\right)$$

and

$$\mathbf{e}_i \sim N(0, \sigma^2 I)$$

We performed F-tests to make inference about β for the three line segments. We rejected the hypothesis that the slopes of all three line segments were zero. We then added `Drug` as another covariate to the above model and made inference about β. The comparison between three drug groups was done with three F-tests (between Drug groups 1 and 2, 2 and 3, and 1 and 3). The p-values for the tests between drugs 1 and 2 and between drugs 2 and 3 were smaller than 0.05, while the p-value for the test between drugs 1 and 3 were slightly greater than 0.05. Thus We rejected the null hypotheses that effects of drugs 1 and 2 as well as 2 and 3 were equal but failed to reject the null hypotheses that the effects of drugs 1 and 3 were equal.

4 Discussion

4.1 Implications

Our findings show that the number of vaccine injections did contribute to higher binding rates, although we did not determine whether the increase could be translated into immunity against HIV. The lack of evidence for effects of Treg inhibitor treatments, on the other hand, suggests that the theory of using immuno-suppressing drugs to enhance the efficacy of HIV vaccines has not been proven.

4.2 Limitations

4.2.1 Independent of antibodies

While our analyses reached some findings, some further investigations could improve our analyses. In our multivariate analyses, we followed the common method of treating antibodies (rows in our data) as independent from each other. In our longitudinal analyses, the models allow measurements from the same observational unit (macaque) to correlate over time. It would be beneficial to formally determine whether the antibodies were correlated or independent, but it would require more biological knowledge and the investigation would be beyond the scope of a final report. This

remains an interesting topic that could be explored.

4.2.2 Equal variance-covariance matrices assumption for MANOVA

For multivariate data analyses, the use of MANOVA was restricted by the assumptions of equal variance-covariance matrices among different populations. We ran the Fligner-Killeen Test of Homogeneity of Variances on all the four response variables `H_CDR3`, `HMuFreq`, `L_CDR3`, and `LMuFreq`. As shown in the output in Section 7.5, most of the p-values are very small, meaning the null hypothesis of equal variance is rejected. In most cases, `H_CDR3`, `HMuFreq`, and `Binding` do not meet the equal variance-covariance matrices assumption.

Comparisons of the variance-covariance matrix of the response variables in different groups using ratios (one matrix divided by another matrix) also reveals that the variance-covariance matrices might not be equal. Some matrices seem quite different. For example, as shown in Table 13, the ratio between variance-covariance matrices of Drug 2 and Drug 3 has numbers as large as 26. Furthermore, the sample sizes of each populations, as shown in Table 1, were unequal. Thus, the results of the MANOVA test should be viewed with caution.

4.2.3 Longitudinal Models

For longitudinal data analyses, we did not try out more combinations of models. For example, we only tried two correlation structures (compound symmetry and AR1); other structures might have achieved better results. When we added drug as another covariate, we did not go back to test which correlation structure might perform better and whether the piecewise model should include two or three line segments. Further, we did not assume different random effects on different line segments. These additional steps could lead to better models.

5 Conclusions

In our project, we performed multivariate data analyses and longitudinal data analyses to understand whether time points and drugs had effects on characteristics of antibodies and enhanced the efficacy of HIV vaccines. Our statistical analyses provided answers to our two research questions.

We performed a MANOVA test to answer our first research question, "Do time points and drugs have effects on the mutation frequency (`HMuFreq` and `LMuFreq`) and the amino acid count in the third complementarity determining region (`H_CDR3` and `L_CDR3`)?" and found significant main effects for time points and drugs. [Kan: please add interpretations of pairwise comparisons here.]

To answer our second research question, "How does the binding strength of the antibodies (`Binding` or `logBinding`) develop in response to the number of vaccine dosages (`Time_Point`) and immuno-suppression treatments (`Drug`)?", we constructed longitudinal models. First we used time point as the only covariate and constructed two general linear models with two and three line segments. We then added random effects for intercept and slope for time point as well as different correlation structure–compound symmetry and AR1–and found the model with the transformed response variable `logBinding`, three line segments, random effects of intercept and slope of time point, AR1 correlation structure, and unequal variances over time performed best. F-tests for inference about β reveals that time point 2 have the highest binding rates, suggesting that two vaccine injections improved the binding rates while the third injection decreased binding rates. We also found that adding drug as a covariate did not greatly improve the model and drug 2 appeared to have different effects from drug 1 and the mock control. The difference between different drugs might be worth exploring for future research.

To date, no HIV vaccines have been found effective in creating immunity against the HIV virus. Our analyses showed that the vaccine injections did increase binding rates, but perhaps the change was not enough. The second type of Treg inhibitor treatments might be worth further examination. We hope that, even if the result did not reveal a solution, more analyses could be performed on data from similar studies to find promising directions for future research.

6 References

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7 Appendix

7.1 List of variables

- Monkey_id: The identity of monkey
- Treatment(Drug): The 7 treatments in the dataset are coded into 3 drugs to simplify the data. Drug 1 and 2 are two different kinds of Treg inhibitor treatments, and drug 3 is the mock control.
- Time_Point: 0 represents before immunization; 1 represents 2 weeks post 1st immunization; 2 represents 2 weeks post 2nd immunization; and 3 represents 2 weeks post 3rd immunization, respectively.
- Isotype: There are 5 kinds of immunoglobulin isotypes: IgG, IgA, IgM, IgE, IgD. The two most important kinds are IgG and IgM. IgM occurs in the acute stage of infection and plays a role in the primary response. The secondary response IgG appears later in serum with higher binding affinity and neutralizing potentials against toxins and virus. IgA is mostly found in mucosal tissues such as Nasal mucosa. Non-dominant IgD and IgE are typically lower than 1% in blood.
- H_ID and L_ID: heavy chain and light chain IDs for the particular observation
- H_VBase: the number of nucleotide of the heavy chain variable region
- H_Substitutions: the number of relative nucleotide mutations in heavy chains
- HMuFreq: calculated by H_Substitutions / H_VBase
- H_CDR3: the number of amino acid of a heavy chain's third complementarity determining region
- L_VBase: the number of nucleotide of a light chain's variable region
- L_Substitutions: the number of relative nucleotide mutations in the light chain
- LMuFreq: calculated by L_Substitutions / L_VBase
- L_CDR3: the number of amino acid of a light chain's third complementarity determining region.`H_CDR3` and `L_CDR3` indicates the length of the third complementarity-determining region on the variable heavy chain and light chain. The longer they are, the more potential there is to produce diverse antibodies.
- Binding: affinity of antibodies against a selected HIV glycoprotein. `Binding` indicates the

rate of neutralizing, meaning how much the antibodies bind with the virus and thus make the virus ineffective. Larger values indicate stronger binding.

7.2 Output for the MANOVA test

```
##             Df   Wilks approx F num Df den Df    Pr(>F)
## tp           3 0.95348   9.8224     12 6487.7 < 2.2e-16 ***
## drug         2 0.97728   7.0843      8 4904.0 2.375e-09 ***
## Residuals 2455
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

7.3 Pairwise comparison by time point

```
## [1] "H_CDR3  pairwise CI's"
##  contrast estimate    SE   df lower.CL upper.CL
##  0 - 1       0.489 0.231 2457   -0.223    1.202
##  0 - 2       0.194 0.237 2457   -0.535    0.923
##  0 - 3      -0.608 0.271 2457   -1.442    0.226
##  1 - 2      -0.295 0.159 2457   -0.784    0.194
##  1 - 3      -1.097 0.206 2457   -1.732   -0.462
##  2 - 3      -0.802 0.212 2457   -1.455   -0.148
##
## Results are averaged over the levels of: rep.meas
## Note: contrasts are still on the [.: scale
## Confidence level used: 0.997916666666667
## [1] "HMuFreq  pairwise CI's"
##  contrast estimate      SE   df  lower.CL upper.CL
##  0 - 1     0.00870 0.00288 2457 -0.000188   0.0176
##  0 - 2     0.01872 0.00295 2457  0.009629   0.0278
##  0 - 3     0.02356 0.00338 2457  0.013153   0.0340
##  1 - 2     0.01002 0.00198 2457  0.003926   0.0161
##  1 - 3     0.01486 0.00257 2457  0.006938   0.0228
##  2 - 3     0.00484 0.00265 2457 -0.003316   0.0130
##
## Results are averaged over the levels of: rep.meas
## Note: contrasts are still on the [.: scale
## Confidence level used: 0.997916666666667
## [1] "L_CDR3  pairwise CI's"
##  contrast estimate     SE   df lower.CL upper.CL
##  0 - 1     -0.0169 0.0652 2457   -0.218    0.184
##  0 - 2     -0.0287 0.0667 2457   -0.234    0.177
```

```
##  0 - 3     -0.0034 0.0764 2457   -0.239    0.232
##  1 - 2     -0.0117 0.0447 2457   -0.150    0.126
##  1 - 3      0.0135 0.0581 2457   -0.166    0.193
##  2 - 3      0.0253 0.0598 2457   -0.159    0.210
##
## Results are averaged over the levels of: rep.meas
## Note: contrasts are still on the [.: scale
## Confidence level used: 0.997916666666667
## [1] "LMuFreq  pairwise CI's"
##  contrast estimate      SE   df  lower.CL upper.CL
##  0 - 1     0.00853 0.00369 2457 -0.002847   0.0199
##  0 - 2     0.01465 0.00378 2457  0.003004   0.0263
##  0 - 3     0.01889 0.00432 2457  0.005567   0.0322
##  1 - 2     0.00611 0.00253 2457 -0.001694   0.0139
##  1 - 3     0.01036 0.00329 2457  0.000213   0.0205
##  2 - 3     0.00424 0.00339 2457 -0.006196   0.0147
##
## Results are averaged over the levels of: rep.meas
## Note: contrasts are still on the [.: scale
## Confidence level used: 0.997916666666667
```

7.4 Pairwise comparison by drug

```
## [1] "H_CDR3  pairwise CI's"
##  contrast estimate    SE   df lower.CL upper.CL
##  1 - 2      0.4907 0.160 2458   0.0316    0.950
##  1 - 3      0.4438 0.175 2458  -0.0589    0.946
##  2 - 3     -0.0469 0.171 2458  -0.5372    0.444
##
## Results are averaged over the levels of: rep.meas
## Note: contrasts are still on the [.: scale
## Confidence level used: 0.995833333333333
## [1] "HMuFreq  pairwise CI's"
##  contrast estimate      SE   df lower.CL  upper.CL
##  1 - 2      -0.006 0.00199 2458 -0.01172 -0.000285
##  1 - 3       0.011 0.00218 2458  0.00476  0.017279
##  2 - 3       0.017 0.00213 2458  0.01091  0.023127
##
## Results are averaged over the levels of: rep.meas
## Note: contrasts are still on the [.: scale
## Confidence level used: 0.995833333333333
## [1] "L_CDR3  pairwise CI's"
##  contrast estimate     SE   df lower.CL upper.CL
##  1 - 2      0.0451 0.0450 2458  -0.0839    0.174
##  1 - 3      0.1018 0.0492 2458  -0.0394    0.243
##  2 - 3      0.0567 0.0480 2458  -0.0810    0.194
##
## Results are averaged over the levels of: rep.meas
## Note: contrasts are still on the [.: scale
## Confidence level used: 0.995833333333333
## [1] "LMuFreq  pairwise CI's"
```

```
##  contrast  estimate      SE   df lower.CL upper.CL
##  1 - 2    -0.000176 0.00256 2458 -0.00752  0.00717
##  1 - 3     0.002320 0.00280 2458 -0.00572  0.01036
##  2 - 3     0.002496 0.00273 2458 -0.00535  0.01034
##
## Results are averaged over the levels of: rep.meas
## Note: contrasts are still on the [.: scale
## Confidence level used: 0.995833333333333
```

7.5 Fligner-Killeen Test of Homogeneity of Variances

```
##
##  Fligner-Killeen test of homogeneity of variances
##
## data:  H_CDR3 by Time_Point
## Fligner-Killeen:med chi-squared = 18.036, df = 3, p-value = 0.0004323

##
##  Fligner-Killeen test of homogeneity of variances
##
## data:  HMuFreq by Time_Point
## Fligner-Killeen:med chi-squared = 50.662, df = 3, p-value = 5.775e-11

##
##  Fligner-Killeen test of homogeneity of variances
##
## data:  L_CDR3 by Time_Point
## Fligner-Killeen:med chi-squared = 0.56825, df = 3, p-value = 0.9037

##
##  Fligner-Killeen test of homogeneity of variances
##
## data:  LMuFreq by Time_Point
## Fligner-Killeen:med chi-squared = 16.585, df = 3, p-value = 0.0008601

##
##  Fligner-Killeen test of homogeneity of variances
##
## data:  H_CDR3 by Drug
## Fligner-Killeen:med chi-squared = 13.463, df = 2, p-value = 0.001193

##
```

```
##  Fligner-Killeen test of homogeneity of variances
##
## data:  HMuFreq by Drug
## Fligner-Killeen:med chi-squared = 48.86, df = 2, p-value = 2.456e-11

##
##  Fligner-Killeen test of homogeneity of variances
##
## data:  L_CDR3 by Drug
## Fligner-Killeen:med chi-squared = 2.4113, df = 2, p-value = 0.2995

##
##  Fligner-Killeen test of homogeneity of variances
##
## data:  LMuFreq by Drug
## Fligner-Killeen:med chi-squared = 0.17372, df = 2, p-value = 0.9168
```

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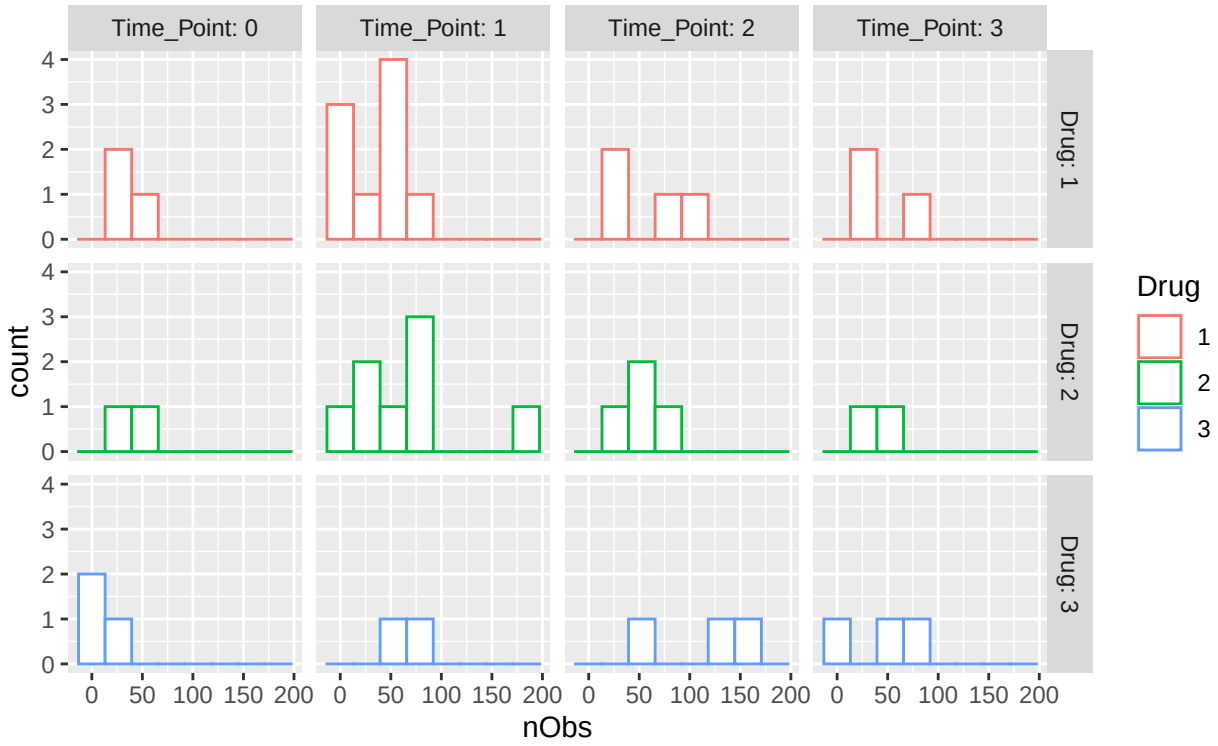

Figure 1: Histograms of Antibodies

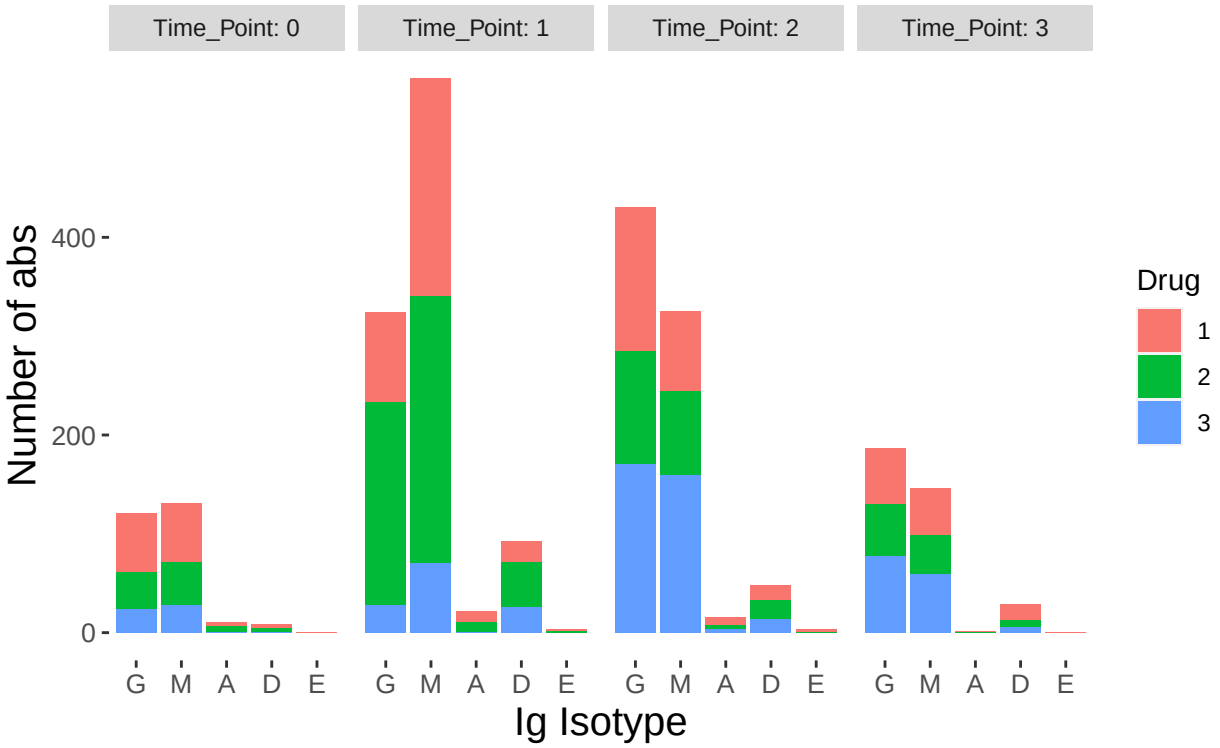

Figure 2: Histograms of Isotypes

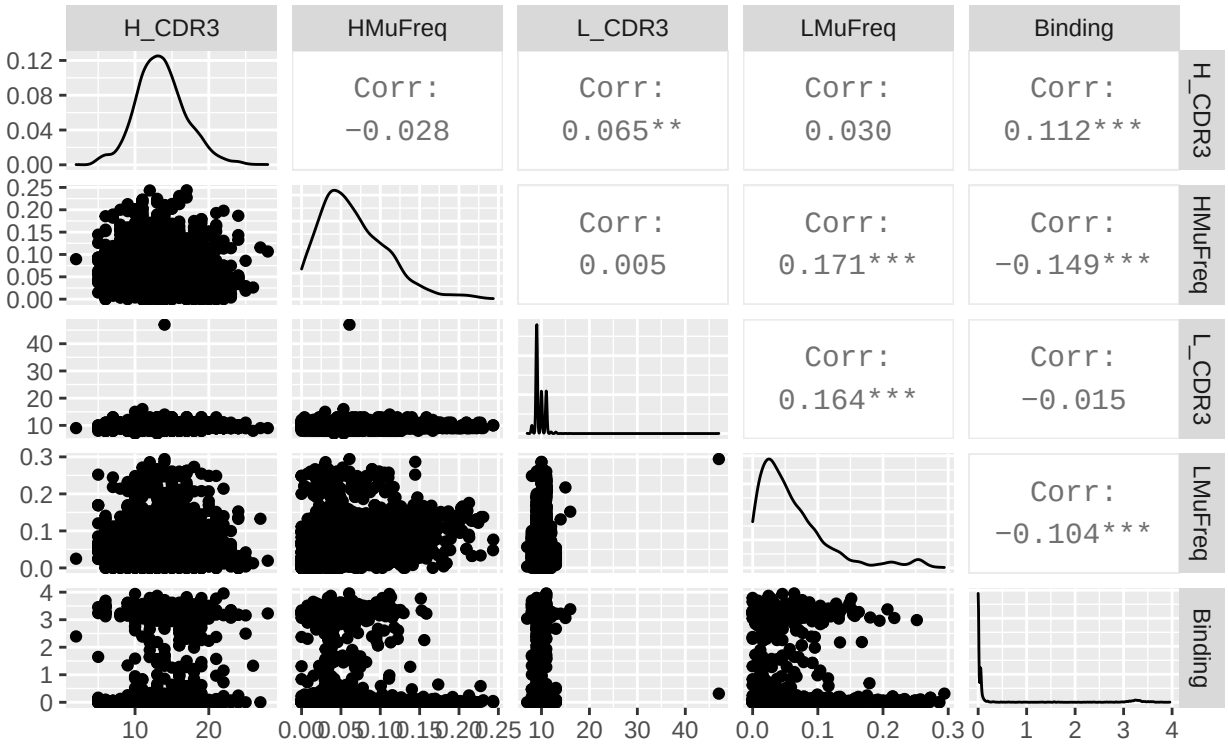

Figure 3: Histogram of Response Variables

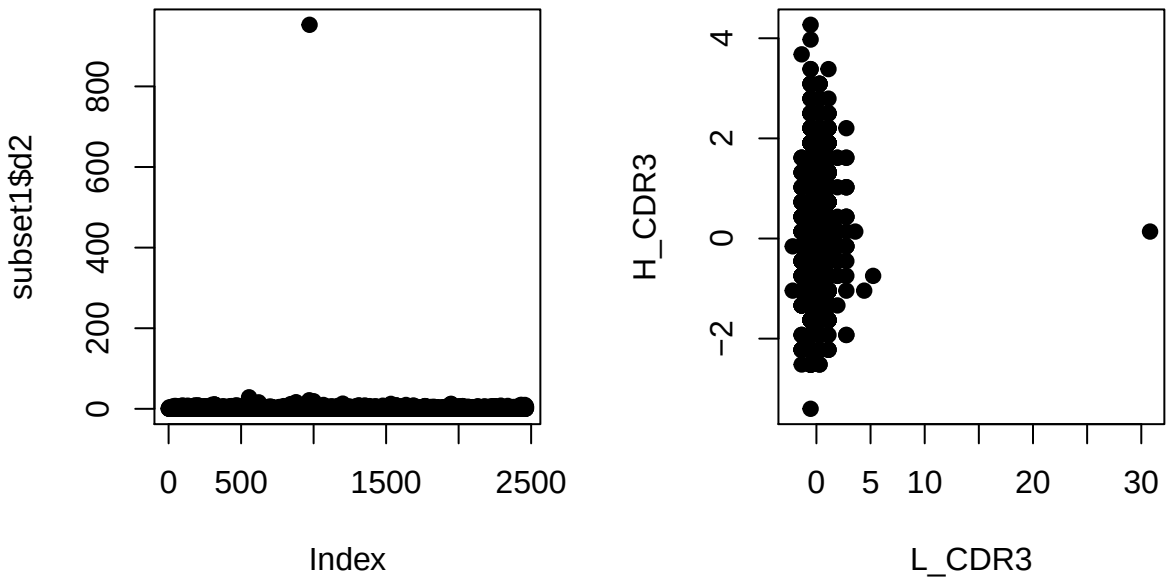

Figure 4: Mahalanobis distances and Z scores

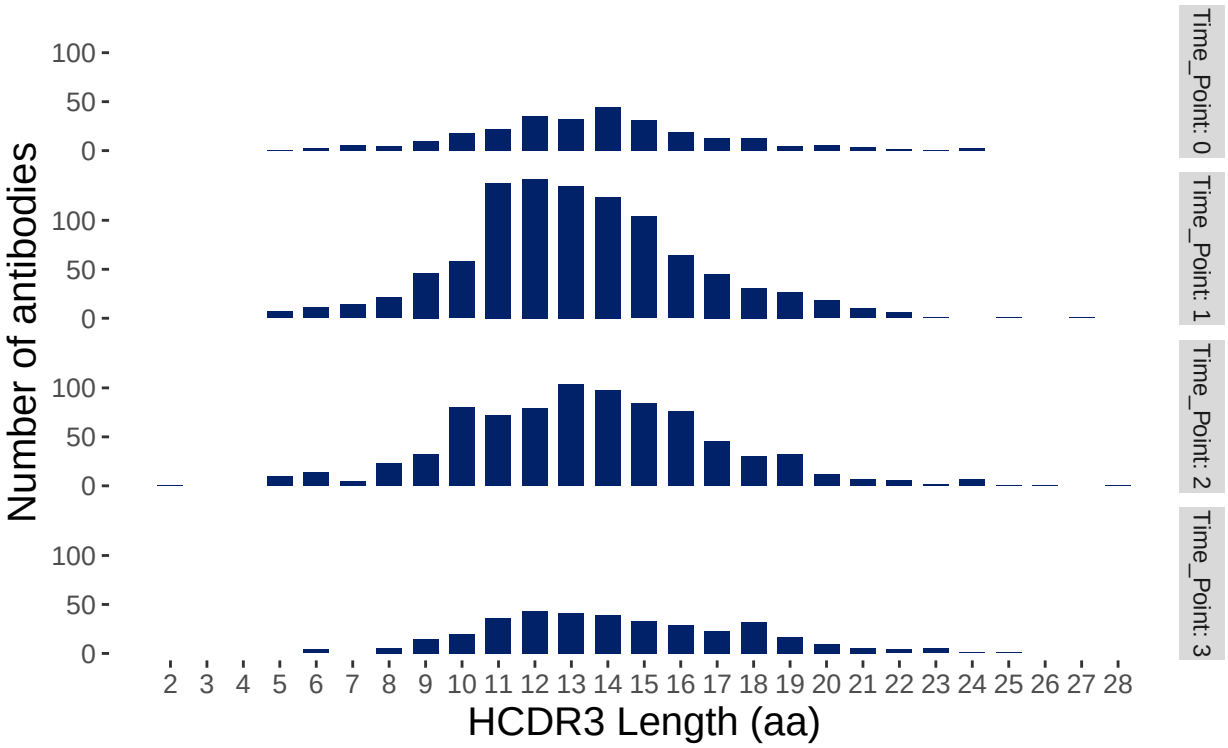

Figure 5: Histogram H_CDR3

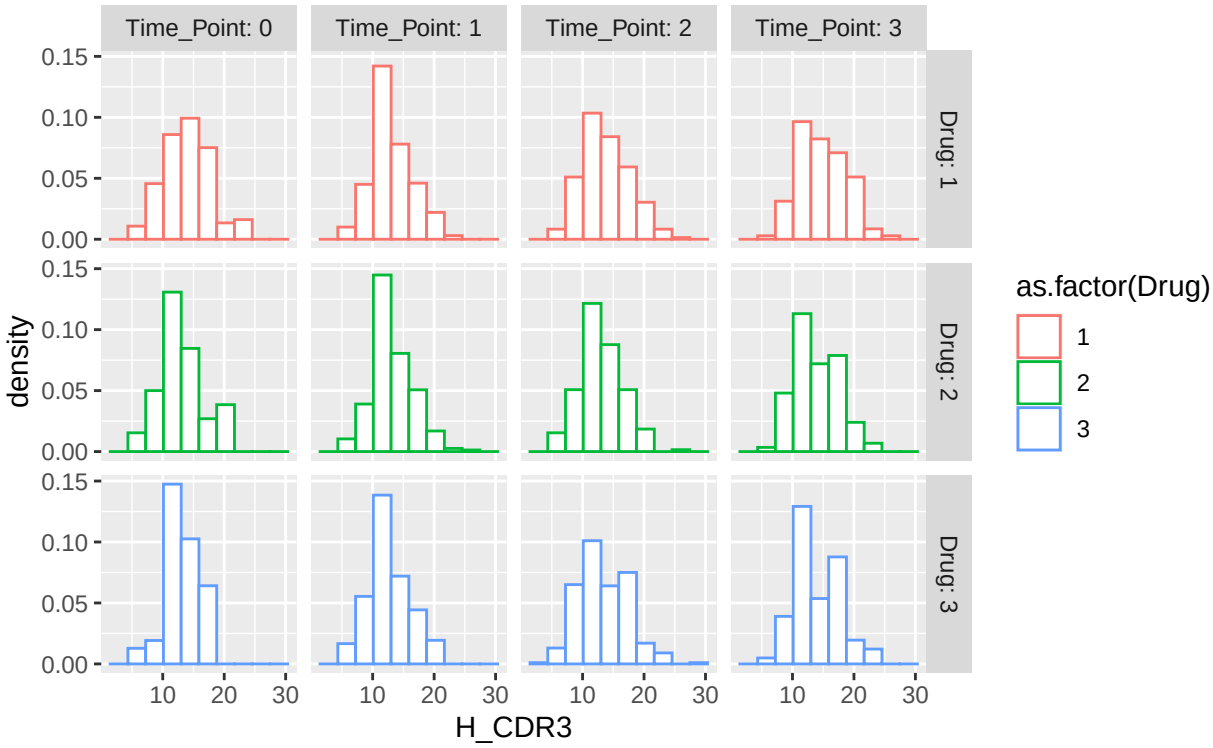

Figure 6: Histograms of H_CDR3 vs Treatment and Timepoint

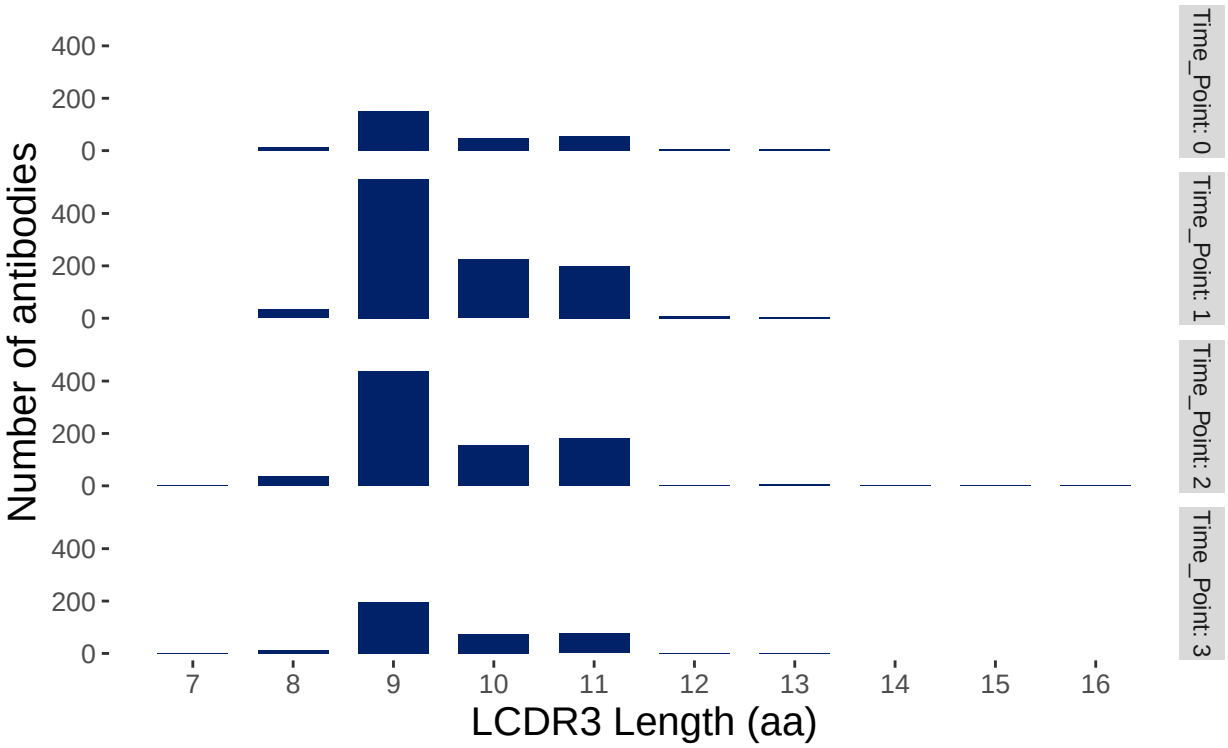

Figure 7: Histogram L_CDR3

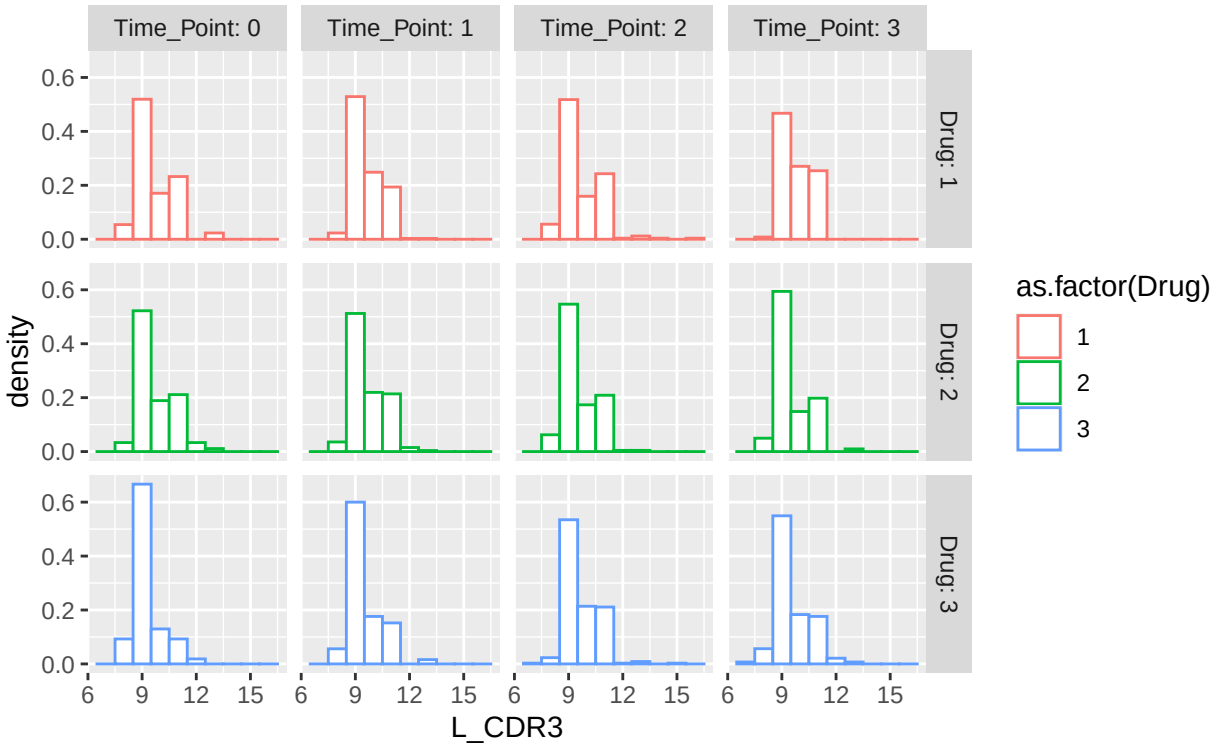

Figure 8: Histograms of L_CDR3 vs Treatment and Timepoint

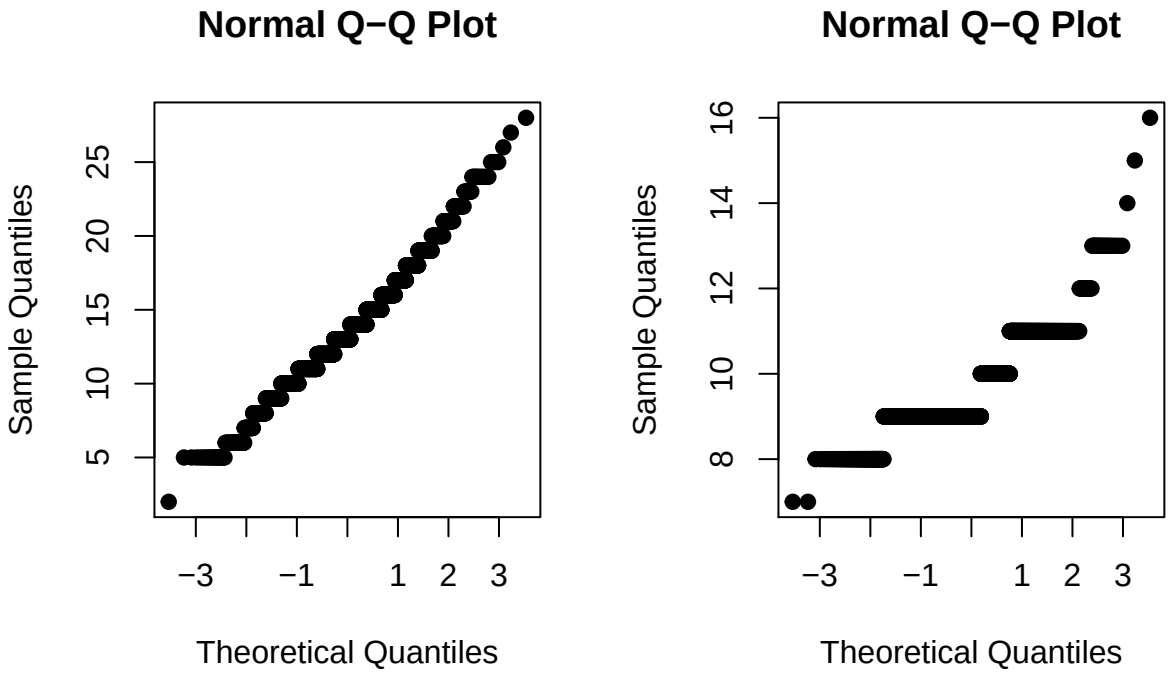

Figure 9: Q-Q Plots of H_CDR3 and L_CDR3

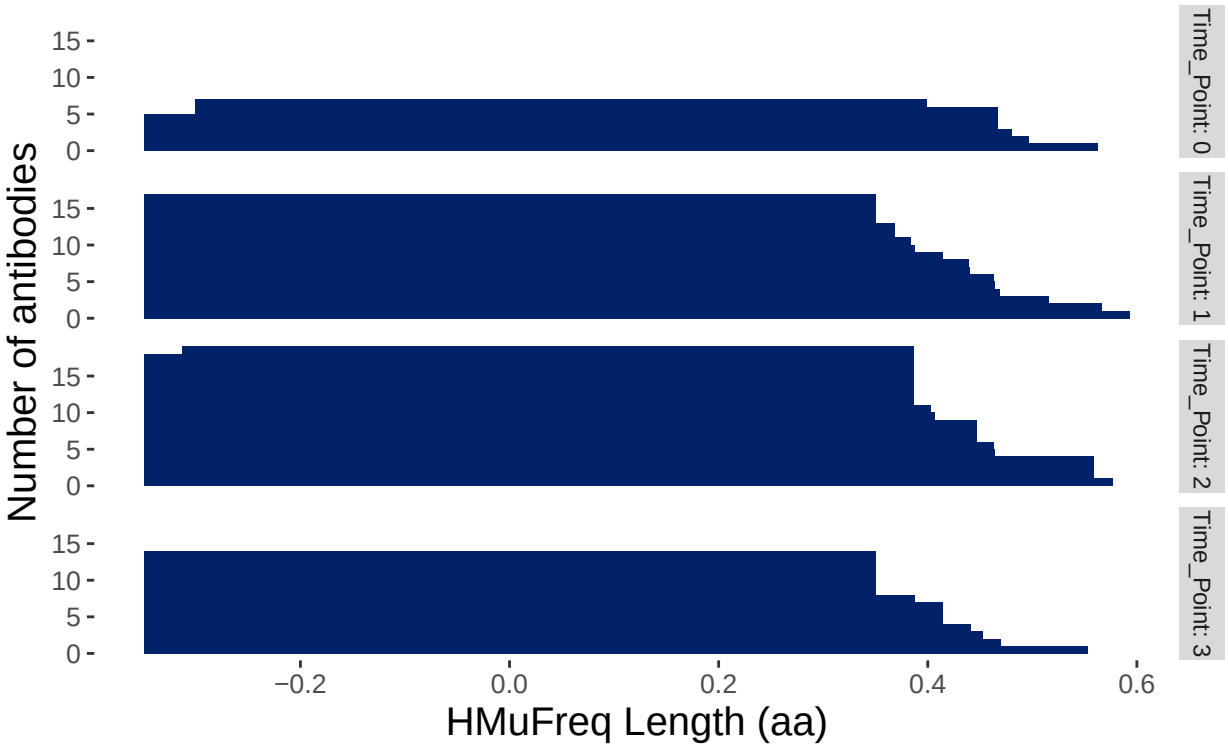

Figure 10: Histogram HMuFreq

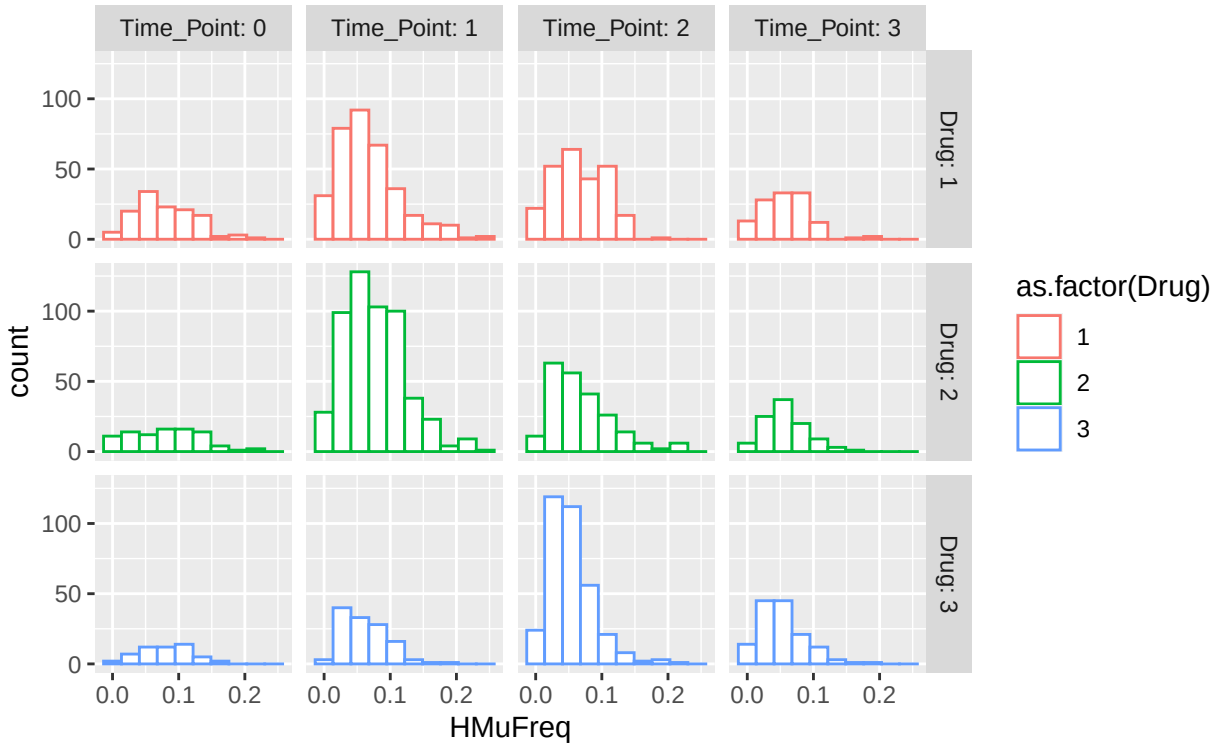

Figure 11: Histograms of HMuFreq vs Treatment and Timepoint

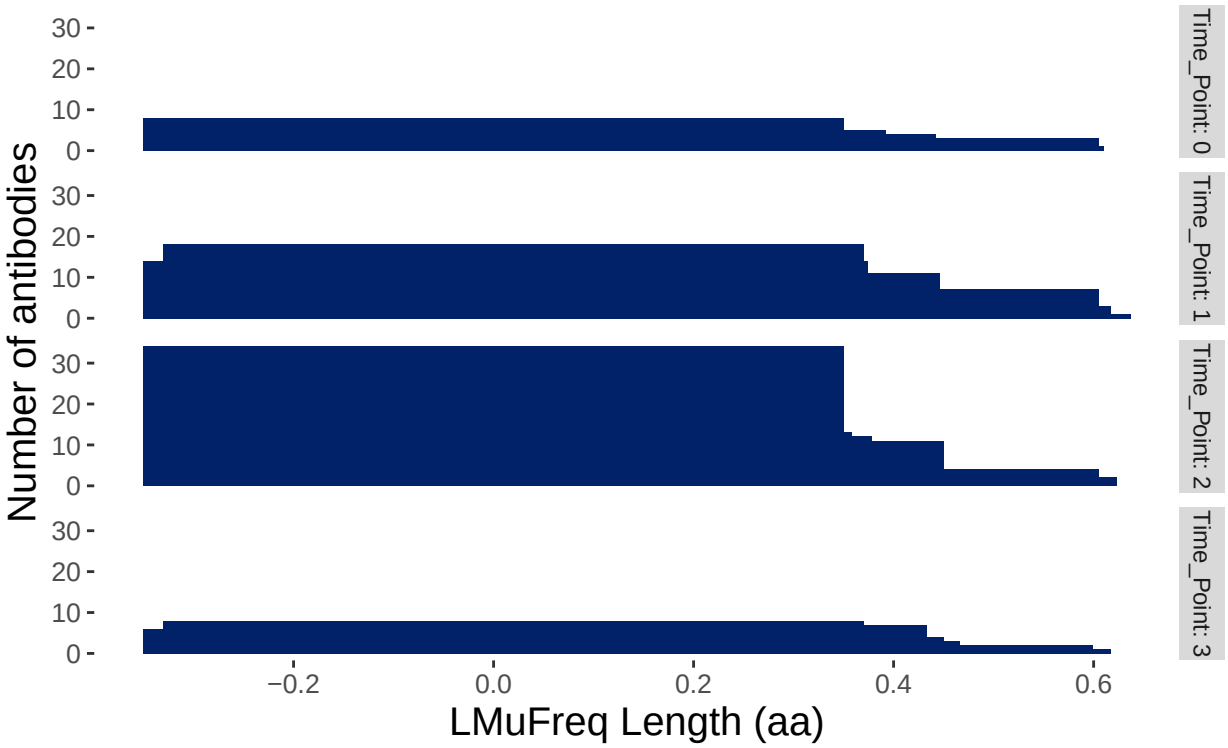

Figure 12: Histogram LMuFreq

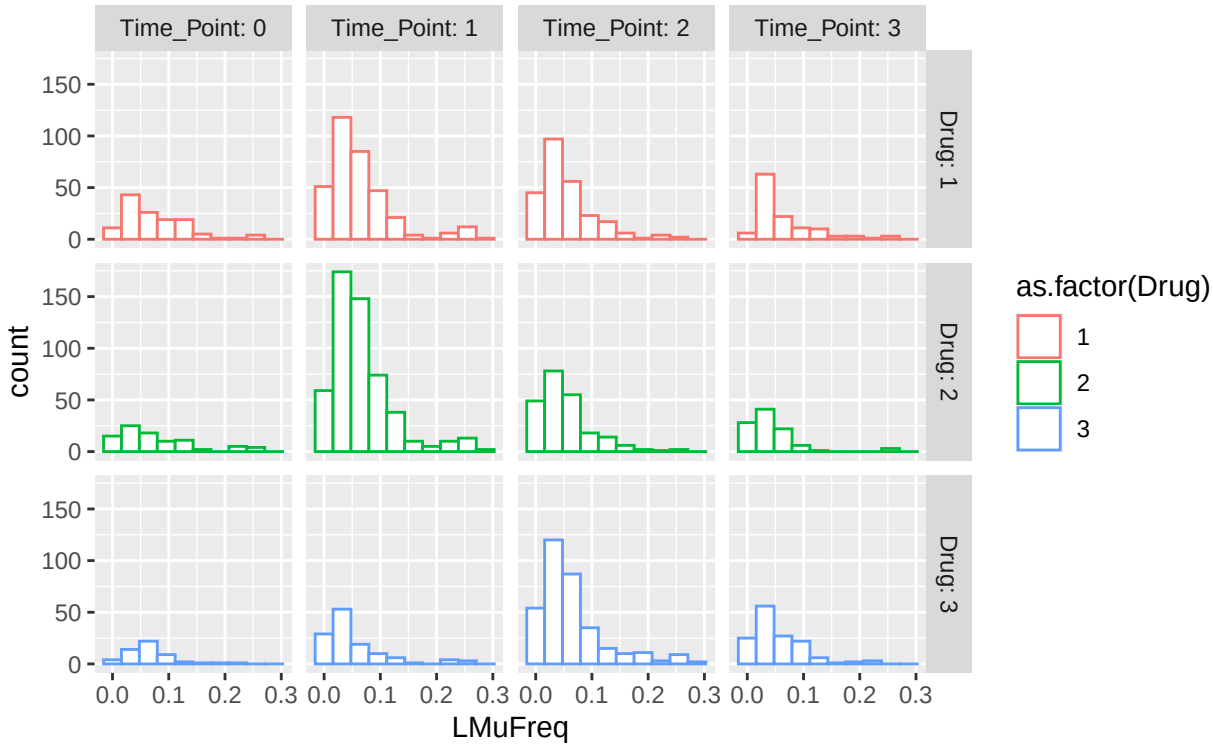

Figure 13: Histograms of LMuFreq vs Treatment and Timepoint

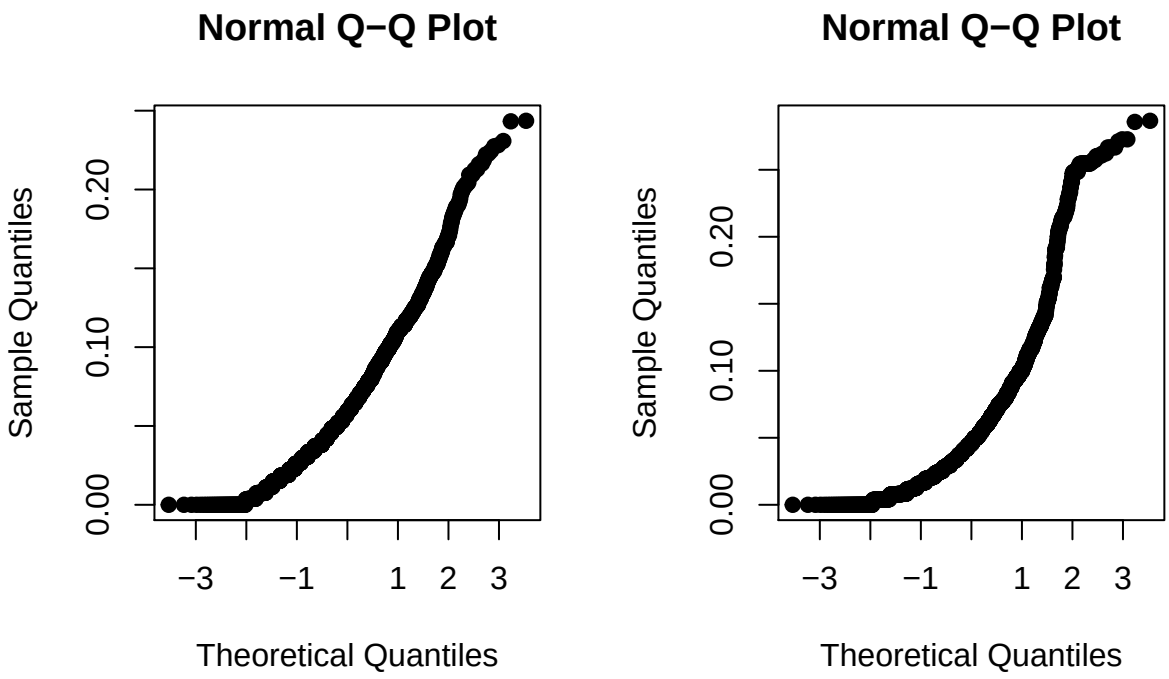

Figure 14: Q-Q Plot of HMuFreq and LMuFreq

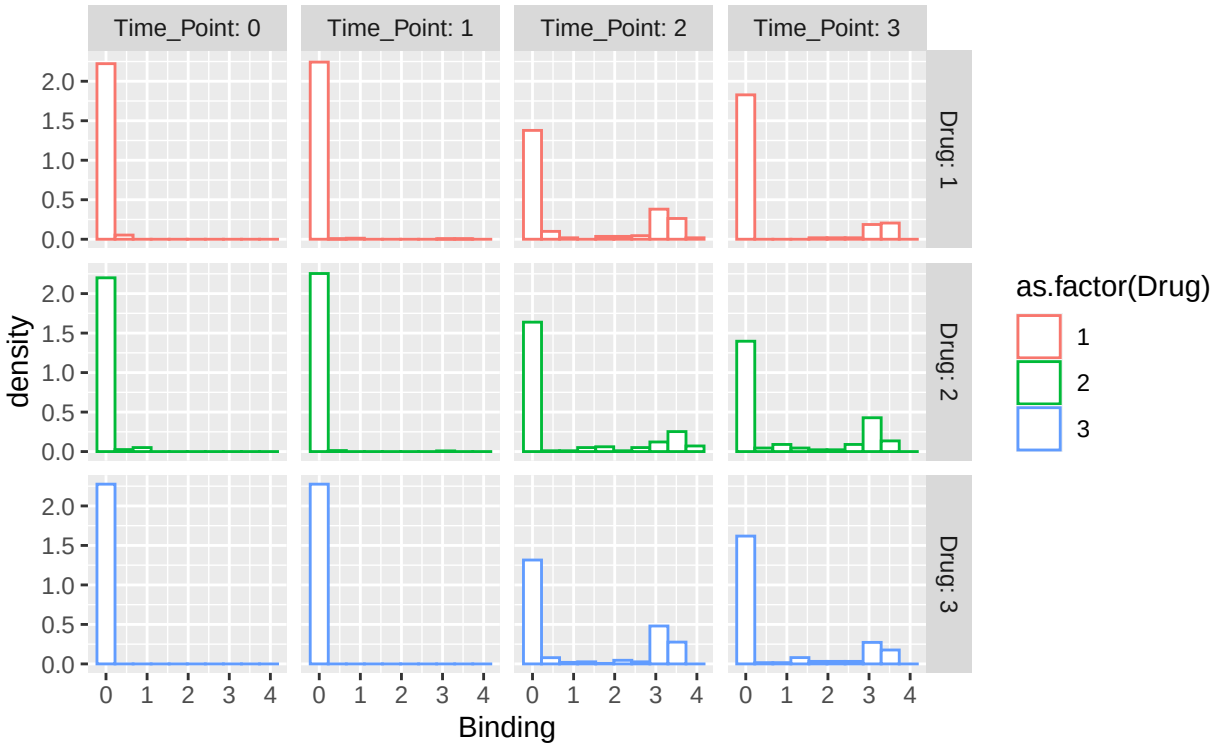

Figure 15: Histograms of Binding Strength vs Treatment and Timepoint

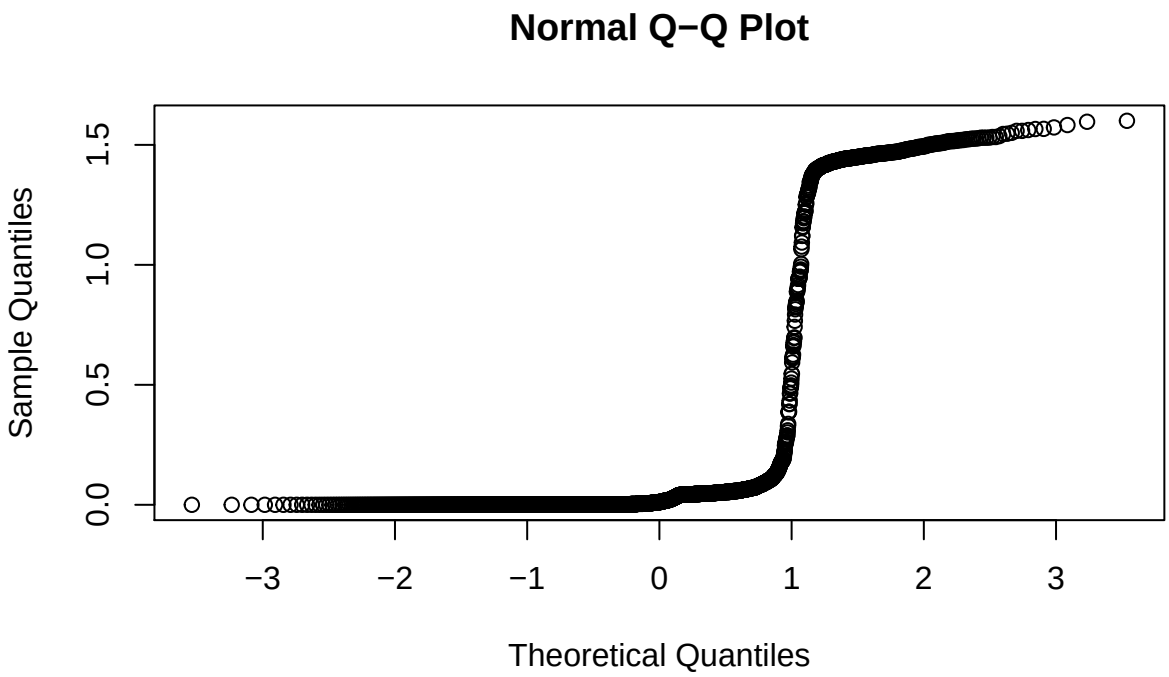

Figure 16: Q-Q plot of logBinding

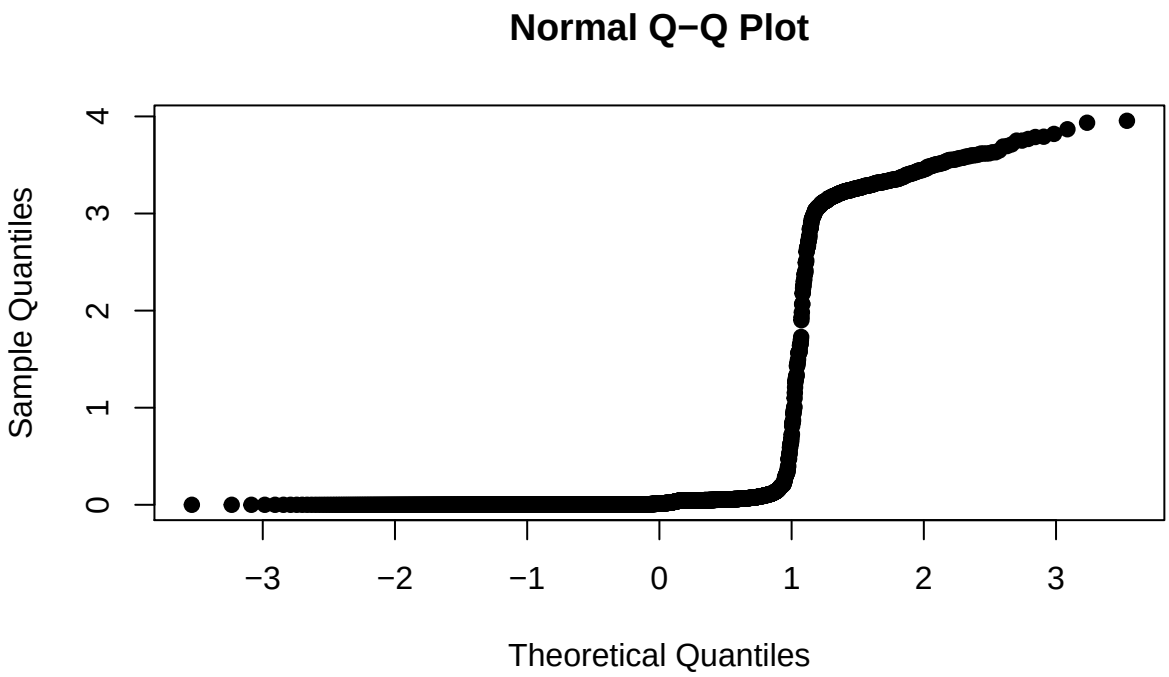

Figure 17: Q-Q Plot of Binding

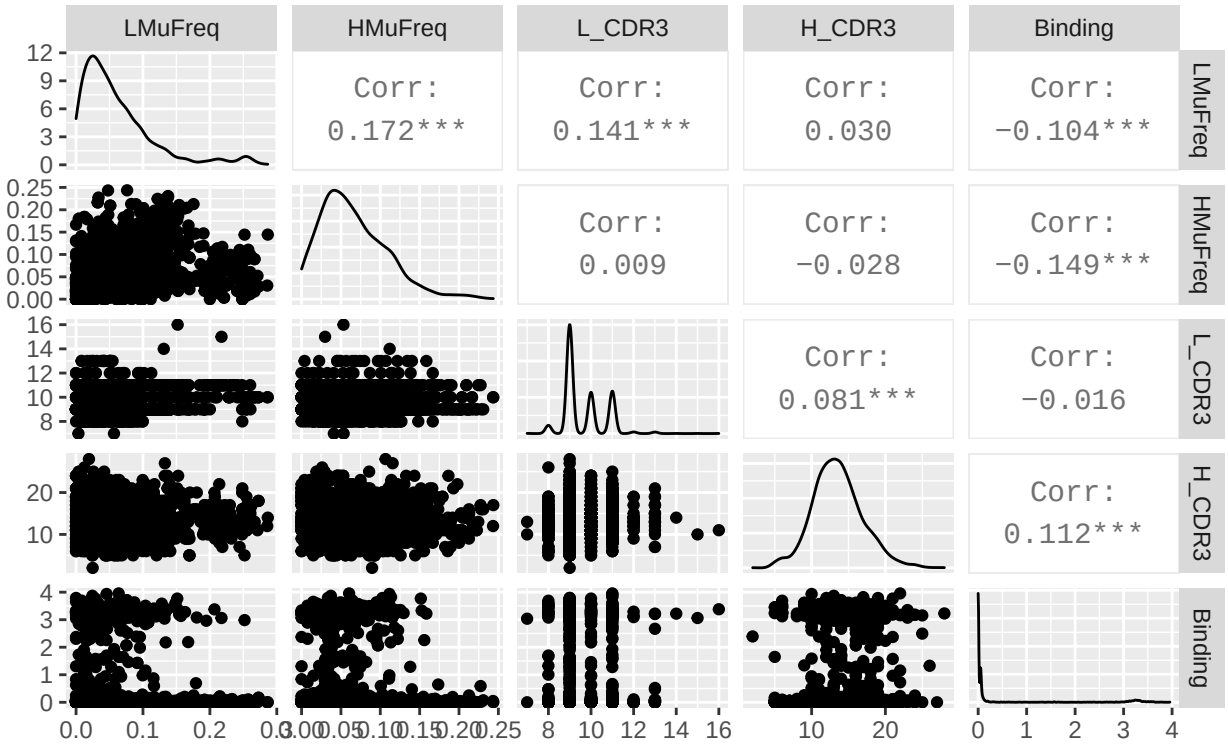

Figure 18: Plots of response variables

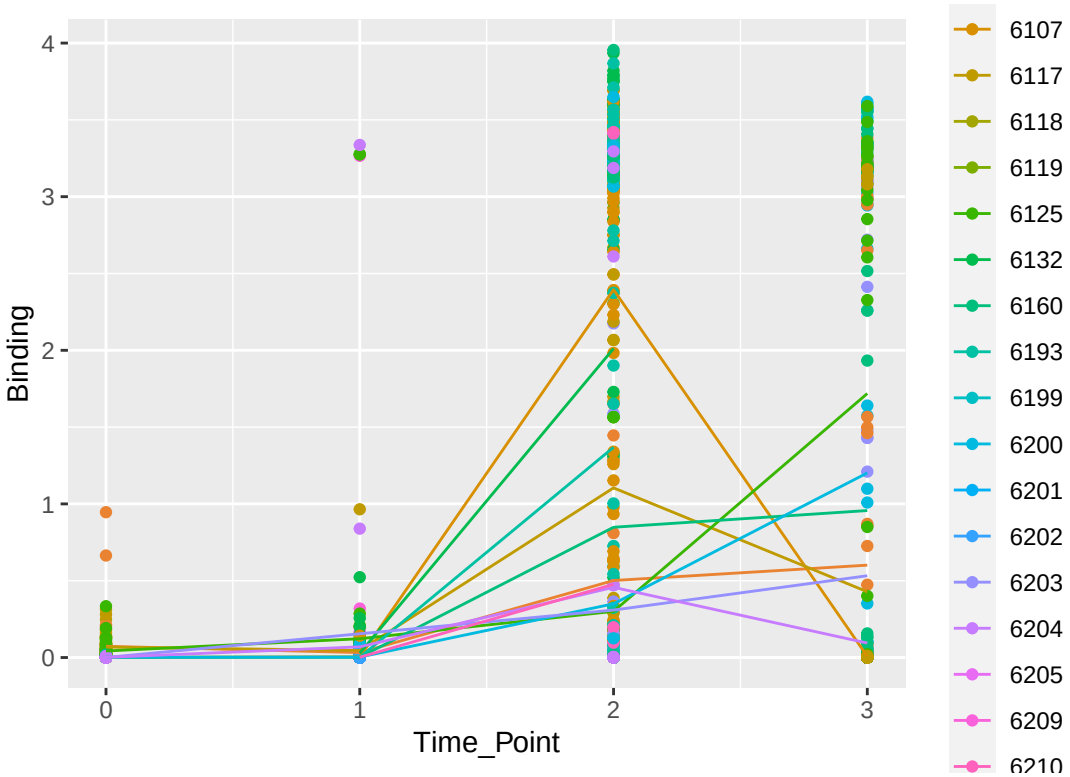

Figure 19: Mean trend by monkey

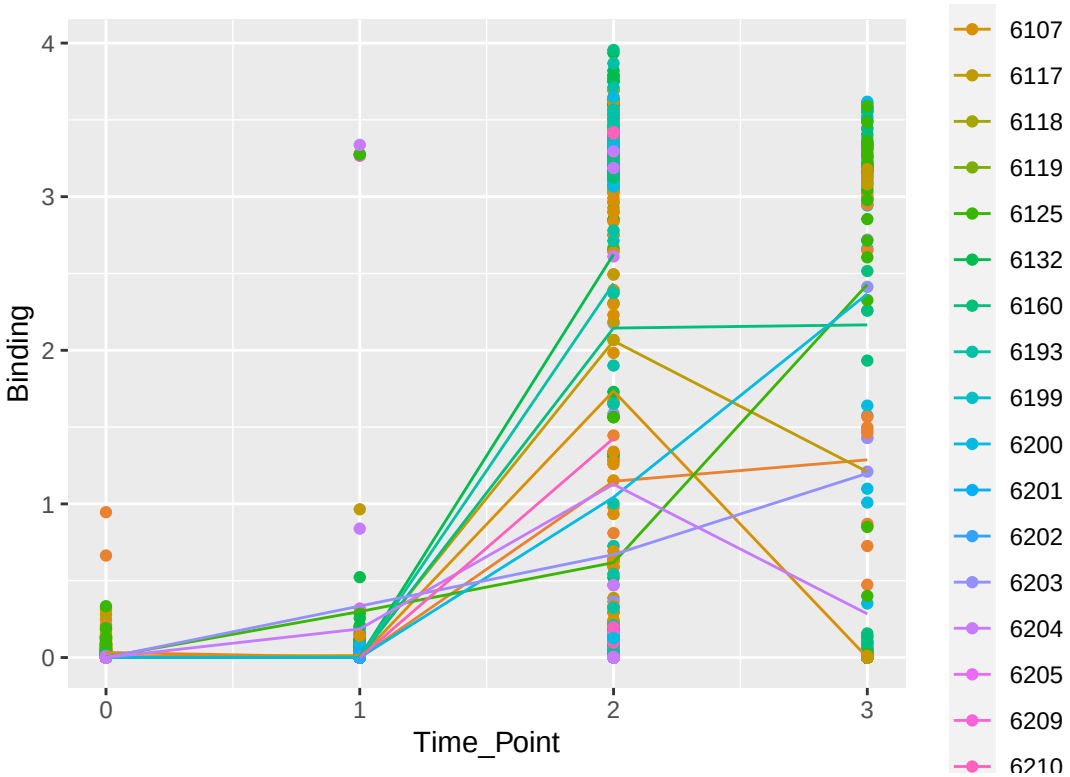

Figure 20: Variances over time by monkey

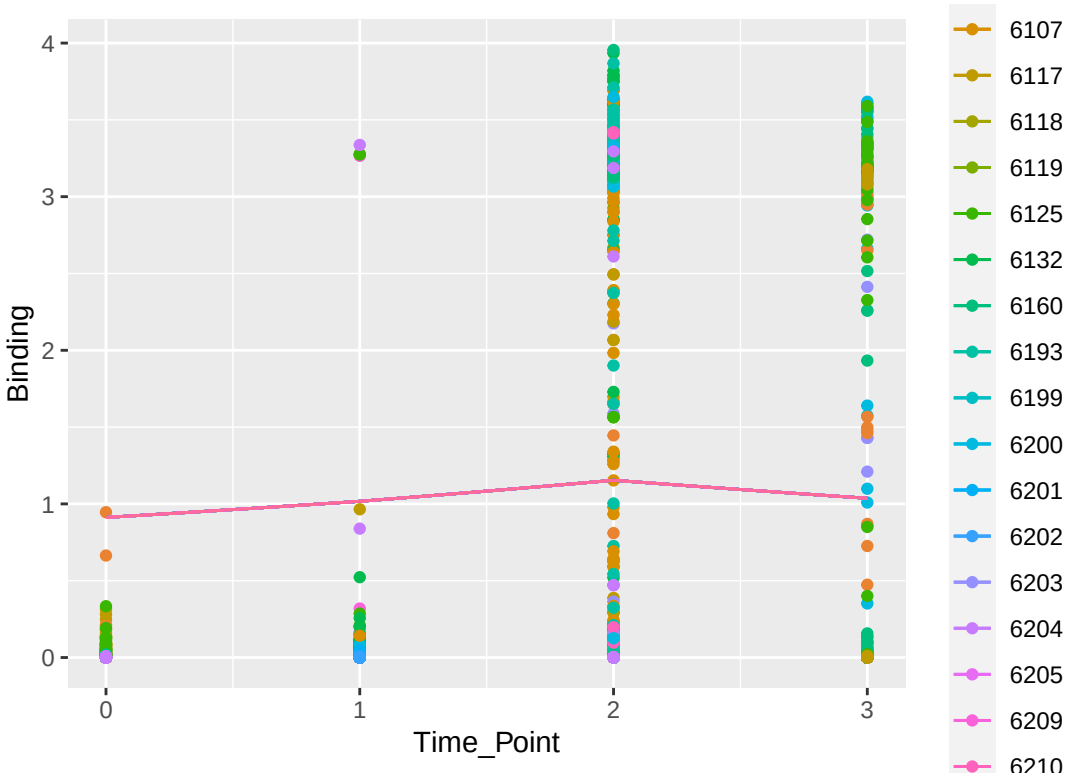

Figure 21: Piecewise Linear Function–three segments

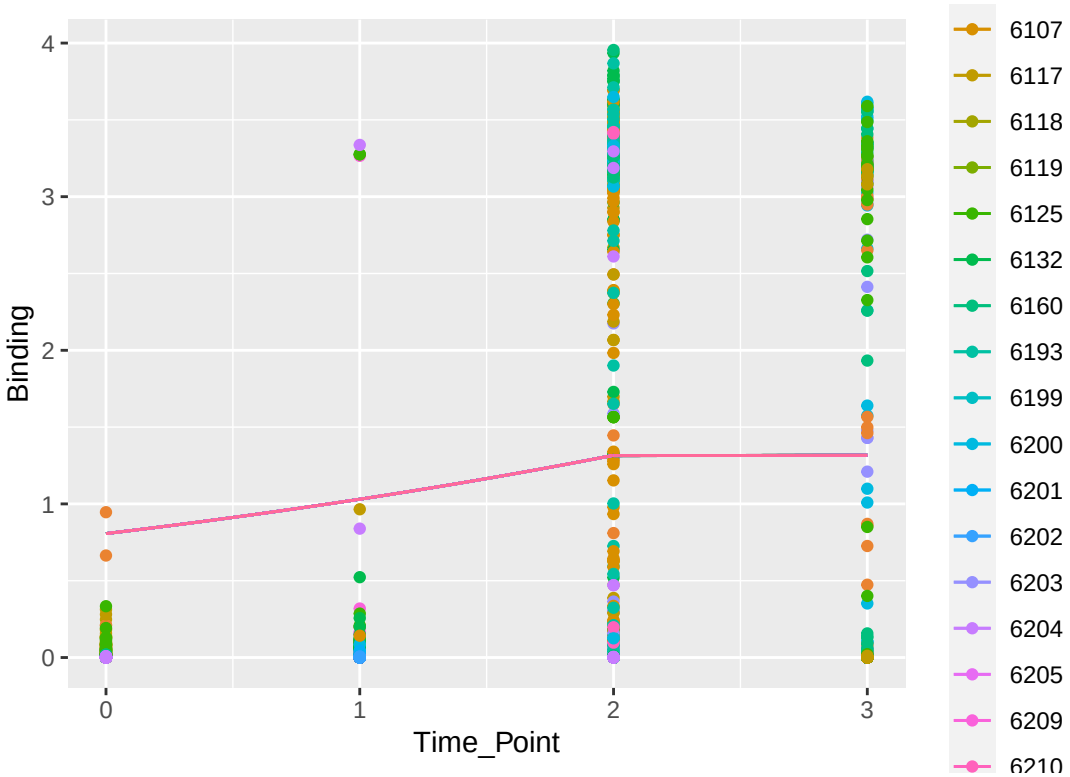

Figure 22: Piecewise Linear Function–two segments

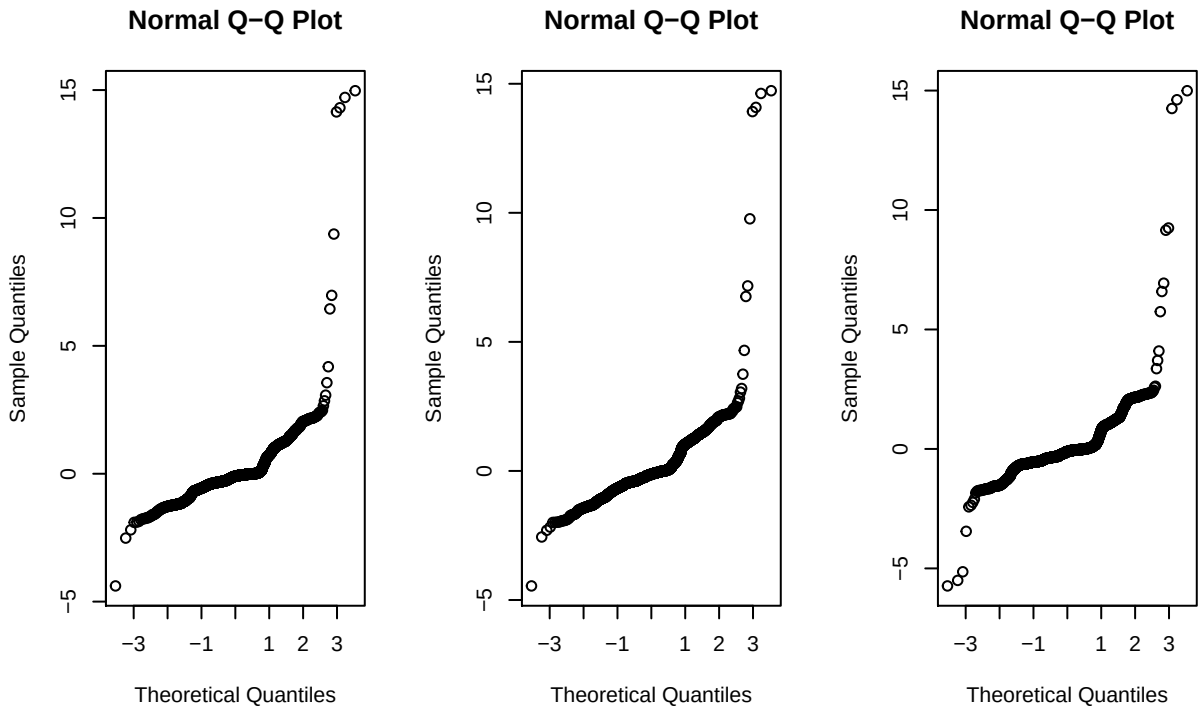

Figure 23: Q-Q plots of models: GLS, compound symmetry, AR1

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Table 1: Frequency tables of drug vs. timepoints

	0	1	2	3
1	129	346	251	122
2	90	533	225	101
3	54	125	347	142

Table 2: Frequency tables of timepoints vs. isotypes for drug = 1 (left), 2 (middle), 3 (right)

	A	D	E	G	M
0	4	4	1	60	60
1	11	22	2	91	220
2	8	15	3	145	80
3	1	16	1	57	47

	A	D	E	G	M
0	6	4	0	37	43
1	10	45	2	205	271
2	4	19	1	115	86
3	1	7	0	53	40

	A	D	E	G	M
0	1	1	0	24	28
1	1	26	0	28	70
2	4	14	0	170	159
3	0	6	0	77	59

Table 3: Summaries of standardized LCDR3

	V1
	Min. :-2.1860
	1st Qu.:-0.5361
	Median :-0.5361
	Mean : 0.0000
	3rd Qu.: 0.2888
	Max. :30.8110

Table 4: Significant Pairs in terms of Time Point and Drug: Significant Pairs

	Time_Point	Drug
H_CDR3	3 > 1, 3 > 2	1 > 2
HMuFreq	0 > 2, 0 > 3, 1 > 2, 1 > 3	2 > 1 > 3
L_CDR3	none	none
LMuFreq	0 > 2, 0 > 3, 1 > 3	none

Table 5: AIC and BIC for meanforms with Binding and logBinding

	df	AIC	df.1	BIC
fit.gls1	9	3293.6959	9	3345.9818
fit.gls2	9	-317.1771	9	-264.8913

Table 6: AIC and BIC between two gls models

	df	AIC	df.1	BIC
fit.gls2	9	-317.1771	9	-264.8913
fit.gls3	8	-310.8418	8	-264.3654

Table 7: AIC and BIC for three models

	df	AIC	df.1	BIC
fit.gls2	9	-317.1771	9	-264.8913
fit.a1	12	-407.9577	12	-338.2432
fit.a2	12	-591.4157	12	-521.7012

Table 8: Inference about S4 ad S5 slopes

numDF	denDF	F.value	p.value
1	2441	58.83591	0
1	2441	137.96033	0
1	2441	56.69165	0

Table 9: AIC and BIC for four models

	df	AIC	df.1	BIC
fit.gls2	9	-317.1771	9	-264.8913
fit.a1	12	-407.9577	12	-338.2432
fit.a2	12	-591.4157	12	-521.7012
fit.a3	20	-599.1739	20	-482.9831

Table 10: Test whether drug 1 = drug 2

Fstat	p_value
2.483881	0.0418059

Table 11: Test whether drug 1 = drug 3

Fstat	p_value
2.111719	0.0768574

Table 12: Test whether drug 2 = drug 3

Fstat	p_value
5.251142	0.0003279

Table 13: Variance-covariance matrices comparison of drug groups 2 and 3

	H_CDR3	HMuFreq	L_CDR3	LMuFreq
H_CDR3	0.8233467	0.0940387	1.7311285	-8.1369695
HMuFreq	0.0940387	1.6459865	-1.2232736	26.2072877
L_CDR3	1.7311285	-1.2232736	0.9656927	0.7827240
LMuFreq	-8.1369695	26.2072877	0.7827240	0.9973451