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ANALYSIS OF DATA FROM A STUDY TO IDENTIFY POTENTIAL BIOMARKERS TO INDICATE RENAL INJURY

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By

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A REPORT

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Definitions

Hydronephrosis – swelling of a kidney due to a build-up of urine.

Ligate – tie up or otherwise close off (an artery or vessel).

Nephrology – a specialty of medicine pertaining to the study of the kidney.

Proximal – situated nearer to the center of the body or point of attachment.

Renography – medical imaging of the kidneys.

Ureter – the duct by which urine passes from the kidney to the bladder.

Urology – a specialty of medicine pertaining to the study of the urinary system.

List of abbreviations

ELISA – Enzyme Linked Immunosorbent Assay

GD – Glomerular Diameter

IRB – Institutional Review Board

KIM-1 – Kidney Injury Molecule-1

NGAL – Neutrophil Gelatinase-Associated Lipocalin

PPG – Percent Positive Glomeruli

PTD – Proximal Tube Diameter

RU – Relative Units

TUNEL – Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

UPJO – Ureteropelvic Junction Obstruction

UUO – Unilateral Ureteral Obstruction

Abstract

Ureteropelvic junction obstruction is a disease in which flow from the kidney to the bladder is obstructed for extended periods of time causing irreversible damage to the kidney. Current tests to detect kidney damage caused by obstruction are not effective until significant damage occurs. The purpose of this report is to identify a panel of biomarkers in urine to detect kidney damage earlier by analyzing data collected from a two-part study. Currently, two established urinary biomarkers to indicate kidney damage are NGAL and KIM-1. Biomarkers of interest in this study are CD13, CD10, and CD26. Results from the linear mixed model, from the murine animal study, determined that these biomarkers express significantly higher concentrations in damaged tissue. Predictive modeling on the clinical data indicated that CD13 and CD10 may provide more accurate predictions on UPJO patients than CD26.

1 Introduction

When a blockage occurs in the kidney or ureter for a short period of time the kidney will be able to recover. However, when the blockage occurs for many days or weeks damage to the kidney can occur; this is called obstructive nephropathy. Ureteropelvic junction obstruction (UPJO), as seen in figure 1.1, is a type of obstructive nephropathy that affects 1 in 500 children [1].

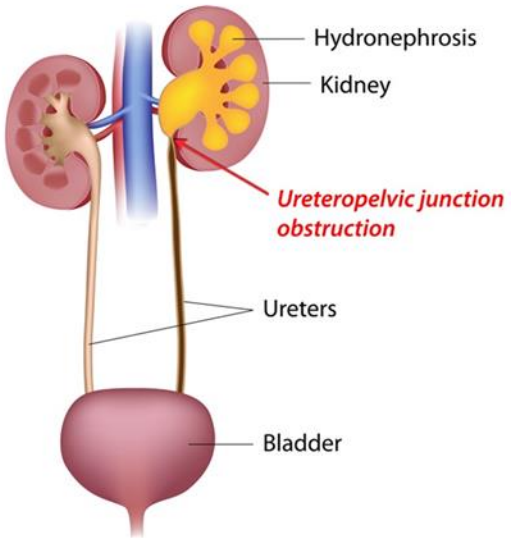

Figure 1.1: Renal system and location of UPJO. Image source: http://www.brighamandwomens.org/Departments_and_Services/surgery/services/urology/ureteropelvic-junction-obstruction.aspx?sub=6.

Currently, urine tests to detect damage caused by obstruction rely on serum creatinine (a waste product that passes through the kidneys), which do not show abnormalities until kidney damage is greater than 50%. Recent studies have identified urinary biomarkers neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1)

as biomarkers in urine that may indicate damage prior to the current method [2-4]. However, the clinical applications of using these biomarkers to asses' kidney damage is still undecided [5]. Therefore, it is a great of importance to develop a panel of biomarkers that can be used to better predict the stage of kidney damage.

This report is a statistical consulting project in which I will be analyzing data collected from two studies aimed at identifying biomarkers in the urine that may indicate kidney damage. The first is an *in vivo* murine animal model study to verify the biomarkers of interest are present in higher quantities for damaged kidneys. The second is a clinical study of UPJO patients using data from a children's hospital. Chapter 2 will give an overview of each study and the biomarkers being investigated, as well as the researchers' needs from the analysis and their hypotheses. Chapters 3 and 4 will discuss specifics for each study and present the results of the murine animal model and clinical studies, respectively. Finally, chapter 5 will discuss the overall results from the study and conclusions to be made.

2 Background

The objective of this study is to develop a panel of biomarkers in urine that could predict kidney damage earlier than the current method. The biomarkers of interest for this study are CD13, CD10, and CD26. These biomarkers were chosen based off a previous pilot study [6]. Both the murine and human studies will have a control group and damaged group, in which the researchers are interested in seeing if the damaged group displays significantly higher concentrations of the biomarkers of interest. Data for these studies can be found in section 7.

The murine study was conducted to provide *in vivo* data for Institutional Review Board (IRB) approval of the clinical study. Furthermore, the murine animal model allowed for a more precise quantification of kidney damage by sacrificing the animal, after urine collection, to allow direct observation of kidney damage using tissue staining methods. Therefore, murine models with significantly higher values for both the biomarkers of interest and tissue damage will support the hypothesis that the novel biomarkers indicate kidney damage. Tissue damage parameters were Trichrome, Percent Positive Glomeruli (PPG), Proximal Tubular Diameter (PTD), Glomerular Diameter (GD), and Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL). Trichrome is a tissue staining technique that will identify damaged kidney tissue, so higher values will be seen in damaged tissue. PPG is another tissue staining technique that will stain live kidney cells, so higher values are expected to be seen in undamaged tissue. PTD is a measurement within the kidney where higher values should associate with reduced flow due to the pressure build up that causes the proximal tubular to dilate. However, GD is

opposite of PTD because scar tissue forms in damaged tissue causing GD to be reduced compared to undamaged tissue. TUNEL is a method to quantify damaged DNA, and hence tissue; this value should be higher in damaged tissue.

The clinical study is the primary study of interest. Animal models give a good preliminary indication and are more cost effective, but do not fully represent what may be seen in humans. Therefore, the predictive model for biomarkers will be built on the data from the clinical study. Both established and novel biomarkers will be analyzed from urine collected in this study. Furthermore, the sex of the patient will also be included in the model because it has recently been made a requirement for NIH-funded research [7].

3 Analysis of Murine Animal Study

3.1 Data

A murine animal model was used for this study, which consisted of four to six mouse samples. Unilateral ureteral obstruction (UUO) was performed on each mouse, in which either the right or left ureter (taken at random) was ligated to simulate renal damage, leaving the other ureter unligated to allow urine to flow freely from the kidney to the bladder on the one side. This allows for collection of paired samples, which were obtained by collecting secreted urine (unligated samples) and urine that had collected proximal to ligation (ligated samples) from the same mouse. Urine for each mouse sample was collected at day 2, 3, 4, 5, 7, and 10 after UUO and the concentrations of each biomarker were then measured. The mouse was sacrificed to assess tissue damage at the end of study, as discussed in Chapter 2.

Data for this study was given to me during the initial meeting. The recommended sample size for each day, calculated by their previous statistical consultant, was ten to provide 80% power to detect a group mean difference of 5,000 relative units (RU). However, the collected data contained at most six paired samples. Some days had only one paired observation due to missing data, as seen in figure 3.1.

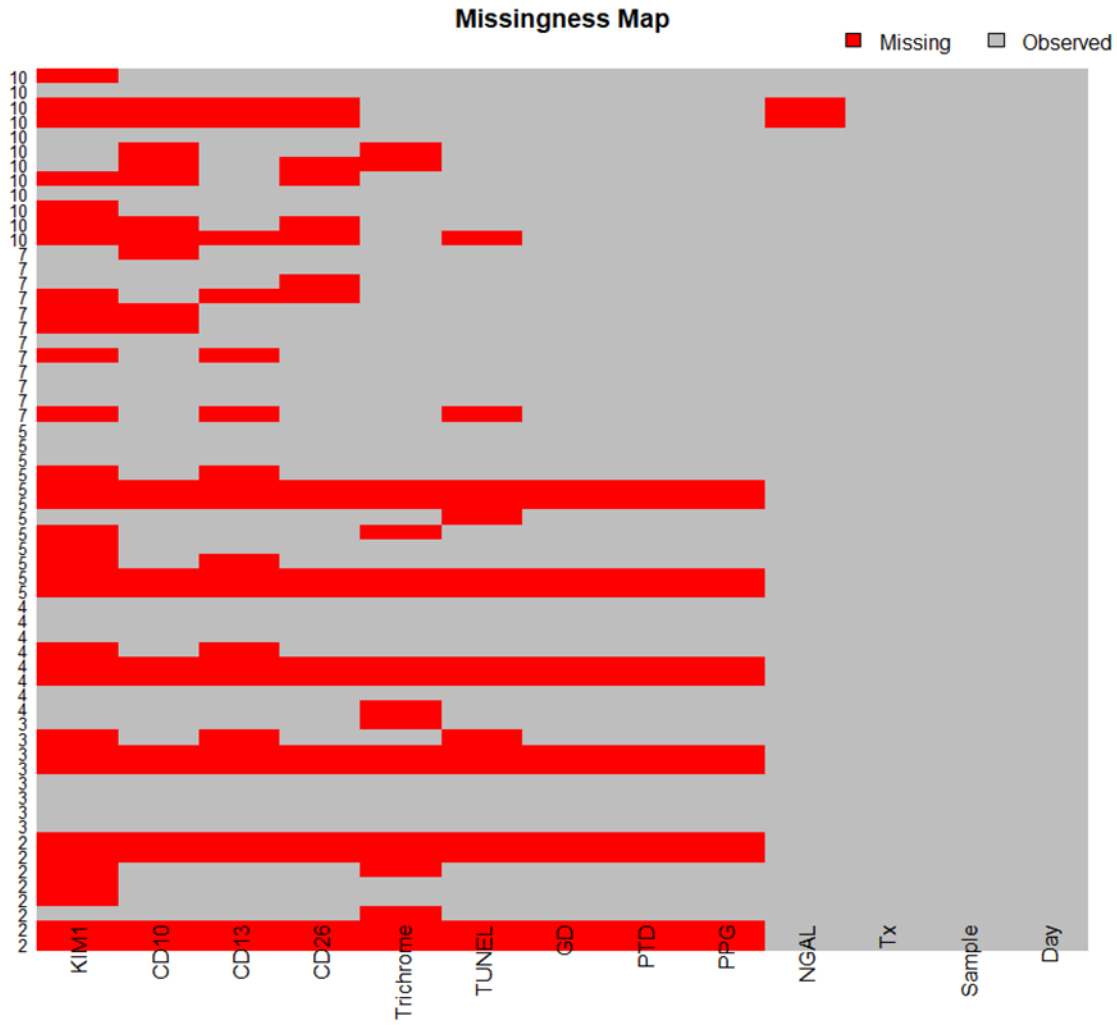

Figure 3.1: Graphical representation of missing data for each variable.

Any samples for a given day with less than three paired observations were not included for the hypothesis testing section (section 3.2). Boxplots were constructed to determine the spread of the data. As seen in figure 3.2, there are many outliers and it is difficult to tell the distribution with a small number of data points. The mean and variance, stratified by the day, can be found in section 9. Due to this observation, non-parametric methods were initially used.

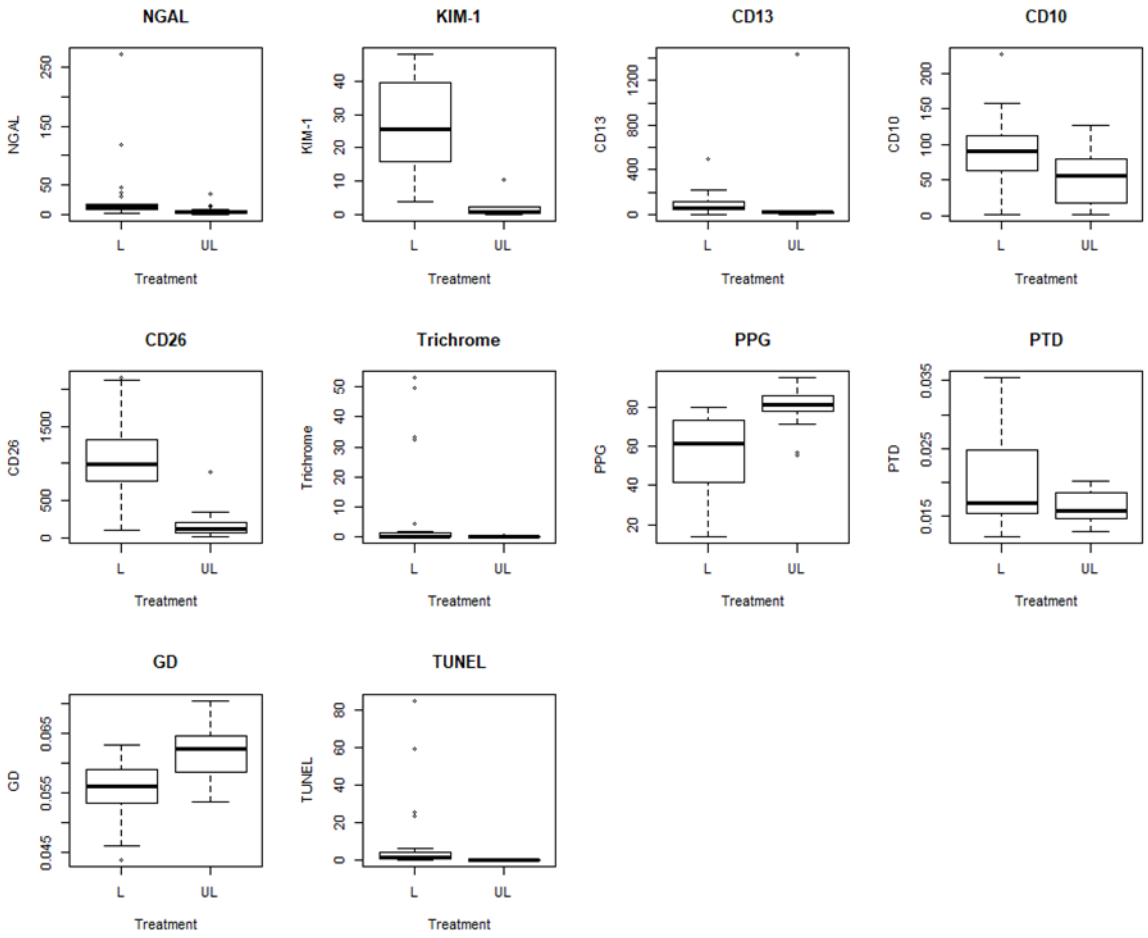

Figure 3.2: Boxplots of each variable separated by treatment.

The hypothesis tests were done by day, so boxplots were made for each day as seen in section 8. From these plots, we do still have some concerns for normality, but there are less outliers (only NGAL). Therefore, paired t-tests were also performed to determine if a significant difference may exist, but it is noted that the *p*-values may not be credible due to the small sample sizes. Therefore, mixed linear models were used to obtain a better indication of how each biomarker relates to the treatment type (ligated or unligated). These results will be discussed in section 3.3.

3.2 Hypothesis Testing

This study is expecting to see higher concentrations for all biomarkers in the ligated (L) samples versus the unligated (UL) samples, as discussed in Chapter 2. Therefore, the null (H_0) and alternative (H_1) hypothesis for the biomarkers discussed in this section are as follows:

$$H_0: Concentrations\ in\ Ligated\ Samples = Concentrations\ in\ Unigated\ Samples$$

$$H_1: Concentrations\ in\ Ligated\ Samples > Concentrations\ in\ Unigated\ Samples$$

The hypotheses are the same for all the tissue damage parameters except for PPG and GD, in which the alternative hypothesis is opposite due to UL values having the higher expected values, as discussed in Chapter 2. Data was collected in pairs, L and UL samples, so the difference between the paired samples was used.

The results in this section are presented with both the standard significance level and also a significance level using a multiple comparsion correction, specifically the Bonferroni correction. This correction is designed to control the probability of rejecting at least one null hypothesis, or familywise error rate, when performing multiple hypotheses tests. For example, in section 3.2.1 we are doing 46 different tests so the probability of reject at least one null hypothesis is much higher than 0.05 even if these 46 null hypotheses are true when we set the significance level for each test as 0.05. For the Bonferroni correction, α/M is used as the significance level for each test, where α is the desired overeall significance level and M is the number of test. In our study, $\alpha=0.05$ was used and the null hypothesis was rejected if the *p*-value was less than 0.05/M.

3.2.1 Non-Parametric Tests

With a small number of observations per day, a non-parametric method, specifically the Wilcoxon Signed-Rank test, was used to determine if the difference is different from zero. Biomedical publications consider the rejection of null hypothesis to be *p*-values less than 0.05. Furthermore, it is typically noted in publications when a *p*-value is less than 0.01 to indicate a stronger conclusion.

For this test, we are assuming that L and UL samples are dependent, each mouse is randomly and independently drawn, the dependent variable is continuous, and the data is at least ordinal. The first two assumptions are met by the method used to collect the data, but it is noted that independence between mice may not be valid due to inbreeding. The dependent variable was a normal approximation to binomial for these tests, so a continuity correction was done to account for the approximation. All the data collected was continuous data, so the data meets the fourth assumption needed.

The statistical software R was used to conduct this test. For the non-parametric test, the built-in function wilcox.test was used. This function inputs the data, alternative hypothesis, and a logical operator to indicate if data is paired, then it returns the test statistic (W) and *p*-value. The continuity correction is applied by default using this function. All the *p*-values from the analysis are summarized in table 3.1, where N/A indicates days that have less than 3 observations so the test was not performed.

Table 3.1: *p*-values from Wilcoxon Signed-Rank tests on mouse data.

Biomarker	**Day**					
	2	**3**	**4**	**5**	**7**	**10**
NGAL	N/A	0.3125	0.125	0.01563*	0.2188	0.01563*
KIM-1	N/A	N/A	N/A	N/A	0.125	N/A
CD13	N/A	0.25	0.125	0.625	0.125	0.0625
CD10	N/A	N/A	N/A	0.0625	0.0625	0.0625
CD26	N/A	N/A	0.0625	0.0625	0.0625	0.0625
Tissue Damage						
Trichrome	0.25	0.0625	0.0625	0.125	N/A	N/A
PPG	0.4375	0.0625	0.0625	0.0625	0.0625	0.0625
PTD	0.9375	0.8125	0.1875	0.125	0.0625	0.0625
GD	0.3125	0.0625	0.1875	0.0625	0.0625	0.0625
TUNEL	0.4375	0.125	0.125	0.125	0.125	0.0625
* = *p*-value < 0.05						

These results indicate that the only statistically significant difference is for the biomarker NGAL at day 5 and 10. This means that for the given data at those time points, NGAL is expressed in significantly higher concentrations for the ligated ureter, compared to the unligated one. The rest of the samples had insufficient evidence to detect a difference between ligated and unligated samples. Using a Bonferroni correction for multiple comparisons we obtain a significance level of 0.00109. Using this significance level, no results are considered significant. However, it is notable that many of the *p*-values are close to 0.05, which leads one to believe that a difference may exist, but is not detectable with the small sample sizes in this study.

3.2.2 Parametric Tests

To see if a difference may exist in some of those, a paired t-test was also performed. DeWinter's paper claims that a paired t-test can be valid for extremely small sample sizes if the within-pair correlation is high [8]. Furthermore, the t-test is robust to the normality assumption. Along with the other assumptions made in section 3.2.1 we must also assume that the L and UL populations have the same skewness and there are no influential outliers.

To perform a paired t-test R's built-in function t.test was used. This function allows for a paired t-test to be performed, which was used for the given data. This function has similar inputs as the wilcox.test function; a continuity correction is not required, but we do assume the variances to not be equal. The output gives the test statistic (t) and *p*-value as in the wilcox.test function. The *p*-values for the given data are presented in table 3.2, again N/A indicates days with less than 3 observations so the t-test was not performed.

Table 3.2: *p*-values from paired t-tests.

Biomarker	**Day**					
	2	**3**	**4**	**5**	**7**	**10**
NGAL	N/A	0.1819	0.0375*	< 0.001***	0.2338	0.0942
KIM-1	N/A	N/A	N/A	N/A	0.0724	N/A
CD13	N/A	0.1036	0.0766	0.7777	0.0262*	0.0997
CD10	N/A	N/A	N/A	0.0226*	0.0034**	0.0364*
CD26	N/A	N/A	0.0121*	0.0216*	0.0147*	0.0025**
Tissue Damage						
Trichrome	0.1501	0.0334*	0.0042**	0.0448*	N/A	N/A
PPG	0.346	0.0968	0.0028**	0.0040**	0.014*	0.0048**
PTD	0.8938	0.6248	0.1702	0.0822	0.0026**	0.0303*
GD	0.1737	0.0152*	0.1655	0.0074**	0.0166*	0.0070**
TUNEL	0.4024	0.0966	0.0573	0.0856	0.0319*	0.0234*
* = *p*-value < 0.05; ** = *p*-value < 0.01; *** = *p*-value < 0.001						

The results in table 3.2 show what was suspected; some of the non-parametric *p*-values that were slightly greater than 0.05 have *p*-values less than 0.05 for the parametric method. Furthermore, only NGAL at day 5 is considered as significant when the Bonferroni correction is used. So, we now see a lot more significant observations compared to the non-parametric test, as indicated by the asterisk in the table. Furthermore, we see that there are no significant observations in day 2 and almost all of the day 10 observations are significant. This aligns with the hypothesized outcome that as time went on these biomarkers would be expressed in significantly higher amounts for the ligated samples. However, the commonly accepted methods for this test make the *p*-values not very credible due to the inability to check assumptions needed for this test.

3.3 Mixed Linear Model

Due to the small sample size and many of the days having observations less than three, mixed linear models were used to help determine the effect of each biomarker. The mixed linear model can consider all data together thus is potentially more powerful than the non-parametric method used in Section 3.2.1 and the t-test used in Section 3.2.2. A mixed linear model consists of the fixed and random effects where the random effects can take account for the correlations of data within each mouse. The following mixed linear model in Equation 1 was used.

$$\text{Equation 1:} \quad Y_{ijk} = \beta_0 + \beta_t * T_k + \left(\alpha_{0i} + \alpha_{1i} * d_j\right) + \varepsilon_{ijk}$$

In this model, subscripts represent the sample (i = 1, 2, . . ., 6), day (j = 2, 3, 4, 5, 7, 10), or the treatment (k = L, UL). The overall intercept (β_0) and treatment effect (β_t) are considered as the fixed effects. The intercept (α_{0i}) and the slope for days α_{1i} are considered as the random effect. We further assume that: (1) α_{0i} and α_{1i} are normally distributed. Specifically, α_{0i} has a mean of 0 and variance of σ_0^2, α_{1i} has a mean of 0 and variance of σ_1^2, and α_{0i} and α_{1i} have a correlation of ρ; (2) α_{0i} and α_{1i} are independent for different mouse. The error terms, ε_{ijk}, are independent and are independent of α_{0i} and α_{1i}. ε_{ijk} is normally distributed and has a mean of and variance of σ_0^2.

The mixed linear models were built using the R package lmerTest [9]. Within this package is the function lmer, which fits a mixed linear model. The formula for the mixed linear model used in R for this analysis is shown below (Equation 2):

$$\text{Equation 2:} \quad Y = Tx + (Day|Sample) + \varepsilon$$

In Equation 2, 'Y' represents the parameter of interest (biomarker or tissue damage). The fixed effect is 'Tx', the treatment (L or UL). The random effect is (Day|Sample), in which 'Day' is evaluated as a linear model and 'Sample' is evaluated as a factor. The *p*-values for the fixed effect were calculated by the lmerTest function using an F statistic. Ten mixed linear models were built for each biomarker and the results for these models are presented in table 3.3.

Table 3.3: Results of the mixed linear models for overall treatment.

Biomarker	**Ligated Treatment Fixed Effect Values**	
	Estimate	***p*-value**
NGAL	21.123	0.017990*
KIM-1	26.009	< 0.001**
CD13	12.57	0.874
CD10	35.08	0.0188*
CD26	884.18	< 0.001**
Tissue Damage		
Trichrome	6.277	N/A
PPG	-25.383	< 0.001**
PTD	0.0038132	< 0.001**
GD	-0.0062047	N/A
TUNEL	9.074	0.0261*
N/A = model did not converge; * = *p*-value < 0.05; ** = *p*-value < 0.001		

The estimate values in table 3.3 indicate the relationship between the biomarker and treatment. A positive estimate value indicates that ligated treatments increase the biomarker (or staining) value. We see that all the estimate values are positive besides PPG and GD, which are expected to be negative. We also notice that *p*-values for NGAL, KIM-1, CD10, CD26, PPG, PTD, and TUNEL are all significant. Using a Bonferroni

correction (significance level of 0.005), we still see significance in KIM-1, CD26, PPG, and PTD. The results from these models support the hypothesized outcome of the study.

4 Analysis of Human Study

4.1 Data

The samples are pediatric patients with Society of Fetal Urology (SFU) grade 3-4 hydronephrosis that are receiving care in the Urology Clinic at Connecticut Children's Medical Center. The two patient types will be control patients and UPJO patients. Control patients consist of patients presenting to the clinic with primary nocturnal enuresis (involuntary urination during the night); generally, they have no complicating urologic or nephrologic abnormalities. UPJO patients are those presenting to the clinic with confirmed UPJO by nuclear renography and with no prior surgical intervention. Urine samples were collected from the patients by clean catch or catheterization at the time of nuclear imaging or surgical intervention.

Data for this study consisted of 12 control patients and 29 UPJO patients. ELISA was performed on urine samples for each patient to quantify the concentration of each biomarker discussed in Chapter 2. Furthermore, the sex of the patient will also be included as a variable in the logistic regression part of this analysis (section 4.3). For the hypothesis testing (section 4.2), boxplots were constructed to look at the spread of the data as seen in figure 4.1.

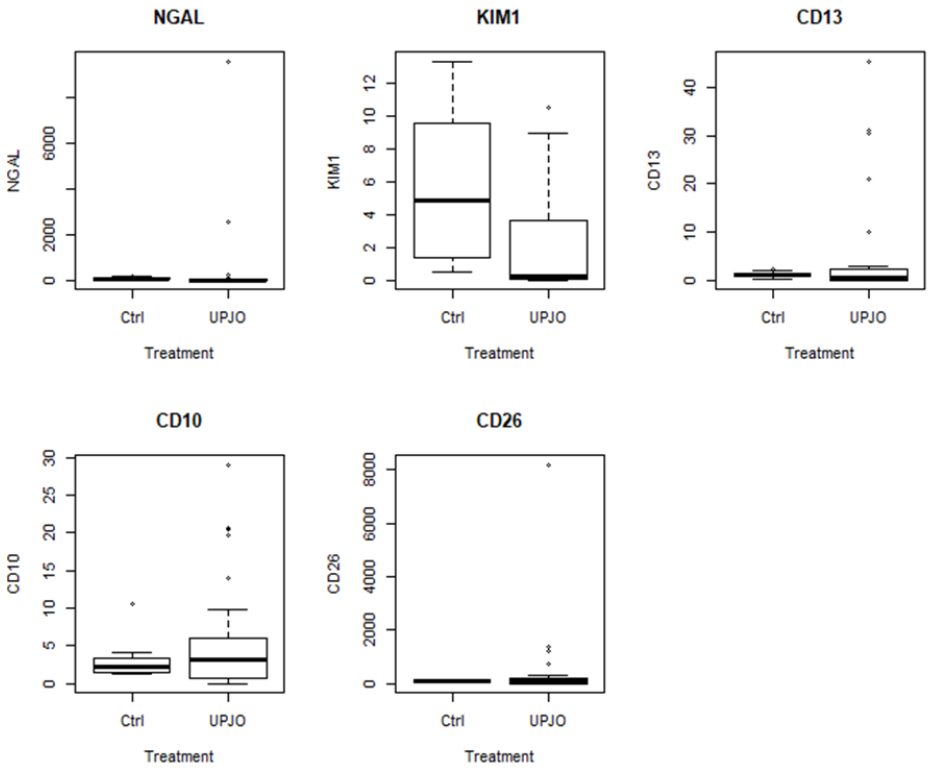

Figure 4.1: Boxplots of biomarkers by treatment.

From figure 4.1, it is obvious that a number of outliers exist and the spread may not be normal. Furthermore, the variance between the two groups may not be equal for each biomarker, as seen in table 4.1. So, equal variance assumption was not used.

Table 4.1: Summary statistics for human study by group.

Group		**NGAL**	**KIM-1**	**CD13**	**CD10**	**CD26**
Control	Mean	66.5	5.58	1.15	2.99	90.6
	Variance	3269	21.5	0.300	6.57	1633
UPJO	Mean	492	2.35	5.29	5.84	467
	Variance	3693939	9.92	133	57.6	2326334

For the logistic regression model, we also checked the collinearity between variables. As shown in figure 4.2, CD10 and CD13 have the highest correlation, but none seem to be greater than 0.7.

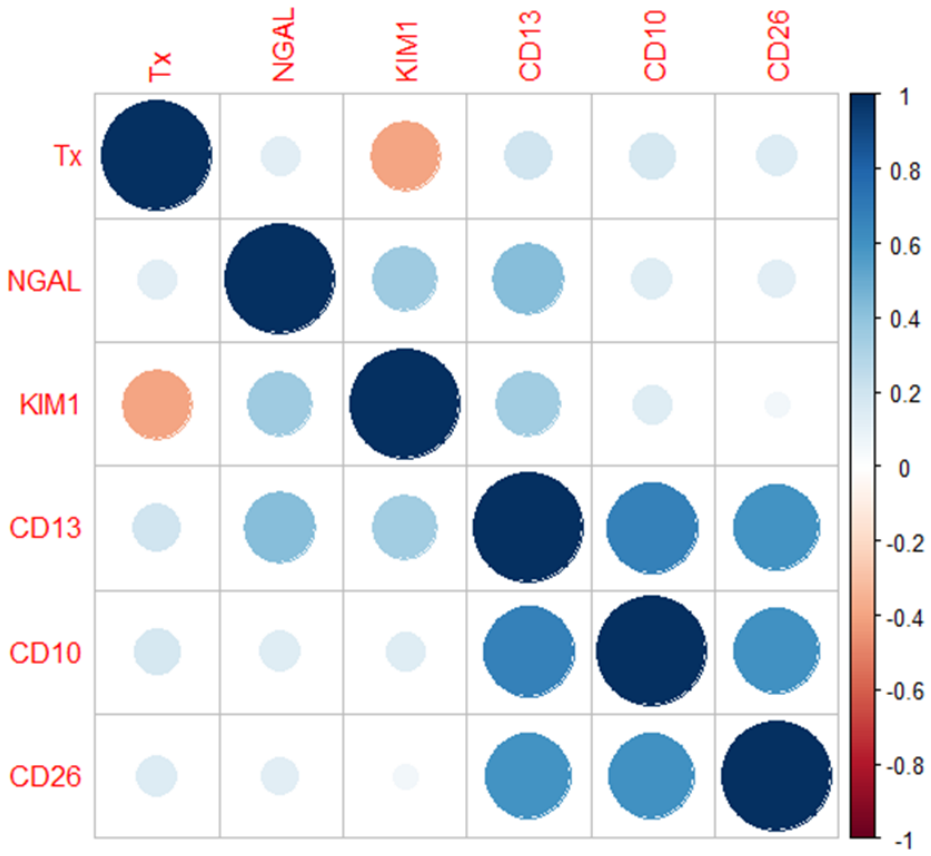

Figure 4.2: Correlation plot for biomarkers.

So, we can see that multicollinearity may not be an issue for logistic regression, but there is a concern with the outliers effecting the results.

4.2 Hypothesis Testing

This study is expecting to see higher values for all the biomarkers in the UPJO patients compared to the control patients. Therefore, the null (H_0) and alternative (H_1) hypothesis for the biomarkers discussed in this section are as follows:

$$H_0: Values\ in\ UPJO\ Patients = Values\ in\ Control\ Patients$$

$$H_1: Values\ in\ UPJO\ Patients > Values\ in\ Control\ Patients$$

For this analysis, a two-sample t-test was performed, without equal variance assumption, for the above hypotheses using the built-in R function t.test. The data was passed into the function with an alternative hypothesis of greater than and the results for each biomarker are summarized in table 4.2.

Table 4.2: Two-sample t-test results for clinical data.

Biomarker	**Test Statistic (t)**	***p*-value**
NGAL	1.1274	0.1351
KIM-1	-2.21	0.9787
CD13	1.9274	0.03201*
CD10	1.7955	0.04025*
CD26	1.3276	0.0975
* = *p*-value < 0.05		

Using the same rejection region as in section 3.2 we see that only CD13 and CD10 are significantly higher in the UPJO patients compared to the control patients. With a Bonferroni correction, a significance level of 0.01 was used to ensure a 0.05 familywise error rate, which results in none of the biomarkers showing significantly higher values in

the UPJO patients. It is interesting to note that NGAL and KIM-1 are not significant even though they are the established biomarkers. Looking at the data there were many values in both NGAL and KIM-1 with values of zero or close to zero (as seen in section 7.2). Although this contradicts the animal model study, it is not too surprising because these markers have been reported to be unreliable in some clinical studies [5].

4.3 Predictive Modeling

Logistic regression models were built to determine if biomarkers with other covariates such as age and gender could be used to predict UPJO patients. Patient type was treated as dummy variables where 0 represented control patients and 1 represented patients with UPJO in the logistic model. Furthermore, patient sex was added as a covariate. The full model was initially used as shown in equation 3.

Equation 3: $\ln\left(\frac{p_i}{1-p_i}\right) = \beta_0 + \beta_1 x_{1,i} + \beta_2 x_{2,i} + \beta_3 x_{3,i} + \beta_4 x_{4,i} + \beta_5 x_{5,i} + \beta_6 x_{6,i} + \varepsilon_i$

For equation 3, p_i indicates the probability of the patient having UPJO. The coefficients of the equation, β_1, β_2, β_3, β_4, β_5, and β_6, are for the variables NGAL, KIM1, CD13, CD10, CD26, and Sex, respectively. ε_i are the error terms and are normally distributed with a mean of 0 and variance of σ^2.

To determine the best model backward elimination was performed with the AIC statistic using the built-in R function, step. The best model has an AIC of 40.798 and contains NGAL, KIM-1, and CD13; however, only KIM-1 was found to be significant at a significance level of 0.05. This model is shown below, with estimated coefficients, in equation 4.

Equation 4: $\ln\left(\frac{p_i}{1-p_i}\right) = 1.5595 + 0.00164 * x_{1,i} - 0.44612 * x_{2,i} + 0.21854 * x_{3,i}$

From this equation (and table 4.3), we can see that only the coefficient of KIM-1 is negative. So, it seems to have an inverse relationship to the outcome of the patient compared to NGAL and CD13.

Table 4.3: Covariate coefficient estimate, 95% C.I., and *p*-values for selected model.

Covariate	**Estimate**	**95% Confidence Interval**	***p*-value**
NGAL	0.00164	(0.0011, 0.0021)	0.2986
KIM-1	-0.44612	(-0.5039, -0.3883)	0.0141
CD13	0.21854	(0.0991, 0.3380)	0.5608

An ROC curve was built for this model, as seen in figure 4.3, to see the tradeoff between sensitivity and specificity. For this study, specificity is more important because the model should reduce the number of false positives to ensure that healthy patients are not being diagnosed with UPJO.

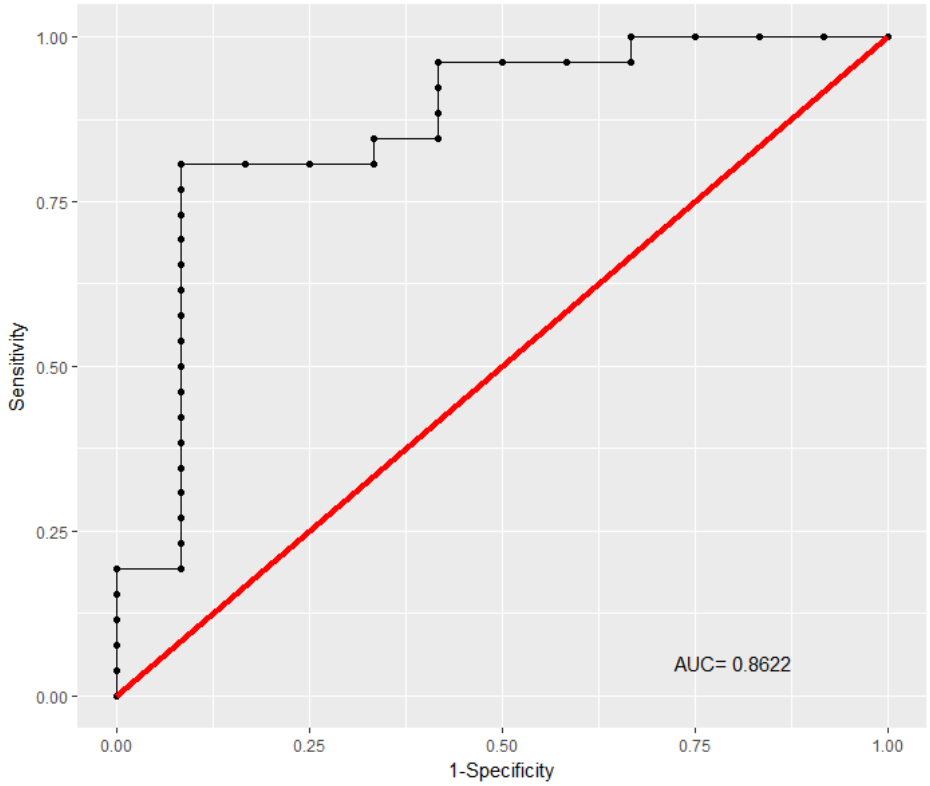

Figure 4.3: ROC plot of selected model.

In addition, the model selection was also determined by the Area Under the Curve (AUC) value from constructed Receiver Operating Characteristic (ROC) plots. The built-in R function, glm, was used to create the models; the link function was specified to be binomial due to a binary response variable. Sixty-three different models, for every combination of the six predictor variables, were built with patient type being the response variable. Table 4.4 shows the models with AUC values larger than 0.85.

Table 4.4: General linear models with AUC values larger than 0.85.

Model	**AUC Value**	**AIC Value**	**Significant Variables**
Pt_Type ~ NGAL + KIM1 + CD13 + CD10 + CD26 + Sex	0.8974	44.693	KIM1*
Pt_Type ~ NGAL + KIM1 + CD13 + CD10 + Sex	0.8974	42.698	KIM1*
Pt_Type ~ NGAL + KIM1 + CD13 + Sex	0.8942	40.829	KIM1*
Pt_Type ~ NGAL + KIM1 + CD13 + CD26 + Sex	0.891	42.792	KIM1*
Pt_Type ~ NGAL + KIM1 + CD10 + CD26 + Sex	0.8846	42.95	KIM1*
Pt_Type ~ NGAL + KIM1 + CD26 + Sex	0.875	41.02	KIM1*
Pt_Type ~ NGAL + KIM1 + CD13 + CD26	0.8718	42.722	Int* KIM1*
Pt_Type ~ NGAL + KIM1 + CD10 + Sex	0.8718	42.029	KIM1*
Pt_Type ~ NGAL + KIM1 + CD13 + CD10 + CD26	0.8654	44.677	Int* KIM1*
Pt_Type ~ NGAL + KIM1 + CD13 + CD10	0.8654	42.705	Int* KIM1*
Pt_Type ~ NGAL + KIM1 + CD13	0.8622	40.798	Int** KIM1*
Pt_Type ~ KIM1 + CD13 + CD10	0.8621	45.514	Int* KIM1*
Pt_Type ~ KIM1 + CD13	0.8621	43.592	Int** KIM1*

From table 4.4 we see that the established biomarkers (NGAL or KIM-1) are in all of the models. It is also notable that both CD13 and CD10 show up in many of the models. This makes sense because those were the two biomarkers that were statistically significant in section 4.2. The significant variable column shows which variables were statistically significant for that model. We see that KIM-1 is significant in every model, so it may have a greater impact than NGAL. However, NGAL seems to be more important than other covariates as indicated by the *p*-values in table 4.5.

Table 4.5: Covariate coefficient estimate, 95% C.I., and *p*-values for the full model.

Covariate	**Estimate**	**Standard Error**	**95% Confidence Interval**	***p*-value**
NGAL	0.00199	0.0016	(0.0015, 0.0025)	0.2164
KIM-1	-0.45651	0.2161	(-0.5252, -0.3878)	0.0347
CD13	0.16616	0.6445	(-0.0388, 0.3711)	0.7966
CD10	0.05197	0.1702	(-0.0021, 0.1061)	0.7602
CD26	0.00086	0.0121	(-0.0030, 0.0047)	0.9432
Sex	1.2971	0.9349	(0.9998, 1.594)	0.1653

An ROC curve was also built for the full model, as seen in figure 4.4, to compare with the selected model.

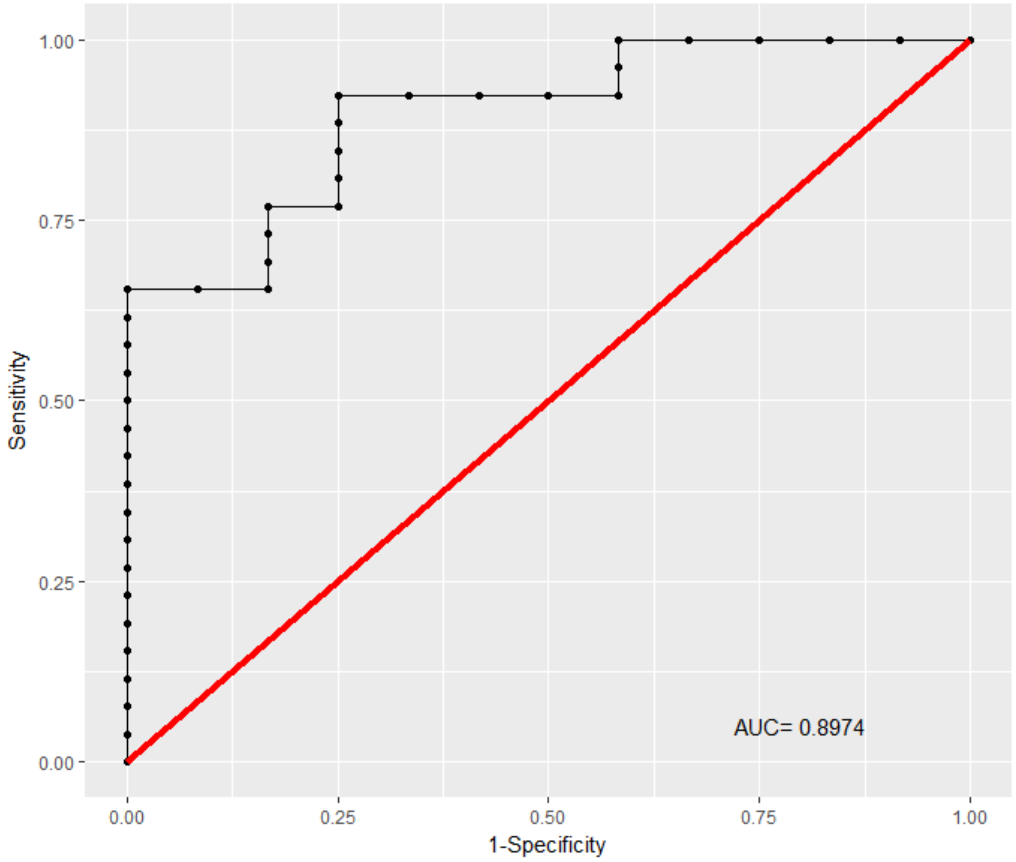

Figure 4.4: ROC plot of the full model.

So, in conclusion table 4.5 may not give us a good indication of what model would be best to predict patient type, but it does give us a good indicator of what biomarkers to look for. Furthermore, when comparing the ROC plots, both models seem to be similar in performance. The selected model has slightly better specificity at lower sensitivity than the full model, but not by much.

5 Conclusion and Discussion

The objective of this study was to develop a panel of biomarkers to help predict kidney damage. Biomarkers of interest were CD13, CD10, and CD26. Established biomarkers included in the study were NGAL and KIM-1. We expected to see the biomarkers expressing significantly higher values for damaged kidneys.

For the murine animal model study, the hypothesis test, using Student's t-test discussed in Chapter 3, had statistically significant results for at least one day in all of the biomarkers of interest. Furthermore, all of the tissue damage parameters showed significant results for at least one time point, which verifies that the models being used were valid because the kidney with a ligated ureter was significantly more damaged than the unligated kidney. Non-parametric methods did not yield any significant results for the biomarkers of interest, but they were all below 0.1. So, the non-parametric results could have not detected a significance due to the small sample sizes. The mixed linear models further verified that the biomarkers of interest are significantly higher in damaged tissue. Therefore, the preliminary murine animal study supported the hypothesized outcome that the biomarkers of interest would be higher in damaged tissue.

The clinical study gave a little more insight into which biomarkers may be a better indicator in predicting kidney damage. Hypothesis tests indicated that, for the given data, there was significant evidence to show that UPJO patients express higher amounts of CD13 and CD10 compared to the control patients. However, the establish biomarkers, NGAL and KIM-1, did not show a significant difference in the hypothesis testing, but was included in almost all the top linear models. This seems to be contradicting.

Overall, the biomarkers of interest all expressed higher values in damaged tissue samples than in the controls, but CD13 and CD10 seemed to be more prominent than CD26 when the linear models were built and tested for prediction accuracy.

6 Reference List

1. Madsen, M.G., et al., Urinary NGAL, cystatin C, beta2-microglobulin, and osteopontin significance in hydronephrotic children. Pediatr Nephrol, 2012. 27(11): p. 2099-106.
2. Cost, N.G., et al., Urinary NGAL levels correlate with differential renal function in patients with ureteropelvic junction obstruction undergoing pyeloplasty. J Urol, 2013. 190(4 Suppl): p. 1462-7.
3. Urbschat, A., et al., Serum and urinary NGAL but not KIM-1 raises in human postrenal AKI. Eur J Clin Invest, 2014. 44(7): p. 652-9.
4. Wasilewska, A., et al., KIM-1 and NGAL: new markers of obstructive nephropathy. Pediatr Nephrol, 2011. 26(4): p. 579-86.
5. Martensson, J., C.R. Martling, and M. Bell, Novel biomarkers of acute kidney injury and failure: clinical applicability. Br J Anaesth, 2012. 109(6): p. 843-50.
6. Gerber, C., et al., Proximal tubule proteins are significantly elevated in bladder urine of patients with ureteropelvic junction obstruction and may represent novel biomarkers: A pilot study. J Pediatr Urol, 2016. 12(2): p. 120 e1-7.
7. National Institutes of Health (NIH). Consideration of sex asa biological variable in NIH-funded research. 2015. Online document at: http://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-102.html
8. DeWinter, J.C.F., Using the Students t-Test with Extremely Small Sample Sizes. Practical Assessment, Research & Evaluation, 2013. 18(10).
9. Alexandra Kuznetsova, Per Bruun Brockhoff and Rune Haubo Bojesen Christensen (2016). lmerTest: Tests in Linear Mixed Effects Models. R package version 2.0-33. https://CRAN.R-project.org/package=lmerTest

7 Clean Data Tables

7.1 Murine Animal Model Data

Table 7.1: Cleaned data used for murine animal model study.

Day	Sample	Tx	NGAL	KIM1	CD13	CD10	CD26	Trichrome	PPG	PTD	GD	TUNEL
2	1	L	16.28464		19.17564	5.907863	172.2124	0.388184	79.16667	0.016479	0.055794	0.042817
2	2	L	119.3126	41.89399	217.0181	37.63961	105.8383	0.058196	80	0.015667	0.06056	0.028251
2	3	L						0.042208	73.33333	0.013198	0.061645	0.028245
2	4	L						0.159239	74.46809	0.015088	0.063107	0.012132
2	1	UL	16.59629	10.52212	20.40063	7.591656	193.8197	0.070986	83.33333	0.017186	0.062711	0.040198
2	2	UL	2.193224	0.739012	6.137465		181.7809		95	0.016667	0.063467	0.013686
2	3	UL	7.047489	2.019812	17.80114				87.23404	0.014733	0.061037	0.02956
2	4	UL	8.240868		27.20539			0.056277	55.26316	0.014579	0.062095	0.022437
3	1	L	37.08096	15.93897	208.5467	12.34244	2151.132	0.102962	66.66667	0.012961	0.059577	0.117675
3	2	L	16.55341		141.268	2.238635	1301.096	0.208161	74.28571	0.011986	0.056815	0.650304
3	3	L	9.5393		3.162459			0.166913	75	0.011947	0.057923	0.2424
3	4	L	3.563585					0.202512	73.33333	0.015718	0.056342	
3	1	UL	8.949155	2.120117	20.33777		103.8271	0.060754	78.78788	0.013392	0.067778	0.029159
3	2	UL	7.060155	2.233234	24.36715	49.80104	87.33597	0.083776	77.77778	0.01327	0.059633	0.01021
3	3	UL	2.962961	0.042881	4.018085	4.494614		0.147088	86.66667	0.015037	0.070576	0.01259
3	4	UL	14.00694			2.337427		0.074184	82.5	0.012701	0.063909	0.020387
4	1	L	9.583429		50.93198		1698.966	0.332803	62.06897	0.016351	0.051037	0.896009
4	2	L	9.94357		83.26117		976.3892	0.253247	68	0.017132	0.062731	2.090032
4	3	L	8.400794	13.90895	22.39525	86.32619	1465.307	0.355463	55.88235	0.015952	0.057826	3.658679
4	4	L	11.09126			89.57775	1123.57	0.43359	70.37037	0.013013	0.061417	0.740973
4	1	UL	9.625606	0.110433	9.402009	114.2932	32.01665	0.139414	77.77778	0.013221	0.069308	0.031506
4	2	UL	6.157225	2.251117	15.85151	120.9203	12.32457	0.132059	93.54839	0.016165	0.058674	0.082687
4	3	UL	6.048851	1.882941	14.66411	60.50397	882.393	0.109357	80	0.015386	0.061923	0.015708
4	4	UL	6.845024			12.84429	345.4728	0.159239	85.18519	0.013911	0.064697	
5	1	L	13.28686	21.91567	47.85022	97.3876	974.7706	0.504416	64.70588	0.023925	0.058174	1.417555
5	2	L	16.45585	39.9746	52.04353	141.8407	2113.052	0.593628	42.85714	0.02432	0.0585	4.67033
5	3	L	15.04944	24.91568	48.33438	158.092	805.4928	0.268116	60	0.016827	0.054824	1.802334
5	4	L	12.99016			82.7636	1334.882	0.298013	42.85714	0.018266	0.0554	0.825796
5	5	L	14.86628									
5	6	L	12.2822									
5	1	UL	1.787073	0.818611	31.82946	89.30498	250.9541	0.073544	90	0.015633	0.0644	
5	2	UL	7.996205		1431.244	85.26763	168.6103		77.77778	0.016063	0.06364	0.015266
5	3	UL	2.49209		12.34136	127.4815	338.8773	0.05308	83.67347	0.014671	0.060538	0.024806
5	4	UL	3.660448			45.33415	24.27742	0.149007	88.88889	0.019575	0.066609	0.009588
5	5	UL	1.675087									
5	6	UL	3.903723									

7	1	L	14.60905	27.27154	52.64234	63.08762	948.4227	0.709007	17.94872	0.025162	0.054231	2.519059
7	2	L	18.33231	47.94893	75.86643	92.95507	445.6144	1.715685	40	0.02754	0.053159	3.816598
7	3	L	4.710246	7.06858	40.30858	87.00018	840.3242	1.204075	39.02439	0.023389	0.053485	6.308345
7	4	L	9.525538			111.9518	1155.908	4.350926	52.38095	0.028109	0.053939	0.595745
7	5	L	10.23885									
7	6	L	17.2886									
7	1	UL	3.783591	0.031258	10.60094	16.38368	89.4902	0.14453	84.84848	0.015329	0.06548	0.032255
7	2	UL	35.41884	0.865871	21.89262	18.28278	217.6204		80.95238	0.019582	0.058333	0.010787
7	3	UL	1.763975	0.170299	18.41144	34.20145	102.8651		71.42857	0.018553	0.056321	0.027437
7	4	UL	5.358177			74.37522	35.8099	0.122786	72	0.020145	0.063	
7	5	UL	3.587321									
7	6	UL	3.985335									
10	1	L	17.77472	25.38217	59.82246	226.0923	996.4028	33.38705	50	0.019373	0.049857	59.31406
10	2	L	31.89781	39.42137	115.5177	104.5474	1117.339	52.97111	18.18182	0.029343	0.046036	23.61717
10	3	L	270.4397	3.91244	493.0896	126.8331	708.0176	32.2217	21.56863	0.035425	0.048649	25.22984
10	4	L	15.33438	37.12438	73.61362	113.2134	731.7444	49.54934	13.63636	0.033425	0.043704	85.1245
10	5	L	45.75411									
10	6	L	10.80205									
10	1	UL	3.083772		22.47939	62.74	34.77489		80.76923	0.018316	0.053517	0.044551
10	2	UL	2.228493		18.96808	68.5004	144.2897	0.37646	56.75676	0.019158	0.056	0.044719
10	3	UL	4.517244		10.66497	74.66811	92.35815	0.121507	74.19355	0.016662	0.056147	0.022758
10	4	UL	1.469772	0.322207	4.6859	51.46267	143.9203		80.55556	0.019143	0.053848	0.031305
10	5	UL	4.163486									
10	6	UL	2.011246									

7.2 Human Analysis Data

Table 7.2: Cleaned data used for human analysis.

Pt_Type	Pt_ID	NGAL	KIM1	CD13	CD10	CD26	Sex
Ctrl	21	55.82375	10.145	1.870833	2.624472	116.9375	M
Ctrl	22	19.01167	4.927741	1.161111	2.403519	93.86667	M
Ctrl	23	29.75051	1.218354	1.091139	2.044228	67.92532	M
Ctrl	24	27.71359	13.36505	0.814563	1.514874	63.03204	F
Ctrl	25	191.1914	12.75109	2.254688	3.830969	151.3602	F
Ctrl	26	14.33121	5.008889	1.186869	1.362848	130.3303	F
Ctrl	27	77.73309	1.068022	0.47518	1.281475	33.43921	M
Ctrl	28	16.00969	4.756875	0.853906	1.861203	52.8625	M
Ctrl	29	100.1384	1.473616	1.29726	2.741178	91.57945	F
Ctrl	32	154.4282	0.464197	0.238028	1.445634	48.32254	F
Ctrl	45	37.21419	2.792323	1.358065	10.56594	82	F
Ctrl	46	74.86727	8.980273	1.186364	4.153509	155.0764	F
UPJO	20	28.88621	5.471931	3.010345	5.768966	181.131	F
UPJO	30	11.93167	7.532524	1.640476	3.455714	113.1714	M
UPJO	31	67.68848	8.922485	45.51212	14.04855	759.1576	M
UPJO	33	118.555	3.5415	0.6175	6.38795	82.0425	M
UPJO	34	19.33333	3.479833	20.88333	19.7065	1229.167	M
UPJO	36	262.998	3.617961	1.129412	2.615922	154.1882	M
UPJO	37		0.838857	10.01429	29.05086	290.3286	M
UPJO	38	10.62719	0.163754	0.247368	3.139018	26.84386	M
UPJO	39	76.5885	2.295	30.495	20.5925	8188.84	M
UPJO	40	6.146429	3.118405	2.415476	3.215548	125.9417	M
UPJO	41		6.104065	2.187097	5.930903	224.4677	F
UPJO	42	2574.209	7.464178	0.604459	1.010153	99.7	F
UPJO	44	9587.328	10.5014	31.1375	9.8939	1398.268	F
UPJO	47		3.896455	1.714545	3.192909	198.4	F
UPJO	57	0	0.019059	0.015802	1.006109	3.473515	M
UPJO	56	1.205136	0.240773	0.020545	4.396591	4.171	F
UPJO	58	7.538	0.322048	0.041952	5.589714	6.824333	M
UPJO	61	1.085044	0.142189	0.006433	0.780711	2.251744	F
UPJO	65	1.2197	0.098	0.1869	2.6179	13.7711	M
UPJO	66	0	0.042422	0.007016	0.001	0.708281	M
UPJO	64	0.267519	0.052308	0.009019	0	2.085058	F
UPJO	60	1.256	0.182879	0.470182	1.685333	207.6241	M
UPJO	62	0	0.038	0.5148	20.41	197.7416	M
UPJO	68	0.158315	0.018074	0.003481	0.287722	1.140491	M
UPJO	59	0	0.012667	0.052267	3.737467	9.802867	M
UPJO	69	0.152909	0.00897	0.252879	0.685409	2.876121	M
UPJO	70	10.91064	0.068694	0.048222	0.198806	2.311306	F
UPJO	71	0	0.059615	0.023346	0.082231	2.188385	M
UPJO	73	0.48075	0	0.0895	0	11.0495	M

8 Boxplots by Day

8.1 Biomarker Boxplots

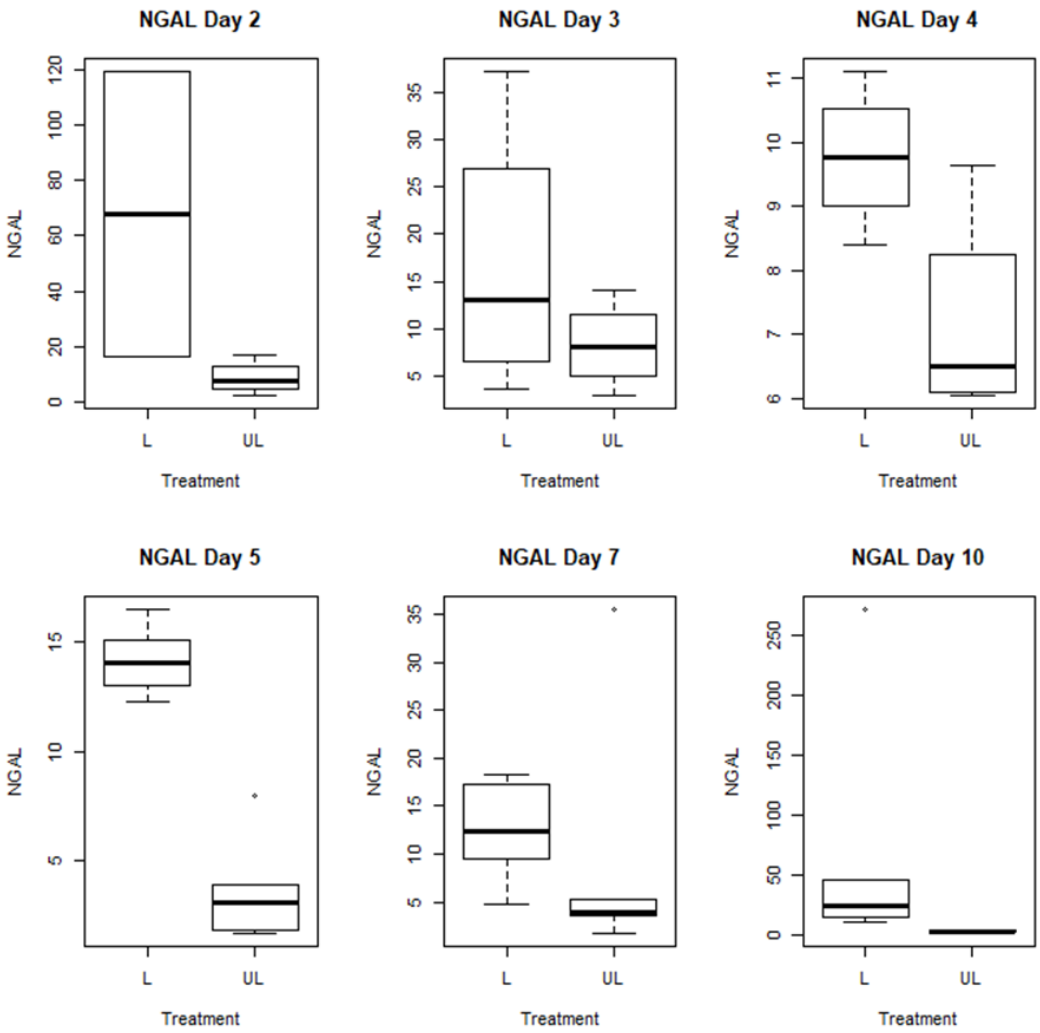

Figure 8.1: Boxplots of NGAL by day.

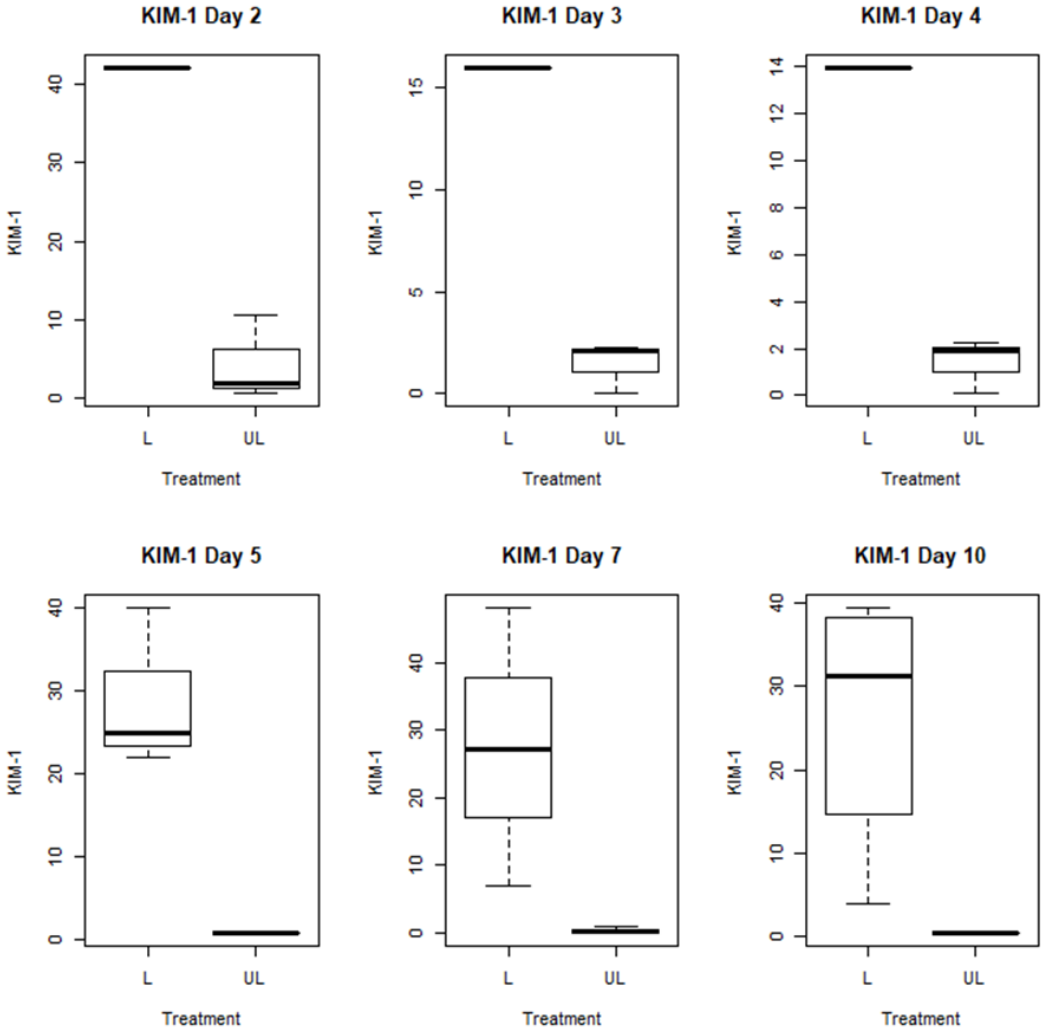

Figure 8.2: Boxplots of KIM-1 by day.

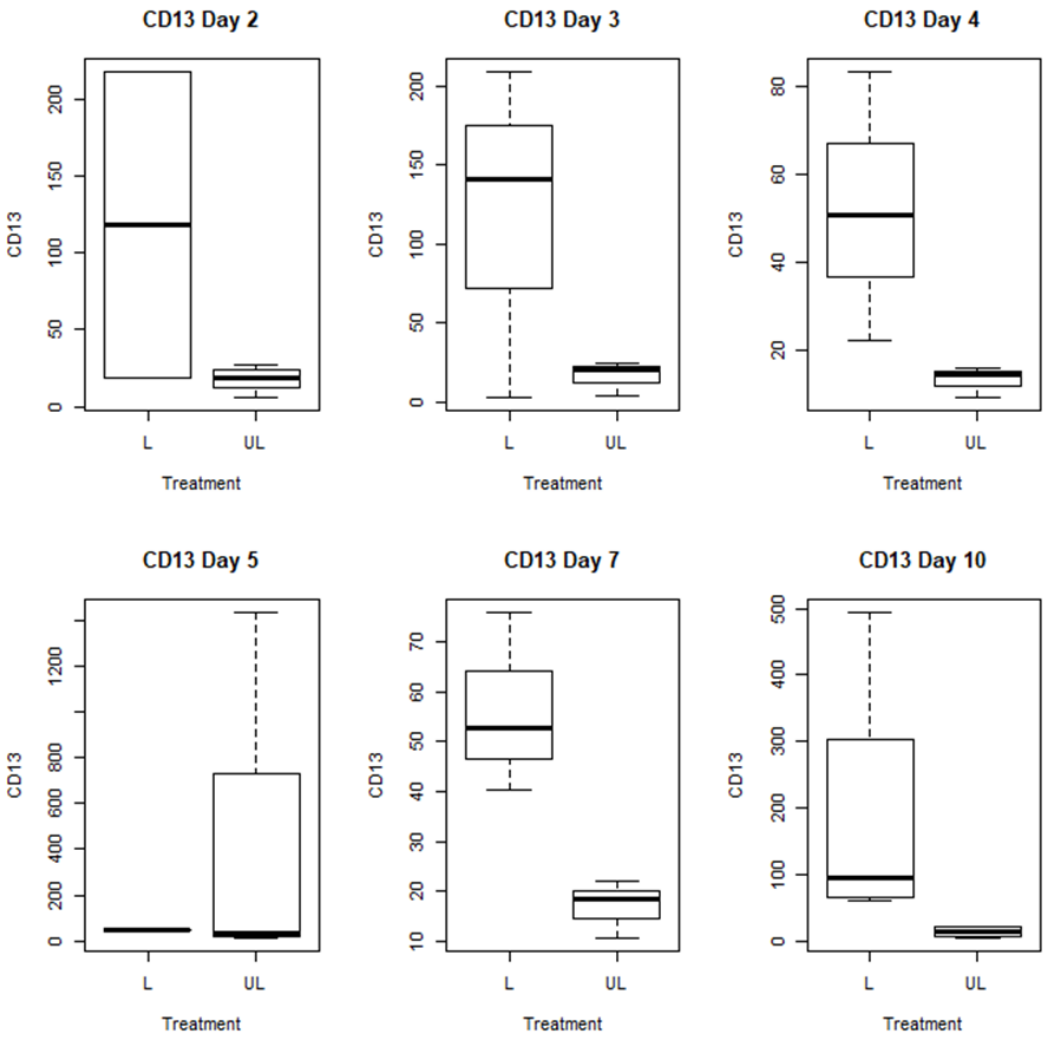

Figure 8.3: Boxplots of CD13 by day.

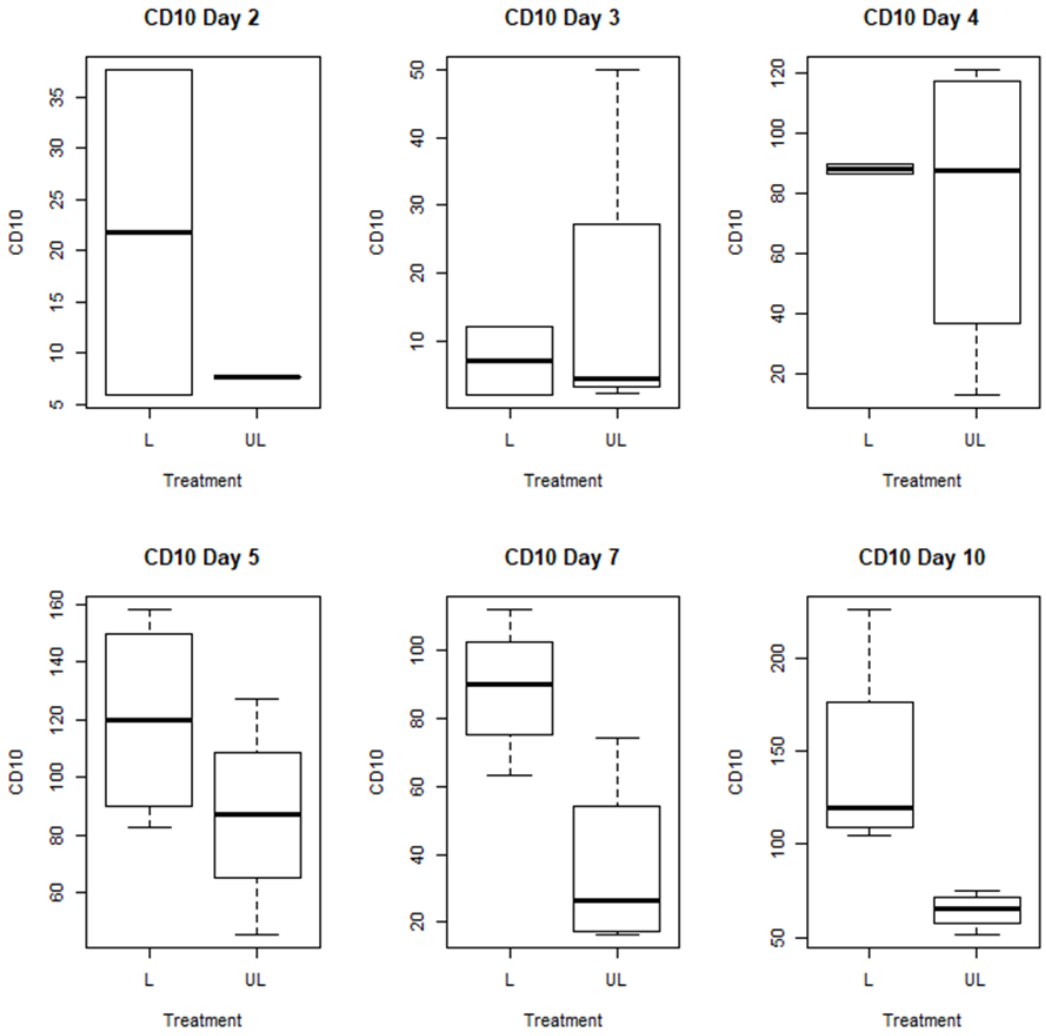

Figure 8.4: Boxplots of CD10 by day.

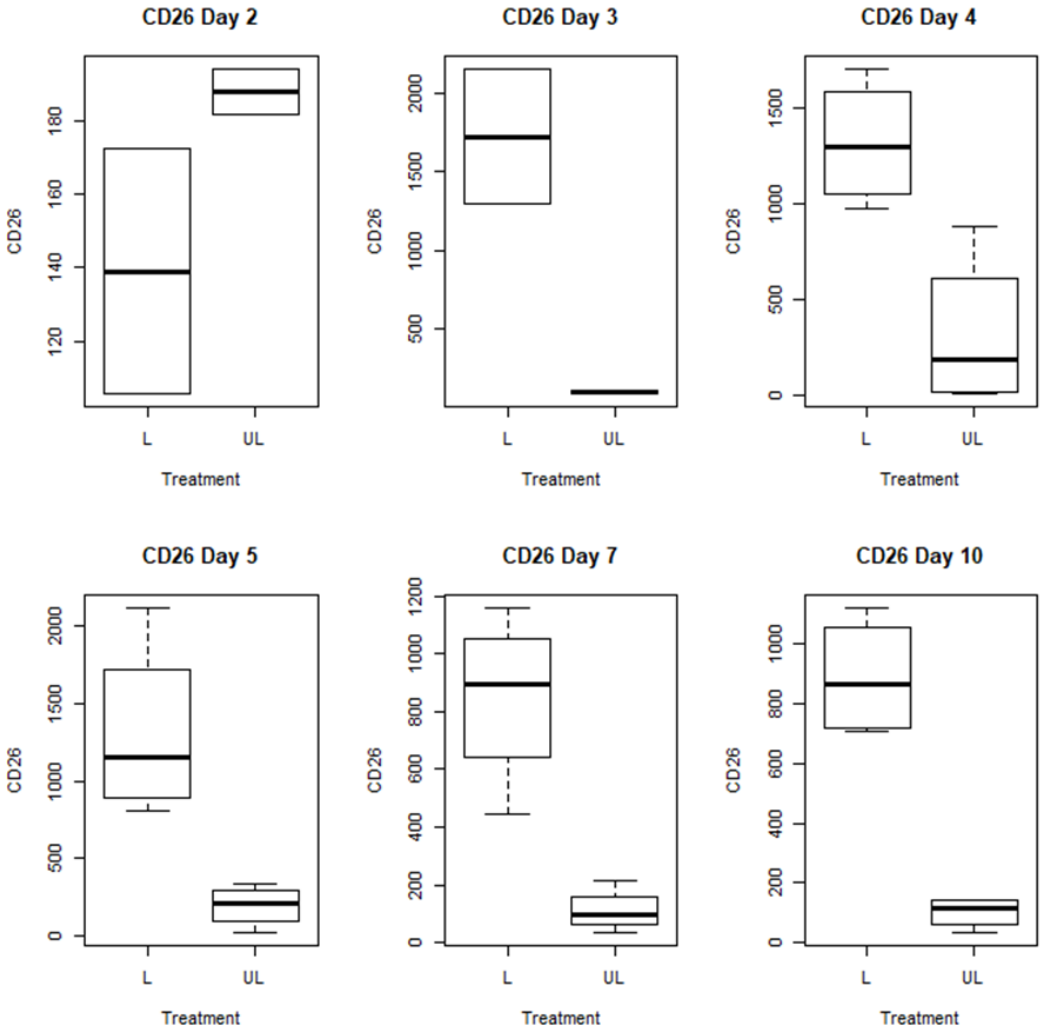

Figure 8.5: Boxplots of CD26 by day.

8.2 Tissue Damage Boxplots

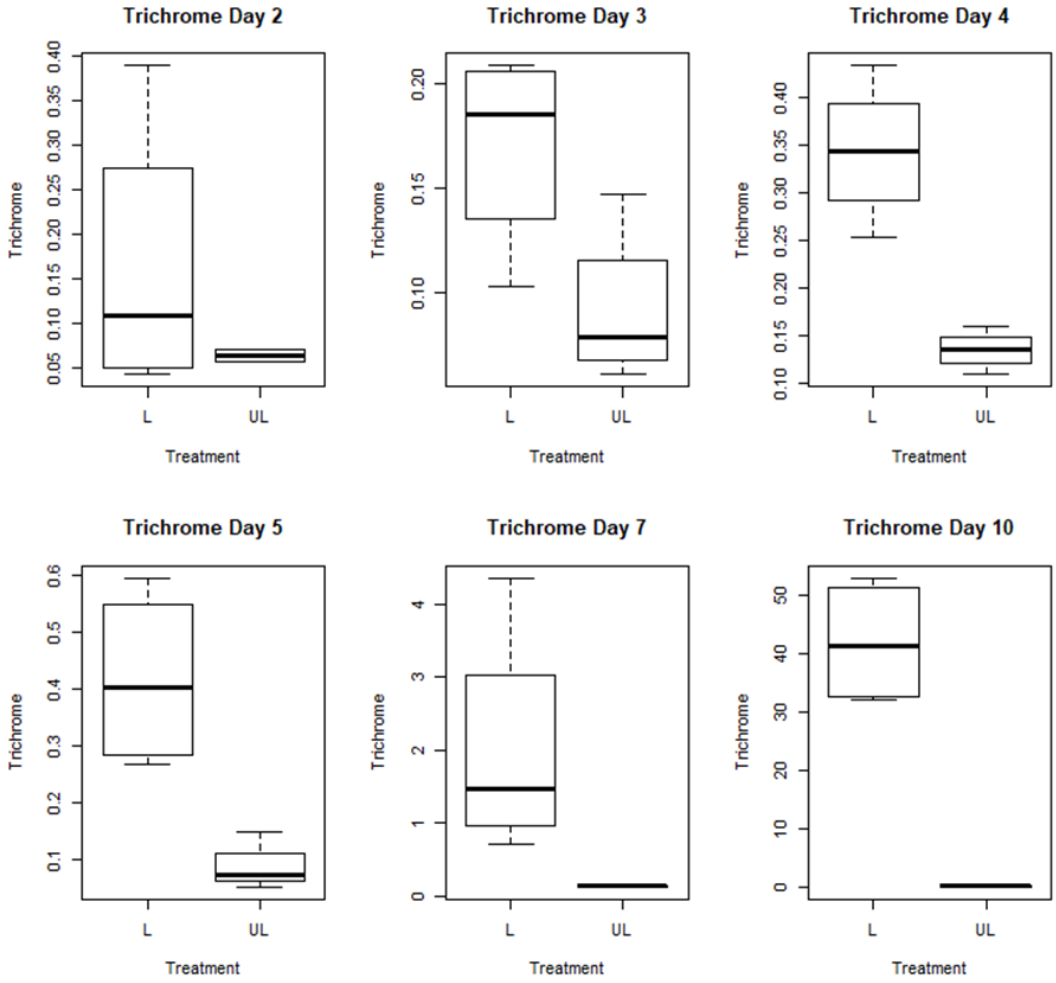

Figure 8.6: Boxplots of trichrome by day.

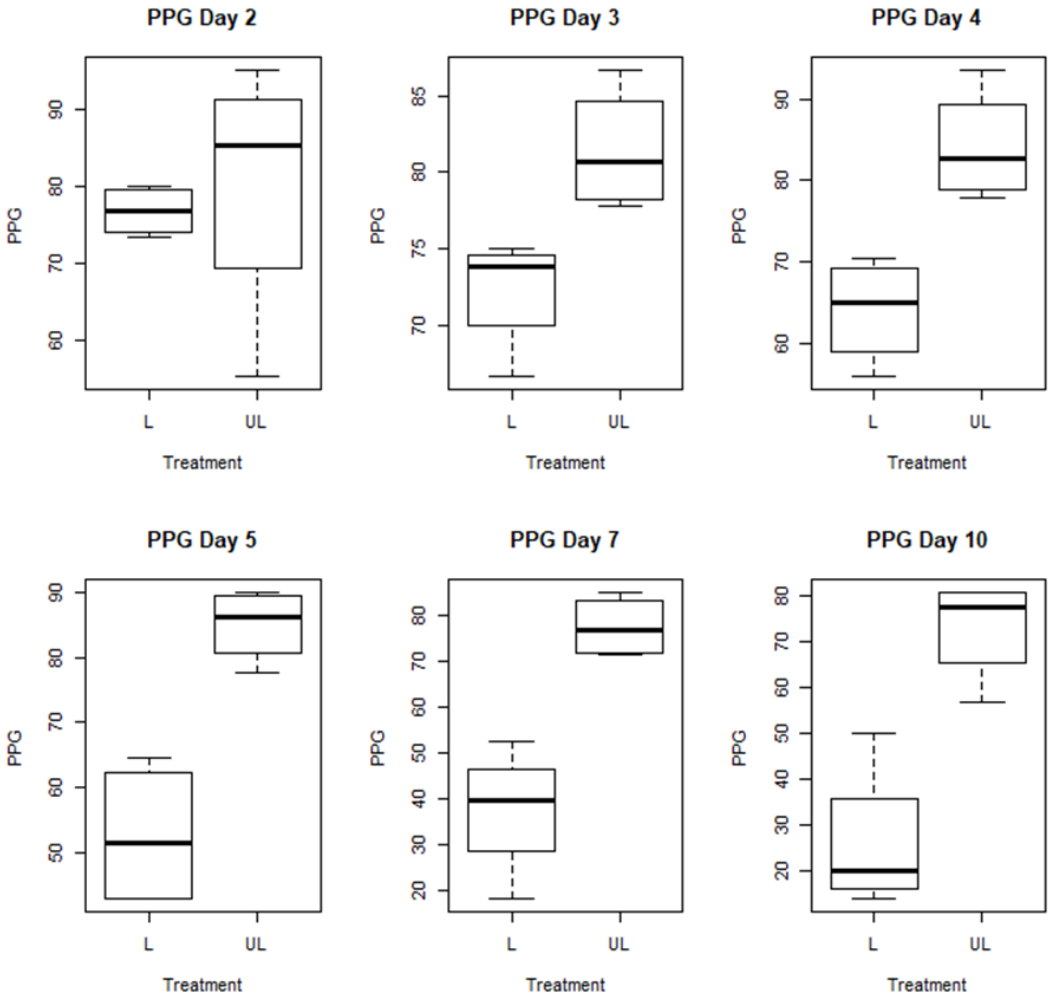

Figure 8.7: Boxplots of PPG by day.

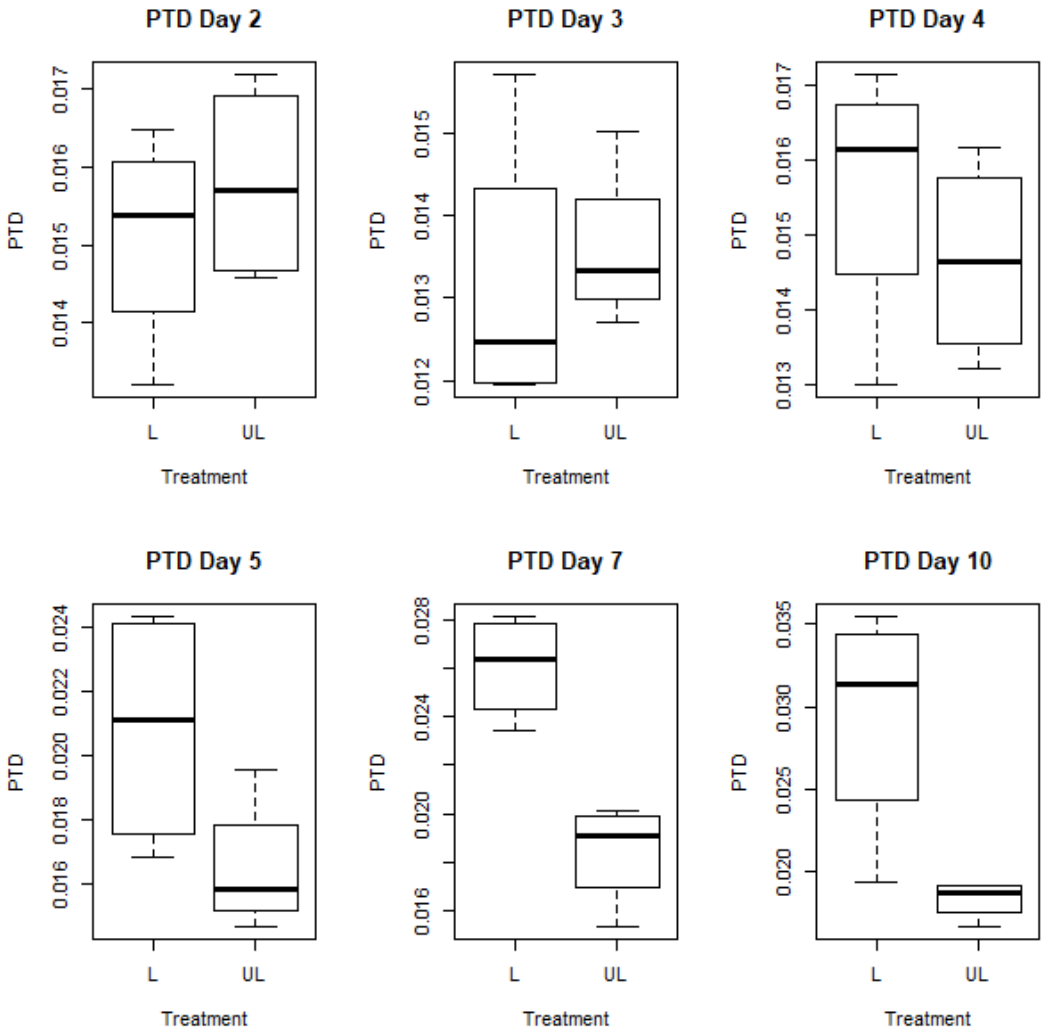

Figure 8.8: Boxplots of PTD by day.

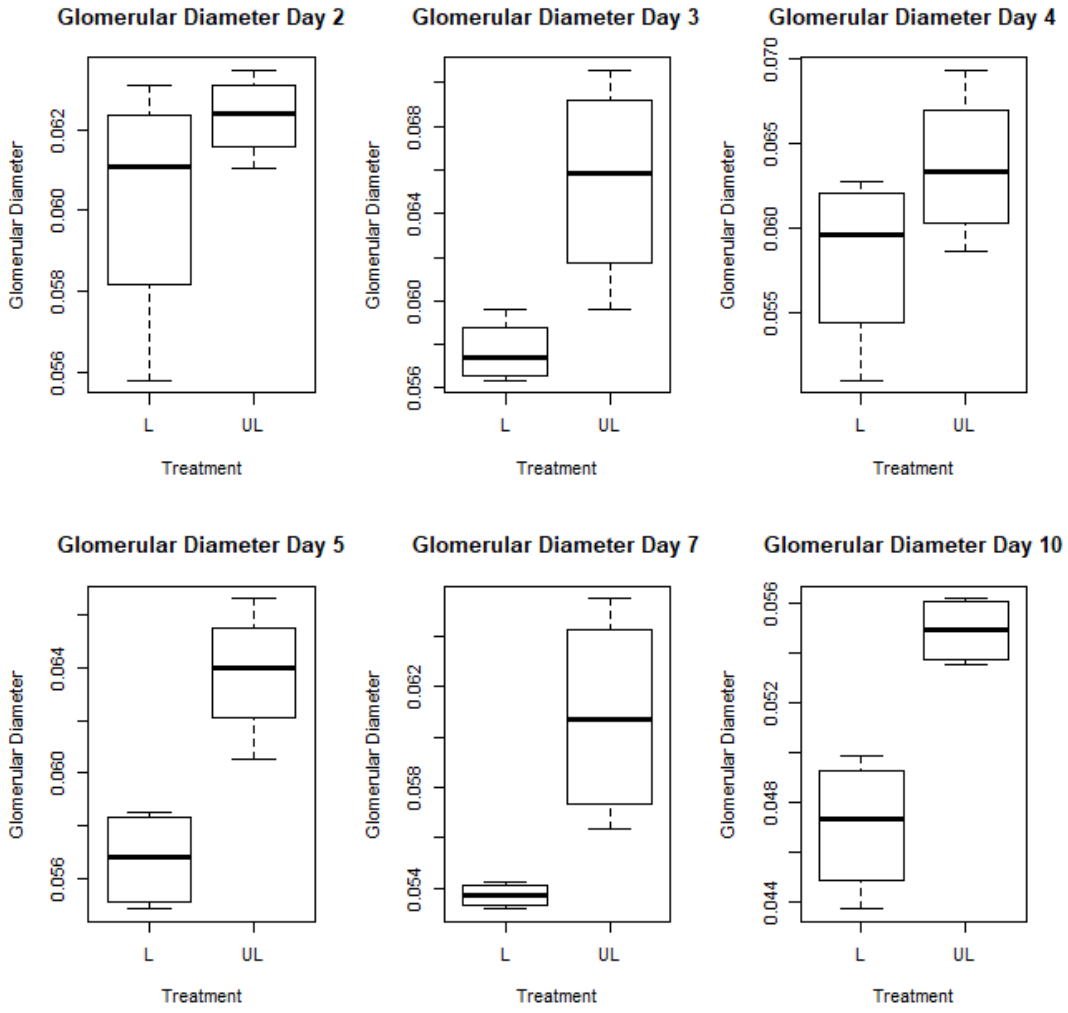

Figure 8.9: Boxplots of GD by day.

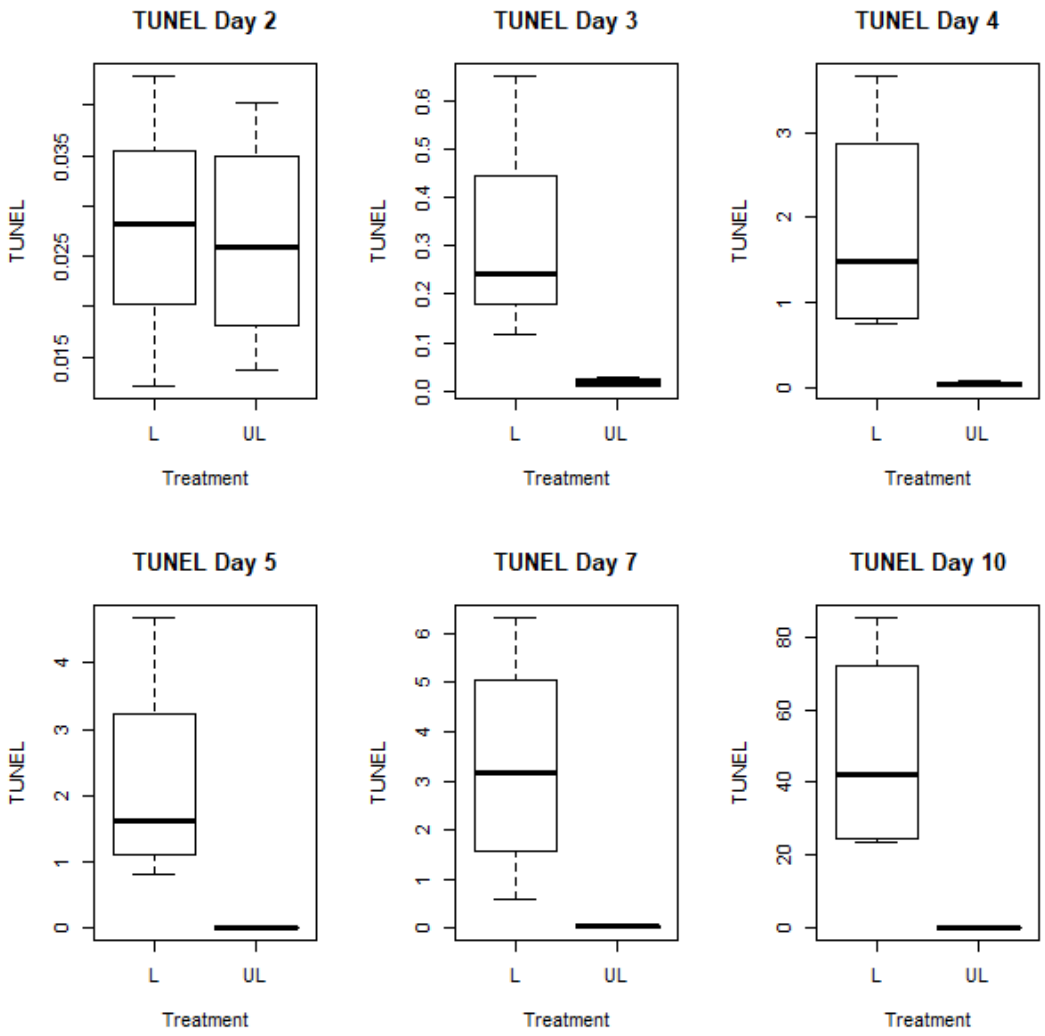

Figure 8.10: Boxplots of TUNEL by day.

9 Summary Statistics for Murine Study Data

9.1 Biomarker Tables

Table 9.1: Summary statistics for L data.

Day	**Biomarkers**					
		NGAL	**KIM1**	**CD13**	**CD10**	**CD26**
2	Mean	67.8	41.9	118	21.8	139
	Variance	5307	-	19571	503	2203
3	Mean	16.7	15.9	118	7.29	1726
	Variance	213	-	10964	51.0	361280
4	Mean	9.75	13.9	52.2	88.0	1316
	Variance	1.23	-	927	5.29	107107
5	Mean	14.2	28.9	49.4	120	1307
	Variance	2.44	93.6	5.26	1275	337461
7	Mean	12.5	27.4	56.3	88.7	847
	Variance	27.2	418	326	406	88955
10	Mean	65.3	26.5	185	142	888
	Variance	10261	264	42607	3177	40385
Overall	Mean	28.3	26.7	100	91.1	1058
	Variance	2718	195	13114	3107	288089

Table 9.2: Summary statistics for UL data.

Day	**Biomarkers**					
		NGAL	**KIM1**	**CD13**	**CD10**	**CD26**
2	Mean	8.52	4.43	17.9	7.59	188
	Variance	35.8	28.3	77.1	-	72.5
3	Mean	8.24	1.47	16.2	18.9	95.6
	Variance	21.0	1.52	116	718	136
4	Mean	7.17	1.41	13.3	77.1	318
	Variance	2.81	1.31	11.8	2569	164839
5	Mean	3.59	0.819	492	86.8	196
	Variance	5.53	-	662005	1128	17891
7	Mean	8.98	0.356	17.0	35.8	111
	Variance	169	0.020	33.4	725	5850
10	Mean	2.91	0.322	14.2	64.3	104
	Variance	1.51	-	64.8	97.5	2715
Overall	Mean	6.29	1.72	87.2	56.0	174
	Variance	43.2	7.19	100142	1525	37211

9.2 Tissue Damage Tables

Table 9.3: Summary statistics for L data.

Day	**Tissue Damage**					
		Trichrome	**PPG**	**PTD**	**GD**	**TUNEL**
2	Mean	0.162	76.7	0.015	0.060	0.028
	Variance	0.025	11.1	0.000002	0.00001	0.0002
3	Mean	0.170	72.3	0.013	0.058	0.337
	Variance	0.002	14.7	0.000003	0.000002	0.078
4	Mean	0.344	64.1	0.016	0.058	1.85
	Variance	0.006	42.1	0.000003	0.00003	1.82
5	Mean	0.416	52.6	0.021	0.057	2.18
	Variance	0.025	130	0.00001	0.000003	2.92
7	Mean	1.99	37.3	0.026	0.054	3.31
	Variance	2.64	204	0.000005	0.0000002	5.75
10	Mean	42.0	25.8	0.029	0.047	48.3
	Variance	116	270	0.00005	0.000008	873
Overall	Mean	7.52	54.8	0.020	0.056	9.73
	Variance	264	439	0.00005	0.00003	450

Table 9.4: Summary statistics for UL data.

Day	**Tissue Damage**					
		Trichrome	**PPG**	**PTD**	**GD**	**TUNEL**
2	Mean	0.064	80.2	0.016	0.062	0.026
	Variance	0.0001	300	0.000002	0.000001	0.0001
3	Mean	0.091	81.4	0.014	0.065	0.018
	Variance	0.001	16.3	0.000001	0. 00002	0.00007
4	Mean	0.135	84.1	0.015	0.064	0.043
	Variance	0.0004	49.1	0.000002	0.00002	0.001
5	Mean	0.092	85.1	0.016	0.064	0.017
	Variance	0.003	31.3	0.000005	0.000006	0.00006
7	Mean	0.134	77.3	0.018	0.061	0.023
	Variance	0.0002	44.3	0.000005	0.00002	0.0001
10	Mean	0.249	73.1	0.018	0.055	0.036
	Variance	0.033	128	0.000001	0.000002	0.0001
Overall	Mean	0.122	80.2	0.016	0.062	0.027
	Variance	0.006	91.6	0.000005	0.00002	0.0003