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## ABSTRACT

### PATHWAY GROUP LASSO FOR INTEGRATING METABOLOMICS AND TRANSCRIPTOMICS

by

SOPHIA A. BANTON

JUNE 17, 2015

**INTRODUCTION:** Transcriptomics and metabolomics are high-throughput technologies that are critical to contemporary biomedical sciences, measuring gene expression levels and metabolite concentrations, respectively. Effective methods of integrating metabolomics and transcriptomics data are highly desired. Gene and metabolic pathways represent accumulated expert knowledge in particular domains. LASSO regression is widely used for feature selection, and group LASSO incorporates prior knowledge of groups of variables.

**AIM:** To address the current need to integrate the two data types, a novel approach in the framework of group LASSO was developed and tested using a set of metabolomics and transcriptomics data on malaria intermittent preventative treatment with pyrimethamine in Rhesus macaques (*Macaca mulatta*).

**METHODS:** Groups are predefined using biological pathways and variables in groups will be standardized separately. The leading principal components were obtained for each pathway for each of the two data types, and then combined into an integrated matrix, which together with the group information served as input for a group LASSO regression model.

**RESULTS:** We identified multiple pathways that were top contributors to the differences due to pyrimethamine exposure in the macaques and jointly predicted the association of member genes and metabolites to plasma hemoglobin levels.

**DISCUSSION:** By applying this integration approach via group LASSO, we identified multiple pathways that are top contributors to the differences due to pyrimethamine exposure in the macaques and jointly predicted the association of member genes and metabolites to plasma hemoglobin levels. Our findings are consistent with current literature, and provide high-quality mechanistic hypotheses. Pathway group LASSO is thus a novel and effective method of integrating metabolomics and transcriptomics data.

PATHWAY GROUP LASSO FOR INTEGRATING METABOLOMICS AND TRANSCRIPTOMICS

by

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B.S., GEORGIA STATE UNIVERSITY  
M.S., GEORGIA STATE UNIVERSITY

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of the  
Requirements for the Degree

MASTER OF PUBLIC HEALTH

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APPROVAL PAGE

PATHWAY GROUP LASSO FOR INTEGRATING METABOLOMICS AND TRANSCRIPTOMICS

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Author's Statement Page

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\_\_\_\_ Sophia A. Banton \_\_\_\_\_

Signature of Author

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## **Introduction:**

Globally, malaria continues to be a major public health concern accounting for three to five hundred million infections and twenty-percent of all childhood deaths annually<sup>1</sup>. Historically, anti-malarial pharmaceutical usage has been largely centered on methods of chemoprophylaxis with chloroquine. However, chloroquine resistance in malaria parasites and poor adherence among drug recipients led to poor effectiveness. To overcome this hurdle, intermittent preventative treatment (IPT) was introduced in the early part of the twenty-first century to replace chemoprophylaxis. Recommendations for its use by the World Health Organization (WHO)<sup>2,3</sup> and the single dose advantage of IPT with pyrimethamine have led to its use as the primary form of IPT in regions in which malaria is endemic<sup>4</sup>.

The Malaria Host-Pathogen Interaction Center<sup>5</sup> has developed the malaria IPT model in Rhesus macaques, and generated detailed data using transcriptomics and metabolomics. Transcriptomics is an established scientific methodology that examines the global expression level of mRNAs using either DNA microarrays or massively parallel sequencing. Metabolomics is an emerging field, where high performance mass spectrometry is used to profile small molecules (metabolites) in biological samples. Statistical analyses of the data produced by both methods typically lead to the identification of genes and metabolites that are associated with the outcome or exposure of interest. Gene and metabolic pathways represent accumulated expert knowledge in particular domains, pivotal to the interpretation of these data. The integration of transcriptomics and metabolomics at pathway level is thus highly desired in many research projects. Thus, we sought to identify key features across both the transcriptome and metabolome that can be used to predict host response to pyrimethamine and specifically its impact on plasma hemoglobin levels.

Within the framework of this study, data integration is taken to mean the process by which multiple types of omic data are combined as predictor variables in statistical models to allow more systematic and complete modelling of multifactorial traits or phenotypes. The reasoning behind such a definition is the supposition that these non-Mendelian traits reflect an intricate interplay in biological system variation at multiple levels of regulation<sup>6</sup>. Thus, the ability to identify significant host factors and their connections that explain or predict biological predisposition to disease or clinical outcome is the primary motivation of omic integration<sup>6</sup>.

Here, we report a novel approach of omic data integration in a framework of group LASSO (least absolute shrinkage and selection operator) regression, and demonstrate its application using a set of metabolomics and transcriptomics data on malaria intermittent preventative treatment with pyrimethamine in Rhesus macaques (*Macaca mulatta*). LASSO regression is widely used for feature selection, and group LASSO incorporates prior knowledge of group information. Groups were predefined using biological pathways and standardized separately. Using this technique, we demonstrate that the significant biological pathways detected by a metabolomics and transcriptomics study can be successfully integrated to identify key genes and metabolites that regulate hemoglobin plasma levels following pyrimethamine exposure.

## **Materials and Methods:**

### **Animals**

Five rhesus macaques (*Macaca mulatta*; age, 2 y.), all males, from the Yerkes National Primate Center were selected for study and each primate was exposed to all treatment conditions in a crossover experimental design. The primates were housed at the Yerkes National Primate Center and maintained in accordance with the Emory University Institutional Animal Care and

Use Committee (IACUC). The study design and the husbandry of the animals have been described elsewhere<sup>7</sup>. Briefly four monkeys were housed in pairs and a fifth monkey was housed alone. These monkeys were followed over a 100 day time course; during which samples for clinical and omic measurements were collected daily.

Study design was to collect samples 1) baseline upon entry into the study, 2) baseline after 20 days, 3) after primary pyrimethamine exposure on day 27, 4) after primary pyrimethamine injection and before secondary pyrimethamine injection on day 52, 5) after secondary pyrimethamine exposure on days 59, 6) after secondary pyrimethamine injection and before tertiary pyrimethamine injection on day 90, and 7) after tertiary pyrimethamine injection on day 98. Pyrimethamine (1 mg/kg) was delivered intramuscularly once on day 20 and for three successive days beginning at days 52 and 90. This constitutes two pre-drug time points (TP1 and TP2), two inter-drug time points (TP4 and TP6), and three post-drug time points (TP3, TP5, and TP7). Samples for transcriptomics analysis were collected at time points 1-7, while samples for metabolomics were collected at time points 3-7.

### **Transcriptomics**

Peripheral blood (PB) samples from each time were point processed and analyzed using a previously described protocol<sup>7</sup>. Briefly, RNA was isolated using a RNeasy minikit from Qiagen. RNA samples (1 µg total RNA) were delivered to the Yerkes National Primate Center Genomics Core, where samples were assessed for quality and prepared for gene expression array analysis as per manufacturer protocol.

### **Metabolomics**

Plasma samples were collected with EDTA after sedation with 10 mg/ml of ketamine given by intramuscular (*i.m.*) injection. Samples were processed and analyzed using a previously described protocol<sup>8</sup>. Briefly, each biological sample was run in triplicate using a 10 µl injection volume with separation by C18 reverse phase chromatography (Higgins Analytical, Targa, 2.1 x 10 cm) using an acetonitrile gradient<sup>8</sup> with electrospray ionization and detection with a Thermo Scientific LTQ-Velos Orbitrap mass spectrometer of  $m/z$  85 to 2000 at 60,000 resolution.

Following liquid chromatography-high resolution mass spectrometry (LC-MS), the data were collected and pre-processed using the XCalibur file converter software. apLCMS<sup>9</sup> and xMSAnalyzer<sup>10</sup> were used for feature detection and extraction. A metabolic feature was defined as a specific mass-to-charge ratio ( $m/z$ ) along with its retention time and associated ion intensity; 14,339 features were detected. Data were log<sub>2</sub> transformed and subjected to standard quality assessment including exclusion of data for technical replicates with greater than thirty percent missing values. For all further analyses, the median of the three technical replicates of each metabolic feature were used.

## **Statistical Analysis**

Statistical analysis was completed in two stages with the primary stage being used for exploratory data analysis. First, Spearman correlation was used to identify features that are associated with hemoglobin levels in the metabolome and transcriptome independently. Then, a mixed-effects ANOVA model was used to identify the genes and metabolites that are differentially expressed across the experimental conditions for each data type. The results of the ANOVA were then subjected to principal component analysis (PCA) to observe whether or not the transcriptomes and metabolomes were correlated across the experimental conditions.

Statistical significance was determined at  $p < 0.05$  for all tests. In the second stage of analysis, a group LASSO regression model was constructed to integrate the two data types by identifying the differences across experimental conditions at the pathway level, and jointly predict the association of the selected pathways to plasma hemoglobin levels.

For Spearman correlation, each feature for each primate at each time point was correlated against the hemoglobin levels recorded for the primate at the same time point. The mixed-effects ANOVA model was constructed using drug-exposure as the fixed effect and animal as the random effect. Drug exposure was treated as a predictor with three levels that reflected pre-drug (TP1 and TP2), inter-drug (TP4 and TP6) and post-drug (TP3, TP5, and TP7) exposures. Following feature selection with ANOVA, the top ten principal components of each platform were subjected to Pearson correlation ( $p < 0.05$ ). These primary analyses were used to establish whether the data set was suitable for integration with LASSO regression.

In order to remove bias from analytical platforms for group LASSO regression, gene expression and metabolite data were standardized separately. The groups of genes and metabolites were defined based on a collection of metabolic pathways. PCA was performed on each pathway of genes or metabolites separately for the data with subjects in drug exposure groups (inter-drug vs. post-drug). The pre-drug measurements were not used because samples were not available for metabolomic data. Filtering by variance explained from PCA reduced the usable number of pathways from 64 to 38. The leading principal components for each pathway were then combined into an ordered integrated matrix, in which rows contain principal components from both the transcriptome and the metabolome *per* subject. These principal component scores are used as new predictors in a group LASSO regression with groups defined by pathways. With group LASSO, the coefficients of group members are either all reduced to

zero or retain non-zero coefficients. Each integrated pathway of genes and metabolites was assigned a chronological number, and all 38 groups were used to test for the association of each pathway with plasma hemoglobin levels. Since a single model is built for the integration of all pathways using this approach, this method answers the question of which biological pathways best predict pathway level association with the clinical outcome. All statistical analyses were conducted and all figures were generated using R.

## Results

### **Features from both the metabolome and transcriptome are associated with plasma hemoglobin levels following pyrimethamine exposure**

Spearman correlation determined that there were 1,074 genes from the whole blood transcriptome and 305 metabolic features from the plasma that were correlated with plasma hemoglobin levels in the macaques for time points 3 to 7 ( $p < 0.05$ ) (Figure 1). The mixed effects ANOVA model, in which drug effect was the fixed term and animal was the random term, determined that 925 genes and 1,660 metabolites were differentially expressed across the treatment conditions ( $p < 0.05$ ) (Figure 2). Pearson correlation of the top ten principal components of the significant features of each data type showed that subsets of features are potentially co-regulated within the biological system (Figure 3).

### **Grouping by LASSO provides pathway level information and reduces the number of significant features**

The group LASSO regression model identified eight pathways associated with hemoglobin levels in macaques (Table 1). The number of genes and metabolites present in these pathways were 182 and 52 respectively. These pathways involve porphyrin and chlorophyll metabolism, redox metabolism, branched-chain amino metabolism, and lipid metabolism.

Multiple vitamin pathways were also selected for association with plasma hemoglobin levels by the group LASSO model including vitamin A (retinol) and vitamin C.

To verify the findings of the group LASSO method, the porphyrin and chlorophyll metabolism pathway was selected for further inspection, because heme itself is one type of porphyrin. From this pathway, the most significant gene in terms of correlation with hemoglobin and differential expression between inter and post-drug exposures was the ALAS2 gene (Pearson's  $r = -0.53$ ,  $p = 0.0069$ ;  $t = -2.225$ ,  $p = 0.0365$ ) (Figure 4). The end product of the ALAS2 gene, aminolevulinic acid ( $m/z = 154.0472$ ) was also selected by the group LASSO model as a member of the porphyrin metabolism pathway (Pearson's  $r = 0.43$ ,  $p = 0.0333$ ;  $t = 3.98$ ,  $p = 0.0010$ ) (Figure 4). This metabolite was also significantly correlated with hemoglobin and differentially expressed between inter and post-drug exposures (Figure 4). Additional genes involved in heme synthesis (ALAD and FECH) from the porphyrin pathway were selected by LASSO, and these genes were both significantly correlated with hemoglobin (ALAD - Pearson's  $r = 0.48$ ,  $p = 0.0145$ ; FECH - Pearson's  $r = -0.49$ ,  $p = 0.0127$ ). While FECH is differentially expressed between inter and post-drug exposures ( $t = -2.673$ ,  $p = 0.0143$ ), the ALAD gene is not ( $t = 0.1883$ ,  $p = 0.8524$ ) (Figure 5). Other genes from the porphyrin metabolism pathway that were significantly correlated with plasma hemoglobin were the HCCS (Pearson's  $r = 0.46$ ,  $p = 0.0209$ ) and MMAB (Pearson's  $r = 0.41$ ,  $p = 0.0396$ ) genes.

## **Discussion and Conclusion**

The present study was designed to investigate the effects of the anti-malarial drug pyrimethamine on the physiology of macaques that have been administered sub-curative doses. This multi-omic approach has enabled us not only to shed light on the impact of pyrimethamine



on plasma hemoglobin levels in Rhesus macaques, but to also develop a novel method for integrating transcriptomics and metabolomics data.

Primary analysis of both omic data types using Spearman correlation and mixed effects ANOVA revealed that there were indeed gene and metabolites that were correlated with plasma hemoglobin levels in the macaque, and that there were genes and metabolites that could separate the samples based on drug exposure. As is typical with most omic studies, the list of potential genes and metabolites that were statistically significant was numerous. Thus we chose to develop a novel methodology of integrating the data types using group LASSO regression by assigning the principal components of the data types to knowledge-based pathways. Prior to integration, the metabolomics data was better at classifying the pyrimethamine exposure level of macaques than the gene expression data (Figures 6 and 7). Our approach allowed us to account for variation between drug exposures (inter-drug vs post-drug) and to use this information to predict associations to hemoglobin at the pathway level.

Our pathway group LASSO regression model selected 8 biological pathways that were associated with hemoglobin levels in the macaques. Among these pathways was the porphyrin and chlorophyll metabolism pathway that contains genes and metabolite that are involved in heme synthesis. Heme is a well-known porphyrin, which functions a cofactor in the hemoglobin protein that distributes oxygen to the cells of animals and humans. Inspection of the members of the porphyrin and chlorophyll metabolism pathway revealed that it contained three genes and one metabolite that are involved in heme synthesis, and that these genes and the metabolite were either statistically significant for correlation with hemoglobin, differentially expressed between inter and post-drug exposures, or both. The *ALAS2* gene (Figure 4A and 4B) produces an erythroid-specific mitochondrially active aminolevulinic acid synthase, which catalyzes the first

step in the heme biosynthesis pathway. The end product of the reaction, aminolevulinic acid (Figure 4C and 4D) was also detected using our methods. The remaining two genes from the pathway were ALAD and FECH. The ALAD gene produces a cytosolic enzyme that catalyzes the second step in the porphyrin and heme biosynthetic pathway, and the FECH gene produces ferrochelatase (FECH, protoheme ferrolyase), an enzyme that catalyses the terminal (eighth) step in the biosynthesis of heme. Other pathway members that met the statistical threshold for significance were the HCSS and MMAB genes. The HCSS gene produces an enzyme called holocytochrome c-type synthase, which is involved in a reaction that adds heme to make mature cytochrome c. Finally, the MMAB gene produces an enzyme that is involved in the formation of a compound called adenosylcobalamin (AdoCbl), which is derived from vitamin B12. The ability of the LASSO model to select the porphyrin metabolism pathway was therefore both biologically and statistically sound.

The remaining pathways selected by group LASSO include a number of pathways that are involved in anemia. The roles of vitamins A and C in anemia have long been established<sup>11</sup>. Heme synthesis, which would be triggered by anemic cellular conditions, originates at the mitochondria, so the selection of the nicotinate and nicotinamide metabolism pathway for association with hemoglobin levels by our group LASSO method is also biologically sound. The branched-chain amino acids (valine, leucine, and isoleucine) are proteogenic, so the involvement of this pathway is likely a reflection of increased hemoglobin synthesis in the macaques following pyrimethamine administration.

The evolution of biological science from a descriptive to a quantitative discipline has ushered in an era of high-throughput omic studies. While the prospect of answering research questions using omic technologies is appealing, sorting through the lengthy targets generated by

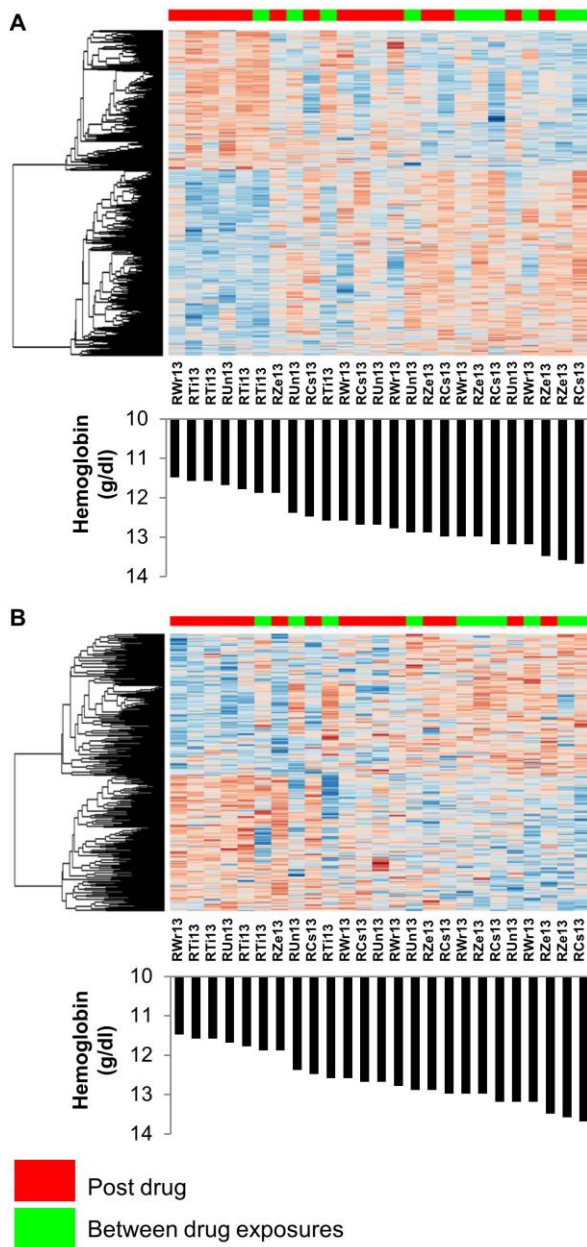
these methods is not. Certainly, the full potential of multi-omic studies has not been reached, and this partly due to a great need to integrate the data generated by these studies. To make study results more manageable, it is not unusual for investigators to subject the results to additional analyses to uncover system-wide associations such as pathway enrichment and network modelling. These methods represent domain-knowledge guided approaches<sup>6</sup>. Our method addresses the challenge of reducing the targets of interest, while preserving pathway level information.

Our findings are consistent with current literature, and our technique provides a means by which this sort of pathway refinement can be performed. As with multiple other strategies for analyzing omic data, it is difficult to assess the statistical power of the methods used here. A major limitation is that our results are based on a small population of five subjects, though they were followed over a 100 day time course. Additional studies must be performed to determine the universal power of our approach. Nonetheless, our findings provide new insights into the mode of action by which pyrimethamine influences hemoglobin metabolism, demonstrating that pathway group LASSO is a novel and effective method of integrating metabolomics and transcriptomics data.

**Table 1.** Pathways selected by group pathway LASSO for association with plasma hemoglobin in macaques between and after pyrimethamine exposure with the numbers of contributing features.

Pathway	Number of Genes	Number of Metabolites
Ascorbate and aldarate metabolism (Vitamin C)	5	4
Glycerophospholipid metabolism	53	7
Linoleic acid metabolism	6	6
Cysteine and methionine metabolism	26	11
Porphyrin and chlorophyll metabolism	20	9
Retinol metabolism (Vitamin A)	16	6
Valine, leucine and isoleucine degradation	38	6
Nicotinate and nicotinamide metabolism	18	3
Total features	182	52

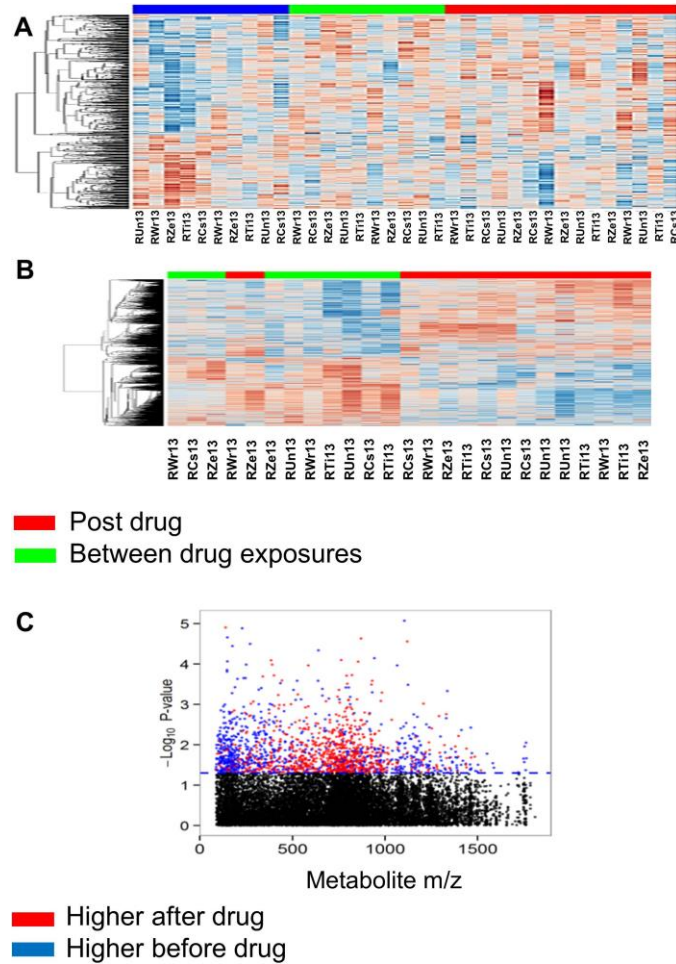
**Figure 1**



**Figure 1.** Features in the Rhesus macaques that are significantly correlated with hemoglobin levels at time points (TP) 3 – 7. (A) Heat map showing the 1,074 genes that are associated with hemoglobin ( $p < 0.05$ ). (B) Heat map showing the 305 metabolites that are associated with

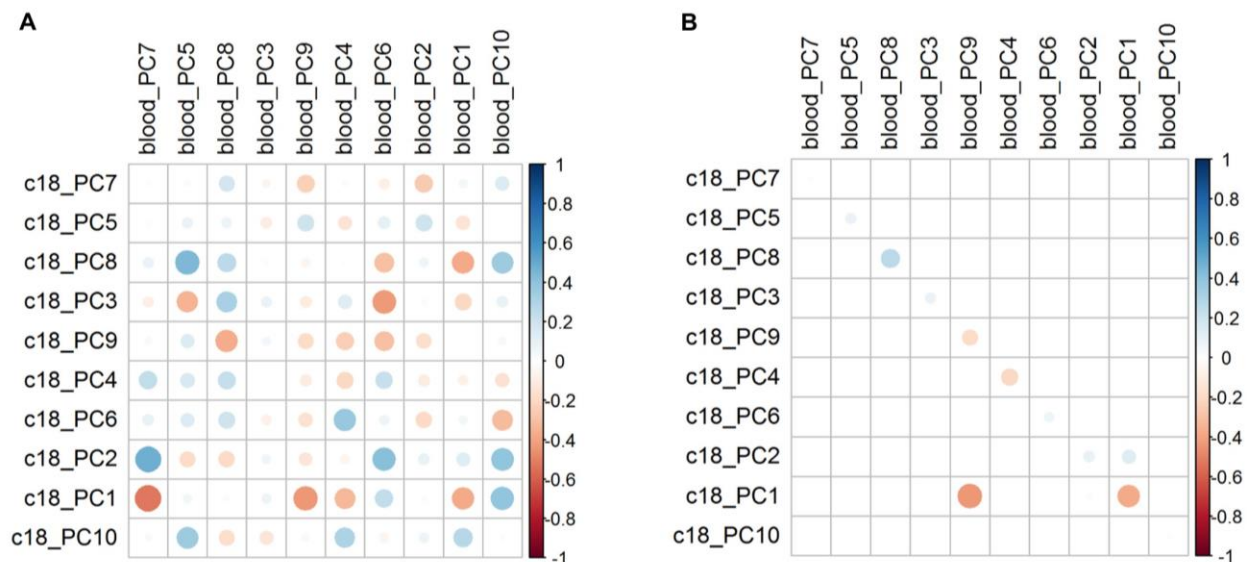
hemoglobin ( $p < 0.05$ ). Inter-drug exposure includes samples from TP4 and TP6. Post-drug exposure includes samples from TP3, TP5, and TP7.

**Figure 2**



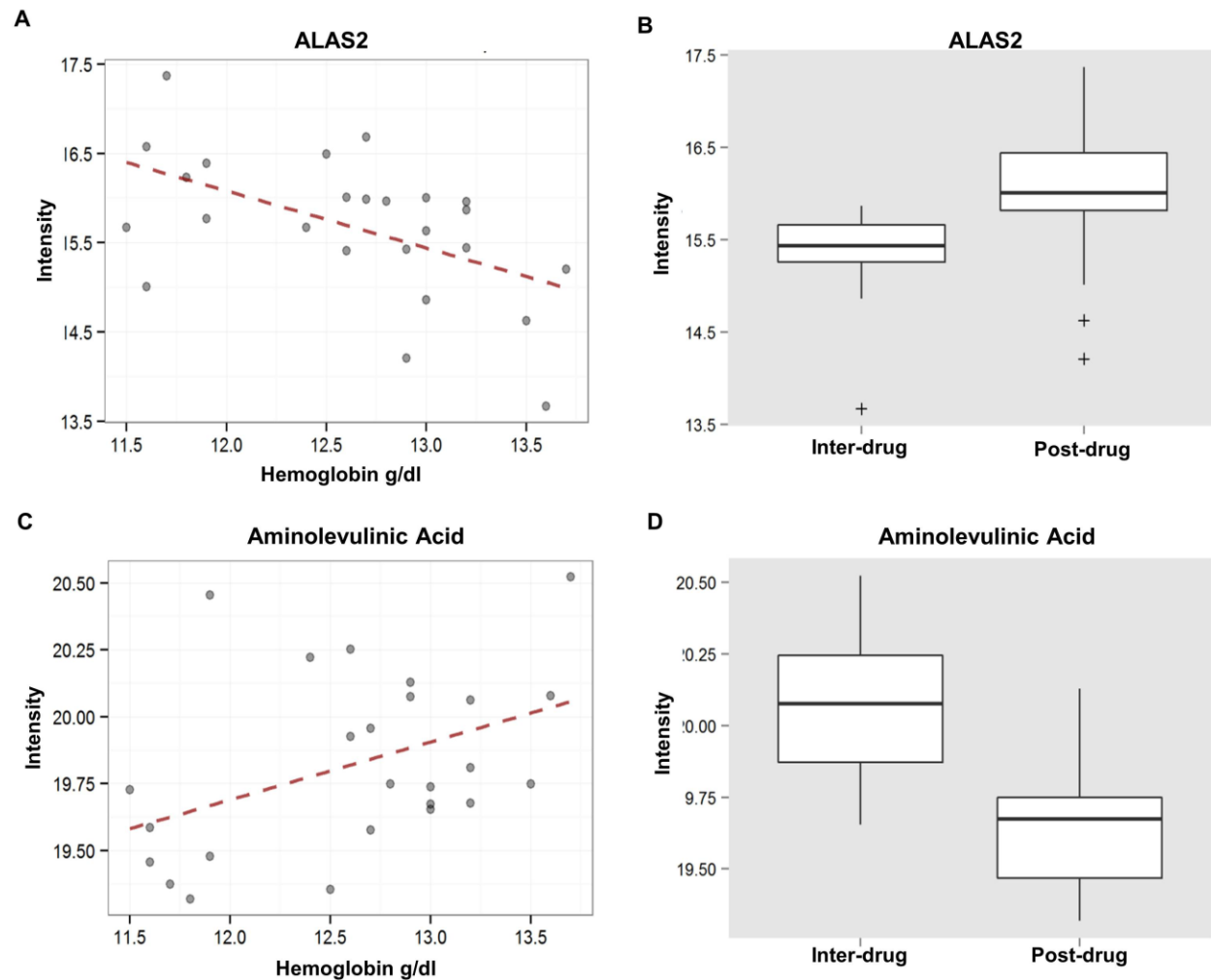
**Figure 2.** Genes and metabolites in the macaques that were differentially expressed across experimental time points (TP) due to drug exposure. (A) The top 925 genes ( $p < 0.05$ ). (B) The top 1660 metabolites ( $p < 0.05$ ). Pre-drug exposure includes samples from TP1 and TP2. Inter-drug exposure includes samples from TP4 and TP6. Post-drug exposure includes samples from TP3, TP5, and TP7.

**Figure 3**



**Figure 3.** Correlation of Principal Components of differentially expressed genes and metabolites before pathway assignment. (A) Pearson correlation of top 10 principal components before testing for statistical significance. Pearson correlation of top 10 principal components after testing for statistical significance ( $p < 0.05$ ).

**Figure 4**

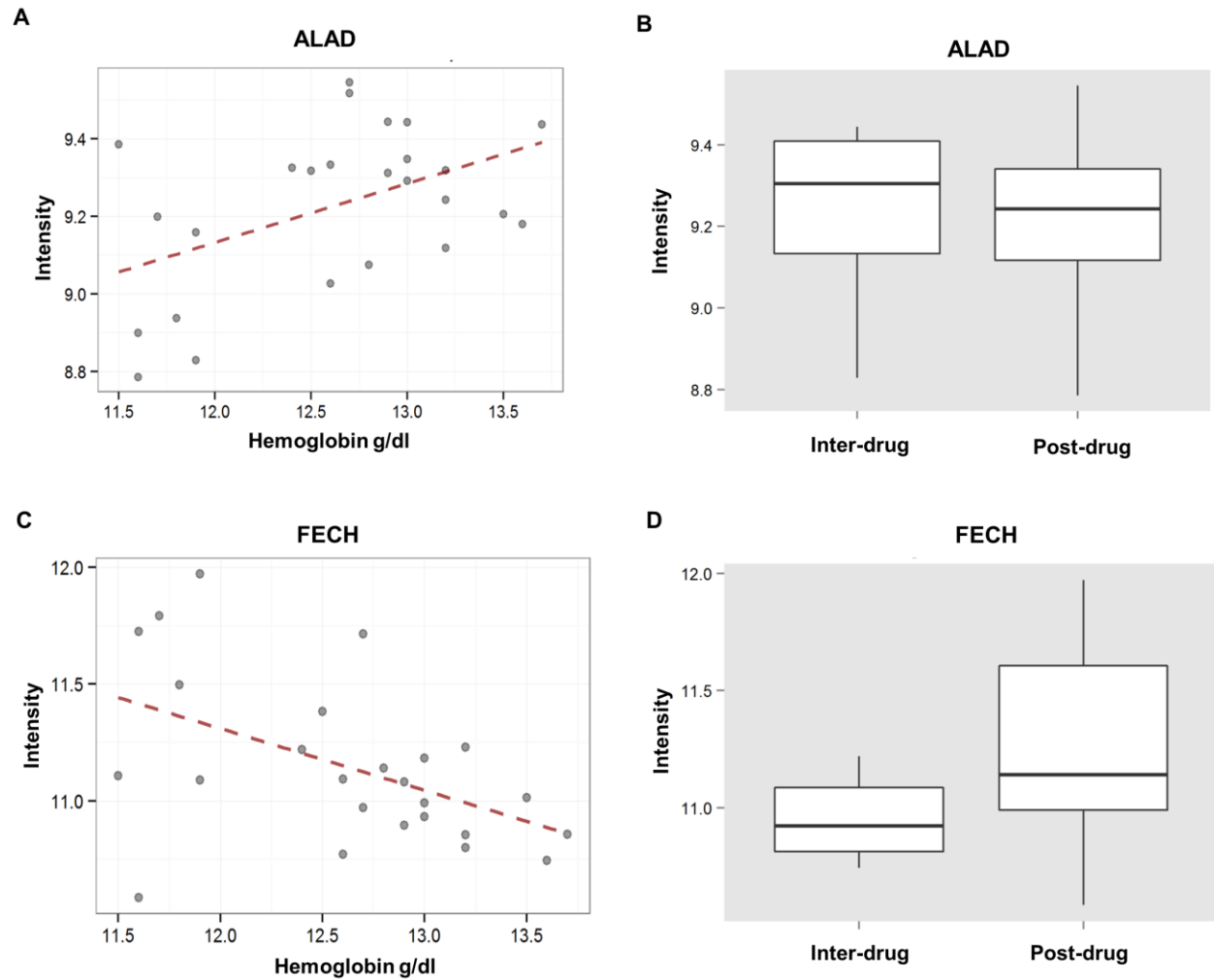


**Figure 4.** The most significant gene and metabolite from the porphyrin metabolism pathway that was selected by Group Pathway LASSO. (A) The ALAS2 gene is correlated with plasma hemoglobin (Pearson's  $r = -0.53$ ,  $p = 0.0069$ ). (B) The ALAS2 gene is differentially expressed between inter and post-drug exposure conditions ( $t = -2.225$ ,  $p = 0.0365$ ). (C) Aminolevulinic acid is correlated with plasma hemoglobin (Pearson's  $r = 0.43$ ,  $p = 0.0333$ ). (D) Aminolevulinic



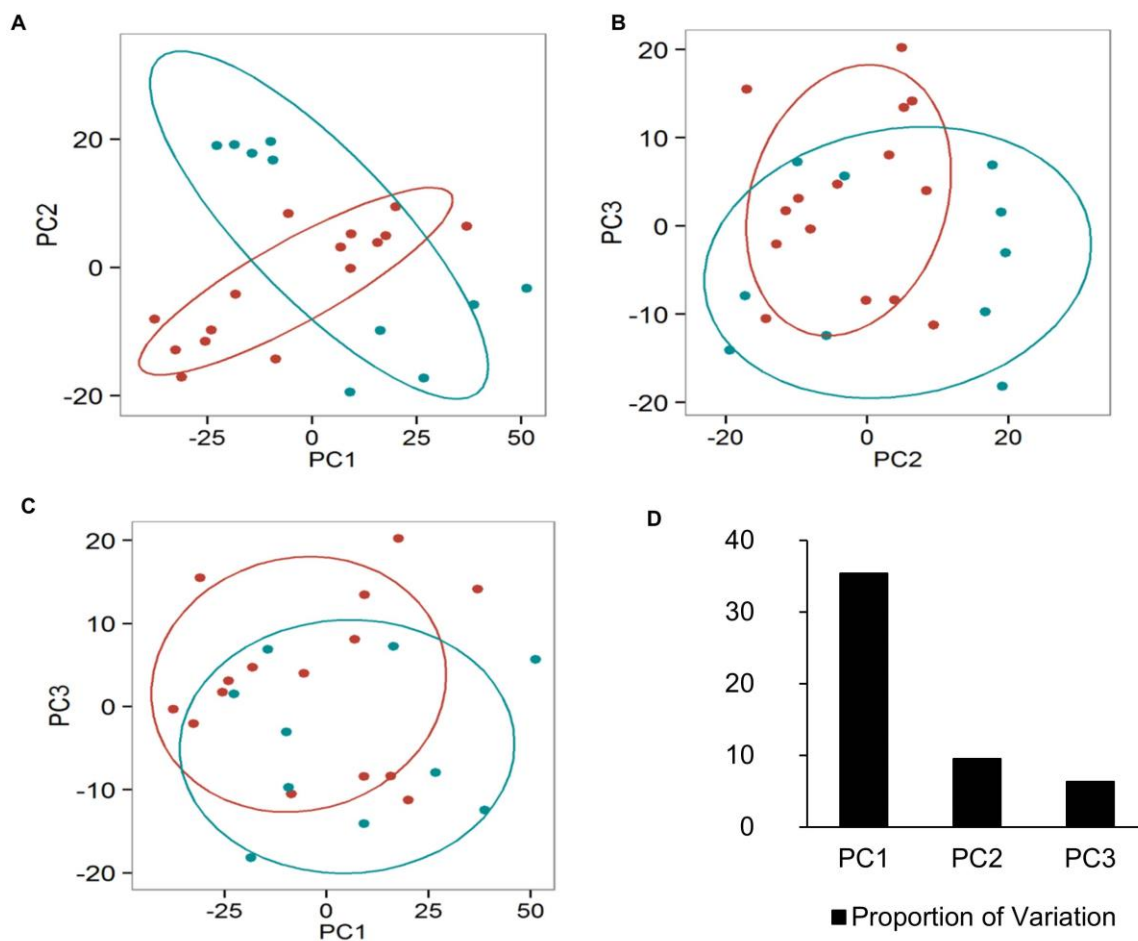
acid is differentially expressed between inter and post-drug exposure conditions ( $t = 3.98$ ,  $p = 0.0010$ ).

**Figure 5**



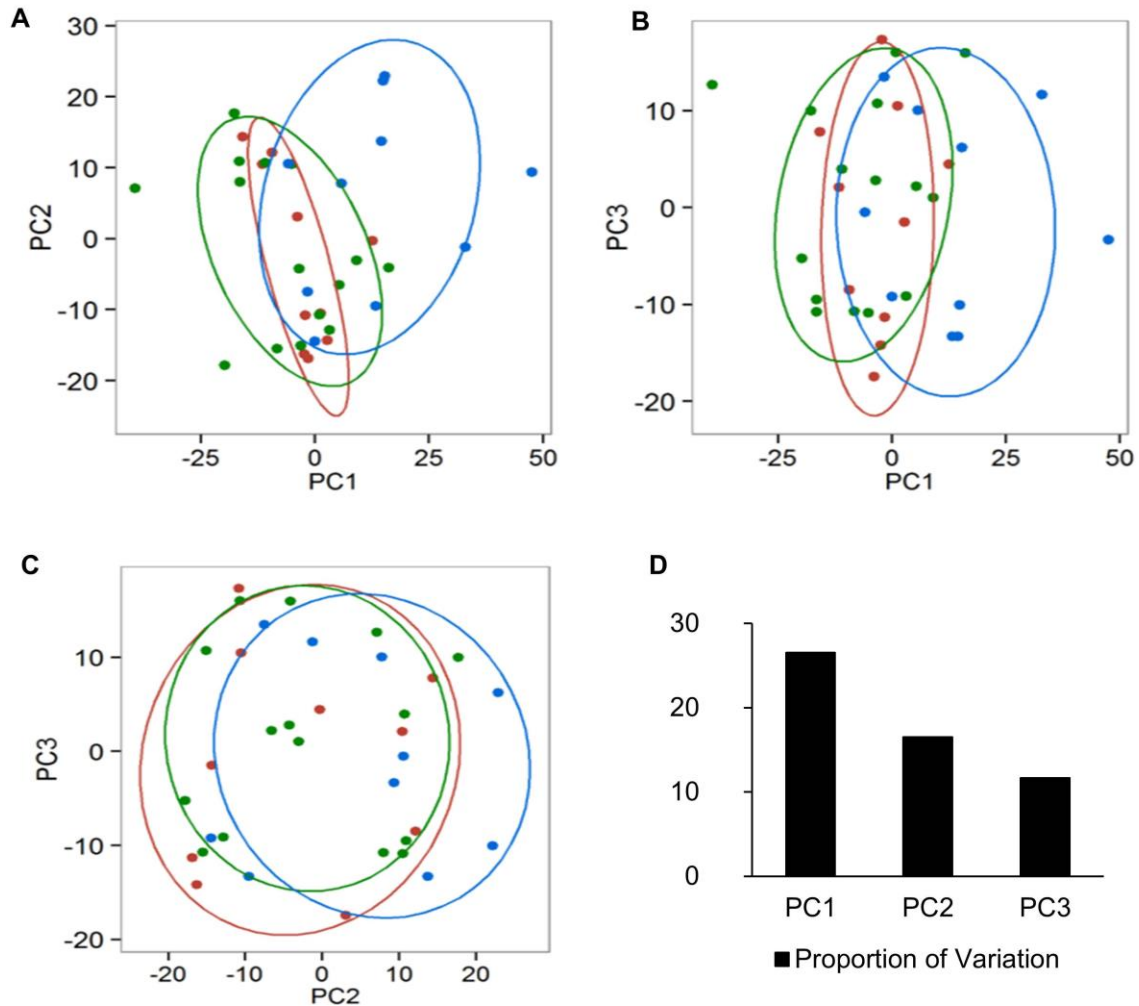
**Figure 5.** Significant genes and metabolites from the porphyrin metabolism pathway that were selected by Group Pathway Lasso. (A) The ALAD gene is correlated with plasma hemoglobin (Pearson's  $r = 0.48$ ,  $p = 0.0145$ ); however the ALAD gene (B) is not differentially expressed between inter and post-drug exposure conditions ( $t = 0.1883$ ,  $p = 0.8524$ ). (C) The FECH gene is correlated with plasma hemoglobin (Pearson's  $r = -0.49$ ,  $p = 0.0127$ ). (D) FECH is differentially expressed between inter and post-drug exposure conditions ( $t = -2.673$ ,  $p = 0.0143$ ).

**Figure 6**



**Figure 6.** Plot of principal component analysis (PCA) of metabolites that are differentially expressed in macaques between and after treatment with pyrimethamine. PC1, principal component 1; PC2, principal component 2; PC3, principal component 3. Each point/circle represents a sample. Samples from inter-drug exposure (TP4 and TP6) are colored in cyan and samples from post-drug exposure (TP3, TP5, and TP7) are colored in red.

**Figure 7**



**Figure 7.** Plot of principal component analysis (PCA) of genes that are differentially expressed in macaques before, between, and after treatment with pyrimethamine. PC1, principal component 1; PC2, principal component 2; PC3, principal component 3. Each point/circle represents a sample. Samples from pre-drug exposure (TP1 and TP2) are colored blue, samples

from inter-drug exposure (TP4 and TP6) are colored in red, and samples from post-drug exposure (TP3, TP5, and TP7) are colored in green.

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