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**CORRELATION BETWEEN IRON AND  
 $\alpha$  AND  $\pi$  GLUTATHIONE-S-TRANSFERASE  
LEVELS IN HUMANS**

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| 14. ABSTRACT Correlations were examined in humans of $\alpha$ and $\pi$ glutathione-S-transferase s (serum and plasma, respectively) and blood levels of iron, transferrin, ferritin, soluble transferrin receptor (sTfR) as well as Total Iron-binding Capacity (TIBC), and percent Transferrin saturation (%TS). Data analysis of ungrouped (unbinned) data indicated significant gender differences in the blood levels of iron, TIBC, ferritin, and %TS, with none seen with transferrin, sTfR, or the GSTs. Regression analysis of ungrouped data with $\alpha$ GST as a response variable identified the interaction between age and iron as well as between age and percent iron saturation (%ISAT) as significant predictors of $\alpha$ GST. Regression analysis using ungrouped $\pi$ GST data, only resulted in a quadratic model with iron as the only significant predictor ( $R^2 = 6.9\%$ ). Using binned data based on published clinical levels, it was seen that elevated levels of iron and sTfR are strong predictors of elevated $\alpha$ GST levels. These associations were not seen using binned normal $\alpha$ GST levels. Unlike regression analyses of the ungrouped data, regression analyses of binned $\pi$ GST data indicated significant associations (elevated iron, normal transferrin) although not as strong as those seen using $\alpha$ GST data. |             |                           |                               |                                                                          |                                              |
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## **PREFACE**

This research was accomplished by a research group in the 711th Human Performance Wing, the Human Effectiveness Directorate under the Molecular Bioeffects Branch (RHDJ) within the Bioeffects (RHD) Division. Prior to 1 October 2011, this Branch was called the Applied Biotechnology Branch (RHPB) and previous reports and approvals on this project are listed under RHPB. This technical report was written for AFRL Work Unit OAFWP001, and is a follow-on study on the work reported in AFRL-HE-WP-TP-2006-0021.

This study was reviewed by the AFRL IRB which determined that it did not meet criteria for human subject research under the definition in 32 CFR 219.102(f). As the samples were obtained in the course of routine patient care, there was no interaction with humans for the purpose of this research, and the samples were de-identified.

## **ACKNOWLEDGEMENTS**

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## 1.0 SUMMARY

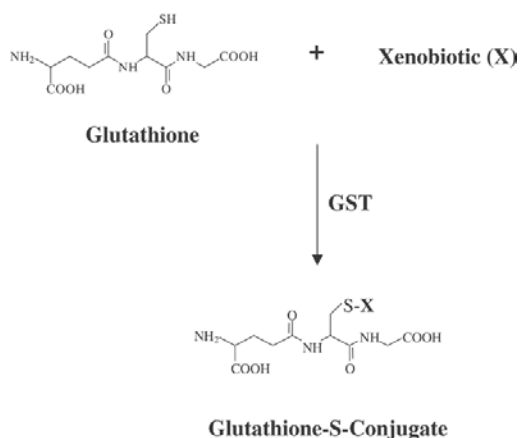
We have identified a number of potential biomarkers indicative of low level liver damage in response to uncharacterized toxin exposures. To conduct pre-validation and validation studies on these markers, blood and/or urine must be obtained in a human cohort with known sub-clinical hepatic damage. The acquisition of such samples is problematic – low level liver damage usually does not present primary or secondary effects that can be used a screen for potential subjects. In order to develop a non-invasive test method for such sample collections, we examined the levels of possible mild to increased liver damage in heterozygote hemochromatosis (HFE) samples as indicated by levels of the known hepatic biomarkers  $\alpha$  and  $\pi$  glutathione-S-transferase s (GST) in the blood. HFE mutations can lead to liver damage caused by hepatocellular iron overload. As part of this research effort, correlations were examined using  $\alpha$  and  $\pi$  glutathione-S-transferases (serum and plasma, respectively) and blood levels of iron, transferrin, ferritin, soluble transferrin receptor (sTfR) as well as Total Iron-binding Capacity (TIBC), and percent Transferrin saturation (%TS). A total of 90 matched samples (48 male, 42 female) of serum and plasma were obtained and tested for concentrations of all components. Data analysis of ungrouped (unbinned) data indicated significant gender differences in the blood levels of iron, TIBC, ferritin, and %TS, with none seen with transferrin, sTfR, or the GSTs. Regression analysis of ungrouped data with  $\alpha$ GST as a response variable identified the interaction between age and iron as well as between age and percent iron saturation (%ISAT) as significant predictors of  $\alpha$ GST. Regression analysis using ungrouped  $\pi$ GST data, only resulted in a quadratic model with iron as the only significant predictor ( $R^2 = 6.9\%$ ). Using binned data based on published clinical levels, it was determined that elevated levels of iron and sTfR are strong predictors of finding elevated  $\alpha$ GST levels. These associations were not observed using binned normal  $\alpha$ GST levels. Unlike regression analyses of the ungrouped data, regression analyses of binned  $\pi$ GST data indicated significant associations (elevated iron, normal transferrin) although not as strong as those seen using  $\alpha$ GST data.

Keywords: glutathione-S-transferase, GST, pi GST, alpha GST, iron, transferrin, ferritin, transferrin receptor, liver damage, biomarker, serum, plasma, blood, biomarker

## 2.0 INTRODUCTION

### 2.1 Glutathione S-Transferases

**2.1.1 Activity.** The large protein family of glutathione S-transferases (GSTs) consists of constitutively expressed multi-role enzymes. These enzymes conjugate glutathione to a diverse array of both endogenous and exogenous xenobiotic compounds, a critical role in cellular detoxification (**Figure 1**).<sup>1,2</sup> GST enzymes catalyze a nucleophilic attack by glutathione to electrophilic xenobiotics by activating a sulfhydryl group on glutathione. This reaction effectively eliminates reactivity seen in the 'free' xenobiotic thus providing a detoxification mechanism within the cell. GSTs are also thought to be critical for removal of lipid peroxide products produced during oxidative stress.<sup>3</sup>



**Figure 1.** The role of GSTs in detoxification via conjugation with glutathione.

Image taken from Townsend *et al.* 2003<sup>4</sup>.

**2.1.2. Subfamilies and expression.** Mammalian cytosolic GSTs exist in several subfamilies (Alpha, Mu, Pi) and are classified based on primary/tertiary/quaternary structure as well as their kinetic properties.<sup>5</sup> Most GST species are expressed in multiple organs.<sup>6</sup> It has been shown that  $\pi$ GST is primarily located within the liver intrahepatic bile duct cells, whereas  $\alpha$ GST is distributed throughout the liver with the highest concentration in hepatocytes.<sup>7</sup> By testing  $\alpha$  or  $\pi$  GST levels in the serum or plasma, respectively, it has been shown that elevated levels of these proteins are indicative of site-specific hepatic injury.<sup>8</sup> Therefore, hepatic injury can be monitored, at a fairly sensitive level, by determining  $\alpha$  and/or  $\pi$  GST concentrations in the blood.

### 2.2 Cellular Iron Status in Humans

As there are no forms of the passive excretion of iron, its cellular levels are especially sensitive to perturbations. Normally, duodenal crypt cells determine the organism's need for iron and

these adsorption requirements are “set” into maturing absorptive enterocytes. Once absorbed through the gut villi on the enterocytes, the divalent metal transporter 1 (DMT1) protein transfers the iron into the cell using a proton-coupled reaction.<sup>9,10</sup> For storage, iron is primarily sequestered in the ubiquitous globular protein complexes, ferritin<sup>11</sup> or hemosiderin.<sup>12</sup>  $\alpha$ GSTs also can sequester forms of iron inside the cell via a nitric oxide-mediated reaction.<sup>13</sup>

Changes to iron uptake or metabolism can play a key role in mammalian health, with numerous disease states affiliated with altered iron homeostasis. There are many effectors of cellular iron concentration such as diet, malabsorption, *Helicobacter pylori* infection, drug interference, and hemorrhage.<sup>14</sup> Variants of hepcidin, considered the main regulator of iron homeostasis, as well as its regulatory proteins have been shown to change iron absorption.<sup>15</sup> Hereditary hemochromatosis type 1 is another well characterized disease of iron absorption. In this ailment, genetic mutations significantly alter iron levels in the blood by effecting either the concentration or the activity of key proteins involved in maintenance of iron status. One of these effectors, the hemochromatosis (HFE) gene, encodes a major histocompatibility complex protein. The HFE protein forms a heterodimer with  $\beta$ 2-microglobulin, and this complex is embedded in the membrane surface of several types of cells, including duodenal crypt cells and macrophages. Several well characterized mutations in this gene have been shown to increase iron levels.<sup>16</sup> Of known HFE mutations, the cysteine to tyrosine change at position 282 (C282Y)<sup>17</sup> and possibly a histidine to aspartic acid at position 63 (H63D)<sup>18</sup> may alter iron absorption or protein concentration to initiate iron overload.<sup>19</sup> Iron overload in the blood can cause excess toxic iron deposition in key organs, namely the liver and heart, as well as lead to bone loss.<sup>20</sup> High levels of iron deposition in these tissues lead to damage due to the catalytic nature of soluble ferrous iron to react with soluble oxygen to produce oxygen free radicals and further down-stream reactive oxygen species (ROS).<sup>21</sup> Therefore an organism which demonstrates iron overload can, among other things, exhibit hepatic damage ranging from low level (pre-clinical) to massive organ failure.<sup>22</sup> Hemochromatosis is usually initially diagnosed by the use of blood tests examining the levels of serum transferrin saturation as well as serum ferritin. The saturation levels of transferrin are indicative of the amount of iron which is bound to transferrin in the blood. The ferritin tests the amount of iron stored in the liver. If suspected, secondary genetic tests can confirm the presence of the HFE mutations.

### **2.3 Modeling Human Sub-Clinical Hepatic Damage**

Evaluation and validation of new biomarkers indicative of sub-clinical hepatic damage is problematic in that current clinical assays for hepatic damage are not sensitive enough to allow detection of low level damage. Past studies<sup>23</sup> have examined the effects of HFE gene mutations<sup>24,25</sup> on the development of low-level liver damage. Causative genetic mutations in the HFE gene were examined to see if the presence of the mutations correlated with low-level liver damage as indicated by elevated levels of serum  $\alpha$ GST. It was hoped that successful correlation of the mutation with sub-clinical liver damage would allow the use of HFE genetic

testing as a non-invasive method for collecting blood samples from persons with undetected liver damage. The identification of such a test model for sample collection is important in the development and validation of blood-based biomarkers capable of detecting early hepatic damage due to toxin exposures.<sup>26</sup> The conclusions of this study indicated that no statistically significant differences were seen in serum  $\alpha$ GST levels in individuals heterozygous for the deleterious HFE H63D or C282Y mutations. Indeed, affected individuals seemed to have lower, not higher,  $\alpha$ GST levels. However, there were several drawbacks to the study conducted. As the samples obtained were pre-existing clinical samples, there was concern that the sample quality may have been suboptimal for  $\alpha$ GST enzyme-linked immunosorbant assay (ELISA) testing due to the length of time between sample draw and  $\alpha$ GST analysis. The sample size also did not exhibit adequate populations of heterozygotes to make a statistically significant argument for the association of GST levels (and presumed hepatic damage) with the two mutations. In addition, only  $\alpha$ GST was examined as only serum samples were collected. A  $\pi$ GST analysis using existing test assays requires plasma.

## **2.4 Correlation of Iron, $\alpha$ GST and $\pi$ GST Levels in the Blood**

An investigation of blood iron and GSTs may give insight into the use of GSTs as indicators of low level liver tissue damage as well as an understanding of their relationship to iron regulation. The purpose of the follow-on work was to examine the relationship between  $\alpha$  and  $\pi$  glutathione-S-transferase and clinical iron levels. Therefore a second study was investigated to examine the possible correlations between  $\alpha$ GST,  $\pi$ GST, iron and iron regulatory elements (transferrin, transferrin receptor, ferritin) using human matched serum/plasma samples. The previous study generated data from a total of 52 samples displaying heterozygote frequencies of 9.9% C282Y and 20.8 % H63D. We felt that, with these distributions, it would be difficult to collect enough matched samples to generate adequate statistical strength. Therefore in the new study, HFE mutations were not analyzed and only possible GST/iron associations examined. The 88<sup>th</sup> Medical group personnel selected matched serum/plasma samples from pre-existing clinical samples, and efforts were made to insure that the samples were received at RHDJ within the limits of assay time.

### 3. MATERIALS AND METHODS

#### 3.1 Sample Collection

This study utilized pre-existing human serum and plasma samples drawn at WPAFB Medical center for other clinical analyses. Once the pre-existing samples were determined to be of no further utility to the Medical Center, matched serum/plasma samples were released to RHDJ. A total of 90 matched serum and plasma samples were collected in either SST or clot tubes without additive. The samples were stored at -80° C after transfer to RHDJ. Researchers did not have access to medical history data, only age/gender of each sample. The limited demographic information is presented in **Table 1**.

**Table 1:** Gender and Average Age of Serum Samples.

|        | Sample Number | Average Age in Years<br>( $\pm$ standard deviation) |
|--------|---------------|-----------------------------------------------------|
| Male   | 48            | 34.3 (11.3)                                         |
| Female | 42            | 33.2 (12.1)                                         |
| Total  | 90            | 33.8 (11.6)                                         |

#### 3.2 Enzyme-Linked Immunosorbent Assay (ELISA) Testing

**3.2.1  $\alpha$ GST ELISA.** Enzyme-linked immunosorbent assays were performed as described in the Biotrin High Sensitivity Alpha GST EIA kit protocol. First, serum samples were diluted 1:10 with wash solution (phosphate buffered saline with Tween 20). A total of 100  $\mu$ L of diluted sample was added to the wells of 96-well microtiter plates coated with IgG directed against  $\alpha$ GST and then incubated at room temperature (20-25° C) for 60 min with uniform shaking. Plates were washed six times with wash solution. One hundred microliters of anti- $\alpha$ GST IgG conjugated to horseradish peroxidase were added to each well and incubated at room temperature for 60 min with uniform shaking. Wells were washed six times, after which 100  $\mu$ L of stabilized liquid TMB solution were added to each well and the plates incubated for exactly 15 min at room temperature. Reactions were stopped by the addition of 100  $\mu$ L of 1 N sulfuric acid. The absorbance (optical density [OD]) was measured at 450-630 nm with a microplate reader (Molecular Devices SpectraMax M2<sup>e</sup>).

**3.2.2  $\pi$ GST ELISA.** Enzyme-linked immunosorbent assays were performed as described in the Biotrin Pi GST EIA kit protocol. First, plasma samples were diluted 1:5 with sample diluent (protein containing solution with added stabilizers). One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with IgG directed against  $\pi$ GST and the plates were incubated at room temperature (20-25° C) for 60 min with uniform shaking. Plates were washed 4 times with wash solution (phosphate buffered saline with Tween 20). One hundred microliters of anti- $\pi$ GST IgG/ horseradish peroxidase conjugate were added and

incubated at room temperature for 60 min with uniform shaking. Wells were washed four times after which 100  $\mu$ L of stabilized liquid TMB solution were added, and the plates were incubated for exactly 15 min at room temperature. Reactions were stopped by the addition of 100  $\mu$ L 0.5 M sulfuric acid. The absorbance (optical density [OD]) was measured at 450-630 nm with a microplate reader (Molecular Devices SpectraMax M2<sup>e</sup>).

**3.2.3 Transferrin.** Enzyme-linked immunosorbent assays were performed as described in the AssayMax Human Transferrin ELISA kit protocol. First, serum samples were diluted 1:2000 with MIX diluent A (containing 0.01% thimerosal). Twenty-five microliters of diluted sample were added to the wells of 96-well microtiter plates coated with polyclonal antibody against human transferrin. Immediately, 25  $\mu$ L of biotinylated transferrin were added to each well and incubated for 60 min at 20-30° C. Plates were washed 5 times with wash buffer (containing 0.01% thimerosal). Fifty microliters of streptavidin-peroxidase conjugate diluted in MIX diluent were added and incubated for 30 min at 20-30° C. Wells were washed five times, 50  $\mu$ L of stabilized peroxidase chromogen substrate (<0.05% w/v 3,3',5,5'-tetramethylbenzidine in H<sub>2</sub>O) were added to each well, and plates were incubated for 10 min at 20-30° C. Reactions were stopped by the addition of 50  $\mu$ L of 0.5 N hydrochloric acid. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2<sup>e</sup>).

**3.2.4 Soluble Transferrin Receptor (sTfR).** Enzyme-linked immunosorbent assays were performed as described in the BioVendor Human sTfR ELISA kit protocol. First, serum samples were diluted 1:50 with dilution buffer. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with mouse monoclonal anti-sTfR antibody and incubated for 60 min at 30-35° C with uniform shaking at 300 rpm. Plates were washed 3 times with wash solution (containing 0.05% thimerosal). One hundred microliters of mouse monoclonal anti-sTfR antibody, horseradish peroxidase conjugate were added to all wells and incubated for 60 min at 30-35° C with uniform shaking at 300 rpm. Wells were washed 3 times, 100  $\mu$ L of TMB substrate solution were added, and plates were incubated 10 min at 20-30° C in the dark. Reactions were stopped by the addition of 100  $\mu$ L of 1.96% sulfuric acid. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2<sup>e</sup>).

### **3.3 Blood Chemistry Analyses**

**3.3.1 Serum Iron and Total Iron-Binding Capacity (TIBC).** Serum iron and TIBC were measured in 65  $\mu$ L of serum using Dade Behring reagents on the Dimension® RxL Max® Integrated Chemistry System. Samples were thawed to room temperature prior to analysis.

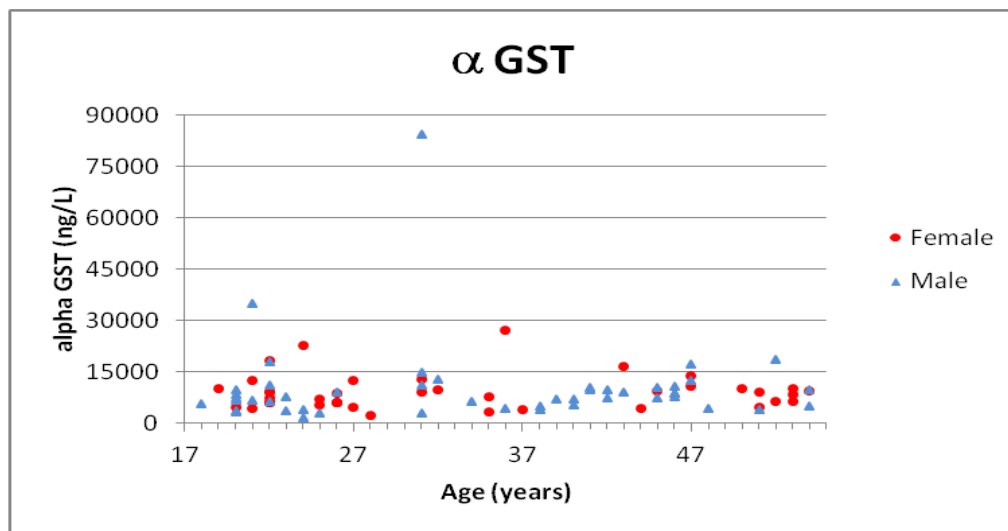
**3.3.2 Ferritin.** Ferritin is commonly used as a clinical indicator of iron storage. Ferritin levels were measured in 25  $\mu$ L of serum using ADVIA ReadyPack® reagents on the ADVIA Centaur® XP Immunoassay System. Samples were thawed to room temperature prior to analysis.

**3.3.3 Percent Transferrin Saturation (%TS).** %TS was calculated using the formula %TS = (Serum Iron / TIBC) x 100%.

## 4. RESULTS

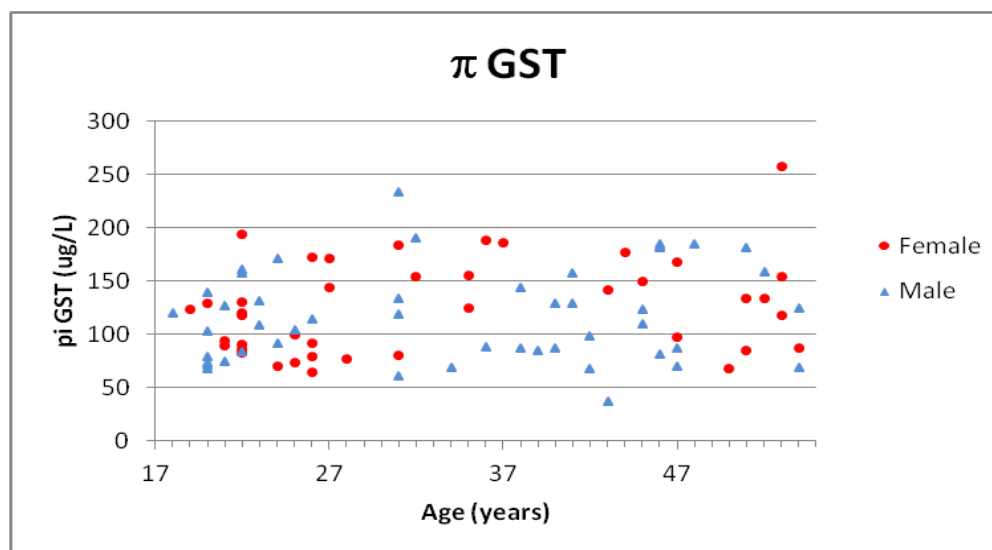
### 4.1 Enzyme-Linked Immunosorbent Assay Testing

**4.1.1  $\alpha$ GST ELISA.** Serum from 48 male and 42 female subjects was analyzed for  $\alpha$ GST concentration (**Figure 2**). Individual  $\alpha$ GST concentrations ranged from 1531 to 84273 ng/L (mean =  $9552 \pm 9554$  ng/L). There were no statistically significant differences ( $p = 0.267$ ) between men (1531 to 84273 ng/L, mean =  $10165 \pm 12233$  ng/L) and women (2123 to 27126 ng/L, mean =  $8956 \pm 5081$  ng/L).



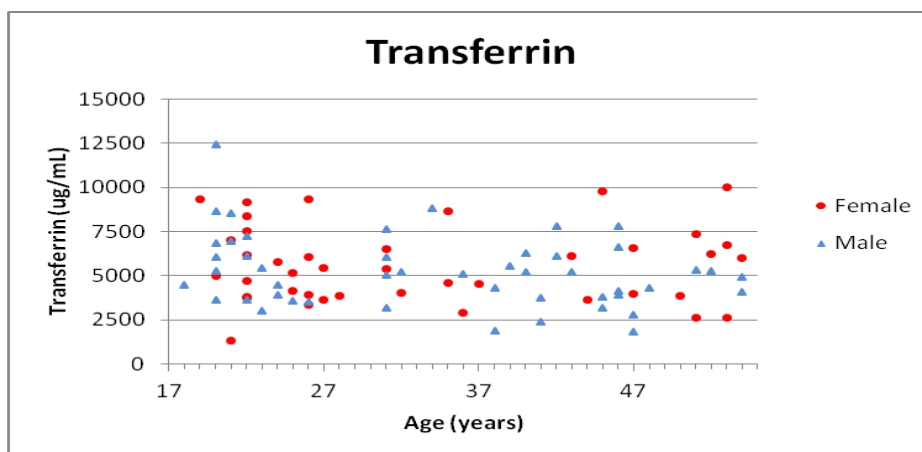
**Figure 2.** Age versus  $\alpha$ GST concentration in sample cohort.

**4.1.2  $\pi$ GST ELISA.** Plasma from 48 male and 42 female subjects was analyzed for  $\pi$ GST concentration (**Figure 3**). Individual  $\pi$ GST concentration ranged from 37 to 257  $\mu$ g/L (mean =  $122 \pm 44$   $\mu$ g/L). There were no statistically significant differences in  $\pi$ GST ( $p = 0.195$ ) between men (37 to 234  $\mu$ g/L, mean =  $117 \pm 44$   $\mu$ g/L) and women (64 to 257  $\mu$ g/L, mean =  $125 \pm 44$   $\mu$ g/L).



**Figure 3.** Age verses  $\pi$ GST concentration in sample cohort.

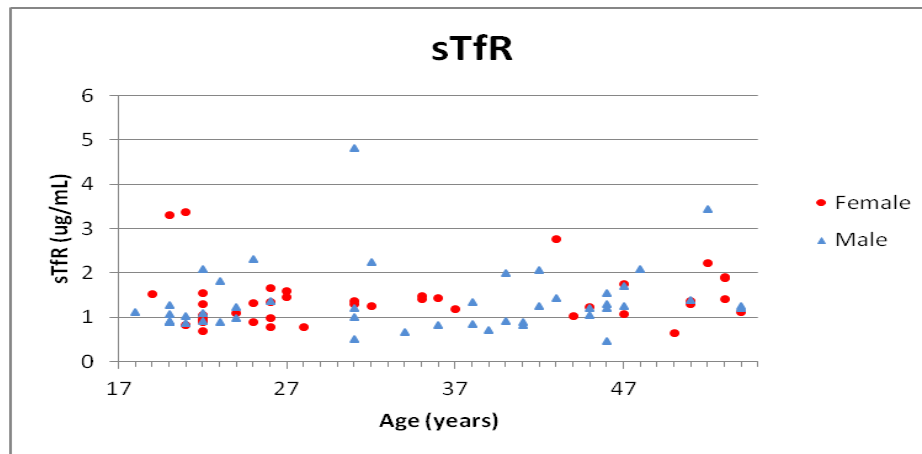
**4.1.3 Transferrin ELISA.** Serum from 48 male and 42 female subjects was analyzed for transferrin concentration (**Figure 4**). Individual transferrin concentration ranged from 1343 to 12468 ug/mL (mean =  $5435 \pm 2104$  ug/mL). There were no statistically significant differences ( $p = 0.226$ ) between men (1824 to 12468 ug/mL, mean =  $5253 \pm 2043$  ug/mL) and women (1343 to 9994 ug/mL, mean =  $5592 \pm 2178$  ug/mL).



**Figure 4.** Age versus transferrin concentration in sample cohort.

**4.1.4 Soluble Transferrin Receptor (sTfR) ELISA.** Serum from 48 male and 42 female subjects was analyzed for sTfR concentration (**Figure 5**). Individual sTfR concentration ranged from 0.46 to 4.82 ug/mL (mean =  $1.37 \pm 0.68$  ug/mL). There were no statistically

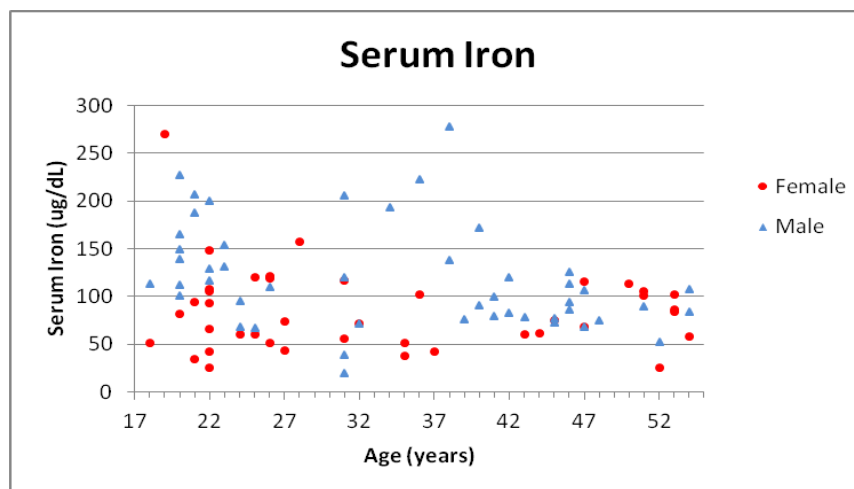
significant differences ( $p = 0.341$ ) between men (0.46 to 4.82 ug/mL, mean =  $1.34 \pm 0.75$  ug/mL) and women (0.64 to 3.37 ug/mL, mean =  $1.40 \pm 0.61$  ug/mL).



**Figure 5.** Age versus sTfR concentration in sample cohort.

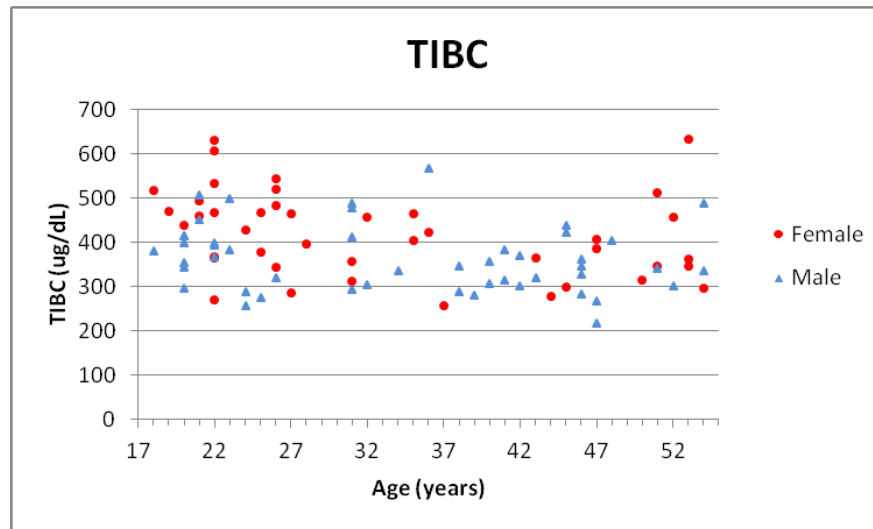
## 4.2 Blood Chemistry Analyses

**4.2.1 Serum Iron and Total Iron-Binding Capacity (TIBC).** Serum from 48 male and 42 female subjects was analyzed for iron concentration (**Figure 6**). Individual serum iron concentration ranged from 20 to 278 mg/dL (mean =  $103 \pm 52$  mg/dL). There were statistically significant differences ( $p = 0.00089$ ) between men (20 to 278 mg/dL, mean =  $119 \pm 54$  mg/dL) and women (26 to 270 mg/dL, mean =  $85 \pm 44$  mg/dL).



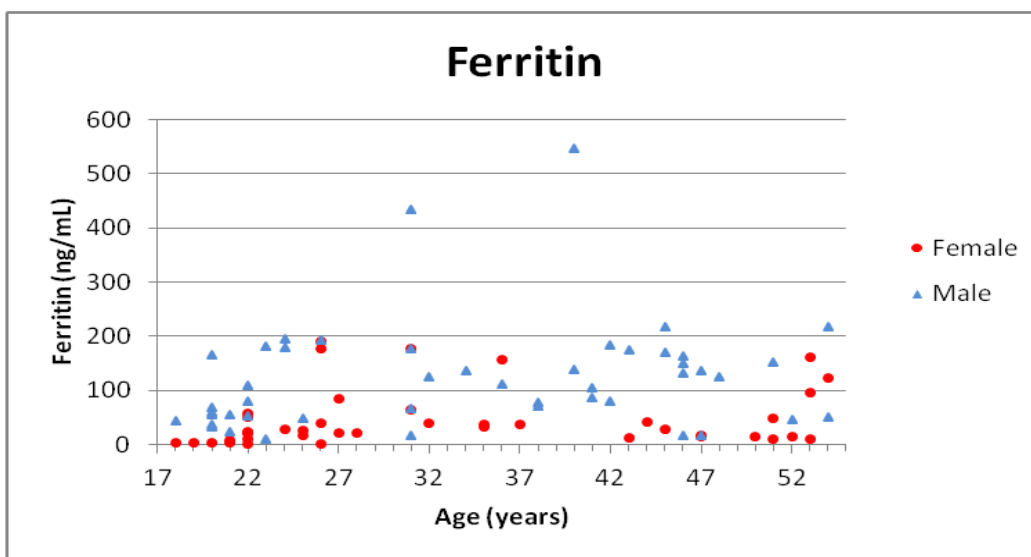
**Figure 6.** Age versus serum iron concentration in sample cohort.

Serum from 48 male and 42 female subjects was analyzed for total iron binding capacity (**Figure 7**). Individual TIBC concentration ranged from 217 to 634 ug/dL (mean =  $389 \pm 90$  ug/dL). There were statistically significant differences ( $p = 0.00132$ ) between men (217 to 568 mg/dL, mean =  $363 \pm 75$  ug/dL) and women (258 to 634 ug/dL, mean =  $419 \pm 97$  ug/dL).



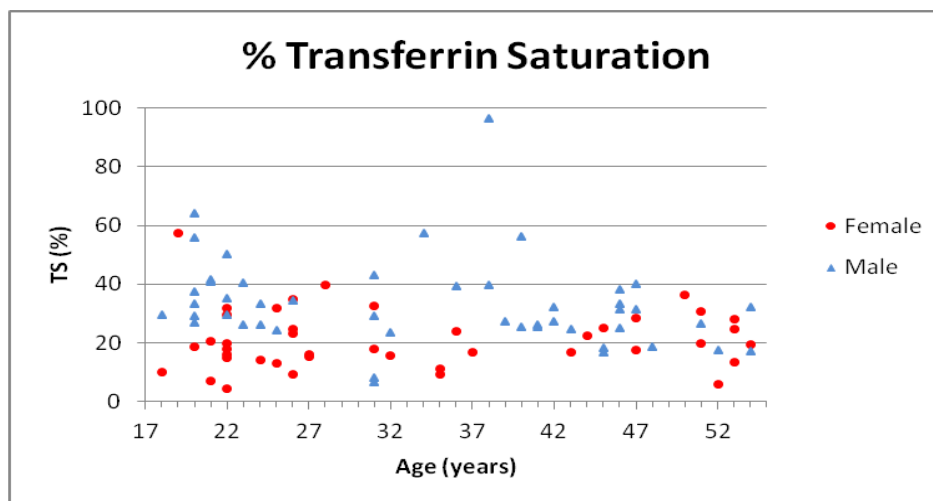
**Figure 7.** Age versus TIBC concentration in sample cohort.

**4.2.2 Ferritin.** Serum from 48 male and 42 female subjects was analyzed for ferritin concentration (**Figure 8**). Individual ferritin concentration ranged from 1.3 to 548.4 ng/mL (mean =  $86.5 \pm 89.3$  ng/mL). There were statistically significant differences ( $p = 0.0000133$ ) between men (10.9 to 548.4 ng/mL, mean =  $86.5 \pm 89.3$  ng/mL) and women (1.3 to 191.5 ng/mL, mean =  $46.8 \pm 53.8$  ng/mL).



**Figure 8.** Age versus ferritin concentration in sample cohort.

**4.2.3 Percent Transferrin Saturation (%TS).** Individual transferring saturations ranged from 4.28 to 96.53% (mean %TS =  $27.55 \pm 14.38$ ) (**Figure 9**). There were statistically significant differences ( $p = 0.0000162$ ) between men (6.78 to 96.53%, mean %TS =  $33.21 \pm 15.08$ ) and women (4.28 to 57.57%, mean %TS =  $21.08 \pm 10.39$ ).



**Figure 9.** Age versus % transferrin saturation (%TS) in sample cohort.

### 4.3 Regression Analysis of Ungrouped Data

All data were the average of duplicate determinations. Analyses were completed using Minitab 15 Statistical Software™ from Minitab Inc and MATLAB® Statistics Toolbox™ from The

MathWorks, Inc. Regression analyses were performed using either  $\alpha$ GST or  $\pi$ GST concentration as response variable and age, gender, transferrin, sTfR, iron, TIBC and %TS as indicator variables. Analyses required transformation of the response variables to satisfy the assumptions of normality and equal variance.

**4.3.1  $\alpha$ GST Data.** Regression analysis was performed with serum  $\alpha$ GST concentrations as the response variable with age, gender, transferrin, sTfR, iron TIBC, %ISAT, and ferritin as the predictors. The analysis required a transformation of the response variable in order to satisfy assumptions of normality and equal variance (Tables 2-4). After transformation of the response variable, the parameters of age, sTfR, iron and %TS were identified as significant predictors. The  $R^2$  value for the regression model was a very low 25.5%. A positive association was identified between age and  $\alpha$ GST concentration, indicating that increased age is associated with higher  $\alpha$ GST concentrations in serum (**Figure 10**). Negative associations were identified between transformed  $\alpha$ GST concentration and serum iron and (**Figure 11**) as well as %TS (**Figure 12**). However, in all four cases the correlation is very weak. Unlike the linear associations observed with age, serum iron and %TS as indicator variables, an increase in transformed  $\alpha$ GST concentration values was only observable using a quadratic regression model with sTfR as an indicator variable (**Figure 13**).

**Table 2.**  $\alpha$ GST Regression Plot Parameters: Summary of Fit.

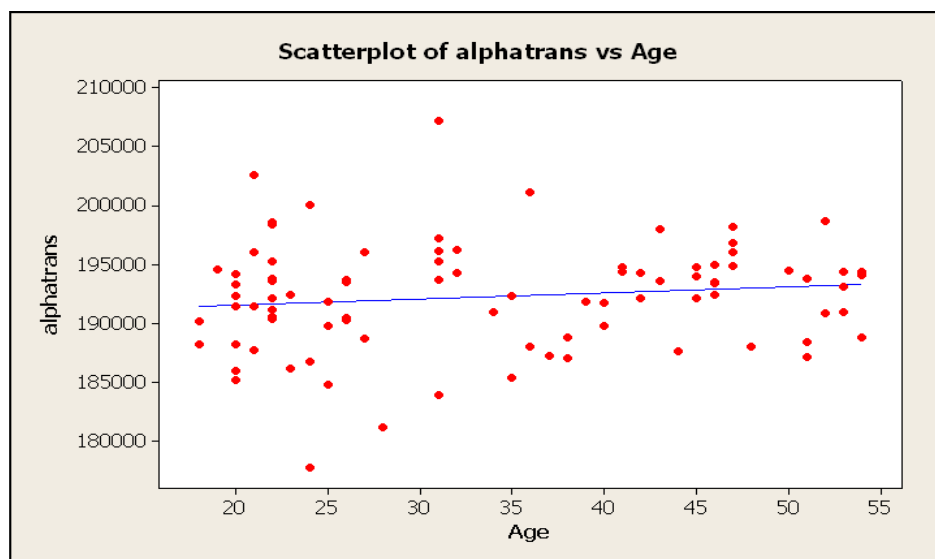
| Summary of Fit                |          |
|-------------------------------|----------|
| RSquare                       | 0.255572 |
| RSquare Adjusted              | 0.192023 |
| Root Mean Square Error        | 4076.842 |
| Mean of Response              | 192214.3 |
| Observations (or Sum Weights) | 90       |

**Table 3.**  $\alpha$ GST Regression Plot Parameters: Analysis of Variance.

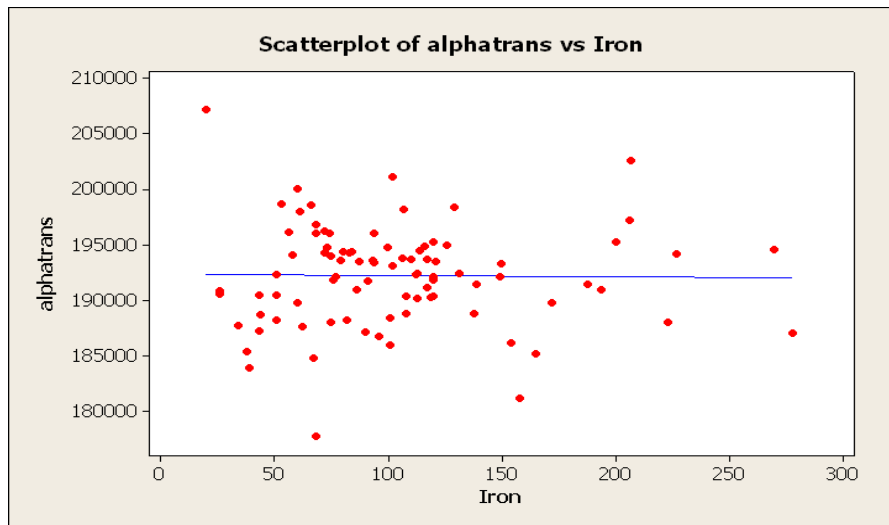
| Analysis of Variance |    |                |             |          |
|----------------------|----|----------------|-------------|----------|
| Source               | DF | Sum of Squares | Mean Square | F Ratio  |
| Model                | 7  | 467898812      | 66842687    | 4.0217   |
| Error                | 82 | 1362892527     | 16620641    | Prob > F |
| C. Total             | 89 | 1830791339     |             | 00.0008* |

**Table 4.**  $\alpha$ GST Regression Plot Parameters: Parameter Estimates.

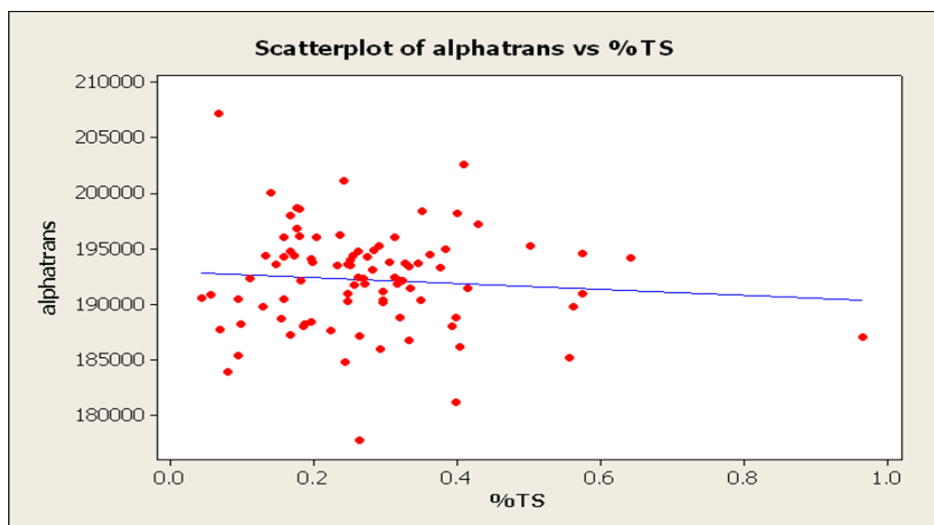
| Parameter Estimates           |           |           |         |           |
|-------------------------------|-----------|-----------|---------|-----------|
| Term                          | Estimate  | Std Error | t Ratio | Prob >  t |
| Intercept                     | 189732.61 | 2438.203  | 77.82   | <0001*    |
| Age                           | 76.891162 | 40.82519  | 1.88    | 0.0632    |
| sTfR                          | -472.39   | 1091.17   | -0.43   | 0.6662    |
| (sTfR-1.3534)*(sRfR-1.36534)  | 1173.0051 | 491.3059  | 2.39    | 0.0193*   |
| Iron                          | 31.164953 | 22.04088  | -3.06   | 0.0030*   |
| (Age-33.7889)* (Iron-103.411) | 0.0020047 | 0.001101  | 1.82    | 0.0720    |
| Age-33.7889)* (%ISAT-0.27548) | 1821.993  | 702.5931  | 2.59    | 0.0113*   |



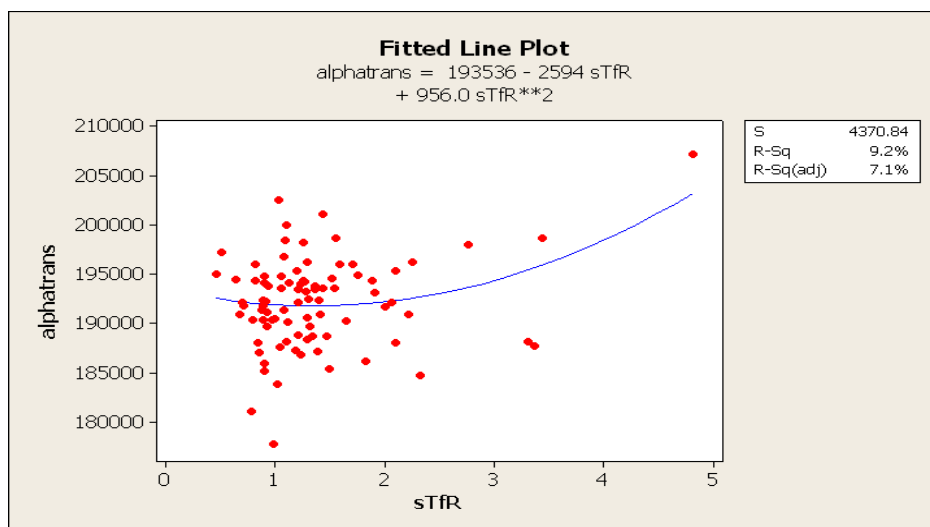
**Figure 10.** Age versus Transformed  $\alpha$ GST concentration values (alphasatrans).



**Figure 11.** Serum iron versus Transformed  $\alpha$ GST concentration values (alphasatrans).

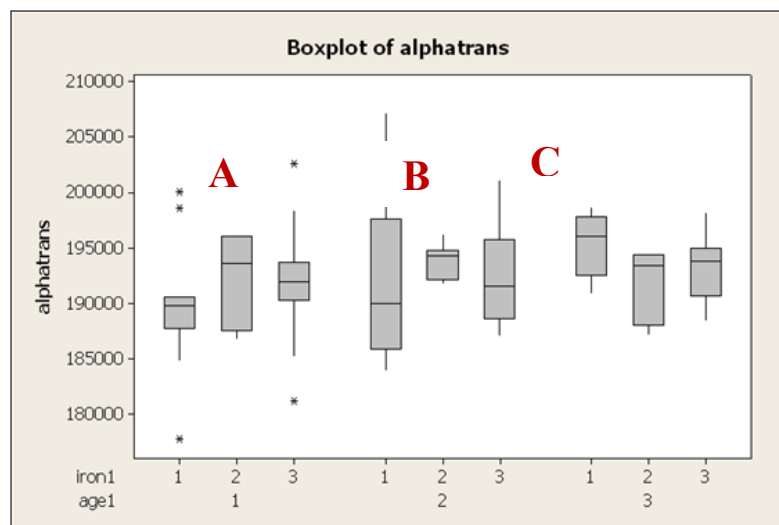


**Figure 12.** %TS versus Transformed  $\alpha$ GST Concentration values (alphasatrans).

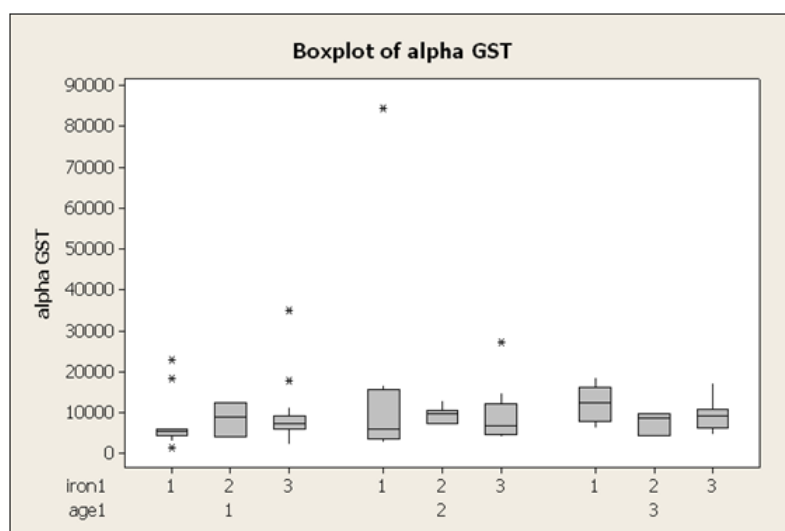


**Figure 13.** sTfR versus Transformed  $\alpha$ GST Concentration values (alphatrans).

**4.3.2 Ungrouped  $\alpha$ GST Concentration Values: Interactions with Age and Iron.** It was shown that the interactions between age and iron, and between age and %ISAT, were significant predictors of  $\alpha$ GST concentrations in the serum. In order to create a pictorial representation of these interactions, age and iron were placed into three groups, numbered 1 to 3 in increasing order against transformed  $\alpha$ GST values (alphatrans) (**Figure 14**). In this graph, boxes indicate interquartile ranges as well as the medians (horizontal line inside box). Outliers are indicated as dots. In Figure 14, nested plots of  $\alpha$ GST by age and iron can be seen. In the first two age groups,  $\alpha$ GST is seen to peak at the middle concentration of iron (**Figures 14A and 14B**). In the oldest age group, the  $\alpha$ GST peaked at the lowest concentration of iron (**Figure 14C**). This trend was also seen using the same parameters against untransformed  $\alpha$ GST values (**Figure 15**).

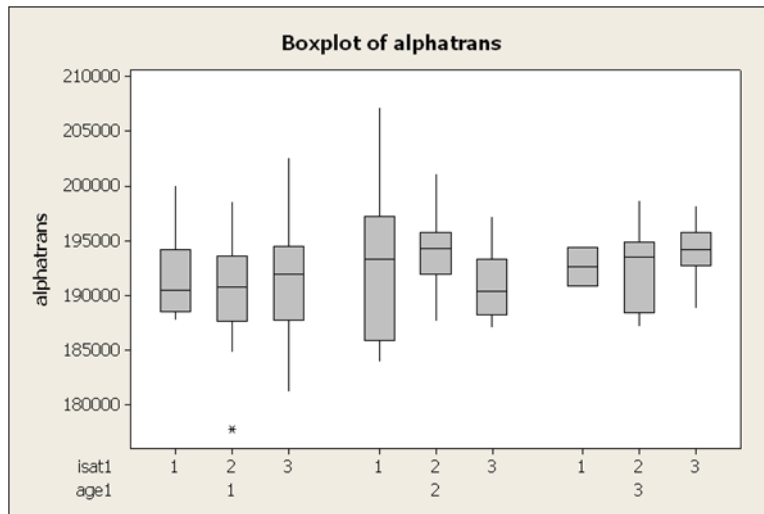


**Figure 14.** Box plot of Transformed  $\alpha$ GST (alphas) with age and iron.



**Figure 15.** Boxplot of  $\alpha$ GST with age and iron.

**4.3.3 Ungrouped  $\alpha$ GST Concentration Values: Interactions with Age and %ISAT.** A similar examination of the interactions of transformed  $\alpha$ GST with age and %ISAT indicate that  $\alpha$ GST levels increased with %ISAT within the first and third age groups (**Figure 16**). In the middle age group,  $\alpha$ GST peaked at the middle level of %ISAT. The same results were also seen using a boxplot analysis of untransformed  $\alpha$ GST with age and %ISAT (data not shown).



**Figure 16.** Boxplot of transformed  $\alpha$ GST (alphasatrans) with age and %ISAT.

**4.3.4 Correlations with Ungrouped Plasma  $\pi$ GST Concentrations.** Similar regression analyses were completed for the  $\pi$ GST data. The analyses required transformation of the response variable ( $\pi$ GST concentration) to satisfy the regression assumptions. Using regression analysis for transformed  $\pi$ GST values resulted in a quadratic model with serum iron levels identified as the only significant predictor of  $\pi$ GST concentration (**Figure 13, Tables 5-7**). In order to obtain this quadratic model, the significance was relaxed to  $\alpha = .01$ . However, the  $R^2$  for the developed  $\pi$ GST model was only 6.9%.

**Table 5.**  $\pi$ GST Regression Plot Parameters: Summary of Fit.

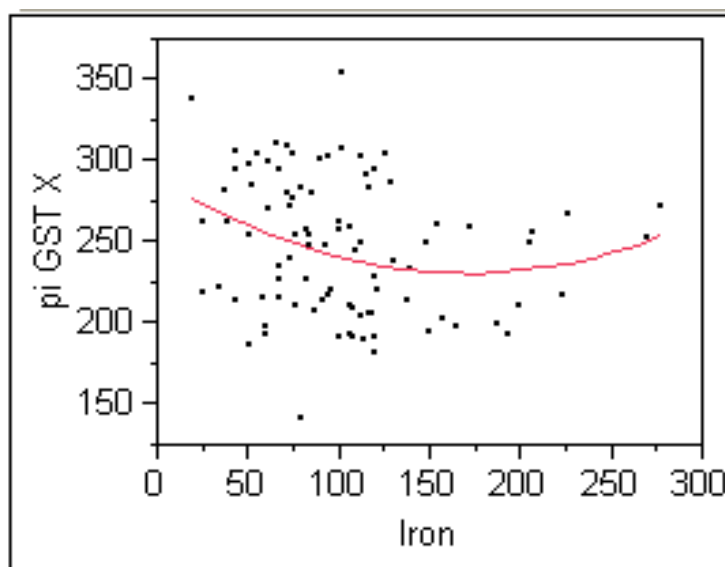
| Summary of Fit             |          |
|----------------------------|----------|
| RSquare                    | 0.06971  |
| RSquare Adjusted           | 0.048324 |
| Root Mean Square Error     | 41.06229 |
| Mean of Response           | 245.0576 |
| Observations (or Sum Wgts) | 90       |

**Table 6.**  $\pi$ GST Regression Plot Parameters: Analysis of Variance

| Analysis of Variance |    |                |             |          |
|----------------------|----|----------------|-------------|----------|
| Source               | DF | Sum of Squares | Mean Square | F Ratio  |
| Model                | 2  | 10992.17       | 5496.09     | 3.2596   |
| Error                | 87 | 146691.73      | 1686.11     | Prob > F |
| C. Total             | 89 | 157683.91      |             | 0.0431*  |

**Table 7.**  $\pi$ GST Regression Plot Parameters: Parameter Estimates.

| Parameter Estimates           |           |           |         |           |
|-------------------------------|-----------|-----------|---------|-----------|
| Term                          | Estimate  | Std Error | t Ratio | Prob >  t |
| Intercept                     | 267.62111 | 10.41809  | 25.69   | .0001*    |
| Iron                          | -0.270286 | 0.106658  | -2.53   | 0.0131*   |
| (Iron-103.411)*(Iron-103.411) | 0.0020047 | 0.001101  | 1.82    | 0.0720    |



**Figure 17.** Regression plot of serum iron versus Transformed  $\pi$ GST Concentration values (pi GST X).

**4.3.5 Ungrouped  $\pi$ GST Data: Other Modeling Efforts.** Multiple iterations of model fitting were run in an attempt to establish associations between plasma  $\pi$ GST concentrations and the remaining indicator variables; however, no statistically significant relationships were identified. While the correlation of sTfR concentration with  $\pi$ GST concentrations were determined to be significant prior to transformation, no such significance was observed applying either linear or quadratic models with transformed  $\pi$ GST concentration values.

#### 4.4 Multiple Linear Regression Analysis of Binned Data.

**4.4.1 Binning Data into Normal and Elevated.** Data were separated into two groups, elevated and normal, for all parameters studied. Published clinical data on normal and elevated concentrations were used to select the cutoff values to delineate between the two groups (Table 8). Analyses were completed using MATLAB® Statistics Toolbox™ from

MathWorks, Inc. Regression analyses were performed using either  $\alpha$ GST or  $\pi$ GST concentration as response variable and age, gender, transferrin, sTfR, iron, TIBC and %TS as indicator variables. The indicator variables were close to normally-distributed, so no transformation was required. All data were the average of duplicate determinations.

**Table 8.** Cutoff Levels Used for Binning. Concentrations above these levels were binned into Elevated.

| Parameter    | Elevated Levels                          |
|--------------|------------------------------------------|
| $\alpha$ GST | > 10 $\mu$ g/L                           |
| $\pi$ GST    | > 100 $\mu$ g/L (plasma)                 |
| sTfR         | >1.5 $\mu$ g/mL                          |
| Transferrin  | >3600 $\mu$ g/mL                         |
| Iron         | > 175 $\mu$ g/L                          |
| TBIC         | >450 $\mu$ g/dL                          |
| %ISAT        | >50% (males)<br>>45% (females)           |
| Ferritin     | >250 ng/mL (male)<br>>160 ng/mL (female) |

**4.4.2  $R^2$  and Regression Coefficients of Binned Data.** The  $R^2$  values were generated using multiple linear regression of the binned data. In examining  $\alpha$ GST values as the response variable, two sets generated an  $R^2 > 0.8$  ( $\alpha$ GST vs. normal transferrin;  $\alpha$ GST vs. normal ferritin), indicating that in these models over 80% of the variance could be accounted for by the indicator parameters (**Table 9**).  $R^2$  values for  $\alpha$ GST regression models were seen to be higher overall than those seen in  $\pi$ GST using similar modeling techniques (**Table 10**). However, both  $\alpha$ GST and  $\pi$ GST statistical models indicate significant associations with GST, normal transferrin, elevated sTfR, and elevated iron.

The regression coefficient values represent a change in the response variable given a 1 unit change in the stated predictor parameter. Interestingly, regression coefficient values from  $\alpha$ GST analyses indicate a greater sensitivity in sTfR and iron than those seen in  $\pi$ GST statistical models.

**Table 9.**  $R^2$  and Correlation Coefficients using  $\alpha$ GST Concentrations as the predictor.

|                                        | $R^2$                                                                                     | $\alpha$ GST Regression coefficients |              |          |          |          |          |             |
|----------------------------------------|-------------------------------------------------------------------------------------------|--------------------------------------|--------------|----------|----------|----------|----------|-------------|
|                                        |                                                                                           | Age                                  | sTfR         | Iron     | IBCT     | Ferritin | ISAT     | Transferrin |
| $\alpha$ GST vs. elevated $\alpha$ GST | 0.7361                                                                                    | -0.22025                             | 4.034467615  | 0.577892 | -0.16586 | 0.071929 | -2.42517 | -0.00109    |
| $\alpha$ GST vs. elevated ferritin     | Too few variables                                                                         |                                      |              |          |          |          |          |             |
| $\alpha$ GST vs. elevated IBCT         | 0.1934                                                                                    | -0.0374                              | -0.644319432 | 0.118352 | -0.02208 | -0.00162 | -0.42234 | 0.000352    |
| $\alpha$ GST vs. elevated iron         | 0.7734                                                                                    | -2.4373                              | -7.513862534 | -0.88889 | 0.507174 | 0.217527 | 3.685244 | 0.003738    |
| $\alpha$ GST vs. elevated ISAT         | Rank deficient - predictor variables are not independent probably due to too few samples. |                                      |              |          |          |          |          |             |
| $\alpha$ GST vs. elevated $\pi$ GST    | 0.5301                                                                                    | -101.915                             | 7977.602985  | 250.6775 | -27.9586 | 47.15244 | -885.503 | -0.45054    |
| $\alpha$ GST vs. elevated sTfR         | 0.6895                                                                                    | 69.73334                             | 8269.712198  | 369.777  | -98.1864 | 67.22313 | -1634.41 | 0.367387    |
| $\alpha$ GST vs. elevated transferrin  | 0.1168                                                                                    | 0.028524                             | 0.828658745  | 0.116978 | -0.01914 | N/A      | -0.41337 | 0.000126    |
| $\alpha$ GST vs. normal $\alpha$ GST   | 0.1511                                                                                    | 0.063258                             | -0.530135475 | 0.022495 | 0.000581 | -0.00024 | -0.07461 | 0.000107    |
| $\alpha$ GST vs. normal ferritin       | 0.0816                                                                                    | 0.049496                             | 0.651131291  | 0.11084  | -0.01736 | -0.00339 | -0.38415 | -0.00016    |
| $\alpha$ GST vs. normal IBCT           | 0.4601                                                                                    | -0.0882                              | 7.288708621  | 0.031478 | -0.01167 | 0.032575 | -0.21583 | -0.00067    |
| $\alpha$ Gst vs. normal iron           | 0.4295                                                                                    | -0.05367                             | 5.2660233    | 0.150148 | -0.03615 | 0.03645  | -0.77781 | -0.00059    |
| $\alpha$ GST vs. normal ISAT           | 0.3924                                                                                    | -0.05288                             | 5.129834215  | 0.257796 | -0.0514  | 0.042947 | -1.12295 | -0.00023    |
| $\alpha$ GST vs. normal $\pi$ GST      | 0.1245                                                                                    | 70.05476                             | -340.7989315 | 12.69368 | -9.32598 | -5.31989 | -119.847 | -0.15562    |
| $\alpha$ GST vs. normal sTfR           | 0.1191                                                                                    | 14.9016                              | 3771.840539  | 127.8873 | -16.3882 | 0.680121 | -416.988 | -0.0799     |
| $\alpha$ GST vs. normal transferrin    | 0.8914                                                                                    | -0.33987                             | 12.84481277  | 0.130527 | 0.00693  | 0.106365 | -0.00686 | -0.01082    |

**Table 10.**  $R^2$  and Correlation Coefficients using  $\pi$ GST concentration as the predictor.

|                                     | $R^2$                                                                                    | $\pi$ GST Regression coefficients |              |          |          |          |          |             |
|-------------------------------------|------------------------------------------------------------------------------------------|-----------------------------------|--------------|----------|----------|----------|----------|-------------|
|                                     |                                                                                          | Age                               | sTfR         | Iron     | IBCT     | Ferritin | ISAT     | Transferrin |
| $\pi$ GST vs. elevated $\alpha$ GST | 0.4387                                                                                   | -0.39104                          | -2.22881228  | 3.151102 | -1.00508 | 0.081409 | -13.4464 | -0.00508    |
| $\pi$ GST vs. elevated ferritin     | Too few variables                                                                        |                                   |              |          |          |          |          |             |
| $\pi$ GST vs. elevated IBCT         | 0.1669                                                                                   | 0.416644                          | -8.511734572 | 0.791773 | -0.28428 | -0.09969 | -4.26459 | 0.003143    |
| $\pi$ GST vs. elevated iron         | 0.7409                                                                                   | -3.98427                          | -28.28382823 | -0.09218 | 0.432    | 0.268939 | 3.243887 | -0.00034    |
| $\pi$ GST vs. elevated ISAT         | Rank deficient - predictor variables are not independent probably due to too few samples |                                   |              |          |          |          |          |             |
| $\pi$ GST vs. elevated $\pi$ GST    | 0.3364                                                                                   | 0.360959                          | 4.680361696  | 0.107789 | -0.17711 | 0.022297 | -0.82216 | -0.00126    |
| $\pi$ GST vs. elevated sTfR         | 0.5762                                                                                   | 0.680597                          | -4.854234531 | 3.595405 | -0.68847 | 0.006954 | -16.1723 | -0.00997    |
| $\pi$ GST vs. elevated transferrin  | 0.1009                                                                                   | 0.337077                          | 6.514631029  | 0.021304 | -0.07948 | N/A      | -0.47716 | -0.00273    |
| $\pi$ GST vs. normal $\alpha$ GST   | 0.1475                                                                                   | 0.783888                          | -3.009125144 | -0.35732 | 0.003646 | -0.02965 | 0.764065 | -0.00393    |
| $\pi$ GST vs. normal ferritin       | 0.0898                                                                                   | 0.479301                          | 5.824376335  | 0.220123 | -0.10381 | -0.04035 | -1.19933 | -0.00266    |
| $\pi$ GST vs. normal IBCT           | 0.1678                                                                                   | 0.175588                          | 13.35431035  | -0.0998  | -0.03999 | 0.042648 | -0.29357 | -0.00482    |
| $\pi$ GST vs. normal iron           | 0.1745                                                                                   | 0.361015                          | 6.267600779  | 0.678581 | -0.22394 | 0.066079 | -3.8235  | -0.00188    |
| $\pi$ GST vs. normal ISAT           | 0.183                                                                                    | 0.379139                          | 6.040854725  | 0.928046 | -0.28543 | 0.037831 | -5.00855 | -0.00191    |
| $\pi$ GST vs. normal $\pi$ GST      | 0.0541                                                                                   | 0.22978                           | 4.312140608  | 0.154746 | -0.01867 | -0.03007 | -0.62846 | -0.00102    |
| $\pi$ GST vs. normal sTfR           | 0.0547                                                                                   | 0.408309                          | -10.26627365 | -0.08468 | -0.04275 | -0.00209 | -0.06139 | -0.00162    |
| $\pi$ GST vs. normal transferrin    | 0.6147                                                                                   | 0.983792                          | 5.755937998  | 6.737295 | -1.51798 | 0.251232 | -21.2758 | -0.01043    |

## 5. CONCLUSIONS

The purpose of the original study was to develop a human model of sub-clinical hepatic damage to be used for pre-validation of in-house biomarker discoveries. In the course of reviewing existing literature on the association of GST and iron components in support of our HFE model, we discovered a lack of relative data on the range of GST levels and associations with iron and related iron parameters. In theory, abnormally high concentrations of cellular iron should result in some level of liver damage which in turn should be signaled by elevated GST levels in the blood. However, for accurate model development it was necessary to examine other factors which impact baseline levels of GST (such as age and gender) as well determine the exact nature of iron status as correlated with  $\alpha$  and  $\pi$  GST levels in the blood. Matched human serum/plasma samples were analyzed for GSTs and iron parameter concentrations to provide additional data in fine tuning this potential testing model for future use in biomarker validation, especially needed for validation of markers to uncharacterized/mixed toxin exposures (original data presented in **Appendix A**).

In the original study, individuals with an elevated %TS did have elevated liver enzyme activity, with the exception of  $\alpha$ GST and alanine aminotransferase (ALT). In this report, significant correlations with iron and iron components were seen with  $\alpha$  and  $\pi$  GST levels. In addition, a basic understanding of the confounding factors of age and gender of GST biomarker levels was established.

### 5.1 Ungrouped Data

**5.1.1 Iron Parameter Sample Analyses.** The iron parameter concentrations in the collected cohort samples were examined for gender differences. Measurements of serum transferrin and sTfR levels indicated no statistically significant differences between male/female samples. However, significant gender differences were seen in the cohort sample set in serum levels of iron, TIBC, ferritin, and %TS.

**5.1.2 GST Sample Analyses.** Examination of both  $\alpha$ GST and  $\pi$ GST demonstrated no statistically significant differences between male and female samples when analyzed from serum and plasma, respectively.

**5.1.3 Regression Analysis of Data Sets.** Regression analysis with ungrouped data using  $\alpha$ GST as a response variable identified the interaction between age and iron as well as between age and %ISAT as significant predictors of  $\alpha$ GST (**Figure 16**). This statement is more relevant than stating that  $\alpha$ GST increases with age and iron but decreases with %ISAT. Plots of untransformed  $\alpha$ GST data indicated the same trends.

Similar analyses for  $\pi$ GST resulted in a quadratic model with only iron as a significant predictor (**Figure 17**). In this model, a transformation of the response variable was required to satisfy the regression assumptions. In order to get this model, the significance was relaxed

to  $\alpha = 0.10$ , giving The output for this model showed that, using transformed  $\pi$ GST data, the  $R^2$  for the quadratic model was only a very low 6.9%.

## 5.2 Binned Data

When the data sets have been binned into ‘Elevated’ versus ‘Normal’ groupings based on current clinical delineations, higher  $R^2$  values and stronger significance is seen using  $\alpha$ GST as a response variable (**Table 9**). Using binned data, elevated levels of iron and sTfR are strongly significant predictors of elevated  $\alpha$ GST levels (0.7734 and 0.6895, respectively), but not normal levels. Circulating sTfR has been seen to increase with iron requirements or cellular growth.<sup>27</sup> and is reflective of the status of tissue iron stores. The sTfR is elevated under conditions of iron deficiency. Therefore increased sTfR may be indicative of a physiological injury reflecting a need for increased cellular iron, and the concomitant increase in  $\alpha$ GST may be reflective of primary or secondary injury. No medical data was collected on the cohort samples, therefore no conclusions can be drawn regarding associations with physiological conditions.

It has been seen that transcriptional induction of a GST isoform upon iron overload is thought to be a consequence of the production of ROS via an antioxidant responsive element (ARE) in the promoter region of the gene. Due to limited data for binned elevated ferritin, no definitive conclusion could be made in regards to its correlation with GST expression. Published data indicates that use of ferritin levels may be of limited value in determining iron status, especially in chronic disease states, unless different cutoff values are used.<sup>28</sup> Therefore, a more thorough examination of the correlation of  $\alpha$ GST and ferritin would require information on subject health for more accurate binning of the ferritin data.

Unlike the ungrouped data analyses, statistically significant associations can be seen with  $\pi$ GST albeit not as stringent (**Table 10**). Analysis also indicates that both  $\alpha$  and  $\pi$  GST levels associate with normal transferrin concentrations in the blood. However, significant association is lost for both GST variants when analyzed against elevated transferrin.

The data presented here does not support a relationship between  $\alpha$ GST and  $\pi$ GST levels as the  $R^2$  values do not indicate correlation of the two GST variant concentrations in the blood.

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## 7. LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

|              |                                   |
|--------------|-----------------------------------|
| %ISAT        | percent iron saturation           |
| ROS          | reactive oxygen species           |
| HFE          | hemochromatosis                   |
| GST          | glutathione-S-transferase         |
| $\pi$ GST    | pi glutathione-S-transferase      |
| $\alpha$ GST | alpha glutathione-S-transferase   |
| sTfR         | soluble transferrin receptor      |
| ELISA        | enzyme-linked immunosorbant assay |
| EIA          | enzyme immunoassay                |
| OD           | optical density                   |
| N            | Normality                         |
| M            | Molar concentration               |
| TIBC         | total iron-binding capacity       |
| %TS          | percent transferrin saturation    |
| Wgts         | weights                           |
| $\mu$ L      | microliter(s)                     |
| mL           | milliliter(s)                     |

## 8. APPENDICE A: COHORT DATA

| Sample number | Age     | Gender | $\alpha$ GST | $\pi$ GST | Transferrin | sTfR    | Iron    | TIBC    | %ISAT     | Ferritin |
|---------------|---------|--------|--------------|-----------|-------------|---------|---------|---------|-----------|----------|
| Units         | (years) |        | (ng/L)       | (mg/L)    | (mg/mL)     | (mg/mL) | (mg/dL) | (mg/dL) | Iron/TIBC | (ng/dL)  |
| 1             | 51      | F      | 4650.4       | 133.51    | 7359.377    | 1.294   | 101     | 513     | 19.69%    | 10.2     |
| 2             | 54      | M      | 9850.3       | 124.58    | 4116.218    | 1.256   | 84      | 489     | 17.18%    | 50.9     |
| 3             | 36      | M      | 4450.0       | 88.46     | 5094.696    | 0.836   | 223     | 568     | 39.26%    | 111.6    |
| 4             | 27      | F      | 12435.7      | 143.88    | 3651.099    | 1.588   | 74      | 466     | 15.88%    | 85.0     |
| 5             | 35      | F      | 3314.5       | 155.39    | 8667.215    | 1.49    | 38      | 404     | 9.41%     | 36.8     |
| 6             | 45      | F      | 9455.6       | 149.61    | 9805.218    | 1.234   | 75      | 300     | 25.00%    | 29.3     |
| 7             | 31      | F      | 8977.7       | 80.21     | 5366.211    | 1.357   | 117     | 358     | 32.68%    | 178.4    |
| 8             | 28      | F      | 2122.6       | 76.98     | 3855.453    | 0.78    | 158     | 396     | 39.90%    | 21.3     |
| 9             | 43      | M      | 8917.2       | 36.97     | 5196.056    | 1.433   | 79      | 320     | 24.69%    | 175.9    |
| 10            | 31      | M      | 11258.8      | 61.14     | 5034.653    | 1.199   | 120     | 413     | 29.06%    | 177.0    |
| 11            | 35      | F      | 7552.2       | 124.79    | 4620.636    | 1.402   | 51      | 464     | 10.99%    | 31.9     |
| 12            | 48      | M      | 4473.9       | 184.88    | 4345.473    | 2.094   | 75      | 405     | 18.52%    | 125.9    |
| 13            | 37      | F      | 4065.6       | 185.45    | 4556.746    | 1.183   | 43      | 258     | 16.67%    | 36.7     |
| 14            | 53      | F      | 6342.1       | 154.03    | 6731.956    | 1.405   | 86      | 347     | 24.78%    | 95.2     |
| 15            | 24      | M      | 3874.8       | 91.36     | 4503.392    | 1.231   | 96      | 288     | 33.33%    | 194.7    |
| 16            | 24      | M      | 1530.9       | 170.88    | 3949.13     | 0.975   | 68      | 258     | 26.36%    | 179.0    |
| 17            | 26      | F      | 5984.5       | 64.48     | 9323.175    | 0.991   | 51      | 545     | 9.36%     | 2.2      |
| 18            | 25      | M      | 3096.4       | 104.66    | 3599.96     | 2.323   | 67      | 275     | 24.36%    | 48.5     |
| 19            | 31      | M      | 2816.1       | 133.30    | 6069.445    | 1.013   | 39      | 489     | 7.98%     | 18.0     |
| 20            | 21      | F      | 4329.2       | 93.65     | 7007.041    | 3.368   | 34      | 493     | 6.90%     | 4.0      |
| 21            | 24      | F      | 22810.2      | 70.28     | 5776.7      | 1.098   | 60      | 427     | 14.05%    | 29.3     |
| 22            | 32      | M      | 12905.7      | 190.35    | 5249.079    | 2.248   | 72      | 305     | 23.61%    | 124.4    |
| 23            | 31      | F      | 12748.3      | 184.04    | 6545.747    | 1.295   | 56      | 311     | 18.01%    | 65.5     |
| 24            | 52      | F      | 6290.0       | 133.69    | 6216.127    | 2.215   | 26      | 456     | 5.70%     | 15.1     |
| 25            | 20      | M      | 3531.5       | 68.26     | 5285.57     | 0.898   | 101     | 345     | 29.28%    | 69.6     |
| 26            | 27      | F      | 4802.9       | 171.72    | 5474.081    | 1.466   | 44      | 285     | 15.44%    | 21.7     |
| 27            | 46      | M      | 7650.7       | 181.27    | 4132.431    | 1.296   | 113     | 361     | 31.30%    | 131.4    |
| 28            | 26      | F      | 5902.3       | 171.9     | 3358.738    | 0.791   | 120     | 344     | 34.88%    | 39.0     |
| 29            | 51      | M      | 4022.7       | 181.08    | 5344.261    | 1.382   | 90      | 341     | 26.39%    | 152.7    |
| 30            | 46      | M      | 10761.9      | 184.89    | 3944.567    | 0.456   | 126     | 329     | 38.30%    | 164.1    |
| 31            | 54      | M      | 4894.1       | 68.42     | 4935.952    | 1.202   | 108     | 337     | 32.05%    | 218.8    |
| 32            | 36      | F      | 27126.4      | 188.60    | 2926.242    | 1.429   | 102     | 423     | 24.11%    | 158.1    |
| 33            | 22      | M      | 17785.4      | 160.70    | 3662.471    | 1.089   | 129     | 367     | 35.15%    | 109.0    |
| 34            | 38      | M      | 3979.3       | 144.14    | 1891.878    | 0.855   | 278     | 288     | 96.53%    | 78.1     |
| 35            | 22      | M      | 11251.4      | 83.93     | 7247.631    | 2.091   | 200     | 398     | 50.25%    | 79.6     |

| Sample number | Age     | Gender | $\alpha$ GST | $\pi$ GST | Transferrin | sTfR    | Iron    | TIBC    | %ISAT     | Ferritin |
|---------------|---------|--------|--------------|-----------|-------------|---------|---------|---------|-----------|----------|
| Units         | (years) |        | (ng/L)       | (mg/L)    | (mg/mL)     | (mg/mL) | (mg/dL) | (mg/dL) | Iron/TIBC | (ng/dL)  |
| 36            | 18      | F      | 4535.253     | 176.06    | 7750.308    | 1.095   | 51      | 517     | 9.86%     | 4.3      |
| 37            | 20      | F      | 4552.85      | 128.86    | 4982.38     | 3.309   | 82      | 439     | 18.68%    | 3.3      |
| 38            | 31      | M      | 14871.911    | 119.42    | 7658.101    | 0.512   | 206     | 479     | 43.01%    | 67.1     |
| 39            | 22      | F      | 9111.033     | 130.23    | 9187.192    | 0.932   | 106     | 533     | 19.89%    | 25.0     |
| 40            | 18      | M      | 5740.209     | 119.51    | 4479.619    | 1.111   | 113     | 381     | 29.66%    | 45.1     |
| 41            | 20      | M      | 8559.355     | 71.18     | 6077.5      | 1.278   | 150     | 399     | 37.59%    | 33.7     |
| 42            | 22      | F      | 7329.698     | 119.96    | 3758.439    | 0.698   | 149     | 468     | 31.84%    | 56.9     |
| 43            | 21      | M      | 6710.714     | 74.05     | 6984.819    | 0.878   | 188     | 453     | 41.50%    | 24.4     |
| 44            | 53      | F      | 9945.528     | 117.86    | 9993.548    | 1.877   | 84      | 634     | 13.25%    | 9.7      |
| 45            | 20      | M      | 6724.279     | 103.02    | 8647.55     | 1.079   | 139     | 415     | 33.49%    | 60.1     |
| 46            | 25      | F      | 7088.018     | 99.28     | 5171.3      | 0.89    | 120     | 379     | 31.66%    | 27.3     |
| 47            | 20      | M      | 3234.214     | 73.33     | 6876.747    | 0.895   | 165     | 296     | 55.74%    | 36.4     |
| 48            | 22      | F      | 8919.644     | 118.18    | 7508.549    | 1.054   | 93      | 630     | 14.76%    | 10.7     |
| 49            | 22      | F      | 6046.732     | 90.21     | 6157.433    | 1.292   | 26      | 607     | 4.28%     | 1.3      |
| 50            | 38      | M      | 4860.962     | 86.75     | 4313.116    | 1.335   | 138     | 346     | 39.88%    | 70.9     |
| 51            | 40      | M      | 6990.016     | 86.62     | 5235.917    | 2       | 91      | 356     | 25.56%    | 139.6    |
| 52            | 21      | M      | 35039.773    | 126.43    | 8533.885    | 1.029   | 207     | 506     | 40.91%    | 55.0     |
| 53            | 50      | F      | 10056.187    | 67.61     | 3878.369    | 0.642   | 114     | 315     | 36.19%    | 15.3     |
| 54            | 46      | M      | 8841.081     | 81.38     | 6609.763    | 1.542   | 87      | 346     | 25.14%    | 18.3     |
| 55            | 22      | F      | 5868.465     | 82.59     | 8368.142    | 0.967   | 108     | 365     | 29.59%    | 21.0     |
| 56            | 42      | M      | 7376.247     | 67.99     | 6150.799    | 2.063   | 120     | 371     | 32.35%    | 183.3    |
| 57            | 32      | F      | 9720.984     | 154.13    | 4045.487    | 1.26    | 72      | 456     | 15.79%    | 39.0     |
| 58            | 45      | M      | 7395.383     | 123.83    | 3183.165    | 1.211   | 77      | 423     | 18.20%    | 171.0    |
| 59            | 47      | F      | 10626.367    | 168.05    | 3975.582    | 1.757   | 116     | 408     | 28.43%    | 14.2     |
| 60            | 53      | F      | 8343.258     | 257.26    | 2613.897    | 1.909   | 102     | 363     | 28.10%    | 162.1    |
| 61            | 22      | F      | 18316.968    | 193.86    | 4737.35     | 1.548   | 66      | 367     | 17.98%    | 22.0     |
| 62            | 47      | M      | 17187.647    | 70.28     | 2824.296    | 1.25    | 107     | 267     | 40.07%    | 135.8    |
| 63            | 41      | M      | 9860.095     | 157.88    | 3762.174    | 0.816   | 80      | 315     | 25.40%    | 86.1     |
| 64            | 19      | F      | 10153.525    | 123.37    | 9346.78     | 1.52    | 270     | 469     | 57.57%    | 4.2      |
| 65            | 26      | F      | 5843.738     | 79.20     | 6065.644    | 1.65    | 119     | 483     | 24.64%    | 191.5    |
| 66            | 26      | M      | 8996.359     | 114.42    | 3559.075    | 1.375   | 110     | 319     | 34.48%    | 192.3    |
| 67            | 45      | M      | 10505.471    | 110.18    | 3816.41     | 1.051   | 73      | 438     | 16.67%    | 217.6    |
| 68            | 26      | F      | 8776.702     | 92.06     | 3919.096    | 1.355   | 121     | 519     | 23.31%    | 177.9    |
| 69            | 21      | F      | 12521.522    | 89.11     | 1342.847    | 0.817   | 94      | 460     | 20.43%    | 8.2      |
| 70            | 23      | M      | 7617.456     | 108.47    | 3019.291    | 0.889   | 131     | 499     | 26.25%    | 10.9     |
| 71            | 41      | M      | 10411.102    | 128.83    | 2427.578    | 0.902   | 100     | 383     | 26.11%    | 104.4    |
| 72            | 20      | M      | 9638.724     | 139.10    | 3668.455    | 0.899   | 227     | 354     | 64.12%    | 55.8     |

| Sample number | Age     | Gender | $\alpha$ GST | $\pi$ GST | Transferrin | sTfR    | Iron    | TIBC    | %ISAT     | Ferritin |
|---------------|---------|--------|--------------|-----------|-------------|---------|---------|---------|-----------|----------|
| Units         | (years) |        | (ng/L)       | (mg/L)    | (mg/mL)     | (mg/mL) | (mg/dL) | (mg/dL) | Iron/TIBC | (ng/dL)  |
| 73            | 51      | F      | 9191.311     | 84.32     | 2622.87     | 1.364   | 106     | 347     | 30.55%    | 48.5     |
| 74            | 20      | M      | 7516.914     | 78.89     | 12467.973   | 0.91    | 112     | 416     | 26.92%    | 166.3    |
| 75            | 23      | M      | 3628.479     | 131.13    | 5474.494    | 1.821   | 154     | 382     | 40.31%    | 182.1    |
| 76            | 22      | M      | 6513.543     | 157.19    | 6106.605    | 0.92    | 117     | 394     | 29.70%    | 53.6     |
| 77            | 43      | F      | 16697.142    | 141.83    | 6139.577    | 2.766   | 61      | 365     | 16.71%    | 13.1     |
| 78            | 47      | F      | 13974.136    | 97.87     | 6560.662    | 1.074   | 68      | 386     | 17.62%    | 16.3     |
| 79            | 34      | M      | 6344.88      | 69.28     | 8842.979    | 0.671   | 194     | 337     | 57.57%    | 137.5    |
| 80            | 52      | M      | 18499.399    | 158.80    | 5266.084    | 3.432   | 53      | 302     | 17.55%    | 47.1     |
| 81            | 46      | M      | 8731.867     | 182.72    | 7829.328    | 1.207   | 94      | 282     | 33.33%    | 151.3    |
| 82            | 54      | F      | 9541.21      | 87.53     | 6035.025    | 1.122   | 58      | 296     | 19.59%    | 122.3    |
| 83            | 47      | M      | 12512.786    | 87.35     | 1823.885    | 1.709   | 68      | 217     | 31.34%    | 16.5     |
| 84            | 42      | M      | 9786.145     | 98.19     | 7825.03     | 1.25    | 83      | 302     | 27.48%    | 79.6     |
| 85            | 39      | M      | 7045.813     | 84.31     | 5576.537    | 0.706   | 76      | 280     | 27.14%    | NA       |
| 86            | 44      | F      | 4246.396     | 177.15    | 3668.335    | 1.037   | 62      | 277     | 22.38%    | 42.5     |
| 87            | 40      | M      | 5475.159     | 128.95    | 6307.169    | 0.916   | 172     | 306     | 56.21%    | 548.4    |
| 88            | 31      | M      | 84273.314    | 234.01    | 3202.919    | 4.818   | 20      | 295     | 6.78%     | 434.2    |
| 89            | 25      | F      | 5467.978     | 73.06     | 4153.129    | 1.318   | 60      | 467     | 12.85%    | 17.9     |
| 90            | 22      | F      | 5938.144     | 86.04     | 3795.758    | 0.89    | 43      | 271     | 15.87%    | 50.9     |