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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Difference in the Urinary Peptide Composition between Children at
High Risk for Autism and Children at Low Risk

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Bioengineering

by

Joon Seok Park

Committee in charge:

Professor Geert Schmid-Schönbein, Chair
Professor Pedro Cabrales
Professor Karen Dobkins

2013

The Thesis of Joon Seok Park is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2013

DEDICATION

To Jessica, my lovely wife,
whose selfless love and care for me humble me every day;

To my parents,
for teaching me to be the man I am today;

To all the other people in my life whom I love dearly;

And above all,
may all the glory be to God the Father and His Son Jesus Christ,
whose sin-atoning sacrifice saved my soul for eternity.

EPIGRAPH

Rejoice always, pray without ceasing, give thanks in all circumstances; for this is the will of God in Christ Jesus for you.

1 Thessalonians 5:16-18

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Finally, I would like to acknowledge Tiffany Lai and Ryota Yoda Shimada for their help in the actual obtaining process of urine samples from participants.

ABSTRACT OF THE THESIS

Difference in the Urinary Peptide Composition between Children at
High Risk for Autism and Children at Low Risk

by

Joon Seok Park

Master of Science in Bioengineering

University of California, San Diego, 2013

Professor Geert Schmid-Schönbein, Chair

Autism spectrum disorder (ASD) is a disorder that hinders the affected individual's language, social, and behavioral development. The causes and origins of ASD have been studied, yet there is no clear consensus. One hypothesis states that ASD is caused by digestive enzymes that escape out of the intestinal lumen due to leaky gut syndrome and enter into the bloodstream, eventually causing damage to the

brain. In this study, I hypothesized that because of the protease activities from these digestive enzymes, urine samples from the High-Risk group contains smaller peptides than those of the Low-Risk group. I devised and tested a method to quantify urinary peptide content, and utilized the method to examine 22 samples in the Low-Risk category and 16 samples in the High-Risk category. The two categories demonstrated a statistically significant difference ($p = 0.044$) in their urinary peptide content, with the High-Risk category containing a higher number of peptides per protein than the Low-Risk. This result suggests the possibility that ASD is associated with proteolytic degradation and production of protein fragments. Further study of a larger number of samples, a more in-depth examination of the urinary peptides, and a consideration of the participants' diet phase are recommended.

CHAPTER 1. INTRODUCTION

1.1 Autism

Most generally, an autism spectrum disorder (ASD) affects an individual by hindering behavioral development, most commonly known for causing deficient language development, poor social abilities, and repetitive behaviors (10). Its prevalence is estimated to be 1.68-4.05 per 1,000 people worldwide, and 4.5-11.0 per 1,000 in the United States; its incidence is estimated at around 1 in 100 children under 17 years old (4). Generally diagnosed by the age of 3 years, ASD has increased in its prevalence significantly in the U.S. in the past few decades, and it is currently the second most common serious developmental disabilities in the United States (6).

The causes and origins of ASD are a topic of immense interest among researchers throughout the world today. Even though the exact causes are unknown, much work has been done to demonstrate genetic as well as environmental factors common to those affected by ASD. For example, it has been shown that increased maternal age is associated with higher odds of an early ASD diagnosis (4). Some other risk factors include the child's sex (boys are at a higher risk for autism than girls), low birth weight, and low gestational age, among others (4). Although the heritability of autism has loosely been established, the exact model of inheritance remains unclear (6). For instance, studies have demonstrated that the sibling recurrence risk of ASD could be as high as 18.7%, compared to the incidence rate of the general population of 1% (7). In other words, among children with an autistic sibling, roughly one in five will be diagnosed with ASD.

1.2 Autism and Gastrointestinal Symptoms

Among the symptoms associated with ASD, the ones most relevant to this study are gastrointestinal (GI) symptoms, such as diarrhea and constipation. Recent studies have found that children diagnosed with autism are 3.5 times more likely to develop GI conditions than children not diagnosed with ASD (8). Other studies have demonstrated that many autistic children suffer from chronic GI conditions such as chronic diarrhea, chronic constipation, and chronic vomiting (5). The possibility of a causal link between ASD and GI conditions has been considered but not thoroughly explored. For example, one study suggested a lack of evidence for causal link between ASD and GI conditions (and diet) because there was no evidence of presence of opioid peptides in the urine of autistic children (2). However, the examination of opioid peptide content in the urine is limited in nature and thus cannot fully disprove the possibility of this causal link.

1.3 Autism and the Autodigestion Theory

In considering the possible link between autism and GI symptoms, one recently developed theory proves especially relevant. The autodigestion theory states that in hemorrhagic shock, which is a medical condition that often leads to multi-organ failure and death, the walls of the small intestine become permeable, allowing digestive enzymes in the lumen of the intestine to pass through and enter the bloodstream (9). In the context of shock, these enzymes then travel to different organs through the bloodstream and cleave essential proteins. Various studies have been conducted within the lab where the theory originated. For example, one study showed

that the mucosal barrier within the lumen of the small intestine loses its integrity during ischemia (which leads to shock) in rats, which demonstrated that digestive enzymes were able to pass through the walls of the intestine, a phenomenon also known as “leaky gut” syndrome (3). Another study observed that in the event of shock in rats, protease activity increased in the plasma as well as vital organs (1). These studies have provided strong evidence for the autodigestion theory as the correct mechanism for the multi-organ failure in shock.

As it relates to ASD, the autodigestion theory may give a significant insight into the etiology of ASD. The overarching hypothesis that this study at least partially seeks to address is that ASD is caused by digestive enzymes that escape out of the intestinal lumen due to leaky gut syndrome and enter into the bloodstream, eventually causing damage to the brain, which leads to ASD.

1.4 Assumptions and Definitions

In order to examine this hypothesis, we first assumed that ASD develops post-birth. This was due to the fact that there is no plausible reason why the fetus’ intestine would undergo leaky gut syndrome. We assumed that if the hypothesis were true, then there exists some factor after birth that causes the infant’s intestine to undergo leaky gut syndrome, factors such as diet.

Secondly, we assumed that any protease activity in the plasma or in organs due to the leaky gut syndrome would leave evidence in the blood, and consequently, in the urine. This is plausible because as the kidneys filter the blood and produce urine, any excess proteins and peptides present in the blood would lead to a corresponding

increase in the peptide concentration of the urine. In order to fully prove the hypothesis, this assumption would need to be demonstrated scientifically; However, for the purpose of this study, this assumption was made because urine samples from infants and children are much easier to obtain than blood samples, and for the scope of this study, examination of urine samples was more appropriate than that of blood samples. This will be discussed further in Chapter 4, Discussion.

Thirdly, we assumed that the samples collected from the subjects were sufficiently randomized. Although samples were volunteered and collected on a case-by-case basis from children whose parents were comfortable with participating in this study, there is no apparent reason to assume a necessary bias one way or another. We do acknowledge, however, that the samples collected were from subjects within the San Diego area. Ideally, the subjects should be chosen truly randomly, but the limitations of time and resources called for a more convenient method of sample collection.

Also, we define two categories in which we divided the samples: High-Risk (HR) and Low-Risk (LR). The HR category consists of infants and children who have at least one autistic older sibling. The LR category consists of all others, who do not have an autistic older sibling. This categorization was necessary because our study seeks to address the cause of ASD, which means that we cannot readily use samples from subjects who are already diagnosed with ASD. In order to examine the cause, we needed to examine samples from subjects who have not yet developed ASD and are at risk for developing it eventually.

Lastly, we define three diet categories under which the subjects fell: breast milk only (BMO), breast milk and formula (MBM), and formula (NBM). As mentioned above, a possible cause of the leaky gut syndrome in children may be their diet, so in order to examine this possibility, a definition of the subjects' typical diet phases was required.

1.5 Goals of This Investigation

Since there currently exists little evidence supporting the hypothesis stated above, this study was designed to serve as a pilot study to examine whether further investigation of the hypothesis would be beneficial in the study of the etiology of ASD. The overall aim of this study was to provide initial evidence for the hypothesis that digestive enzymes from the lumen of the small intestines are involved in the mechanism that leads to ASD.

1.5.1 Objective

The objective of this study was to compare the composition of urinary peptides of LR children versus HR children.

1.5.2 Hypothesis

We hypothesized that the HR children's urine contains smaller peptides than LR children's urine due to protease activity in the HR children.

1.5.3 Specific Aims

Our specific aims were as follows:

- 1) Develop an inexpensive, efficient method to determine the mass concentration of protein as well as number of peptides in a urine sample.

- 2) Utilize this method to measure the peptide composition of LR and HR urine samples.

CHAPTER 2. MATERIALS AND METHODS

2.1 Collection and Storage of Urine Samples

The collection of samples occurred as part of a separate study by a different group within University of California, San Diego. Permission to collect urine samples was obtained from parents of the subjects who were involved in this study. Upon collection, the urine sample was transported to the laboratory and stored at -80°C.

2.2 Preparation of the Urine Sample via Acetone Precipitation

The method chosen to prepare the urine sample was precipitating the protein out of the sample using acetone. 250 µl of urine samples were pipetted into 2-ml Eppendorf tubes, along with 1 ml of acetone (Acetone, 177170010, Thermo Fisher Scientific, Rockford, IL) that was previously stored at -20°C. After mixing thoroughly via pipetting and vortexing, the samples were stored at -20°C for one hour, after which they were removed from the freezer and centrifuged at approximately 15,000 g. The tubes were then taken out of the centrifuge, and the supernatant of the samples were removed via pipetting, carefully avoiding the visible pellet on the tube. Then the tubes were left open to allow the protein pellet on the tube wall to dry for 30 minutes, after which the pellets were reconstituted in 500 µl of deionized water directly in the tubes (final dilution of 1:2). After carefully mixing via pipetting and vortexing, the samples were then ready for further experiments.

2.3 Coomassie Assay

Coomassie® Protein Assay Kit (Coomassie® Protein Assay Reagent Kit, 23200, Thermo Scientific, Rockford, IL) was used to determine the mass

concentration of protein in the prepared urine samples. Coomassie reagent reacts with specific amino acids (Lys, Arg, and His), which allows for a measurement of the mass concentration of protein in a sample, assuming that a particular sample does not contain an abnormally low or high percentage of those amino acids.

First, a set of nine protein standards (25, 20, 12.5, 10, 5, 2.5, 1, 0 mg/l) was prepared using deionized water and bovine serum albumin (BSA) while allowing about 14 ml of the Coomassie® Reagent to warm up to room temperature in a 15-ml tube. Then, 150 µl of each of the nine protein standards as well as the prepared urine samples were pipetted into 96-well microplate wells (Corning, New York, NY) in duplicates. To each of the wells, 150 µl of the Coomassie® Reagent was added using a multi-channel pipette, and the microplate was mixed on a plate shaker for 30 seconds. After allowing an incubation period of 10 minutes at room temperature, the absorbance of the wells was measured at 595 nm using a plate reader (FilterMax F-5 Multi-mode, Molecular Devices, Sunnyvale, CA; in absorbance units). The data gathered was then imported into Microsoft® Excel and analyzed.

2.4 Fluoraldehyde (OPA) Assay

The chemistry of the OPA Reagent allows for an estimate of the molar concentration of proteins in solution because it interacts with primary amines. Every peptide contains an N-terminus, which ends with a primary amine group. This allows for a measurement of how many primary amines there are in a sample, which is a good estimate of how many peptides there are. First, a set of nine protein standards (500, 400, 250, 100, 50, 25, 10, 1, 0 mg/l) was prepared while equilibrating about 14 ml of

the OPA Reagent to room temperature in a 15-ml tube. Then, 20 μ l of each of the nine protein standards as well as the prepared urine samples were pipetted into 96-well microplate wells in duplicates. Then, a small amount of β -mercaptoethanol (2-Mercaptoethanol, Sigma-Aldrich, St. Louis, MO) equivalent to 1/2000 of the total OPA Reagent volume was added to the OPA Reagent inside a fume hood and mixed gently by inverting the tube. Then, to each of the wells, 200 μ l of the OPA Reagent was added using a multi-channel pipette, and the microplate was allowed an incubation period of 5 minutes at room temperature. Finally, the fluorescence of the wells was measured at excitation 360 nm and emission 465 nm using a plate reader. The data gathered was then imported into Microsoft® Excel and analyzed.

2.5 Control Experiments

Several control experiments were conducted using various compounds in order to test the validity of the methods described above; in particular, Coomassie and OPA Assays were examined.

2.5.1 Linear Range of Coomassie and OPA Assays

In order to examine the linear range for the two assays, the absorbance/fluorescence values for the BSA standards from both assays were imported into Microsoft® Excel. The value corresponding to the blank standard (0 mg/l) was subtracted from each of the values from the standards. The result was plotted on a graph, with the protein concentration on x-axis and the adjusted absorbance/fluorescence values on y-axis, and the plot was examined for linearity. A linear regression equation was found along with its R^2 value.

2.5.2 Protein Retention Rate of Acetone Precipitation

From the urine samples, five samples were randomly chosen. 250 μ l of each sample was set aside and diluted 1:1 with 250 μ l of deionized water. Another 250 μ l of each sample was pipetted into separate tubes, and acetone precipitation was performed as described above. Both the precipitated and unprecipitated samples were analyzed via Coomassie and OPA assays as described above.

2.5.3 Addition of a Known Amount of BSA as Control

Next, an experiment was conducted to show the effect of adding a known amount of protein into a urine sample. One urine sample was randomly chosen from all the samples, and 1 ml of it was set aside in a 2 ml tube, and another 1 ml was mixed with 0.005 μ l of 1,000 mg/l BSA solution, effectively increasing the concentration of the protein by 5 mg/l. 250 μ l from both solutions were set aside and diluted 1:1 with 250 μ l of deionized water, and another 250 μ l of both solutions were used to perform acetone precipitation as described above. All four solutions were then analyzed via Coomassie and OPA assays, as described above.

2.5.4 Addition of Other Known Compounds as Control

In addition to BSA, other compounds were used for control experiments. Solutions of urea (29700, Thermo Scientific, Rockford, IL), dimethyl urea (Sigma-Aldrich, St. Louis, MO), and glycine (G8898, Sigma-Aldrich) were prepared at various concentrations (50, 25, 12.5, 1.25, 0.125, and 0.0125 g/l for urea and dimethyl urea; 10,000, 5,000, 2,500, 250, 25, and 2.5 mg/l for glycine). The Coomassie and OPA assays were performed for all of these samples as described above.

2.6 Analysis of Urine Samples

Once all control experiments were complete, the peptide composition of all the samples in the LR and HR categories was measured by preparing the samples via precipitation and analyzing via Coomassie and OPA assays, as described above.

2.7 Data Analysis

For the Coomassie and OPA experiments, a standard curve was constructed as described in 2.5.1 above and was used to determine the mass protein concentration (in mg/l) of the samples as well as the primary amine molar concentration (in mM).

For the analysis of urine samples, a ratio (r) of the Coomassie and OPA assay values was used to compare the peptide composition of the samples:

$$r \left(\frac{\text{mmol}}{\text{g}} \right) = \frac{\text{Primary Amine Concentration (mM)}}{\text{Protein Mass Concentration} \left(\frac{\text{mg}}{\text{l}} \right)} \times 1000 \frac{\text{mg}}{\text{g}}$$

In addition to r , the mass concentration of protein was also compared, as well as the primary amine concentration.

2.8 Statistical Analysis

All measurements shown with an error bar are Mean $\pm$ Standard Deviation. Two-tailed Student's t-test was used for comparison between the LR and HR groups. A probability of $p < 0.05$ was considered statistically significant.

CHAPTER 3. RESULTS

3.1 Aim 1: Development of Method

3.1.1 Coomassie and OPA Working (Linear) Ranges

Using BSA protein standards, the linear range for the Coomassie protein assay was shown to be 1-25 mg/l, with an R^2 value of 0.99977 (Figure 1). For the OPA assay, the linear range was 0.01-0.5 mM primary amine, with an R^2 value of 0.9997 (Figure 2). The R^2 values for both assays are close to 1, meaning that the linear regression line is a good fit for the given ranges.

3.1.2 Protein Retention Rate for Acetone Precipitation

The protein concentrations of five random samples as measured via Coomassie assay before and after precipitation were compared (Figure 3). On average, the protein concentration of the samples after precipitation decreased by 16.0%, giving us a mean retention rate of 84.0%. The same comparison of the primary amine concentrations as measured via OPA assay yielded a mean retention rate of just 9.1%, suggesting the presence of interfering molecules in urine before precipitation (Figure 4).

From the Coomassie assay, the mean protein concentration before precipitation was calculated to be 16.7 mg/l, and the OPA assay yielded a mean primary amine concentration before precipitation of 1.62 mM (Figure 4). From these mean values, a mean molecular weight of the theoretical “protein” that the two assays detected was calculated as follows:

$$\begin{aligned} \text{mean "protein" MW} &= \frac{\text{protein concentration}}{\text{primary amine concentration}} = \frac{16.7 \frac{\text{mg}}{\text{l}}}{1.62 \text{ mM}} \\ &= 10.3 \text{ g/mol} \end{aligned}$$

For comparison, the molecular weight of the smallest peptide, glycine, is 75.1 g/mol.

3.1.3 Coomassie Control Experiments with Various Compounds

The protein concentrations of the urine sample without BSA added were 42.8 mg/l before precipitation and 41.1 mg/l after precipitation. With the BSA added, they were 49.1 mg/l and 45.1 mg/l, respectively, showing a corresponding increase in protein concentration. The measured increase in the protein concentration was 6.3 mg/l before precipitation and 4.0 mg/l after precipitation (Figure 5).

Coomassie measurements of urea solutions yielded very small readings. It ranged from about 1 mg/l for lower urea concentrations to 2.9 mg/l for higher concentrations (Figure 6). For the urea concentration typical in urine, between 1.25 and 12.5 g/l, the Coomassie assay reading was between 1.3 and 1.8 mg/l, which is at the bottom end of the Coomassie assay's working range.

Coomassie measurements of dimethyl urea samples were similarly low. It ranged from below detectable range to 4.0 mg/l (Figure 7).

Glycine samples also produced small Coomassie readings relative to their concentrations. The measured concentrations ranged from below detectable range to 7.4 mg/l (Figure 8). These concentrations were significantly lower (about 3 orders of magnitude lower) than the expected concentration if Coomassie were able to detect glycine.

3.1.4 OPA Control Experiments with Various Compounds

Dimethyl urea was used as a negative control for the OPA assay, since it lacks primary amines. For all of the concentrations of dimethyl urea used in the experiment, the OPA readings were below detectable range (Figure 9). Glycine served as a positive control for the OPA due to its small size, simple structure, and the presence of a primary amine. At the lowest concentration used (2.5 mg/l), the OPA measurement was below detectable range. With a concentration similar to that of protein in urine (25 mg/l), the OPA measurement was within the range, at 0.28 mM. All other samples with the higher concentrations were above detectable range (Figure 9).

3.2 Aim 2: Analysis of HR and LR Urine Samples

In total, 38 samples were collected and analyzed. In the High Risk (HR) category, a total of 16 samples were collected from subjects ranging from 3.1 to 14.7 months of age. In the Low Risk (LR) category, a total of 22 samples were obtained from subjects ranging from 3.1 to 36.3 months of age. Because of the apparent discrepancy in the age range of the two groups, two analyses were carried out: one with all 22 samples in the LR category, and one without the three oldest subjects' samples, for a total of 19 samples with an age range of 3.1 to 18.2 months of age.

3.2.1 Primary Amine to Protein Ratio (r)

The ratio of the OPA measurement (primary amine concentration) to the Coomassie measurement (protein mass concentration) was calculated. The two groups (LR and HR) showed a statistically significant difference ($p = 0.044$), with HR group having a higher average r than LR (Figure 10). The mean of r for the LR group as well

as the standard deviation decreased slightly with the exclusion of three oldest subjects (Figure 11, $p = 0.031$). The distribution of r within each group was also plotted (Figure 12).

3.2.2 Protein Mass Concentration

The protein mass concentration of the samples as measured by Coomassie was also compared but no significant difference was found between the two groups, with or without the three oldest subjects in the LR category, with $p = 0.55$ and $p = 0.677$ respectively (Figures 13, 14). The distribution of protein concentration for each group was also plotted (Figure 15).

3.2.3 Primary Amine Concentration

The primary amine concentrations of the two groups were also compared but were not significantly different ($p = 0.081$ with three oldest, $p = 0.072$ without) (Figures 16, 17). The distribution of the primary amine concentrations for each group was plotted, and a presence of a possible outlier was detected (Figure 18).

3.2.4 Diet Phase and Ages

Lastly, the r value each of the samples was plotted by age and diet phase (Figure 19). Although difficult to conclude from such a small sample size, the LR, BMO category seemed to have the lowest r values and also youngest. In addition, the r value seemed to increase more sharply with age in the HR, NBM group than in the LR, NBM group.

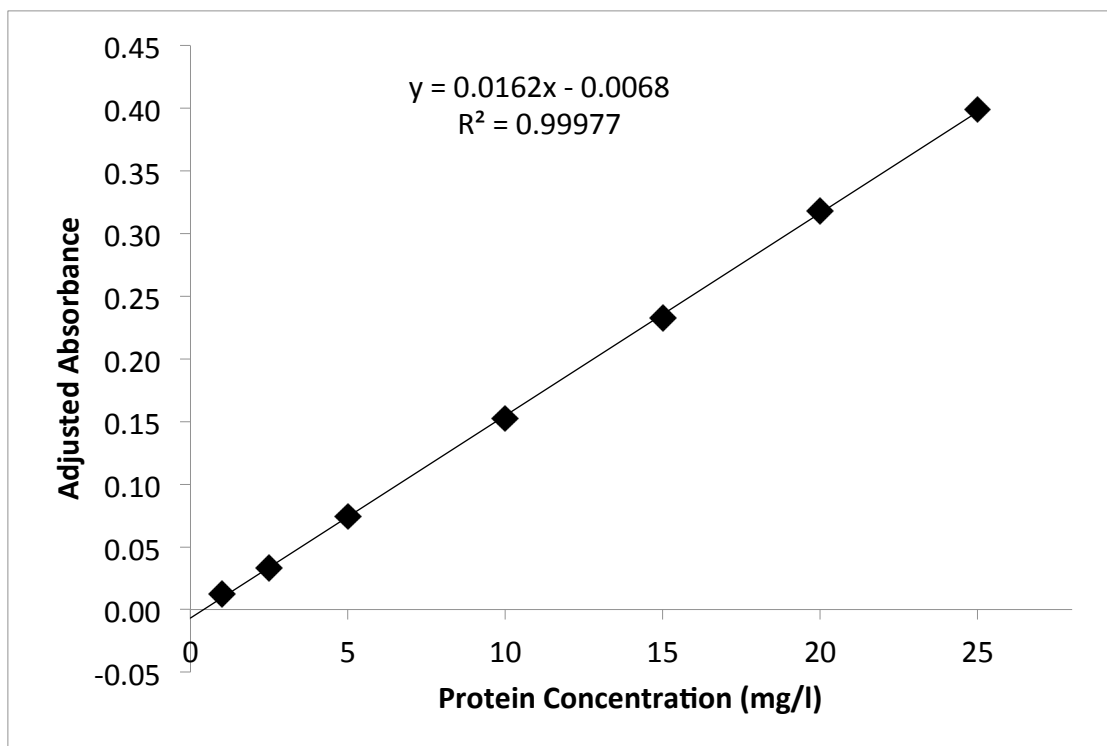


Figure 1. Coomassie Protein Assay Standard Curve. x-axis represents the calculated protein concentrations of BSA standards, and y-axis is the adjusted absorbance values of each sample. Between 1 and 25 mg/l, the assay is strongly linear ($R^2 = 0.9998$).

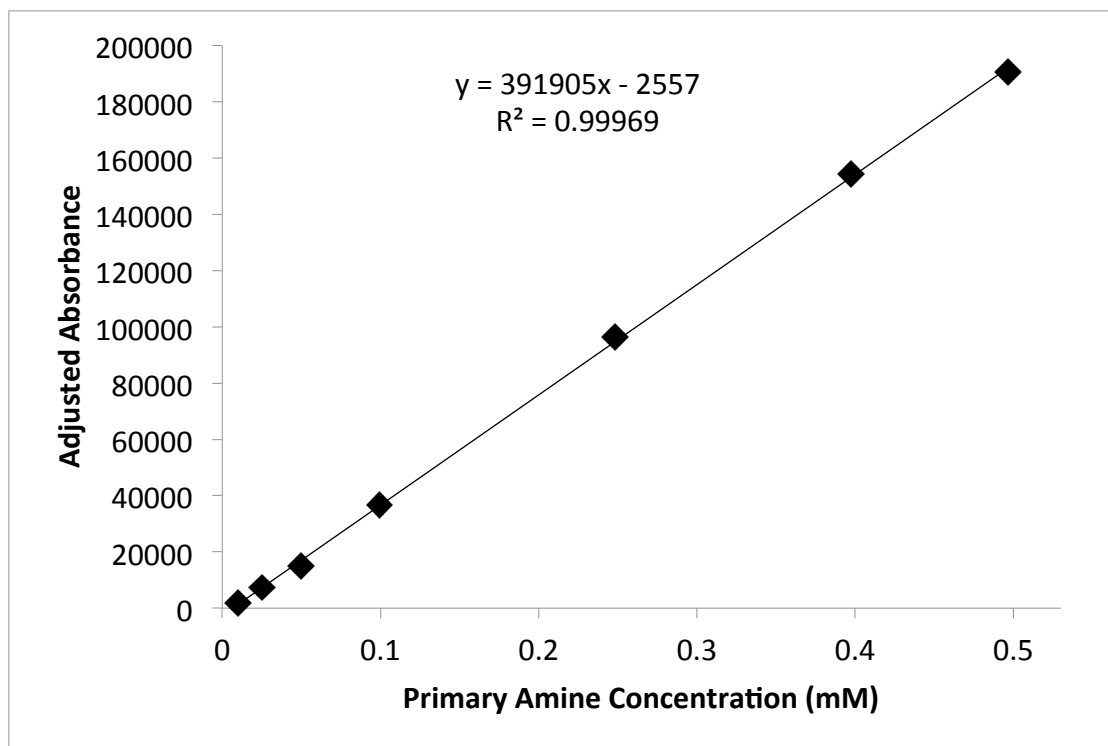


Figure 2. OPA Assay Standard Curve. Between 0.01 and 0.5 mM of primary amines, the assay is linear ($R^2 = 0.9997$).

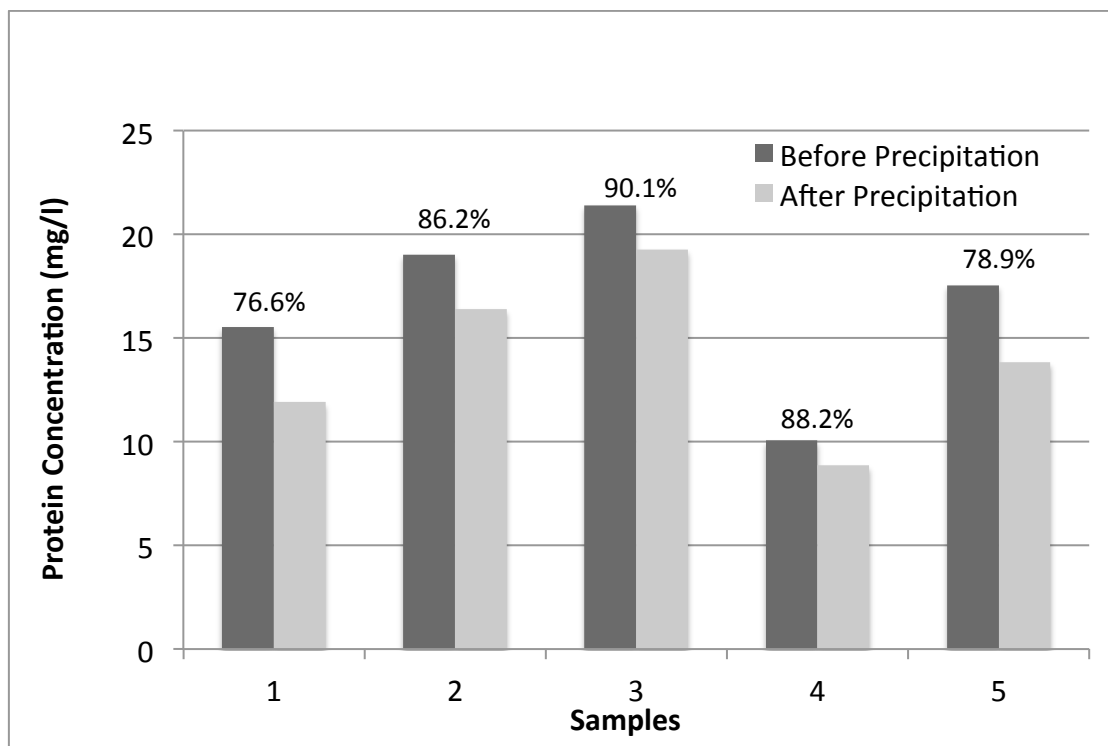


Figure 3. Protein Concentration of Urine Samples before and after Precipitation.

The average concentration before precipitation was 16.7 mg/l, and after precipitation was 14.1 mg/l. The mean retention rate was 84.0%.

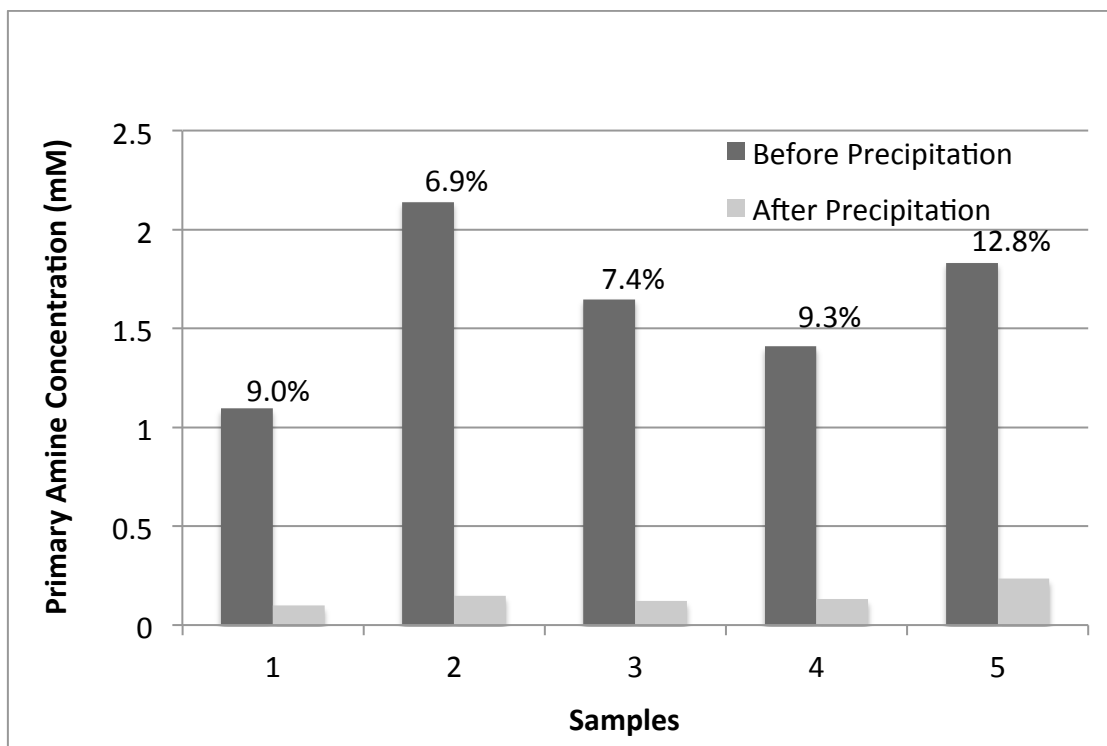


Figure 4. OPA Assay Measurements of Urine Samples before and after Precipitation. The average concentration before precipitation was 1.62 mM, and after precipitation was 0.147 mM. The mean retention rate was 9.1%.

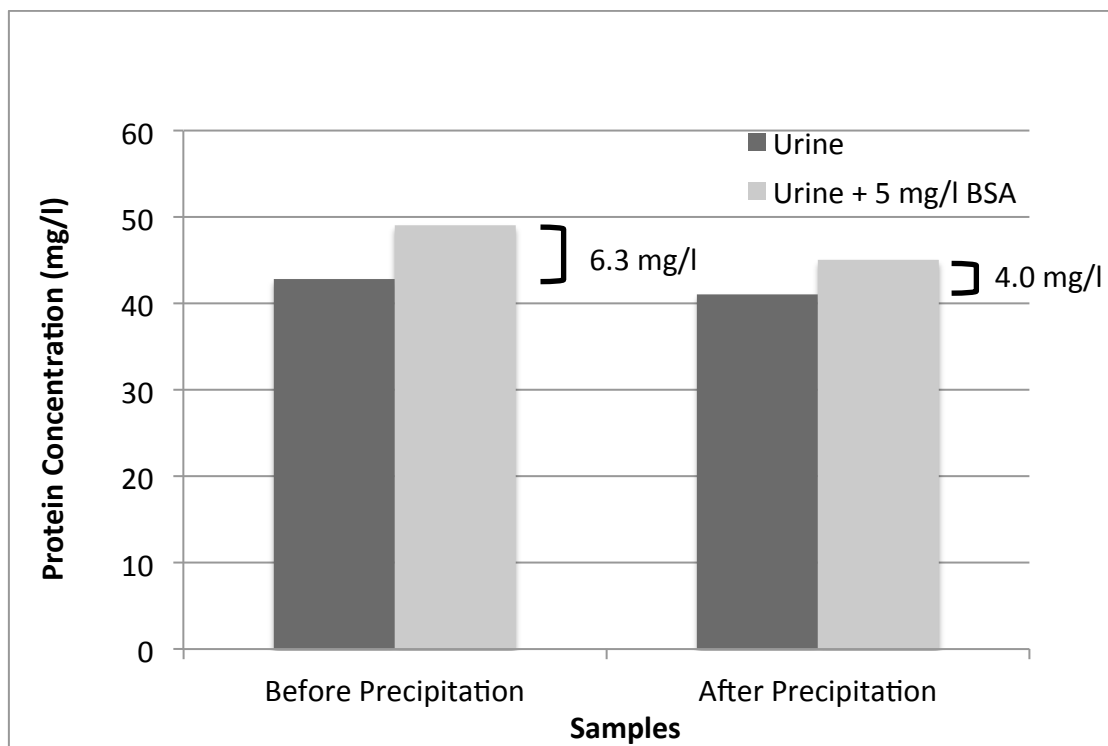


Figure 5. The Effect of the Addition of BSA into Urine. As expected, the addition of a small amount of BSA leads to a corresponding increase of protein concentration, as measured by Coomassie assay.

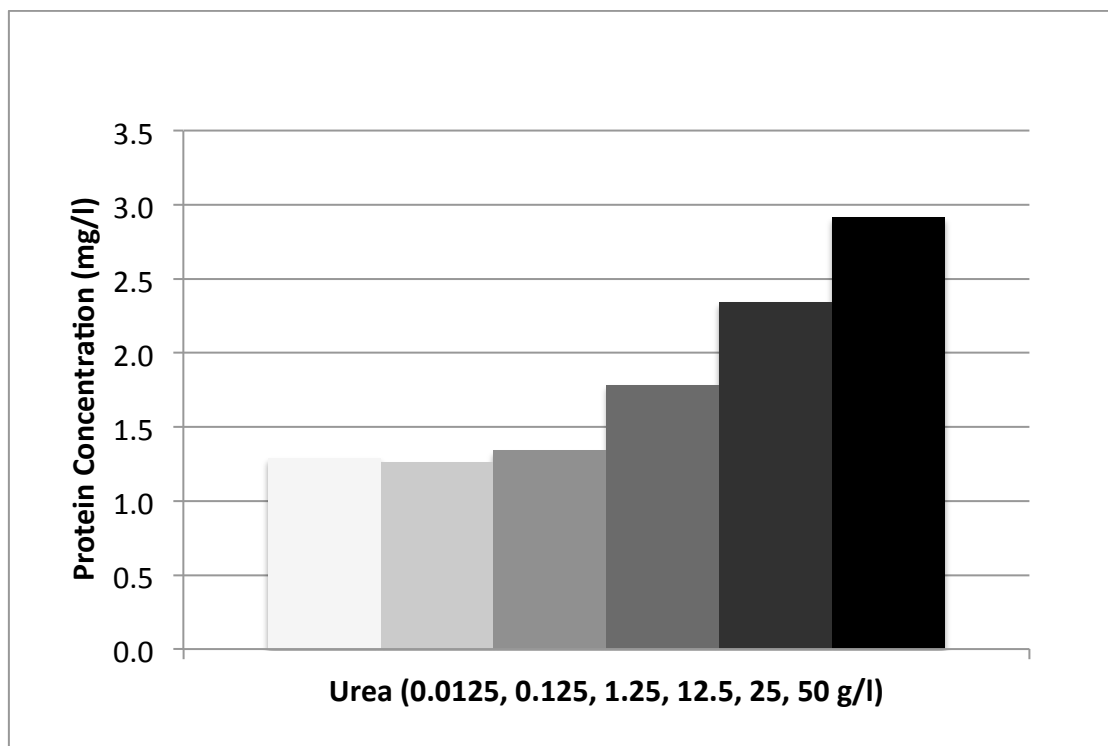


Figure 6. The Effect of Urea on the Protein Concentration Measurement.

Although higher urea concentration leads to a correspondingly increased absorbance value, the amount increased is about 1 mg/l, which is a small fraction (at least one order of magnitude smaller) and is also at the lowest end of the linear range of the assay. The urinary concentration of urea is somewhere in between 1.25 and 12.5 g/l.

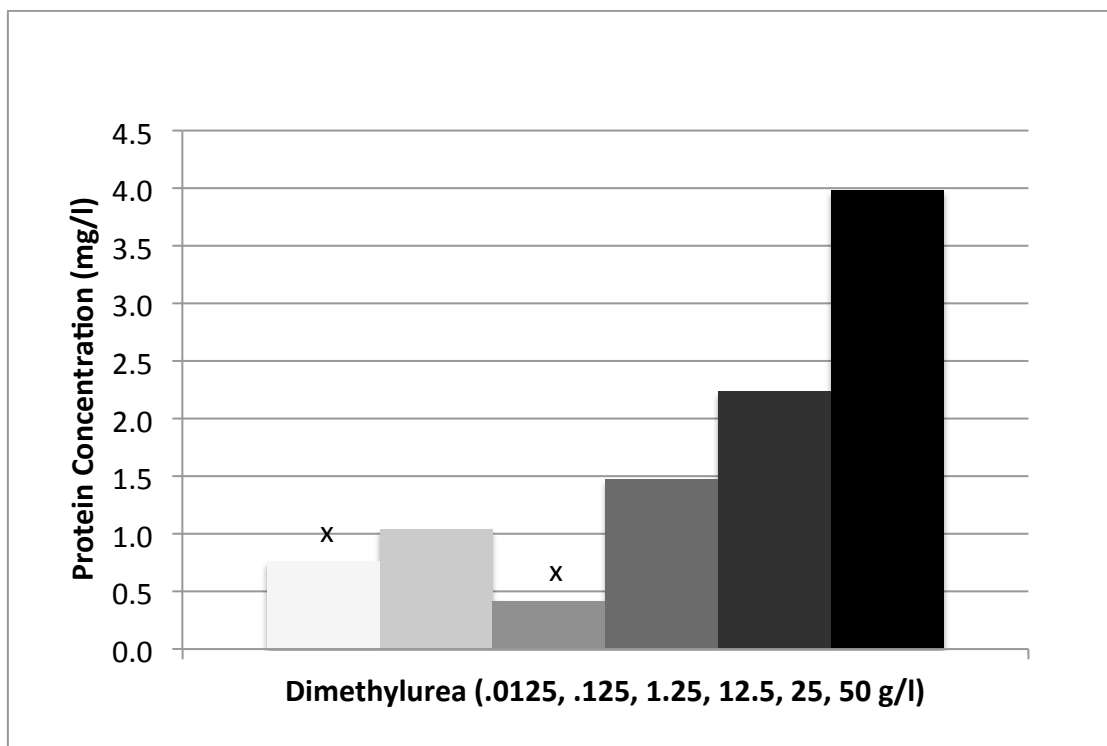


Figure 7. The Effect of Dimethyl Urea on the Protein Concentration Measurement. The increase in the Coomassie assay measurement is inconsequential at a low concentration. “x” indicates absorbance values that were out of the linear range.

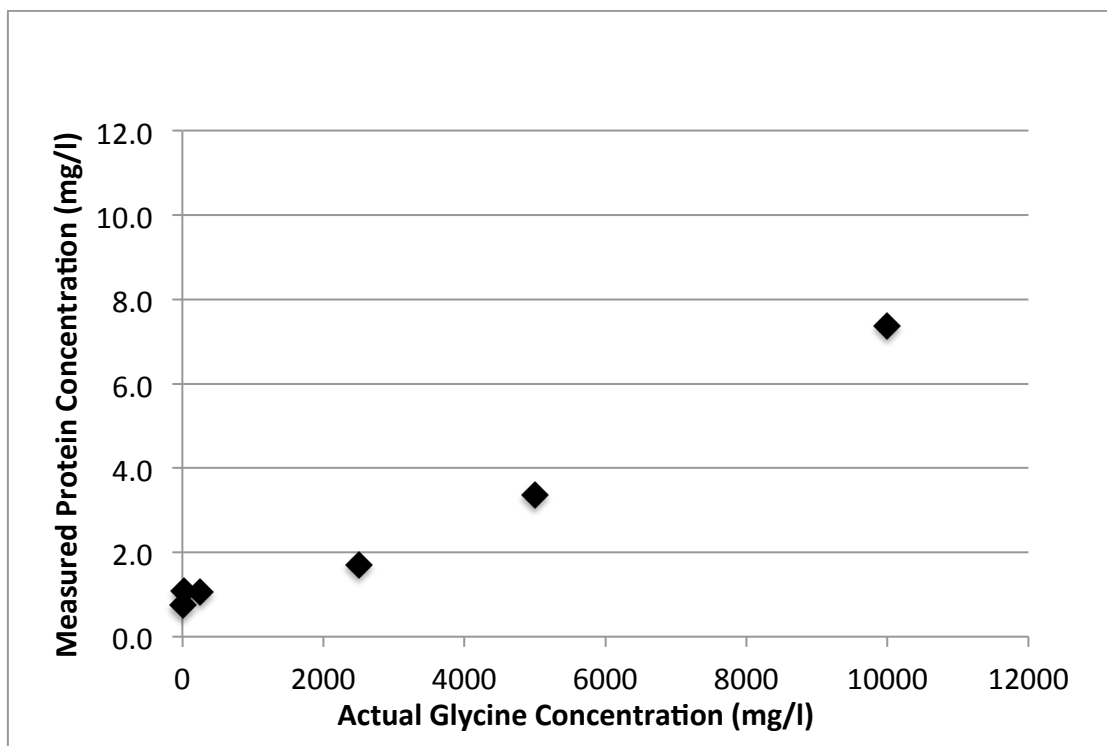


Figure 8. Measured and Actual Glycine Concentrations. The x-axis shows the actual concentration of glycine used. The y-axis shows the measured concentration via Coomassie. Even at 10,000 mg/l, the measured concentration does not exceed 8 mg/l.

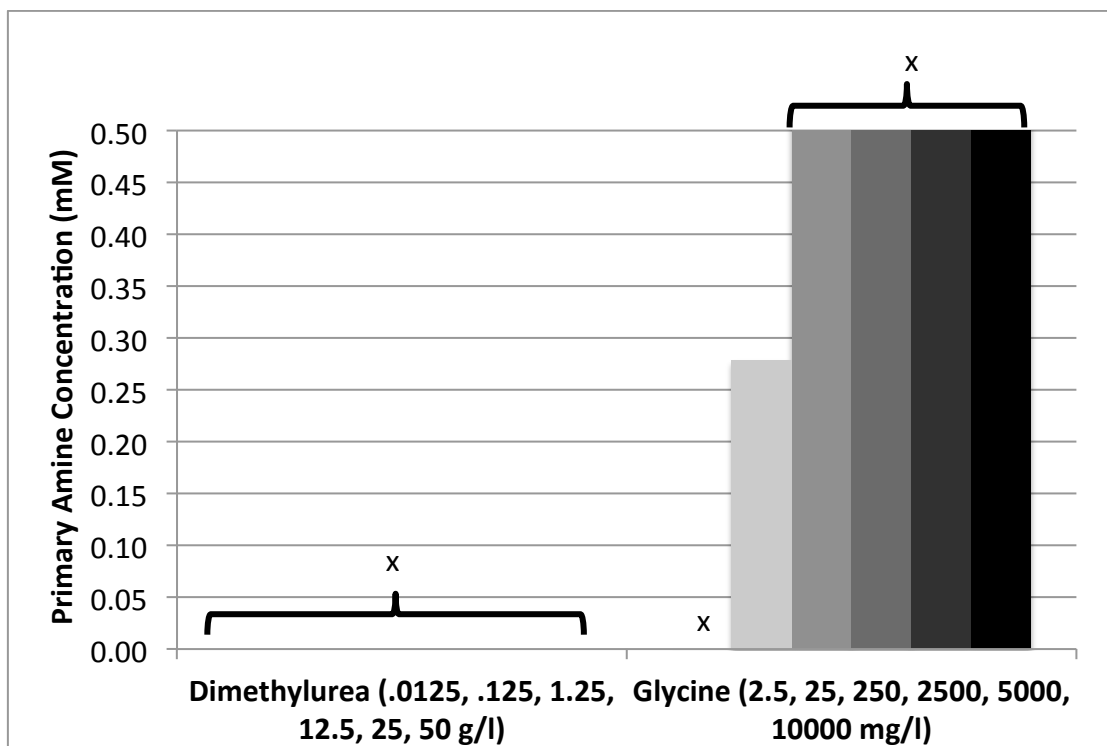


Figure 9. The Effects of Dimethylurea and Glycine on the OPA Measurements.

Dimethylurea did not increase the OPA measurements above the minimum detection level at all concentrations, and glycine affected the OPA measurements at certain concentrations. “x” indicates that the measurement was out of the linear range of the assay. For the glycine samples with concentrations of 250, 2,500, 5,000, and 10,000 mg/l, the true measured concentrations were above 0.5 mM and are not shown.

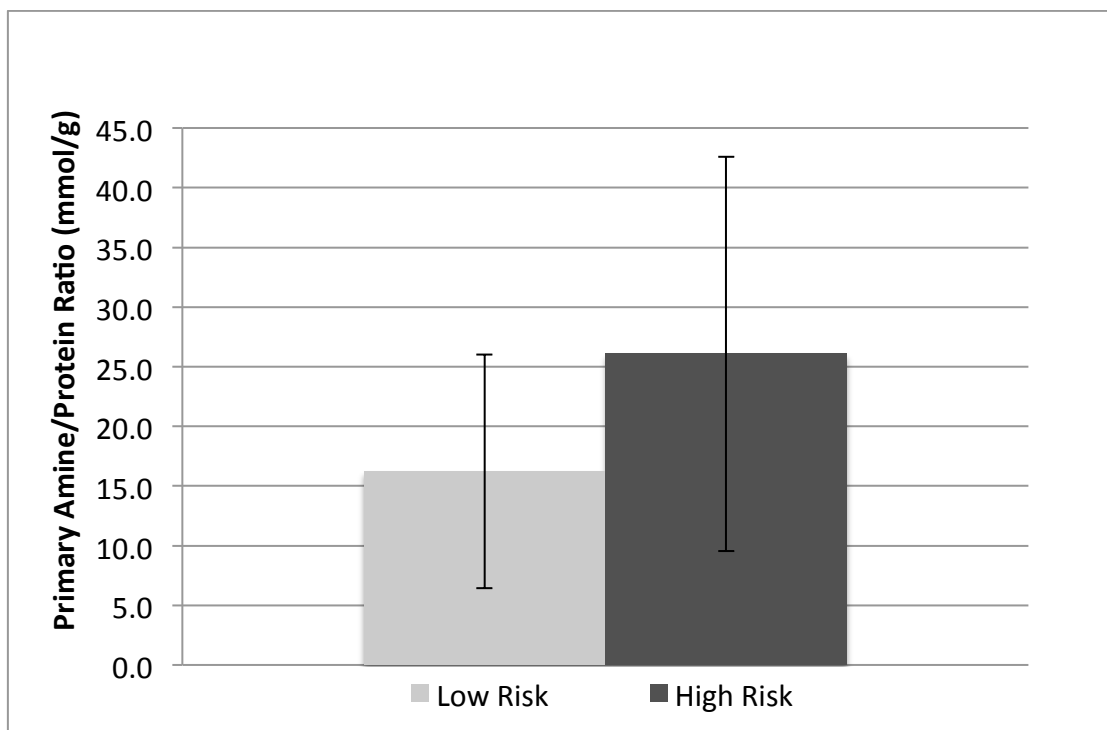


Figure 10. Comparison of r Values for LR and HR Groups. $n = 22$ for LR and $n = 16$ for HR ($p = 0.044$).

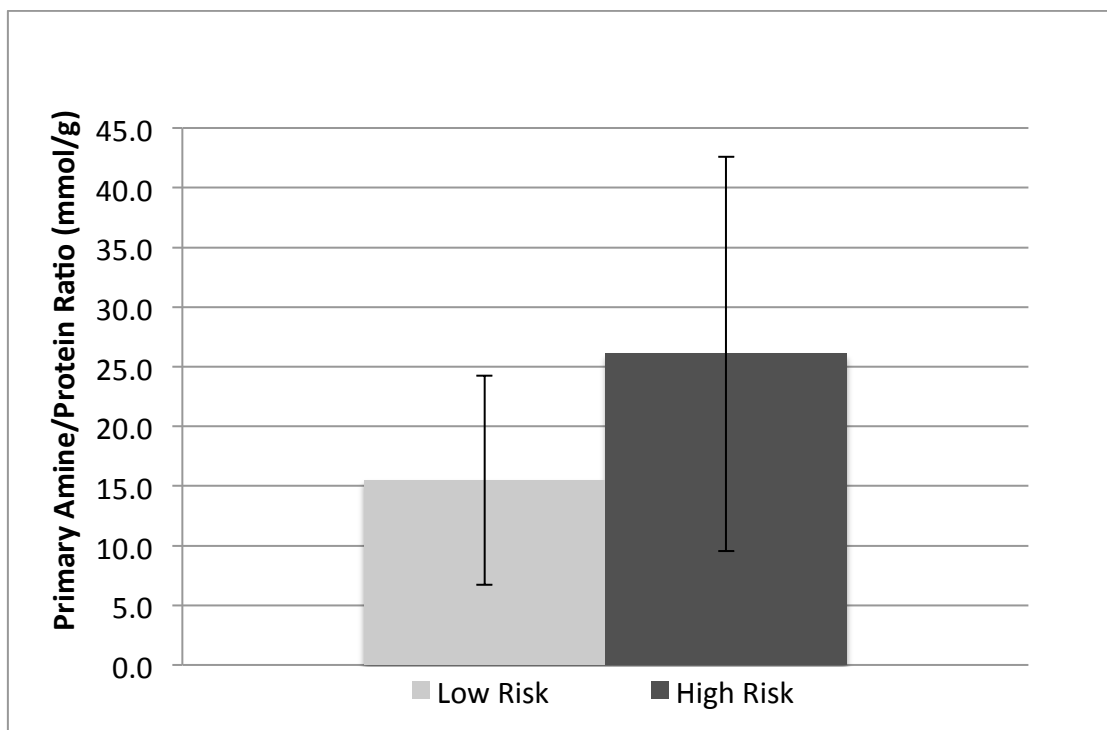


Figure 11. Comparison of r Values for LR and HR Groups, Three Oldest Subjects Removed. $n = 19$ for LR and $n = 16$ for HR ($p = 0.031$).

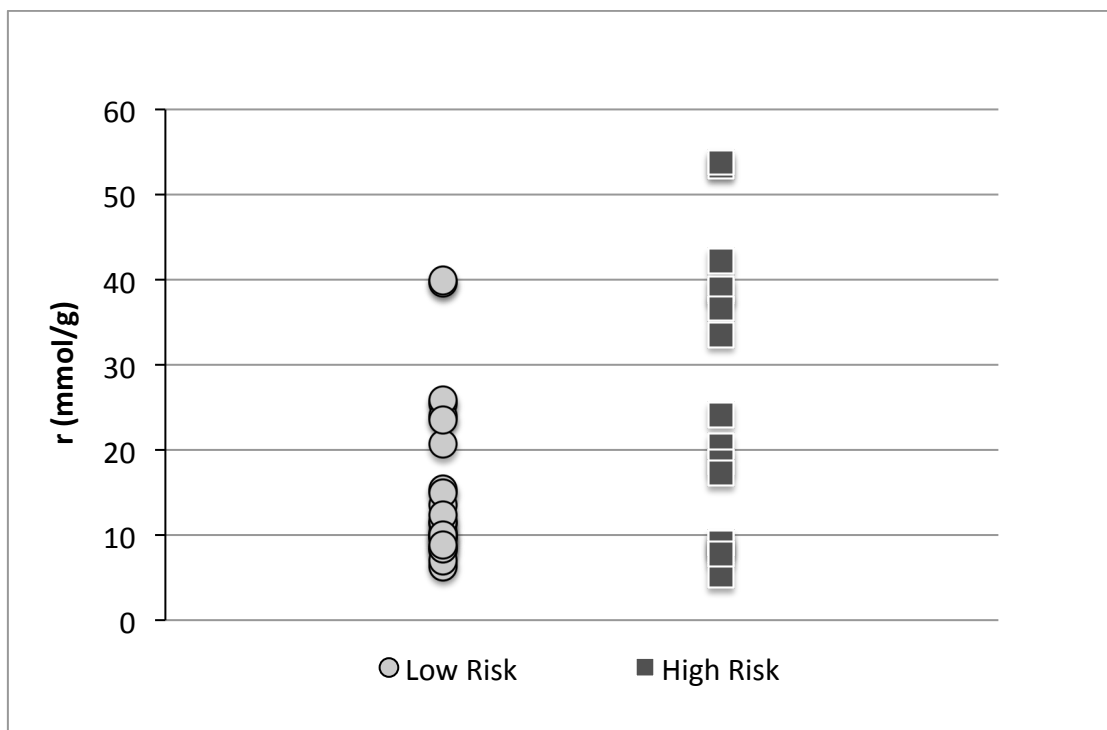


Figure 12. Distribution of r Values for LR and HR Groups. The distribution for LR is shown on the left, and HR on the right. Both groups contain possible outliers on the higher end.

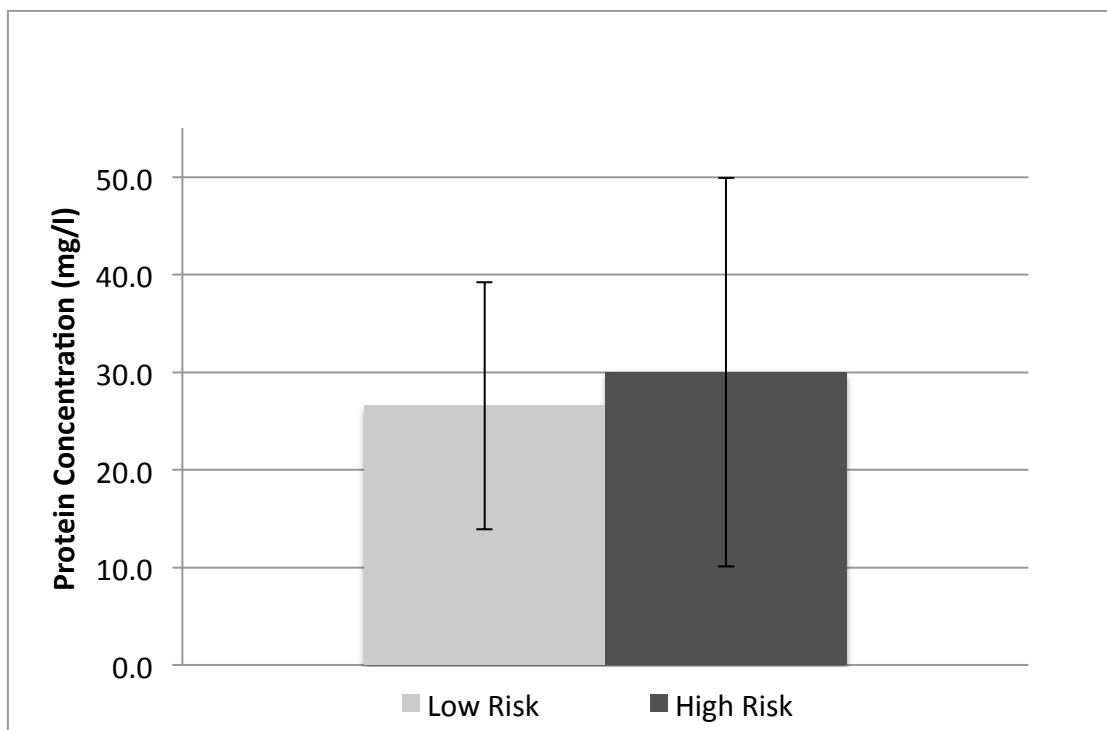


Figure 13. Comparison of Protein Mass Concentration for LR and HR Groups. $n = 22$ for LR and $n = 16$ for HR ($p = 0.55$).

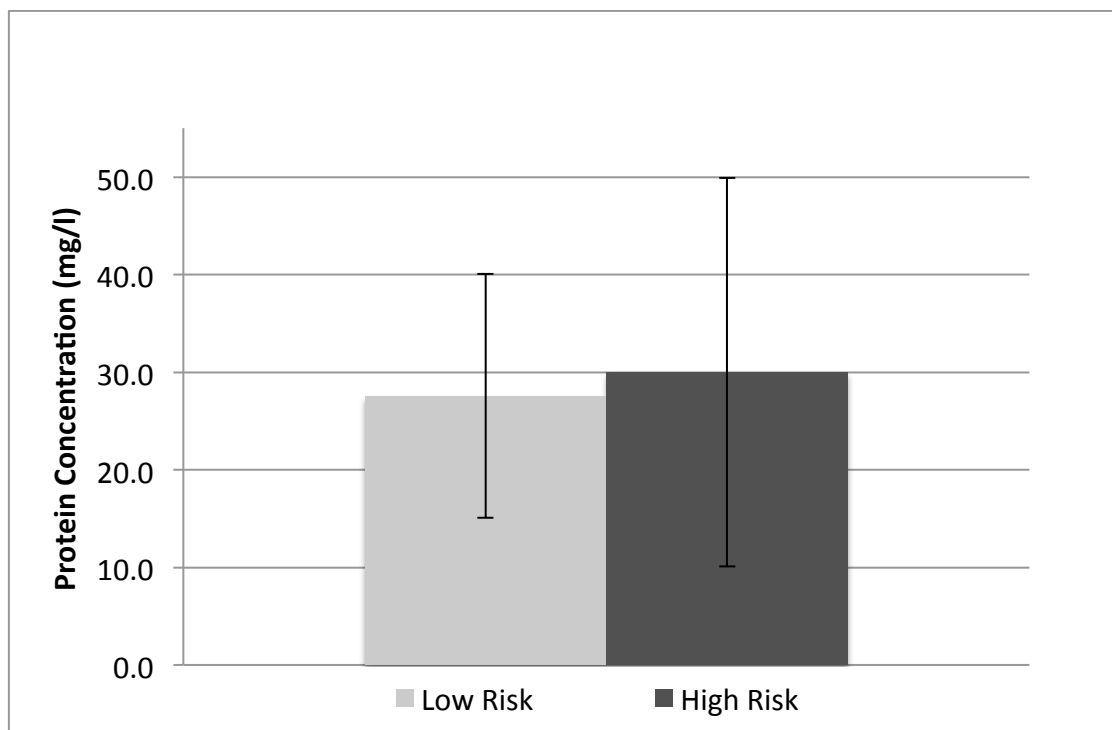


Figure 14. Comparison of Protein Mass Concentration for LR and HR Groups, Three Oldest Subjects Removed. $n = 19$ for LR and $n = 16$ for HR ($p = 0.678$).

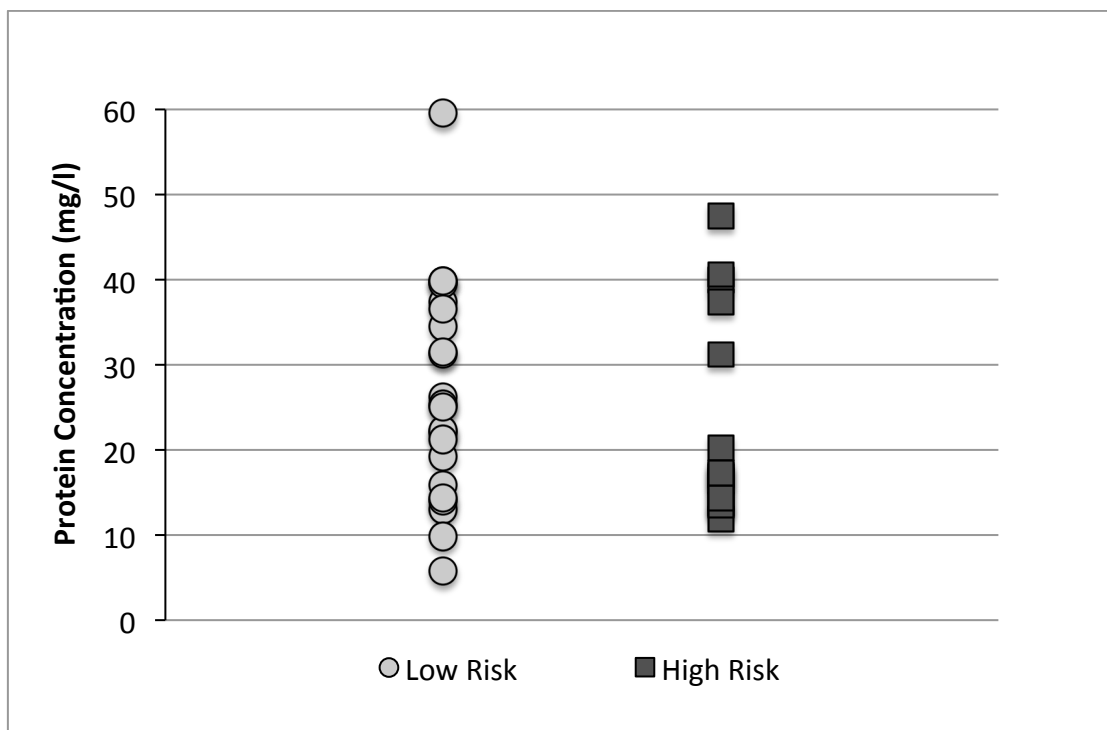


Figure 15. Distribution of Protein Concentration for LR and HR Groups. The distribution for LR seems to contain an outlier. HR distribution seems bimodal.

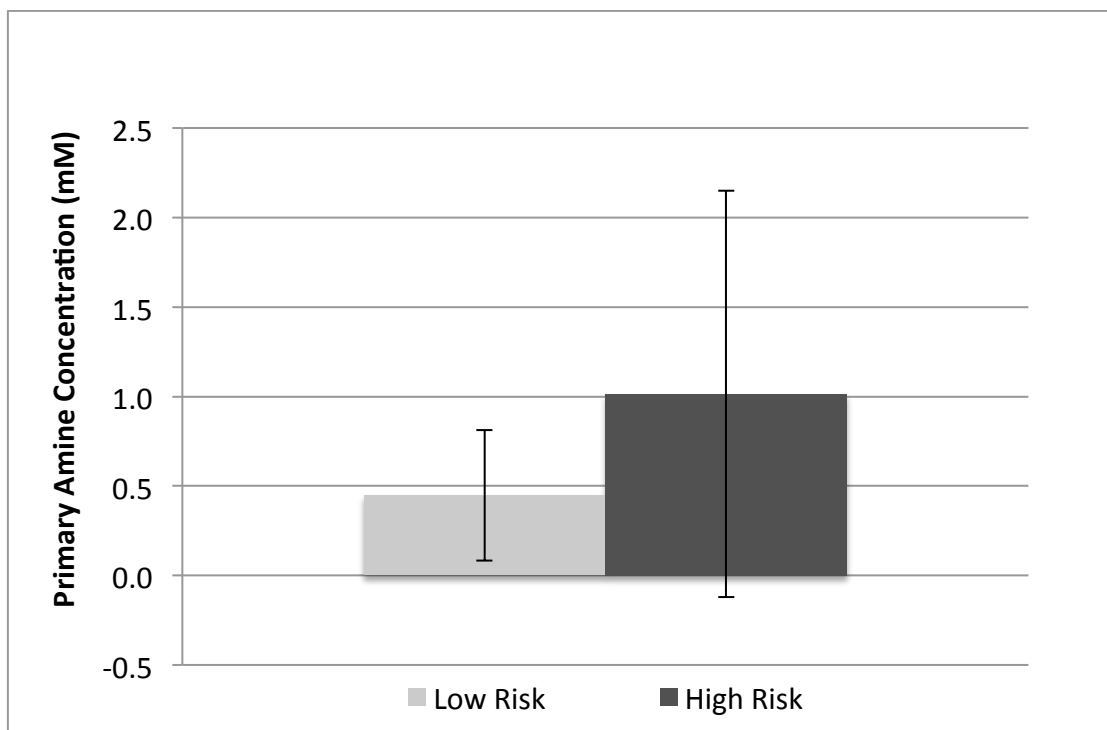


Figure 16. Comparison of Primary Amine Concentration for LR and HR Groups. $n = 22$ for LR and $n = 16$ for HR ($p = 0.081$).

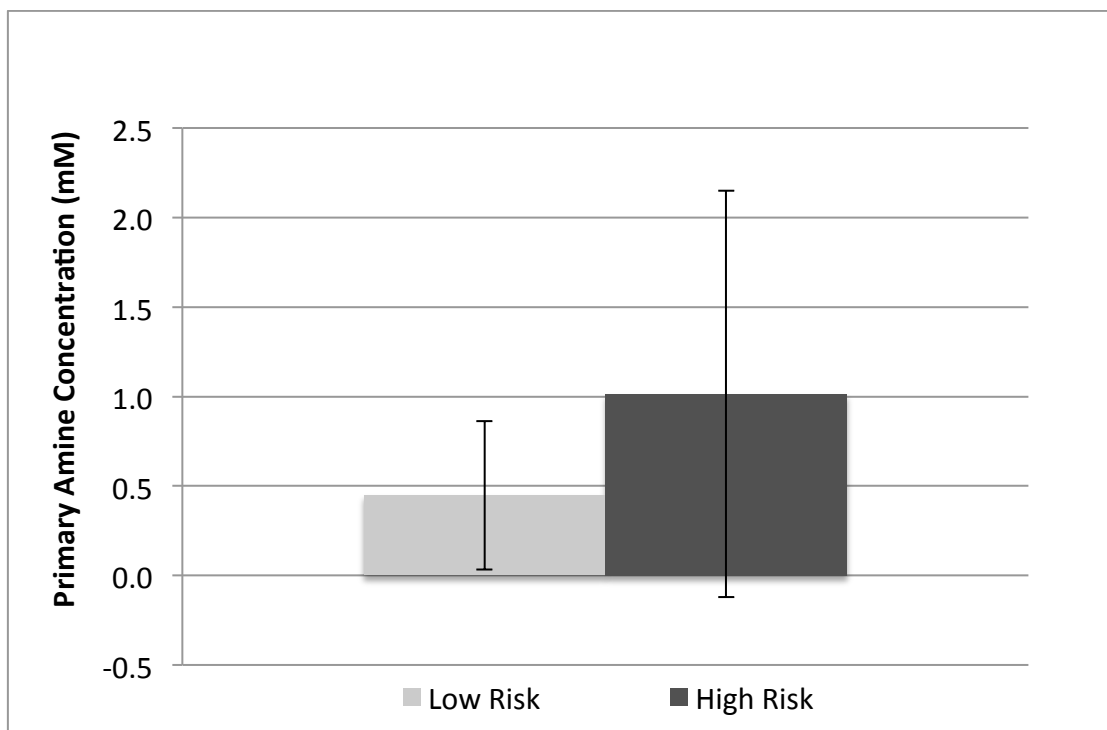


Figure 17. Comparison of Primary Amine Concentration for LR and HR Groups, Three Oldest Subjects Removed. $n = 19$ for LR and $n = 16$ for HR ($p = 0.072$).

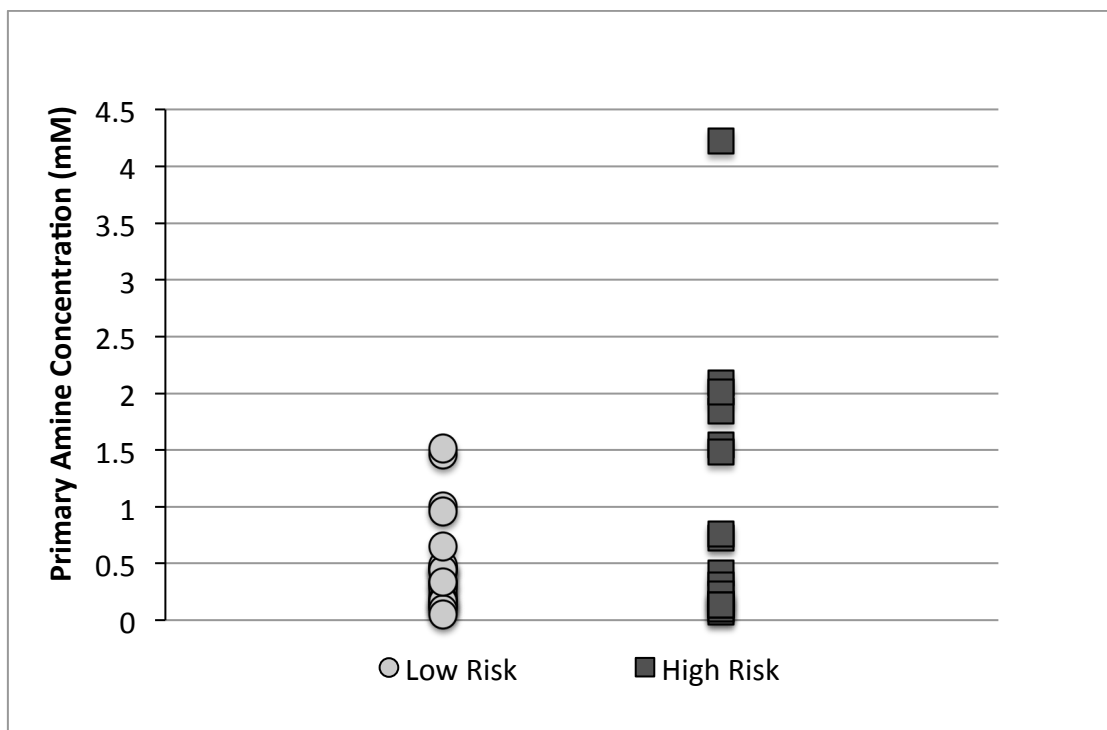


Figure 18. Distribution of Primary Amine Concentration for LR and HR Groups.

The HR distribution seems bimodal and contains an outlier.

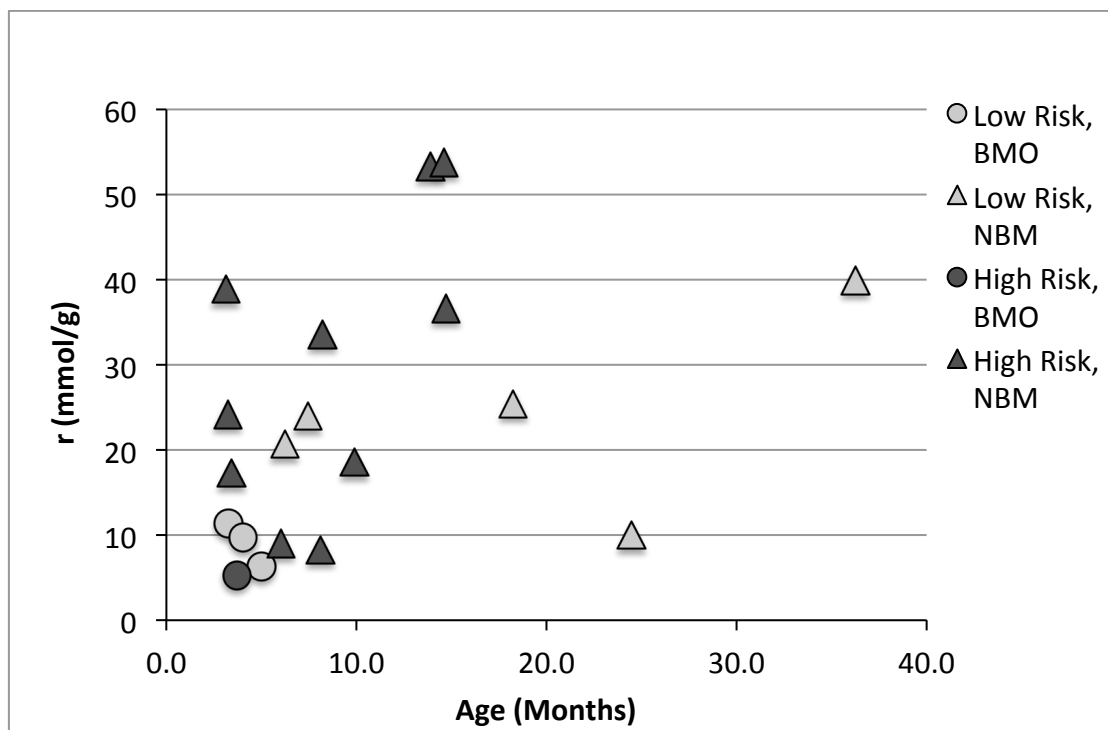


Figure 19. Distribution of r Values by Age, Diet Phases, and HR/LR. Small sample size is due to some subjects being categorized as neither BMO nor NBM (mixed).

CHAPTER 4. DISCUSSION

4.1 Summary

Overall, the results indicate that there is a difference in the urinary peptide composition between LR and HR groups ($p = 0.044$). Specifically, the HR group's r value was larger than that of LR, which implies smaller peptides in the HR group's urine. Both groups contained about the same amount of protein by mass concentration. This result supports the hypothesis that there exists protease activity in the development of ASD.

4.2 Developed Method

A series of experiments were conducted to validate the assays chosen for this study, and the results have confirmed their validity. The retention rate of 84% for the acetone precipitation procedure is considered high for such an inexpensive, quick assay because although the ideal rate is 100%, the loss of protein is inevitable in all kinds of sample preparation procedures. Furthermore, the linear range for the Coomassie and OPA was confirmed with very high R^2 values ($R^2 > 0.999$), which validated the usefulness of these assays in their linear range of concentrations.

The OPA levels of urine samples before precipitation validated a need for the purification step. As reported above, if the entirety of the signal in the OPA assay were due to peptides, then it follows that those particular “peptides” have a mean molecular weight of 10.3 g/mol. However, this is impossible, since the smallest peptide, glycine, has a molecular weight of 75.1 g/mol. That is, even the smallest amino acid has 75.1 g of mass per one mole of primary amines; therefore, the extremely high signals that

urine samples produce for the OPA assay must be due to noise from interfering compounds that are present in urine. The significant drop in the signal after precipitation suggests that the precipitation step achieves its intended effect of removing such compounds.

The Coomassie protein assay demonstrated results that were consistent with its chemistry. Adding a known amount of protein led to an increase in the protein concentration as measured by Coomassie. Urea, dimethylurea, and glycine all served as negative controls for the assay, since Coomassie is known to only react with basic amino acids. Specifically, results for the Coomassie assay on urea samples ranged from 1.0 to 3.0 mg/l, which is at the lower end of the linear range for the assay. Also, at concentrations of urea that are common in urine, the protein concentration readings were below 2.0 mg/l, or no more than 10% of a typical urinary protein concentration level. The small amount of signal that is present in the urea samples may be due to the fact that urea is a weak base. Regardless, any amount of noise that urea contributes to the Coomassie assay is also expected to be removed in the precipitation step; however, the results show that even if the acetone precipitation procedure failed to remove urea from the samples, its effect on the Coomassie results would be relatively small. A conservative estimate for the error in the Coomassie results due to the presence of urea is 5%.

Similarly, dimethylurea solutions produce relatively small signals for the Coomassie assay. Even at a very high concentration of 50 g/l, dimethylurea solution only produced signals equivalent to 4.0 mg/l of protein. Since there is no presence of

dimethylurea in urine, the exact amount of noise it produces is not significant; however, its results confirm that the Coomassie assay is a reliable protein assay that is not greatly affected by interfering compounds that may be present.

Lastly, results for the glycine samples demonstrated that the Coomassie reagent is indeed specific in its affinity for certain amino acids. Even a concentration as high as 10,000 mg/l only produced a signal equivalent to 8 mg/l of protein, which implies that Coomassie reagent does not react with glycine in the way it is expected to react with proteins in general.

The reliability of the OPA assay was also demonstrated by the results. The absence of signal in the dimethylurea samples, as well as the presence of signal in the glycine samples, confirms that OPA can be used reliably to detect primary amine groups. Since dimethylurea contains no primary amine groups, it is not expected to produce any signal in the OPA assay, which was confirmed by the results. A glycine molecule contains one primary amine and thus was expected to produce signals accordingly, and the results confirmed this. In summary, Coomassie and OPA assays behaved in a predictable manner, and all three assays (acetone precipitation, Coomassie, and OPA) fit the criteria of being inexpensive, reliable, and quick.

4.3 The Peptide Composition of HR and LR Groups

As shown above, the HR group's r value was significantly higher than that of the LR group. This implies that the peptides found in the HR urine are smaller because r is a measure of the number of primary amines per mass of protein. Higher r value implies more primary amines per mass of protein, which means that there are more N-

termini. This implies that the peptide content found in HR was smaller in size. There is a possibility that the HR group contained more peptides with Lysine in them, since Lysine contains a primary amine group, but there is no scientific reason to conclude so.

It is also significant that there was no difference between the mass concentration of protein in HR and LR samples because it confirms that the difference in the r value was not simply because of variations in the sheer amount (in mass) of protein in the urine. In other words, there is no difference in how much peptide is found in these samples, but the size of the found peptides is necessarily different.

The removal of the three oldest subjects in the LR group was included in order to account for the possibility that age may be a confounding variable in this study. However, removing them did not lead to a different result in any of the three parameters considered.

Therefore, the initial results from the samples that we collected seem to confirm the hypothesis that the HR group would contain smaller peptides, and thus provide initial evidence confirming the overall hypothesis that ASD is caused by digestive enzymes.

4.4 Limitations

A few factors presented themselves as limitations to this study. First, the sample sizes presented in this study are small, and therefore the conclusions drawn from it are not as strong as ones drawn from studies with a larger sample size. We

recommend that the study be repeated with a larger sample size from a more varied context (location, demographics, etc.).

Secondly, the categorization of HR and LR was necessary for this study but is not the most ideal. A better categorization would be ASD-diagnosed and Control. That is, ideally, we would be able to compare the urinary peptide compositions of subjects that never develop ASD versus those who are eventually diagnosed with ASD. That way, we can more certainly attribute any differences to ASD and rule out other confounding variables.

Thirdly, the inherent goal of this study was to serve as a pilot study, which meant that depth was sacrificed for efficiency in some aspects. The conclusions that we drew regarding the peptide composition serves their purpose of providing initial evidence to the hypothesis, but there are other methods available (such as HPLC or Mass Spectrometry) that allows for a deeper analysis of peptides in a sample.

4.5 Future directions

The most immediate direction that one could take is to obtain more samples from subjects from a variety of backgrounds to obtain a more representative, randomized sample. The larger sample size should also lead to stronger conclusions. Secondly, the effect of diet was mentioned in this study but was not fully analyzed due to small sample sizes. We recommend that information regarding the diet of the subjects be collected and analyzed. Lastly, we recommend a larger-scope study to further investigate the urinary peptide content of HR as well as LR children in order to explore the possibility of being able to identify some of these peptides.

CHAPTER 5. CONCLUSION

This study for the first time presented evidence in favor of the hypothesis that digestive enzymes escaping from the lumen of the small intestine may be involved in the development of ASD. Although limited in scope, the study shows a difference in the peptide composition of those who are at risk for ASD and those who are not. This observation makes a possible contribution to the etiology of ASD, and suggests concrete directions for future investigations. A more involved investigation of the overarching hypothesis may possibly lead to a stronger understanding of the etiology of ASD and even open the doors for the prevention of ASD.

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