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# Dysregulation of Human Phase I Enzymes in APAP Overdose Subjects with Low ALT Levels

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# SENIOR THESIS APPROVAL

This Honors Thesis entitled

## "Dysregulation of Human Phase I Enzymes in APAP Overdose Subjects with Low ALT Levels"

written by

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meets the criteria for acceptance
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# Dysregulation of Human Phase I Enzymes in
# APAP Overdose Subjects with Low ALT Levels

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**Background:** Acetaminophen (APAP) is a common analgesic that can cause liver injury and death in high doses. Changes in gene expression following APAP overdose may be more sensitive indicators of liver injury than the current clinical indicator, alanine aminotransferase (ALT). The aim of this study was to examine gene expression of Phase I enzymes in pediatric APAP overdose patients with low ALT levels (<75 IU/L), in order to understand the mechanism of APAP toxicity.

**Methods:** Blood samples were collected from control patients (no APAP exposure; N=5) and APAP overdose patients (N=5). Using the PAXgene system, RNA was extracted from the blood samples and cDNA was synthesized. Quantitative polymerase chain reaction (qPCR) was performed and samples were profiled with an array containing 84 Phase I Enzyme drug metabolism genes.

**Results:** The low ALT pediatric APAP overdose patients had higher (median [range]) ALT levels (35 [25,48] IU/L) compared to controls (16 [7, 20] IU/L). Three genes had significant downregulation that was later confirmed: GZMB (-2.86 fold, p<0.01), ALDH6A1 (-2.13 fold, p<0.01), and CYP4F12 (-4.04 fold, p<0.05). Pathway analysis for the three genes pointed to signaling pathways connected to apoptosis, metabolism of valine and leucine (involved in the immune response), and hydroxylation of leukotriene B4 (involved in inflammation).

**Conclusion:** Dysregulation of Phase I Enzyme drug metabolism pathways has relevance for understanding mechanisms of cell injury in APAP toxicity. Pathway analysis points to the potential for a weakened immune response associated with APAP overdose.

## INTRODUCTION

Acetaminophen (APAP) is a mild analgesic found in many over-the-counter drugs. Taken in normal doses, it is generally regarded as safe and non-toxic, though liver injury is common. APAP overdose is the leading cause of acute liver failure in the United States [6].

Acetaminophen overdose is a common problem in the United States. A 2015 study (conducted from 2006-2010) showed that in the United States alone, 82,362 people visited the emergency room every year for APAP overdose. Every year, there were roughly 494 deaths caused by acetaminophen toxicity [1].

APAP toxicity does not have a clearly defined limit. Tylenol, the largest and most well-known distributer of APAP, recommends a maximum daily dosage of 3,000 mg for adults. For children, this amount greatly varies by weight and age [10]. Nonetheless, the acetaminophen toxicity limit varies from person to person.

The clinical biomarker for APAP overdose is alanine aminotransferase (ALT). Raised ALT levels indicate liver injury, but this is not specific to acetaminophen [6]. APAP adducts are formed in the metabolism of acetaminophen. APAP is metabolized by Phase I enzymes in the cytochrome P450 family (namely CYP2E1) into N-acetyl-p-benzoquinone imine (NAPQI), a toxic reactive intermediate metabolite. When

NAPQI binds to the cysteine group on a protein, APAP adducts are formed [7]. In a therapeutic dose of APAP, this material is detoxified by glutathione and is excreted. Thus, these adducts are only present in patients who have exceeded the normal dosage of APAP and present liver injury [6, 7].

The lab previously studied APAP overdose in patients with severe liver injury, defined as having ALT levels >75 IU/L. The five high ALT blood samples were run in qPCR against five control samples, and three genes were identified as being downregulated (using a 2-fold and p<0.05 cut-off): ALDH1A1, CYP27A1, and GZMB. The downregulation of all three genes was confirmed by individual analysis.

This study functioned as a follow-up to the high ALT experiment and a conclusion to the lab's PAXgene APAP overdose project. In this study, we investigated gene dysregulation of APAP overdose patients who exhibited mild liver injury, or low ALT levels, to better understand acetaminophen's pathway to cell injury.

## METHODS

### Sample Collection

The study focused on pediatric APAP overdose patients with low ALT levels from Arkansas Children's Hospital. Patients who were in the hospital due to APAP overdose and presented a mild liver injury, defined as ALT levels <75 IU/L, were identified and selected for the study. Blood samples were collected by nurses who were aware of the study and delivered to the lab for research. ALT levels and APAP adduct levels were measured for each sample. Blood samples were stored in PAXgene Blood RNA tubes (PreAnalytiX, GmbH, Switzerland). This study included five APAP overdose samples (low ALT levels) and five control samples (randomly identified and

selected with no APAP influence in the last two weeks).

### RNA Isolation and cDNA Synthesis

The PAXgene Blood miRNA system was used for the purification of total RNA from peripheral blood collected in PAXgene Blood RNA Tubes. PAXgene-extracted RNA was quantified using spectrophotometer (NanoDrop™ 1000, Thermo Fisher Scientific, Wilmington, DE). cDNA synthesis was performed using the RT² First Strand kit (Qiagen).

### Phase I PCR Array

For target gene expression analysis, Phase I Enzymes RT² Profiler™ PCR Array (PAHS-068Z), which contains 84 genes involved in Phase I drug metabolism, were used with the SYBR®Green system (Qiagen). The ABI QuantStudio 6 Flex Real Time PCR cycler (Applied Biosystems) was used to perform quantitative real-time PCR (qPCR). All experiments were performed in DNase/RNase free conditions and normalized to GAPDH. The analysis was performed by Threshold Cycle Method and the fold change obtained by the delta CT method. Qiagen's qPCR analysis tool, available on http://pcrdataanalysis.sabiosciences.com/pcr/arr ayanalysis.php, was used for analysis. The Volcano plot considered genes that had $\geq 2$ fold change and a p-value <0.05 in the t-test.

### cDNA Expression Analysis

Quantitative real time PCR (qPCR) was performed for all samples in triplicate on QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific, Carlsbad, CA). qRT-PCR quantification of cDNA expression was performed using assays and accompanying reagents from Qiagen cDNA analyses were performed using Primer Assays for aldehyde dehydrogenase 6 family, member A1 (ALDH6A1; PPH16308B), Cytochrome P450 family 4, subfamily F, polypeptide 12

(CYP4F12, PPH01237F), Granzyme B (GZMB; PPH02594A), and Beta Actin (ACTB; PPH00073G). For each primer assay, negative controls were run with water and cDNA samples. For no template controls (NTC) a master mix with no cDNA was used. Data normalization was performed with ACTB. Relative quantitation was calculated using the delta CT method. Pathway analysis was performed for each of the identified genes using The Human Gene Database available at http://www.genecards.org/.

## RESULTS

The ALT levels and APAP adduct levels in the APA overdose low ALT group were significantly higher than those in the control group, both with p-values <0.01 (**Figure 1**).

The qpCR array, analyzed with the Qiagen web portal qPCR analysis tool, identified three genes as dysregulated, defined as having a fold change $\geq 2$ and a p-value <0.05. They were identified on a volcano plot that plots −log(fold change) against −log(p-value) for each of the 84 genes profiled in the qPCR array (Figure 2).ALDH6A1, GZMB, and CYP4F12 were all identified as downregulated in the low ALT APAP overdose samples. The individual qPCR validation assays confirmed the downregulation of all three genes (Figure 3).

GeneCards' reported pathway analysis shows that ALDH6A1 is involved in metabolic pathways in the degradation of valine, leucine, and isoleucine [2]; GZMB is involved in the Granzyme-B pathway of the immune response, specifically in apoptosis [4]; and CYP4F12 catalyzes leukotriene B4 omega-hydroxylation [3].

| | Sample ID | ALT level (IU/L) | Adducts (moles/mL) |
|---|---|---|---|
| **Control** | 24 | 7 | 0.0001 |
| | 30 | 10 | 0.0009 |
| | 32 | 16 | 0.0038 |
| | 36 | 18 | 0.008 |
| | 38 | 14 | |
| Mean | | 13 | 0.0032 |
| **Low ALT** | 59 | 48 | 0.4403 |
| | 60 | 25 | 0.6983 |
| | 61 | 35 | 0.6663 |
| | 69 | 33 | 0.7767 |
| | 74 | 35 | |
| Mean | | 35.2 | 0.6454 |

**Figure 1:** Sample data for control and APAP overdose (low ALT) groups. The test group had higher ALT levels (p=0.0017) and significantly higher APAP adduct levels (p=0.0030).
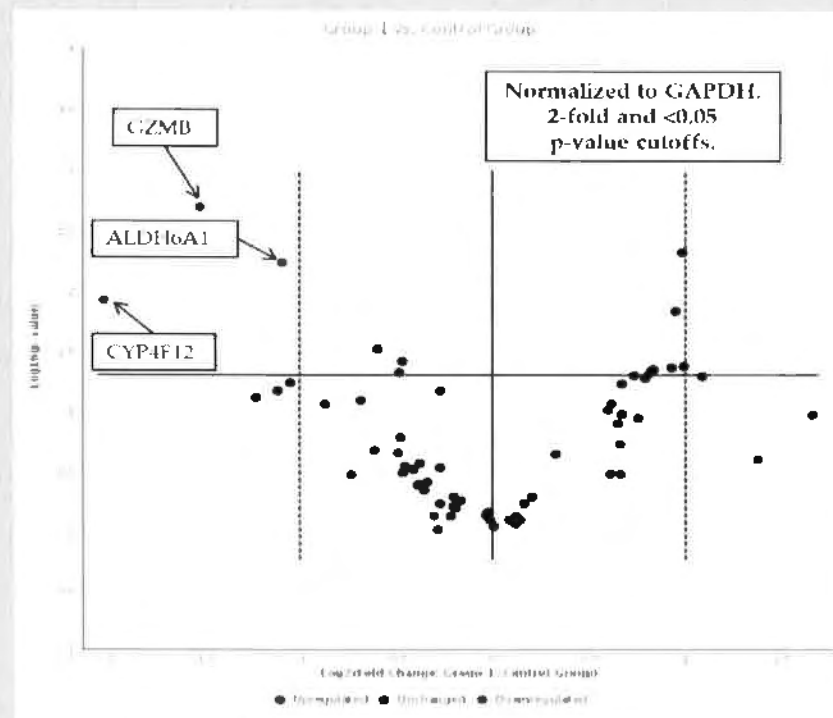
**Figure 2:** Volcano plot that identifies dysregulated genes. Plots −log(fold change) against −log(p-value). Three genes were identified as downregulated: GZMB, ALDH6A1, and CYP4F12.

| Gene | Initial Fold Change | Initial p-value | Validation Fold Change |
|---|---|---|---|
| ALDH6A1 | -2.1281 | 0.00588 | -1.5945 |
| GZMB | -2.8555 | 0.00201 | -1.3148 |
| CYP4F12 | -4.0393 | 0.01208 | -6.6555 |

**Figure 3:** Fold change values for dysregulated genes. Initial fold change values and p-values identified with volcano plot from initial qPCR array. Validation fold change values identified using delta CT method from individual validation qPCR assays.

## DISCUSSION

Based on this study, ALDH6A1, GZMB, and CYP4F12 are all downregulated in pediatric APAP overdose patients with mild liver injury from Arkansas Children's Hospital. Further research that expands the PAXgene database is

needed to confirm these results, as the sample size was limited to the patients available in the hospital.

The most interesting results come from the pathway analysis of the three genes, and the related damage that APAP overdose may cause.

ALDH6A1 is involved in the metabolism of three branched chain amino acids (BCAAs) [2]. Valine, leucine, and isoleucine are the three essential BCAAs that facilitate glucose uptake in the liver and aid in protein synthesis. Research suggests that impairing the degradation of BCAAs leads to their buildup and can produce toxic materials. BCAAs are also essential for the growth of lymphocytes and cytotoxic T cell activity, meaning that they play an important role in the immune response [9]. With the downregulation of an enzyme that metabolizes these amino acids, their activity and role in the immune response would potentially be weakened. Further research to find a link between APAP overdose and the downregulation of ALDH6A1 is needed, but this study may suggest a pathway of liver injury through BCAAs, as well as a weaker immune response as a result.

GZMB is involved in the immune response [4]. It is released by natural killer cells and cytotoxic T cells to induce target cell apoptosis. GZMB also plays a role in chronic inflammation and wound healing [5]. A downregulation of this gene may cause a weakened immune response. GZMB was also found to be downregulated in a previous study, mentioned above, of this lab with APAP overdose patients who presented severe liver injury (ALT levels >75 IU/L). A comprehensive study will be published in the near future. Further research that focuses specifically on the role of GZMB in APAP metabolism is needed to confirm these results and establish a connection between the gene dysregulation and potential cell injuries.

CYP4F12 is involved in the hydroxylation of leukotriene B4 (LTB4) [3]. LTB4 is an epoxide that is an inflammatory mediator [8]. Inflammation is also an important part of the immune response. This suggests that the downregulation of CYP4F12 may affect the effectiveness of inflammation and the overall immune response. Further research into

differences of inflammatory responses in pediatric APAP overdose patients would be interesting.

The downregulated genes point to a potential weakening of the immune response related to APAP overdose, especially in the liver. To confirm these suspicions, further research would need to be done to confirm the initial downregulation of the Phase I enzymes. Extensive experimentation would need to be conducted with APAP overdose patients that tested their immune response. Though not conclusive, this study points to a potential for cell injury associated with APAP overdose outside of the known liver damage. It also gives insight into potential biomarkers through the affected enzymes.

## ARKANSAS CHILDREN'S RESEARCH INSTITUTE

My summer interning at Arkansas Children's Research Institute was an educational and highly beneficial experience. Working in Dr. Laura James' lab under Dr. Prit Gill, I learned valuable laboratory skills and improved upon my professional communication. As part of the Summer Science Program, I was also able to hear from and shadow medical professionals in various departments of the hospital. These experiences expanded my knowledge and helped to prepare me to enter a physician assistant program in the coming year, and to one day be a health care provider myself.

While working in the laboratory, I learned new lab techniques, including plating samples in preparation for qPCR, operating the PCR cycler, and data analysis. In addition to my project, I had the opportunity to assist in other projects that the lab was working on. For a related study, researching the effects of acetaminophen exposure on mouse Leydig cells, I learned how to perform cell culture. This involved splitting the cells, preparing acetaminophen doses, treating the cells with the drug, and extracting

RNA to perform qPCR similar to that done with the PAXgene blood samples.

Working closely with Dr. Gill improved my professional communication skills. I learned how to contribute to meetings by staying up-to-date on reading articles and learning more about the subject, which was all relatively new to me. I also spent a lot of my time on my own, so I learned to work independently in a lab setting, another new experience for me. My internship allowed me to network and to develop a professional relationship with my mentors that will greatly benefit me in the future.

Aside from researching, the Arkansas Children's Research Institute Summer Science Program provided twenty-two other students and me with opportunities to meet medical professionals. Most of us were in college with aspirations to attend a medical graduate program and practice in the health profession. We enjoyed a lecture series twice a week during the eight-week program where we heard from different professionals, including a neonatologist, a geneticist, a pediatric psychologist, an emergency medicine physician, a neurosurgeon, and others from diverse backgrounds and areas. This introduced me to areas of medicine that I knew close to nothing about, and helped me continue to decide what area of medicine I wanted to enter.

Along with the lectures, the shadowing opportunities were immensely beneficial and essential to the overall experience. One of the most interesting experiences I had was shadowing a medical ethicist during his pediatric intensive care unit rounds. We discussed ethical questions that arose during hospital care, and how he worked with the physicians to ensure that patients were cared for with regard to the best medical practices as well as the patient's wishes. It opened my eyes to ethical dilemmas I had never considered. I also had the chance to shadow in the general pediatric surgery unit and the emergency department. Both of these were areas I had never experienced from the side of the provider. I became very interested in both of these high-stakes fields and excited at the possibility of one day working in one of them.

However, the most important shadowing experience I had was with the physician assistant team in cardiovascular surgery. Shadowing with the physician assistants confirmed my desire to apply for physician assistant school and enter the field. I had the opportunity to sit in a pre-operation consultation day, two different open-heart surgeries, and post-operation intensive care rounds. The physician assistants worked with their patients throughout their entire treatment and I had the chance to see all of the aspects. This experience inspired me and helped me be accepted into the physician assistant program.

Overall, my summer internship at Arkansas Children's Research Institute was highly beneficial. I would strongly suggest that those who wish to enter to medical field apply for similar programs or experiences, as they expanded my knowledge and gave me invaluable opportunities. Because of the opportunity to independently research and my time spent shadowing and networking with medical professionals, I feel better prepared to enter into a career in the health care field.

## ACKNOWLEDGMENTS

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*In accordance with Dr. Prit Gill and his pending manuscript, "Granzyme B and miR-738a interaction in acetaminophen toxicity," which is awaiting publication.