THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

EXPLORING THE CAUSES OF MUSCLE DYSFUNCTION ASSOCIATED WITH ADENYLOSUCCINATE LYASE DEFICIENCY IN THE MODEL ORGANISM *CAENORHABDITIS ELEGANS*

JUDY JAN
SPRING 2022

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biochemistry and Molecular Biology
with honors in Biochemistry and Molecular Biology

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ABSTRACT

Adenylosuccinate Lyase Deficiency (ASLD) is caused by a mutation in a gene encoding for the adenylosuccinate lyase enzyme, which functions twice in the purine metabolic pathway, converting metabolites succinylaminoimidazole carboxamide ribotide (SAICAR) to 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and succinyladenosine monophosphate (S-AMP) to adenosine monophosphate (AMP). Elevated levels of succinylaminoimidazole carboxamide riboside (SAICAr) and succinyladenosine (S-Ado)—the dephosphorylated forms of SAICAR and S-AMP, respectively—in the body fluids of patients with ASLD have been observed. Previous research studying ASLD using the model organism *C. elegans* has looked at how muscle function is affected by decreased or eliminated *adsl-1* expression; however, the cause of the changes in muscle function remains in contention. In this study, I further the research begun by the Hanna-Rose lab regarding the role that increased levels of SAICAR play in worms with reduced expression of *adsl-1*. I supplement *adsl-1*(RNAi) knockdown worms with water-soluble lometrexol, a drug inhibiting the *de novo* purine biosynthesis pathway upstream of where the adenylosuccinate lyase enzyme functions. I demonstrate that supplementation with lometrexol fully restores muscle function in *adsl-1*(RNAi) animals, suggesting that inhibition of the *de novo* purine biosynthesis pathway does improve muscle function in animals that have reduced *adsl-1* expression. I also perform a metabolomics analysis to explore if there are any differences in mitochondrial function in worms with decreased *adsl-1* expression to further the research on the additional possible contributing factors of muscle dysfunction in ASLD.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

5'-PRA	5'-Phosphoribosylamine
AICAR	5-Aminoimidazole-4-carboxamide Ribonucleotide
AMP	Adenosine Monophosphate
APRT	Adenine Phosphoribosyltransferase
ASLD	Adenylosuccinate Lyase Deficiency
EV	Empty Vector
GARFT	Glycinamide Ribonucleotide Formyltransferase
GMP	Guanosine Monophosphate
HGPRT	Hypoxanthine-guanine Phosphoribosyltransferase
IMP	Inosine Monophosphate
LC-MS	Liquid Chromatography-Mass Spectrometry
LMX	Lometrexol
N^{10}-formyl-THF	10-Formyltetrahydrofolate
PRPP	Phosphoribosyl Phosphate
S-Ado	Succinyladenosine
SAICAR	Succinylaminoimidazole Carboxamide Ribotide
SAICAr	Succinylaminoimidazole Carboxamide Riboside
S-AMP	Succinyladenosine Monophosphate
TCA	Tricarboxylic Acid Cycle
THF	Tetrahydrofolate
TIC	Total Ion Chromatogram

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Hanna-Rose, for her guidance throughout my time in the lab. I would also like to thank my mentor, Latisha Franklin, for educating me on how to perform several of the lab and analysis techniques used throughout my research. I want to thank Latisha Franklin and Corinna Moro for providing me with their metabolomics data set for analysis in this thesis. I am extremely grateful for the help that all the members of the Hanna-Rose lab were always willing to offer me when I had a question. Lastly, I want to thank my parents for making my educational journey possible and for supporting me along the way.

Chapter 1

Introduction

Adenylosuccinate Lyase Deficiency

Adenylosuccinate lyase deficiency (ASLD) is a rare metabolic disease that was first characterized by presence of succinyladenosine (S-Ado) and succinylaminoimidazole carboxamide riboside (SAICAr) in the body fluids of patients (Jaeken et al., 1984). These patients exhibited phenotypes such as psychomotor delay, autistic-like behaviors, and hypotonia (Jaeken et al., 1984). Due to the presence of S-Ado and SAICAr, it was proposed that this disease was due to lack of proper functioning of the adenylosuccinate lyase enzyme, which acts twice in the purine metabolic pathway (Jaeken et al., 1984). This was supported by findings of decreased or non-existent adenylosuccinate lyase enzyme activity in particular organs, such as the liver or kidney, of a patient with ASLD (Jaeken et al., 1984). This hypothesis was later confirmed when a point mutation in the gene encoding for the adenylosuccinate lyase enzyme was found to be associated with ASLD, and several other mutations of the adenylosuccinate lyase gene have also since been discovered (Marie et al., 1999; Stone et al., 1992).

Both the phenotypes and severity of ASLD vary clinically, and surveys of ASLD patients have reported a delay of several years when it comes to diagnosis (Mastrogiorgio et al., 2021). While traditional diagnosis methods for ASLD, such as testing for S-Ado and SAICAr in the body fluids (such as cerebral spinal fluid, urine, etc.), are still being used, newer techniques, like genetic testing and amniotic fluid testing, may help to lead to quicker diagnosis of ASLD in

patients as this would reduce the time spent testing the patient for other phenotypically similar diseases (Lee & Campion, 2021; Mastrogiorgio et al., 2021).

In a recent web-based survey of 18 patients confirmed to have ASLD, patients' symptoms could largely be separated into three different categories, which have been previously documented (Mastrogiorgio et al., 2021). The first category is associated with newborns and is severe and fatal in nature, with patients exhibiting symptoms including difficulty breathing, encephalopathy, and seizures (Jurecka et al., 2008a; Mastrogiorgio et al., 2021; Mouchegh et al., 2007; Van den Bergh et al., 1998). The second category is also severe and begins early on in a patient's life, with patients experiencing seizures (Jurecka et al., 2008a). Patients with this 'type I' ASLD also tend to exhibit autistic-like behaviors and psychomotor delay (Jurecka et al., 2008a). The third category ('type II' ASLD) is less severe and begins slightly later compared to 'type I' ASLD, but this category still features psychomotor delay and, in some cases, seizures (Jurecka et al., 2008a). Each category of ASLD is also associated with a different ratio of S-Ado to SAICAr, with the most severe forms, which are associated with fatality and earlier onset, featuring a higher concentration of SAICAr compared to that of S-Ado, while the least severe form ('type II'), associated with a later onset, features a higher concentration of S-Ado compared to SAICAr (Mastrogiorgio et al., 2021; Van den Bergh et al., 1993). Because age of onset and severity of ASLD are associated with one another (Lee & Campion, 2021; Mastrogiorgio et al., 2021), further evidence may be needed to prove that the differing ratios of S-Ado to SAICAr associated with each category of ASLD are responsible for the differing levels of severity as the effect that the patient's age at diagnosis has on the ratio of S-Ado to SAICAr should be further explored.

Focusing on the muscular phenotypes of ASLD, the first few patients diagnosed with ASLD when the disease was first characterized exhibited axial hypotonia (reduced muscle tone) (National Institute of Neurological Disorders and Stroke, 2019), with some of the patients' performance of motor processes being comparable to that of children younger in age; however, tendon reflexes did not appear to be affected (Jaeken et al., 1984). In the more recent web-based survey, some patients also exhibited hypotonia while others did not (Mastrogiorgio et al., 2021). Patients also displayed differing levels of psychomotor delay (Mastrogiorgio et al., 2021).

Currently, there are no definitive treatments for ASLD; there are only medications designed to alleviate symptoms of ASLD, such as epilepsy, or medications that require further testing in their efficacy (Jurecka et al., 2015). Some proposed treatments, such as administering D-ribose, have demonstrated variable results that require further testing (Jurecka et al., 2008b; Salerno et al., 1998). Currently, one clinical trial is examining the effects of treating ASLD patients with allopurinol, specifically testing the drug's ability to decrease SAICAr levels as well as studying its effects on epilepsy and the autistic-like behaviors associated with ASLD (De Lonlay et al., 2021). Ultimately, given the varying symptoms and levels of severity of ASLD, a closer look into the role of the adenylosuccinate lyase enzyme in the purine metabolic pathway may help to better elucidate the reasons underlying the differences in the disease's phenotypic expression.

Adenylosuccinate Lyase Enzyme and the Purine Metabolic Pathway

The purine metabolic pathway is broken down into the *de novo* purine biosynthesis pathway and the purine salvage pathway, with the adenylosuccinate lyase enzyme acting twice in

the purine metabolic pathway (Figure 1) (Yin et al., 2018). The *de novo* purine biosynthesis pathway is a series of ten steps, starting with the conversion of phosphoribosyl phosphate (PRPP) to 5'-phosphoribosylamine (5'-PRA) and concluding with the production of inosine monophosphate (IMP) (Yin et al., 2018). Following the production of IMP, an additional series of steps can lead to the generation of adenosine monophosphate (AMP) or guanosine monophosphate (GMP) (Yin et al., 2018). Adenylosuccinate lyase catalyzes the conversion of succinylaminoimidazole carboxamide ribotide (SAICAR) into 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) as well as succinyladenosine monophosphate (S-AMP) into AMP, with fumarate being a byproduct of both steps (Yin et al., 2018).

Once AMP and GMP are produced, AMP can be converted to adenine and GMP can be converted to guanosine in a series of reactions. The enzymes adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) catalyze the conversion of adenine and guanine to AMP and GMP, respectively, in the purine salvage pathway. AMP and GMP can be further converted back to IMP, and these two reactions are catalyzed by AMP deaminase and GMP reductase, respectively.

In the two reactions catalyzed by adenylosuccinate lyase in the purine metabolic pathway, the reactants can be further dephosphorylated. SAICAR can be converted to SAICAr, and S-AMP can be converted to S-Ado. Therefore, mutations in the adenylosuccinate lyase enzyme would be expected to cause an increase in the metabolites SAICAr and S-Ado, which is seen in patients with ASLD (Jaeken et al., 1984).

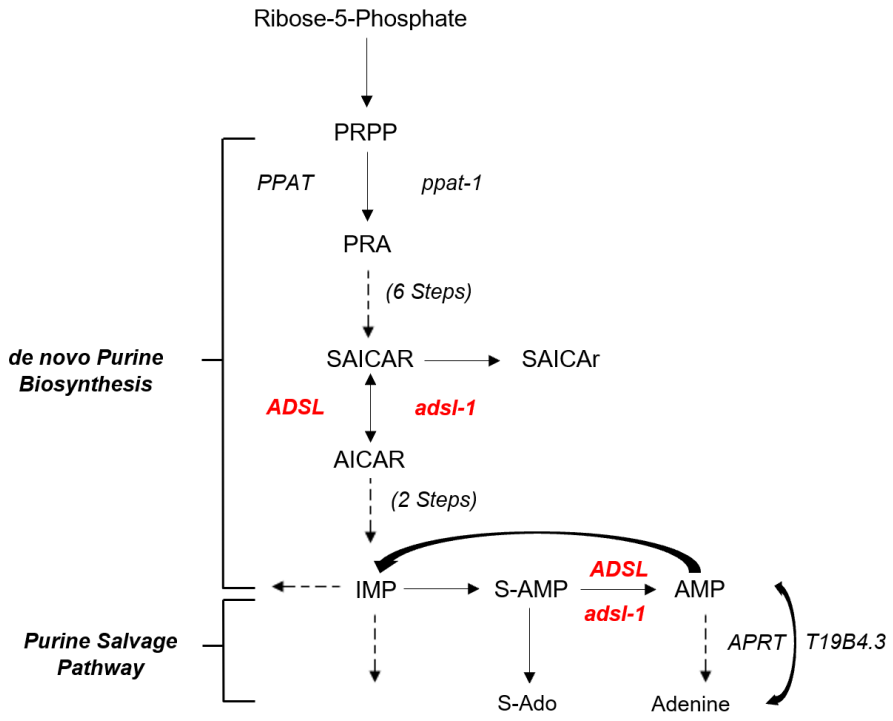

Figure 1. The Purine Metabolic Pathway

The purine metabolic pathway consists of both the *de novo* purine biosynthesis pathway and the purine salvage pathway. The adenylosuccinate lyase enzyme functions twice in the purine metabolic pathway, converting SAICAR to AICAR and converting S-AMP to AMP. The human gene names are located on the left or on the top of the arrows. For example, the human gene encoding for adenylosuccinate lyase is *ADSL*. The *C. elegans* gene encoding for adenylosuccinate lyase is *adsl-1*.

Adenylosuccinate Lyase Deficiency in *C. elegans*

Previous studies of ASLD have used the nematode *Caenorhabditis elegans* as a model organism (Fenton et al., 2017; Marsac et al., 2019). One such study justified the usage of *C. elegans* as a model organism for studying purine biosynthesis by first using sequence homology to identify conserved genes, with *adsl-1* being one such gene that was predicted to encode for the

adenylosuccinate lyase enzyme (Marsac et al., 2019). This was followed by a heterologous rescue assay where *adsl-1* of *C. elegans* was expressed in *ade13(Δ) S. cerevisiae*, where *ade13* is the gene in yeast encoding for the adenylosuccinate lyase enzyme (Marsac et al., 2019). This assay demonstrated the ability of the adenylosuccinate lyase enzyme of *C. elegans* to restore growth of *ads13(Δ) S. cerevisiae* in the absence of adenine, providing further evidence that *adsl-1* of *C. elegans* has conserved function to the *ADSL* gene of *H. sapiens* (Marsac et al., 2019). An additional test looking at *adsl-1* function in *C. elegans* was then performed in which high-performance liquid chromatography was used in a metabolomics analysis of *adsl-1(RNAi)* worms (Marsac et al., 2019). This data revealed that a knockdown of *adsl-1* using RNAi did, indeed, lead to an increase in SAICAR and SAICAr as well as S-AMP and S-Ado, as predicted (Marsac et al., 2019). Thus, *C. elegans* is an appropriate model organism for studying the purine biosynthesis pathway and particularly the role of the adenylosuccinate lyase enzyme (Marsac et al., 2019).

Research shows that *adsl-1* mutants as well as *adsl-1*(RNAi) worms display several phenotypes (Fenton et al., 2017; Marsac et al., 2019). *adsl-1* mutants are sterile, lacking oocytes and sperm cells (Marsac et al., 2019). Some of these *adsl-1* mutants as well as *adsl-1*(RNAi) animals also display a protruding vulva (Marsac et al., 2019). Moreover, *adsl-1* mutants also have a delay in post-embryonic development and are smaller in size compared to wild-type (N2) worms (Marsac et al., 2019). Furthermore, *adsl-1* mutants and *adsl-1*(RNAi) animals have a decreased speed or thrashing rate compared to wild-type animals or control animals (Fenton et al., 2017; Marsac et al., 2019). Additionally, *adsl-1* mutants have a different muscular fiber structure compared to wild-type animals as well as decreased muscle mass (Marsac et al., 2019).

Proposed Hypotheses for the Muscle Dysfunction Phenotype Associated with ADSL Deficiency

In two studies that observed either decreased speed or thrashing rate in either *adsl-1* mutants and/or *adsl-1*(RNAi) animals, differing conclusions were reached regarding the likely cause of muscle dysfunction (Fenton et al., 2017; Marsac et al., 2019). One study assessed the speed of *ppat-1(Δ);adsl-1(Δ)* double mutants (Marsac et al., 2019); the *ppat-1* gene encodes for the amidophosphoribosyltransferase enzyme which functions upstream of the adenylosuccinate lyase enzyme, converting PRPP to PRA (Figure 1). If the decrease in speed of *adsl-1* mutants was attributed to changes in levels of metabolites involved in *de novo* purine biosynthesis, especially an increase in SAICAR, then the *ppat-1(Δ);adsl-1(Δ)* double mutants would be predicted to have at least a partial recovery in muscle function and thus an increased speed compared to *adsl-1* mutants. However, the study observed the opposite finding, noting that the *ppat-1(Δ);adsl-1(Δ)* mutants had a slower speed compared to *adsl-1* mutants (Marsac et al., 2019). A metabolomics analysis of *adsl-1*(RNAi) animals did find slightly increased levels of SAICAR (Marsac et al., 2019). Although a metabolomics analysis was not performed for *ppat-1(Δ);adsl-1(Δ)* animals, metabolomics was performed on *ppat-1(Δ);adsl-1*(RNAi) animals, and it was observed that SAICAR levels were negligible, indicating that the deletion of *ppat-1* did appear to lower SAICAR levels in *adsl-1*(RNAi) animals (Marsac et al., 2019). This study thus concluded that the decreased speed in *adsl-1* mutants was not attributed to increased levels of SAICAR (Marsac et al., 2019).

However, in another study, performed by the Hanna-Rose lab, *adsl-1* mutants and *adsl-1*(RNAi) animals were supplemented with methotrexate, an antimetabolite that results in inhibition of parts of the *de novo* purine biosynthesis pathway (Fenton et al., 2017; Tjong et al.,

2021). The Hanna-Rose lab found that both *adsl-1* mutants and *adsl-1*(RNAi) animals supplemented with methotrexate showed increased thrashing rates, with *adsl-1* mutants showing a partial restoration of muscle function compared to wild-type and the *adsl-1*(RNAi) animals showing a full rescue of muscle function when compared to control animals (Fenton et al., 2017). This study used liquid chromatography-mass spectrometry (LC-MS) to demonstrate that *adsl-1*(RNAi) animals supplemented with methotrexate did have decreased levels of SAICAR compared to untreated *adsl-1*(RNAi) animals, leading them to conclude that accumulation of SAICAR due to decreased *adsl-1* expression is at least partially responsible for the muscle dysfunction phenotype (Fenton et al., 2017). However, it is important to note that this study used DMSO-soluble methotrexate, with DMSO being identified as affecting purine metabolism, by itself (Zoref-Shani et al., 1994). In a metabolomics analysis performed by the Hanna-Rose lab, it was noted that DMSO treatment of *adsl-1*(RNAi) animals also led to a significant decrease in SAICAR. However, DMSO, by itself, was not capable of rescuing the muscle dysfunction phenotype (Fenton et al., 2017). Therefore, it is uncertain as to whether the decrease in SAICAR levels is responsible for the partial and full restoration of muscle function in *adsl-1* mutants and *adsl-1* (RNAi) animals, respectively. Further experimentation using a water-soluble drug inhibiting the *de novo* purine biosynthesis pathway upstream of where adenylosuccinate lyase functions may better elucidate whether increased SAICAR levels lead to muscle dysfunction in *C. elegans* with decreased levels of *adsl-1* expression.

The Purine Biosynthesis Pathway and Mitochondrial Function

Within the last decade, scientists have proposed the existence of a purinosome, a complex containing nine enzymes that catalyze fourteen reactions of the purine metabolic pathway (Pareek et al., 2020). Through a combination of metabolomics and mass spectrometry, the purinosome has been found to be capable of producing both AMP and GMP, though the conversion of IMP to AMP (rather than GMP) is suggested to be favored (Pareek et al., 2020). Furthermore, scientists have gathered data supporting the possible interaction between the purinosome and the mitochondria (French et al., 2016). Through the use of 3D stochastic optical reconstruction microscopy (3D STORM), scientists determined that when purinosomes form, they are not found evenly throughout the cytoplasm of cells; rather, most purinosomes are located near the mitochondria (French et al., 2016). When these scientists sought to identify proteins that coprecipitated with the mitochondria, one of the enzymes was adenylosuccinate lyase and another was phophoribosyl formylglycinamidine, two enzymes of the *de novo* purine biosynthesis pathway (French et al., 2016). This again supported that the purinosome likely colocalizes with the mitochondria (French et al., 2016).

Other potential connections between the purine biosynthesis pathway and the mitochondria can be hypothesized based on the substrates needed as well as some of the byproducts produced in the reactions of the purine biosynthesis pathway. For example, the 3rd and 9th reactions of the *de novo* purine biosynthesis pathway require 10-formyltetrahydrofolate (N^{10}-formyl-THF) as a substrate, which can be made from formate produced in the tetrahydrofolate (THF) cycle of the mitochondria (Ben-Sahra et al., 2016). The THF cycle of the mitochondria thus serves as another connection between the mitochondria and the *de novo* purine biosynthesis pathway. Furthermore, in both reactions that adenylosuccinate lyase catalyzes,

fumarate is produced as a byproduct. Fumarate is one of the key metabolites of the tricarboxylic acid (TCA) cycle, which occurs in the mitochondria. Therefore, one might hypothesize that a mutation in adenylosuccinate lyase may potentially have some effect on the TCA cycle (Figure 2); however, both metabolomic analysis as well as experiments assessing mitochondrial function in animals with decreased *adsl-1* expression are required before any conclusions can be drawn.

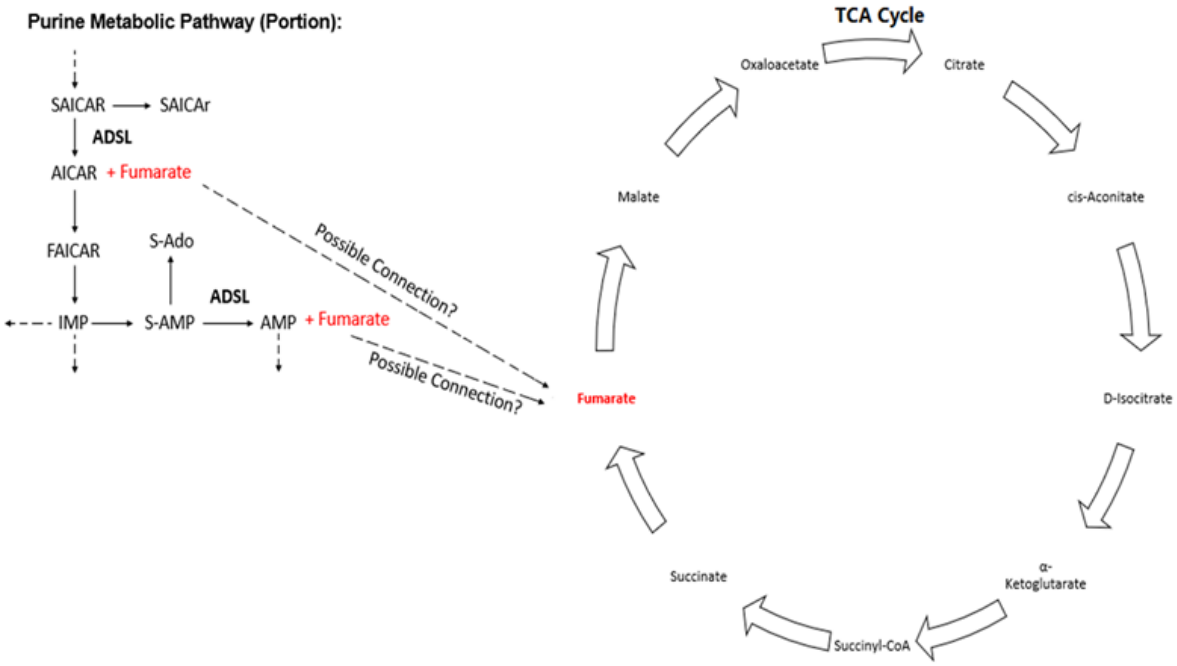

Figure 2. Possible Connection Between the Purine Metabolic Pathway and the TCA Cycle

In the two reactions of the purine metabolic pathway that adenylosuccinate lyase catalyzes, fumarate is produced as a byproduct. Fumarate is a key metabolite of the TCA cycle; therefore, an analysis of TCA cycle metabolite levels in samples of worms with decreased *adsl-1* expression should be performed to elucidate any possible connections between the purine metabolic pathway and the TCA cycle.

Ultimately, I hypothesize that imbalances in *de novo* purine biosynthesis due to lack of proper function of the adenylosuccinate lyase enzyme as well as impaired mitochondrial function both contribute to the muscle dysfunction phenotype observed in *C. elegans* with lowered *adsl-1*

expression. To test the role that imbalances in *de novo* purine biosynthesis play in muscle function, I will be treating *adsl-1*(RNAi) worms with water-soluble lometrexol, which will inhibit the *de novo* purine biosynthesis pathway upstream of where adenylosuccinate lyase functions, theoretically preventing accumulation of SAICAR in the *adsl-1*(RNAi) worms. To test the effect that lowered *adsl-1* expression has on mitochondrial function, I will analyze a metabolomics data set and determine if metabolites of the TCA cycle are significantly altered in *adsl-1*(RNAi) samples.

Chapter 2

Results

Inhibition of *de novo* Purine Biosynthesis Restores Muscle Function in *adsl-1*(RNAi) *C. elegans*

A previous study of the effects of *de novo* purine biosynthesis inhibition on muscle function in *adsl-1*(RNAi) animals used DMSO-soluble methotrexate (Fenton et al., 2017). To determine the sole effects of the inhibition of the *de novo* purine biosynthesis pathway upstream of where adenylosuccinate lyase functions, without any contributing effects by DMSO, I used water-soluble lometrexol (LMX), which inhibits the enzyme glycinamide ribonucleotide formyltransferase (GARFT) (Avendaño et al., 2008), which catalyzes the 2nd, 3rd, and 5th reactions of the *de novo* purine biosynthesis pathway (Figure 3).

After supplementing RNAi plates with water-soluble LMX and performing a knockdown of *adsl-1* on N2 (wild-type) animals, I performed a thrashing assay and determined the thrashing rates of *adsl-1*(RNAi) and empty vector (EV)(RNAi) animals treated with 54 nM LMX as well as the thrashing rates of untreated *adsl-1*(RNAi) and EV(RNAi) animals. Untreated *adsl-1*(RNAi) animals (n=36) were found to have a significantly lower thrashing rate compared to untreated EV(RNAi) animals (n=41) ($p<0.0001$) (Figure 4), confirming that *adsl-1*(RNAi) animals have decreased levels of muscle function. EV(RNAi) animals treated with 54 nM LMX (n=39) were not found to have a significantly different thrashing rate compared to untreated EV(RNAi) animals ($p=0.967$) (Figure 4), indicating that LMX, alone, likely does not affect the muscle function of control animals. On the other hand, *adsl-1*(RNAi) animals treated with 54 nM LMX (n=30) had a significantly higher thrashing rate compared to *adsl-1*(RNAi) animals

($p<0.0001$), indicating that 54 nM LMX was able to significantly increase levels of muscle function in *adsl-1*(RNAi) animals (Figure 4).

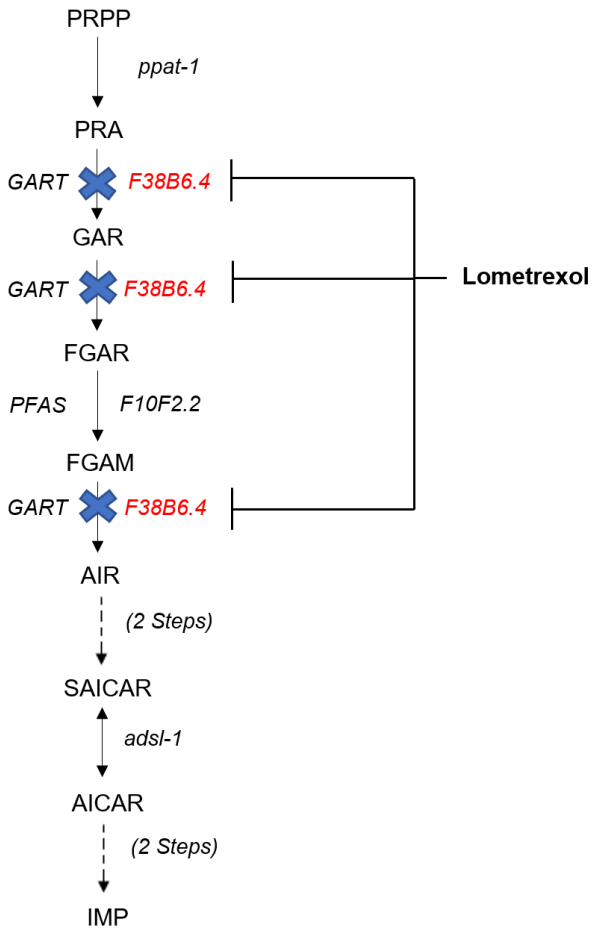

Figure 3. Lometrexol Inhibits the *de novo* Purine Biosynthesis Pathway Upstream of *adsl-1*

Lometrexol inhibits the GARFT enzyme, preventing the catalyzation of the second, third and fifth reactions of the *de novo* purine biosynthesis pathway upstream of where adenylosuccinate lyase functions (Avendaño et al., 2008).

Additionally, when comparing the thrashing rates between treated EV(RNAi) animals and treated *adsl-1*(RNAi) animals, the thrashing rates were not found to be significantly different ($p=0.664$), indicating that 54 nM LMX is capable of fully recovering muscle function in *adsl-*

1(RNAi) animals (Figure 4). This experiment indicates that inhibiting *de novo* purine biosynthesis in *adsl-1*(RNAi) animals fully recovers muscle function.

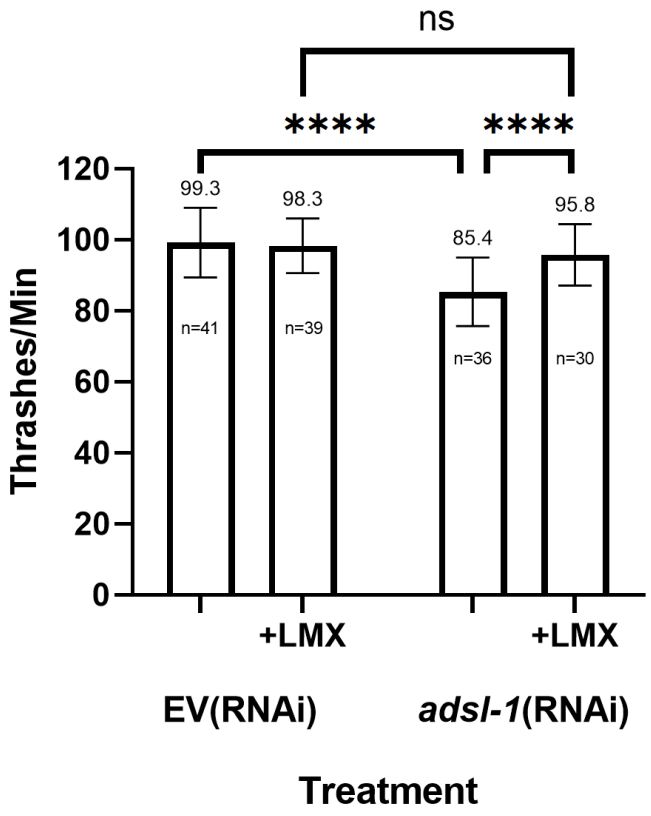

Figure 4. Lometrexol Supplementation Restores Muscle Function of *adsl-1*(RNAi) Worms

adsl-1(RNAi) animals treated with lometrexol have a significantly higher thrashing rate compared to untreated *adsl-1*(RNAi) animals ($p<0.0001$), indicating improved muscle function. Furthermore, treated *adsl-1*(RNAi) and treated EV(RNAi) animals have comparable thrashing rates ($p=0.664$), indicating a full recovery of muscle function in treated *adsl-1*(RNAi) animals. **** indicates that the p-value is less than or equal to 0.0001. P-values were deemed non-significant (ns) if they were greater than 0.05.

Reduced Expression of *adsl-1* Affects Levels of Some Metabolites of the TCA Cycle

Both of the previous studies looking at the causes of muscle dysfunction associated with a lack of *adsl-1* expression indicated that inhibition of the *de novo* purine biosynthesis pathway upstream of where adenylosuccinate lyase functions either only partially rescued (Fenton et al., 2017) or even worsened muscle function of *adsl-1* mutants (as was the case with *ppat-1(Δ);adsl-1(Δ)* mutants)(Marsac et al., 2019). Therefore, to explore additional contributing factors to the muscle dysfunction phenotype, I performed an analysis of a metabolomics data set collected by fellow Hanna-Rose lab members, Latisha Franklin and Corinna Moro. Metabolites were considered to be 'identified' if a standard for that metabolite had been run; otherwise, possible metabolite candidates were found using a combination of the metabolite's mass-to-charge ratio, literature value for retention time (Lu et al., 2010), and fill percentage. While a total of 61 samples were collected and assessed, I analyzed only EV(RNAi) and *adsl-1*(RNAi) samples (which together made up 11 of the 61 total samples).

I first performed an analysis of the metabolite SAICAR. When a knockdown of *adsl-1* in *eri-1* worms—worms that have an elevated sensitivity to RNAi treatment (Kennedy et al., 2004)—was performed, SAICAR levels were significantly higher in these worms compared to EV control animals, which showed no detectable evidence of SAICAR (Fenton et al., 2017). Similarly, for this data set, SAICAR levels were also found to be elevated in N2 worms with a knockdown in *adsl-1* compared to EV control animals ($p=0.013$), which showed no detectable levels of SAICAR (Figure 5). This indicates that reduced *adsl-1* expression does affect the purine metabolic pathway, leading to an accumulation of SAICAR.

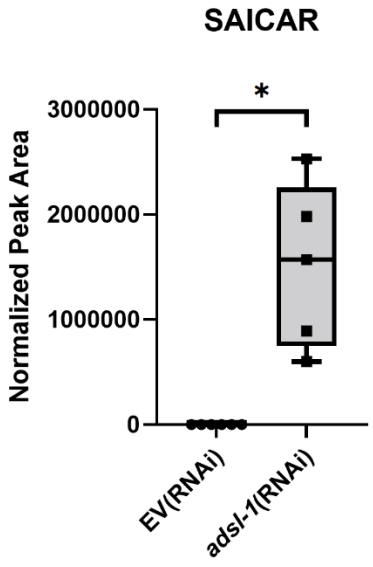

Figure 5. SAICAR Levels are Elevated in *adsl-1*(RNAi) Samples

SAICAR levels in *adsl-1*(RNAi) samples (n=5 samples) are significantly higher (p=0.013) compared to those of EV(RNAi) samples (n=6 samples), which show no detectable levels of SAICAR. * indicates a p-value less than or equal to 0.05.

After confirming that decreased *adsl-1* expression led to changes in SAICAR levels as expected, I then assessed the levels of metabolites in the TCA cycle of the mitochondria to determine if there are any signs of changes in this key pathway that occur with decreased expression of *adsl-1*. Beginning with citric acid, an identified metabolite in which citrate is a derivative of, the normalized peak area of this metabolite was significantly higher (p=0.0016) in *adsl-1*(RNAi) samples compared to EV(RNAi) samples (Figure 6). A potential candidate for aconitate did not have significantly different normalized peak areas between *adsl-1*(RNAi) and EV(RNAi) samples (p=0.59) (Figure 7). A potential candidate for alpha-ketoglutarate had a significantly lower normalized peak area in *adsl-1*(RNAi) samples compared to EV(RNAi) samples (p=0.023) (Figure 6). Succinate, an identified metabolite, did not appear to have a

significantly different normalized peak area between *adsl-1*(RNAi) and EV(RNAi) samples (p=0.33) (Figure 7).

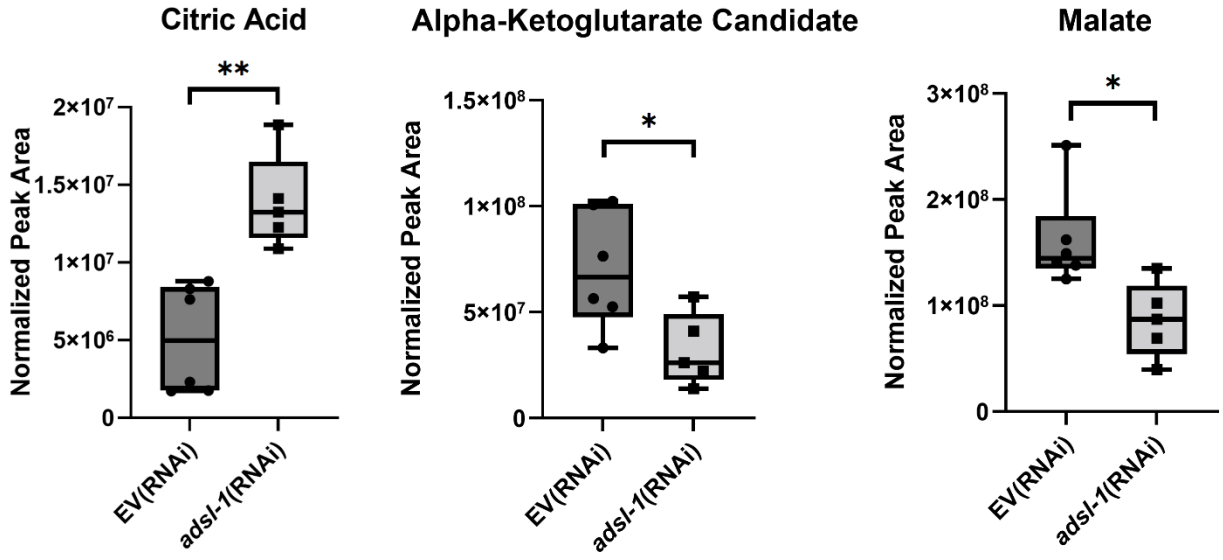

Figure 6. Citric Acid, Alpha-Ketoglutarate, and Malate Levels are Significantly Different in *adsl-1*(RNAi) Samples

The normalized peak area for citric acid in *adsl-1*(RNAi) samples (n=5 samples) is significantly higher compared to that of EV(RNAi) samples (n=6 samples) (p=0.0016). The normalized peak area for alpha-ketoglutarate in *adsl-1*(RNAi) samples is significantly lower compared to that of EV(RNAi) samples (p=0.023). The normalized peak area for malate in *adsl-1*(RNAi) samples is also significantly lower compared to that of EV(RNAi) samples (p=0.015). ** indicates a p-value less than or equal to 0.01. * indicates a p-value less than or equal to 0.05.

Fumarate was a metabolite proposed to serve as a potential connection between the purine metabolic pathway and the TCA cycle as fumarate is produced as a byproduct in reactions catalyzed by adenylosuccinate lyase. However, when identifying the metabolite with the closest mass-to-charge ratio and retention time to the fumarate standard run, the data suggested that fumarate was a metabolite where peak area could not be easily detected using LC-MS.

Malate, an identified metabolite, had a significantly decreased normalized peak area in *adsl-1*(RNAi) samples compared to EV(RNAi) samples (p=0.015) (Figure 6). Lastly, a candidate for oxaloacetate did not show significantly different normalized peak areas between *adsl-1*(RNAi) and EV(RNAi) samples (p=0.71) (Figure 7).

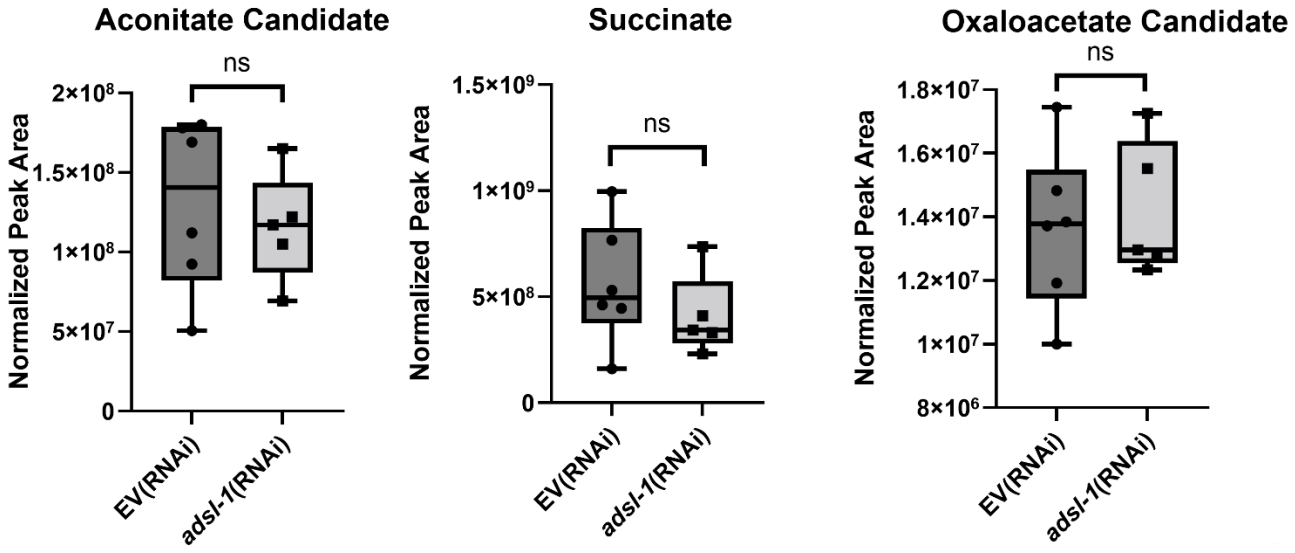

Figure 7. Aconitate, Succinate, and Oxaloacetate Levels are not Significantly Different in *adsl-1*(RNAi) Samples.

The normalized peak areas for the candidates of the metabolites aconitate, succinate, and oxaloacetate of *adsl-1*(RNAi) samples (n=5 samples) were not observed to be significantly different from those of EV(RNAi) samples (n=6 samples) ($p > 0.05$; ns=non-significant).

Overall, it appears that there are significantly different normalized peak areas of three metabolites of the TCA cycle in *adsl-1*(RNAi) samples: citric acid, alpha-ketoglutarate, and malate (Figure 8). For the rest of the TCA cycle, metabolites showed no significant differences in normalized peak areas between *adsl-1*(RNAi) and EV(RNAi) samples, or the peak area of

some metabolites could not be easily determined using LC-MS, or a potential candidate could not be easily identified using mass-to-charge and literature retention time values.

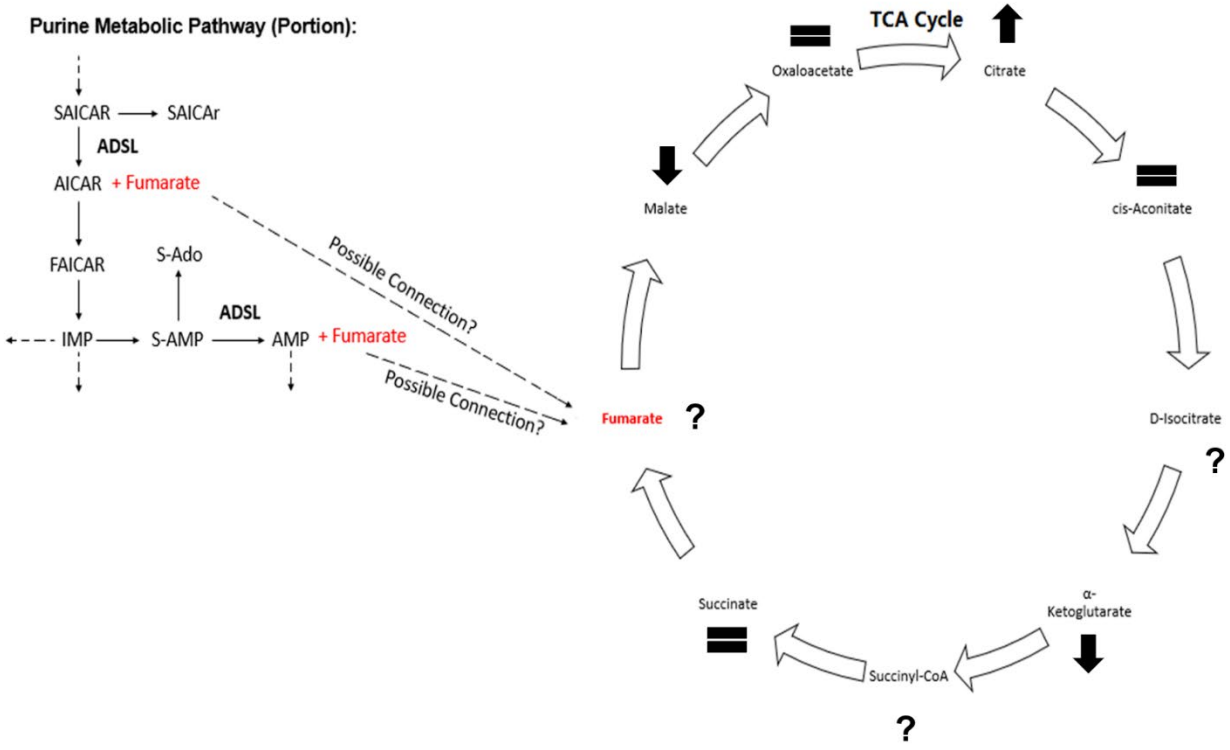

Figure 8. Overview of Normalized Peak Areas of TCA Cycle Metabolites Associated with *adsl-1* Knockdown

Metabolites citric acid (citrate), alpha-ketoglutarate, and malate had significantly different normalized peak areas in *adsl-1*(RNAi) samples compared to EV(RNAi) samples, as indicated by the black arrows, where arrows pointing up indicate that the metabolite had a significantly higher normalized peak area in *adsl-1*(RNAi) samples while arrows pointing down indicate that the metabolite had a significantly lower normalized peak area in *adsl-1*(RNAi) samples. If the normalized peak areas of metabolites were not significantly different between *adsl-1*(RNAi) and EV(RNAi) samples, these metabolites were designated with an equal-sign. If a potential candidate for the metabolite was not identified, or if LC-MS methods could not easily and accurately identify a metabolite's peak area, these metabolites were designated with a question mark.

Chapter 3

Discussion

In this study, I explored the possible contributing factors for muscle dysfunction seen in *adsl-1*(RNAi) animals to address the conflicting conclusions reached by two separate studies looking at the role that SAICAR accumulation plays in the muscle dysfunction phenotype (Fenton et al., 2017; Marsac et al., 2019). An RNAi knockdown of *adsl-1* led to a significant decrease in thrashing, as expected based on previous research (Fenton et al., 2017; Marsac et al., 2019). Although previous research demonstrated that *adsl-1(tm3328)* mutants (lack any *adsl-1* expression) display a more significant decrease in thrashing (reported to be approximately 77% lower than the thrashing rate of control animals) compared to *adsl-1*(RNAi) animals (Fenton et al., 2017), they were not used for this experiment due to the difficulty in handling this strain that makes achieving high enough sample sizes challenging. Furthermore, because previous research had indicated that DMSO-soluble methotrexate fully restored muscle function in *adsl-1*(RNAi) animals (knockdown performed on *eri-1* worms) (Fenton et al., 2017), the efficacy of water-soluble LMX in recovering muscle function would likely be easier to see in *adsl-1*(RNAi) worms.

I found that treating *adsl-1*(RNAi) worms with LMX did increase the thrashing rate of *adsl-1*(RNAi) worms to levels comparable to treated control animals, indicating that muscle function was fully restored. Because previous studies either showed that inhibition of *de novo* purine biosynthesis could only partially restore the muscle function of *adsl-1* mutants (Fenton et al., 2017) or even led to decreased muscle function in the case of *ppat-1(Δ);adsl-1(Δ)* double mutants (Marsac et al., 2019), I hypothesize that residual *adsl-1* expression is required for

inhibition of upstream *de novo* purine biosynthesis to rescue muscle function in animals with decreased *adsl-1* expression.

Further experiments are required to support my hypothesis that residual expression of *adsl-1* is needed for inhibition of *de novo* purine biosynthesis to recover muscle function. RT-PCR should be performed on untreated *adsl-1*(RNAi) and EV(RNAi) worms as well as *adsl-1*(RNAi) and EV(RNAi) worms treated with water-soluble LMX. This would provide quantitative evidence that *adsl-1* expression is significantly reduced when a knockdown of *adsl-1* is performed on N2 animals. For my experiments, I solely relied on the presence of the protruding vulva phenotype that *adsl-1*(RNAi) animals have been previously documented to have to indicate that the *adsl-1* RNAi knockdown had worked (Marsac et al., 2019). Furthermore, by quantifying the relative expression level of *adsl-1* in *adsl-1* (RNAi) animals, I can ensure that treatment with LMX, itself, did not influence *adsl-1* expression, and I can gain a better understanding of how much expression of *adsl-1* is required for inhibition of *de novo* purine biosynthesis to influence muscle function. Another future experiment that should be performed is an LC-MS metabolomics analysis of *adsl-1*(RNAi) animals treated with water-soluble LMX. This analysis would ensure that treatment of *adsl-1*(RNAi) animals with LMX did inhibit the expected steps of *de novo* purine biosynthesis, leading to an expected decrease in SAICAR levels. If *adsl-1*(RNAi) animals treated with LMX were found to have lower levels of SAICAR compared to untreated *adsl-1*(RNAi) animals, I could then conclude that decreasing SAICAR levels in *adsl-1*(RNAi) animals restores muscle function.

Furthermore, it is possible that the HT115 *E. coli* strain used for the RNAi knockdown is metabolizing water-soluble LMX and that the metabolized byproducts are contributing to the recovery in muscle function. Although a metabolomics analysis of SAICAR levels in treated

worms would determine if LMX does lead to a decrease in SAICAR levels, another experimental approach would be to grow the worms on UV-killed bacteria that are incapable of metabolizing LMX. Because an RNAi knockdown would require the HT115 *E. coli* to incubate on the RNAi plates a few days before plating the worms (Ahringer, 2006), UV treatment of the RNAi bacteria would not be a feasible pathway to pursue. Instead, *adsl-1(tm3328)* mutants could be grown on plates containing UV-killed OP50 *E. coli* and supplemented with the water-soluble LMX. However, before this experiment is performed, an analysis of the developmental timeline of *adsl-1(tm3328)* mutants on UV-killed OP50 should be performed first as it is critical to perform the thrashing assay on control and *adsl-1(tm3328)* mutants that are at the same developmental stage.

A past study has already indicated that inhibition of the *de novo* purine biosynthesis pathway cannot fully restore muscle function in *adsl-1(tm3328)* mutants (Fenton et al., 2017); therefore, I also explored other possible contributing factors to the muscle dysfunction phenotype associated with animals with reduced *adsl-1* expression. In both steps of the purine metabolic pathway in which adenylosuccinate lyase functions, fumarate is produced as a byproduct. Because fumarate is a metabolite of the TCA cycle, I performed an analysis of the TCA cycle metabolites to determine if any metabolite levels were altered due to a decrease in *adsl-1* expression. Ultimately, only citric acid, alpha-ketoglutarate, and malate were found to have significantly different normalized peak areas in *adsl-1*(RNAi) samples compared to EV(RNAi) samples, with citric acid having a significantly higher normalized peak area and malate and alpha-ketoglutarate having significantly lower normalized peak areas in *adsl-1(*RNAi) samples.

Citrate accumulation is thought to lead to the redirection of the cell to perform anabolic reactions rather than catabolic reactions such as the TCA cycle (Frezza, 2017). Thus, the significantly higher levels of citric acid in *adsl-1*(RNAi) samples could indicate that energy-

producing catabolic reactions are not being run as often, leading to lower energy reserves to fuel processes required for proper muscle function. An analysis of other catabolic reactions, such as glycolysis, should be performed to determine if all catabolic reactions are affected by decreased *adsl-1* expression.

Previous research has indicated that alpha-ketoglutarate increases the lifespan of *C. elegans* (Chin et al., 2014); therefore, the decreased normalized peak area of alpha-ketoglutarate in *adsl-1*(RNAi) samples may help to explain why past studies have observed that *adsl-1* mutants do not live as long as wild-type worms (Marsac et al., 2019). Furthermore, when scientists supplemented mice used to model Duchenne muscular dystrophy with alpha-ketoglutarate, they observed that the mice had recovered muscle function and an increase in muscle mass (Cai et al., 2018). When researchers studied the muscle structure of *adsl-1* mutants, they noted that the mutants displayed both abnormal muscle structure, and the muscle mass also appeared to be decreased (Marsac et al., 2019). Therefore, a valuable future experiment could involve supplementing *adsl-1*(RNAi) animals and *adsl-1* mutants with alpha-ketoglutarate and assessing the resulting muscle structure, muscle mass, and thrashing rates of these treated worms.

Although a standard was run for fumarate, the results of the data suggested that it may be difficult to accurately assess levels of fumarate using LC-MS. However, levels for malate were able to be analyzed and were shown to be significantly decreased in *adsl-1*(RNAi) samples, indicating that it is possible that inhibition of purine metabolism through decreased *adsl-1* expression could affect the TCA cycle. However, because only three TCA cycle metabolites appeared to be significantly affected by the decreased *adsl-1* expression, further experiments testing mitochondrial function in animals with decreased *adsl-1* expression should be performed. A luciferase assay could be performed to assess the level of ATP in *C. elegans* to determine if

decreased *adsl-1* expression is associated with decreased ATP levels (possibly due to lower ATP production or higher ATP consumption). An experiment testing oxygen consumption rates of animals with decreased *adsl-1* expression using a Seahorse XF Analyzer is another method for testing differences in mitochondrial function. To accurately identify and assess the levels of metabolites of the THF cycle of the mitochondria, standards of the THF cycle metabolites could be run prior to another metabolomics analysis of *adsl-1*(RNAi) samples. Ultimately, while the metabolomics analysis performed in this study indicates that some metabolites of the TCA cycle may be influenced by decreased *adsl-1* expression, further experimentation is needed before any conclusions regarding differences in mitochondrial function can be made.

Overall, my data suggests that imbalances in the *de novo* purine biosynthesis pathway upstream of where adenylosuccinate lyase functions could contribute to the muscle dysfunction phenotype seen in worms with decreased *adsl-1* expression as LMX supplementation was able to recover muscle function. Furthermore, my analysis of metabolomics data also shows that some of the metabolites of the TCA cycle have significantly different normalized peak areas in *adsl-1*(RNAi) samples, suggesting that parts of the TCA cycle are affected by decreased *adsl-1* expression. Therefore, future experiments exploring possible connections between *de novo* purine biosynthesis, mitochondrial function, muscle function, and neural transmission could better elucidate the exact causes of the muscle dysfunction phenotype associated with ASLD.

Chapter 4

Materials and Methods

C. elegans strains

The N2 (wild type) strain, from the *Caenorhabditis* Genetics Center (CGC), was used for both the RNAi knockdown experiments and for the metabolomics experiment. Prior to plating the worms on RNAi plates, N2 worms were maintained under standard conditions (Brenner, 1974), and they were kept at 20°C.

Lometrexol Supplementation

A stock solution of 1.5 x 10^{-5} M lometrexol (Sigma) was prepared in Milli-Q water and then filter sterilized. The filter sterilized lometrexol was then added to 8 mL RNAi plates to a final concentration of 54 nM and left to dry at room temperature overnight.

RNAi Treatment

RNAi treatment methods were adapted from previously published literature as well as protocols detailed by Fenton et al. (2017), using the feeding method to induce a knockdown (Ahringer, 2006). Both *adsl-1* and empty vector RNAi were used. The *adsl-1* clone originated from the *C. elegans* RNAi Library (Source BioScience, Nottingham, UK), and the empty vector bacteria used was the HT115 *E. coli* strain with the L4440 plasmid. A single bacterial colony was inoculated in 5 mL of liquid broth and then incubated for approximately 18 hours at 37°C with shaking. 200 µL of liquid culture was then spotted onto each RNAi plate and then left to incubate at room temperature for approximately 2 days. Approximately 5 L4 N2 worms were then placed onto the RNAi plates, and these worms were left to develop at 20°C. The next day,

the egg-laying adults were removed, and the resulting progeny were left to develop on the RNAi plates at 20°C until they reached the young-adult stage. *adsl-1*(RNAi) animals were classified as young adults when they displayed a protruding vulva. This typically occurred around 8 hours after EV(RNAi) animals were determined to be young adults. Therefore, a delay of at least 8 hours occurred before thrashing assays were performed on treated and untreated *adsl-1*(RNAi) young adults, and only *adsl-1*(RNAi) worms with a protruding vulva were used for the thrashing assays.

Phenotypic Analysis: Thrashing Assay

This thrashing assay protocol was adapted from the protocol described by Fenton et al. (2017). NGM plates at room temperature were spotted with 30 µL of M9 solution. Young adult hermaphrodites of each condition, in sets of 10, were placed in the M9 solution. After approximately 5 minutes in the M9 solution, one-minute recordings of the worms thrashing in the M9 solution were made using WormLab software (WormLab, 2020). When performing the thrashing assay on *adsl-1*(RNAi) worms (treated and untreated), only worms with a protruding vulva were used.

Metabolomics

All metabolomics data was collected by Latisha Franklin and Corinna Moro, performed according to methods detailed by Fenton et al. (2017) and done at the Penn State Metabolomics Core Facility. N2 worms were used for all RNAi knockdowns performed. Only EV(RNAi) and *adsl-1*(RNAi) samples were considered for analysis. To normalize the data, chlorpropamide normalization factors were first calculated by averaging the raw peak areas of chlorpropamide

across EV(RNAi) and *adsl-1*(RNAi) samples and then dividing this average by each sample's chlorpropamide peak area. The raw peak areas of the metabolites for each sample were then normalized using each sample's respective chlorpropamide normalization factor. The peak area for chlorpropamide was then removed from each sample. Then, total ion chromatogram (TIC) was determined by adding up the normalized peak areas for each respective sample. A TIC normalization factor was determined by averaging the TICs across all samples and then dividing this average by each sample's TIC. The peak area data was again normalized, this time using the TIC normalization factor. For all metabolites in which a standard had been run, the metabolite matching the standard's mass-to-charge ratio and retention time the closest was used for analysis. For metabolites in which a standard had not been run, I first looked at the mass-to-charge ratio and then the retention time matching the closest to literature values (Lu et al., 2010). Lastly, fill percentage was also considered, with metabolites with fill percentages above 40% being considered for analysis before those with fill percentages below 40%.

Statistical Analysis

A student's t-test was run using Microsoft Excel to compare two groups. A one-way ANOVA test, followed by a Tukey's multiple comparison test, was run using GraphPad Prism version 9.3.1 to compare multiple groups.

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- Design experimental procedures and conduct behavioral assays using model organism *C. elegans* (nematode) to evaluate role of purine metabolism in muscle function
- Streamlined code using R Programming to analyze maximum bending angle and muscle function of *C. elegans*
- Collaborated with lab members in project using CRISPR to induce knockout of *adsl-1* in muscle cells of *C. elegans*
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