Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

05/03/2021

Savannah Hardin Date

Metabolomic alterations in Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) premutation carriers

By

Savannah Lee Hardin

MPH

Epidemiology

Emily G. Allen

Committee Chair

Metabolomic alterations in Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) premutation carriers

By

Savannah Lee Hardin

Bachelor of Science in Biology and Chemistry

Young Harris College

2019

Thesis Committee Chair: Emily G. Allen, Ph.D.

An abstract of

A thesis submitted to the Faculty of the

Rollins School of Public Health of Emory University

in partial fulfillment of the requirements for the degree of

Master of Public Health in Epidemiology

2021

Abstract

Metabolomic alterations in Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) premutation carriers

By Savannah Lee Hardin

Carriers of the Fragile X mental retardation 1 (*FMR1*) gene premutation are at risk of developing Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS). 38 early onset FXTAS cases, 41 premutation (PM) control males, as well as 20 noncarrier controls were selected for sphingolipid profiling and identification of any further metabolomic differences for this study. Previous studies found that there were a wide variety of pathways affected such as carbohydrate, amino acid, and fatty acid pathways. Our research supports these findings and demonstrates that premutation men with FXTAS are metabolically dissimilar to noncarriers, but metabolically similar to premutation carriers without FXTAS symptoms. In the comparison between FXTAS men and noncarrier men we found significant pathways to include multiple amino acid metabolism pathways (alanine and aspartate (p=0.01); glycine, serine, alanine, and threonine (p=0.01); tyrosine (p=0.04); methionine and cysteine (p=0.04); and tryptophan (p=0.049)), some vitamin metabolism pathways (vitamin B_1 (0.01), B_2 (p=0.02), and H (p=0.02)), several oxidation pathways (phytanic acid and peroxisomal oxidation (p=0.009) and mono-unsaturated fatty acid beta-oxidation (p=0.04)), as well as a few other biosynthesis and metabolic pathways (alkaloid biosynthesis II (p=0.01), ubiquinone biosynthesis (p=0.01), carnitine shuttle (p=0.03), phosphatidylinositol phosphate metabolism (p=0.04), selenoamino acid metabolism (p=0.04), and dynorphin metabolism (p=0.047)). In the comparison between FXTAS men and premutation men without FXTAS symptoms we found selenoamino acid metabolism (p=0.047), Vitamin A (retinol) metabolism (p=0.03), and arachidonic acid metabolism (p=0.04) to be statistically different. Lastly, in the comparison between all premutation men and noncarrier men we identified mono-unsaturated fatty acid beta-oxidation (p=0.01), vitamin B_1 (thiamin) metabolism (p=0.03), tyrosine metabolism (p=0.03), vitamin B_5 – CoA biosynthesis from pantothenate (p=0.003), di-unsaturated fatty acid beta-oxidation (p=0.006), vitamin B_3 (nicotinate and nicotinamide) metabolism (p=0.02), and glutathione metabolism (p=0.04) to be significant pathways. There is much research that still needs to be done, but through the use of metabolomics, we will identify pathways for potential treatments of FXTAS.

Metabolomic alterations in Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) premutation carriers

By

Savannah Lee Hardin

Bachelor of Science in Biology and Chemistry

Young Harris College

2019

Thesis Committee Chair: Emily G. Allen, Ph.D.

A thesis submitted to the Faculty of the

Rollins School of Public Health of Emory University

in partial fulfillment of the requirements for the degree of

Master of Public Health in Epidemiology

2021

Metabolomic alterations in Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) premutation carriers

1. Introduction

1.1. Brief description of FXTAS

Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) has been recognized in older males of Fragile X Syndrome (FXS) families, but it is not common in individuals with the neurodevelopmental Fragile X disorder[1]. The primary features of FXTAS are intention tremor and gait ataxia. Although both disorders involve repeat expansions in the *FMR1* gene, the clinical presentation and molecular mechanisms underlying each disease are distinct and depend on the length of the expanded repeat; a repeat size of less than 50 typically shows no symptoms, between 50-200 repeats can lead to FXTAS, and over 200 repeats are associated with the neurodevelopmental disorder (FXS). At the molecular level, *FMR1* premutation carriers exhibit a 2 to 8-fold increase in FMR1 mRNA compared to control individuals. Expression of mutant mRNAs containing long (~100) CGG triplets has been shown to be toxic in cell and animal models. Currently, data support two non-mutually exclusive molecular pathogenesis mechanisms for FXTAS: 1) RNA gain-of-function, in which rCGG repeat-binding proteins (RBPs) become functionally limited through sequestration by lengthy rCGG repeats, and 2) repeat-associated non-AUG (RAN) translation, whereby translation through the CGG (or antisense CCG) repeats leads to the production of toxic homo-polypeptides, such as FMR polyG, which in turn interfere with a variety of cellular functions. Multiple mouse models have been developed to study these

mechanisms. The development of biomarkers and effective treatments for these premutation carriers is still in its infancy. However, recent studies have identified a few novel findings among asymptomatic *FMR1* premutation carriers and FXTAS carriers, which has provided promising insight into this field of study.

1.2.Previous sphingolipid and metabolomic study findings

Much remains unknown regarding the metabolic alterations associated with FXTAS, especially in the brain, and the most affected region, the cerebellum. Investigating the metabolic changes in FXTAS will aid in the identification of biomarkers as well as in understanding the pathogenesis of disease. In previous papers, researchers identified alterations in the intermediates of the Krebs Cycle and Glycolysis[9]. One paper found that 200 out of 506 metabolites were altered in various comparisons in young wildtype, young FXTAS, old wildtype, and old FXTAS mice[3]. Over half of the metabolites found to be significantly different were lipids[3]. Many different amino acids and their derivatives were affected such as proline, glycine, hydroxyproline, citrulline, and glutamylvaline[9]. These particular amino acids and amino acid derivatives have also been correlated to CGG expansion size. Lastly, there are a few fatty acid pathways that seem to be affected as well[9]. The purpose of our study is to take a deeper look into these metabolic alterations and to determine if we are able to identify similar results within our cohort of individuals.

Table 1. Characteristics of study participants categorized by study group

Study Group	***N (#)***	***Age (mean ± sd)***
Premutation men with symptoms of FXTAS	38	64.6 ± 7.6
Premutation men without symptoms of FXTAS	41	72.8 ± 5.0
Noncarrier men	20	61.1 ± 10.8

2. Materials and methods

2.1. Plasma samples from FXTAS cases, PM carriers without FXTAS, and noncarrier men

As part of a previous study by the Emory National Fragile X Center, whole genome sequencing was performed on 100 PM males with early onset of FXTAS symptoms (FXTAS cases) and 100 PM males without significant symptoms of FXTAS by age 68 (PM controls). Of these subjects, 80 PM men have provided a blood sample where plasma has been stored for future studies. Of the stored plasma samples, we selected 38 of the early onset FXTAS cases, 41 PM control males, as well as 20 noncarrier controls for sphingolipid profiling and identification of any further metabolomic differences for this study.

2.2. High resolution liquid chromatography-mass spectrometry

Sample analysis was performed similarly to methods that have been previously described[6]. Plasma samples were analyzed on a Thermo Scientific LTQ Velos Orbitrap mass spectrometer,

coupled with dual liquid chromatography, alternating data collection between HILIC and C18 columns with three technical replicates each[6]. The analyses were performed on the positive electrospray ionization mode with a mass-to-charge ratio (*m/z*) scan range of 85-2,000, an injection volume of 10 μL, and a resolution of 60,000. Plasma samples were randomized and run in batches of 20; in addition, - pooled reference plasma (Q-Standard) samples were run before and after each batch for metabolite quantification, quality assurance, and control[6]. xmsPANDA in R was used to retrieve raw data and run a PCA analysis in order to check for batch effects; these were compensated for by using ComBat[5].

The resulting data contained individual samples defined by accurate mass *m/z*, retention time (RT), and ion intensities. Each ion intensity from the three technical replicates were averaged and log transformed for each person to produce one set of intensities per m/z and RT per study participant. Metabolites were identified based on their m/z and RT through the usage of xMSannotator[4]. We restricted these metabolite matches to M+H and M+Na adducts with a mass error between ±10 ppm and within the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[5].

2.3. Significant features and pathway analysis

First, xmsPANDA was utilized to identify features and metabolites of interest to look at in the comparison of the various study groups (noncarrier men, premutation men without FXTAS symptoms, and premutation men with FXTAS) and these results were used in a pathway enrichment analysis in Mummichog. Three different analyses were performed in this study:

premutation men with FXTAS versus noncarrier men (figure 1), premutation men with FXTAS versus premutation men without symptoms of FXTAS (figure 2), and all premutation carrier men (premutation men with and without FXTAS symptoms) versus noncarrier men (figure 3. Significant pathways ($p \leq 0.05$) were annotated and identified by using Mummichog and xMSannotator[4]. For each of these comparisons, we were able to combine the output from the C18 and HILIC columns and analyze the results for a combined pathway analysis using Mummichog.

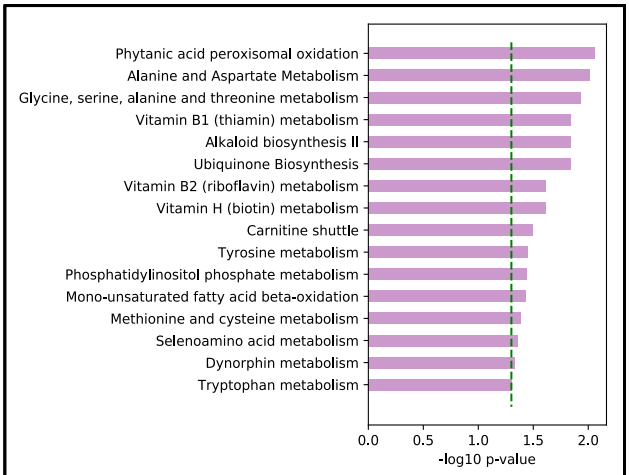

Figure 1. Statistically significant pathways identified in the comparison between premutation men with FXTAS and noncarrier men.

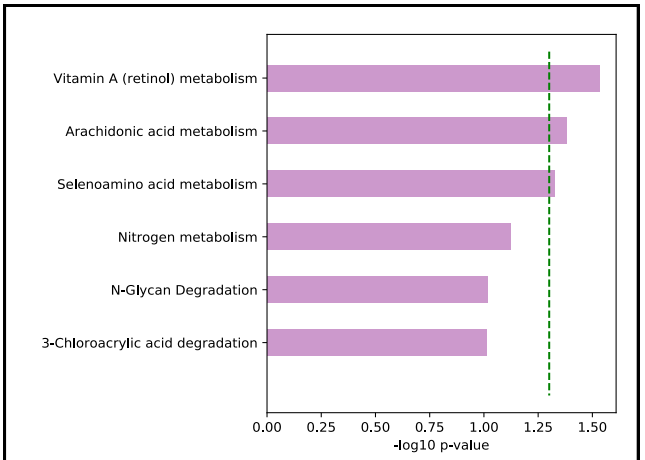

Figure 2. Statistically significant pathways identified in the comparison between premutation men with FXTAS symptoms and premutation men without FXTAS symptoms.

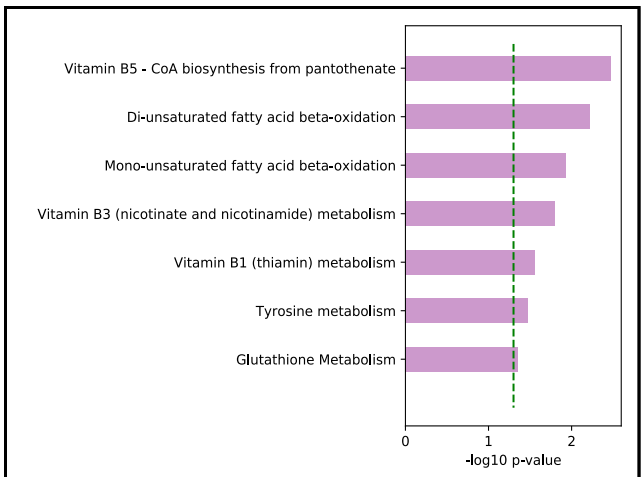

Figure 3. Statistically significant pathways identified in the comparison between all premutation men (with and without FXTAS symptoms) and noncarrier men.

3. Results

Characteristics of the study population, stratified by each study group, can be found in Table 1. Several significant differences were found in each of the comparisons between the different study groups (figure 1, 2, & 3). The highest number of significant pathways were identified in the comparison between premutation men with FXTAS and noncarrier men (figure 1). Significant pathways in this comparison include multiple amino acid metabolism pathways (alanine and aspartate (p=0.01); glycine, serine,

alanine, and threonine (p=0.01); tyrosine (p=0.04); methionine and cysteine (p=0.04); and tryptophan (p=0.049)), some vitamin metabolism pathways (vitamin B_1 (0.01), B_2 (p=0.02), and H (p=0.02)), several oxidation pathways (phytanic acid and peroxisomal oxidation (p=0.009) and mono-unsaturated fatty acid beta-oxidation (p=0.04)), as well as a few other biosynthesis and metabolic pathways (alkaloid biosynthesis II (p=0.01), ubiquinone biosynthesis (p=0.01), carnitine shuttle (p=0.03), phosphatidylinositol phosphate metabolism (p=0.04), selenoamino acid metabolism (p=0.04), and dynorphin metabolism (p=0.47)) (figure 1). The comparison with the second highest number of significant pathways is between all premutation carrier men (those with FXTAS and without FXTAS symptoms) and noncarrier men (figure 3). Some of the pathways that were found to be significant in this comparison overlapped with those found to be significant in the previous one: such as, mono-unsaturated fatty acid beta-oxidation (p=0.01), vitamin B_1 (thiamin) metabolism (p=0.03), and tyrosine metabolism (p=0.03). Other pathways that were found to be significant in this comparison include vitamin B_5 – CoA biosynthesis from pantothenate (p=0.003), di-unsaturated fatty acid beta-oxidation (p=0.006), vitamin B_3 (nicotinate and nicotinamide) metabolism (p=0.02), and glutathione metabolism (p=0.04) (figure 3). The comparison between premutation carriers with FXTAS symptoms and premutation men without FXTAS symptoms yielded the least number of significant results (figure 2). One of these pathways overlapped with the first comparison (FXTAS vs noncarriers) namely, selenoamino acid metabolism (p=0.047). Vitamin A (retinol) metabolism (p=0.03) and arachidonic acid metabolism (p=0.04) were the other two pathways that were found to be significant in this particular comparison (figure 2).

4. Discussion

As stated above, the comparison between premutation men with FXTAS symptoms and noncarrier men yielded the highest number of significantly different pathways, while the comparison between premutation men with FXTAS symptoms and premutation men without FXTAS symptoms yielded the least. This could be due to the fact that phenotypically and genotypically, FXTAS cases are the most dissimilar to noncarrier men and the least dissimilar to premutation men without FXTAS symptoms. In addition, because this is a cross sectional study, it is possible that premutation men without symptoms of FXTAS could potentially go on to develop symptoms of FXTAS in the future. When we take a closer look at each of the affected pathways, we notice that several of the pathways have been previously linked to various neurodegenerative diseases and typical phenotypes that surround premutation carriers affected by FXTAS.

4.1 Comparing the metabolomic profiles of noncarriers and FXTAS men (figure 1)

Many interesting pathways were found to be significantly different among these two groups. For example, changes in the metabolism of tryptophan and tyrosine, which are precursors for many neurotransmitters such as serotonin and dopamine[7], have been linked to various neurodegenerative diseases. Phytanic acid peroxisomal oxidation has been linked to ataxia (the loss of full control of bodily movements) in diseases such as FXTAS and Parkinson's Disease. Vitamin B_1 and H metabolisms have been linked to peripheral neuropathy and dementia respectively. Lastly, there are several pathways that were found that are connected to

mitochondrial dysfunction such as phytanic acid peroxisomal oxidation, vitamin B_2 metabolism, carnitine shuttle, and ubiquinone biosynthesis[3].

4.2 Comparing the metabolomic profiles of premutation men with and without FXTAS symptoms (figure 2)

There were three pathways that were determined to be significant in the comparison between these two groups: selenoamino acid metabolism, vitamin A (retinol) metabolism, and arachidonic acid metabolism. Previous studies have found that selenoamino acids, as well as selenium itself, may play a role in normal neurological and mitochondrial function[8]. Arachidonic acid metabolism has been linked to playing a role in many neurological, neurodegenerative, and psychiatric disorders[9]. Lastly, for years, vitamin A has been targeted as a potential treatment for a number of illnesses including cancer and neurodegenerative diseases because of its antioxidant abilities. The selenoamino acid pathway was found to be significantly different in both comparisons containing FXTAS cases vs another group (i.e., noncarriers or premutation carriers without FXTAS). This data suggests that the selenoamino acid pathway might be involved in premutation carriers developing FXTAS and warrants further study.

4.3 Comparing the metabolomic profiles of all premutation men (with and without FXTAS) and noncarrier men (figure 3)

We found several pathways to be significant when comparing all premutation men and noncarrier men, three of which were also significant when looking at the comparison between

premutation men with FXTAS and noncarrier men. These three pathways were mono-unsaturated fatty acid beta-oxidation, vitamin B_1 metabolism, and tyrosine metabolism. Because we identified these three pathways to be altered in both comparisons, this might indicate that these pathways are affected in all premutation men (i.e., those with FXTAS symptoms and those without FXTAS symptoms) and not just FXTAS cases. Importantly, all of the pathways identified as statistically different in this comparison of our cohort have been previously identified as altered pathways in premutation carriers, which further validates our findings[9].

5. Conclusion

Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) has been identified in older males of FXS families, but it is not common in individuals with Fragile X Syndrome. The development of biomarkers and effective treatments for these premutation carriers is still in its infancy. However, recent studies have identified a few novel findings among asymptomatic *FMR1* premutation carriers and FXTAS carriers, which has pushed research efforts towards the examination of metabolomics in these individuals. Previous studies found that there were a wide variety of pathways affected such as carbohydrate, amino acid, and fatty acid pathways. Our research supports these findings and demonstrates that premutation men with FXTAS are metabolically dissimilar to noncarriers, but metabolically similar to premutation carriers without FXTAS symptoms. There is much research that still needs to be done, but through the use of metabolomics, we will identify pathways for potential treatments of FXTAS.

6. References

1. Hagerman RJ, Hagerman P. Fragile X-associated tremor/ataxia syndrome -features, mechanisms and management. Nat Rev Neurol. 2016;12(7):403-12. doi: 10.1038/nrneurol.2016.82. PubMed PMID: 27340021.
2. Giulivi C, Napoli E, Tassone F, Halmai J, Hagerman R. Plasma metabolic profile delineates roles for neurodegeneration, pro-inflammatory damage and mitochondrial dysfunction in the FMR1 premutation. Biochem J. 2016;473(21):3871-88. doi: 10.1042/BCJ20160585. PubMed PMID: 27555610.
3. Kong HE, Lim J, Zhang F, Huang L, Gu Y, Nelson DL, Allen EG, Jin P. Metabolic pathways modulate the neuronal toxicity associated with Fragile X-Associated Tremor/Ataxia Syndrome. Hum Mol Genet. 2018. doi: 10.1093/hmg/ddy410. PubMed PMID: 30476102; PMCID: PMC6400045.
4. Uppal K, Walker DI, Jones DP. xMSannotator: An R Package for Network-Based Annotation of High-Resolution Metabolomics Data. Anal Chem. 2017;89(2):1063-7. doi: 10.1021/acs.analchem.6b01214. PubMed PMID: 27977166; PMCID: PMC5447360.
5. Johnson, W.E., Li, C., Rabinovic, A., 2007. Adjusting batch effects in microarray ex-pression data using empirical Bayes methods. Biostatistics 8 (1), 118–127. http://dx.doi.org/10.1093/biostatistics/kxj037.
6. Fischer, S.T., Lili, L.N., Li, S., Tran, V.T., Stewart, K.B., Schwartz, C.E., Jones, D.P., Sherman, S.L., Fridovich-Keil, J.L., 2017. Low-level maternal exposure to nicotine associates with significant metabolic perturbations in second-trimester amniotic fluid. Environment International 107 (2017) 227-234. http://dx.doi.org/10.1016/j.envint.2017.07.019.

7. Rajagopal S., Sangam S.R., Singh S., Joginapally V.R. 2016. Modulatory Effects of Dietary Amino Acids on Neurodegenerative Diseases. In: Essa M., Akbar M., Guillemin G. (eds) The Benefits of Natural Products for Neurodegenerative Diseases. Advances in Neurobiology, vol 12. Springer, Cham. https://doi.org/10.1007/978-3-319-28383-8_22.
8. Cardoso BR, Roberts BR, Bush AI, Hare DJ. Selenium, selenoproteins and neurodegenerative diseases. Metallomics. 2015 Aug;7(8):1213-28. doi: 10.1039/c5mt00075k. PMID: 25996565.
9. Cao, Y., Peng, Y., Kong, H.E., Allen, E.G., Jin, P., 2020. Metabolic alterations in FMR1 Premutation Carriers. Front. Mol. Biosci. 7:571092. doi: 10.3389/fmolb.2020.571092.
10. Allen EG, Hunter JE, Rusin M, Juncos J, Novak G, Hamilton D, Shubeck L, Charen K, Sherman SL. Neuropsychological findings from older premutation carrier males and their noncarrier siblings from families with fragile X syndrome. Neuropsychology. 2011;25(3):404-11. doi: 10.1037/a0021879. PubMed PMID: 21443343; PMCID: 3086936.