University of Nevada, Reno

Antibody-Based Detection of 3-Methylglutaconic Acid

A thesis submitted in partial fulfillment of the requirements

for the degree of Master of Science in

Biochemistry

by

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May 2020

THE GRADUATE SCHOOL

We recommend that the thesis
prepared under our supervision by

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entitled

Antibody-Based Detection of 3-Methylglutaconic Acid

be accepted in partial fulfillment of the
requirements for the degree of

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1 ABSTRACT

The organic acid, 3-methylglutaconic acid (3MGA), is known to accumulate in urine of patients with disorders associated with compromised mitochondrial energy metabolism. Although the biochemical basis for this excretion is currently under investigation, there is evidence this metabolite can serve as a biomarker of disease. With the intent of creating a biomarker assay that can be used for disease diagnosis, monitoring progression of disease, or treatment efficacy, the development of a simple antibody-based assay was explored.

Using immune serum from the inoculation of rabbits with a 3MGA-conjugated keyhole limpet hemocyanin (KLH) immunogen, several enzyme-linked immunosorbent assay (ELISA) formats were investigated to find the most sensitive assay. From this study, a competitive ELISA was chosen and optimized. The assay was then tested for reproducibility, specificity, and matrix effects in urine. In seven of 11 assays analyzed for inter-assay precision, the limit of detection (LOD) for measurement of 3MGA averaged 1.51 mg/mL with a coefficient of variation (CV%) of 31%. Specificity testing of similar dicarboxylic acids and a non-similar amino acid, showed that the anti-3MGA antibody had notably reduced binding activity compared to 3MGA. There was a noted decrease in signal when urine was used as the matrix, but measurement of free 3MGA spiked in urine was possible.

Taken together, these data indicate that a quick and easy immunoassay to measure 3MGA is feasible. Future experiments could further refine reproducibility and sensitivity.

2 ACKNOWLEDGEMENTS

To:

My committee members, Dr. Bob Ryan, Dr. Trish Ellison, and Dr. Mike Teglas, for all of their support and encouragement.

Irina Romenskaia and Sharon Young who supplied me with reagents, lab space and interesting conversation.

Dr. Marina Maclean who forged the path that I'm following. Thanks for the guidance!

My mom, dad, brother, and sister: love you all!

For Ethan, who is the funniest young man I know and who will do great things one day.

Cooper and The Stig who reminded me that barking for no reason is fun and usually results in playtime or food.

Amanda Jones and Floyd who meowed at me until I got up to see what was the matter, but nothing was. The point was I was not sitting anymore…I think...it's hard to tell with cats.

And, to Jeff, my partner through everything.

This work was supported by a grant from the National Institutes of Health (R37 HL-64159).

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6 ABBREVIATIONS

3MGA or 3MGC	3-methylglutaconic acid
3MG	3-methylglutaric acid
HMG	3-hydroxy-3-methylglutaric acid
CoA	Coenzyme A
3MGAuria	3-methylglutaconic aciduria
ELISA	Enzyme-Linked Immunosorbent Assay
TMB	3,3',5,5'-tetramethylbenzidine
ATP	Adenosine triphosphate
TCA	The citric acid cycle
BSA	Bovine serum albumin
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
IgG	Immunoglobulin G
HRP	Horseradish peroxidase
KLH	Keyhole limpet hemocyanin
GC	Gas chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
MEGDEL	3MGAuria with sensori-neural deafness, encephalopathy, and Leigh-like syndrome
DCMA	Dilated cardiomyopathy with ataxia
MELAS	Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke
ACOT	Acyl CoA thioesterase
ETC	Electron Transport Chain
Sufo-NHS	sulfo-*N*-hydroxysuccinimide
OVA	Ovalbumin
kDA	Kilodalton

LOD	Limit of detection
LLOQ	Lower limit of quantitation
LFA	Lateral flow assay
LOC	Lab-on-a-chip
AUH	gene for: 3-Methylglutaconyl CoA hydratase
HMGCL	gene for: 3-hydroxy-3-methylglutaryl lyase
NADH	Nicotinamide adenine dinucleotide
FADH2	Flavin adenine dinucleotide
FDA	Food and Drug Administration
NBS	Nucleotide binding site

7 INTRODUCTION

7.1 BACKGROUND

For centuries physicians have used the urine of patients to diagnose disease. In ancient times it was considered "the divine fluid" as it was the most important, and often only, diagnostic tool physicians had. They heated it, poured vinegar in it, observed it for bubbles, smelled it, and tasted it. Though their methods were crude by today's standards, surprisingly accurate diagnoses could be made. For example, heat and vinegar precipitated protein indicating kidney disease, cloudy urine indicated an infection, and a sweet taste indicated diabetes mellitus (Armstrong, 2007). Modern doctors now have sophisticated laboratories that can test for many types of metabolites and have access to databases, such as The Urine Metabolome (Bouatra S, 2013), that compile normal and abnormal urine profiles. Although we know more about the metabolites associated with disease, it's not always understood how and why they are there. 3-Methylglutaconic aciduria (3MGAuria) is one such disorder. It is known to occur in several seemingly dissimilar metabolic diseases. For some of these diseases the excretion of 3MGA is obvious, but in others its appearance is a mystery. It is this last point that is of significant interest and the focus of current research.

Three-methylglutaconyl coenzyme A (CoA) is a metabolite of the leucine degradation pathway found in the mitochondria (Figure 1). The end product of this pathway, acetyl CoA is used in the citric acid (TCA) cycle and ultimately produces ATP for energy in the cell (Ikon & Ryan, 2016). Defects in 3-hydroxy-3-methylglutaryl CoA (HMG CoA) lyase, which catalyzes the last reaction in the pathway, or

3-methylglutaconyl CoA (AUH) hydratase, which catalyzes the second to last reaction, ultimately cause a buildup of 3MGA CoA as the reversing of the reaction pathway stops at 3-methyl crotonylCoA carboxylase; an enzyme that cannot catalyze the reverse reaction. The CoA on 3MGA is then removed by acyl CoA thioesterase (ACOT) leaving the free acid to be excreted in urine. Because there is a defect in the pathway itself this is a primary 3MGAuria and can be confirmed by the ingestion of large amounts of leucine which causes an increase in excretion of 3MGA in the urine (Jones, Perez, & Ryan, 2020).

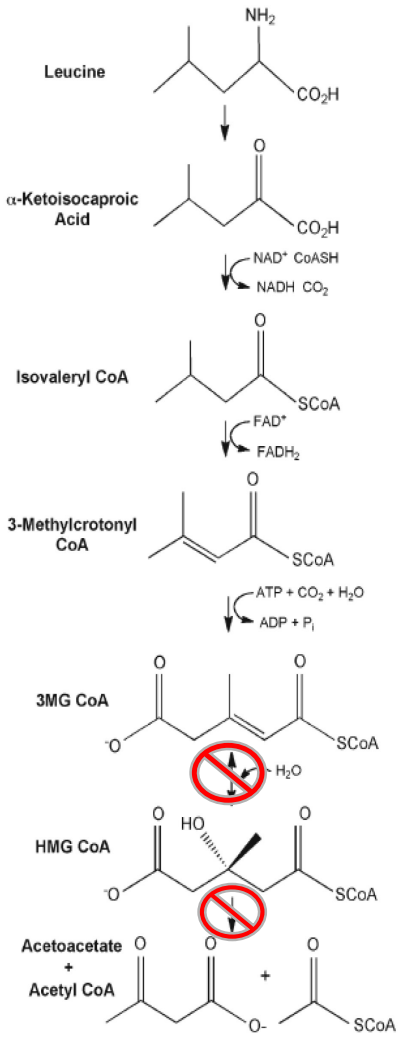

Figure 1. Leucine degradation pathway. Enzyme defects at the red circles cause primary 3MGAuria (Su & Ryan, 2014).

However, there are a secondary set of disorders that cause 3MGAuria that are not associated with an enzyme defect in leucine catabolism (Table 1). All of these, in some way, disrupt mitochondrial function.

Table 1. Gene mutations causing secondary 3MGAuria. MEGDEL=3MGAuria with sensori-neural deafness, encephalopathy, and Leigh-like syndrome, DCMA=dilated cardiomyopathy with ataxia, MELAS=mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke. Adapted from (Su & Ryan, 2014).

Disease	Mutated Gene	Gene Product Function
Barth Syndrome	TAZ	Cardiolipin remodeling
Costeff Syndrome	OPA3	Unknown
MEGDEL Syndrome	SERAC1	Phosphatidylglycerol trafficking
DCMA	DNAJC19	Mitochondrial protein import
	TMEM70	ATP synthase assembly
MELAS	m.3243A>G	tRNALeu
	POLG1	Mitochondrial DNA polymerase
Statin-induced myopathy	HMG CoA reductase	Drug target, CoenzymeQ10 biosynthesis

The source of 3MGA in urine from these diseases is currently being investigated, but an "acetyl CoA diversion pathway" has recently been proposed. The main driver of the diversion pathway is a slowing of the electron transport chain (ETC) resulting in the accumulation of acetyl CoA in the mitochondrial matrix. At a certain point, the amount of acetyl CoA triggers the diversion pathway (Figure 2) (Jones, Perez, & Ryan, 2020).

Since the presence of 3MGA in urine is an indicator of either a defect in leucine catabolism or interruption of the proper functioning of the ETC, it can be used as a

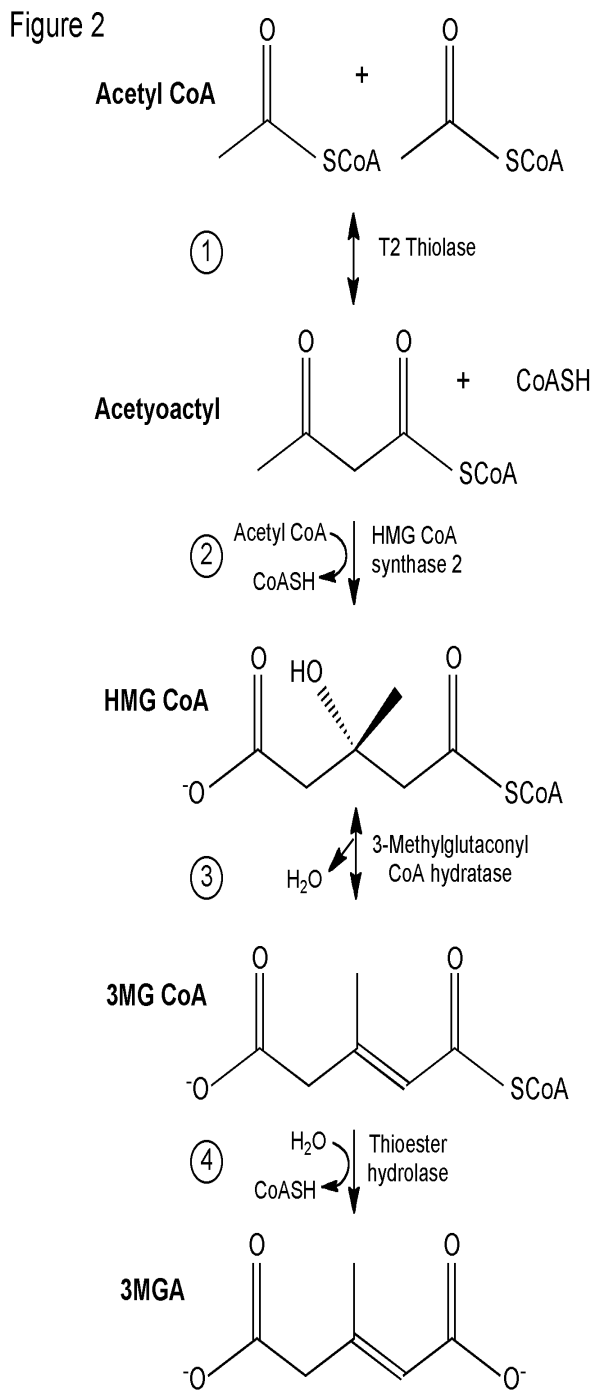

Figure 2. The acetyl CoA diversion pathway (Su & Ryan, 2014)

biomarker to aid in diagnosis or as a way to monitor disease progression or treatment. However, current methods of measurement are either by gas chromatography followed by

mass spectrometry (GC-MS) or by nuclear magnetic resonance (NMR). There are major drawbacks to using either method: the instrumentation is complex and expensive, they require highly trained personnel, sample preparation requires solvent extraction (GC-MS) or isotopes (NMR), and data analysis is complicated especially in a complex matrix like urine (Engelke, et al., 2006) (Suh, Seon, & Chung, 1997).

Neither GC-MS or NMR is conducive to patient diagnosis and monitoring in a clinical setting. Immunoassays, however, are a common assay format for analyte measurement in clinics, they do not require expensive equipment, and are relatively quick to run. The defining feature of an immunoassay is the use of an antibody to capture and/or detect the desired analyte (Figure 3). Antibodies are very specific and can measure with great accuracy a single analyte out of a complex matrix.

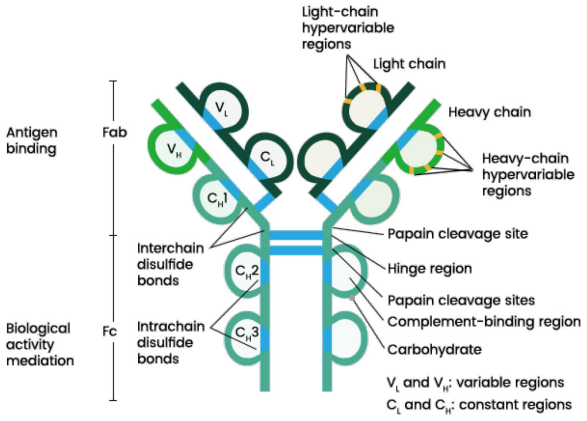

Figure 3. Antibody structure (Sino Biological, n.d.)

7.2 IMMUNOASSAYS

7.2.1 ELISA

Enzyme-linked immunosorbent assays (ELISA) are the most common type of immunoassay. There are several formats of ELISA and the choice depends on the reagents available, the type of analyte, the matrix being measured, and the type of result (positive/negative or quantitative). Figure 4 illustrates several types of ELISA formats. In the case of 3MGA, a sandwich ELISA is not an option as the molecule is too small to have two antibodies bind to it at the same time. Therefore, this approach was not used in this research. A typical approach to measurement of small molecules by ELISA is to use a competitive ELISA, where the antigen competes with an inhibitor antigen or competitor for binding to the antibody. This results in a signal that is inversely proportional to the amount of free antigen in the sample.

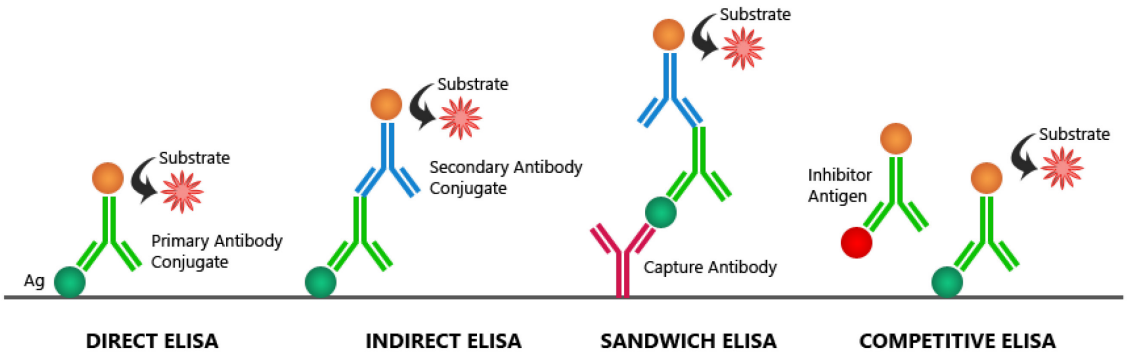

Figure 4. Illustrations of the types of ELISA (Boster, 2017).

7.2.2 ELISA Results

There are two main types of data that can be obtained from an ELISA: quantitative and qualitative. Quantitative assay results provide a specific number or concentration measured in a sample of the analyte (i.e. mg/mL), a qualitative result is a simple positive or negative (is the antigen there or not). The decision on which type to use, and,

therefore, how the assay is designed is usually based on the biology of the analyte being measured. Does it matter how much of the antigen is there or just that it is there at all? In a healthy person, with proper functioning mitochondria, 3MGA should not be present, in which case a qualitative assay is useful and sufficient.

7.2.3 Antibody Generation

The most important first step to develop an immunoassay is the generation of an antibody. Because small molecules themselves cannot cause an immune response in an animal, they must be conjugated to an immunogenic molecule, usually a protein such as ovalbumin (OVA) or bovine serum albumin (BSA). In the case of this research, it is keyhole limpet hemocyanin (KLH), a highly immunogenic protein in mammals. Since 3MGA is a dicarboxylic acid, carbodiimide chemistry can be used to conjugate the hapten (3MGA) to primary amines on the KLH protein. An overview of the reaction can be seen in Figure 4 (Grabarek & Gergely, 1990). The intermediate o-acylisourea is extremely unstable and it is common to utilize sulfo-*N*-hydroxysuccinimide (sulfo-NHS) to stabilize the reaction.

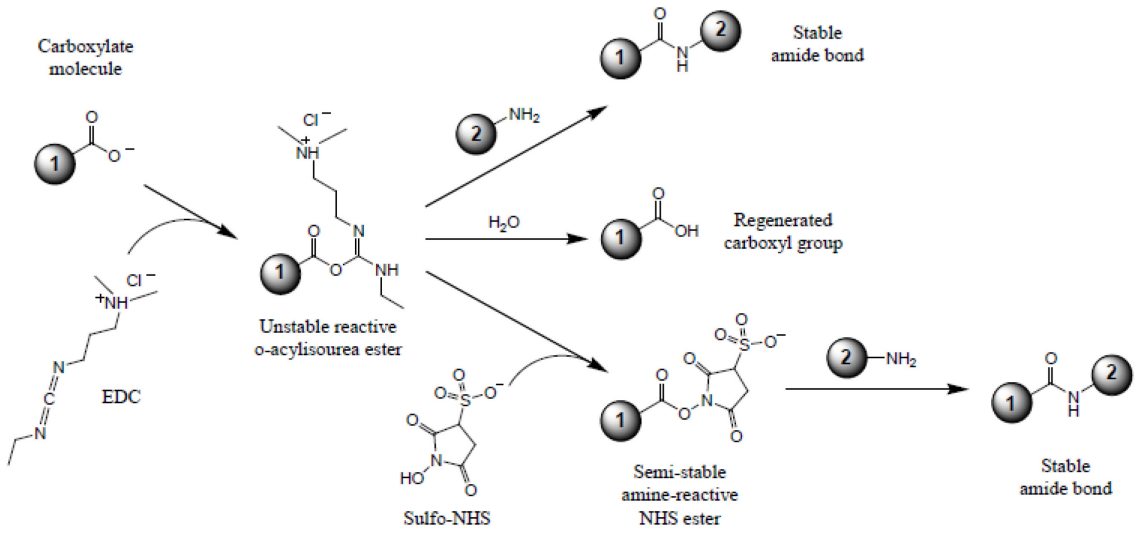

Figure 5. Conjugation of a carboxylate to a protein with primary amines using a carbodiimide reaction (Thermo Scientific: EDC, 2017).

The molar ratio of hapten to protein is very important as the number of molecules conjugated to the protein can determine the type of immunogenic response. It has been noted in some instances that too many hapten molecules per carrier protein can elicit a higher titer IgM antibody response which has low affinity for the antigen, whereas a high titer IgG is desired. This means that as far as developing a good reagent for this type of assay, a strong, fast immune response (IgM) is not as good as a slower response which produces higher affinity antibodies (IgG) (Singh, Kaur, Varshney, Raje, & Suri, 2004).

Once an immune response has occurred, the animal (usually rabbit) serum contains polyclonal antibodies against both the hapten and the conjugated protein. It is important not to use the immunogenic protein in downstream assays as the antiserum will bind and cause false results.

7.2.4 Other ELISA Reagents

Depending on the ELISA design, several other reagents will be necessary. One of the most important ones is the detection reagent, as seen in Figure 4, that is usually an enzyme-conjugated antibody. When a substrate, such as 3,3',5,5'-tetramethylbenzidine (TMB) reacts with the enzyme horseradish peroxidase (HRP), a colored product is produced which correlates to the amount of antigen being measured. The most common enzyme is HRP, but alkaline phosphatase, or urease can also be used (Winston, Fuller, Evelegh, & Hurrell, 2001).

In the case of several of the assay formats in this research, biotin-streptavidin (a bacterial avidin protein) binding was utilized for detection. The bond between biotin and streptavidin is one of the strongest non-covalent bonds in biology. It is unbreakable under most ELISA conditions and amplifies signal as four biotin molecules can bind to one streptavidin molecule. In addition, it is quite easy and cheap to biotinylate proteins (Figure 6). When used with streptavidin-HRP, a biotinylated reagent produces a reliable and strong detection system (Chivers, Koner, Lowe, & Howarth, 2011).

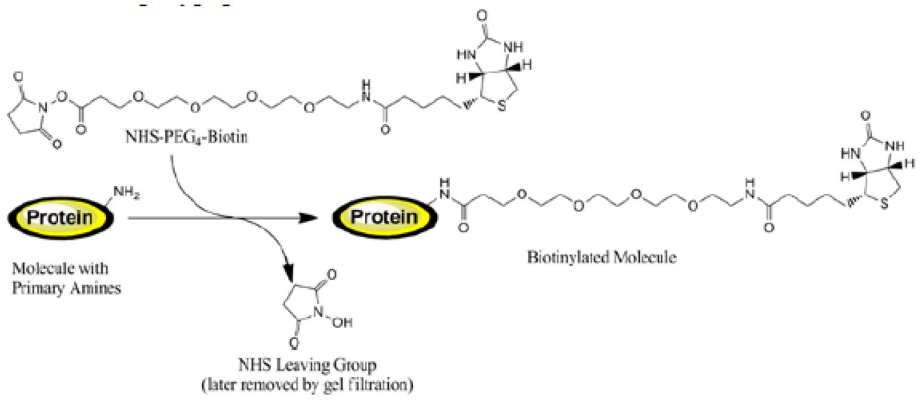

Figure 6. Biotinylation of a protein (Thermo Scientific, 2016).

During assay development it might be necessary to tag molecules other than proteins with biotin. In such cases carbodiimide chemistry (as in Figure 4) can be used to label carboxyl molecules with a biotin molecule, provided that the biotin molecule contains an amine group. Direct tagging of a molecule might be necessary in systems where the surface chemistry of the protein (carboxyls or amines) might react with other reagents in the system or if the size of a protein might cause steric hindrances or interference (Bayer, Ben-Hur, & Wilchek, 1988).

For a competitive ELISA, the assay will need a competitor reagent. The reagent must be able to bind to the antibody (compete with the free antigen in a sample) and be detectable. Since competitive assays are inversely correlated with the amount of antigen, the samples with no antigen will have the highest signal. In this research, two types of competitors were used: biotinylated BSA-conjugated 3MGA and 3MGA directly labeled with biotin.

8 RESEARCH GOALS

The main goal of this research was to develop an immunoassay for the detection of 3MGA in urine. To this end, several ELISA formats were designed and tested to find one with the highest sensitivity. A competitive ELISA was chosen and evaluated for repeatability, selectivity, and for matrix effects caused by urine.

The work herein is a first step toward an easy, reliable clinical test to monitor disease or treatment in patients suffering from primary or secondary 3MGAuria or to monitor effects of drug therapies.

9 ANTIBODY-BASED DETECTION OF 3-METHYLGLUTACONIC ACID

This chapter is written in manuscript form in preparation for journal submission.

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9.1 ABSTRACT

3-methylglutaconic acid (3MGA) is a singular organic acid associated with a growing number of defects in mitochondrial energy metabolism. The presence of 3MGA in urine provides diagnostic evidence of disease. The most common method of 3MGA detection is gas chromatography-mass spectrometry, although NMR spectroscopy methods also exist. In the present study, an antibody based approach to detection / quantitation of 3MGA in biological fluids has been pursued. 3MGA-conjugated keyhole limpet hemocyanin was inoculated into rabbits, generating an anti-3MGA polyclonal serum. Western blot analysis revealed that immune rabbit serum recognizes 3MGA-conjugated bovine serum albumin. A competitive ELISA yielded a limit of detection for free 3MGA of approximately 0.185 mg/ml. To optimize assay sensitivity, different ELISA formats were evaluated. The IgG fraction was isolated from immune serum and used to investigate ELISA specificity and reproducibility. Compared to 3MGA, other short chain dicarboxylic acids showed lower binding activity, providing evidence of IgG specificity for 3MGA. The assay measured an average of 1.51 mg/mL free 3MGA in

seven of 11 independent assays, with four of 11 assays measuring <0.2 mg/mL. Experiments using neat urine as matrix led to a reduction in signal intensity compared to buffer but, otherwise, yielded similar results. Taken together, the data indicate that, if assay sensitivity and reproducibility can be improved, antibody-based detection of 3MGA may offer an alternative method to detect this organic acid in urine. In a growing number of mitochondrial disorders 3MGA is generated from acetyl CoA via a diversion pathway that reflects compromised aerobic energy metabolism. The present data support the premise that a high affinity monoclonal antibody could allow self-monitoring of the effects of exercise, nutrition and/or drug therapy on 3MGA levels in urine.

9.2 INTRODUCTION

3-methylglutaconic aciduria (3MGAuria) is a prominent phenotypic characteristic of numerous inborn errors of metabolism that affect a) leucine metabolic enzymes (primary 3MGAuria) or b) mitochondrial energy metabolism (secondary 3MGAuria). Primary 3MGAuria arises from mutations in either 3-methylglutaconyl (3MGC) CoA hydratase (*AUH*) or 3-hydroxy,-3-methylglutaryl CoA (HMG CoA) lyase (*HMGCL*) (Jones, Perez, & Ryan, 2020). Both enzymes function in degradation of the branched chain amino acid, leucine, and the absence of their activity leads to a buildup of upstream pathway intermediates. One such intermediate is 3-methylglutaconyl CoA (3MGC CoA), a metabolite that is unique to this metabolic pathway. 3MGC CoA is formed by the ATP dependent carboxylation of 3-methylcrotonyl CoA by 3-methylcrotonyl CoA carboxylase (Jones, Perez, & Ryan, 2020).

Of increasing interest, however, is 3MGAuria that arises from mutations that, directly or indirectly, affect mitochondrial energy metabolism (i.e. secondary 3MGAuria). Approximately a dozen distinct inborn errors of metabolism manifest secondary 3MGAuria (Su & Ryan, 2014) (Jones, Perez, & Ryan, 2020). A common feature of these disorders is that the leucine degradation pathway is intact. It has been hypothesized that, in muscle and perhaps brain tissue, 3MGA arises *de novo* from acetyl CoA under conditions of defective electron transport chain (ETC) function. This "acetyl CoA diversion pathway" is initiated when the ETC malfunctions and NADH / $FADH_2$ generated by oxidative metabolism of fuel molecules is unable to efficiently donate electrons to the ETC. As NADH levels rise in the mitochondrial matrix, enzymes that generate NADH as a product (including three TCA cycle enzymes) are subject to end product inhibition. In turn, this leads to inhibition of the TCA cycle and accumulation of acetyl CoA. When acetyl CoA levels in the mitochondrial matrix rise, it is converted to acetoacetyl CoA via a T2 thiolase-catalyzed condensation reaction. Acetoacetyl CoA subsequently condenses with another acetyl CoA via HMG CoA synthase 2 to form HMG CoA. Under these conditions, HMG CoA is dehydrated to 3MGC CoA by the leucine pathway enzyme, AUH. Once formed via this route, 3MGC CoA is unable to proceed up the leucine pathway because the reaction catalyzed by 3-methylcrotonyl CoA carboxylase is irreversible. Under these metabolic conditions, as 3MGC CoA is produced via the acetyl CoA diversion pathway, its major fate is conversion to the corresponding organic acid, 3MGA, by acyl CoA thioesterase-mediated hydrolysis of its CoA moiety. Once formed, 3MGA is a dead end product whose ultimate fate is excretion in urine.

3MGA levels in urine of subjects with primary 3MGAuria are strongly affected by leucine catabolism with leucine loading resulting in markedly increased urinary 3MGA levels (Wortmann S. B., Kluijtmans, Sequeira, Wevers, & Morava, 2014). In cases of secondary 3MGAuria, however, leucine loading has little or no effect on urinary 3MGA levels. While this result is consistent with the fact that no leucine pathway defects exist in secondary 3MGAurias, it raises the possibility that urinary 3MGA content is a reflection of the relative efficiency of aerobic respiration and mitochondrial energy metabolism. Based on this potential correlation it may be anticipated that a low cost, rapid, easy to use assay method for detection / quantitation of urinary 3MGA would allow for levels of this organic acid to be correlated with physical activity, drug intervention, nutritional status or otherwise. Toward that end, in the present study the hypothesis that an antibody-based assay could serve as an alternative to the labor-intensive, expensive and time-consuming method of gas chromatography-mass spectrometry (GC-MS), was tested. The results obtained represent a first step toward development of an assay capable of monitoring 3MGA levels in urine outside of a clinical setting. When employed in a competitive ELISA with urine, the rabbit polyclonal antibody reported herein detected 344 mmol 3MGA/mmol creatinine. However, most secondary 3MGAurias have urine 3MGA concentrations of <200 (Jones, Perez, & Ryan, 2020). By transitioning to a monoclonal antibody, screened for enhanced antigen binding properties, the detection limits of 3MGA may be reduced. Moreover, it is conceivable that a lateral flow assay, or similar method, can be developed for home use.

9.3 MATERIALS AND METHODS

9.3.1 Antigen preparation

Mariculture keyhole limpet hemocyanin (KLH) was obtained from ThermoScientific. 3MGA-conjugated KLH antigen was synthesized from activated (E)-3-methylglutaconic acid (3MGA; Sigma) and KLH (Grabarek and Gergely, 1990; Hermason, 2008). Briefly, 3MGA sodium salt was activated with N-Hydroxysuccinimide (2.5 molar excess) in the presence of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC; 4.5 molar excess). The reaction was buffered with 50 mM MES, pH 6 containing 0.65 M NaCl. After 15 min the reaction was quenched with 2-mercaptoethanol prior to addition of KLH solution in 0.1 M sodium phosphate, pH 7.5, 0.65 M NaCl. Reaction pH was adjusted to 7.2 if necessary, and the sample was maintained at room temperature. After 2 h another aliquot of activated 3MGA was added and the sample incubated overnight at 4 °C. Reaction product was purified and buffer exchanged to 10 mM sodium phosphate, pH 7.4, 0.9 M NaCl using spin columns and the protein concentration determined by the BCA method (Thermo-Pierce). The percentage of KLH lysine residues derivatized was determined using the OPA method (Peng, et al., 2005). 3MGA-KLH used for antibody production had 26% of its lysine side chains derivatized. 3MGA conjugated bovine serum albumin (BSA) was prepared according to the same protocol (39% lysines derivatized) and stored in phosphate-buffered saline (PBS) until use.

9.3.2 Polyclonal antibody production in rabbits

Antibody production was contracted to ProSci Inc (Poway, CA). Prior to inoculation with 3MGA-KLH antigen, a pre-immune bleed (yielding 8 ml serum) was

collected from each of two rabbits. Subsequently, the rabbits were inoculated with 3MGA-KLH conjugate in complete Freund's adjuvant. After two weeks the rabbits were inoculated with 3MGA-KLH conjugate in incomplete Freund's adjuvant (IFA) followed two weeks later by a third inoculation in IFA. One week after the third inoculation, 25 ml of serum was collected from each rabbit. One week later, a fourth and final inoculation with 3MGA-KLH conjugate (in IFA) was administered and, after 3 weeks, a terminal bleed yielded 60 ml serum and this material was used in experiments.

9.3.3 Western blot analysis

Samples were loaded into the wells of a 4-20% polyacrylamide gradient gel and proteins separated by SDS-PAGE. The gel contents were then electrophoretically transferred to a PVDF membrane on a Trans-Blot Turbo RTA Mini apparatus (Bio-Rad Labs). The membrane was washed in 1xPBS-Tween (0.1%) and blocked with 5% non-fat milk in 1xPBS-Tween (0.1%). The membrane was then incubated with immune serum (1:40,000 in blocking buffer) followed by goat anti-rabbit IgG HRP-conjugated secondary antibody (1:5,000 dilution in blocking buffer) for 1 h at room temperature. The membrane was washed 3x with 1xPBS-Tween (0.1%) before and after antibody incubations. Antibody binding was detected by chromophore development using TMB substrate reagent (Genscript), followed by picture acquisition.

9.3.4 Isolation of the IgG fraction of immune rabbit serum

Rabbit serum from the final bleed was centrifuged at 10,000 x g for 10 min, the supernatant was diluted 1:1 with 20 mM sodium phosphate, pH 7 and filtered through a 0.45 µm filter. Column storage buffer (20% ethanol) was washed out of an S. areus Protein A column (GE Healthcare) with 20 mM sodium phosphate, pH 7 prior to

application of filtered immune serum. After loading the column and washing with 20 mM sodium phosphate, pH 7, IgG was eluted with 0.1 M citrate buffer, pH 3 with collection of 1 mL fractions in tubes containing 100 µL neutralization buffer (1 M Tris-HCl, pH 9). Following elution, samples were buffer exchanged to 1xPBS on a desalting column and protein concentrations determined by BCA assay. SDS-PAGE was performed on fractions (10 µg protein) under reducing and non-reducing conditions. Gels were stained with GEL Blue (G Biosciences) and destained in H_2O.

9.3.5 Biotinylation

Two-hundred µg 3MGA-conjugated BSA or anti-3MGA IgG was labeled with a 20-fold molar excess of biotin using an antibody biotinylation kit (Thermo-Pierce). Buffer was exchanged to 1xPBS by desalting column and protein concentration measured by BCA assay. In another reaction, 3MGA was directly labeled with biotin by adding 25 µL 50 mM biotin hydrazide (Cayman Chemicals) in DMSO to 1 mL of a 5 mg/mL 3MGA solution in 0.1 M MES, pH 5. Following this, 12.5 µL EDC reagent (100 mg/mL) in 0.1 M MES buffer was added and the sample incubated overnight at room temperature with agitation.

9.3.6 Competition ELISA formats

Specific ELISA format variations are described in Results but, generally, high-bind ELISA plates (Nunc or Greiner Bio-one) were coated with 3MGA-BSA or the IgG fraction of rabbit anti-serum. Coating reagents were diluted in 0.05 M carbonate-bicarbonate buffer and incubated overnight at 4°C. Free 3MGA was incubated on plates, or separately in tubes, with 3MGA-BSA, biotinylated 3MGA-BSA, biotinylated IgG fraction, or biotinylated 3MGA, depending on the format, for 1-2 h at room temperature

or 4°C. Plates were blocked for at least 1 h at room temperature with blocking buffer [5% non-fat milk in 1xPBS-Tween (0.1%)] or CaseinBlocker (Thermo Scientific). Sample dilutions were made with blocking buffer. Plates were washed between incubations 3x with 1xPBS-T (0.1%). Goat anti-rabbit HRP secondary antibody (Bio-Rad) or streptavidin-HRP (Genscript) was used for detection with TMB substrate (Life Technologies). 2 N sulfuric acid was added to stop reaction after 10 min and absorbance was measured on a Molecular Devices SpectraMax M5 microplate reader equipped with Softmax v5.4 software. All samples were assayed a minimum of two times, with replicates having a coefficient of variation of <10%.

Competition ELISA for assessment of competitor specificity was conducted using alternate short chain dicarboxylic acids, glutaric acid, HMG acid; 3-methylglutaric acid and succinic acid. These acids, as well as L-lysine, were from Sigma Chemical Co.

9.3.7 Creatinine Assay

A creatinine microplate assay (Eagle Biosciences) was conducted per manufacturer's protocol. Briefly, 25 µL of each standard and sample was diluted 1:8 in water and plated in duplicate. Alkaline picrate reagent (180 µL) was added to each well and the plate was mixed and incubated for 10 min at room temperature. Absorbance at 490 nm was measured on a Molecular Devices SpectraMax M5 microplate reader prior to the addition 15 µL acid reagent. The plate was agitated and, after 5 min, sample absorbance read at 490 nm. Second reading values were subtracted from the first and the difference in absorbance plotted versus standard concentration. Sample values were interpolated from a linear regression plot.

9.3.8 Data Analysis

The limit of detection (LOD) of competition ELISAs were calculated by establishing a non-competition cutoff point that was calculated according to the formula: mean of non-competition values – (standard deviation of non-competition values x 1.645). After calculation, LOD values were normalized to the mean of the non-competition sample and interpolated off a standard curve (normalized values versus concentration of free acid) using a four-parameter logistic equation. All curves used had R^2 >0.9 and there was <10% variation between replicates. Analysis was conducted using SoftMax Pro and the mean, standard deviation, and % coefficient of variation (%CV) were calculated in Excel. Inhibition curves were calculated by plotting B/B_0*100, where B = OD_{450} of the sample with competitor and B_0 = OD_{450} of the sample absent competitor versus the concentration of free acid using a four-parameter logistic equation using GraphPad Prism v8.

9.4 RESULTS

9.4.1 Antiserum characterization by Western blot

Immune rabbit serum was employed in Western blot analysis to evaluate its ability to recognize the hapten, 3MGA. Insofar as the rabbits were inoculated with 3MGA conjugated to KLH, this protein is unsuitable for use in a Western blot assay designed to detect 3MGA. Instead, 3MGA was conjugated to bovine serum albumin (3MGA-BSA) by EDC crosslinking. As seen in **Figure 7**, immune rabbit serum failed to recognize unconjugated control BSA yet showed a strong positive response against 3MGA conjugated BSA. The light positive banding at higher molecular weight in lane 3 may be attributed to BSA crosslinking via the two carboxylic acid groups of 3MGA or between BSA molecules during the crosslinking reaction. Dilution experiments revealed

that anti-3MGA serum gave rise to a positive signal at dilutions as low as 1:200,000 while no signal was observed with pre-immune serum at any dilution (data not shown). These data indicate that antibodies that recognize the hapten, 3MGA, are present in the rabbit immune serum.

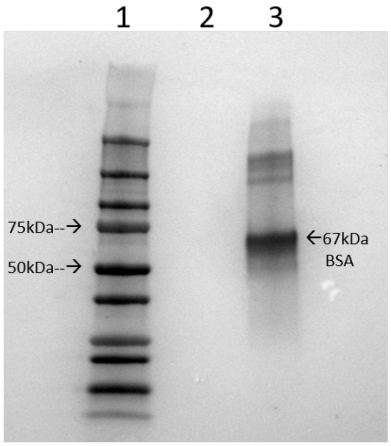

Figure 7. Immunoblot analysis of rabbit immune serum. Unmodified BSA or 3MGA-BSA (25 ng) were loaded into the wells of a 4-20% polyacrylamide gradient gel and proteins separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was washed, blocked and probed with rabbit immune serum (1:40,000 dilution). Antibody binding was detected with HRP-conjugated goat anti-rabbit IgG (1:5,000 dilution) and TMB substrate. Lane 1) molecular weight standards; Lane 2) unmodified control BSA; Lane 3) 3MGA conjugated BSA.

9.4.2 Indirect ELISA

In order to determine if anti-3MGA serum recognizes antigen in an ELISA format, checkerboard titer assays were conducted to determine optimal reagent dilutions. Optimal dilutions were considered to be when the positive signal was at least one order of magnitude greater than background signal. Using this approach, the data revealed that, when 0.31 µg/mL 3MGA-BSA (0.1 ml/well) was coated on the plate and a 1:100,000 antiserum dilution used along with a secondary antibody dilution of 1:3000, an optimized signal (Abs at 450 nm = 2.8 versus background Abs at 450 nm = 0.13) was observed. These conditions were then used in competition ELISAs described below.

9.4.3 Competition ELISA assay

To determine the ability of anti-3MGA immune serum to detect free 3MGA at levels comparable to those present in urine of subjects presenting with 3MGAuria, a competitive ELISA was performed. Using optimized conditions derived from indirect ELISA studies, the wells of a 96-well microtiter plate were coated with 100 µL of 0.31 µg/mL 3MGA-BSA and incubated overnight at 4°C. The plate was then washed and blocked for at least 1 h at room temperature. While the plate was in blocking buffer, increasing concentrations of free 3MGA (0.039 to 2.5 mg/mL, 2-fold serial dilutions, in 1xPBS containing 0.5 mg/mL BSA) were incubated with a 1:100,000 dilution of antiserum for 1 h at room temperature. Following incubation, anti-3MGA serum / free 3MGA solution was added to wells of the plate and incubated for 1 h. After washing, HRP-conjugated goat anti-rabbit secondary antibody was added and the plate incubated for a further 1 h. After washing, the amount of HRP-secondary antibody bound to each well was measured following addition of TMB substrate reagent and absorbance measurement at 450 nm (Format 1 in **Table 2**). The normalized absorbance signal observed was inversely proportional to the amount of free 3MGA present and, thus, serves as a measure of its ability to compete with 3MGA-BSA for antibody (**Figure 8**). The LOD calculated indicates 3MGA at concentrations as low as 0.474 mg/mL are detectable under these conditions.

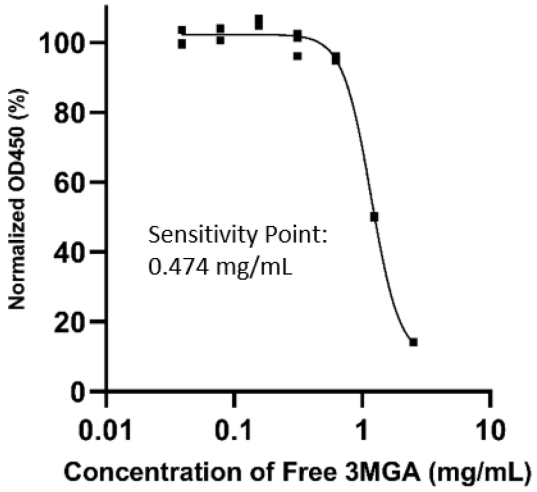

Figure 8. Competition ELISA assay with immune serum. 3MGA-BSA (0.31 µg/mL) was coated on a plate and incubated overnight at 4°C. Rabbit immune serum (1:100,000 dilution), incubated with 0, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25 or 2.5 mg/mL free 3MGA for 1 h was then added. Goat anti-rabbit HRP secondary antibody (1:3000 dilution) and TMB substrate reagent were used for detection. Data are presented as an inhibition curve (B/B_0*100 versus concentration) each replicate (n=3) was plotted using a 4-parameter logistic equation. B = OD_{450} of the sample with competitor and B_0 = OD_{450} of the sample with no competitor.

Table 2. Assay formats with their corresponding limits of detection.

	Format 1	Format 2	Format 3	Format 4	Format 5
Coating[1]	0.31 µg/mL 3MGA-BSA	0.31 µg/mL 3MGA-BSA	0.31 µg/mL 3MGA-BSA	2 µg/mL IgG	2 µg/mL IgG
Primary Ab/ Competitor[2]	1:100,000 antiserum	1:750 IgG	1:320 Biotin-IgG	0.25 µg/mL Biotinylated 3MGA-BSA	1 mg/mL Biotinylated-3MGC
Detection[3]	1:3000 GAR-HRP	1:3000 GAR-HRP	1:1000 Streptavidin-HRP	1:2000 Streptavidin-HRP	1:2000 Streptavidin-HRP
LOD	0.474 mg/mL	0.344 mg/L	0.19 mg/mL	0.185 mg/mL	0.679 mg/mL
SD of non-competitor	0.003	0.007	0.031	0.039	0.038

[1]Coating was incubated overnight at 4°C.

[2]Primary antibody/competitor was incubated with free acid for 1 h at room temperature either on the plate or separately in tubes, depending of format.

[3]Plates were washed prior to detection reagent being added.

9.4.4 Isolation of IgG fraction from anti-3MGA immune serum

To optimize the ELISA for increased 3MGA detection sensitivity, the IgG fraction of rabbit immune serum was isolated on a *S. areus* Protein A column. Flow-through and IgG elution fractions were collected and analyzed by SDS-PAGE (**Figure 9**). The results reveal that this procedure removed the bulk of contaminating serum proteins and, under reducing conditions, gave rise to two major bands, corresponding to the heavy and light chains of IgG, respectively. Under non-reducing conditions a single major band, corresponding to the size of intact IgG, was observed.

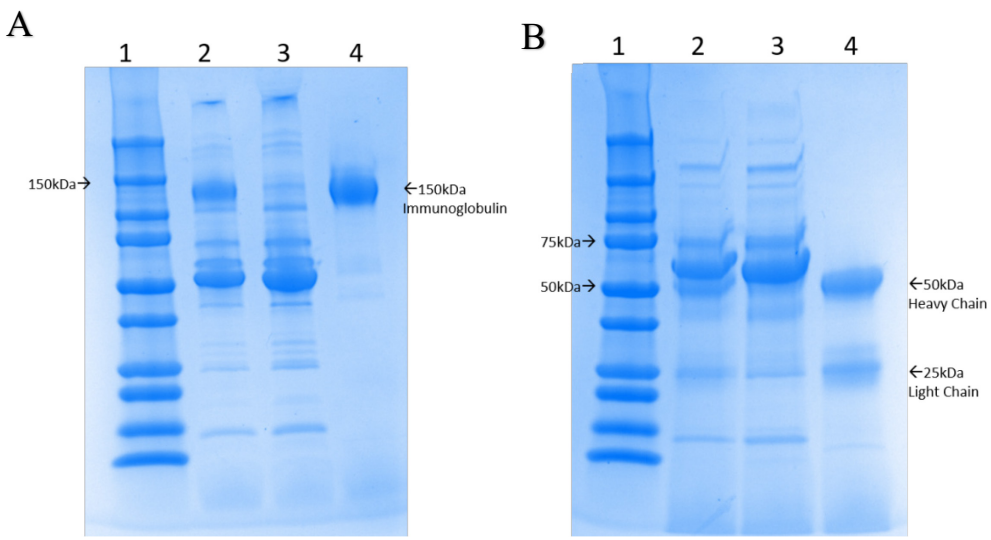

Figure 9. SDS-PAGE of isolated rabbit immune serum IgG fraction. The IgG fraction of rabbit immune serum was isolated by chromatography on an *S. aureus* Protein A affinity column as described. Ten µg protein aliquots of different column elution fractions were electrophoresed on a 4%-20% SDS-PAGE gel in the absence (A) or presence (B) of ß-mercaptoethanol, and stained with GELBlue. A) Non-reducing conditions; Lane 1) molecular weight standards; Lane 2) immune serum; 3) column flow through and Lane 4) eluted IgG. B) Reducing conditions; lane assignments same as panel A.

9.4.5 ELISA optimization studies

Initially, isolated anti-3MGA IgG was employed in an ELISA similar to that presented in Figure 8 (3MGA-BSA coated plate incubated with anti-3MGA IgG followed by detection of bound antibody with HRP-conjugated secondary antibody). Using this assay format (Format 2 in **Table 2**), the concentration of free 3MGA detected decreased to 0.344 mg/mL. In an effort to improve the limits of detection, additional variations on the ELISA format were investigated. For example, a biotin/streptavidin detection system was employed for signal amplification (Format 3 in **Table 2**) wherein IgG was biotinylated and streptavidin-HRP used for detection. This format increased free 3MGA detection sensitivity to 0.190 mg/mL. In another variation, a "flipped assay" format was employed (Format 4 in **Table 1**). In this case, the wells of the plate were coated with anti-3MGA IgG and incubated with biotinylated 3MGA-BSA as competitor. When used in a

competitive ELISA, 3MGA detection sensitivity was 0.185 mg/mL. A final format examined involved direct biotinylation of 3MGA, in case steric hindrance was a factor that affects the use of protein-conjugated 3MGA as competitor. The LOD in this case was 0.679 mg/mL. Inhibition curves, and corresponding LODs, for all assay formats tested are presented in **Figure 10** and **Table 1**, respectively.

The data obtained revealed the most sensitive ELISA format was the 'flipped' version (Format 4). Thus, this assay format was employed in all subsequent experiments. Various buffers and incubation times used in competition ELISAs were also assessed using this format in an effort to further optimize the assay. However, no differences in sensitivity were observed (data not shown).

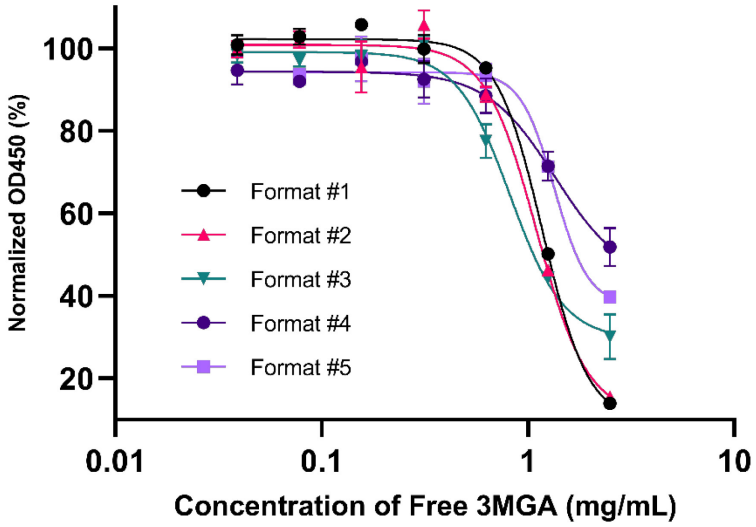

Figure 10. Optimization of ELISA plate format. Competitive ELISAs were performed using one of 5 alternate formats (see Table 2). Data are presented as inhibition curves (B/B_0*100) using a 4-parameter logistic equation. Each value represents the mean ±SD. (n = 3), except format 5 which is the average of two determinations. LOD for each assay format, calculated as described in Materials and Methods are given in Table 1. SD=standard deviation.

9.4.6 Reproducibility of the assay

A key aspect of an ELISA relates to reproducibility of measurement. In order to test this, inter-assay precision was examined on 11 independent competition ELISAs using Format 4. In addition, intra-assay precision was assessed on a single run of three independently prepared standard curves, in replicates of four. The assay conditions used were the same for each experiment. The mean, standard deviation and %CV were calculated and the LOD of each assay is summarized in **Table 3**. Analysis of inter-assay precision using data from all 11 independent assays yielded a %CV = 78.7%, indicating high variability with respect to LOD measurements. However, if the four lowest LOD values are removed, the seven remaining assays gave rise to a %CV = 30.9%. It is noteworthy that the four assays removed are the same assays that yielded the highest non-competition absorbance values, suggesting the dynamic range of the curve is an important factor. A similar degree of variability and average LOD was observed for the intra-assay precision data (33.9% among the three sets at 1.30 mg/mL). In addition, all three independently prepared data sets had lower no competition absorbance values, suggesting the lower dynamic range, which increases the LOD, occurs plate-to-plate rather than during sample preparation. Hence, it is possible to evaluate data from a single plate, but less accurately than with several plates.

Table 3. Inter and intra-assay precision.

Inter-Assay Precision

	Assay 1[1]	Assay 2[1]	Assay 3	Assay 4	Assay 5[2]	Assay 6
n=	2	5	5	5	12	5
LOD (mg/mL)	0.046	0.043	1.197	1.54	2.05	0.978
Mean Non-Competitor Absorbance Value	1.115	0.972	0.924	0.796	0.684	0.652
r^2	0.987	0.988	0.975	0.966	0.995	0.992

	Assay 7	Assay 8[1]	Assay 9	Assay 10	Assay 11[1]
n=	5	4	2	2	2
LOD (mg/mL)	1.868	<0.039	0.864	2.057	0.19
Mean Non-Competitor Absorbance Value	0.796	1.052	0.745	0.886	1.059
r^2	0.966	0.999	0.983	0.997	1

Summary Assays 1-11		**Summary of Assays: 3-7, 9-11[1]**	
Mean	0.990 mg/mL	**Mean**	1.508 mg/mL
SD	0.780	**SD**	0.466
%CV	78.7%	**%CV**	30.9%
Range	0.039 to 2.057 mg/mL	**Range**	0.864 to 2.057 mg/mL

Intra-Assay Precision[3]

	Set 1	**Set 2**	**Set 3**	**Summary Sets 1-3**	
n=	4	4	4	**Mean**	1.304
LOD (mg/mL)	1.093	1.919	0.901	**SD**	0.442
Mean Non-Competitor Absorbance Value	0.741	0.676	0.635	**%CV**	33.9%
r^2	0.995	0.99	0.993	**Range**	0.901 to 1.919 mg/mL

[1]After removal of four lowest points.

[2]Assay 5 was composite data from the intra-assay precision plate.

[3]Three independently prepared curves, in replicates of four, on one plate.

9.4.7 Evaluation of competitor specificity

Competition ELISAs were performed to determine the extent to which anti-3MGA IgG crossreacts with structurally related short chain dicarboxylic acids and L-lysine. The organic acids tested included glutaric acid, succinic acid, 3-methylglutaric acid and HMG acid. Assays were conducted using ELISA Format 4 and the results obtained compared to assays performed using 3MGA as competitor. As seen in **Figure 11**, whereas L-lysine showed little or no ability to compete, the short chain dicarboxylic acids examined did manifest weak competition. Compared to 3MGA, however, these compounds were clearly less effective. The LOD for 3MGA calculated from this assay was 0.185 mg/mL. The other short chain dicarboxylic acids, and L-lysine, did not yield data that could be plotted on a curve and, therefore, their LOD could not be calculated. This result provides evidence that these analytes do not share the same binding characteristics for anti-3MGA IgG as 3MGA itself.

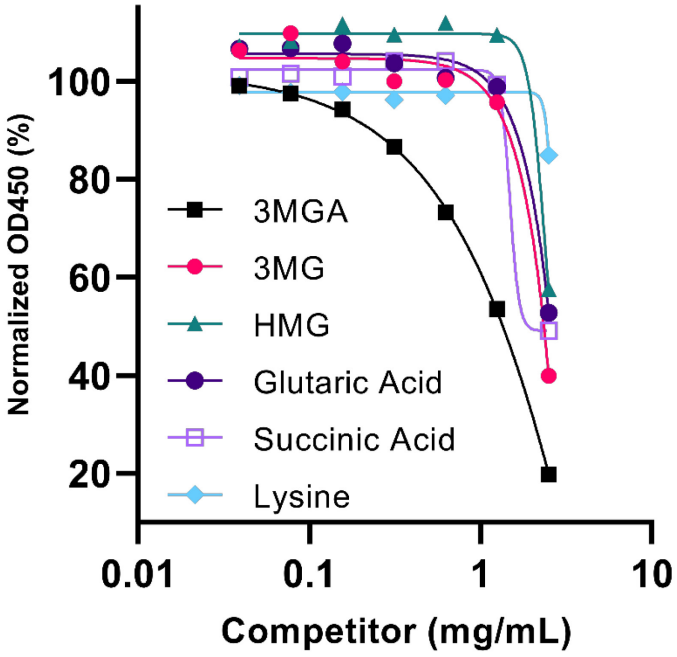

Figure 11. Evaluation of competitor specificity. Using competition ELISA assay format 4, inhibition curves were generated for 3MGA. Four structurally related short chain dicarboxylic acids and L-lysine were evaluated. Values plotted correspond to the mean of duplicate determinations. HMG=3-hydroxy-3-methylglutaric acid, 3MG=3-methylglutaric acid.

9.4.8 Studies with pooled human urine

To simulate conditions for detection of 3MGA in subjects with 3MGAuria, matrix effect studies were conducted by indirect ELISA using a sample of pooled human urine (Innovative Research). As seen in **Figure 12A**, data obtained with neat urine, and indicated urine dilutions, revealed a decrease in signal intensity as a function of increasing urine concentration, compared to a buffer control. Given that a long-term goal of this research is to accurately detect the lowest concentration of 3MGA in urine possible, neat urine and buffer were compared in a competition ELISA using Format 4 (**Figure 12B**). LODs calculated from inhibition curves corresponded to 0.237 mg/mL for urine and 0.978 mg/mL for buffer. It should be noted that the curves for urine and buffer

have very different shapes and this likely accounts for the large differences in LOD observed. When buffer was used as matrix, the calculated LOD was in the same range as that observed in inter- and intra-assay precision assessments (see Table 2). Because analyte measurements in urine are routinely calculated per the concentration of creatinine (to account for urine concentration), the creatinine content of the pooled urine sample used was measured. When correlated to the concentration of creatinine (4.791 mM), 3MGA concentrations as low as 344 mmol/mmol creatinine were detected.

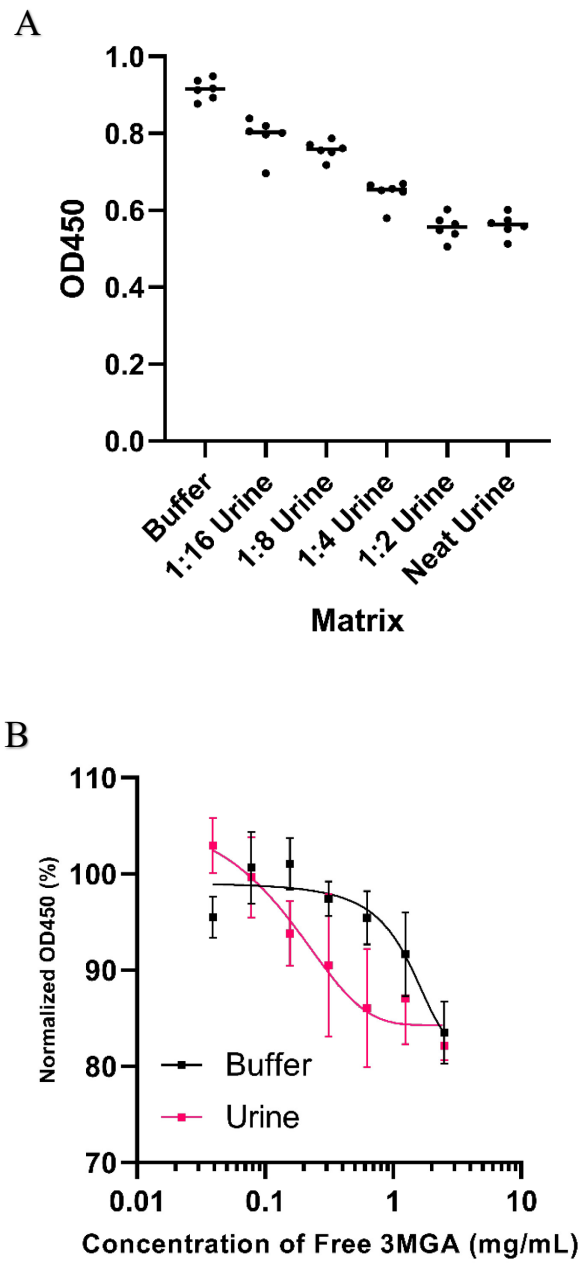

Figure 12. Effect of assay matrix on indirect and competitive ELISA. Panel A) Pooled human urine was assayed neat and at indicated dilutions in assay buffer by indirect ELISA. Each of 6 replicate measurements are plotted with the mean value depicted by a horizontal line. Panel B) Competition ELISA inhibition curves obtained for free 3MGA in buffer and neat urine. Values reported represent the mean ±SD (n = 3). SD=standard deviation.

9.5 DISCUSSION

3MGAuria is a phenotypic feature of a growing number of inborn errors of metabolism that affect mitochondrial energy metabolism (Wortmann, et al., 2013) (Su & Ryan, 2014) (Jones, Perez, & Ryan, 2020). Evidence suggests 3MGA arises from one of two distinct metabolic pathways. On one hand, in individuals with mutations in specific leucine degradation pathway enzymes (AUH or HMGCL) 3MGA is a byproduct of incomplete leucine metabolism. The presumed immediate precursor of 3MGA, 3MGC CoA, is an intermediate in the leucine degradation pathway and when downstream pathway enzymes are deficient, this metabolite accumulates. In Primary 3MGAurias leucine loading leads to exceptionally high levels of 3MGA (e.g. 3,000 mmol/mol creatinine; (Wortmann S. B., Kluijtmans, Sequeira, Wevers, & Morava, 2014). Above a threshold level of 3-methylglutaconyl CoA, this metabolite is subject to thioester hydrolysis, providing a means to preserve the CoA pool in the mitochondrial matrix (Roschinger, et al., 1995). The identity of the thioester hydrolase involved in this reaction has yet to be identified.

By contrast, secondary 3MGAurias have no defects in leucine metabolism and, in these disorders, 3MGC CoA is considered to arise from acetyl CoA via a recently described Acetyl CoA Diversion Pathway (Su & Ryan, 2014) (Ikon & Ryan, 2016) (Jones, Perez, & Ryan, 2020). This pathway is only initiated under conditions of compromised mitochondrial energy metabolism. Interestingly, an increasing number of disorders have been described wherein seemingly unrelated gene defects give rise to this phenotypic feature.

Among the disorders that manifest secondary 3MGAuria is Barth Syndrome, caused by mutations in the cardiolipin transacylase, tafazzin (Ikon & Ryan, 2017). Also, Costeff optic atrophy is the result of mutations in the *OPA3* gene, whose gene product of unknown function, localizes to the mitochondria (Anikster, Kleta, Shaag, Gahl, & Elpeleg, 2001). Dilated cardiomyopathy with ataxia syndrome is caused by mutations in DNAJC19, which encodes a chaperone protein (Richter-Dennerlein, et al., 2014). Likewise, subjects with mutations in CLPB (*c*aseino*l*ytic *p*eptidase *B*), a AAA+ protease family member (Wortmann, et al., 2015), manifest 3MGAuria. Other examples include high temperature requirement protein A2 (*HTRA2*) encoding a mitochondria-localized serine protease (Kovacs-Nagy, et al., 2018). Mutations in TMEM70 (transmembrane protein 70), a protein located in the mitochondrial inner membrane involved in the assembly of the F_1 and F_0 structural subunits of ATP synthase, manifests 3MGAuria (Tort, et al., 2011). Likewise, mutations in TIMM50 (translocase of inner mitochondrial membrane 50), encoding a subunit of the TIM23 inner mitochondrial membrane translocase complex, cause 3MGAuria (Tort, et al., 2019). Finally, SERAC1, a causative gene for MEGDEL syndrome (3-methylglutaconic aciduria with deafness, encephalopathy and Leigh-like syndrome); (Wortmann, et al., 2012) encodes a protein involved in phospholipid remodeling that when mutated, manifests 3MGAuria.

Each of these mutant genes encodes a protein that localizes to mitochondria. Studies have revealed widely variable amounts of 3MGA in urine in these disorders. In Barth Syndrome, for example, sometimes this organic acid is near the lowest detectable

level by GC-MS while at other times its levels are dramatically increased. When considered in light of the proposed pathway to 3MGA in secondary 3MGAurias, this pattern is consistent with the hypothesis that levels of 3MGA in urine, directly or indirectly, reflect the physiological status of energy metabolism in affected individuals. In order to test whether levels of 3MGA in urine of subjects harboring mutations in these genes correlate with disease symptoms or response to therapy, a rapid detection method would be very useful.

In addition to the gene mutations described above, another cause of secondary 3MGAuria is statin therapy (Phillips, et al., 2002). The underlying rationale for this effect is that statin-mediated inhibition of HMG CoA reductase activity results in inhibition of coenzyme Q biosynthesis and this, in turn, decreases ETC efficiency. In the case of statin-induced 3MGAuria, and perhaps other disorders, the concept has emerged that the severity of the physiological defect positively correlates with 3MGA levels in urine.

Insofar as GC-MS is time consuming and requires highly specialized equipment, routine examination of variability in the 3MGA content of urine as a function of exercise, diet or medication has not been explored. In an effort to facilitate this approach, the hypothesis that an antibody-based assay could be used for detection of this short chain dicarboxylic acid in biological fluids was tested. As a six carbon unsaturated organic acid, 3MGA is too small to elicit an immune response on its own. To overcome this, 3MGA was covalently attached to lysine side chains of KLH using EDC-based chemical crosslinking. Results obtained following immunization of rabbits with 3MGA-KLH revealed the generation of antibodies against the 3MGA hapten. Moreover, in a

competitive ELISA with immune rabbit serum, free 3MGA was able to compete for binding to 3MGA-BSA bound to wells of the plate. This result provides evidence that antibodies present in immune rabbit serum recognize and bind to free 3MGA. Indeed, immune rabbit serum gave rise to a readily detectable positive signal at dilutions as low as 1:200,000 in Western blot analysis.

To optimize the detection of 3MGA, a series of steps were performed. First, the IgG fraction of immune rabbit serum was isolated and characterized in ELISA plates. A competitive ELISA using anti-3MGA IgG led to a measurable improvement in the limits of detection of 3MGA and this was further optimized by testing a number of alternative ELISA formats (see Table 2). Following further optimization of reagent concentrations, the detection limits of the assay for 3MGA were observed to be in the range of 0.185 mg/ml. In studies of assay reproducibility, evidence was obtained that the ELISA format is not only sensitive but yielded acceptable intra- and inter- assay variability values. With regard to assay specificity, when several related short chain dicarboxylic acids were compared to 3MGA in competitive ELISAs, the data indicated a high degree of specificity for 3MGA. The amino acid, L-lysine, was a very poor competitor.

In order to adapt this assay method to a more biological setting, studies were performed with pooled human urine. In this case, there was an overall decrease in signal, as compared to using buffer as matrix. Despite this, inhibition was still evident suggesting the decrease in signal is due to non-specific interference. Moreover, according to intra-assay precision results with buffer, when the signal of the no competition control is lower, the LOD is higher suggesting that the dynamic range of the assay is important in

determining sensitivity. This is not the case when urine is the matrix, however, where the no competition control was significantly less than buffer on the same plate yet the sensitivity (LOD) was greater (0.237 mg/mL in urine versus 0.978 mg/mL in buffer). This suggests there might be some matrix effect but it is difficult to distinguish it from variability in the assay.

When the limits of detection for 3MGA are presented on the basis of mmoles creatinine in urine, the detection limits for 3MGA were in the range of 344 mmol / mmol creatinine. Given that this value falls in the range of many 3MGAurias, these data provide evidence for the potential utility of this method of analysis.

Based on the data presented, it is reasonable to consider that an immunological approach to measuring 3MGA in human urine samples is feasible. Indeed, metabolic aspects of secondary 3MGAuria suggest a rapid testing method may benefit subjects with these disorders. It is also clear that assay sensitivity may present a problem in cases where urinary 3MGA levels are borderline. To improve sensitivity, it is conceivable that screening a panel of monoclonal antibodies directed against 3MGA-KLH may be fruitful. Alternatively, recombinant antibodies may be an option to enhance sensitivity. If so, it is reasonable to consider that a test kit could be developed that would allow for detection of 3MGA in subject urine samples without the need for expensive and time consuming GC-MS analysis.

10 DEVELOPMENT OF A DIRECT ELISA

10.1 INTRODUCTION

To explore the possibility that a sensitive immunoassay could be developed in a non-competitive format, a study was initiated to measure free 3MGA in a matrix by first conjugating it to BSA using a carbodiimide reaction (Figure 5) before detection via direct ELISA. Initially, the assay was to be used for measurement of 3MGA in cell culture samples, therefore cell culture media was used as the matrix and 3MGA was spiked in to mimic a sample. The carbodiimide conjugation reaction is highly dependent on the ratio of protein to small molecule (Hermanson, 2008), therefore it was necessary to remove any proteins in the media prior to conjugation. A known amount of BSA could then be added along with EDC. After conjugation, a direct ELISA could be performed where the sample is coated on a plate and detected with an anti-3MGA antibody. Signal would then correspond to the amount of 3MGA in the sample. The assay could be developed as either a quantitative assay by using samples with known 3MGA amounts as standards, or as qualitative assay where any signal above a certain level would be positive.

10.2 MATERIALS AND METHODS

10.2.1 Optimized Carbodiimide Conjugation of BSA

3MGA-conjugated BSA was synthesized from activated (E)-3-methylglutaconic acid (3MGA; Sigma) and BSA (Grabarek & Gergely, 1990) (Hermanson, 2008). Briefly, 3MGA sodium salt was activated with N-Hydroxysuccinimide (NHS, 2.5 molar excess) in the presence of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC; 4.5 molar excess). The reaction was buffered with 50 mM MES, pH 6 containing

0.65 M NaCl. After 15 min the reaction was quenched with 2-mercaptoethanol prior to addition of BSA solution in 0.1 M sodium phosphate, pH 7.5, 0.65 M NaCl. Reaction pH was adjusted to 7.2 if necessary, and the sample was maintained at room temperature. After 2 h another aliquot of activated 3MGA was added and the sample incubated overnight at 4 °C. Reaction product was purified and buffer exchanged to 10 mM sodium phosphate, pH 7.4, 0.9 M NaCl using spin columns and the protein concentration determined by the BCA method (Thermo-Pierce). The degree of BSA lysine derivatization (39%) was determined using the OPA method (Peng, et al., 2005).

10.2.2 Carbodiimide Conjugation

Amicon, 4mL, 7k MWCO concentrators (Millipore) were washed prior to use by adding 1 mL of water and centrifuging for 5 min at 4,000 x g. Before filters dried, 2 mL of 3MGA in cell culture media was added and centrifuged for 40 min at 4,000 x g. The flow-through was collected (~1.5 mL) and 447 µL was added to two tubes. BSA diluted in 0.1M MES, pH 5.3 was added to a final amount of 0.05 mg and 0.01 mg with 1 mg of 3MGA in each reaction. Three hundred microliters of EDC were added to the BSA:3MGA solution (final volume of 1 mL) and incubated overnight at 4°C while rocking.

10.2.3 Western blot analysis.

Samples were loaded (25 ng) into the wells of a 4-20% polyacrylamide gradient gel and proteins separated by SDS-PAGE. The gel contents were then electrophoretically transferred to a PVDF membrane on a Trans-Blot Turbo RTA Mini apparatus (Bio-Rad Labs). The membrane was washed in 1xPBS-Tween (0.1%) and blocked with 5% non-fat milk in 1xPBS-Tween (0.1%). The membrane was then incubated with immune serum

(1:40,000 in blocking buffer) followed by goat anti-rabbit IgG HRP-conjugated secondary antibody (1:5,000 dilution in blocking buffer) for 1 h at room temperature. The membrane was washed three times for five min each with 1xPBS-Tween (0.1%) before and after antibody incubations. Antibody binding was detected by chromophore development using TMB (Genscript), followed by picture acquisition.

10.2.4 Direct ELISA

High-bind ELISA plates (Nunc, Thermo Fisher) were coated with 3MGA-BSA samples that were serially diluted in 0.05 M carbonate buffer and 100 µL of each dilution (in triplicate) was plated. An optimized 3MGA-BSA and a BSA only sample were diluted to 0.31 µg/mL in 0.05M carbonate buffer and 100 µL were plated as controls, prior to overnight incubation at 4°C. Plates were blocked for at least one hour at room temperature with 5% non-fat milk. Plates were washed three times with 300 µL 1XPBS-T (0.1%). One hundred microliters of biotinylated anti-3MGA IgG (1.56 µg/mL) was added and incubated for one hour at room temperature. Streptavidin-HRP (1:1000 dilution) was used for detection after TMB substrate was added. 2N sulfuric acid was added to stop the reaction. Plates were read on a Molecular Devices Spectromax M5 microplate reader with Softmax v5.4 software. All samples were run in triplicate, with replicates having a coefficient of variation of <10%.

10.2.5 Statistical Analysis

Student's unpaired t-test was used to compare BSA only samples to a mock 3MGA-BSA cell culture sample (Figure 15). Statistical test was performed using GraphPad Prism version 8.4.2 for Windows (GraphPad Software, San Diego, CA).

10.3 Results and Discussion

The hypothesis that 3MGA-BSA can be detected at a lower concentration in a direct ELISA than free 3MGA in competition ELISA was tested. In this initial experiment a cell culture media sample was spiked with 3MGA and 0.05 mg or 0.01 mg BSA and subjected to carbodiimide coupling prior to ELISA and immunoblot. Western blot data shows that 1 mg of 3MGA can be detected if coupled to either 0.05 mg or 0.01 mg BSA, though not as intensely as a 3MGA conjugated to BSA in an optimized carbodiimide reaction (Figure 13).

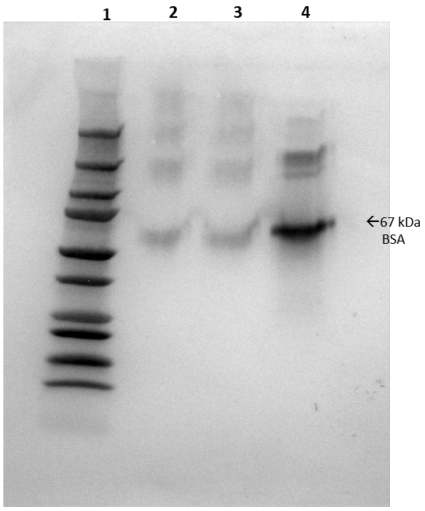

Figure 13. Western blot analysis of spiked 3MGA cell culture samples. Carbodiimide conjugated 3MGA-BSA cell culture samples or optimized 3MGA-BSA (25 ng) were loaded into the wells of a 4-20% polyacrylamide gradient gel and proteins separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was washed, blocked and probed with rabbit immune serum (1:40,000 dilution). Antibody binding was detected with HRP-conjugated goat anti-rabbit IgG (1:5000 dilution) and TMB substrate. Lane 1) molecular weight standards; Lane 2) 1.0mg 3MGA and 0.05 mg BSA; Lane 3) 1 mg 3MGA and 0.01 mg BSA, Lane 4) optimized 3MGA-BSA.

To test the feasibility of utilizing a direct ELISA to measure 3MGA in cell culture samples, each mock sample (1 mg 3MGA:0.05 mg BSA and 1 mg 3MGA:0.05 mg BSA)

were plated neat and then diluted in a 2-fold serial dilution with 0.05M carbonate buffer. The optimized 3MGA-BSA and BSA only were used as controls. The ELISA results show a decrease in signal at very high concentrations of BSA (Figure 14). This was also observed during the checkerboard titers done prior to the competitive ELISA development suggesting that the same steric hindrance to the antibody binding 3MGA is at work here. When 0.31µg/mL of protein was used for coating, BSA, mock sample 3MGA-BSA, and optimized 3MGA-BSA, there was marked difference between the mock sample 3MGA-BSA and optimized 3MGA-BSA. Although the signal is much reduced from the optimized 3MGA-BSA, it is still significantly higher than BSA alone (Figure 15). These data confirm the Western blot data and suggest that it is possible to measure cell culture samples via this method.

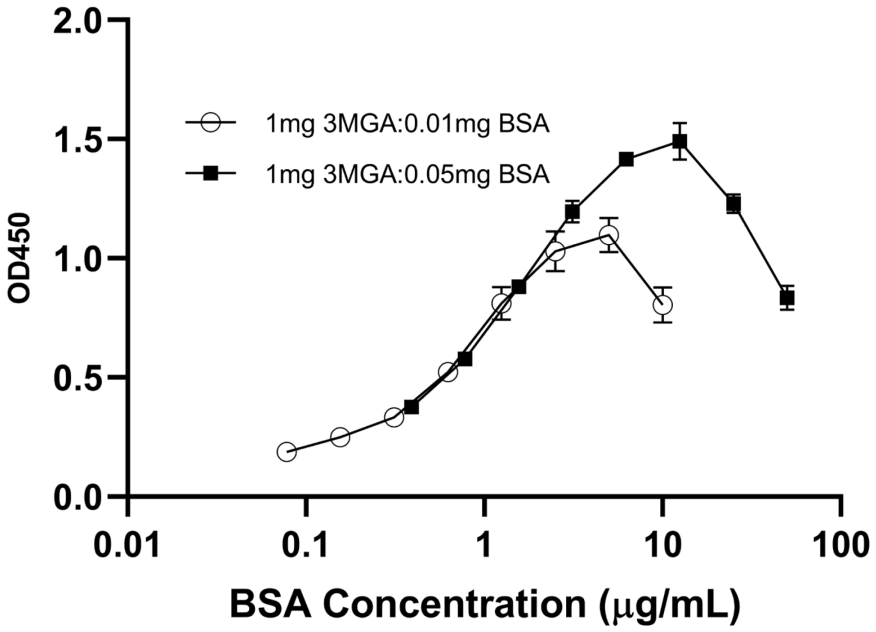

Figure 14. Serial dilution of 1mg 3MGA:0.05 BSA and 1mg 3MGA: 0.01mg BSA samples in a direct ELISA. Eight, 2-fold serial dilutions were plated in triplicate for each sample and incubated

overnight at 4°C. Biotinylated anti-3MGA IgG (1.56 µg/mL) was incubated for 1 h. Streptavidin-HRP (1:1000 dilution) and TMB substrate reagent were used for detection. Data is presented as the absorbance value at 450 nm versus BSA concentration on a logarithmic scale.

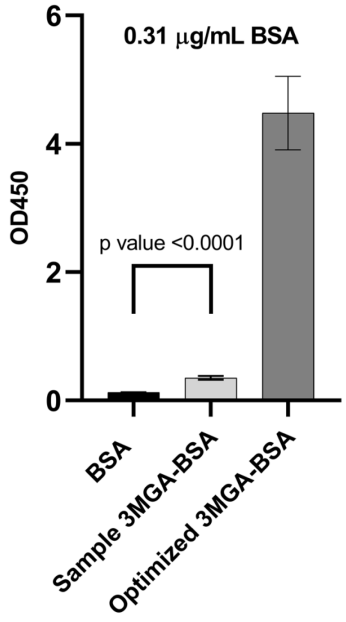

Figure 15. Comparison of 3MGA absorbance signal when the same amount of BSA is coated on an ELISA plate. A direct ELISA was performed as in Figure XX using 0.31 µg/mL of BSA (n=21), mock sample 3MGA-BSA (n=6), and optimized 3MGA-BSA (n=27) for a comparison of 3MGA signal. Values reported are the mean ± standard deviation, $p < 0.0001$ versus BSA only.

The competitive ELISA measures around 1 mg/mL of free 3MGA in a sample fairly accurately and this direct ELISA method has shown that it can at least measure that same amount. Further optimization and testing is needed, however, and testing lower 3MGA sample concentrations is necessary for determining the limits of the assay. An inherent difficulty in this method is finding the best ratio of 3MGA, BSA, and EDC for the carbodiimide conjugation, however, because each sample will have an unknown amount of 3MGA. This problem might be mitigated by spiking in several concentrations of BSA (with each using an optimized amount of EDC) to each sample, running them all

on the ELISA, and choosing the data from the dilution that meets criteria set *a priori*. It is also difficult to know how much of the 3MGA in a sample might be lost during the filtration step, or how much will be derivitized and available for measurement. Because of this, the limit of detection would need to be well-defined and only relative measurements could be made in a qualitative fashion.

11 Other Immunoassay Formats

11.1 Introduction

Development of other types of immunoassay were attempted in the course of this research that were not pursued beyond the initial experiments. This was mostly due to time constraints and not because the assays were without merit. Following similar formats as the assays in the previous chapter, two further variations were attempted: an ELISA using a Fab fragment instead of a whole antibody and the covalent attachment of 3MGA directly to an amine-coated plate.

The overall goal of this immunoassay is to develop the most sensitive assay possible to measure 3MGAuria for the purpose of patient monitoring during disease progression or drug therapy. One way to increase sensitivity is to reduce the size of the components in the assay to increase density and to decrease steric hindrances from neighboring molecules (Ylikotila, et al., 2006) (Peluso, et al., 2003). Antibodies are large proteins, about150 kDa, and make up the largest component of the assay. In addition, the bivalent nature of the antibody means that in a competitive assay, it can bind both the free analyte in the sample and the labeled competitor thereby needing twice as much of the free analyte to fully inhibit the signal. By splitting the antibody into just its Fab

fragments, the size is reduced to about 50 kDa per fragment with only a single antigen binding site (Figure 16). The fragments could then be used in ELISA formats as previously described instead of the intact IgG antibody.

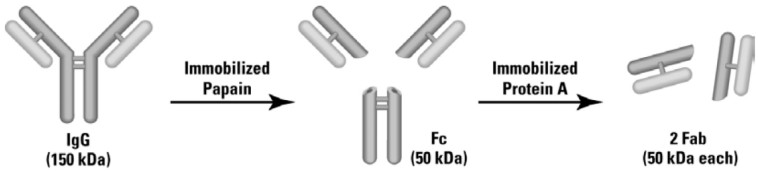

Figure 16. The results of papain digestion on an IgG antibody (Thermo Scientific: Fab Fragments, 2017).

The fragments are generated using a proteolytic enzyme of the peptidase C1 family called papain in the presence of cysteine (a reducing agent). The enzyme is specific for the arginines and lysines after a hydrophobic residue (Thermo Scientific: Fab Fragments, 2017).

Direct conjugation of 3MGA to an amine-coated plate would allow for a direct ELISA format instead of a competitive ELISA. Theoretically, a urine sample from a patient with 3MGAuria could be filtered to remove protein, the 3MGA present would then be activated with sulfo-NHS followed by EDC in the amine-coated plate and detection by an anti-3MGA antibody. The signal would be proportional to the amount of 3MGA in the sample. Furthermore, the 3MGA molecules would be more uniformly orientated through a linker, which would increase antibody access to the antigen (Figure. 17). 3MGA conjugated to BSA would be in random orientation with some of the BSA hidden between the plate and protein, reducing antibody access.

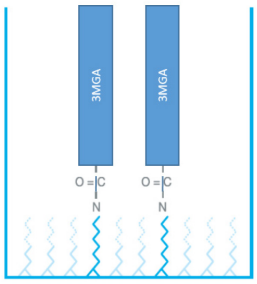

Figure 17. Amine linker and orientation of 3MGA on a CovaLink plate. Adapted from (Andersen, 2012).

11.2 Materials and Methods

11.2.1 Generation of anti-3MGA IgG Fab Fragment

Fab fragments were generated from protein A purified anti-3MGA IgG using a Fab fragmentation kit (G Biosciences) according to the manufacturer's protocol. Briefly, 250 µg of anti-3MGA IgG in 1x PBS was exchanged to 20mM cysteine-HCl with papain using a desalting column and incubated overnight in a resin column with vigorous shaking. Digested antibody was collected and applied to a protein A column. The Fab fragments from the flow-through and eluted Fc fragments were collected. The concentration was measured using A280 absorbance on a NanodropOne prior to SDS-PAGE (with and without β-mercaptoethanol) and Coomassie staining (28 µg of protein per lane).

11.2.2 Fab Fragment ELISA

High-bind Nunc ELISA plates (Thermo Fisher) were coated with 3MGA-BSA or with anti-3MGA Fab fragment in 0.05M carbonate buffer and incubated at 4°C overnight. Plates were blocked for at least 1 hour at room temperature with 5% non-fat milk in 1xPBS-Tween (0.1%). Plates were washed between incubations three times with 300µL 1XPBS-Tween (0.1%). Goat anti-rabbit HRP secondary antibody or streptavidin-

HRP was used for detection. TMB substrate was added to induce color change. 2N sulfuric acid was added to stop reaction.

11.2.3 Covalink™ ELISA

A 4 mg/mL 3MGA solution in cell culture media was diluted 1:2 in DMSO which was then added to 10 mL of freshly prepared 0.184 mg/mL sulfo-N-hydroxysuccinimide (sulfo-NHS) solution and mixed. The 3MGA:sulfo-NHS mixture was added (50 µL) to the Nunc™ Covalink™ NH plates (Thermo Fisher). Following this, 50 µL of freshly prepared 1.23mg/mL EDC solution was added to the wells and incubated for 2 hours at room temperature. The plate was then washed in CovaBuffer (2M NaCl, 40mM $MgSO_4$, 0.05% tween) and 1.56 µg/mL of biotinylated anti-3MGA IgG was added and incubated for 1 hour at room temperature. Following a wash, 1:1000 dilution of streptavidin-HRP was added and incubated another hour. TMB substrate was added after washing and the enzymatic reaction stopped with 2N sulfuric acid.

11.3 Results and Discussion

SDS-PAGE and Coomassie staining indicated that the Fc portion of the antibody was successfully separated from the Fab fragments (Figure 18). However, when used in an ELISA as either the coating reagent or primary antibody, it did not bind to BSA-conjugated 3MGA at any concentration tested. The anti-3MGA IgG bound quite strongly to BSA-3MGA, this suggests that during papain digestion the antigen binding site of the antibody was compromised.

Likewise, the ELISA using the Covalink plate where 3MGA was covalently linked to the amine-coated surface, did not produce results. This could be due to the

large dilution of 3MGA into the sulfo-NHS solution (500 µL of 3MGA into 10 mL of sulfo-NHS solution) as per the manufacturer's recommendations (Andersen, 2012).

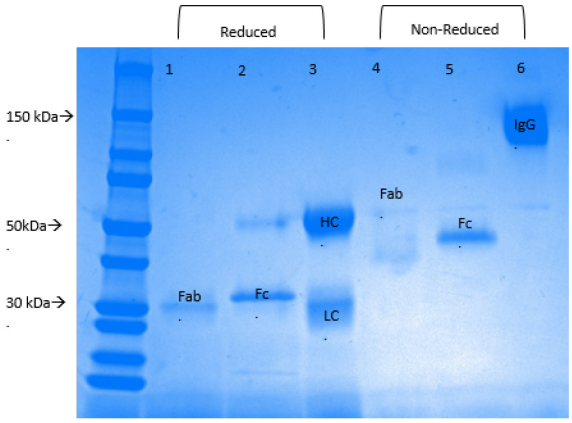

Figure 18. Coomassie stained SDS-PAGE of Fab fragment digest. Gel is a 4-20% SDS-PAGE with 28 µg of samples run with (reduced) and without β-mercaptoethanol (non-reduced). Lane 1 and 4 are the flow-through from the protein A column, lanes 2 and 5 are the eluted Fc fragments from the protein A column, lanes 3 and 6 are the intact IgG. HC=Heavy Chain, LC=Light Chain.

In both instances, further work should be done to investigate the feasibility of the assay. Especially in the case of optimizing an immunoassay with Fab fragments. This one experiment digested a polyclonal IgG overnight using a proprietary concentration of papain with cysteine. This might have over digested the antibody. A papain digestion can produce heterogeneous fragments, some smaller than the ~25 kDa Fab fragment, that are not obvious in a Coomassie stain. In addition, different isotypes of antibodies are more or less sensitive the papain enzyme (Adamczyk, Gebler, & Wu, 2000). Since this is a polyclonal antibody, and it is unknown the isotype(s) that were generated against 3MGA, further optimization of incubation time and papain concentration should be done. Studies in the literature have stated that concentrations of papain and cysteine, along with the length and temperature of incubation can affect the size and exact placement of the

enzymatic cut. In cases where there is an excess of cysteine, the cleavage of the disulfide bond holding the heavy and light chain of the Fab fragment together would result in a loss of the antigen binding site (Adamczyk, Gebler, & Wu, 2000) (Bennett, Smith, Truscott, & Sheil, 1997).

The use of Fab fragments in an assay have an additional advantage of being able to be tagged with biotin in such a way as to ensure the orientation of the antigen binding site is available for binding. The nucleotide binding site (NBS) on the Fab fragment is a conserved region of the variable domain. When UV cross-linked with a indole 3-butyric acid-labeled biotin molecule and coated on a streptavidin-plate, the Fab fragment is orientated in all the same way (Mustafaoglu, Alves, & Bilgicer, 2015). Not only could a higher density of "antibody" be coated on a plate, but more antigen binding sites would be available. Both improvements would increase sensitivity.

There are drawbacks to using an amine-coated plate for a clinical immunoassay, not the least of which is the cost of the plates themselves. If the desire is to have a quick and easy assay to use in the clinic, then using an expensive plate plus the effort of attaching 3MGA using a EDC-mediated conjugation reaction might not be worth it. The use of this assay, however, might be useful to further elucidate the binding characteristics of the antibody as this would be a "protein-free" system. All the other assays attempted, used a BSA-conjugated 3MGA molecule as a competitor (with the exception of Format 5 in Table 2, that directly biotinylated 3MGA. However, that is a competitive ELISA and this version is a direct ELISA). There is some evidence (data generated in the course of this research but not shown) that the polyclonal antibody generated for this assay

development, binds more strongly to protein conjugated 3MGA. This might not be a true representation of how it binds to free 3MGA, however. It is conceivable that a protein-free version of the assay would give a more accurate measurement of 3MGA in a sample.

12 DISCUSSION

The excretion of 3MGA in urine from a primary 3MGAuria can be >1000 µmol/mmol creatinine (Bouatra S, 2013), therefore, this assay which measures 3MGA around 344 mmol/mmol creatinine would only be adequate for detecting primary 3MGAuria at the higher end of the known range for secondary 3MGAuria. Excretion of 3MGA in urine from secondary 3MGAuria is also highly variable in each patient, from trace amounts to about 500 µmol/mmol creatinine, requiring a much more sensitive assay for monitoring (Jones, Perez, & Ryan, 2020). Statin usage, which disrupts the efficient function of the ETC via inhibition of coenzyme Q10 synthesis, is reported to have an even lower amount 3MGA at <20 µmol/mmol (Phillips, et al., 2002). In either secondary 3MGAuria or statin-induced 3MGAuria, an assay with increased sensitivity would be needed.

Several formats for a more sensitive assay, such as a competitive and direct ELISA were tested with varying results. One competitive ELISA format (Format 4, Table 2) was investigated further for reproducibility, specificity, and feasibility of 3MGA measurement in a urine matrix. The parameters chosen for the additional evaluation of the Format #4 assay were to establish the amount of variability in the assay, the specificity of the antibody for its antigen, and an assessment of urine matrix interference. Comparison of these results to established criteria for clinical biomarker assays from the

Food and Drug Administration (FDA) and other organizations involved in validating assays of this type, would be helpful in assessing future work needed on a 3MGA immunoassay.

The type of assay needed, qualitative or quantitative, would determine how the assay is developed. It might be useful, in the case of this biomarker, to develop both a quantitative and a qualitative assay. A qualitative, "dipstick"-type assay (positive/negative) for home monitoring of disease progression or treatment and a quantitative ELISA assay, for a more precise measurement in the clinic. In both cases, a reliable method that gives reproducible results is needed. It is possible that further optimization of one of the other assay formats would result in a more sensitive assay that meets all criteria of the federal guidelines.

12.1 Reproducibility

Reproducibility of the assay at the limit of detection was high (77%) when all 11 assays were included in the calculation for inter-assay precision. Federal guidelines and industry standards for quantitative biomarker assays state the LOD should be significantly above the background signal, but recognizes that at this level results can be variable. True reproducibility of the assay is assessed by the lower limit of quantitation (LLOQ); the lowest point that an assay measures precisely and accurately (usually a value higher than the LOD). At this level, the variability should be no more than 30%. (Bioanalytical Method Validation: Guidance for Industry, May 2018) (Lowes & Ackermann, 2016) (Lee, et al., 2006). Intra- and inter-assay accuracy, which was not assessed at all in this research, would also need to be done to meet federal guidelines.

Samples, spiked with low, middle, and high concentrations of 3MG, would be interpolated off the curve and percent of theoretical concentration would be analyzed. The direct ELISA format or the CovaLink format, where there is inherent loss of analyte due to processing of the samples, would not pass this assessment and could not be developed as a quantitative assay.

For a qualitative assay, the positive signal simply needs to be sufficiently above background to give a reliable positive signal, and close enough to background variation that 5% of the time a false positive is given. This positive cutoff would be determined by measuring 50 individual lots of urine (25 male and 25 female) that do not have 3MGA to define the background variation. To determine the sensitivity, several individual lots would be spiked with 3MGA and diluted down passed the positive cutoff. Because a 5% false positive rate is built into the cutoff, a confirmatory test by another method would be needed when positive (Shankar, et al., 2008). All the formats attempted could be further developed as a qualitative assay.

There was much work done to improve the reproducibility of the assay when it was evident that there were quite disparate LOD values. The observation that four of the inter-assay precision plates had a large dynamic range and markedly more sensitive LOD, is interesting and worth investigating further. The larger dynamic range was due to the much higher absorbance values of the non-competitor samples, so was not a function of free 3MGA competition. All assays were processed in the same way, with the same reagents, the only difference being they were conducted on different days. Troubleshooting into possible points of variation were examined (length of time it took to

prepare samples, sample preparation on ice versus at room temperature, type of ELISA plate, length of incubations, and different types of blocking and assay buffers), but no definitive source of the variation could be determined. This would need to remedied prior to this version of the assay moving forward. In addition, the other assays would need to be similarly evaluated in case this variability is due to the antibody itself.

12.2 SPECIFICITY

The specificity measurement clearly showed that the antibody does not bind other small, organic dicarboxylic acids as well as 3MGA. There is inhibition, though, at the highest concentration of the other acids as well as, to a minor extent, L-lysine. The pH of 3MGA at the highest concentration was measured in case the acidic solution would inhibit binding of the antibody, and though it was fairly low (>5), adjustment of the solution to pH 7 made no difference in the assay. Most likely, the inhibition at the highest concentrations are the result of steric hindrances that non-specifically inhibit antibody binding. Of course, further investigation should be done to evaluate higher concentrations of these metabolites, especially of 3-methylglutaric acid (3MG) which is often found in higher concentrations in secondary 3MGAuria than 3MGA (Jones, Perez, & Ryan, 2020). Further, other metabolites found in urine with similar structure to 3MGA should be tested, as well any drug (pharmaceutical or recreational) that could potentially be in patient urine should be evaluated as well.

Another avenue that should be explored is the specificity of the antibody to bind the two isomers of 3MGA. Measurement using GC-MS and NMR have recorded the presence of both *cis* and *trans* 3MGA in patient urine samples. It is unknown if the

isomerization stems from how the samples are treated prior to measurement on these platforms or if there is a natural flipping between *cis* and *trans* of the free acid. The enzyme that produces 3MGA-CoA only produces the *trans* isomer so the presence of *cis* isomers is hard to explain (Engelke, et al., 2006). Since the antibody was generated using the *trans* version as the hapten, it might be possible that the shape of the *cis* isomer, which is not as linear as a *trans* isomer, would not fit the binding pocket of the antigen binding site of the antibody.

12.3 MATRIX INTERFERENCE

Neat urine showed a definitive reduction in no competition signal as compared to buffer, however, a 3MGA competition ELISA still generated an inhibition curve. It is interesting that the LOD in urine is lower than that of buffer. Given the trend in the intra- and inter-assay precision data that lower no competition signal results in less sensitive assays (higher LODs), the urine data is opposite. This suggests that there may be matrix interference but the variability of the assay is masking the effect. In addition, the curves between the two matrices are quite different. The buffer inhibition curve does not plateau at high concentrations only low ones, whereas the urine curve plateaus at both ends. Suggesting that a sigmoidal inhibition curve results in lower LOD values. Matrix interference might be more of a problem in some assay formats and not others. It might be interesting to test urine as a matrix in several formats to see if it will be a problem prior to moving forward with a specific assay design.

The concentration of urine that might be seen in patients should also be evaluated in the assay for interference. Does more concentrated urine show more assay interference? If yes, dilutions of the matrix should be considered (Lee, et al., 2006). Normalization of 3MGA to creatinine should also be evaluated in cases of 3MGAuria (primary, secondary, or drug induced) as there is literature stating that creatinine excretion might not be consistent among individuals in a disease state (Tang, Toh, & Teo, 2015). Normalization with creatinine would only be necessary if doing a quantitative assay, a simple positive/negative readout would not require this additional measurement.

12.4 Future Directions

Both specificity and reproducibility could be improved, and matrix interference minimized, by the use of a monoclonal or a recombinant antibody. Monoclonal antibodies are generated from B-cell hybridomas against a single antigen resulting in higher affinity for its antigen and also reduces the range of epitopes recognized resulting in a greater concentration of specific antibodies to (Lipman, Jackson, Trudel, & Weis-Carcia, 2005). A recombinant antibody is generated through molecular biology techniques and a phage library is used to isolate, evolve, and grow the phage particles that express the single chain variable region that best binds the antigen (Smith, et al., 2004). This is in contrast to a polyclonal antibody generated against a hapten-conjugated protein, which has affinity to the hapten, the protein, and the combination of a hapten and protein, making affinity variable. A monoclonal or recombinant antibody would be necessary to develop a robust and reliable assay.

Regarding assay types, a well-designed quantitative ELISA is probably the simplest type of assay for clinical use. Clinics would readily be able to conduct a variety of the tests, including creatinine measurement, from a single urine sample. However, to truly monitor if diet, exercise, or therapy is affecting mitochondrial function (for better or for worse) from day-to-day or even hour-to-hour, an at home test is needed. The simplest at home test format would be a "dipstick" or lateral flow assay (LFA). The most common of this kind is the pregnancy test, but there are other technologies that are becoming more readily available for home use. Cell phone-based technologies include attachments that can be added to any phone that will, for example, fluoresce detection molecules on a test strip and digitally read them, and even send the results to a doctor (Vashist, Luppa, Yeo, Ozcan, & Luong, 2015). Lab-on-a-chip (LOC) technologies are an all-in-one types system where a sample is placed in a cassette which is then inserted into a small desktop analyzer that then prepares and processes the sample before giving a readout. LOC platforms currently are being used in doctor's offices, but could easily be adapted for home use in the future (Vashist, Luppa, Yeo, Ozcan, & Luong, 2015).

In summary, the work conducted herein provides important first steps towards developing an immunoassay for the detection of 3MGA in patient samples. The eventual availability of immunoassays for 3MGA will help patients with self-monitoring, will provide improved options for medical and research professionals, and may aid the eventual development of improved therapeutic options for the affected patients.

13 REFERENCES

Adamczyk, M., Gebler, J. C., & Wu, J. (2000). Papain digestion of different mouse IgG subclasses as studied by electrospray mass spectrometry. *Journal of Immunological Methods*, 237: 95-104.

Andersen, T. (2012). Coupling of peptides to Thermo Scientific Nunc CovaLink Surfaces via their carboxylic groups. *Application Note*. Roskilde, Denmark.

Anikster, Y., Kleta, R., Shaag, A., Gahl, W., & Elpeleg, O. (2001). Type III 3-methylglutaconic aciduria (optic atrophy plus syndrome, or Costeff optic atrophy syndrome): identification of the OPA3 gene and its founder mutation in Iraqi Jews. *Am J Hum Genet.*, 69: 1218-1224.

Armstrong, J. (2007). Urinalysis in Western Culture: A Brief History. *Kidney International*, 71:384-387.

Bayer, E. A., Ben-Hur, H., & Wilchek, M. (1988). Biocytin Hydrazide-A selective label for Sialic Acids, Galactose, and Other Sugars in Glyconconjugates using Avidin-Biotin Technology. *Analytical Biochemistry*, 170: 271-281.

Bennett, K. L., Smith, S. V., Truscott, R. J., & Sheil, M. M. (1997). Monitoring papain Digestion of a Monoclonal Antibody by Electrospray Ionization Mass Spectrometry. *Analytical Biochemistry*, 245:17-27.

Boster. (2017, July 28). *Which ELISA Is For You?* Retrieved April 2020, from www.bosterbio.com: https://www.bosterbio.com/newsletter-archive/20170728-which-elisa

Bouatra S, A. F. (2013). Urine Metabolome. PLoS ONE 8(9):e73076. doi:10.1371/journal.pone.0073076

Chivers, C. E., Koner, A. L., Lowe, E. D., & Howarth, M. (2011). How the biotin-streptavidin interaction was made even stronger: investigation via crystallography and a chimaeric tetramer. *Biochem. J.*, 435: 55-63.

Engelke, U. F., Kemer, B., Kluijtmans, L. A., van der Graaf, M., Morava, E., Loupatty, F. J., . . . Wevers, R. A. (2006). NMR spectroscopic studies on the late onset form of 3-methylglutaconic aciduria type 1 and other defects in leucine metabolism. *NMR Biomed.*, 19:271-278.

Grabarek, Z., & Gergely, J. (1990). Zero-Length Crosslinking Procedure with the use of Active Esters. *Analytical Biochemistry*, 185: 131-135.

Hermanson, G. T. (2008). *Bioconjugate Techniques* (second edition ed.). Amsterdam: Academic Press.

Ikon, N., & Ryan, R. O. (2016). On the origin of 3-methylglutaconic acid in disorders of mitochondrial energy metabolism. *J Inherit Metab Dis*, 39:749-756.

Ikon, N., & Ryan, R. O. (2017). Barth Syndrome: Connecting Cardiolipin to Cardiomyopathy. *Lipids*, 52: 99-108.

Jones, D. E., Perez, L., & Ryan, R. O. (2020). 3-Methylglutaric acid in energy metabolism. *Clinica Chimica Acta*, 233-239.

Kovacs-Nagy, R., Morin, G., Nouri, M., Brandau, O., Saadj, N., Nouri, M., . . . Wortmann, S. (2018). HTRA2 Defect: A Recognizable Inborn Error of Metabolism with 3-Methylglutaconic Aciduria as Discriminating Feature Characterized by Neonatal Movement Disorder and Epilepsy—Report of 11 Patients. *Neuropediatrics*, 49(06): 373-378.

Lee, J. W., Devanarayon, V., Barrett, Y., Weiner, R., Allinson, J., Fountain, S., . . . Wagner, J. A. (2006). Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement. *Pharmaceutical Research*, 1-17.

Lei, R., Huo, R., & Mohan, C. (2020). Current and emerging trends in point-of-care urinalysis. *Expert Review of Molecular Diagnostics*, 20: 69-84.

Lipman, N. S., Jackson, L. R., Trudel, L. J., & Weis-Carcia, F. (2005). Monoclonal Versus Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources. *ILAR Journal*, 46 (3): 258-268.

Lowes, S., & Ackermann, B. L. (2016). AAPS and US FDA Crystal City VI workshopon bioanalytical method validation for biomarkers. *Bioanalysis*, 8(3): 163-167.

Mustafaoglu, N., Alves, N. J., & Bilgicer, B. (2015). Oriented Immobilization of Fab Fragments by Site-Specific Biotinylation at the Conserved Nucleotide Binding Site for Enhanced Antigen Detection. *American Chemical Society*, 31: 9728-9736.

Peluso, P., Wilson, D. S., Do, D., Tran, H., Venkatasubbaiah, M., Quincy, D., . . . Nock, S. (2003). Optimizing antibody immobilization strategies for the construction of protein microarrays. *Analytical Biochemistry*, 312: 113-124.

Peng, D.-Q., Wu, Z., Brubaker, G., Zheng, L., Settle, M., Gross, E., . . . Smith, J. D. (2005). Tyrosine Modification is Not Required for Myeloperoxidase-induced Loss of Apolipoprotein A-I Functional Activities. *J. Biol. Chem.*, 280: 33775-33784.

Phillips, P. S., Haas, R. H., Bannykh, S., Hathaway, S., Gray, N. L., Kimura, B. J., . . . Scripps Mercy Clinical Resarch Center. (2002). Statin-Associated Myopathy with Normal Creatinine Kinase Levels. *Annals of Internal Medicine*, 137:7 581-585.

Richter-Dennerlein, R., Korwitz, A., Haag, M., Tatsuta, T., Dargazanli, S., Baker, M., . . . Langer, T. (2014). DNAJC19, a mitochondrial cochaperone associated with cardiomyopathy, forms a complex with prohibitins to regulate cardiolipin remodeling. *Cell metab.*, 20: 157-171.

Roschinger, W., Millington, D. S., Gage, D. A., Huang, Z. H., Iwamoto, T., Yano, S., . . . Sweetman, L. (1995). 3-Hydroxyisovalerylcarnitine in patients with deficiency of 3-methylcrotonyl CoA carboxylase. *Clin Chim Acta*, 240: 35-51.

Shankar, G., Devanarayan, V., Amaravadi, L., Barrett, Y., Bowsher, R., Finco-Kent, D., . . . Koren, E. (2008). Recommendations for the validation of immunoassays used for detecction of host antibodies against biotechnology products. *Journal of Pharmaceutical and Biomedical Analysis*, 48:1267-1281.

Singh, K., Kaur, J., Varshney, G. C., Raje, M., & Suri, C. (2004). Synthesis and Characterization of Hapten-Protein Conjugates for Antibody Production against Small Molecules. *Bioconjugate Chem.*, 15: 168-173.

Sino Biological. (n.d.). *Antibody Structure and Function*. Retrieved April 2020, from Sinobiological.com: https://www.sinobiological.com/resource/antibody-technical/antibody-structure-function

Smith, K. A., Nelson, P. N., Warren, P., Astley, S. J., Murray, P. G., & Greenman, J. (2004). Demystified...recombinant antibodies. *J Clin Pathol*, 57: 912-917.

Su, B., & Ryan, R. O. (2014). Metabolic biology of 3-methylglutaconic acid-uria: a new perspective. *J Inherit Metab Dis*, 37:359-368.

Suh, J., Seon, H., & Chung, B. (1997). GC-MS determination of organic acids with solvent extraction after cation-exchange chromatography. *Clinical Chemistry*, 43:12 2256-2261.

Tang, K. W., Toh, Q. C., & Teo, B. W. (2015). Normalization of urinary biomarkers to creatinine for clinical practice and research-when and why. *Singapore Med J*, 56(1): 7-10.

Thermo Scientific. (2016). Pierce Antibody Biotinylation Kit for IP. *Pub. Part No. 2162590.1 Rev. A.O, Pub. No. MAN016152*.

Thermo Scientific. (2018). NHS and Sulfo-NHS. *Pub. Part No. 2160650, MAN0011309 Rev. B.0*.

Thermo Scientific: Fab Fragments. (2017). Pierce Fab Preparation Kit. *Pub. Part No. 2162088.8, MAN0011651 Rev. B.0*.

Thermo Scientific: EDC. (2017). EDC. *Pub. Part No. 2160475.7, MAN0017125 Rev. A.0*.

Tort, F., del Toro, M., Lissens, W., Montoya, J., Fernandez-Burriel, M., Font, A., . . . Ribes, A. (2011). Screening for nuclear genetic defects in the ATP synthase-associated genes TMEM70, ATP12 and ATP5E in patients with 3-methylglutaconic aciduria. *Clinical Genetics*, 80: 297-300.

Tort, F., Ugarteburu, O., Texido, L., Gea-Sorli, S., Gacrcia-Villoria, J., Ferrer-Cortex, X., . . . Ribes, A. (2019). Mutations in TIMM50 cause severe mitochondrial dysfunction by targeting key aspects of mitochondrial physiology. *Hum Mutat*, 40: 1700-1712.

U.S. Depart. of Health and Human Services, FDA, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). (May 2018). *Bioanalytical Method Validation: Guidance for Industry.* Silver Spring, MD: Biopharmaceutics.

Vashist, S. K., Luppa, P. B., Yeo, L. Y., Ozcan, A., & Luong, J. H. (2015). Emerging Technologies for Next-Generation Point-of-Care Testing. *Trends in Biotechnology*, 33 (11): 692-705.

Winston, S. E., Fuller, S. A., Evelegh, M. J., & Hurrell, J. G. (2001). Conjugation of Enzymes to Antibodies. *Current Protocols in Molecular Biology, Section 1: Unit 11.1*(Supplement 10).

Wortmann, S. B., Duran, M., Anikster, Y., Barth, P. G., Sperl, W., Zschocke, J., . . . Wevers, R. A. (2013). Inborn errors of metabolism with 3-methylglutaconic aciduria as discriminative feature: proper classification and nomenclature. *Journal of Inherited Metabolic Disease*, 36: 923-928.

Wortmann, S. B., Kluijtmans, L. A., Engelke, U. F., Wevers, R. A., & Morava, E. (2012). The 3-methylglutaconic acidurias: what's new? *Journal of Inherited Metabolic Disease*, 35: 13-22.

Wortmann, S. B., Kluijtmans, L. A., Sequeira, S., Wevers, R. A., & Morava, E. (2014). Leucine Loading Test is Only Discriminative for 3-Methylglutaconic Aciduria Due to AUH Defect. *JIMD Reports*, 16: 1-6.

Wortmann, S., Rodenburg, R., Huizing, M., Loupatty, F., de Koning, T., Kluijtmans, L., . . . Morava, E. (2006). Association of 3-methylglutaconic aciduria with sensori-neural deafness, encephalopathy, and Leigh-like syndrome (MEGDEL association) in four patients with a disorder of the oxidative phosphorylation. *Moleculr Genetics and Metabolism*, 88: 47-52.

Wortmann, S., Vaz, F., Gardeitchik, T., Vissers, L., Renkema, G., Schuurs-Hoeijmakers, J., . . . Brouwer, A. (2012). Mutations in the phospholipid remodeling gene SERAC1 impair mitochondrial function and intracellular cholesterol trafficking and cause dystonia and deafness. *Nat Genet*, 44: 797-802.

Wortmann, S., Zietkiewics, S., Kousi, M., Szklarczyk, R., Haack, T., Gersting, S., . . . Wevers, R. (2015). CLPB mutations cause 3-methylglutaconic aciduria progressive brain atrophy, intellectual disability, congenital neutropenia, cataracts, movement disorder. *Am J Hum Genet*, 96: 245-257.

Ylikotila, J., Hellstrom, J. L., Eriksson, S., Vehniainen, M., Valimaa, L., Takalo, H., . . . Pettersson, K. (2006). Utilization of Recombinant Fab fragments in a cTnI immunoassay conducted in spot wells. *Clinical Biochemistry*, 39:843-850.