GLUCOSE SUPPLEMENTATION AND MEASURES OF OXIDATIVE STRESS IN PATIENTS WITH GLYCOGEN PHOSPHORYLASE DEFICIENCY (MCARDLE'S DISEASE).

THE EFFECT OF ORAL GLUCOSE SUPPLEMENTATION PRIOR TO NON-ISCHEMIC FOREARM EXERCISE TESTING ON EXERCISE PERFORMANCE AND MEASURES OF OXIDATIVE STRESS IN PATIENTS WITH GLYCOGEN PHOSPHORYLASE DEFICIENCY (MCARDLE'S DISEASE).

By

Nicholas J. Mocellin B.Sc. Kin.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Masters of Science in Medical Science

McMaster University

MASTER OF SCIENCE (2011) McMaster University
(Medical Sciences) Hamilton, Ontario

TITLE: **The Effect of Oral Glucose Supplementation Prior to Non-Ischemic Forearm Exercise testing on Exercise Performance and Measures of Oxidative Stress in Patients with Glycogen Phosphorylase Deficiency (McArdle's Disease).**

AUTHOR: Nicholas Joseph Mocellin, B.Sc. Kin. (McMaster University)

SUPERVISOR: Dr. Mark A. Tarnopolsky

NUMBER OF PAGES: xi, 90

ABSTRACT

Objective: We evaluated the potential effect of oral glucose supplementation on: (1) exercise performance and tolerance, (2) the concentrations of plasma uric acid and ammonia (NH_3) and (3) blood plasma markers of oxidative stress in patients with McArdle's disease (MCD) after non-ischemic forearm exercise testing (non-ischemic forearm exercise test). *Methods:* Blood samples and exercise performance measures were performed on from 16 patients with MCD and 17 control subjects (CON) matched for age, sex and physical activity status. Subjects performed 2 exercise bouts 30 minutes apart and received oral glucose or placebo supplementation between tests. Blood samples were analyzed for concentrations of 8-isoprostanes (8-ISO), malondialdehyde (MDA) and total anti-oxidant capacity (TEAC). Exercise performance was assessed using a handgrip dynamometer to measure force of contraction over time. Exercise tolerance was assessed based on subject's self-reported perception of pain and perception of exertion during exercise. *Results:* MCD was associated with greater fatigue, perceived pain, perceived exertion, and higher uric acid during non-ischemic forearm exercise test ($P < 0.05$), and higher concentrations of plasma NH_3 post exercise ($P < 0.05$). Glucose did not influence plasma uric acid or NH_3 and had no effect on exercise measures in MCD patients. Baseline plasma markers of oxidative stress were not different between MCD patients and CON; however, MCD patients who ingested glucose between non-ischemic forearm exercise tests had lower plasma 8-ISO

concentrations ($P < 0.05$). CON who ingested glucose between non-ischemic forearm exercise tests had lower plasma 8-ISO concentrations ($P < 0.05$) at +1 min post exercise compared to initial non-ischemic forearm exercise test. The TEAC of control subjects was lower following non-ischemic forearm exercise test ($P < 0.05$), with no change in MCD patients. *Conclusion:* Glucose may have a protective effect on oxidative stress following exercise that may be due to attenuated flux through xanthine oxidase.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank many of the individuals who were inspirational in assisting me with this achievement. Firstly, I would like to thank my girlfriend Diana. Without your positivity, support and motivation this journey would have been even more difficult. Dr. Mark Tarnopolsky, your patience, guidance and intelligence is world class. Thank you for opening up your lab and making it a positive environment for academic achievement and growth. My other committee members, Dr. Martin Gibala and Sandy Raha, thank you for your guidance and patience over the last few years. Andrew and Lidia Charabin, thank you for the support and flexibility to allow me to achieve this. Finally my family, Denise, Ric, and Danica, you have been pillars of support and have always believed in me. Thank you to all.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF APPENDICIES

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Myophosphorylase deficiency also known as McArdles Disease (MCD) or glycogen storage disease type V is a genetic disorder affecting the muscle specific form of the enzyme glycogen phosphorylase (GP). The deficiency is due to mutations of the *PYGM* gene that encodes for this specific enzyme [1]. GP is responsible for removing the 1,4 glycosyl residues of glycogen within the muscle and the subsequent release of glucose which can then enter the glycolytic pathway [2]. Other organs such as liver and brain have their own specific PYGM isoforms both of which are unaffected by MCD [3].

1.2 Clinical Symptoms and Presentation of McArdle's disease

While the onset of MCD is usually noted in childhood, a formal diagnosis may not occur until after adolescence when symptoms become severe enough to impact an individual's quality of life [3]. A lifetime of exercise intolerance is a typical feature of MCD with common symptoms including: muscle fatigue, stiffness, cramping, pigmenturia (myoglobin) and weakness [4]. A phenomenon known as the "second wind" is reported in the majority of patients where patients endure through the initial phases of exercise and reach a steady state where the perceived exertion and heart rate starts to decrease [5] [6]. This phenomenon has been attributed to the fact MCD patients begin to rely on glycolysis for fuel

via the availability of blood-borne glucose [7]. A high plasma creatine kinase (CK) activity level is common in MCD patients and indicates skeletal muscle damage and a loss of membrane integrity [8] [9]. More strenuous bouts of activity may lead to contractures in which the muscle undergoes a metabolic cramp characterized by no electrical activity. The metabolic crisis associated with the contracture can lead to rhabdomyolysis and myoglobinuria, particularly after higher intensity anaerobic exercise. Myoglobinuria occurs when the intracellular muscle enzymes leak out of the damaged sarcolemma enter the bloodstream and are excreted into the urine and potentially lead to renal damage [10] [11] [12]. Patients often report of tea or “Coca-Cola” coloured urine (pigmenturia) during these episodes.

The process of aging brings a decline in muscle mass and strength [13]; however, the fixed weakness seen with MCD and with aging may also be the result of a lifetime of muscle damage and repair as indicated by the persistently elevated CK levels [14, 15].

1.3 Diagnosis of McArdle’s Disease

There are multiple diagnostic procedures used in the screening process for MCD. As discussed, chronically high plasma CK levels are common among patients. The muscle biopsy is a useful technique to test for suspected MCD. It allows for laboratories to perform a histochemical reaction for PYGM activity and determine the presence or absence of PYGM in the sectioned muscle [1]. An

increase in non-membrane bound glycogen can be seen using the Periodic acid - Shiff stain.

A common diagnostic test for ruling in or ruling out MCD is the forearm ischemic exercise test (FIT). While the FIT does not specifically test for MCD, it can be used to determine if there is a block in glycogenolysis and aid in assessing a patient with suspected MCD [3]. The FIT assesses an individual's response to anaerobic exercise by restricting blood flow to the forearm while completing a series of repetitive hand-grip exercises [16]. In healthy individuals, venous blood collected from the exercising arm shows a significant increase in lactate while plasma ammonia is moderately elevated. However, findings consisted with a diagnosis of MCD show a lack of venous lactate production and an exaggerated response in ammonia, due to impaired glycogenolysis and enhanced flux through myoadenylate deaminase, respectively [17]. The FIT proves to be a useful tool and aids clinicians; however, patients with MCD often report muscle pain and cramps during testing. In an attempt to create a more tolerable test for patients, Kazemi-Esfarjani et al (2002) compared the traditional FIT testing to a non-ischemic forearm exercise testing (non-ischemic forearm exercise test) protocol. The researchers found the non-ischemic forearm exercise test to be better tolerated by patients and was just as accurate as a diagnostic tool [16]. The protocol for the present study in this thesis was a based on the non-ischemic forearm exercise test described in 2002 [15].

1.4 Genetics of McArdle's disease

MCD is an autosomal recessive disorder. It is one of the most common genetic myopathies with an estimated prevalence of approximately 1 in 100 000 [8]. Diagnosis can be confirmed by sequencing the *PYGM* gene (chromosome 11) that codes for the PYGM enzyme of the muscle. The *PYGM* gene contains 20 exons and 19 introns. Though multiple mutations have been reported the most common continues to be in patients who are homozygous for the R49X mutation [1, 18]. While this mutation continues to be the most common, as of August 2011 an estimated 133 mutations in all 20 exons of the *PYGM* gene have been reported [19]. Most recently, six additional novel mutations have been reported in the *PYGM* gene with three missense mutations, and one of each nonsense, frame-shift and an amino acid deletion [19]. Previous studies are in agreement with their conclusions that there is no genotype – phenotype correlation [3].

1.5 Treatments and Therapy of McArdle's Disease

In general, individuals with MCD present with symptoms during exercise that is primarily anaerobic in nature, isometric, or performed and at high intensities [3] [5]. Therefore, when developing a suitable nutritional intervention or exercise prescription, it is clear that these strategies need to be targeted toward these specific areas. It is well established [20] [21] that MCD patients experience the majority of their acute symptoms in the transition period between the creatine – phosphocreatine system and oxidative phosphorylation. With this in mind, when

applying nutritional interventions or a supplementation strategy a critical concept may be to take advantage of and promote a greater flux through the metabolic pathways that remain intact or take advantage of compensatory adaptations. In doing so, the dietary manipulation may decrease the transition time across the gap as a result of absent anaerobic glycolysis (Figure 1) and diminish some of the symptoms. In addition, when deciding on a suitable exercise prescription for MCD patients, it is fundamental that the design permits a gradual progression from anaerobic to aerobic metabolism to reduce the symptoms during this transition [22, 23].

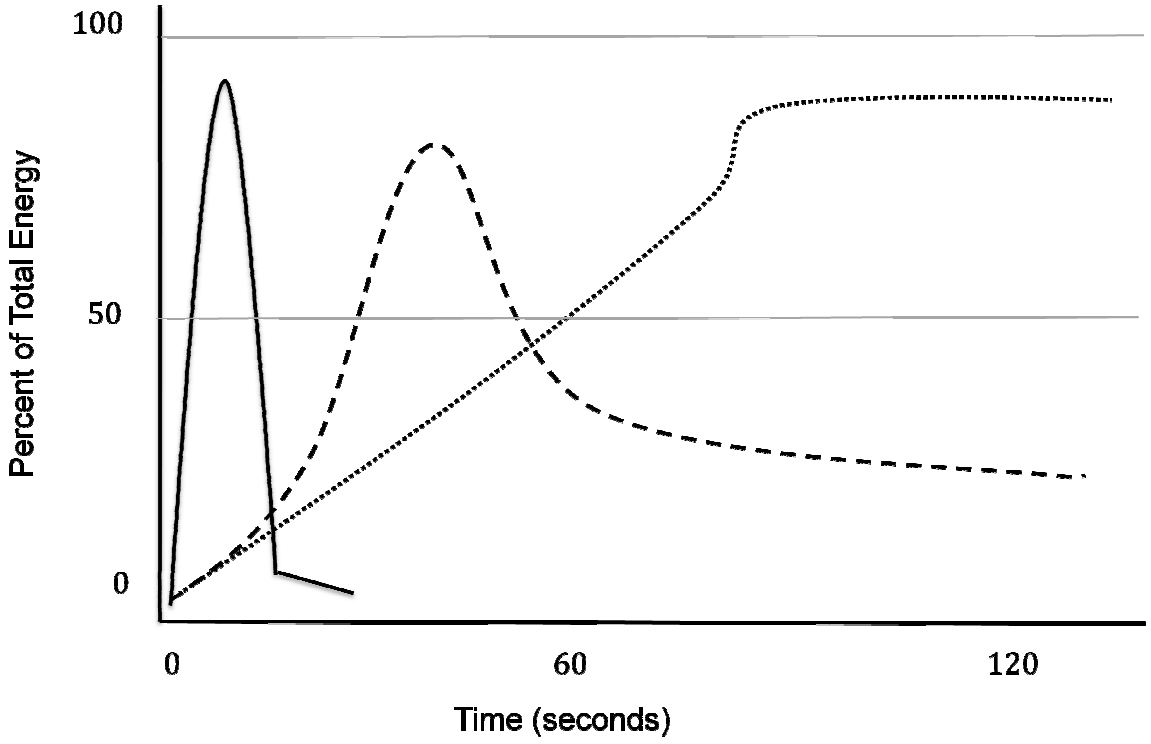

Figure 1: Contributions from different energy sources during exercise (adapted from [24])
_____ Creatine-Phosphocreatine system
---------- Glycolytic system (Anaerobic Glycolysis absent in MCD)
……. Aerobic metabolism

1.6 McArdle's Disease and Exercise

Individuals with MCD may adjust their lifestyle in an attempt to avoid participating in activities that require high intensity and short bursts, as most of their symptoms are generated in these instances [11] . It is critical for patients to select the appropriate exercise type and intensity. MCD patients experience a process called the second wind phenomenon [25, 26]. The process involves initially tolerating exercise, followed by a period of fatigue and cramping and finally exercise is tolerated again. This secondary stage of exercise tolerance is only achievable if the intensity is lowered or a period of rest is taken [27] [20]. Therefore, when prescribing exercise for MCD patients, it is suggested that cautious and progressive aerobic conditioning be undertaken [22]. This type of training has shown to improve functional capacity in MCD patients and if incorporated into a healthy lifestyle has reduced muscle pain and cramping [28].

1.7 Aerobic Metabolism and McArdle's Disease

In an attempt to compensate for or bypass the metabolic defect in MCD, specific nutritional interventions or supplementations have been tested. From a longer-term nutritional perspective, diets high in relative amounts of protein and low in carbohydrates have been suggested but have not resulted in success [29] [23]. The intention of these strategies is to increase protein and lipid oxidation to compensate for the absent glycogenolysis.

In addition to the habitual dietary interventions, short-term supplementation strategies have also been suggested. Specifically two strategies, the consumption of glucose or sucrose prior to exercise and creatine monohydrate supplementation have been examined.

1.7.1 Carbohydrate Metabolism in McArdle's Disease

Glucose metabolism is limited by the activity of certain enzymes along the glycolytic pathway. Specifically, PFK is a key enzyme in the control of glycolysis. Without this enzyme the phosphorylation of fructose-6-P would not occur and would inhibit glucose from entering glycolysis. In addition, pyruvate kinase and hexokinase appear to be other regulatory enzymes based on the large ΔG. In an attempt to take advantage of the increases in GLUT4 protein content and PFK protein content and enzyme activity in skeletal muscle [30] of MCD patients, acute carbohydrate ingestion approximately fifteen to twenty minutes prior to the onset of exercise has been suggested [31]. It has been demonstrated by Anderson et al [32] and others [33] showed this technique may ease the transition from anaerobic into aerobic metabolism and by bypassing the metabolic block. Specifically, Anderson et al have demonstrated that the ingestion of oral sucrose, prior to exercise, diminished some symptoms and may improve exercise capacity by increasing VO_2 max and lowering heart rates [32].

It is commonly recommended that MCD patients follow a diet high in carbohydrate in order to keep stores of liver glycogen high and to maintain

circulating blood glucose at optimal levels [2]. Determining the proper dosage of carbohydrate and the timing of that dosage appears to be an important consideration in managing this disease. Administering too much carbohydrate may lead to unwanted weight gain and too little may not have a beneficial affect on exercise tolerance [34].

GLUT4, GLUT1,GLUT5 and GLUT12 are all present in skeletal muscle. GLUT4 and it's translocation to the membrane are sensitive to muscle contraction stimulus as well as insulin concentration. GLUT1 and GLUT5 are present in smaller quantities and are located in the plasma membrane. GLUT1 facilitates the transport of glucose across the plasma membrane while GLUT5 facilitates the transport of fructose across the plasma membrane [35]. GLUT12 has been suggested to be part of a second insulin responsive glucose transport system [36]. Glucose uptake can be stimulated by both contractions in the muscle as well as insulin. It has been demonstrate in the muscles of rats that muscle contraction promotes the translocation of GLUT4 to the plasma membrane and subsequently the uptake of glucose even in the absence of insulin [37]. It has also been suggested that 2 pools of glucose transporters are present in the muscle. 1 group being sensitive to insulin the other activated by exercise and contractions [37].Potentially the localization of GLUT4 to the plasma membrane in MCD patients is stimulated to a greater extent than healthy individuals as MCD patients are known to have intense contractions of the muscle on a regular basis. Vissing and Haller (2003) examined the effects of glucose delivery in MCD patients

participating in bicycle exercise [31]. The study involved sucrose-loading 30 - 40 minutes prior to exercise; all subjects completed 2 bicycle exercise trials, one after consuming 75 grams of sucrose before exercise, the other after consuming a sweetened placebo. This crossover design allowed for direct comparison of heart rate and perceived exertion on the 15 minute cycling test. With placebo, the mean peak heart rate and exertion occurred in the 7^{th} minute of exercise, followed by a drop in both measures as subjects settled into a natural second wind. However, after sucrose ingestion heart rate was significantly lowered during the 7^{th} minute of exercise and a second-wind was effectively abolished. These results demonstrated that exercise tolerance could be improved by ensuring sufficient supply of blood-borne fuels at a point earlier then could be supplied by normal physiology.

Despite the promising nature of the findings by Vissing and Haller (2003), being prepared to ingest such a large amount of sucrose 30 - 40 minutes before anticipated exercise may not be realistic or convenient for MCD patients. Patients may begin to experience weight gain over time from the amount of calories consumed in 75 grams of sucrose. To explore this further, a similar study [34] was conducted that reduced the amount of sucrose by ~ 50 % (37 grams), and the time of the dose prior to exercise to only 5 minutes. Consistent with the prior study, exertion and heart rate peaked at 7 minutes of exercise followed by a second-wind. This second-wind was again abolished by sucrose ingestion, in both 40 minute - 75 gram and 5 minute - 37 gram trials. However, at the same

workload, both exertion and heart rate were reduced to a greater extent when sucrose was ingested 5 minutes prior to exercise compared to 40 minutes before. This observation correlated well with measures of blood glucose, which decreased throughout the 15 minute exercise test when sucrose was ingested 40 minutes prior to exercise, but consistently increased when ingested 5 minutes prior exercise. The results were successful in modifying glucose supplementation recommendations for MCD patients, to make them both more practical and at a reduced caloric intake.

Overcoming the blockage in muscle glycogenolysis through increasing blood-borne glucose has been the most successful approach to overcome exercise intolerance in McArdle's patients [38, 39].

1.7.2 *Free fatty Acid Metabolism in McArdle's Disease*

Free fatty acid (FFA) oxidation contributes a considerable amount to aerobic metabolism and is used as a dominant fuel source second during lower intensity activity, particularly in the fasted state [40]. The time period to deliver this blood- borne fuel to muscle is delayed to some extent due to the time for lipolysis, blood transport to and uptake by the muscle. This has particularly important implications for patients with MCD since it is during this delay that the majority of muscle symptoms and damage may occur. In contrast, recent research suggested that increasing FFA in the blood attenuated blood borne glucose delivery and metabolism and resulted in a net negative effect on exercise

capacity. Although MCD patients rely more on FFA as an energy source during low-intensity exercise than healthy controls [41], when blood levels were increased three-fold from lipid infusion, there was no elimination of a second-wind effect and heart rate peaked at higher levels in patients during exercise compared to placebo. As hypothesized, glucose infusions significantly lowered peak heart rate and essentially abolished the second-wind. Similar to branch chain amino acids (BCAAs), elevating FFAs may attenuate delivery of blood borne glucose to skeletal muscle and could potentially attenuate the energy that could be derived from glucose in these patients. In addition, fat metabolism may already be at a peak level in McArdle's patients and further increases in delivery may not lead to enhanced uptake and oxidation. Due to a consistently low flux through glycolysis, it has been identified that TCA cycle intermediates are lower in McArdle's patients as compared to healthy persons [41]. Therefore, despite the large spike in plasma FFA levels resulting from intralipid infusion, metabolism of this fuel may still not be able to keep up with energy demands of the working muscle [42].

1.7.3 *Protein Metabolism and McArdle's Disease*

It is well documented that BCAA oxidation represents a small proportion of total energy supply to working muscle and are used only under aerobic conditions [43], challenging its usefulness in MCD patients [44] [45]. In addition, BCAA

require the vitamin B6 for metabolism. The majority of B6 is bound to GP therefore the and overall body stores of this vitamin are depleted in MCD [3].

Of the 20 amino acids, 9 are considered essential meaning the body cannot synthesize them and therefore must be consumed in the diet. When consumed as part of the diet, 8 of the 20 amino acids are oxidized by skeletal muscle for a source of energy. The BCAA's valine, leucine and isoleucine are unlike other amino acids as they are primarily metabolized in skeletal muscle [46]. Following removal of nitrogen groups through deamination or transanimation, the amino acid skeletons are converted into intermediates of the citric acid cycle. The intermediate is dependant upon the original amino acid. Branched-chain oxo-acid dehydrogenase (BCOAD) is a rate-limiting enzyme for BCAA oxidation. The activity of BCOAD increases from approximately 5% (at rest) to approximately 25 % (during exercise). The oxidation of the BCAA leucine has been the most widely studied amino acid in endurance exercise studies [47]. Ultimately, BCAA's are metabolized by skeletal muscle, liver and a smaller extent the kidneys.

Increasing blood levels of BCAAs prior to exercise could provide sufficient energy to alleviate exercise intolerance in MCD patients. MacLean et al (1998) had patients ingest 77 mg of BCAAs per kilogram body mass 30 minutes prior to cycling 20 minutes at the maximal capacity that could be tolerated without experiencing pain. Plasma levels of BCAAs tripled after ingestion; however, performance was equal or worse in trials after consumption of BCAAs compared

to trials with placebo. BCAA ingestion did not have an effect on second-wind occurrence. These results may be explained by the finding that free fatty acids and glucose levels in plasma decreased in response to BCAA administration, perhaps due to inhibition of glycogenolysis in the liver and the resultant plasma glucose [48].

1.8 Anaerobic Metabolism and McArdle's Disease

In MCD, the absent energy production from anaerobic glycogenolysis leads to a greater reliance on other anaerobic pathways [38]. This greater reliance is most apparent upon the initiation of exercise (the rest-exercise transition) and has been reflected in the term "myogenic hyperuricemia" indicating the greater flux through adenylate kinase > myoadenylate deaminase (see below) [26, 49].

1.8.1 *The Creatine-Phosphocreatine Energy Buffer System*

The creatine – phosphocreatine system is one of the compensatory anaerobic pathways that are up regulated. The creatine kinase reaction (Figure 2) that catalyzes the transfer of a phosphate from PCr to ADP is a reaction close to equilibrium. Creatine monohydrate supplementation has been suggested as a therapeutic strategy [50], to promote flux in the creatine kinase pathway in the direction to generate ATP [51]. One trial found that a low dose (0.06 g/kg/day) of creatine monohydrate was of some benefit [52]; however, with a higher dose (0.15 g/kg/day) of creatine monohydrate, there was an impairment in exercise

capacity in MCD patients [53], perhaps due to inhibition of the PFK reaction by free creatine [54]. Another issue that is likely limiting the usefulness of creatine supplementation strategies is the fact that patients with McArdle's disease do not generate a proton during exercise and this would limit the forward flux of the CK reaction (see Figure 2). From a daily symptom/functional perspective there were no significant differences between creatine supplementation and placebo on the frequency and severity of muscle pain and cramping episodes in the low dose study but EMG measures determined a greater ability to sustain muscle contraction. In the high dose study, EMG did not identify positive effects and in addition there were increases in the severity and frequency of muscle pain during normal daily activities with creatine usage [55].

Sahlin et al (1990) have demonstrated that there is an abnormally steep decline in PCr in MCD patients with fatiguing exercise. This reflects a higher flux to compensate for lower ATP generation by glycogenolysis. The metabolism of Cr/PCr obviously cannot fully compensate for the loss of this major anaerobic ATP generating system [56]. This impaired capacity would promote the transient increases in ADP and impairment in ATP generation [14]. Elevated levels of free ADP and P_i may decrease the rate of muscle relaxation by slowing the rate of Ca 2^+ uptake into the sarcoplasmic reticulum and attenuate muscle force development [20]. These are important concepts because they could help explain the muscle contractures that are apparent in many clinical cases of MCD.

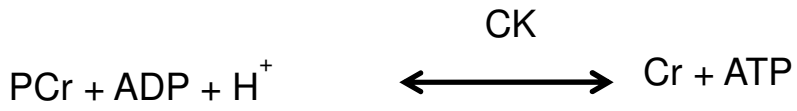

Figure 2: Creatine phosphocreatine metabolism
Adenosine tri-phosphate (ATP), Adenosine di-phosphate (ADP), Phosphocreatine (PCr), Creatine (Cr), Creatine Kinase (CK) and proton (H^+).

1.8.2 *The Adenylate Kinase and AMP Deaminase Reactions*

When the production of ATP cannot match its consumption in working muscle, which is accentuated in MCD patients due to limited glycolysis, two ADP molecules can combine to form 1 ATP and 1 AMP molecule via the adenylate kinase (AK) reaction in an attempt to keep up with ATP demand. To keep the reaction moving in the forward direction, the AMP is removed by the enzyme myoadenylate deaminase (AMPD1) resulting in the production of inosine monophosphate (IMP) and a molecule of ammonia. The IMP is converted to inosine > hypoxanthine > xanthine >uric acid (Figure 3). The xanthine oxidase enzyme is responsible for the final two steps in this pathway and generates the free radical superoxide O_2^- [57].

The first examination of this pathway in MCD was a comparison of blood levels of several intermediates and products in two MCD patients compared with six controls [58]. After a hand-grip exercise test involving maximal hand contractions every second for 2 minutes, the production of ammonia and inosine

was at least 5 times greater in MCD subjects, and hypoxanthine levels were over 3 fold higher in MCD subjects as compared to controls. Of interest, no changes in blood uric acid were detected in response to exercise. In a similar study [59], involving only 1 subject with MCD disease participating in a cycling test, ammonia, inosine and hypoxanthine were all elevated in plasma immediately following exercise. Uric acid; however, reached peak elevation 1 hour after exercise. The delayed increase in uric acid, presumably due its lower position in the purine catabolism pathway and/or slowed diffusion from muscle to blood.

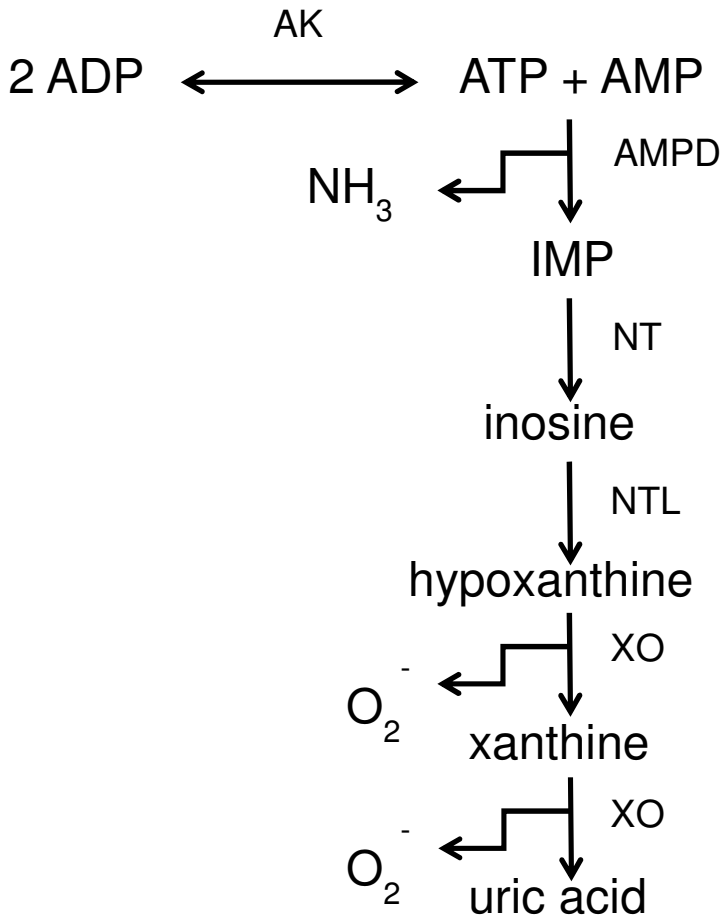

Figure 3: The uric acid pathway
Adenosine tri-phosphate (ATP), Adenosine di-phosphate (ADP), Adenosine mono-phosphate (AMP), Adenylate kinase (AK), AMP- deaminase (AMPD) Inosine mono-phosphate, Ammonia (NH_3), Nucleotidase (NT), nucleotide lyase (NTL), Xanthine oxidase (XO), and Superoxide (O_2^-).

1.8.3 *Anaerobic Glycogenolysis*

The predominant mechanism for anaerobic ATP regeneration in healthy people is through anaerobic glycogenolysis. Anaerobic glycogenolysis is inhibited in patients with MCD. This is a series of ten reactions that generate 2 ATP per molecule of glucose derived from glycogen. Under physical activity, the creatine –

phosphocreatine energy buffer is quickly depleted and it is this system along with AK that are responsible for the generation of ATP during the time prior to blood-borne fuels becoming available in patients with MCD [2].

1.9 Reactive Oxygen Species

Reactive oxygen species are highly unstable molecules with an unpaired electron that attracts electrons from other molecules. This process may cause a reaction in which each unstable molecule influences an adjacent molecule in a feed-forward mechanism leading to a chain reaction resulting in damage to many molecules, including lipid, protein DNA and RNA. The free radical theory of aging suggests that an accumulation of damage from these particles over time may lead to cell damage, disease and death [60]. In fact, higher levels of reactive oxygen species have been implicated with a variety of diseases and disorders affecting many different parts of the body [61, 62]. Reactive oxygen species are also generated to a greater extent during exercise [63, 64]. However, it has been demonstrated by Parise and others that progressive exercise training programs actually promote increases in certain anti-oxidant enzymes [65-67]. Anti-oxidants gain electrons from free radical by becoming reduced but remain stable [68]. Anti-oxidants are differentiated into enzymatic and non-enzymatic anti-oxidants. Three groups of enzymatic anti-oxidants that play a significant role in protecting against oxidative stress are superoxide dismutases (SOD), catalase and glutathione peroxidase. The major non-enzymatic anti-oxidants are vitamin E, vitamin C and glutathione (part of the glutathione peroxidase system). Anti- oxidants may play

an important role in the treatment of MCD due to the fact patients may experience elevated reactive oxygen species production from an increased flux through xanthine oxidase**.** In addition, if muscle activity continues in an environment of low ATP generation, it may promote the progression of the disease by increasing susceptibility to oxidative stress [69] (Figure 4). A drastic decrease in ATP concentration during anaerobic type exercise is observed. ATP is needed for the release of the myosin head from the actin filament after muscle contraction. If the muscle activity continues, contractures may occur and damage the sarcolemma. Subsequently, the phospholipid bilayer is exposed making it vulnerable to oxidative damage from reactive oxygen species. The sarcolemma has now become more permeable due to lipid peroxidation and may result in the inhibition of Ca2+ transport into the sarcoplasmic reticulum due to the fact that ATPases are contained within this membrane. The increased level of Ca2+ in the sarcoplasm activates proteases and promotes contractures. Under normal muscular activity the activated voltage gated calcium channel interact with calcium release channels to activate them causing the sarcoplasm to release calcium. Deficiencies in the receptors responsible for the intracellular release of calcium within the muscle, such as ryanodine receptor, will increase intracellular calcium release and promote muscular contractions [70].

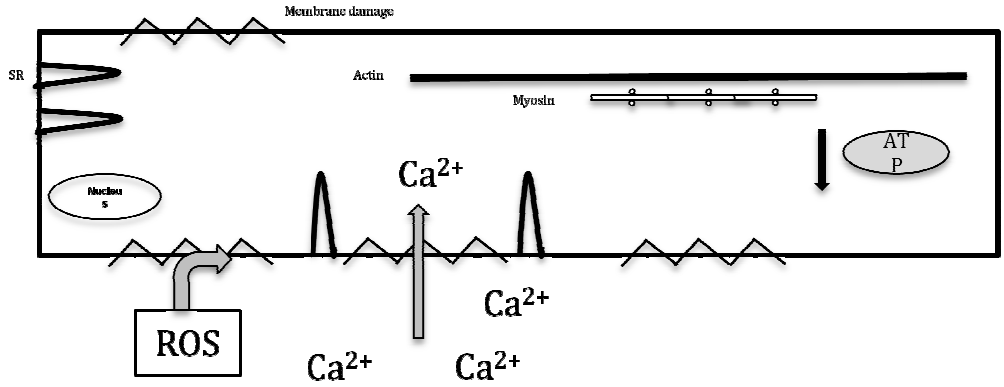

Figure 4: Muscle cell of an individual with McArdle's disease
Adenosine tri-phosphate (ATP), Sarcoplasmic Reticulum (SR), Reactive Oxygen Species (ROS) and Calcium Ion (Ca2+).

1.10 Exercise Studies and McArdle's Disease

There have been several studies that demonstrate that exercise is a very effective therapy for patients with MCD. The focus of these studies was however, aerobic exercise. Haller in 2006 trained MCD patients over the course of 14 weeks with an aerobic type regiment and was able to increase average work capacity (36%), oxygen uptake (14%) and cardiac output (15%) in subjects [32]. Similar results were also found in another study with an aerobic training component over the course of 8 months. This intervention was able to increase peak power output by 25% and VO_{2peak} by 44% [71]. This increase in performance could be beneficial to the lifestyle of individuals with MCD but they

may also derive benefit from an induction of endogenous anti- oxidant enzymes as shown in exercise training in healthier older adults [67].

1.11 Overall Conclusions

In summary, nutritional strategies and dietary manipulation have been developed to offer MCD patients the opportunity to benefit from exercise while suppressing some of the severe symptoms and negative consequences of certain types of activity. Overcoming the blockage in muscle glycogenolysis through increasing blood-borne glucose has been the most successful approach in overcoming exercise intolerance in McArdle's patients. Avoiding carbohydrate fuels altogether and increasing availability of alternative fuels have failed to demonstrate such improvements. Individuals with MCD rely heavily on blood-borne fuels since glycogenolysis within the muscle is essentially absent. Although not a response to exercise, elevated GLUT4 and PFK protein content and increased PFK activity appear to be an adaption to the altered metabolic environment found in MCD patients and may facilitate the up-take and use of glucose from the blood stream [30]. In an attempt to take advantage of the increases in GLUT4 and PFK protein content and enzyme activity, acute carbohydrate ingestion approximately fifteen to twenty minutes prior to the onset of exercise has been suggested. The heightened capacity of these specific enzymes may be a source of optimism for MCD patients as it may be exploited

when attempting nutritional interventions or supplementation strategies. Acute carbohydrate ingestion prior to exercise may also help reduce the production of reactive oxygen species/oxidative stress via decreasing the flux through xanthine oxidase that has been shown to occur in MCD (Figure 5). Reactive oxygen species may contribute to the myopathy of MCD and may eventually lead to muscle weakness in patients with MCD. Additionally, it is well known that aging results in an increase in oxidative stress markers in muscle [72, 73] and this may be even more accelerated in people with McArdle disease and contribute to their known accelerated weakness.

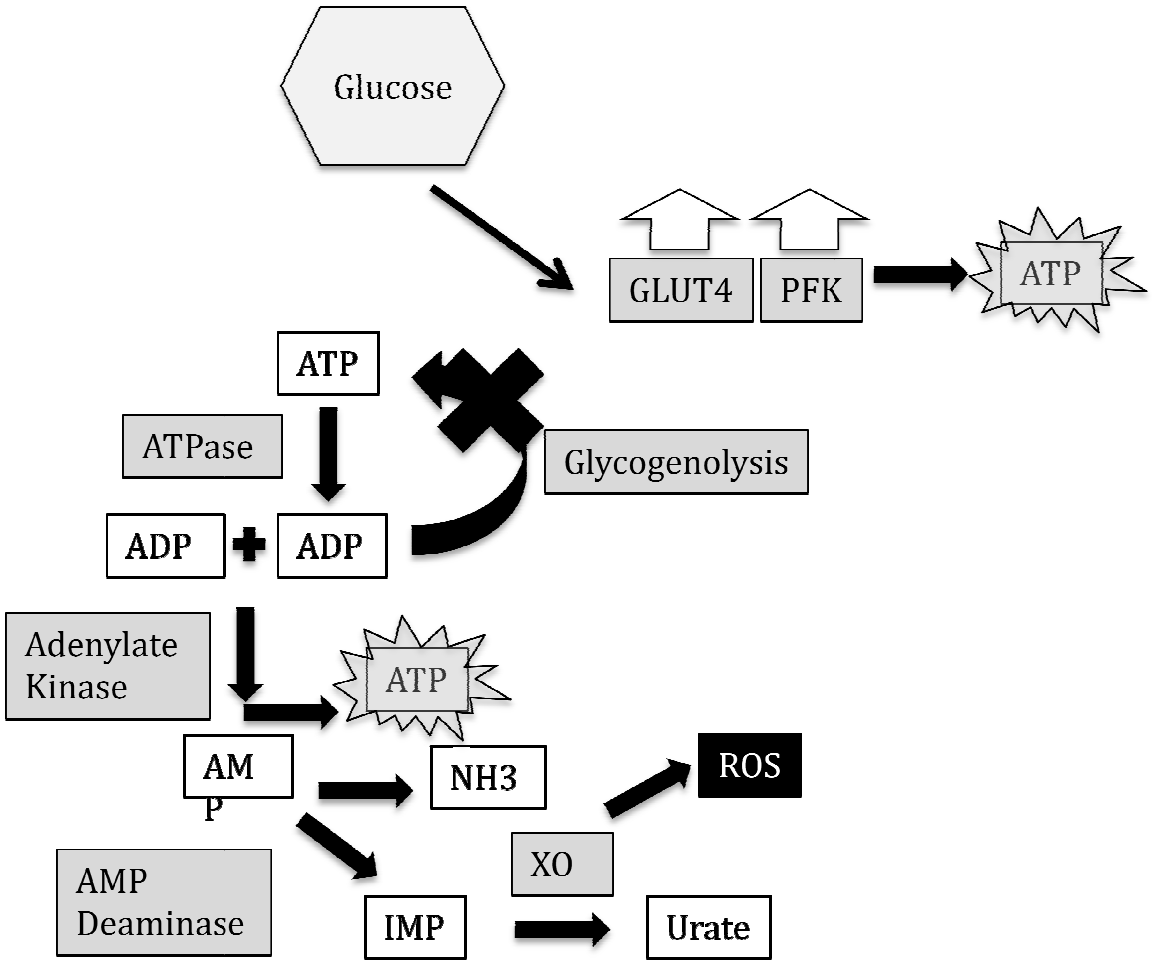

Figure 5: Study Hypothesis
Acute carbohydrate ingestion prior to exercise may also help reduce the production of reactive oxygen species/oxidative stress via decreasing the flux through the uric acid cycle and xanthine oxidase by taking advantage of elevated GLUT4 and PFK protein content and increased PFK activity that has been shown to occur in MCD.
Adenosine tri-phosphate (ATP), Adenosine di-phosphate (ADP), Inosine mono-phosphate (IMP), Ammonia (NH^3), Xanthine oxidase (XO), Phosphofructokinase (PFK) and Reactive oxygen species (ROS).

1.12 Study Objective and Hypotheses

1.12.1 *Study Objectives*

Previous studies involving MCD and oral glucose supplementation have focused primarily on aerobic exercise. In the present study, the immediate goal was to evaluate the effect of oral glucose on exercise tolerance during predominantly anaerobic exercise and to determine the potential for oral glucose to attenuate the hypothesized greater reactive oxygen species production in the MCD patients.

1.12.2 *Study Hypotheses*

Given that MCD patients experience the majority of symptoms with anaerobic type exercise/activity when there is a severe deficit of ATP and that skeletal muscle of patients appears to be primed for the uptake of glucose, it was hypothesized that:

1) MCD patients would have chronically higher plasma oxidative stress as a consequence of a sustained flux through the uric acid cycle.

2) Ingesting oral glucose prior to exercise testing would attenuate symptoms of exercise intolerance and fatigue in MCD patients.

3) Ingesting oral glucose prior to exercise would attenuate the production of ROS.

CHAPTER 2

THE EFFECT OF ORAL GLUCOSE SUPPLEMENTATION PRIOR TO NON-ISCHEMIC FOREARM EXERCISE TESTING ON EXERCISE PERFORMANCE AND MEASURES OF OXIDATIVE STRESS IN PATIENTS WITH MCARDLE'S DISEASE.

2.1 INTRODUCTION

Myophosphorylase deficiency also known as MCD or glycogen storage disease type V is an autosomal recessive condition affecting the muscle specific form of the enzyme glycogen phosphorylase (GP) [74] [75]. The deficiency is due to mutations of the *PYGM* gene that encodes for GP. GP is responsible for removing the 1,4 glycosyl residues of glycogen within the muscle and the subsequent release of glucose which can then enter the glycolytic pathway [2]. Patients with this disorder often experience symptoms such as muscle pain, stiffness and cramps and myoglobinuria caused by rhabdomyolysis where intra-muscular enzymes are released from the muscle and may result in kidney damage [3, 12]. These symptoms individually or in combination may ultimately promote inactivity in these individuals [20]. In MCD, the major deficit in energy production from impaired anaerobic glycogenolysis suggests there may be an increased reliance on alternative anaerobic pathways. The adenylate kinase reaction in which 2 molecules of ADP are converted to one molecule of ATP and AMP may be utilized by the cell in an attempt to generate necessary energy in a state of energy depletion [7]. The adenylate kinase reaction is coupled with AMP deaminase in which AMP is broken down to IMP and ammonia (NH_3). Ultimately, this pathway leads to a greater flux through xanthine oxidase that generates

superoxide O_2^-, a reactive oxygen species, and uric acid (Kaczor et al. 2012, manuscript in preparation). The enhanced flux through this compensatory anaerobic pathway is termed myogenic hyperuricemia and may contribute to gout [76]. As a result of the xanthine oxidase mediated superoxide production, MCD patients may experience elevated levels of oxidative stress at rest and with acute bouts of exercise [69]. It has been proposed that the observed increases in oxidative stress in MCD patients during exercise and at rest may be due in part to an increase flux through the uric acid cycle ending with uric acid [50, 58].

Previous research suggested that skeletal muscle in MCD is primed for glucose uptake and glycolysis as reflected by higher GLUT4 and PFK protein content and increased PFK enzyme activity in skeletal muscle [30]. Additionally, there are multiple lines of evidence that suggest glucose significantly attenuates the onset and severity of MCD associated symptoms during aerobic activity [33, 34]. Our purpose was to examine the effect glucose had on measures of oxidative stress on MCD patients undergoing forearm exercise testing.

We hypothesized that oral glucose supplementation prior to non-ischemic forearm exercise test would attenuate reactive oxygen species production in plasma and increase the exercise performance and tolerance in patients with MCD by lowering the flux through the AK > AMPD1 > xanthine oxidase pathway and enhancing flux through the glycolytic pathway, respectively.

2.2 METHODS

2.2.1 Study Design

This study was approved by the McMaster University Hamilton Health Sciences Human Research Ethics Board and conformed to the Declaration of Helsinki guidelines. All participants arrived at the clinical research centre after an overnight (12 h) fast, between the times of 0800h-1000h. Study personnel met with the participants and explained the study and the participants signed the consent form. A review of medical history, review of the patient's medication and nutritional supplements, and measurement of height and weight was performed. Participants were also issued and instructed in the completion of the Baecke Physical Activity Frequency Questionnaire (AFQ) [77]. Finally, participants completed the non-ischemic forearm exercise testing (non-ischemic forearm exercise test) (see below) and were randomized to receive a glucose/fructose or placebo drink between the exercise testing. Both researchers and subjects were blinded to which drink was being administered. All study assessments and procedures were completed in a single (1) visit which lasted ~ 2 h.

2.2.2 Study Participants

McArdle's disease patients

Sixteen patients (54.2±17.6 y; N=4 women and N=12 men) with confirmed MCD were studied. Participants with MCD were confirmed through biochemical and/or genetic diagnoses. Fasting plasma creatine kinase (CK)

levels were significantly elevated above normal reference levels (N = < 220 iU) in all patients.

Control subjects

MCD patients were sex and age-matched with seventeen control subjects (57.3± 20.0 y; N=5 women and N=12 men) who did not have MCD. Healthy controls were included if they were not clinically symptomatic for any neuromuscular or metabolic disease and were not taking any medications that would alter metabolism.

Inclusion Criteria

Male and female subjects were recruited being 18 years of age or greater. MCD patients had been formerly diagnosed (biochemically and/or genetically) and had **not** experienced muscle contractures for a period of 1 month prior to the study visit. Control subjects had **not** been previously diagnosed with a neuromuscular or metabolic disorder. Participants were living a sedentary lifestyle. Participants could **not** be taking anti-oxidant (AO) supplements or allopurinol for a period of 1 month prior to the study visit.

2.2.3 Anthropometrics

Height was measured without shoes, in centimeters to the nearest 0.5-centimeter. Body weight was measured on a mechanical scale (Detecto, Webb

City, MO.) in kilograms to the nearest 0.1-kilogram. The same scale was used for all weight measurements. Body mass index (BMI) was calculated as the participant's weight in kilograms divided by the participant's height in meters squared (kg/m^2) and was calculated to the nearest one-tenth unit. BMI calculations were made using the following formula:

$$\textbf{BMI } (kg/m^2) = \textbf{weight (kg)} / \textbf{height } (m)^2$$

Dual Energy X-ray Absorptiometry (DXA) (Lunar; GE, Madison, WI) was used to determine body composition in participants. On the morning of the participant's visit and prior to participant data acquisition, the DXA was calibrated by performing a quality control scan. The test/re-test coefficients of variation (CV) for this scanner was < 2.6% for lean mass and < 3.4% for fat mass.

2.2.4 Forearm Exercise Testing Procedure

Subjects performed 2 non-ischemic forearm exercise test (60 seconds each) approximately 25 minutes apart using opposite hands. Hand dominance was accounted for as an equal number of participants completed the testing starting with their dominant hand and non-dominant hands. During the testing, the participant was required to sit upright with the arm of interest positioned on a wedge shaped pillow on an adjustable table. The handle of the handgrip dynamometer was placed in a vertical position so that the forearm was in a mid-

position between supination and pronation and the elbow was at a flexion angle of 110 to 120 degrees. Participants squeezed the handgrip dynamometer at maximum voluntary contraction during each contraction (contraction 1 sec; rest 1 second) over a total test time of 60 seconds for a total of 30 contractions. The handgrip dynamometer with transducer was connected to a custom voltmeter. The voltmeter signal was amplified on an amplifier (National Instruments Corp., Austin, TX.). A custom computer interface was created using LabVIEW 7 Express, v.7 (National Instruments Corp., Austin, TX.). This interface was able to provide visual and audible feedback to the subjects during testing. Force of contraction was recorded every 0.01 seconds. Data from this test was subsequently exported and managed with microsoft excel. Blood was taken from an antecubital vein of the involved arm during testing (see below). Between the 2 tests (10 minutes prior to the 2nd test) subjects consumed 34 grams of glucose/fructose or a placebo within 5 min (illustration 1). Heart rate (HR) was monitored with an optical HR monitor (Welch Allyn Inc. Skaneateles Falls, NY.) and recorded during and after the non-ischemic forearm exercise test. Finally, participants were asked to rate their perceived exertion and perceived pain after the non-ischemic forearm exercise test on a Likert type numerical scale (0-10) [78] .

Illustration 1: Subject visit protocol

2.2.5 Glucose and Placebo Supplementation

An orange flavoured drink containing 34 grams of glucose/fructose was used for the supplementation (591 mL, Gatorade, PepsiCo). The placebo group received 1 pouch Crystal Light® Tangerine Grapefruit Low Calorie Drink Mix mixed in ~ 591 mL of water.

2.2.6 Blood Sampling

During and after the non-ischemic forearm exercise test, blood samples were taken from an antecubital vein of the exercised arm at baseline (BASE), post exercise (POST), 1 min after exercise (+1), 3 min after exercise (+3) and 15 min after exercise (+15). Blood was collected in 4 mL heparinised vacutainers and 4mL vacutainers containing ethylenediaminetetraacetic acid. Blood was analyzed using a radiometer (ABL 800 Flex – Radiometer, Denmark) shortly after collection for glucose, lactate, pO_2, pCO_2 and pH. Remaining blood was put

immediately on ice and centrifuged at 1750 rpm for 5 minutes. Plasma was aliquoted into 1 mL polypropylene tubes and stored at -80°C for subsequent analysis of markers of oxidative stress (see below). The CORE lab (see below) at MUMC analyzed plasma samples for CK (BASE), uric acid (all time points) and NH_3 (BASE and +1). The same sampling procedure was repeated in the opposite arm of participants for the second non-ischemic forearm exercise test.

2.2.7 CORE Lab Analyses

uric acid was determined using an enzymatic colorimetric test. NH_3 was determined using an enzymatic kinetic assay. CK was determined using an ultraviolet test (see appendix VI).

2.2.8 Plasma Oxidative Stress

2.2.8.1 *Plasma Malondialdehyde*

At the time of sampling, 200 μL of plasma was aliquoted and stored at -80°C. Subsequent analysis of plasma malondialdehyde (MDA) was determined using a Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit (Caymen Chemical Co., Ann Arbor, MI.) according to manufacturer instructions. The plates were read at a wavelength of 535 nm on a micro plate absorbance spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA.) and data was managed with Microplate Manager v.6 software (Bio-Rad Laboratories, Inc., Hercules, CA.).

2.2.8.2 Plasma 8-isoprostanes

At the time of sampling, 200 μL of plasma was aliquoted with 0.005% Butylated hydroxytoluene (BHT) to prevent further oxidation in the sample and was stored at -80°C. Subsequent analysis of total plasma 8-isoprostanes (8-ISO) was performed using an immunoassay kit (Caymen Chemical Co., Ann Arbor, MI.). Plasma samples were purified prior to performing the assay utilizing an 8-isoprostane-affinity purification kit (Caymen Chemical Co., Ann Arbor, MI.). The plates were read at a wavelength of 420 nm on a microplate absorbance spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA.) and data was managed with Microplate Manager v.6 software (Bio-Rad Laboratories, Inc., Hercules, CA.).

2.2.9 Total Antioxidant Capacity

Plasma samples collected at baseline and immediately post initial non-ischemic forearm exercise test were analyzed for total anti-oxidant capacity (TAC) using an assay based on the decolorizing of a solution of 2,2-azinobis-3-ethylbenzothiazo -6-sulfonic acid (ABTS) radical cations (ABTS·□) by antioxidant solutions [79], as previously described by our lab [80]. Briefly, after preparing stock solutions of ABTS (5.00 X 10 M) and sodium persulfate (6.89 X 10^{-3} M) (Sigma Aldrich, St. Louis, MO) in PBS (pH 7.4), 1 ml of sodium persulfate solution was added to 99 ml of ABTS solution and allowed to equilibrate in the dark for 16 h. All measurements were taken in triplicate by adding 40 μl of each

sample or standard to 160 μl ABTS·, incubating at 37°C for 30 min and measuring for absorbance at 734 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA). Serial dilutions of the vitamin E analog Trolox (Sigma Aldrich) were prepared in ethanol and used to generate a standard curve. Plasma samples were diluted 80-fold to ensure they were within the linear portion of the standard curve. All results are expressed as millimole Trolox equivalents.

2.2.10 Statistical Analyses

Data was analyzed using a two-way analysis of variance (ANOVA) with repeated measures utilizing Statistica, v.7 (Statsoft, Inc., Tulsa, OK.). Specific comparisons were selected that reflected predictions based on our original hypotheses. Between groups (McArdle vs. Controls) and within group (Glucose/Fructose vs. Placebo) differences were compared over time (BASE, POST +1, +3, +15 minutes) for blood measures of uric acid, PO2, PCO2, pH, glucose, lactate, 8-ISO and MDA. When a main effect was identified, Tukey's *HSD* post-hoc analysis was used. A 1-tailed t-test was used for between group difference in the initial testing measures of perceived exertion, perceived pain, CK, uric acid, NH_3 and markers of oxidative stress since *a priori* hypotheses were made such that these measures would be higher in MCD patients versus control subjects. For the remaining analyses, a two-tailed t-test was used. Results were considered statistically significant when $P \leq 0.05$. Results were expressed as mean ± SD.

2.3 RESULTS

2.3.1 *Group Characteristics*

The weight, FM, B.M.I. and plasma CK concentrations were higher in MCD patients compared to controls ($P \leq 0.05$) (Table 1). The activity status- measure of work activity was higher ($P \leq 0.05$) in MCD patients receiving placebo than in MCD patients receiving glucose (Table 2). There were no other significant differences between groups.

2.3.2 *Classic Presentation of McArdle's Disease*

Plasma lactate concentrations were lower after initial non-ischemic forearm exercise test in MCD patients compared with control subjects except for baseline sampling ($P \leq 0.05$) (Figure 4A). Plasma lactate concentrations in MCD patients were not different from baseline at any time point after non-ischemic forearm exercise test (Figure 6A).

Blood $_{P}CO_2$ concentrations were lower after initial non-ischemic forearm exercise test in MCD patients compared with control subjects except for baseline and +15 min sampling ($P \leq 0.05$) (Figure 6B). Blood $_{P}CO_2$ concentrations in MCD patients were not different from baseline at any time after non-ischemic forearm exercise test (Figure 6B). Blood pH values were higher after initial non-ischemic forearm exercise test in MCD patients compared with control subjects except for baseline and +15 min sampling ($P \leq 0.05$) (Figure 6C). Blood pH values in MCD

patients were not different from baseline at any time after non-ischemic forearm exercise test (Figure 6C). Plasma uric acid concentrations were higher at all time points in MCD patients compared with control subjects ($P \leq 0.05$) (Figure 7A). Plasma uric acid concentrations in MCD patients were not different from baseline at any time point after non-ischemic forearm exercise test (Figure 7A). Plasma NH_3 concentrations were higher after non-ischemic forearm exercise test in MCD patients ($P \leq 0.05$) (Figure 7B). Plasma NH_3 concentrations were higher in MCD patients compared to control subjects after non-ischemic forearm exercise test ($P \leq 0.05$) (Figure 7B).

2.3.3 *Exercise Performance and Tolerance*

The amount of force generated over the course of the initial non-ischemic forearm exercise test was lower in MCD patients compared to control subjects ($P \leq 0.05$) at 7 sec, 9 sec and for the duration between 20 sec - 60 sec (Figure 8A). Self-reported measures of perceived pain and perceived exertion were higher after non-ischemic forearm exercise test in MCD patients compared to control subjects ($P \leq 0.05$) (Figure 8B).

2.3.4 *Baseline Oxidative Stress*

Plasma 8-ISO concentrations were not different between MCD patients and controls subjects before or after the initial non-ischemic forearm exercise test (Figure 9A). Changes in plasma MDA concentrations were not different between

MCD patients and controls subjects before or after the initial non-ischemic forearm exercise test (Figure 9B).

2.3.5 *Glucose supplementation on Features of McArdle's Disease*

Plasma glucose concentrations were higher after the 2nd non-ischemic forearm exercise test compared to the 1st non-ischemic forearm exercise test in MCD patients receiving oral glucose ($P \leq 0.05$) (Figure 10A). Plasma lactate concentrations were higher after the 2nd non-ischemic forearm exercise test compared to the 1st non-ischemic forearm exercise test in MCD patients receiving oral glucose except for ($P \leq 0.05$) indicating that the ingested glucose did flux through glycolysis (Figure 10B). Plasma uric acid levels were not different in the 2nd non-ischemic forearm exercise test compared to the 1st non-ischemic forearm exercise test in MCD patients receiving oral glucose (Figure 11A). Plasma NH_3 concentrations in the 1st and 2nd non-ischemic forearm exercise test were not different in MCD patients receiving oral glucose (Figure 11B)

The amount of force generated over the course of the 1st and 2nd non-ischemic forearm exercise test was not different in MCD patients receiving oral glucose (Figure 12A) and no difference in MCD patients receiving the placebo. Self-reported measures of perceived pain and perceived exertion were not different between the 1st and 2nd non-ischemic forearm exercise test in MCD patients receiving oral glucose (Figure 12B).

2.3.6 *Glucose supplementation on Oxidative Stress*

Baseline plasma 8-ISO concentrations decreased ($P \leq 0.05$) in MCD patients receiving glucose between the 1st and 2nd non-ischemic forearm exercise test. Plasma 8-ISO concentrations decreased ($P \leq 0.05$) at +1 minute post exercise in control subjects receiving glucose between the 1st and 2nd non-ischemic forearm exercise test (Figure 13A).

Changes in plasma MDA concentrations were not different between MCD patients and controls subjects receiving oral glucose during the 1st and 2nd non-ischemic forearm exercise test (Figure 13B).

2.3.7 *Total Anti-oxidant Capacity*

Plasma TEAC decreased ($P \leq 0.05$) after initial non-ischemic forearm exercise test in control subjects. Plasma TEAC was unchanged after the initial non-ischemic forearm exercise test in MCD patients (Figure14).

	Between Group Characteristics		
	MCARDLE (N=16)	CONTROLS (N=17)	Effect
Age (y)	54.2 ± 17.6	57.3 ± 20.0	NS
Sex (M/F)	12/4	12/5	NS
Height (cm)	169.5 ± 8.6	172.6 ± 8.7	NS
Weight (kg)	91.4 ± 21.8	78.1 ± 12.0	P<0.05
FFM (kg)	58.3 ± 13.5	53.1 ± 11.7	NS
FM (kg)	32.5 ± 11.9	24.1 ± 5.5	P<0.02
B.M.I.	31.5 ± 5.2	26.0 ± 2.5	P<0.001
C.K. (μmol/L)	2295.5 ± 3292.7 (192-12128)	117.6 ± 61.5 (38-246)	P<0.05
Activity Status			
1. Work	2.3 ± 0.6	2.4 ± 0.6	NS
2. Sport	2.3 ± 0.8	2.2 ± 0.7	NS
3. Leisure	2.9 ± 0.6	3.0 ± 0.7	NS

Table 1. Between Group Subject Characteristics, Body Composition, and Activity Status. *Values are means ± standard deviation. NS = not significant; CK = creatine kinase; FFM = fat free mass; FM = fat mass; BMI = body mass index.*

	Within Group Characteristics					
	McArdles Patients			Control Subjects		
	GLUCOSE (N=8)	PLACEBO (N=8)	Effect	GLUCOSE (N=9)	PLACEBO (N=8)	Effect
Age (y)	55.2 ± 18.0	53.2 ± 18.3	NS	56.6 ± 23.1	58.1 ± 17.4	NS
Sex (M/F)	6/2	6/2	NS	7/2	5/3	NS
Height (cm)	169.7 ± 8.9	169.3 ± 8.9	NS	173.3 ± 7.1	171.7 ± 10.7	NS
Weight (kg)	93.5 ± 26.5	89.3 ± 17.5	NS	79.1 ± 9.0	77.1 ± 15.4	NS
FFM (kg)	58.1 ± 15.6	58.5 ± 12.1	NS	53.0 ± 9.4	53.2 ± 14.6	NS
FM (kg)	34.5 ± 13.6	30.5 ± 10.5	NS	25.1 ± 3.7	23.1 ± 7.2	NS
B.M.I.	32.1 ± 6.7	30.9 ± 3.5	NS	25.9 ± 1.8	26.3 ± 3.3	NS
C.K. (μmol/L)	3003.8 ± 4471.0	1587.1 ± 1447.9	NS	109.2 ± 60.3	127.0 ± 65.7	NS
Activity Status						
1. Work	2.0 ± 0.4	2.6 ± 0.6	P<0.03	2.6 ± 0.6	2.3 ± 0.6	NS
2. Sport	2.1 ± 0.8	2.6 ± 0.8	NS	2.4 ± 0.7	2.0 ± 0.6	NS
3. Leisure	2.8 ± 0.6	3.0 ± 0.7	NS	3.1 ± 0.4	2.8 ± 0.9	NS

Table 2. Within Group Subject Characteristics, Body Composition, and Activity Status. *Values are means ± standard deviation. NS = not significant; CK = creatine kinase FFM = fat free mass; FM = fat mass; BMI = body mass index.*

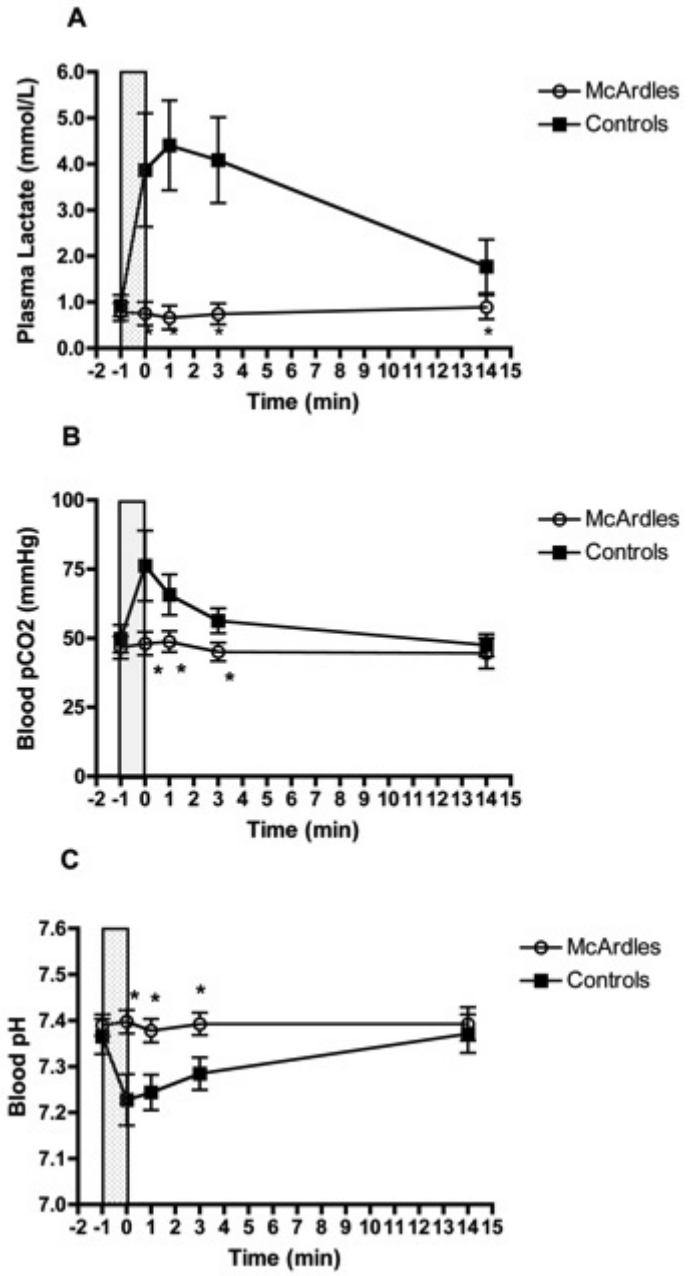

Figure 6. Classic response to exercise in patients with McArdle's disease compared to control subjects (1).

*Blood lactate response to non-ischemic forearm exercise test (Plasma lactate; panel A), blood PCO2 response to non-ischemic forearm exercise test (Plasma PCO2; panel B) and blood pH response to non-ischemic forearm exercise test (Blood pH) in MCD patients and control subjects. Data are expressed as mean ± SD. * P ≤ 0.05 vs. controls.*

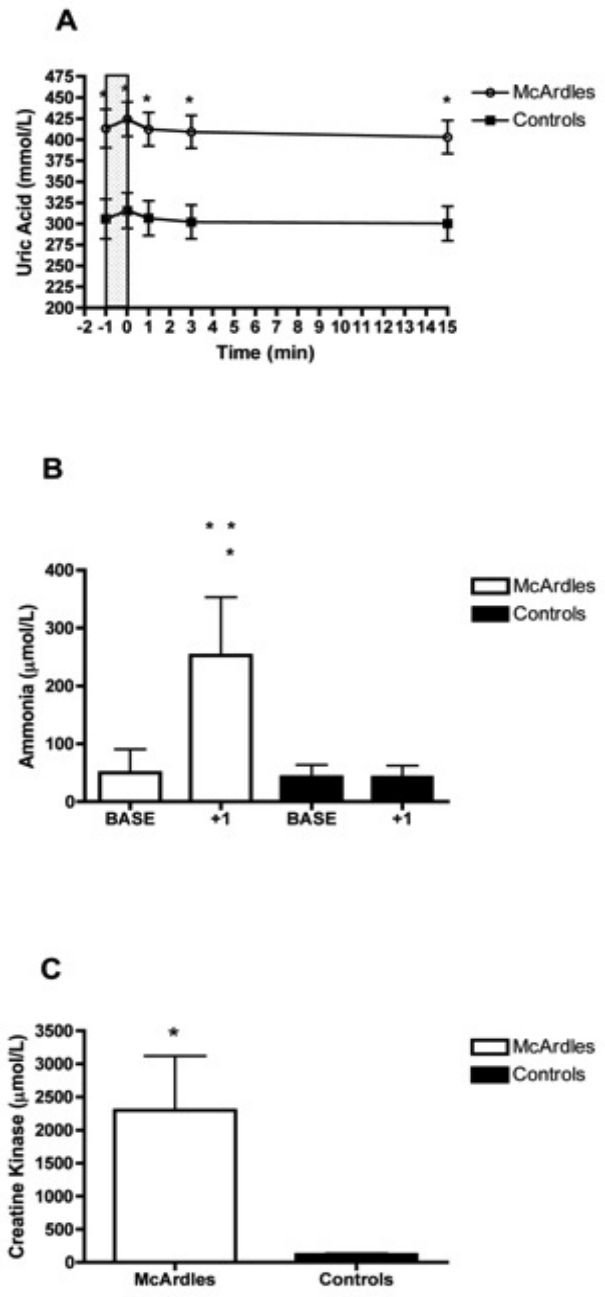

Figure 7. Classic response to exercise in patients with McArdle's disease compared to control subjects (2).

*Blood uric acid response to non-ischemic forearm exercise test (uric acid; panel A), blood ammonia response before and after non-ischemic forearm exercise test (ammonia; panel B) and baseline plasma CK (creatine Kinase; panel C) in MCD patients and control subjects. Data are expressed as mean ± SD. * P ≤ 0.05 vs. controls.*

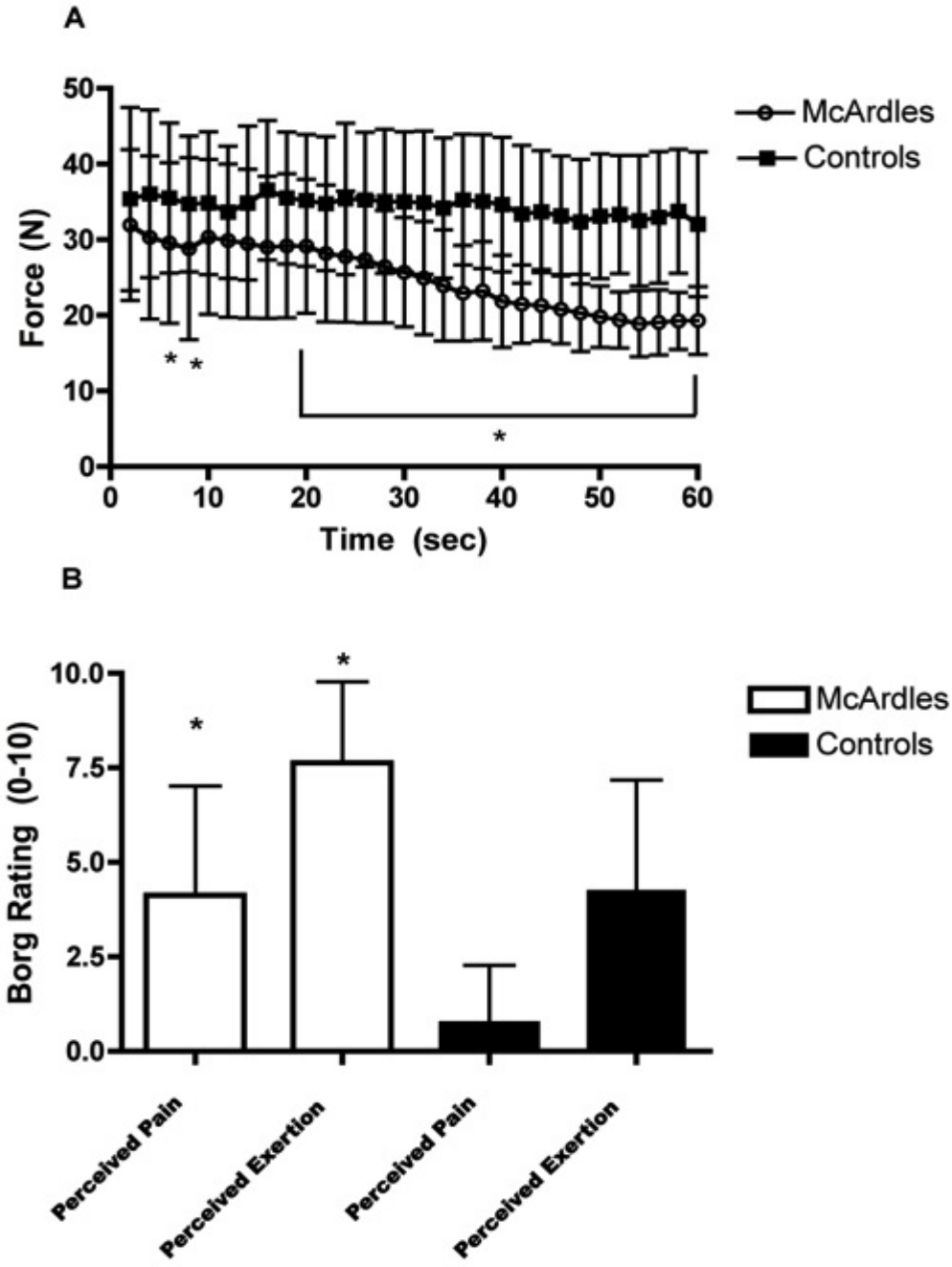

Figure 8. Initial non-ischemic forearm exercise test performance and tolerance in patients with McArdle's disease and control subjects.
*Force generation during non-ischemic forearm exercise test (Force; panel A) and rating of perceived pain and exertion after non-ischemic forearm exercise test (Borg scale; panel B) in MCD patients and control subjects. Data are expressed as mean ± SD. * P ≤ 0.05 vs. controls.*

Grouping	Baseline/ McArdles vs. Controls	McArdles/ Glucose	McArdles/ Placebo	Controls/ Glucose	Controls/ Placebo
Result	Differences at 7, 9, 20 - 60 sec, ($P \leq 0.05$)	No differences	No differences	No differences	No differences

Table 3. Between and within groups Non- ischemic forearm exercise testing results. *Force generation during non-ischemic forearm exercise test. Maximum voluntary contraction N.*

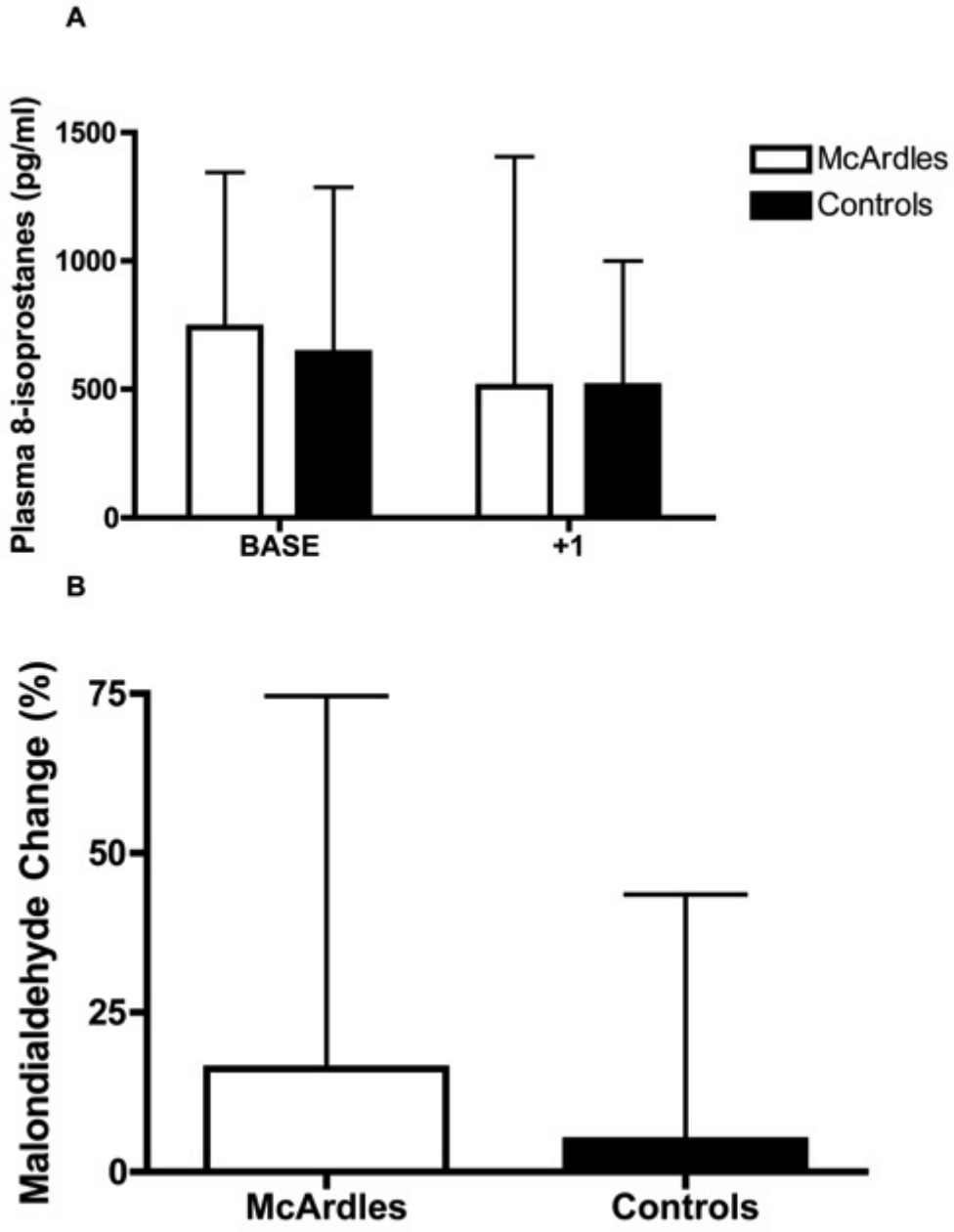

Figure 9. Initial oxidative stress response to non-ischemic forearm exercise test in patients with McArdle's disease compared to control subjects.
*Plasma 8-ISO response to non-ischemic forearm exercise test (plasma 8-isoprostane; panel A) and %change in plasma MDA before and after non-ischemic forearm exercise test (malondialdehyde; panel B) in MCD patients and control subjects. Data are expressed as mean ± SD. * P ≤ 0.05 vs. controls.*

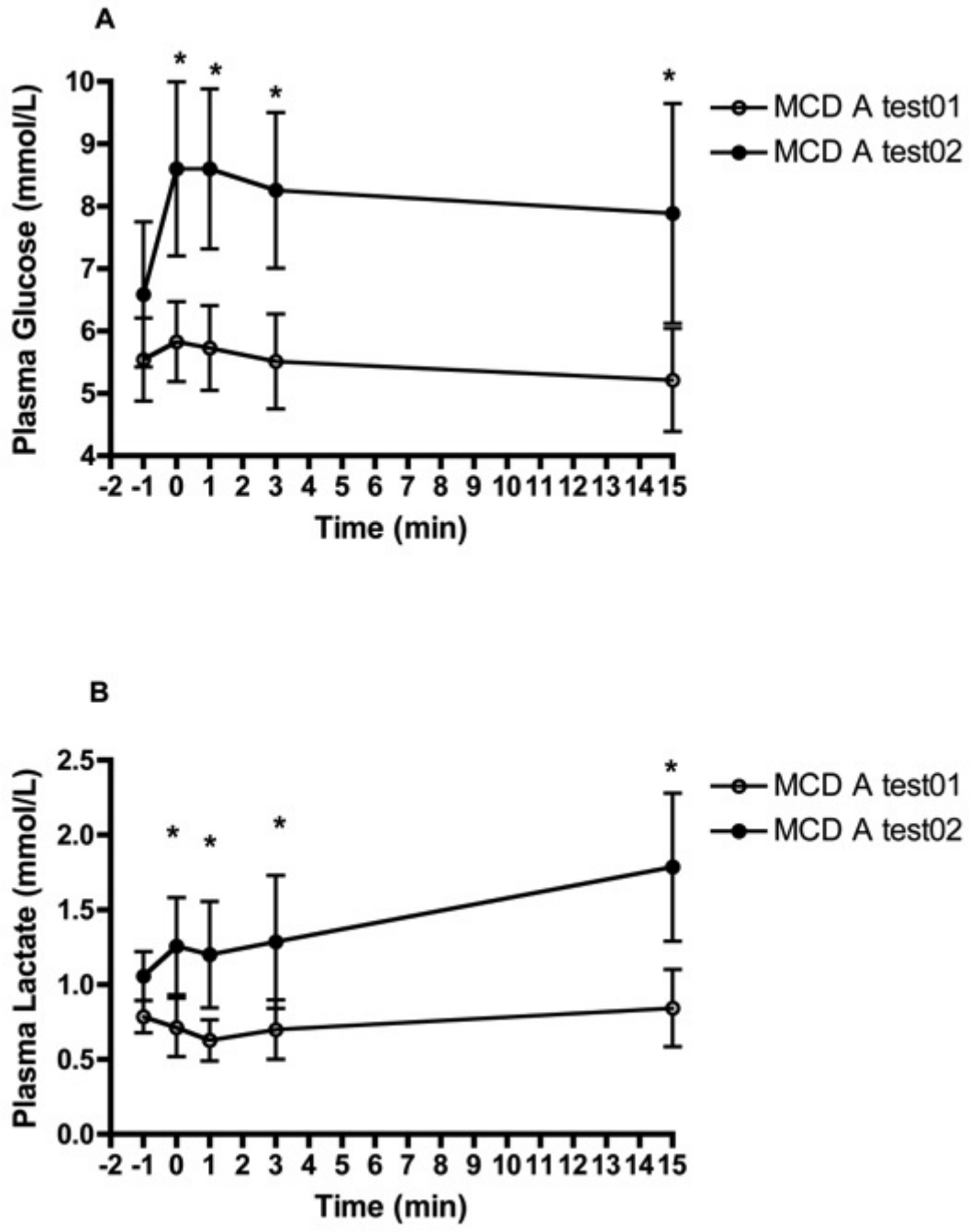

Figure 10. Effect of glucose supplementation in patients with McArdle's disease (1).
*Plasma glucose response to non-ischemic forearm exercise test (plasma glucose; panel A), plasma lactate response to non-ischemic forearm exercise test (plasma lactate; panel B) in MCD patients before and after receiving oral glucose supplementation. Data are expressed as mean ± SD. * P ≤ 0.05 vs. controls.*

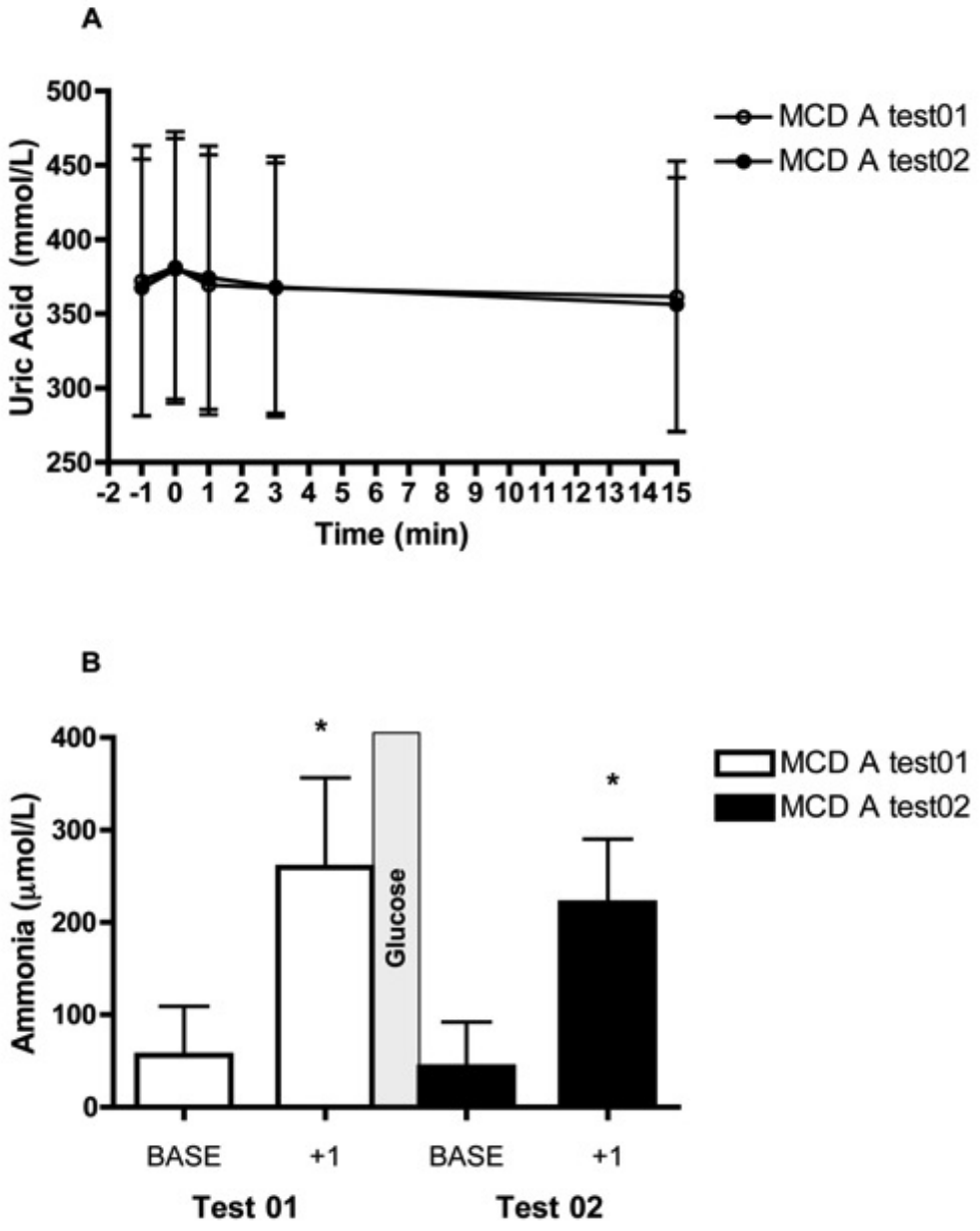

Figure 11. Effect of glucose supplementation in patients with McArdle's disease (2).

*Plasma uric acid response to non-ischemic forearm exercise test (plasma uric acid; panel A), plasma ammonia response to non-ischemic forearm exercise test (plasma ammonia; panel B) in MCD patients before and after receiving oral glucose supplementation. Data are expressed as mean ± SD. * P ≤ 0.05 vs. glucose.*

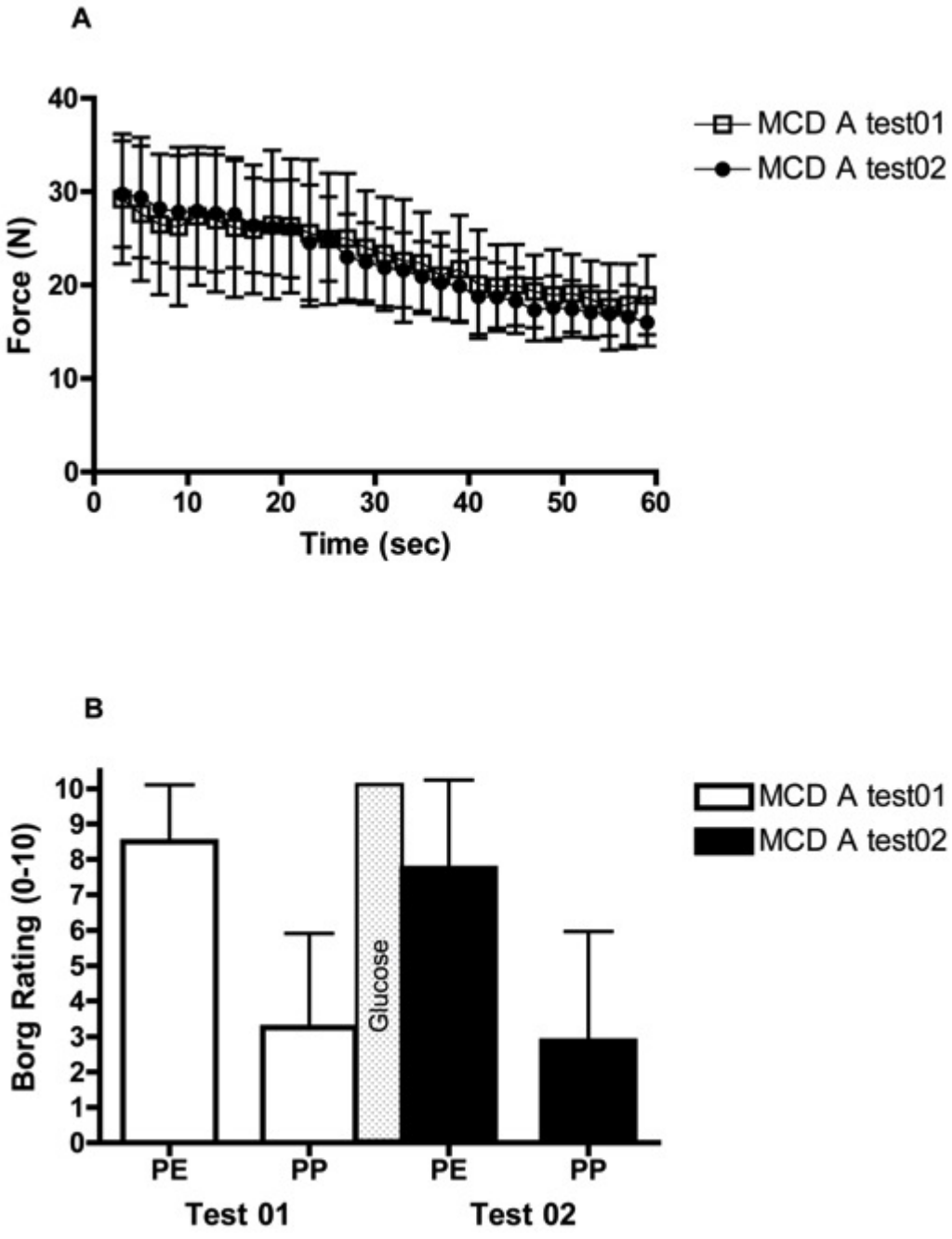

Figure 12. Effect of glucose supplementation on non-ischemic forearm exercise test performance and tolerance in patients with McArdle's disease. *Force generation during non-ischemic forearm exercise test (Force; panel A) and rating of perceived pain and exertion after non-ischemic forearm exercise test (Borg scale; panel B) in MCD patients before and after receiving oral glucose supplementation. Data are expressed as mean ± SD. * P ≤ 0.05 vs. glucose*

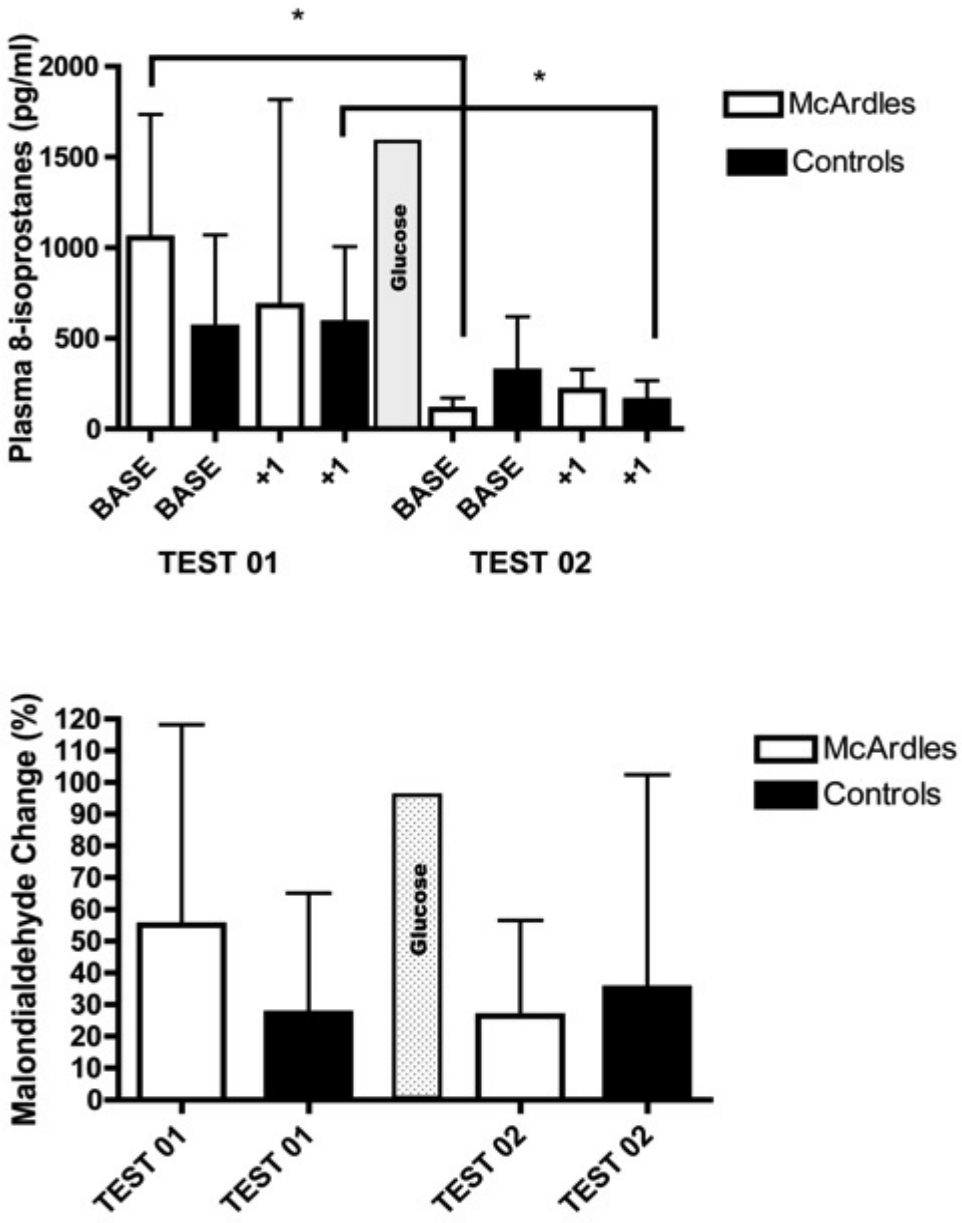

Figure 13. Effect of glucose supplementation on blood markers of oxidative stress in McArdle's disease patients compared to control subjects after non-ischemic forearm exercise test.

*Plasma 8-ISO response to non-ischemic forearm exercise test (plasma 8-isoprostane; panel A) and % change in plasma MDA before and after non-ischemic forearm exercise test (malondialdehyde; panel B) in MCD patients and control subjects receiving oral glucose supplementation. Data are expressed as mean ± SD. * P ≤ 0.05 vs. controls.*

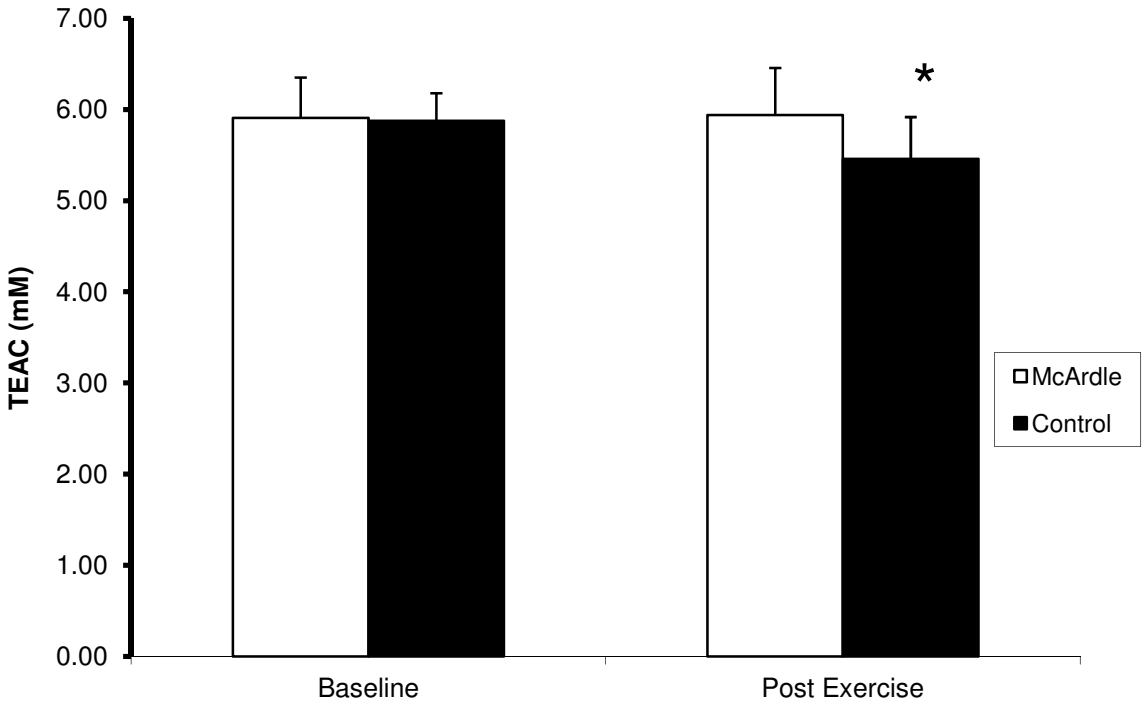

Figure 14. Total anti-oxidant capacity in patients with McArdle's disease and control subjects before and after non-ischemic forearm exercise test. *Plasma total anti-oxidant capacity before and after non-ischemic forearm exercise test (TEAC) in MCD patients and control subjects. Data are expressed as mean ± SD. * P ≤ 0.05 vs. controls.*

2.4 DISCUSION

The purpose of this study was to determine the effect of oral glucose supplementation on exercise performance and tolerance and plasma concentrations of oxidative stress in patients with MCD. In addition, we wanted to determine if the production of oxidative stress was a direct result of an increased flow through xanthine oxidase, an enzyme responsible for the generation of superoxide free radicals O_2^- and uric acid. In addition, we wanted to determine if we could lower the production of these radicals after exercise with glucose supplementation. It has been shown that MCD patients utilize more glucose while exercising compared to control subjects [81]. In the present study, greater changes in plasma lactate were observed in the MCD patients receiving glucose as compared to the CON subjects. In addition, the enzymes associated with glucose uptake and glycolytic flux, specifically GLUT 4 and PFK, have been shown to be up regulated in the skeletal muscle of patients with MCD [30] and would in theory facilitate a greater uptake of glucose from the blood into exercising muscle and less reliance on the adenylate kinase/AMPD1 reaction for ATP regeneration. It was predicted that the increased availability of blood glucose would have improved the exercise tolerance and performance in MCD patients after ingestion. However, rates of perceived exertion and perceived pain were not improved and the rate of fatigue during non-ischemic forearm exercise test did not change in response to glucose supplementation. The lack of improvement in exercise may be attributed to a couple of factors 1. The non-ischemic forearm

exercise test protocol used for this study was based on a protocol that was developed to be better tolerated in MCD patients and was stated to be an aerobic testing protocol [16]. However, because of the multiple high intensity contractions (30) the non-ischemic forearm exercise test may be partially ischemic (lactate went up significantly in the control subjects) and this could have restricted blood flow in the exercising arm of patients and consequently limit the uptake of glucose from the blood during exercise. However, the fact that we saw an increase in lactate in MCD patients receiving glucose (figure 10) suggests glucose did get into the muscle and provided some glycolytic flux: 2. The skeletal muscle of MCD patients may be primed for glucose uptake, apparent by the previously observed higher GLUT4 and PFK enzyme content [30]. However, the amount of glucose taken up in the first minute of exercise is likely very small compared to aerobic type exercise lasting 20 minutes and beyond. Additionally, it is well known that insulin resistance is associated with high stores of glycogen in skeletal muscle. Since, MCD patients have increased stores of glycogen, the glucose uptake in study patients could have been impaired but may have been countered by the known higher GLUT4 in MCD patients. Nielsen et al. (2002) concluded the MCD patients in their study were insulin resistant in terms of glucose uptake [82]. However, there is no evidence supporting that contraction stimulated glucose uptake is impaired in patients with MCD. Additionally, in the present study MCD patients also had greater fat mass (FM) and a higher B.M.I. compared to matched controls. Both characteristics are associated with insulin

resistance [83]. These two factors individually or in combination could have impaired glucose uptake during exercise of MCD patients.

A novel finding in the present study was the sustained TEAC in the plasma of MCD after exercise while the TEAC of matched controls decreased significantly after exercise. It appears MCD patients may have developed an enhanced buffering capacity to reactive oxygen species demonstrated by the sustained TEAC after the non-ischemic forearm exercise test. This adaptation could be the result of a lifetime of chronic reactive oxygen species production.

In the present study we hypothesized that glucose supplementation would be associated with a decline in oxidative stress post non-ischemic forearm exercise test in MCD patients. In addition, we wanted to determine if the production of reactive oxygen species was a direct result of an increased flow through xanthine oxidase, an enzyme responsible for the generation of superoxide free radicals O_2^- and uric acid. Mitochondria in muscle [84] and cytoplasmic xanthine oxidase [85] are likely the main cellular source of reactive oxygen species generation in skeletal muscle of MCD patients. Uric acid is the final product of the xanthine oxidase pathway and we used it as a marker of the activity in this pathway [43]. We found an attenuated production of oxidative stress, specifically 8-ISO, in MCD patients and controls who had received glucose between their 1st and 2nd non-ischemic forearm exercise test. However, we feel due to the relative short time period between the 2 exercise tests there

may have been an effect of the first testing on the second, therefore making the result unreliable. The trend in plasma 8-ISO was also observed in our placebo group and therefore could be attributed to the effect of test/ re-test. Recent research has demonstrated that an increase in anti-oxidant enzyme activity was detected in plasma after maximal and sub maximal exercise [86]. These results are in agreement with our data, specifically 8-ISO in the plasma. In the present study we observed the highest levels at the initial baseline time point followed by a continuous decrease over time and the 2nd exercise testing. The initial testing could have increased the total anti-oxidant status of subject's plasma and subsequently lowered 8-ISO markers at all subsequent time points. Glucose was not associated with lower levels of uric acid in patients; therefore, we were not able to ascertain if the production of oxidative stress was due to a decreased flow through the uric acid cycle and xanthine oxidase. Glucose may have not had an effect. Alternatively, we could have measured more proximal components of the uric acid cycle. Components that may have come out of the muscle more rapidly and we may have been able to establish a link. Previous work conducted by Sahlin demonstrated that uric acid was not significantly elevated beyond baseline until 60 minutes after onset of aerobic exercise [87]. However, other components of the uric acid cycle (NH_3 and xanthine) were detected much sooner [87].

In the present study the markers of oxidative stress chosen where MDA and 8- ISO samples from participants blood plasma. MDA is a commonly employed measure of oxidative stress. Many studies have demonstrated that

MDA concentrations increase of resistance type exercise [88] and exhaustive aerobic exercise [89]. We have also demonstrated reliable results in our lab using a similar protocol [90]. Other research suggests MDA may not be specific enough and reliable for repetitive results. MDA is the by-product of polyunsaturated fatty acid peroxidation and readily combines with other molecules such as proteins and DNA and therefore we may have been measuring uncharacterized products of reactive oxygen species [91]. Other TBARS could have been measured in the assay in addition to MDA. Perhaps this marker was not specific enough in this study design. Previous research suggests both plasma and urinary 8-ISO are reliable markers to assess oxidative stress in vivo [92, 93]. While we were able to get a partial predictable response to glucose supplementation, the 8-ISO markers of oxidative stress may not have been giving us real time status on their production but perhaps a more dynamic process involving their formation, accumulation and removal at the time of sampling [91]. In addition to our choice of oxidative stress markers our blood sampling time course may not have been optimal. We took samples prior to non-ischemic forearm exercise test and immediately after and 1 minute after non-ischemic forearm exercise test. If the oxidative damage was occurring within the muscle there may have been a delay before it was seen in the blood plasma of participants. Additionally, the concentration of the marker of oxidative stress may have been diluted because blood markers are not as localized as a muscle biopsy for instance [94] and the muscle is where these markers originate. Reactive oxygen species are transient

and local and operate over small distances, however the markers of oxidative stress are stable. The markers we were detecting likely needed more time to diffuse out of the muscle and accumulate. These factors likely added variance that was apparent in our data that led to the inability to detect the hypothesized changes.

Another potential reason we may have not observed the reactive oxygen species generation to be higher in the MCD patients is the fact that uric acid may be acting as an anti-oxidant. Uric acid accounts for approximately 60% of plasma's free radical buffering capacity [95]. The plasma concentration of uric acid in the MCD subjects was significantly higher than that of the control subjects at all time points. The elevated concentrations may have added an enhanced buffering capacity in the plasma of the MCD subjects and auto buffered the rise of oxidative stress.

2.4.1 Future Considerations

The results from this current study may direct subsequent research in the acute and chronic responses in MCD patients. Future studies could centre on:

1. The implication of reactive oxygen species and oxidative stress on muscle atrophy and weakness in MCD patients, which may be a contributor to muscle wasting seen in many MCD patients with age.
2. A more comprehensive characterization of the aging process, free radicals and oxidative stress in MCD patients. This may provide support to the

theory that suggests organism's age because cells accumulate free radical damage over time.

3. Supplementation with allopurinol, a specific inhibitor of xanthine oxidase, discretely or in conjunction with antioxidant supplementation.
4. The skeletal muscle response to chronic oxidative stress in MCD patients, specifically a compensatory up-regulation of antioxidant enzymes.
5. Determining the blood insulin and glucose profiles in MCD patients and the response to exercise.

2.4.2 Conclusions

The observations that MCD patients had sustained TAEC after exercise when healthy individual's TAEC decreased may suggest an increase in another molecule that has anti-oxidant properties. Perhaps a more rapidly effluxing molecule, such as xanthine or hypoxanthine or the chronic elevation in uric acid could have buffered the reactive oxygen species. Xanthine has shown to have a quenching effect on the production of hydroxyl radicals and the oxidation of DNA [96]. This would be a beneficial in the skeletal muscle of patients with MCD, as it would highlight the adaptive capacity of the muscle in response to a constant systemic stress.

During the non-ischemic forearm exercise test it was observed that control subjects had a much lower blood pH than control subjects. The fact that the MCD patients did not get intra- cellular acidosis potentially may attenuate the magnitude of the effect of reactive oxygen species. This theory has been

described in hop compounds in beer, where lowering the intra-cellular pH caused cellular oxidative damage [97]. It has also been demonstrated that oxidative stress and oxidative DNA damage can be induced in cells by short exposures to low pH [98]

By continuing exercise in the absence of available substrate for energy it may promote the progression of the MCD by relying on energy producing pathways that are also a source of increased reactive oxygen species production. The observation that lower levels of oxidative stress were measured in the plasma of MCD patients after ingesting glucose is promising but inconclusive based on the fact that the initial bout of exercise testing could have influenced the second bout and ultimately influenced the concentrations of plasma markers of oxidative stress. In addition we were not able to establish a link to an attenuated flux through the uric acid pathway.

In conclusion, trials with glucose supplementation for MCD patients have mostly focused on relief of exercise intolerance during aerobic exercise in which the second-wind phenomenon would occur. However, minimal investigation has been conducted into providing symptomatic relief for acute, anaerobic exercise. Glucose ingestion may help alleviate exercise intolerance in patients during aerobic exercise, however it is unclear if it has a protective effect during anaerobic exercise and the production of reactive oxygen species.

2.5 REFERENCES

1. Nogales-Gadea, G., J. Arenas, and A. Andreu, *Molecular genetics of McArdle's disease.* Current Neurology and Neuroscience Reports, 2007. 7(1): p. 84-92.
2. Roach, P.J., *Glycogen and its Metabolism.* Current Molecular Medicine, 2002. 2(2): p. 101-120.
3. Dimaur S, A.A., Bruno C, Hadjigeorgiou GM. 2002, *Myophosphorylase deficiency (glycogenosis type V; McArdle disease).* Curr Mol Med, 2002. 2(2):189-196.
4. Rommel, O., et al., *Muscle pain in myophosphorylase deficiency (McArdle's disease): The role of gender, genotype, and pain-related coping.* Pain, 2006. 124(3): p. 295-304.
5. Perez, M., et al., *The second wind phenomenon in very young McArdle's patients.* Neuromuscular Disorders, 2009. 19(6): p. 403-405.
6. Riley, M., et al., *Respiratory gas exchange and metabolic responses during exercise in McArdle's disease.* J Appl Physiol, 1993. 75(2): p. 745-754.
7. Hargreaves, M., *Muscle glycogen and metabolic regulation.* The Proceedings of the Nutrition Society, 2004. 63(2): p. 217.
8. Lucia, A., Nogales-Gadea, G., Perez, M., Martin, MA., Andreu, AL., Arenas, J., *McArdle disease: what do neurologists need to know?* Nature Clinical Practice Neurology, 2008. 4 (10), 568-577.

9. Gospe, S., et al., *Asymptomatic McArdle's disease associated with hyper-creatine kinase-emia and absence of myophosphorylase.* Neurology, 1998. 51(4): p. 1228-9.

10. .Fattah SM, R.A., Faloon WW. , *McArdle's disease: Metabolic studies in a patient and review of the syndrome. .* The American Journal of Medicine, 1970. 48(6):693-699.

11. Ollivier, K., et al., *Exercise tolerance and daily life in McArdle's disease.* Muscle & Nerve, 2005. 31(5): p. 637-641.

12. Pillarisetti, J. and A. Ahmed, *McArdle Disease Presenting as Acute Renal Failure.* Southern Medical Journal, 2007. 100(3): p. 313-316 10.1097/01.smj.0000242355.27078.f3.

13. Candow, D., et al., *Effect of nutritional interventions and resistance exercise on aging muscle mass and strength.* Biogerontology, 2012: p. 1-14.

14. McArdle, A., A. Vasilaki, and M. Jackson, *Exercise and skeletal muscle ageing: cellular and molecular mechanisms.* Ageing Research Reviews, 2002. 1(1): p. 79-93.

15. Jackson, M.J. and A. McArdle, *Age-related changes in skeletal muscle reactive oxygen species generation and adaptive responses to reactive oxygen species.* The Journal of Physiology, 2011. 589(9): p. 2139-2145.

16. Kazemi-Esfarjani, P., et al., *A nonischemic forearm exercise test for McArdle disease.* Annals of Neurology, 2002. 52(2): p. 153-159.

17. Coakley, J.H., A.J. Wagenmakers, and R.H. Edwards, *Relationship between ammonia, heart rate, and exertion in McArdle's disease.* American Journal of Physiology - Endocrinology And Metabolism, 1992. 262(2): p. E167-E172.

18. McConchie, S.M., et al., *Molecular heterogeneity in McArdle's disease.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1990. 1096(1): p. 26-32.

19. Wu, Y., et al., *Six novel mutations in the myophosphorylase gene in patients with McArdle disease and a family with pseudo-dominant inheritance pattern.* Molecular Genetics and Metabolism, 2011(0).

20. Lewis, S.F. and R.G. Haller, *The pathophysiology of McArdle's disease: clues to regulation in exercise and fatigue.* J Appl Physiol, 1986. 61(2): p. 391-401.

21. Ronald, G.H., et al., *Myophosphorylase deficiency impairs muscle oxidative metabolism.* Annals of Neurology, 1985. 17(2): p. 196-199.

22. Perez M, M.M., Cardona C, et al. , *Can patients with McArdle's disease run?* Br J Sports Med., 2007. 41(1):53-54. 10.1136/bjsm.2006.030791.

23. Ronald, G.H., et al., *Aerobic conditioning: An effective therapy in McArdle's disease.* Annals of Neurology, 2006. 59(6): p. 922-928.

24. ; Available from: http://www.medbio.info/Horn/Time%206/muscle2.jpg.

25. Lewis, S.F., et al., *Metabolic control of cardiac output response to exercise in McArdle's disease.* J Appl Physiol, 1984. 57(6): p. 1749-1753.

26. O'Dochartaigh, C.S., et al., *Oxygen consumption is increased relative to work rate in patients with McArdle's disease.* European Journal of Clinical Investigation, 2004. 34(11): p. 731-737.

27. Vissing, J., et al., *Effect of deficient muscular glycogenolysis on extramuscular fuel production in exercise.* J Appl Physiol, 1992. 72(5): p. 1773-1779.

28. Mate-Munoz JL, Moran M, and P. M, *Favorable responses to acute and chronic exercise in McArdle patients. .* Clin J Sport Med, 2007. 17(4): p. 297-303.

29. Wagenmakers AJ, C.J., Edwards RH, *Metabolism of branched-chain amino acids and ammonia during exercise: Clues from McArdle's disease.* Int J Sports Med, 1990. 11 Suppl 2:S101-13.

30. Robertshaw, H.A., et al., *Increased PFK activity and GLUT4 protein content in McArdle's disease.* Muscle & Nerve, 2008. 37(4): p. 431-437.

31. Vissing, J. and R.G. Haller, *The Effect of Oral Sucrose on Exercise Tolerance in Patients with McArdle's Disease.* N Engl J Med, 2003. 349(26): p. 2503-2509.

32. Haller, R., et al., *Aerobic conditioning: An effective therapy in McArdle's disease.* Annals of Neurology, 2006. 59(6): p. 922-928.

33. Haller, R.G. and J. Vissing, *Spontaneous "Second Wind" and Glucose-Induced Second "Second Wind" in McArdle Disease: Oxidative Mechanisms.* Arch Neurol, 2002. 59(9): p. 1395-1402.

34. Andersen, S.T., R.G. Haller, and J. Vissing, *Effect of Oral Sucrose Shortly Before Exercise on Work Capacity in McArdle Disease.* Arch Neurol, 2008. 65(6): p. 786-789.

35. Hayashi, T., J.r.F.P. Wojtaszewski, and L.J. Goodyear, *Exercise regulation of glucose transport in skeletal muscle.* American Journal of Physiology - Endocrinology And Metabolism, 1997. 273(6): p. E1039-E1051.

36. Rogers, S., et al., *Identification of a novel glucose transporter-like protein,ÄîGLUT-12.* American Journal of Physiology - Endocrinology And Metabolism, 2002. 282(3): p. E733-E738.

37. Lund S Fau - Holman, G.D., et al., *Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin.* Proc Natl Acad Sci U S A., 1995. 20(0027-8424 (Print)): p. 5817-21.

38. Fattah SM, R.A., Faloon WW. , *McArdle's disease: Metabolic studies in a patient and review of the syndrome. .* The American Journal of Medicine, 1970. 48(6):693-699.

39. Quinlivan, R., A. Martinuzzi, and B. Schoser, *Pharmacological and nutritional treatment for McArdle disease (Glycogen Storage Disease type V).* Cochrane Database of Systematic Reviews: Reviews 2010 Issue 12 John Wiley & Sons, Ltd Chichester, UK DOI: 10.1002/14651858.CD003458.pub4, 2010.

40. Thompson, D., et al., *Physical Activity and Exercise in the Regulation of Human Adipose Tissue Physiology.* Physiological Reviews, 2011. 92(1): p. 157-191.

41. Orngreen, M.C., et al., *Fat metabolism during exercise in patients with McArdle disease.* Neurology, 2009. 72(8): p. 718-724.

42. Andersen, S.T., et al., *Effect of Changes in Fat Availability on Exercise Capacity in McArdle Disease.* Arch Neurol, 2009. 66(6): p. 762-766.

43. Stathis, C.G., et al., *Purine loss after repeated sprint bouts in humans.* Journal of Applied Physiology, 1999. 87(6): p. 2037-2042.

44. Andersen, S.T. and J. Vissing, *Carbohydrate- and protein-rich diets in McArdle disease: effects on exercise capacity.* J Neurol Neurosurg Psychiatry, 2008. 79(12): p. 1359-1363.

45. Devries, M.C. and M.A. Tarnopolsky, *Muscle Physiology in Healthy Men and Women and Those with Metabolic Myopathies.* Physical medicine and rehabilitation clinics of North America, 2009. 20(1): p. 101-131.

46. Fujita, S. and E. Volpi, *Amino Acids and Muscle Loss with Aging.* The Journal of Nutrition, 2006. 136(1): p. 277S-280S.

47. Tarnopolsky, M., *Protein requirements for endurance athletes.* Nutrition, 2004. 20(7,Äì8): p. 662-668.

48. MacLean, D., et al., *Oral branched-chain amino acids do not improve exercise capacity in McArdle disease.* Neurology, 1998. 51 (5), 1456-1459.

49. Mineo I Fau - Tarui, S. and S. Tarui, *Myogenic hyperuricemia: what can we learn from metabolic myopathies?* Muscle Nerve, 1995. 3(0148-639X (Print)): p. S75-81.

50. Tarnopolsky, M., *What can metabolic myopathies teach us about exercise physiology?* Appl Physiol Nutr Metab, 2006. 31(1):21-30.

51. Devries MC, T.M., *Muscle physiology in healthy men and women and those with metabolic myopathies.* Physical Medicine and Rehabilitation Clinics of North America. , 2009. 20(1):101-131.

52. Vorgerd, M., et al., *Creatine Therapy in Myophosphorylase Deficiency (McArdle Disease): A Placebo-Controlled Crossover Trial.* Arch Neurol, 2000. 57(7): p. 956-963.

53. Vorgerd, M., et al., *Effect of High-Dose Creatine Therapy on Symptoms of Exercise Intolerance in McArdle Disease: Double-blind, Placebo-Controlled Crossover Study.* Arch Neurol, 2002. 59(1): p. 97-101.

54. Hochachka, P.W. and G.B. McClelland, *Cellular metabolic homeostasis during large-scale change in ATP turnover rates in muscles.* Journal of Experimental Biology, 1997. 200(2): p. 381-6.

55. Vorgerd M, Z.J.K.R. and et al., *Effect of high-dose creatine therapy on symptoms of exercise intolerance in mcardle disease: Double-blind, placebo-controlled crossover study.* Archives of Neurology, 2002. 59(1): p. 97-101.

56. Sahlin, K., et al., *Impaired oxidative metabolism increases adenine nucleotide breakdown in McArdle's disease.* J Appl Physiol, 1990. 69(4): p. 1231-1235.

57. George, J. and A. Struthers, *Role of urate, xanthine oxidase and the effects of allopurinal in vascular oxidative stress.* Vascular Health and Risk Management,, 2009. 5: p. 265-272.

58. Mineo I Fau - Kono, N., et al., *Excess purine degradation in exercising muscles of patients with glycogen storage disease types V and VII.* J. Clin. Invest., 1985. 76(0021-9738 (Print)): p. 556-560.

59. Mineo, I., Kono, N., Hara, N., Shimizu, T., Yamada, Y., Kawachi, M., Kiyokawa, H., Wang, YL., Tarui, S. , *Myogenic hyperuricemia: A common pathophysiologic feature of glycogenosis types III, V and VII.* The New England Journal of Medicine,, 1987. 317(2): p. 75-79.

60. Harman, D., *Origin and evolution of the free radical theory of aging: a brief personal history, 1954-2009.* Biogerontology, 2009. 6(1389-5729 (Print)).

61. Groleau, J., et al., *Accelerated Vascular Aging in CuZnSOD-Deficient Mice: Impact on EPC Function and Reparative Neovascularization.* PLoS ONE, 2011. 6(8): p. e23308.

62. Cai, Z., B. Zhao, and A. Ratka, *Oxidative Stress and β-Amyloid Protein in Alzheimer's Disease.* NeuroMolecular Medicine, 2011: p. 1-28.

63. Nalcakan, G.R., et al., - *Acute oxidative stress and antioxidant status responses following an American.* - J Sports Med Phys Fitness. 2011 Sep;51(3):533-9., 2011(- 0022-4707 (Print)): p. - 533-9.

64. Jackson, M.J., *Reactive oxygen species and redox-regulation of skeletal muscle adaptations to exercise.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2005. 360(1464): p. 2285-2291.

65. De Lisio M Fau - Kaczor, J.J., et al., *Exercise training enhances the skeletal muscle response to radiation-induced oxidative stress.* Muscle & nerve, 2011. 43(1)(1097-4598 (Electronic)): p. 58-64.

66. Alessio, H., *Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training.* J Appl Physiol, 1988. 64.

67. Parise, G., et al., *Antioxidant enzyme activity is up-regulated after unilateral resistance exercise training in older adults.* Free Radical Biology and Medicine, 2005. 39(2): p. 289-295.

68. Helen Hermana M. Hermsdorff, K.B.F.B., Ana Carolina P. Volp, Blanca Puchau, Josefina Bressan, M. Ángeles Zulet and J. Alfredo Martínez *Vitamin C and fibre consumption from fruits and vegetables improves oxidative stress markers in healthy young adults.* British Journal of Nutrition, Available on CJO 2011. doi:10.1017/S0007114511004235.

69. Russo, P.J., J.W. Phillips, and N.W. Seidler, *The role of lipid peroxidation in McArdle's disease: Applications for treatment of other myopathies.* Medical Hypotheses, 1992. 39(2): p. 147-151.

70. Wehrens, X.H.T., et al., *FKBP12.6 Deficiency and Defective Calcium Release Channel (Ryanodine Receptor) Function Linked to Exercise-Induced Sudden Cardiac Death.* Cell, 2003. 113(7): p. 829-840.

71. Mate-Munoz Jl Fau - Moran, M., et al., *Favorable responses to acute and chronic exercise in McArdle patients.* Clin J Sport Med, 2007. 17(4): p. 297-303.

72. Harman, D., *Aging: A Theory Based on Free Radical and Radiation Chemistry.* Journal of Gerontology, 1956. 11(3): p. 298-300.

73. Navarro, A. and A. Boveris, *The mitochondrial energy transduction system and the aging process.* American Journal of Physiology - Cell Physiology, 2007. 292(2): p. C670-C686.

74. McArdle, B., *Myopathy due to a defect in muscle glycogen breakdown.* Clin Sci, , 1951. 10: p. p. 13.

75. Andersen, S.T., et al., *Do carriers of PYGM mutations have symptoms of McArdle disease?* Neurology, 2006. 67(4): p. 716-718.

76. Mineo, I., et al., *Myogenic hyperuricemia. A common pathophysiologic feature of glycogenosis types III, V, and VII.* N Engl J Med, 1987. 317(2): p. 75-80.

77. Baecke, J., J. Burema, and J. Frijters, *A short questionnaire for the measurement of habitual physical activity in epidemiological studies.* The American Journal of Clinical Nutrition, 1982. 36(5): p. 936-942.

78. Borg, G., *Perceived exertion as an indicator of somatic stress.* Scand J Rehabil Med. , 1970. 2(2):92-8(0036-5505 (Print)).

79. Walker, R.B. and J.D. Everette, *Comparative Reaction Rates of Various Antioxidants with ABTS Radical Cation.* Journal of Agricultural and Food Chemistry, 2009. 57(4): p. 1156-1161.

80. MacNeil, L.G., et al., *17Œ≤-estradiol attenuates exercise-induced neutrophil infiltration in men.* American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 2011. 300(6): p. R1443-R1451.

81. Vissing, J., et al., *Effect of deficient muscular glycogenolysis on extramuscular fuel production in exercise.* Journal of Applied Physiology, 1992. 72(5): p. 1773-1779.

82. Nielsen, J.N., et al., *Decreased insulin action in skeletal muscle from patients with McArdle's disease.* American Journal of Physiology - Endocrinology And Metabolism, 2002. 282(6): p. E1267-E1275.

83. Galletti, F., et al., *Hyperleptinemia is associated with hypertension, systemic inflammation and insulin resistance in overweight but not in normal weight men.* Nutrition, Metabolism and Cardiovascular Diseases, (0).

84. Grivennikova, V.G. and A.D. Vinogradov, *Generation of superoxide by the mitochondrial Complex I.* Biochimica et Biophysica Acta (BBA) - Bioenergetics. 1757(5-6): p. 553-561.

85. Ji, L.L., M.-C. Gomez-Cabrera, and J. Vina, *Exercise and Hormesis.* Annals of the New York Academy of Sciences, 2006. 1067(1): p. 425-435.

86. Berzosa C Fau - Cebrian, I., et al., *Acute exercise increases plasma total antioxidant status and antioxidant enzyme activities in untrained men.* J Biomed Biotechnol., 2011(1110-7251 (Electronic)).

87. Sahlin, K., M. Tonkonogi, and K. Söderlund, *Plasma hypoxanthine and ammonia in humans during prolonged exercise.* European Journal of Applied Physiology and Occupational Physiology, 1999. 80(5): p. 417-422.

88. McBride Jm Fau - Kraemer, W.J., et al., *Effect of resistance exercise on free radical production.* Med Sci Sports Exerc., 1998. 1(0195-9131 (Print)): p. 67-72.

89. Akova B Fau - Surmen-Gur, E., et al., *Exercise-induced oxidative stress and muscle performance in healthy women: role of vitamin E supplementation and endogenous oestradiol.* Eur J Appl Physiol., 2001. 84(1-2): p. 1439-6319 (Print).

90. Rodriguez Mc Fau - Rosenfeld, J., M.A. Rosenfeld J Fau - Tarnopolsky, and M.A. Tarnopolsky, *Plasma malondialdehyde increases transiently after ischemic forearm exercise.* Med Sci Sports Exerc., 2003. 35(0195-9131 (Print)): p. 1859-1865.

91. Vaya, J., *Exogenous markers for the characterization of human diseases associated with oxidative stress.* Biochimie, 2012(0).

92. Vassalle C Fau - Botto, N., et al., *Evidence for enhanced 8-isoprostane plasma levels, as index of oxidative stress in vivo, in patients with coronary artery disease.* Coron Artery Dis., 2003. 14(3): p. 0954-6928 (Print).

93. Vassalle C Fau - Botto, N., et al., *Evidence for enhanced 8-isoprostane plasma levels, as index of oxidative stress in vivo, in patients with coronary artery disease.* Acta Cardiol, 2008. 63(4): p. 0954-6928 (Print).

94. Nikolaidis, M.G., et al., *The Effect of Muscle-Damaging Exercise on Blood and Skeletal Muscle Oxidative Stress: Magnitude and Time-Course Considerations.* Sports Medicine, 2008. 38(7): p. 579-606.

95. Lippi, G., et al., *The paradoxical relationship between serum uric acid and cardiovascular disease.* Clinica Chimica Acta, 2008. 392(1,Äì2): p. 1-7.

96. Azam S Fau - Hadi, N., et al., *Antioxidant and prooxidant properties of caffeine, theobromine and xanthine.* Med Sci Monit. , 2003. 9(1234-1010 (Print)): p. 325-330.

97. Behr J Fau - Vogel, R.F. and R.F. Vogel, *Mechanisms of hop inhibition include the transmembrane redox reaction.* Appl Environ Microbiol., 2010. 76(1): p. 1098-5336 (Electronic).

98. Dvorak, K., et al., *Bile acids in combination with low pH induce oxidative stress and oxidative DNA damage: relevance to the pathogenesis of Barrett,Äôs oesophagus.* Gut, 2007. 56(6): p. 763-771.

APPENDIX I: Expanded Methods

Protocol

Quantification of plasma 8-Isoprostanes

Plasma 8-ISOPROSTANE (Purification and Quantification)

8-Isoprostane affinity purification kit (cat.No 10367, Cayman chemical)
8-Isoprostane EIA Kit

Total Isoprostanes

- Add 100 µl KOH 40% (wt/vol) to 100 µl sample
- Incubate 60 min 40°C
- Add 2 volumes EtOH 0.01% BHT and mix
- Incubate 5 min 4°C
- 10 min 1.500 xg
- Keep the supernatant in a clean tube
- Evaporate the EtOH (evaporate until the sample is aprox the same volume it was before add the EtOH) in the speed vac (30 min aprox)
- Neutralize (to pH 7-7.4) the sample with KH2PO4 1M solution. Start with 2 equivalents of KH2PO4 (this is 100 µl of KH2PO4 for 100 µl KOH) and check pH with litmus paper.
- Add 1500 µl of Eicosanoid Affinity Column buffer.

Purification

- Samples: the total isoprostanes are diluted in column buffer, we dilute the free purified isoprostanes 1/5 with column buffer (100 µl and 400 µl column buffer)
- Open the column and wash 2 times with 2 ml column buffer
- Add the sample
- Wash the column with 2ml of column buffer
- Wash the column with 2ml of water
- Elute the sample with 2ml x 2 times of elution buffer (we can save the sample in the elution buffer at –80 °C)
- Reconstitute the column washing with 5 ml of water and 5 ml of column buffer.

(we can use each column 4 times)

- Evaporate all the liquid in the samples with the speed vac centrifuge
- Resuspend the samples in 500 µl of EIA buffer (this is the dilution 1/5)

Measure (plate)

Samples:
- Total and free purified: after purification we have 1/5 dilution, make to 1/10 (250 µl of 1/5 + 250 µl EIA buffer)
- Total unpurified: suspend in 1 ml of column buffer (100 µl of original sample), so it's 1/10 dilution.
- Free unpurified: dilution 1/5 and 1/10 with EIA buffer.

Plate:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	S								
B	Blk	S2	S2		A							
C	NSB	S3	S3			M						
D	NSB	S4	S4				P					
E	B0	S5	S5					L				
F	B0	S6	S6						E			
G	B0	S7	S7							S		
H	Ta	S8	S8									

Samples and Standards: add 50 µl in each well.

Well	EIA buffer	Standard/sample	tracer	antibody
Blk				
Ta			5 µl (at the develop step)	
NSB	100 µl		50 µl	
Bo	50 µl		50 µl	50 µl
Std/Sample		50 µl	50 µl	50 µl

18 h at 4°C

- Wash 5 times with 200 µl wash buffer each well
- Add 200 µl of Ellman's reagent in each well
- 90-120 min room temperature, agitation in dark.
- Read the plate at 415 nm (the Bo absorbance must be between 0.3-1 A.U)

- SOLUTIONS

KOH 15%
(15 gr in 100 ml) Ex. 15 ml water + 2.25 gr KOH

Ethanol BHT 0.01%
(0.01gr in 100 ml)

KH2PO4 1M
Mw 136.09. Ex. 50 ml water + 6.8 gr KH2PO4

EIA buffer
66.5 gr K2HPO4 + 16.075 gr KH2PO4 in 500 ml of water.
Take 50 ml of the above solution and add 50 mg Sodium Azide, 11.7 gr Sodium Chloride, 185 mg EDTA and 0.5 gr BSA, and take to 500 ml with de-ionized water.

Quantification of plasma MDA

MDA-TBARS
(Assay kit, 10009055, Cayman Chemical)

All the reagents must be equilibrate at room temperature before the assay (SDS will take at least an hour to stabilize)

Standards:
125 µM stock solution (250 µl MDA standard+750 µl water)

Tube	MDA (µl)	Water (µl)	MDA concentration (µM)
A	0	1000	0
B	5	995	0.625
C	10	990	1.25
D	20	980	2.5
E	40	960	5
F	80	920	10
G	200	800	25
H	400	600	50

- In a 5 ml polypropylene tube, add 100 µl sample or standard
- Add 100 µl SDS solution

- Add 4 ml color reagent
- Boil the tubes 1 h
- 10 min in ice
- 1.600 xg at 40C 10 min
- Load the plate (150 µl/well)
- Read 530-540 nm (535 nm)

Reagents:

- TBA Acetic Acid: add the 40 ml of concentrated acetic acid to 160 ml of water (stable at least 3 months at room temperature)
- TBA Sodium Hydroxide: dilute the 20 ml sodium hydroxide in 180 ml of water (stable at least 3 months at room temperature)
- Color Reagent (for 24 samples- 100 ml color reagent):

50 ml TBA Acetic Acid
50 ml TBA Sodium Hydroxide
530 mg TBA

APPENDIX II: Physical Activity Questionnaire

The Questionnaire of Baecke et al for Measurement of a Person's Habitual Physical Activity

Overview:

Baecke et al developed a questionnaire for evaluating a person's physical activity and separating it into three distinct dimensions. The authors were from the Netherlands.

Indices for physical activity:

(1) work activity

(2) sports activity

(3) leisure activity

Work Index

Question	Response	Points
What is your main occupation?	low activity	1
	moderate activity	3
	high activity	5
At work I sit	never	1
	seldom	2
	sometimes	3
	often	4
	always	5
At work I stand	never	1
	seldom	2
	sometimes	3
	often	4
	always	5
At work I walk	never	1
	seldom	2
	sometimes	3
	often	4
	always	5

At work I lift heavy loads	never	1
	seldom	2
	sometimes	3
	often	4
	always	5
After working I am tired	very often	5
	often	4
	sometimes	3
	seldom	2
	never	1
At work I sweat	very often	5
	often	4
	sometimes	3
	seldom	2
	never	1
In comparison of others of my own age I think my work is physically	much heavier	5
	heavier	4
	as heavy	3
	lighter	2
	much lighter	1

where: • The work activity is according to the Netherlands Nutrition Council with (1) low activity including clerical work driving shopkeeping teaching studying housework medical practice and occupations requiring a university education; (2) middle activity including factory work plumbing carpentry and farming; (3) high activity includes dock work construction work and professional sport.

work index = ((6 – (points for sitting)) + SUM(points for the other 7 parameters)) / 8

Sport Index

Question	Response	Points
Do you play sports?	yes then calculate sport score	(see below)
	• sport score >= 12	5
	• sport score 8 to < 12	4
	• sport score 4 to < 8	3
	• sport score 0.01 to < 4	2
	• sport score = 0	1
	No	1
In comparison with others of my own age I think my physical activity during leisure time is	much more	5
	More	4
	the same	3
	Less	2
	much less	1
During leisure time I sweat	very often	5
	Often	4
	sometimes	3
	Seldom	2
	Never	1
During leisure time I play sport	Never	1
	Seldom	2
	sometimes	3
	Often	4
	very often	5

Data on Most Frequently Played Sport	Finding	Value
What sport do yo play most frequently	low intensity	0.76
	medium intensity	1.26
	high intensity	1.76
How many hours do you play a week?	< 1 hour	0.5
	1-2 hours	1.5
	2-3 hours	2.5
	3-4 hours	3.5
	> 4 hours	4.5
How many months do you play in a year?	< 1 month	0.04
	1-3 months	0.17
	4-6 months	0.42
	7-9 months	0.67
	> 9 months	0.92

where: • The sport intensity is divided into 3 levels: (1) low level (billiards sailing bowling golf etc) with an average energy expenditure of 0.76 MK/h; (2) middle level (badminton cycling dancing swimming tennis) with an average energy expenditure of 1.26 MJ/h; (3) high level (boxing basketball football rugby rowing) with an average energy expenditure of 1.76 MJ/h

Data on Second Most Frequently Played Sport	Finding	Value
What sport do you play most frequently	low intensity	0.76
	medium intensity	1.26
	high intensity	1.76
How many hours do you play a week?	< 1 hour	0.5
	1-2 hours	1.5
	2-3 hours	2.5
	3-4 hours	3.5
	> 4 hours	4.5
How many months do you play in a year?	< 1 month	0.04
	1-3 months	0.17
	4-6 months	0.42
	7-9 months	0.67
	> 9 months	0.92

simple sports score = ((value for intensity of most frequent sport) * (value for weekly time of most frequent sport) * (value for yearly proportion of most frequent sport)) * ((value for intensity of second sport) * (value for weekly time of second sport) * (value for yearly proportion of second sport))

sport index = (SUM(points for all 4 parameters)) / 4

Leisure Index

Question	Response	Points
During leisure time I watch television	never	1
	seldom	2
	sometimes	3
	often	4
	very often	5
During leisure time I walk	never	1
	seldom	2
	sometimes	3
	often	4
	very often	5
During leisure time I cycle	never	1
	seldom	2
	sometimes	3
	often	4
	very often	5
How many minutes do you walk and/or cycle per day to and from work school and shopping?	< 5 minutes	1
	5-15 minutes	2
	15-30 minutes	3
	30-45 minutes	4
	> 45 minutes	5

leisure index = ((6 – (points for television watching)) + SUM(points for remaining 3 items)) / 4

References:

Baecke JAH Burema J Frijters ER. A short questionnaire for the measurement of habitual physical activity in epidemiological studies. Am J Clin Nutr. 1982; 36: 936-942.

APPENDIX III: Participants Case Report Form

AN INVESTIGATION: McArdle Disease, Aging, Sugar Ingestion & Oxidative Stress
CASE REPORT FORM

STUDY ID:_____ **DATE:** _____/_____/_____
DAY MONTH YEAR

GROUP:
- ❑ **MCD**
- ❑ **Control**

Complete this form after patient has given informed consent. Signed: _____*Y*_____*N*

1.0 Demographic Information

1.1 Age: _____years or DOB: _____D/_____M/_____Y

1.2 Sex: _____Male _____Female

2.0 Anthropometric Data

2.1 Weight: _____ (kg) Height: _____ (cm**) - no shoes**

2.2 BMI: _____ (kg/m^2)

3.0 Diagnosis of McArdle Disease

3.1 Has the participant been formally diagnosed with McArdle disease?

O Yes O No

If "NO", continue to section 5.0.

3.2 Date of formal diagnosis of McArdle disease: _____ (d/m/y)

3.3 Years since formal diagnosis: _____years

3.4 How was the participant diagnosed with McArdle disease?

- ❑ Genetic Testing
- ❑ Biochemical measures (absent myophosphorylase)
- ❑ Forearm exercise test (lack of increase in venous lactate)
- ❑ Other: _____

AN INVESTIGATION: McArdle Disease, Aging, Sugar Ingestion & Oxidative Stress
CASE REPORT FORM

STUDY ID:_____ **DATE:** _____/_____/_____
DAY MONTH YEAR

3.5 What were the diagnostic values?

- ❑ Yes, results documented in chart (see below)
- ❑ No, results NOT documented in chart

Diagnostic test	Value	Units	Date Documented
Genetic testing			
Myophosphorylase staining			
Venous lactate (post forearm/ grip testing)			
Other: _____			

4.0 Symptomatic History

4.1 Have you experienced contractures or severe muscle pain in the past 12 months? **O** Yes **O** No

4.2 When was the last time you experienced a period? _____ (d/m/y)
OR (*use checklist if unable to recall exact date)*

- ❑ 1 year ago
- ❑ 2 years ago
- ❑ 3 years ago
- ❑ 4 years ago
- ❑ 5 years ago
- ❑ 6 years ago
- ❑ 7 years ago
- ❑ 8 years ago
- ❑ 9 years ago
- ❑ 10 years ago

AN INVESTIGATION: McArdle Disease, Aging, Sugar Ingestion & Oxidative Stress
CASE REPORT FORM

STUDY ID:_____ **DATE:** _____/_____/_____
DAY MONTH YEAR

5.0 Current Medications

Participant is currently taking the following (Check all that apply):

Currently Taking	Class	Dose	Frequency	Comments
❑	Anti-hyperuricemia (allopurinol) • Zyloprim • Allohexal • Allosig • Progout • Zyloric			

6.0 Additional Medications:

Supplements:

Anti-oxidants ❒ currently taking ❒ NOT currently taking ❒ Unsure
Dose: _____ Frequency _____

Vit.C ❒ currently taking ❒ NOT currently taking ❒ Unsure
Dose: _____ Frequency _____

Vit. A ❒ currently taking ❒ NOT currently taking ❒ Unsure
Dose: _____ Frequency _____

Vit. E ❒ currently taking ❒ NOT currently taking ❒ Unsure
Dose: _____ Frequency _____

CoQ10 ❒ currently taking ❒ NOT currently taking ❒ Unsure
Dose: _____ Frequency _____

α Lipoic Acid ❒ currently taking ❒ NOT currently taking ❒ Unsure
Dose: _____ Frequency _____

Selenium ❒ currently taking ❒ NOT currently taking ❒ Unsure
Dose: _____ Frequency _____

AN INVESTIGATION: McArdle Disease, Aging, Sugar Ingestion & Oxidative Stress
CASE REPORT FORM

STUDY ID:_____ **DATE:** _____/_____/_____
DAY MONTH YEAR

Multivitamin ❒ currently taking: _____ (brand)
❒ NOT currently taking ❒ Unsure
Dose: _____ Frequency _____

Other Medications:
_____
_____
_____

7.0 Baecke questionnaire *Measurement of a Person's Habitual Physical Activity*
Completed **O** Yes **O** No

Score _____
Score _____
Score _____

8.0 BIODEX Isometric Contraction
1._____ 2._____ 3._____

9.0 DXA Scan **O** Yes **O** No Time_____

10.0 Biopsy **O** Yes **O** No Time_____

11.0 Reimbursement
❑ Parking Voucher
❑ Food Voucher (10$)
❑ 50$ (time/travel//biopsy)

Participants Signature _____ Date_____

AN INVESTIGATION: McArdle Disease, Aging, Sugar Ingestion & Oxidative Stress
CASE REPORT FORM

STUDY ID:_____ **DATE:** _____/_____/_____
DAY MONTH YEAR

NOTES

APPENDIX IV: CORE Lab Methodology

11965352001V16

UA plus

Uric Acid plus

cobas®

● indicates Roche/Hitachi analyzer(s) on which kit(s) can be used

Cat. No.	Bottle	Contents	902	912	MODULAR P	MODULAR D
11875426 216	1	REAGENT 6 x 66 mL			●	
	2	REAGENT 6 x 16 mL				
11929429 216	1	REAGENT 6 x 258 mL			●	●
11929437 216	2	REAGENT 6 x 68 mL				
11929445 216	1	REAGENT 4 x 641 mL				●
11929453 216	2	REAGENT 4 x 278 mL				
11661850 216	1	REAGENT 12 x 50 mL	●	●		
	2	REAGENT 6 x 22 mL				
11661868 216	1	REAGENT 6 x 100 mL		●		
	2	REAGENT 3 x 46 mL				

Some analyzers and kits shown may not be available in all countries. For additional system applications, contact your local Roche Diagnostics representative.

English

System information
For Roche/Hitachi 912/MODULAR P/D analyzers: ACN 700.

Intended use
Enzymatic in vitro test for the quantitative determination of uric acid in human serum, plasma and urine on Roche automated clinical chemistry analyzers.

Summary[1,2,3,4,5,6,7,8,9,10,11,12,13,14,15]
Uric acid is the final product of purine metabolism in the human organism. Uric acid measurements are used in the diagnosis and treatment of numerous renal and metabolic disorders, including renal failure, gout, leukemia, psoriasis, starvation or other wasting conditions, and of patients receiving cytotoxic drugs. The oxidation of uric acid provides the basis for two approaches to the quantitative determination of this purine metabolite. One approach is the reduction of phosphotungstic acid in an alkaline solution to tungsten blue, which is measured photometrically. The method is, however, subject to interferences from drugs and reducing substances other than uric acid.
A second approach, described by Praetorius and Poulson, utilizes the enzyme uricase to oxidize uric acid; this method eliminates the interferences intrinsic to chemical oxidation. Uricase can be employed in methods that involve the UV measurement of the consumption of uric acid or in combination with other enzymes to provide a colorimetric assay.
Another method is the colorimetric method developed by Town, et al. The sample is initially incubated with a reagent mixture containing ascorbate oxidase and a clearing system. In this test system it is important that any ascorbic acid present in the sample is eliminated in the preliminary reaction; this precludes any ascorbic acid interference with the subsequent POD indicator reaction. Upon addition of the starter reagent, oxidation of uric acid by uricase begins.
The Roche assay described here is a slight modification of the colorimetric method described above. The modifications were developed by Siedel. In this reaction, the peroxide reacts in the presence of peroxidase (POD), TOOS[a] and 4-aminophenazone to form a quinone-imine dye. The intensity of the red color formed is proportional to the uric acid concentration and is determined photometrically.
a) TOOS = N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline

Test principle[15]
Enzymatic colorimetric test
- Sample and addition of R1
- Addition of R2 and start of reaction:

$$\text{uric acid} + 2\ H_2O + O_2 \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2$$

Uricase cleaves uric acid to form allantoin and hydrogen peroxide.

$$2\ H_2O_2 + H^+ + \text{TOOS} + \text{4-aminophenazone} \xrightarrow{\text{peroxidase}} \text{quinone-diimine dye} + 4\ H_2O$$

Reagents – working solutions
R1 Phosphate buffer: 0.05 mol/L, pH 7.8; TOOS: 7 mmol/L; fatty alcohol polyglycol ether: 4.8 %; ascorbate oxidase (EC 1.10.3.3; zucchini; 25 °C) ≥ 83.5 µkat/L

R2 Phosphate buffer: 0.1 mol/L, pH 7.8; potassium hexacyanoferrate (II): 0.30 mmol/L; 4-aminophenazone ≥ 3 mmol/L; uricase (EC 1.7.3.3; Arthrobacter protophormiae; 25 °C) ≥ 83.4 µkat/L; peroxidase (POD) (EC 1.11.1.7; horseradish; 25 °C) ≥ 50 µkat/L

Precautions and warnings
For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Safety data sheet available for professional user on request.
Disposal of all waste material should be in accordance with local guidelines.

Reagent handling
R1: Ready for use
R2: Ready for use

Inaccurate pipetting of reagent, leading to potentially erroneous results, may be caused by excessive foaming of reagent. Ensure that foam is removed from the surface of the reagent prior to setting the reagent in the analyzer.

Storage and stability
Unopened kit components: Up to the expiration date at 2–8 °C
R1: 28 days opened and refrigerated on the analyzer, if protected from light
R2: 28 days opened and refrigerated on the analyzer, if protected from light

Specimen collection and preparation
For specimen collection and preparation only use suitable tubes or collection containers.
Centrifuge samples containing precipitates before performing the assay.
Only the specimens listed below were tested and found acceptable.
Serum
Plasma: Heparin or EDTA plasma.
The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Stability:[16] 5 days at 2–8 °C
6 months at (-15)–(-25) °C

Urine
Stability: Assay urinary uric acid as soon as possible. Do not refrigerate.
Stability upon NaOH addition (pH > 8.0): 4 days at 15–25 °C.[17]
To achieve stated uric acid stability, add NaOH prior to sample collection.

Roche/Hitachi analyzers with automatic sample dilution:
Urine samples are diluted 1 + 10 with distilled/deionized water or 0.9 % NaCl. This dilution is taken into account in the calculation of the results.

05021120001V4

NH3

Ammonia

cobas®

● indicates Roche/Hitachi analyzer(s) on which kit(s) can be used

Cat. No.	Bottle	Contents	902	912	917	MODULAR P	MODULAR D
11877984 216	1	REAGENT 2 x 15 mL	●	●	●	●	
	2	REAGENT → 2 x 4.65 mL					
	2a	REAGENT 2 x 10 mL					
	2b	REAGENT → 2 x 0.5 mL					

Some analyzers and kits shown may not be available in all countries. For additional system applications, contact your local Roche Diagnostics representative.

English

System information
For Roche/Hitachi 912/917/MODULAR P analyzers: ACN 478

Intended use
For the quantitative determination of ammonia (NH_3) in plasma on Roche automated clinical chemistry analyzers.

Summary
In 1859, Berthelot[1] described a reaction between ammonia and an alkaline solution of phenol hypochlorite suitable for the determination of ammonia. The assay, however, proved to be subject to interferences, and several alternatives have been proposed to eliminate the problems inherent in the method.
In 1963, Kirsten et al. introduced an enzymatic method for ammonia determination based on the action of glutamate dehydrogenase.[2] Although the enzymatic method proved to be highly specific and utilized direct evaluation based on the molar absorptivity of NADH, several problems, including difficulties in stabilizing the end reaction, were encountered.
The method presented here is based on Da Fonseca-Wollheim's modification of the Kirsten reaction.[3] The original enzymatic method is improved by the addition of ADP to the reaction mixture, the use of NADPH in place of NADH to eliminate interference from the reaction of endogenous LDH with endogenous pyruvate, and the substitution of plasma for deproteinized supernatant.
Ammonia measurements are used in the diagnosis and treatment of severe liver disorders such as cirrhosis, hepatitis and Reye's Syndrome.

Test principle
Enzymatic kinetic assay
In the reaction catalyzed by glutamate dehydrogenase (GLDH), ammonia reacts with α-ketoglutarate and NADPH to form glutamate and $NADP^+$.

- Sample and addition of R1
- Addition of R2 and start of reaction:

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADPH \xrightarrow{GLDH} \text{L-glutamate} + NADP^+ + H_2O$$

The inclusion of ADP in the reaction mixture causes an acceleration of the rate of conversion and stabilizes the GLDH in the indicated pH range.
The amount of NADPH oxidized during the reaction is equivalent to the amount of ammonia in the specimen and can be measured photometrically by the resulting decrease in absorbance.[4,5]

Reagents - working solutions
R1 Triethanolamine buffer: 151 mmol/L, pH 8.6; α-ketoglutarate: 16.6 mmol/L; ADP: ≥ 1.2 mmol/L; preservatives
R2 NADPH: ≥ 458 µmol/L; GLDH (EC 1.4.1.3; bovine liver; 25 °C): ≥ 24.3 U/mL; triethanolamine buffer: 151 mmol/L, pH 8.6; α-ketoglutarate: 16.6 mmol/L; ADP: ≥ 1.2 mmol/L; preservatives

Precautions and warnings
For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

Reagent handling
R1: Ready for use.
R2: Step 1: Reconstitute the contents of one bottle 2b with 0.5 mL of reagent from bottle 2a. Let stand for 10 minutes at room temperature, occasionally swirling gently.
Step 2: Reconstitute contents of one bottle 2 with 4.5 mL of reagent from bottle 2a. Mix by gentle inversion.
Step 3: Add 150 µL of reconstituted reagent from bottle 2b to contents of bottle 2 from step 2. Mix by gentle inversion. Use adapter to transfer into the R2 empty bottle supplied. Remove any bubbles from the surface of the working solution prior to use.

Storage and stability
Unopened kit components: Up to the expiration date at 2–8 °C.
Reconstituted bottle 2b: 42 days at 2–8 °C when stored tightly closed.
R1: 28 days opened and refrigerated on the analyzer when protected from contamination.
R2: 14 days opened and refrigerated on the analyzer when protected from contamination.

Specimen collection and preparation
For specimen collection and preparation, only use suitable tubes or collection containers.
Only the specimens listed below were tested and found acceptable.

EDTA plasma (free from hemolysis and lipemia)

IMPORTANT
Do not use plasma prepared with other anticoagulants.
Do not use serum since ammonia can be generated during clotting.

Collect blood from stasis-free vein of fasting patient. Smoking should be avoided prior to sampling. Tubes should be filled completely and kept tightly stoppered at all times. Place immediately on ice and centrifuge, preferably at 4 °C. Perform analysis within 20 to 30 minutes of venipuncture.

Avoid ammonia contamination of samples with smoke or traffic fumes from e.g. laboratory, patient's room, glassware or water.

Ammonia concentrations can increase in vitro due to breakdown of nitrogen-containing plasma components. One known source of spontaneous ammonia formation is an increased γ-glutamyltransferase activity leading to decomposition of glutamine.[6]

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Materials provided
- See "Reagents - working solutions" section for reagents.

12132567001V11

CK

Creatine Kinase liquid, acc. to IFCC

cobas®

● Indicates Roche/Hitachi analyzer(s) on which kit(s) can be used

Cat. No.	Bottle	Contents	902	912	917	MODULAR P	MODULAR D
12132672 216	1 2	REAGENT 6 x 60 mL REAGENT 6 x 15 mL			●	●	
04580591 190 **04580613** 190	1 2	REAGENT 6 x 250 mL REAGENT 6 x 63 mL				●	●
12132524 216	1 2	REAGENT 12 x 22 mL REAGENT 6 x 10 mL	●	●			
12132605 216	1 2	REAGENT 6 x 100 mL REAGENT 3 x 46 mL		●			

Some analyzers and kits shown may not be available in all countries. For additional system applications, contact your local Roche Diagnostics representative.

English

System information
For Roche/Hitachi analyzers: ACN 057.

Intended use
In vitro assay for the quantitative determination of creatine kinase (CK) in human serum and plasma on Roche automated clinical chemistry analyzers.

Summary[1,2,3,4,5,6,7,8,9]
Creatine kinase (CK) is a dimeric enzyme occurring in four different forms: a mitochondrial isoenzyme and the cytosolic isoenzymes CK-MM (skeletal muscle type), CK-BB (brain type) and CK-MB (myocardial type).
The determination of CK and CK-isoenzyme activities is utilized in the diagnosis and monitoring of myocardial infarction and myopathies such as the progressive Duchenne muscular dystrophy. Following injury to the myocardium, such as occurs with acute myocardial infarction, CK is released from the damaged myocardial cells. In early cases, a rise in the CK-activity can be found just 4 hours after an infarction. The CK-activity reaches a maximum after 12–24 hours and then falls back to the normal range after 3–4 days.
The assay method using creatine phosphate and ADP was first described by Oliver, modified by Rosalki and further improved for optimal test conditions by Szasz. CK is rapidly inactivated by oxidation of the sulfhydryl groups in the active center. The enzyme can be reactivated by the addition of acetylcysteine (NAC). Interference by adenylate kinase is prevented by the addition of diadenosine pentaphosphate and AMP.
Standardized methods for the determination of CK using the "reverse reaction" and activation by NAC were recommended by the German Society for Clinical Chemistry (DGKC) and the International Federation of Clinical Chemistry (IFCC) in 1977 and 1990 respectively. In 2002 the IFCC confirmed their recommendation and extended it to 37 °C.[8] The method described here is derived from the formulation recommended by the IFCC and was optimized for performance and stability.

Test principle[7,9]
UV-test
- Sample and addition of R1
- Addition of R2 and start of reaction:

$$\text{creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{creatine} + \text{ATP}$$

$$\text{glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-P} + \text{ADP}$$

$$\text{glucose-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{gluconate-6-P} + \text{NADPH} + \text{H}^+$$

Equimolar quantities of NADPH and ATP are formed at the same rate. The photometrically measured rate of formation of NADPH is directly proportional to the CK-activity.

Reagents - working solutions
R1 Imidazole buffer: 123 mmol/L, pH 6.5 (37 °C); EDTA: 2.46 mmol/L; Mg^{2+}: 12.3 mmol/L; ADP: 2.46 mmol/L; AMP: 6.14 mmol/L; diadenosine pentaphosphate: 19 µmol/L; NADP (yeast): 2.46 mmol/L; N-acetylcysteine: 24.6 mmol/L; HK (yeast): ≥ 36.7 µkat/L; G6P-DH (E. coli): ≥ 23.4 µkat/L; preservative; stabilizer; additive.

R2 CAPSO[a] buffer: 20 mmol/L, pH 8.8 (37 °C); glucose: 120 mmol/L; EDTA: 2.46 mmol/L; creatine phosphate: 184 mmol/L; preservative; stabilizer; additive.

a) CAPSO: 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid

Precautions and warnings
For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Safety data sheet available for professional user on request.
Disposal of all waste material should be in accordance with local guidelines.

Reagent handling
R1: Ready for use
R2: Ready for use

Storage and stability
Unopened kit components: Up to the expiration date at 2–8 °C. Do not freeze.
R1: 28 days opened and refrigerated on the analyzer
R2: 28 days opened and refrigerated on the analyzer

Specimen collection and preparation
For specimen collection and preparation, only use suitable tubes or collection containers.
Only the specimens listed below were tested and found acceptable.
Serum
Plasma: Heparin (Li-, NH_4^+) or EDTA (K_3-) plasma.
Do not use other anticoagulants.
The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer. Centrifuge samples containing precipitates before performing the assay.

Please note: Differences in the degree of hemolysis resulting from the blood sampling procedure used can lead to deviating results in serum and plasma.

Stability:[10] 2 days at 15–25 °C
7 days at 2–8 °C
4 weeks at (-15)–(-25) °C

Materials provided
See "Reagents - working solutions" section for reagents.

Materials required (but not provided)
- Calibrator: C.f.a.s. (Calibrator for automated systems), Cat. No. 10759350 190, 10759350 360 (for USA)
- Controls: Precinorm U, e.g. Cat. No. 10171743 122 or Precinorm U plus, Cat. No. 12149435 122, 12149435 160 (for USA); Precipath U, e.g. Cat. No. 10171778 122 or Precipath U plus, Cat. No. 12149443 122, 12149443 160 (for USA)
- 0.9 % NaCl
- General laboratory equipment