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Malondialdehyde (MDA) and Glutathione Peroxidase (GPx) are Elevated in Crohns Disease-Associated with Mycobacterium Avium Subspecies Paratuberculosis (MAP)

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MALONDIALDEHYDE (MDA) AND GLUTATHIONE PEROXIDASE (GPX) ARE ELEVATED IN CROHN'S DISEASE-ASSOCIATED WITH MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP)

by:

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for the degree of Master of Science in Biotechnology
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ABSTRACT

Inflamed tissue in Crohn's disease (CD) are continuously producing toxic oxygen metabolites leading to cellular injury and apoptosis. Here, we are evaluating the role of Mycobacterium avium subspecies paratuberculosis (MAP) in oxidative stress in CD by evaluation of lipid peroxidation and antioxidant defense activity. Specifically, we measured malondialdehyde (MDA) level and selenium-dependent glutathione peroxidase (GPx) activity in the plasma from patients and cattle infected with MAP. The level of MAP antibodies in bovine sera was determined by IDEXX kit whereas detection of MAP DNA was performed by IS900-based nPCR. A total of 42 cattle (21 infected with MAP and 21 healthy controls), 27 CD subjects, 27 of CD-healthy relatives, 66 subjects with various diseases and 34 non-related healthy subjects were investigated. Overall, GPx activity was significantly higher in MAP infected humans (0.80941±0.521) versus MAP (-ve) samples (0.42367±0.229 units/ml), $P<0.01$. Similarly, the average of GPx activity in cattle infected with MAP was 1.59±0.65 units/ml compared to 0.46907±0.28 units/ml in healthy cattle ($P<0.01$). Although it was not statistically significant, MDA average level was higher in MAP infected human samples versus MAP (-ve) controls (1.11±0.185 nmol/ml versus 0.805±0.151 nmol/ml, respectively). Similarly, MDA average level in CD samples that are MAP+ (1.703±0.231 nmol/ml) was higher than CD samples that are MAP (-ve) (1.429±0.187 nmol/ml). In cattle, MDA average level in MAP infected samples was significantly higher at 3.818±0.45 nmol/ml compared to 0.538±0.18 nmol/ml in healthy cattle ($P<0.01$). Clearly, the data demonstrated that MAP infection is associated with oxidative stress and resulting in the pathophysiology of worsening of the condition of CD patients.

This is dedicated to the memory of my father, Sa'id Qasem. I miss him every day and he is always in my thoughts. Many thanks to my mother for dealing with the fact that I am worlds away. I am also grateful to my family back home in Jordan and my friends here in the US, who continuously support me while I pursue my life ambitions.

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LIST OF ABBREVIATIONS & ACRONYMS

CD: Crohn's Disease.

DM: Diabetes Mellitus.

GPx: Glutathione Peroxidase.

IBD: Inflammatory Bowel Disease.

MAP: *Mycobacterium avium* subspecies *paratuberculosis.*

MDA: Malondialdehyde.

nPCR: Nested Polymerase Chain Reaction.

PCR: Polymerase Chain Reaction.

ROS: Reactive Oxygen Species.

TE: Tris-ethylenediaminetetraacetic Acid.

TNF-α: tumor Necrosis Factor α.

CHAPTER ONE: INTRODUCTION

Note: This section has been published in part and the citation link is: Qasem, Ahmad, Ahmad Abdel-Aty, Huda Abu-Suwa, and Saleh A. Naser. "Oxidative stress due to Mycobacterium avium subspecies paratuberculosis (MAP) infection upregulates selenium-dependent GPx activity." *Gut pathogens* 8, no. 1 (2016): 1.

Mycobacterium avium subspecies *paratuberculosis* (MAP) is implicated in the etiology of multiple diseases including Crohn's disease (CD) and both type I and type II diabetes mellitus (DM) in humans [1][2]. It is also known to be a causative agent of Johne's disease, a bovine disease similar to CD in humans [3]. MAP is an obligate intracellular pathogen, living inside the macrophages of the infected host [4]. MAP increases the suitability of the macrophage as a host and prevents its own destruction by preventing the acidification of the phagosome [5][6]. This is done by preventing the fusion of the lysosome and the phagosome into the phagolysosomal complex [5][6]. MAP is also resistant to destruction even in an acidified, mature phagolysosome [7]. The primary mechanism for the destruction of MAP resistant to phagolysosomal degradation is the induction of apoptosis of the infected macrophage through a tumor necrosis factor α (TNF-α) dependent mechanism [8][9]. There is evidence that *Mycobacteria* evade this host response by inhibiting apoptosis, and by stimulating necrosis, which allows the bacteria to disseminate [10][11]. Furthermore, in an active infection the body's ability to clear apoptotic cells may be outpaced. The delay in clearance results in the apoptotic cell bodies losing their membrane integrity and becoming secondary necrotic cells [12]. In the case of the apoptosis of an active macrophage, this includes the leaking of lysosomal content. This includes reactive oxygen species (ROS), which leads to inflammation and oxidative stress.

Selenium is an important trace element that has many biological functions, particularly through its incorporation into multiple selenoproteins. There are 25 such proteins in humans [13].

One of these proteins is glutathione peroxidase (GPx), an antioxidant enzyme found in all eukaryotes. This enzyme uses glutathione to reduce hydrogen peroxide, lipid peroxides, and hydroperoxides [14]. Though selenium has been shown to have insulin mimetic properties, elevated selenium has been associated with diabetes [15][16][17]. This is possibly due to the selenium found in GPx. Elevated GPx, as well as the corresponding elevation in selenium, is associated with type I diabetes [18]. GPx has also recently been implicated in the pathophysiology of type II diabetes. McClung et al. found that overexpression of GPx in mice resulted in the development of hyperinsulinemia, hyperglycemia, and decreased insulin sensitivity, all of which are indicators of type II diabetes [19]. Though the mechanism of this is poorly understood, they proposed that excessive GPx quenched peroxides too quickly, resulting in less ROS-mediated inhibition of protein-tyrosine phosphatases [19]. Inhibition of these phosphatases, which dephosphorylate insulin receptors, increases insulin sensitivity [20]. Xi Yan et al. found that decreasing selenium intake in mice overexpressing GPx decreases the hyperinsulinemia, hyperglycemia, and insulin resistance caused by the elevated GPx expression [21]. Excessive dietary selenium, on the other hand, has been found to upregulate GPx and result in higher insulin resistance [22][23]. A similar trend was found in humans [24]. Selenoproteins are also upregulated as a result of oxidative stress [25].

The inflamed mucosal tissues in Crohn's disease patients have overactive inflammatory cells, which are subsequently producing toxic oxygen metabolites such as hydrogen peroxide and nitric oxide leading to impairment of cellular membrane integrity and finally cell death occurs by lipid peroxidation [36][37]. Studies have shown that oxidative stress levels are elevated and antioxidant defenses are declined in intestinal tissues of patients with inflammatory bowel

disease (IBD) [38]. On the other hand, Bhasker et al. did not find any difference in antioxidant enzymes activities or lipid peroxidation products in the mucosa of IBD patients and controls [39].

The association between MAP infection and oxidative stress is poorly understood. The objective in our studies was to assess the potential association between MAP infection and the activity of selenium-dependent GPx and quantifying the lipid peroxidation product malondialdehyde (MDA) in bovine and human samples. We hypothesize that the oxidative stress caused by MAP infection will result in elevated GPx activity and elevate MDA production. The oxidative stress associated with MAP infection may be clinically relevant to diseases including CD and diabetes.

CHAPTER TWO: MATERIALS AND METHODS

Note: This section has been published in part and the citation link is: Qasem, Ahmad, Ahmad Abdel-Aty, Huda Abu-Suwa, and Saleh A. Naser. "Oxidative stress due to Mycobacterium avium subspecies paratuberculosis (MAP) infection upregulates selenium-dependent GPx activity." *Gut pathogens* 8, no. 1 (2016): 1.

Bovine Samples

Sera samples from healthy and MAP infected cattle were kindly provided by Dr. Michael Collins (University of Wisconsin). Bovine samples were confirmed for MAP infection using the IDEXX *Mycobacterium paratuberculosis (M. pt.)* Antibody Test Kit (IDEXX Laboratories, Westbrook, ME, USA) following manufacturer instructions. A S/P less than or equal to 0.60 was considered negative and a S/P greater than or equal to 0.70 was considered positive. Sera from 21 MAP infected cattle and 21 healthy cattle were then included in this study.

Human Samples

Sample Processing

Human blood samples were collected in two separate sets where each subject provided three 6.0-mL K_2-EDTA tubes. All clinical samples were collected following Institutional Review Board approval. A total of 27 human blood samples were collected from CD patients along with 27 samples of their healthy biological family members (parents or siblings), those samples were collected at the University of Florida (UF). An additional randomized 100 blood samples used in earlier studies were also included. Clinical samples were collected blindly with no prior knowledge of MAP diagnosis or other health conditions. Buffy coat preparations and plasma samples were separated and stored at -20^{o} C.

Table 1: Demographics of Crohn's patients and healthy relatives.

Group	*Age range*	*Average age*	*Gender ratio (M/F)*
Relatives	12-65	45	9/18
Crohn's	16-56	32	8/19

DNA extraction and nested PCR analysis

DNA extraction for PCR analysis was performed on purified buffy coat samples. Each sample was re-suspended in 100 µL of TE buffer and then incubated at 100 °C for 30 minutes. The re-suspended solution was then placed in an ice bath for 15 minutes, after which it was centrifuged for 10 minutes at 4°C at 12,000 rpm (18500 g). After centrifugation, the supernatant was extracted in 200 µL of phenol/chloroform/isoamyl alcohol (1:1:24 v/v; Acros Organics, Morris Plains, NJ, USA) was added. The solution was mixed and centrifuged for five minutes at 4°C at 12,000 rpm (18,500g). The pellet, containing the nucleic acid, was then washed, dried, and re-suspended in 50 µL of sterile water [3].

Detection of MAP DNA using nested PCR (nPCR) was based on the MAP-specific IS900 derived oligonucleotide primers [3]. As shown in Table 2, P90 and P91 primers were used for the amplification of 398 bp in the first round of amplification and AV1 and AV2 primers, were used to amplify a 298 bp internal sequence. Each primary PCR reaction used 10 µL of DNA template and 40 µL of PCR buffer, which consists of 5 mM MgCl2, 0.2 mM dNTP, 2 µM primers, and 2.5 U Platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA) or 1 U TFL DNA polymerase (Promega, Madison, WI, USA). Each secondary round of PCR used the same ingredients, except different primers were used and 5 µL of the product of the primary round was

used instead of the DNA template. Negative controls for the PCR were prepared in which sterile water or TE buffer was added instead of the DNA template (in the primary amplification) or the primary product (in the secondary amplification). These negatives were prepared in parallel with the samples. Positive controls were also prepared using MAP DNA from strain ATCC 43015. The amplification product size was assessed on 2% agarose gel.

Table 2: Primers and amplification conditions used for PCR.

Primer	*Oligonucleotide sequence (5'-3')*	*Gene*	*Amplification conditions*	*Product size (bp)*	*Reference*
P90, P91	GTTCGGGGCCGTCGCTTAGG, GAGGTCGATCGCCCACGTGA	IS900	95°C for 5 min, then 34 cycles of 95°C for 1 min, 58°C for 1.5 min, 72°C for 1.5 min. Final extension of 10 min at 72°C.	398	Naser et al. [3]
AV1, AV2	ATGTGGTTGCTGTGTTGGATGG, CCGCCGCAATCAACTCCAG	IS900	95°C for 5 min, then 34 cycles of 95°C for 1 min, 58°C for 1.5 min, 72°C for 1.5 min. Final extension of 10 min at 72°C.	298	Naser et al. [3]

Selenium-dependent Glutathione Peroxidase (Gpx) activity measurement

Glutathione peroxidase works by reducing peroxides by oxidizing glutathione. The glutathione is then restored for further cycles of catalysis (Figure 1). The selenium-dependent glutathione peroxidase activity was measured by using the Sigma-Aldrich Glutathione Peroxidase Cellular Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). Glutathione peroxidase works by reducing peroxides by oxidizing glutathione. The glutathione is then restored for further cycles of catalysis (Figure 1). The rate-limiting step of this reaction is that in which the oxidized glutathione used to reduce the peroxide is restored via the oxidation of NADPH. NADPH absorbs at 340 nanometers, the decrease in absorbance over time is determinative of the activity of serum glutathione peroxidase. Tert-butyl hydroperoxide in particular was used because its breakdown depends specifically on selenium-containing glutathione peroxidase.

All reagents were brought to room temperature before beginning the assay. The NADPH was resuspended with sterile water. The glutathione peroxidase standard was re-suspended with assay buffer. A blank was made using 940 µL of assay buffer, 50 µL of NADPH, and 10 µL of tert-butyl peroxide, so that the uncatalyzed breakdown of NADPH could be subtracted from the catalyzed rate. Standards containing 0.05 and 0.125 units of enzyme were made as positive controls to ensure the assay was working. For each sample 930 µL of buffer, 50 µL of NADPH, and 10 µL of sample were mixed. 10 µL of t-butyl hydroperoxide were added last, since its addition initiated the reaction. The absorbance at 340 nm was measured over the course of a minute.

The activity was determined according to the following equation provided by the manufacturer, derived from Beer's Law:

$$\text{Activity} = \frac{\Delta\ \text{Absorbance} * \text{Dilution Factor}}{6.22 * \text{Volume of sample}}$$

Where 6.22 is the extinction coefficient of NADPH in $mL*mmol^{-1}*cm^{-1}$ and ΔAbsorbance is the change in absorbance at 340 nm over the course of one minute, not including the natural rate of NADPH degradation.

Quantification of Lipid Peroxidation product (MDA)

Lipid peroxidation forms malondialdehyde (MDA) and 4-hydroxynonel (4-HNE), as natural biproducts. Measuring MDA in plasma or body tissues is a useful indication of oxidative damage. MDA quantification was done by using Lipid Peroxidation Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, CA, USA). This test is known as TBARS assay. 10uL of plasma was mixed with 500uL H_2SO_4 in a microcentrifuge tube. 125uL of Phosphotungstic acid solution was added into each tube and vortexed. After 5 minutes of incubation, the tubes were centrifuged for 3 minutes at 13,000 X *g.* The pellet was collected and resuspended on ice with 2uL of Butylated hydroxytoluene (BHT 100X). Final volume was adjusted to 200uL with ddH_2O. Standards with different concentrations were prepared from MDA standard solution (4.17M). 600uL of Thiobarbituric Acid (TBA) was added into each tube to generate the MDA-TBA adduct. Tubes were incubated at 95°C for 60 min then cooled down in an ice bath for 10 minutes. 200uL from each reaction 800uL mixture were pipetted into 96-well microplate for analysis. The MDA-TBA adduct was quantified colormetrically (OD 532 nm) by reading absorbance. MDA

standard curve was generated and used to determine the MDA amount in each test sample in nmol/ml. The mechanism of lipid peroxidation is shown in figure 2.

CHAPTER THREE: RESULTS

Note: This section has been published in part and the citation link is: Qasem, Ahmad, Ahmad Abdel-Aty, Huda Abu-Suwa, and Saleh A. Naser. "Oxidative stress due to Mycobacterium avium subspecies paratuberculosis (MAP) infection upregulates selenium-dependent GPx activity." *Gut pathogens* 8, no. 1 (2016): 1.

MAP Prevalence in Human Samples

We performed nPCR on DNA extracts isolated from all human blood samples in order to analyze for the presence of MAP-specific IS900 gene according to Naser et al. protocol [3]. The overall prevalence of MAP among 154 human blood samples was 32%. MAP was positive in the blood of 40% of CD patients compared to 29.9% in non-CD patients. Specifically MAP was also positive in 11/27 (40%) of CD patients and in 2/27 (7%) in healthy biological family members. Interestingly, 33% (7 out of 21) of patients with type II diabetes and 44% (7 out of 16) pre-diabetic patients were also MAP positive. Patients were considered to be pre-diabetic if they had a fasting blood sugar level between 100 and 125 mg/dl, if the two-hour glucose levels was between 140 and 199 mg/dl in an oral glucose tolerance test, or if they had a glycated hemoglobin (A1C) level between 5.7 and 6.4. Figure 3 illustrates the detection of MAP IS900 gene on 2% agarose gel following nPCR analysis of 100 randomized human blood samples (lanes 1 to 100).

Selenium-dependent GPx levels were elevated in MAP infected bovine samples

Bovine sera were confirmed for presence of anti-MAP IgG. Consequently, a total of 21 cattle sera samples from animals diagnosed with Johne's disease (MAP positive) and 21 sera from healthy cattle (MAP negative) were selected for the study. All 42 sera were analyzed for of

GPx activity. The average level of GPx was 0.46907±0.28 units/ml in healthy cattle sera control compared to 1.590±0.65 units/ml in sera from cows infected with MAP, where a unit was defined as one mmol/minute. The MAP positive samples had a significantly higher activity level, with a difference in means of 1.122 (95% confidence interval 0.810 to 1.435; $P < 0.01$) [Table 3]. Figure 4a shows a scatter plot of selenium-dependent GPx activity for MAP negative and MAP positive samples.

Selenium-dependent GPx activity was elevated in MAP infected humans among Crohn's patients and their healthy relatives

The average level of GPx activity was 0.80941±0.521 units/ml in the MAP positive samples, while the average enzyme activity in MAP negative samples was found to be 0.42367±0.229 units/ml. This result reveals that MAP infection has a significant influence on GPx activity, with a difference in means of 0.387 (95% confidence interval 0.182 to 0.592; $P < 0.01$) [Figure 4b].

The difference between selenium-dependent GPx activity in Crohn's Disease and in Healthy individuals was not significant

In order to confirm that the elevation of GPx activity level was due to MAP infection alone, and not due to CD status, we measured the average of GPx activity in healthy individuals and CD patients separately. The average GPx activity was found to be 0.54±0.414 units/ml and 0.493±0.301 units/ml in CD and healthy patients respectively. While the mean GPx enzymatic activity in CD patients was higher by 0.0469, our results showed that there was no significant difference between both groups (95% confidence interval -0.245 to 0.151; $P= 0.636$) [Figure 4c].

Selenium-dependent GPx activity was elevated in MAP infected Crohn's patients:

As mentioned earlier, out of 27 CD patients, a total of 11 were tested as MAP positive, while 16 were MAP negative. The average GPx activity in CD patients who had the MAP infection was 0.7593±0.537 units/ml, while the GPx activity was found to be 0.389±0.213 units/ml in CD patients without MAP infection. The difference in means was 0.37 (95% confidence interval 0.07 to 0.675; P = 0.019). (P value = 0.019) [Figure 4d]. Furthermore only 2 of the 27 healthy relatives used as controls, or 7.4%, were infected with MAP.

Selenium-dependent GPx activity was elevated among MAP infected humans in randomized field study

Among randomized blood samples from 100 subjects, 36 were determined to be MAP positive as shown in Figure 3. The average of GPx activity level in 36 MAP positive clinical samples was 0.6510±00.1665 units/ml compared 0.4702±0.1299 in 64 MAP negative clinical samples ($P < 0.01$) [Table 3]. The GPx activity in each clinical sample is illustrated in Figure 4E. We further examined the difference in GPx activity according to disease diagnosis, but there was no significant difference in MAP negative clinical samples between healthy controls and subjects with diseases.

It is notable, however, that in all disease states MAP positive individuals still have higher enzymatic activity than MAP negative individuals [Figure 5].

Table 3: GPx Enzyme Average Activity and MAP presence in Bovine and Human Blood Samples.

Number of Samples/Total	*Source*	*MAP Diagnosis*	*Average GPx Activity (Units/ml)*	*P value*
21/42	Bovine	Negative	0.469±0.28	P< 0.01
21/42	Bovine	Positive	1.590±0.65	
105/154	Human	Negative	0.452±0.176	P< 0.01
49/154	Human	Positive	0.693±0.30	
16/27	CD Patients	Negative	0.389±0.213	P<0.05
11/27	CD Patients	Positive	0.7593±0.537	

MDA levels are significantly elevated in MAP infected bovine samples

Sera from 21 cattle infected with MAP and 21 healthy cattle were analyzed for the level of MDA. The average level of MDA was 0.538±0.18 nmol/ml in healthy cattle sera control compared to 3.818±0.45 nmol/ml in sera from cows infected with MAP. The MAP positive samples had a significantly higher MDA level ($P < 0.05$) [Figure 6]. This result shows higher level of lipid peroxidation in cattle infected with MAP.

MDA levels are elevated in Crohn's patients:

The average level of MDA in MAP positive CD patients was 1.703±0.231 nmol/ml, while the average MDA level in MAP negative CD patients was found to be 1.429±0.187 nmol/ml. The MDA average level in the healthy relatives was 0.527±0.158 nmol/ml. This shows significant elevation of lipid peroxidation in CD patients in comparison to healthy subjects ($P < 0.05$). However there was no significant difference ($P > 0.05$) among CD patients according to MAP

Infection but it is still slightly elevated in MAP positive samples [Figure 6]. We also included 20 healthy controls in this study and we found MDA average level to be 0.562 nmol/ml. Table 4 illustrates the average MDA level between different group sets.

Table 4: MDA Average level in Bovine and Human Samples.

Number of Samples	*Sample source*	*Diagnosis and MAP status*	*MDA average level (nmol/ml)*	*P value*
21	Bovine	Johne's/MAP +	3.818±0.451	$P < 0.05$
21	Bovine	Johne's/MAP -	0.538±0.182	
11	Human	CD/MAP +	1.703±0.231	$P > 0.05$
16	Human	CD/MAP -	1.429±0.187	
35	Human	Healthy/MAP -	0.521±0.135	$P > 0.05$
12	Human	Healthy/MAP +	0.567±0.126	

CHAPTER FOUR: DISCUSSION

Note: This section has been published in part and the citation link is: Qasem, Ahmad, Ahmad Abdel-Aty, Huda Abu-Suwa, and Saleh A. Naser. "Oxidative stress due to Mycobacterium avium subspecies paratuberculosis (MAP) infection upregulates selenium-dependent GPx activity." *Gut pathogens* 8, no. 1 (2016): 1.

Oxidative stress and the resulting GPx up-regulation may have significant implications on disease pathophysiology. In particular, long-term up-regulation of GPx may cause insulin resistance and disruptions in insulin signaling [19]. Furthermore, oxidative damage has recently been an area of focus for research into inflammatory conditions such as CD and inflammatory bowel disease (IBD). Despite this, the relationship between MAP infection and oxidative stress has not been clearly established. The purpose of our study was therefore to elucidate the relationship between MAP infection and GPx activity in plasma. This makes it possible to find the differences in oxidative stress level in CD patients with the presence and absence of MAP infection, which may have implications on the treatment of these patients. We acquired bovine and human samples, tested them for MAP infection, and measured their GPx activity.

We found that the enzymatic activity of GPx was significantly higher in cows as well as among two separate groups of human subjects that are infected with MAP, where significance was defined as $P<0.05$. This matched the expected trend. We chose to keep the data for the two cohorts of human patients separate, as the samples for these two groups were collected separately and for different reasons. The CD patients and their healthy relatives were used primarily to determine if the observed trend was caused by CD status, or if it was caused by MAP infection. We propose to exclude CD as a potential cause of the observed trend due to the previously established relationship between MAP and CD [1][3][26-29]. The biological relationships present in this group was ideal for determining the effects of CD, as any observable difference in

GPx activity could be attributed to the disease status, rather than hereditary environmental factors. However, in determining the effect of MAP on GPx activity, we determined that a randomized study would be preferable, and would better reflect the prevalence of MAP in subjects at large. It is worth noting, however, that when all human clinical samples were combined, MAP positive samples still had significantly elevated GPx activity when compared to MAP negative samples ($P < 0.01$).

We found that there was there no significant difference in GPx activity in MAP negative samples from CD and non-CD samples. However, GPx activity was elevated in all clinical samples which were positive for MAP regardless of the source of samples. While it was surprising that an inflammatory condition like CD has no significant effect on GPx, this makes a stronger case that MAP infection may be playing a role in the elevation in enzymatic activity. We further excluded CD status as a potential confounding factor in the human samples by comparing CD patients infected with MAP with those not infected with MAP. We found that there was still a significant correlation between MAP infection and GPx even when CD status was controlled for ($P<0.05$).

The prevalence of MAP in CD patients was 40% compared to 7.4% MAP in healthy relatives ($P < 0.01$), indicating that the CD patients were more susceptible to MAP infection. This is consistent with published reports [3, 26-28, 30-32]. This study is the first to investigate the association of MAP and CD did using healthy relatives as controls. Our findings strengthen the growing body of literature supporting the correlation between MAP and CD. Of course the role of MAP in CD etiology remains debated but genetic susceptibility especially single nucleotide polymorphism in key genes in patients with CD may promote MAP infection [3, 30-32].

The pathogenesis mechanism of CD involves interaction between environmental agents, genetic and immunologic abnormalities [33]. Recently, the role of reactive oxygen species has been an area of interest to study IBD pathophysiology [34]. Granulocyte accumulation is increased in the gut mucosa where inflammation is active in IBD patients and those cells secrete different inflammatory mediators [33]. It has been shown that mucosal inflammation impairs antioxidant defense and the tissues become more liable to oxidative damage [35]. Increasing GPx antioxidant activity is a result of elevation in free radical levels. It is unknown if granulocyte accumulation is increased in CD patients who are infected with MAP in comparison to CD patients who are not. This will lead to different phenotypes of CD patients who are MAP positive or negative with differences in oxidative stress and free radical levels in according to the disease state which will be reflected in the clinical status of those patients.

We also considered smoking, gender, and age as potential confounding factors. There was no significant difference in GPx activity between males and females or smoking habits. The GPx enzymatic activity of patients above 40 was overall lower, but the difference was not significant. None of these factors proved to have an effect on our data.

None of the studies took MAP infection status into consideration when they measured lipid peroxidation or antioxidant enzymes in CD patients. MAP infection causes systemic inflammation and oxidative stress. It is possible that free radicals production is up-regulated after MAP infection. This will be reflected in elevation of lipid peroxides level including MDA.

In our studies we found significant elevation of MDA level in bovine sera infected with MAP in comparison to healthy ones. We chose bovine as our animal model since Johne's disease shares very similar signs and symptoms to CD in humans. In addition to that, there are less confounding factors in bovine samples, as they are fed with a similar diet and they are living in

similar conditions, as opposed to humans in which these factors might vary widely. Thus, cows may be considered a pure model for CD and MAP studies, and the results from the bovine samples may be more representative than human ones. MAP is also under extensive investigation as a causative agent of CD in humans. Elevation of lipid peroxides is an indication of oxidative stress.

We measured MDA level in plasma of 27 CD patients with both MAP positive and negative status. MDA level was significantly elevated in those patients ($P < 0.05$) when we compared them to their 27 healthy biological relatives and 20 healthy nonrelated controls. Despite this fact, MDA in CD patients was still within normal range (< 2 nmol/ml) which could be justified as lipid peroxidation in those patients is contained. This might be due to medications that CD are continuously taking such as sulfasalazine which works as free radicals scavenger. We did not find any significant difference in MDA level between CD patients when MAP status was considered as a factor. However, MDA was slightly elevated in CD patients infected with MAP ($P > 0.05$).

While significant in both human and bovine samples, the difference in average GPx activity between MAP infected and non-infected samples was much more extreme in the bovine samples. This is possibly because bovine are capable of a more robust response, or because they have a larger bacterial load, than humans. There are also less confounding factors in bovine samples, as cattle used as livestock are fed similar diets and live in similar conditions, as opposed to humans in which these factors vary widely. As such, cows may be a purer model for study, and the results from the bovine samples may be more representative.

The unique progression of MAP infection causes systemic inflammation and oxidative stress. It is possible that GPx production is up-regulated in order to compensate for this. Though

on the short term this may offset the negative effects of the infection, on the long term, particularly in chronic infections, it may cause its own problems. Future studies will further elucidate the relationship between MAP infection, GPx up-regulation, glucose homeostasis, as well as the clinical status of CD patients.

CHAPTER FIVE: CONCLUSION

The enzymatic activity of selenium dependent glutathione peroxidase was significantly higher in both bovine and human serum samples infected with MAP. We hypothesize that the presence of this bacterium causes systemic inflammation and oxidative stress, which on the long-term may cause disruptions in insulin signaling and have a deleterious effect on insulin sensitivity. [40]

Lipid peroxidation level, which is characterized by measuring MDA in plasma, was significantly higher in both bovine infected with MAP and CD patients. During this process, MAP infection could be involved in the pathophysiology of worsening the condition of CD patients who are MAP positive when we compare them to MAP negative CD patients. Future studies need to be done on intestinal mucosal tissues of CD patients to confirm the presence of MAP and to compare lipid peroxides production level among different phenotypes of CD.

APPENDIX A: FIGURES

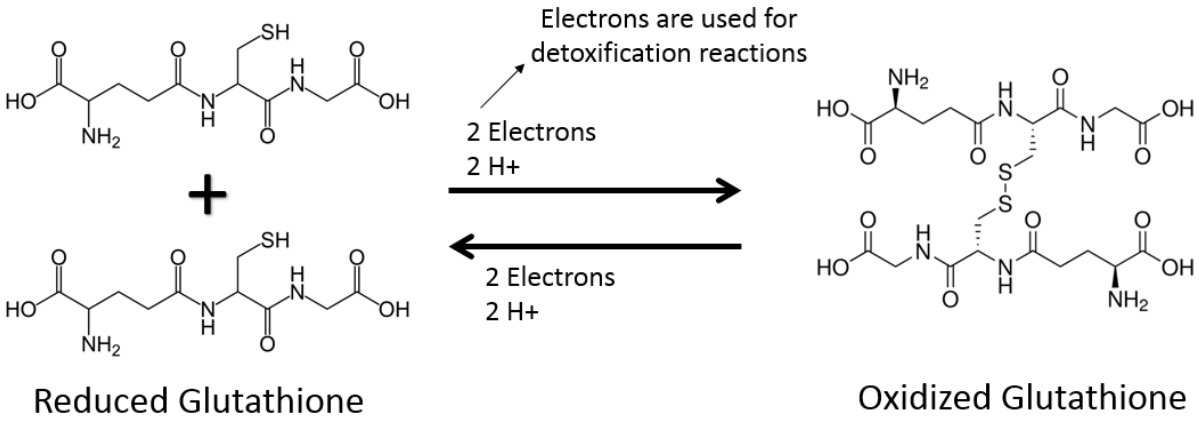

Figure 1: Reduced and oxidized states of glutathione. (Reference 40)

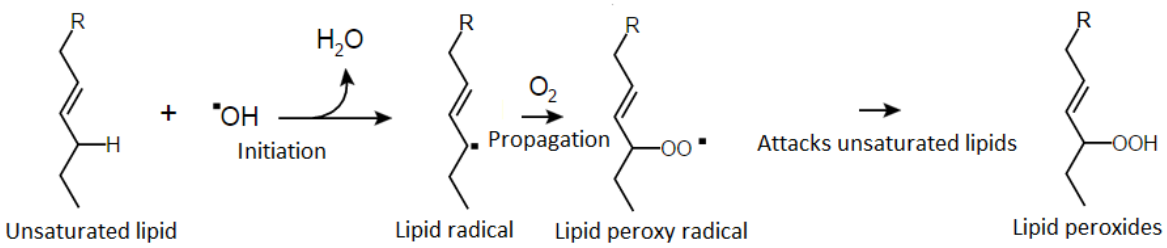

Figure 2: Mechanism of lipid peroxidation.

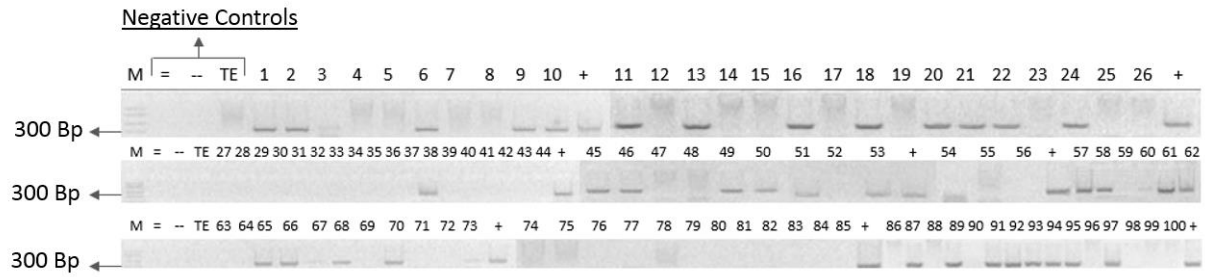

Figure 3: Agarose gels illustrating the presence or absence of MAP-IS900 gene following nPCR.

The PCR products following the second round of nPCR were analyzed on 2% agarose gel. (M): represents molecular weight marker in bp. (=): represents negative control from second round of amplification. (-): represents negative control from first round of amplification. (TE): represents TE buffer negative control. (+): represents positive control prepared from MAP DNA strain ATCC 43015. 1-100: represents patient samples. (Reference 40)

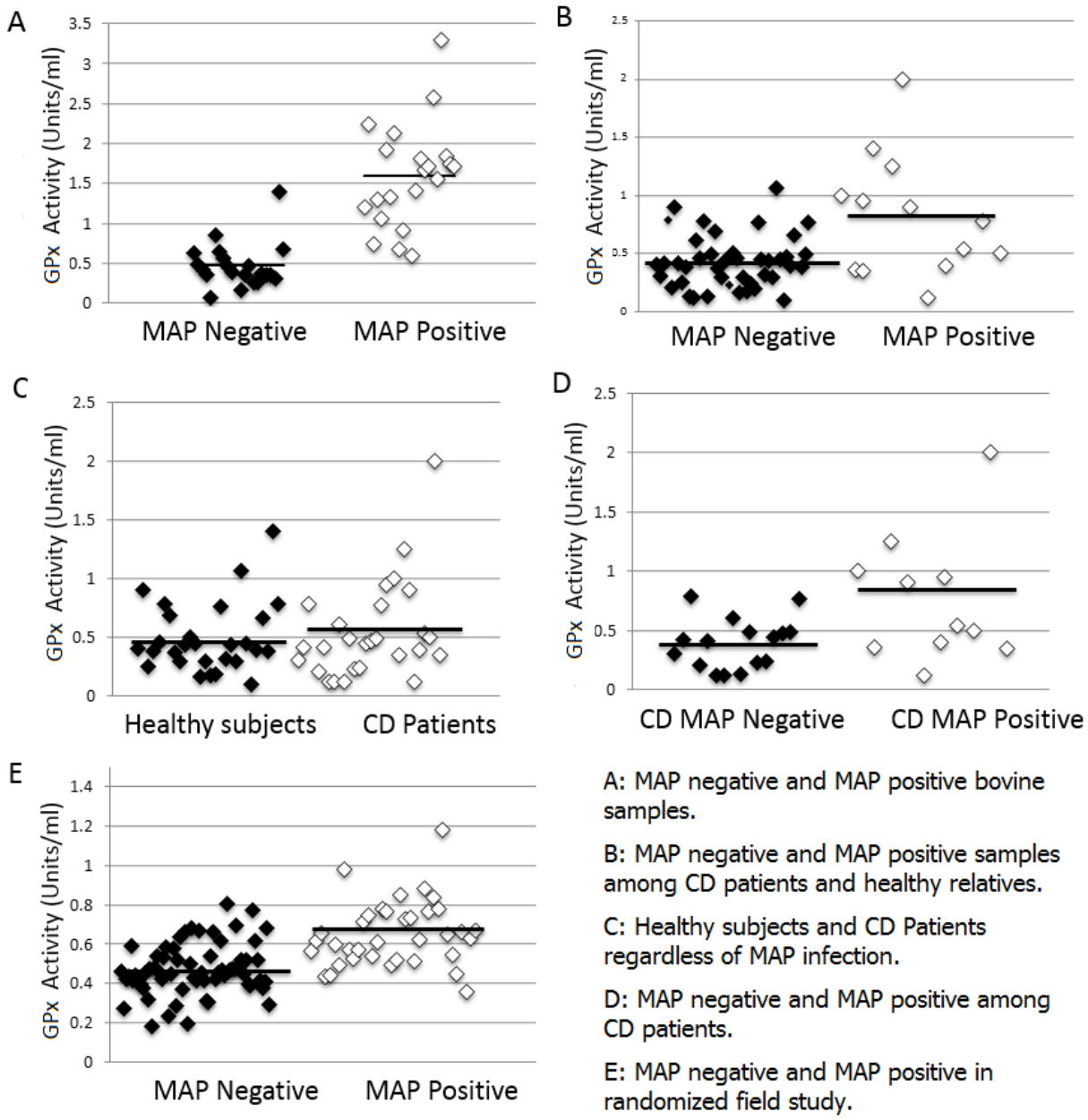

Figure 4: Scatter plot of selenium-dependent GPx activity for all MAP negative and MAP positive samples. (Reference 40)

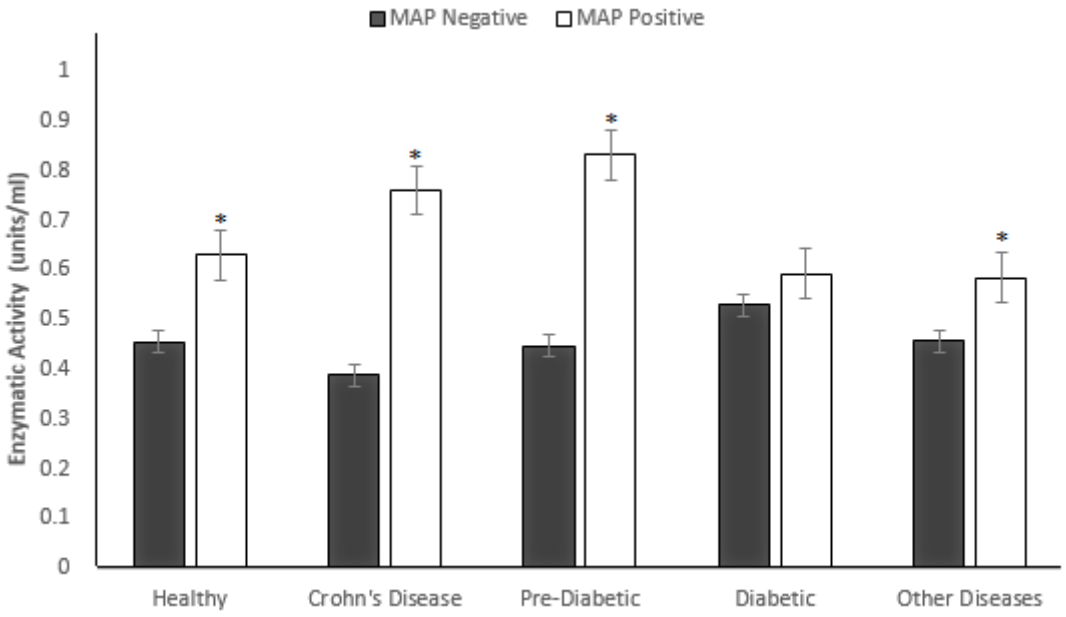

Figure 5: Average GPx activity levels in plasma samples from blood samples identified as MAP negative and positive individuals according to according to disease status. (Reference 40)

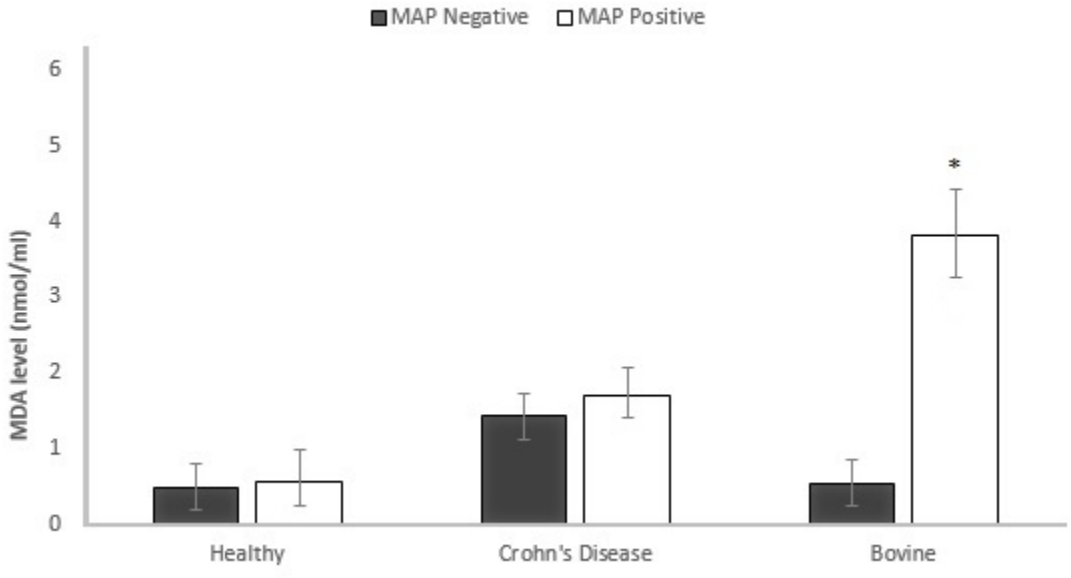

Figure 6: Average MDA levels of MAP negative and positive in human and bovine samples to according to disease status.

APPENDIX B: SUPPLEMENTAL TABLES

Table 5: GPx Enzyme Activity, MDA level and MAP presence in Bovine Samples.

Sample	*MAP Diagnosis*	*GPx Activity (units/ml)*	*MDA level (nmol/ml)*
N1	Negative	0.635	0.714266
N2	Negative	0.485	0.585686
N3	Negative	0.423	0.377979
N4	Negative	0.356	0.38787
N5	Negative	0.069	0.714266
N6	Negative	0.858	0.961536
N7	Negative	0.641	0.585686
N8	Negative	0.562	0.526341
N9	Negative	0.454	0.526341
N10	Negative	0.392	0.437324
N11	Negative	0.167	0.565904
N12	Negative	0.361	0.526341
N13	Negative	0.467	0.50656
N14	Negative	0.262	0.417542
N15	Negative	0.255	0.437324
N16	Negative	0.367	0.496669
N17	Negative	0.362	0.486778
N19	Negative	0.352	0.437324
N20	Negative	0.310	0.51645
N30	Negative	1.392	0.397761
N35	Negative	0.673	0.556014
P2	Positive	1.200	5.906936
P3	Positive	2.233	2.069306
P5	Positive	0.745	2.306685
P6	Positive	1.295	1.673674
P8	Positive	1.059	1.060444
P9	Positive	1.927	5.916827
P15	Positive	1.329	2.207777
P16	Positive	2.127	2.821007
P22	Positive	0.668	0.694485
P24	Positive	0.916	2.277013
P25	Positive	0.588	0.931864
P33	Positive	1.406	0.714266
P38	Positive	1.813	2.999041
P39	Positive	1.670	3.384782
P41	Positive	1.712	13.31515

Sample	*MAP Diagnosis*	*GPx Activity (units/ml)*	*MDA level (nmol/ml)*
P43	Positive	2.575	4.898075
P44	Positive	1.553	7.479574
P45	Positive	3.290	4.779385
P46	Positive	1.847	4.255173
P49	Positive	1.740	3.493581
P50	Positive	1.714	4.947529

Table 6: Age, GPx Enzyme Activity, MDA level, Diagnosis and MAP infection in Crohn's Patients and their healthy relatives.

Sample	*Diagnosis*	*Age*	*MAP diagnosis*	*GPx Activity (Units/ml)*	*MDA level (nmol/ml)*
BF147	Healthy	12	Negative	0.40	0.692
BF156	Healthy	25	Negative	0.90	0.521
BF139	Healthy	28	Negative	0.25	0.568
BF135	Healthy	23	Negative	0.38	0.217
BF163	Healthy	31	Negative	0.456	0.189
BF136	Healthy	31	Negative	0.78	0.513
BF149	Healthy	32	Negative	0.69	0.642
BF154	Healthy	46	Negative	0.37	0.439
BF123	Healthy	48	Negative	0.289	0.385
BF150	Healthy	49	Negative	0.436	0.472
BF158	Healthy	42	Negative	0.497	0.593
BF141	Healthy	59	Negative	0.452	0.233
BF125	Healthy	55	Negative	0.163	0.739
BF126	Healthy	55	Negative	0.289	0.652
BF131	Healthy	57	Negative	0.174	0.581
BF175	Healthy	59	Negative	0.19	0.539
BF134	Healthy	57	Negative	0.315	0.483
BF129	Healthy	61	Negative	0.293	0.591
BF116	Healthy	65	Negative	1.06	0.171

Sample	*Diagnosis*	*Age*	*MAP Diagnosis*	*GPx Activity (Units/ml)*	*MDA Level (nmol/ml)*
BF165	Healthy	62	Negative	0.449	0.647
BF140	Healthy	61	Negative	0.10	0.545
BF128	Healthy	N/A	Negative	0.39	0.513
BF148	Healthy	N/A	Negative	0.66	0.637
BF109	Healthy	N/A	Negative	0.383	0.478
BF159	Healthy	24	Positive	1.40	0.753
BF155	Healthy	40	Positive	0.779	0.672
BF105	Crohn's	19	Negative	0.307	1.147
BF151	Crohn's	16	Negative	0.417	1.492
BF160	Crohn's	18	Negative	0.782	1.389
BF144	Crohn's	19	Negative	0.21	1.423
BF142	Crohn's	28	Negative	0.412	1.336
BF161	Crohn's	27	Negative	0.123	1.421
BF117	Crohn's	31	Negative	0.12	1.548
BF108	Crohn's	34	Negative	0.606	1.587
BF121	Crohn's	33	Negative	0.126	1.563
BF111	Crohn's	33	Negative	0.489	1.237
BF157	Crohn's	43	Negative	0.225	1.531
BF112	Crohn's	51	Negative	0.235	1.592
BF133	Crohn's	56	Negative	0.442	1.283
BF127	Crohn's	N/A	Negative	0.473	1.356
BF113	Crohn's	N/A	Negative	0.487	1.475
BF114	Crohn's	N/A	Negative	0.77	1.492
BF143	Crohn's	19	Positive	0.948	1.852
BF124	Crohn's	20	Positive	1.00	1.741
BF122	Crohn's	20	Positive	0.354	1.344
BF137	Crohn's	35	Positive	1.25	1.471
BF101	Crohn's	32	Positive	0.902	1.905
BF102	Crohn's	38	Positive	0.12	1.656
BF103	Crohn's	44	Positive	0.394	1.703
BF107	Crohn's	44	Positive	0.535	1.846
BF130	Crohn's	52	Positive	0.5	1.466
BF100	Crohn's	N/A	Positive	2.00	1.812
BF110	Crohn's	N/A	Positive	0.35	1.941

Table 7: Age, GPx Enzyme Activity, Diagnosis and MAP infection in field study.

Sample	*Diagnosis*	*Age*	*MAP Diagnosis*	*GPx Activity (Units/ml)*
Sample 1	Healthy	54	Positive	0.5635
Sample 2	Healthy	20	Positive	0.6185
Sample 3	Anxiety, Migraine	46	Negative	0.4625
Sample 4	Pre-diabetes	46	Negative	0.2750
Sample 5	Anemia	47	Negative	0.4200
Sample 6	Thyroid disease	20	Positive	0.6525
Sample 7	Migraine	22	Negative	0.4315
Sample 8	Diabetes Type II, Migraine	63	Negative	0.5900
Sample 9	Diabetes Type II	38	Positive	0.4335
Sample 10	Lung cancer	74	Positive	0.4410
Sample 11	Pre-diabetes	23	Positive	0.7630
Sample 12	Thyroid disease	20	Negative	0.3720
Sample 13	Anemia	31	Positive	0.7800
Sample 14	Anemia	76	Negative	0.5205
Sample 15	Anemia	43	Negative	0.6950
Sample 16	Diabetes Type II	45	Positive	0.4945
Sample 17	Diabetes Type II	38	Negative	0.4135
Sample 18	Diabetes Type II	61	Positive	0.5215
Sample 19	Diabetes Type II	60	Negative	0.6145
Sample 20	Diabetes Type II	63	Positive	0.5440
Sample 21	Diabetes Type II	57	Positive	0.6515
Sample 22	Diabetes Type II	74	Positive	0.6270
Sample 23	Diabetes Type II	43	Negative	0.5820
Sample 24	Diabetes Type II	80	Positive	0.6635
Sample 25	Diabetes Type II	60	Negative	0.4515
Sample 26	Diabetes Type II	63	Negative	0.4500
Sample 27	Diabetes Type II	51	Negative	0.6615
Sample 28	Diabetes Type II	57	Negative	0.5285
Sample 29	Diabetes Type II	63	Negative	0.2900
Sample 30	Diabetes Type II	44	Negative	0.8045
Sample 31	Diabetes Type II	59	Negative	0.4070
Sample 32	Diabetes Type II	43	Negative	0.4215
Sample 33	Diabetes Type II	55	Negative	0.6810
Sample 34	Esophageal cancer	69	Negative	0.3080
Sample 35	Fatty liver	31	Negative	0.4380

Sample	*Diagnosis*	*Age*	*MAP Diagnosis*	*GPx Activity (Units/ml)*
Sample 36	Healthy	28	Negative	0.5000
Sample 37	Healthy	49	Negative	0.1955
Sample 38	Diabetes Type II, Myositis	65	Positive	0.7105
Sample 39	Healthy	16	Negative	0.2315
Sample 40	Healthy	62	Negative	0.3105
Sample 41	Healthy	14	Negative	0.3925
Sample 42	Healthy	47	Negative	0.4550
Sample 43	Healthy	31	Negative	0.1790
Sample 44	Healthy	51	Negative	0.4710
Sample 45	Gout	61	Positive	0.8505
Sample 46	Healthy	49	Positive	0.5140
Sample 47	Healthy	19	Negative	0.3985
Sample 48	Healthy	44	Negative	0.4480
Sample 49	Healthy	21	Positive	0.6125
Sample 50	Urinary Tract Infection	20	Positive	0.5700
Sample 51	Healthy	52	Positive	0.5360
Sample 52	Healthy	45	Negative	0.4185
Sample 53	Healthy	27	Positive	0.7780
Sample 54	Healthy	24	Negative	0.4120
Sample 55	Healthy	62	Negative	0.6700
Sample 56	Healthy	77	Negative	0.5405
Sample 57	Healthy	20	Positive	0.7345
Sample 58	Healthy	30	Positive	0.7435
Sample 59	Healthy	26	Negative	0.4760
Sample 60	Healthy	43	Negative	0.7685
Sample 61	Healthy	52	Positive	0.4905
Sample 62	Healthy	25	Positive	0.4455
Sample 63	Healthy	54	Negative	0.4755
Sample 64	Healthy	51	Negative	0.6350
Sample 65	Healthy	45	Positive	0.7620
Sample 66	Healthy	84	Positive	0.7275
Sample 67	Thyroid disease	37	Negative	0.3770
Sample 68	Healthy	62	Positive	0.6675
Sample 69	Healthy	41	Negative	0.5195
Sample 70	Myositis	52	Positive	0.5940
Sample 71	Healthy	53	Negative	0.6600
Sample 72	Healthy	53	Negative	0.4650
Sample 73	Thyroid disease	19	Negative	0.4770
Sample 74	Healthy	25	Negative	0.4520

Sample	*Diagnosis*	*Age*	*MAP Diagnosis*	*GPx Activity (Units/ml)*
Sample 75	IBS	46	Negative	0.4950
Sample 76	Myositis	44	Negative	0.5800
Sample 77	Pre-diabetes	72	Negative	0.5180
Sample 78	Pre-diabetes	70	Negative	0.3170
Sample 79	Pre-diabetes	38	Negative	0.4685
Sample 80	Pre-diabetes	53	Negative	0.4430
Sample 81	Healthy	20	Negative	0.4125
Sample 82	Pre-diabetes	23	Negative	0.6805
Sample 83	Pre-diabetes	68	Negative	0.5375
Sample 84	Pre-diabetes, Myositis	30	Negative	0.4195
Sample 85	Rheumatoid arthritis, Pre-diabetes	55	Negative	0.3745
Sample 86	Thyroid Disease	32	Negative	0.5170
Sample 87	Pre-diabetes	37	Positive	0.8805
Sample 88	Thyroid disease	59	Negative	0.4275
Sample 89	Pre-diabetes	49	Positive	0.8330
Sample 90	Thyroid disease	17	Negative	0.3895
Sample 91	Pre-diabetes	53	Positive	1.1785
Sample 92	Pre-diabetes	69	Positive	0.5720
Sample 93	Pre-diabetes	----	Positive	0.9810
Sample 94	Pre-diabetes	34	Positive	0.6205
Sample 95	Rheumatoid arthritis	56	Positive	0.3550
Sample 96	Thyroid disease	46	Negative	0.4125
Sample 97	Thyroid disease	52	Positive	0.5265
Sample 98	Thyroid disease	29	Negative	0.6170
Sample 99	Thyroid disease	30	Negative	0.2835
Sample 100	Thyroid disease	27	Negative	0.4565

APPENDIX C: CONSENT FOR PUBLICATION

Consent for Publication

We give our permission to include data and materials described in Qasem A et al, 2016 (below) in the thesis contents of Mr. Ahmad Qasem for his MS degree of science in Biotechnology at the University of Central Florida.

Article Title: "Oxidative stress due to *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection upregulates selenium-dependent GPx activity"

Authors: Ahmad Qasem, Ahmad Abdel-aty, Huda Abu-suwa, and Saleh Naser.

Journal: Gut Pathogens 20168:12 DOI: 10.1186/s13099-016-0090-8

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Co-authors:

1. Saleh Naser 5/25/16
2. Ahmad Abdel-aty 5/25/16
3. Huda Abu-suwa .....

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