Beyond the Antibodies:

Sera Metabolomic Biomarker Signatures

Discriminate Myasthenic and Healthy Cohorts

by

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ABSTRACT

Myasthenia Gravis (MG) is a chronic, potentially debilitating autoimmune disease characterized by weakness and rapid fatigue of the voluntary muscles that worsens on exertion and improves on rest. Left untreated, MG symptoms may cause significant morbidity, affecting occupational performance, social activities, and family life. In severe cases, death may occur. To date, no robust biological marker is available to follow the course of the disease. Therefore, new diagnostic approaches and biological markers are essential not only for improved diagnosis of the disease but for improved outcomes.

The research presented here attempts to provide an alternative biomarker model for the pathogenesis of myasthenia gravis and humoral autoimmune disease in general. The underlying hypothesis was that the metabolomic profile of myasthenia gravis would provide fundamental clues about the functioning of the disease and potential biomarkers to monitor it. The three papers provided have offered proof of concept that metabolomics can profile a disease, differentiate it from other similar diseases and correlate with clinical parameters. These results suggest a new mechanism for the diagnosis and clinical management of myasthenia gravis.

PREFACE

This thesis is an original work by Derrick Blackmore. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Metabolomic Profiling of Serum in Myasthenia Gravis: A Pilot Study”, Pro00030698, Approved May 24, 2012.

D. Blackmore developed the concept and designed the experiment, acquired all blood samples but those from rheumatoid arthritis patients, conducted all databasing, bioinformatics analyses and metabolite-database matching. Further, D. Blackmore prepared all charts, images and tables and wrote the paper. Z. Siddiqi provided project oversight as the clinical expert in myasthenia gravis. Z. Siddiqi also offered conceptual advice, supervised project analysis and edited the manuscript. Liang Li developed the chemical labelling process and several chemometric analysis tools used in this study as well as the chemical identification libraries used in the positive identification of observed metabolites. Liang Li provided oversight in the choice of chemical and statistical techniques used to characterize the metabolome. Liang Li also offered conceptual advice, supervised project analysis and edited the manuscript. Nan Wang performed the sample preparation, chemical labelling and mass spectrometric analysis. Walter Maksymowych provided the rheumatoid arthritis samples for the disease control cohort.

DEDICATION

"… what we are concerned with here is the fundamental interconnectedness of all things. I see the solution to each problem as being detectable in the pattern and web of the whole. The connections between causes and effects are often much more subtle and complex than we, with our rough and ready understanding of the physical world, might naturally suppose…"

— Douglas Adams, Dirk Gently's Holistic Detective Agency

I would like to dedicate this thesis to my wonderful wife Katherine, who listens patiently and understands my crazy. A gifted wordsmith, Katherine read endless proofs. Her insightful edits allowed my work to shine. I am incredibly lucky to have you in my life; without you, I could not have completed this degree.

To Ryan and Heide, who have showed me how to be so much better than I thought I could be. As lifelong mentors, my aunt and uncle reminded me it was ok to not be ok, to be different and aspire to greater philosophical and intellectual challenges than my peers.

And finally, to my parents who never wavered in their support of my pursuits, wherever my idiosyncrasies took me. Especially my mother, who taught me to love language and to learn everything. Your subtle efforts to keep me engaged have at last come to their fruition; I miss you dearly.

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Lastly, I would like to thank Dr. Jeff Guptill for serving as my external examiner. I deeply appreciate the time you took to review my work. The thoughtful comments you offered provided valuable enhancements to the work. I look forward to further discussions regarding this undertaking and hope it generates new and exciting directions to pursue.

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3-MT - 3-methoxytyramine

4-HPPA - 4-Hydroxyphenylpyruvic acid

AA - Amino acid

ACh - Acetylcholine

ACh - Acetylcholine

AChe - Acetylcholinesterase

AChR - Acetylcholine receptor

AIRE - Autoimmune regulator gene

ALDH - Aldehyde dehydrogenase

ANOVA - Analysis of variance

APC - Antigen presenting cells

ATP - Adenosine triphosphate

AUROC - Area under a ROC curve

Ca2+ - Calcium

CAIII - Carbonic anhydrase III

CCL4 - Chemokine (C-C motif) ligand 4

ChAT - Choline acetyltransferase

CIL - Chemical isotope labelling

CMAP - Compound muscle action potential

CNS - Central nervous system

COMT - Catechol-o-methyl transferase

COX-2 - Cyclooxygenase-2 induction

DC - Dendritic cell

DMII - Diabetes mellitus

DNA - Deoxyribonucleic acid

EAMG - Experimentally acquired myasthenia gravis

EOM - Extraocular muscles

EOMG - Early-onset form

ETC - Electron transport chain

FAO - Fatty acid oxidation

FDR - False discovery rate

FOXP3 - Forkhead box P3

GC - Germinal centers

gMG - Generalized myasthenia

HC - Healthy controls

HIF1a - Hypoxia-reactive transcription subunit

HLA - Human leukocyte antigen

HMDB - Human metabolome database

IgG - Immunoglobulin G

INEN - Immune-neuroendocrine network

INEN - The immune-neuroendocrine network

IVIG - Intravenous immunoglobulin

K+ - Potassium

LC-MS - Liquid chromatography-mass spectroscopy

LDH-A - Lactate dehydrogenase A

LOMG - Late-onset form

LPS - Lipopolysaccharide

MAO-B - Monoamine oxidase B

MG - Myasthenia gravis

MHC - Major histocompatibility complex

MHC - Major histocompatibility complex

MMA - Methylmalonic acid

MMA-coa - D-methylmalonyl-coa

MMT - Manual muscle testing

MS - Mass spectrometry

MS - Multiple sclerosis

mTOR - Mechanistic target of rapamycin

MuSK - Muscle-specific kinase

Na+ - Sodium

nAChR - Nicotinic acetylcholine receptor

NAD+ - Nicotinamide adenine dinucleotide

NADPH - Nicotinamide adenine dinucleotide phosphate

NIH - National Institutes of Health

NK - Natural killer

NMJ - Neuromuscular junction

NMJ - Neuromuscular junctions

NMR - Nuclear magnetic resonance

NPP - Negative predictive power

oMG - Ocular myasthenia

OP - Oxidative phosphorylation

OPLS-DA - Orthogonal partial least squares discriminant analysis

p Treg - Peripherally-induced regulatory T-cell

PCA - Principal component analysis

PKU - Phenylketonuria

PLA2 - Phospholipase A2

PLD - Phospholipase D

PLS-DA - Partial least squares discriminant analysis

PPP - Pentose phosphate pathway

PPP - Positive predictive power

PPP - Positive predictive power

pSS - Primary Sjögrens syndrome

RA - Rheumatoid arthritis

RF - Rheumatoid factor

RNS - Nerve stimulation

RNS - Repetitive nerve stimulation

RNS - Repetitive nerve stimulation

ROC - Receiver operating characteristic

ROS - Reactive oxygen species

S1P - Sphingosine-1-phosphate

SE - Sensitivity

SELDI - Surface-enhanced laser desorption/ionization

SFEMG - Single-fiber electromyography

SFEMG - Single-fiber electromyography

SLE - Systemic lupus erythematosus

SNP - Single nucleotide polymorphisms

SP - Specificity

SSc - Systemic sclerosis

STAT4 - Transcription factor signal transducer and activator of transcription 4

T3 - Triiodothyronine

T4 - Thyroxine

TCA - Tricarboxylic acid

TCR - T-cell receptor proteins

TH17 - T helper 17

TLR - Toll-like receptor

TLR4 - Toll-like receptor 4

TLR4 - Toll-like receptor 4

TNFAIP3 - Tumour necrosis factor α-induced protein 3 genes

TNF-α - Tumour necrosis factor-alpha

TOFMS - Time-of-flight mass spectrometry

Treg - Regulatory T-cell

UMS - Universal metabolite standard

VLOMG - Very late-onset form

CHAPTER 1.

INTRODUCTION

INTRODUCTION

MG is a humoral autoimmune disease caused by the presence of antibodies against components of the muscle membrane at the neuromuscular junction. In most cases, autoantibodies against the acetylcholine receptor (AChR) can be found.

The origin of the autoimmune dysfunction in MG patients is unknown, but thymic abnormalities, defects in immune regulation and sex hormones play major roles in patients with anti-AChR antibodies. Genetic predisposition is also likely to influence the occurrence of the disease.

Here, we will review the pathophysiology of MG and provide a description of the roles of immunological, genetic, hormonal and environmental factors in the development of this disease. The inclusion criteria of this study were restricted in scope; patients with thymoma, comorbid autoimmune disease or classed as Musk-positive or seronegative were excluded. For that reason, immunopathology will be limited to discussions of AChR seropositive MG and will not describe thymoma involvement in detail.

1.1 History of Myasthenia Gravis

The first written description of myasthenia gravis (MG) is found in *De anima brutorum* (1672), a work by the English anatomist Thomas Willis, who wrote about "a woman who temporarily lost her power of speech and became mute as a fish." [1] The first modern description was given by Samuel Wilks in 1877, published in a paper in the journal *Guy's Hospital Reports*. Wilks describes a case of "Cerebritis, Hysteria, and Bulbar Paralysis, as illustrative of arrest of function of the cerebro-spinal centres." [1] The patient was a girl diagnosed with bulbar paralysis, whose weakness fluctuated in course, but ultimately ended in death. In the report, the case was summarized as "Bulbar paralysis; fatal; no disease found." [1] The first formally described accounts of MG were those of Erb in 1878 and Goldflam in 1893, [2,3] earning the disorder the name ErbGoldflam syndrome.

In 1895, Friedrich Jolly first used the name myasthenia gravis, or in his words, myasthenis gravis pseudo-paralytica. Jolly was also the first to demonstrate the "myasthenic reaction" of muscle repeatedly stimulated by Faradism,[4] introducing the basic criteria of repetitive nerve stimulation (RNS), the fundamental instrumental

technique of MG diagnosis; later, RNS would be further improved by Desmedt.[5] In 1934, Scottish physician Mary B. Walker discovered the beneficial effects of physostigmine, a cholinesterase inhibitor, on myasthenic symptoms. She further postulated that the neuromuscular junctions (NMJ) were the focus of the disease.[6] Two years later, Henry Dale demonstrated the effect of acetylcholine (ACh) as a neurotransmitter at the NMJ and confirmed the anticholinesterase inhibiting effects of physostigmine.[7]

The association of MG with thymic tumours and hyperplasia was first established in 1901 by Carl Weigert, who described a myasthenic patient with a thymic mass[8]; in 1911 the first thymectomy was performed on a female MG patient.[9] In 1949, a series of patients with thymic hyperplasia and thymoma related to MG were reported by Castleman and Norris.[10] It wasn't until the 1970s, however, that the autoimmune nature of MG was described in animal models by Patrick, Lindstrom, Fambrough, and Lennon.[11–13] Modern research has identified the presence of ACh-receptor (AChR) antibodies in serum of patients affected by MG and the production of antigen-specific CD4+ and CD8+ T-cells in cases with thymomas.[14]

CHAPTER 2.

METABOLOMICS

2.1 Definition

Metabolomics refers to the systematic identification and quantification of the small molecule metabolic products (the metabolome) of a biological system (cell, tissue, organ, biological fluid, or organism) at a specific point in time.[15] Whereas genomics involves the study of gene expression and proteomics involves the expression of proteins, metabolomics describes the consequences of the activity of these genes and proteins. A hypothesis-forming approach, it is driven by the nondiscriminant analysis of low-molecular-weight metabolites present in biological samples. All organs and tissues in the body contribute to the metabolites observed in blood, tissue and other biological samples that comprise a metabolomic profile or fingerprint; the sum of this metabolic flux describes at once their function and dysfunction at any point in time, providing a unique global picture of the patient and her or his condition.

2.2 Metabolomic Analysis and Statistics

2.2.1 Metabolomic analysis

2.2.1.1 Chemical Analysis Techniques

To identify the constituent metabolites contained within a biological sample and construct a profile of molecular species present, two analytical techniques are primarily used: Nuclear Magnetic Resonance (NMR) and Mass spectrometry (MS). Employing strong magnetic fields, NMR allows the identification of hydrogen-containing compounds with a sample. Every chemically distinct hydrogen or group of hydrogens will give a resonance in the NMR spectra. MS is an analytical technique that ionizes chemical species and sorts the ions based on their mass-to-charge ratio. In simpler terms, a mass spectrometer measures the chemical masses within a biological sample. The earliest technology employed for metabolomic analysis, NMR has several benefits. NMR is nondestructive; samples can be recovered and stored for a long time and several analyses can be carried out on the same sample. Samples also require minimal preparation and can be either fluid or tissue, allowing for *in vivo* studies as well. Finally, all metabolites present at NMR detectable levels can be detected at once, with very high reproducibility. NMR presents with some significant disadvantages as well. Sensitivity is low compared to MS and relatively low numbers of different compounds

can be detected. Depending on methods, spectral resolution and spectral libraries, approximately 40–200 compounds can be detected. While there are drawbacks to MS analysis as well (moderate reproducibility, more involved sample preparation; needs different columns and optimization of ionization conditions and no *in vivo* study) the benefits have established it as the technique of choice. MS has a very high sensitivity with a nanomolar detection limit and, while destructive to samples, the amount of sample required is very small (~100-200 ul). Excellent for targeted and untargeted analysis, MS can identify many compounds of several classes, over 500 depending on techniques and available chemical libraries. MS is also less susceptible to matrix effects.[16] LC-MS, the chaining of liquid column chromatography with MS, in particular, allows for improved analysis and is widely adopted throughout the literature.

2.2.1.2 Chemical Isotope Labelling (CIL)

Traditional liquid chromatography-mass spectrometry (LC-MS) analysis techniques are susceptible to artifacts resulting from matrix effects, ion suppression, or MS instrumental drift in MS detection. To overcome these potential inaccuracies and effect broad metabolite coverage, this study applied the chemical isotope labelling (CIL) LC-MS strategy as described by Liang et al.[17,18]

In preparation for LC-MS analysis, each 12C-labeled serum sample is intermixed with an equal amount of subject-corresponding 13C-labelled pooled sample which served as the UMS. Using the intensity ratio of each sample 12C/13C peak pair, the relative concentration of each metabolite to that of the corresponding metabolite in the UMS is measured. The use of 13C-labeled UMS as internal standard libraries endorses more accurate quantification of 12C-labeled metabolites. Since the same pooled UMS is present in all comparative samples, the peak ratio values of a given metabolite in each individual sample reflected the concentration differences between these samples. This technique offers significant advantages over unlabelled chemometric analyses, with the benefit of ongoing sample addition. Future samples may simply be aliquoted, 13C-labelled, and added cumulatively to the existing UMS pool, then 12C-labelled and compared to the modified 13C-labeled UMS, thereby allowing expansion of the current dataset to a larger cohort. Further, the dansylation and acid-labelling LC-MS strategies employed here also have the advantage of improved chromatographic separation and

enhanced electrospray ionization (ESI) response; a 10- to 1000-fold increase in detection sensitivity can be expected over traditional approaches.

2.3 Statistics

Metabolomics allows the measurement of thousands of metabolites simultaneously. The sheer volume of data and complexity of high-throughput small molecule measurements now constitutes a substantial challenge to the researchers. Given the thousands of chemically distinct metabolites measured in a specific experiment, robust statistical methods are required to analyze and meaningfully interpret the complex information informed by thousands of analytes. In order to fully understand metabolomic analyses, it's useful to be aware of the statistical methods commonly endorsed.

2.3.1 Q-value

The *q*-value is a *p*-value that has been adjusted for the False Discovery Rate (FDR), the proportion of false positives you can expect to get from a test.[19] A result is considered significant if the *p*-value is less than the chosen cutoff value, normally 0.05. Therefore, a false positive emerges when you get a significant difference where none exists.; choosing a cutoff of 0.05 means there is a 5% chance that a result is identified as significant, when in fact it is not. While 5% is acceptable for one test, if many tests are conducted on the data, then this 5% can result in a large number of false positives. For example, if 9000 compounds are measured in an experiment and an Anova or t-test is applied to each, then we would expect to get 450 (i.e. 5%) false positives by chance alone. This is known as the multiple testing problem.[20] There are several approaches to overcoming the multiple testing problem. Many traditional techniques such as the Bonferroni correction are too conservative; while they reduce the number of false positives, they also reduce the number of true discoveries.[21] The False Discovery Rate approach is a more popular method. This approach controls the number of false discoveries only in those tests that result in a positive discovery (i.e. a significant result). Because of this, it is less conservative than the Bonferroni approach and has greater ability (i.e. power) to find truly significant results. Put another way, a p-value of 0.05 implies that 5% of all tests will result in false positives. An FDR adjusted *p*-value (or *q*-value) of 0.05 implies that 5% of significant tests will result in false positives.

Because only truly significant results are included, the *q*-value will result in fewer false positives.

2.3.2 Multivariate Analysis

Analysis of metabolomics data requires working with very large data sets, where the observed variables significantly outnumber the recorded observations. Often referred to as "High dimensional" data, these datasets can be unwieldly to analyze due to their large size. Further, the high number of variables relative to the number of observations makes it very likely that redundant information is distributed throughout. In other words, there is high collinearity between the variables. To efficiently manage such large data, it is desirable to reduce the amount of data present as much as possible while retaining as much information contained, or variance, as possible. Multivariate techniques allow the performance of data reduction, without losing a significant amount of the information contained within.

2.3.2.1 PCA, PLS-DA, OPLS-DA

There are three main methods used to explore the variability, or statistical dispersion, present in metabolomic data: principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA). PCA is the simplest of these. Simply put, PCA seeks out the direction that best describes the most variability in the data. The weighted variables constituting the most variability describe a latent variable, hidden in the data, called a *principal component*. Next, another direction is chosen, perpendicular, or orthogonal, to the first component, that contains the most variability present in the remaining, undescribed data. This is the second component. This process continues until all variance in the data has been described by the observed variables (i.e. metabolites). The maximum number of components possible is equal to the number of metabolites. This process is illustrated by Figure 2.1.

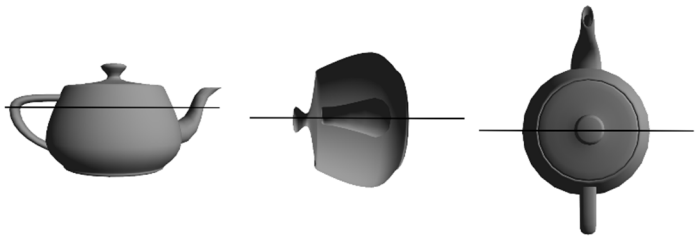


Figure 2.1 Utah teapot example of principal component vectors

PCA is described as an unsupervised method. This means that class information (i.e. MG vs. control) is not taken into account by the analysis. PLS-DA and OPLS-DA are supervised methods. This means that the group to which each data set belongs to is taken into account. Sample noise is a potential confound in any data set, but particularly in biological data. PCA does not account for this noise, but rather makes the best decision on the data as a whole. PLS-DA also makes no accommodation for noise, but using class as a secondary discriminating variable, between-group separation is generally better. OPLS-DA methods take into account the noise unrelated to the variance present in each class, also called orthogonal variation, as well as class membership. Ideally, this will produce the best model of the data, as the effects of class and unrelated variation in the data are accounted for. One downside to this method, however, is the risk of overfitting the data. To overfit the data is to match the data so closely that the predictive model constructed by the analysis is no longer generalizable to data outside the sample. This severely hampers the utility of potential biomarkers, which must remain robust throughout a heterogeneity of collected samples.

CHAPTER 3.

ANATOMY AND PHYSIOLOGY: A PRIMER FOR MYASTHENIA GRAVIS

3.1 The Thymus

3.1.1 Structure and Function

The thymus plays a major role in the pathogenesis of MG with anti-AChR antibodies; marked pathologic alterations are present in most AChR-positive patients. Functionally, the thymus contains the elements required to initiate and sustain an autoimmune reaction (AChR autoantigen, AChR-specific T cells, and autoantibody-secreting plasma cells).

The thymus is an encapsulated primary lymphoid organ, divided into subcapsular cortical, cortical and medullary regions within each lobe. The thymus is structured such that a darker cortical region surrounds a lighter medullary compartment. The colour of the darker cortical region (also called the dark zone) is attributed to the high density of thymocytes. Therefore, the darker cortex has more T-lymphocytes when compared to the lighter medulla (called the light zone).

There are two major categories of cells within the thymus: the thymic epithelial cells and thymocytes. The thymic epithelial cells are endodermal derivatives of the third pharyngeal pouch that further differentiates into specialized epithelium within the cortex and medulla. Overall, these cells are characterized by an eosinophilic cytoplasm containing intermediate filament bundles with pale, ovoid nuclei.

The supporting structure of the cortex is called the cytoreticulum. A collagenous reticular network, the cytoreticulum facilitates attachment of maturing lymphocytes and surrounding macrophages. Cytoreticular, or epithelial reticular, cells are antigen presenting cells (APC) that express both class I and class II major histocompatibility complex (MHC I and MHC II) proteins that participate in the thymic education program. The major histocompatibility complex (MHC) is a set of cell surface proteins essential for the acquired immune system to recognize foreign molecules, which in turn determines histocompatibility, or compatibility with self. The primary function of MHC molecules is to bind to antigens derived from pathogens and display them on the cell surface for recognition by the appropriate T-cells.[22] Epithelial reticular cells are also the primary cells involved with ensuring no autoreactive cells survive the maturation process. Thymocytes, also called T-lymphocytes, evolve from bone marrow

hematopoietic progenitor cells that migrate to the cortex of the thymus. Maturing T-cells migrate from the cortex (dark zone) of the thymus to the medulla (light zone), where they come into contact with many epithelial reticular cells. Here, if T-cell MHC-presented self-proteins are recognized as autoreactive, the epithelial cells remove these T-cells.

Within the medulla, a cytoreticulum also provides a similar microenvironment for resident dendritic cells, macrophages and more mature thymocytes. A collection of thymic epithelial cells known as Hassall corpuscles are responsible for the release of cytokines that regulate dendritic cell activity. Further, they program a subset of thymocytes, regulatory T-cells, that facilitate peripheral tolerance.

3.1.1.1 Education of Thymic T-cells

Early T-lymphoblasts that enter the thymus do not express T-cell receptor proteins (TCR) or CD4 or CD8 proteins. T-cell receptors are responsible for recognizing fragments of foreign antigen. CD4 and CD8 proteins both help to identify the type of T-cell and are co-receptors of the T-cell receptor (TCR) that assist the TCR in communicating with antigen-presenting cells (i.e. dendritic cells, macrophages and B-cells). Within the cortex, as the cells replicate, there is activation of the T-cell receptor alpha and beta (TCR- α and TCR-β) genes that result in the surface expression of the receptor proteins, and both CD4 and CD8 surface proteins.

Following this activation, the thymocytes undergo quality control testing to ensure that receptor binding is effective and selective. Within the cortex, cytoreticular cells present MHC I and MHC II proteins to the maturing thymocytes. The TCR proteins that bind to MHC I will predominantly express CD8 proteins at the end of the thymic education program (i.e. cytotoxic T-lymphocytes). Similarly, those that bind to MHC II will express CD4 proteins at the end of maturation (i.e. helper T-lymphocytes). If the binding is successful, the maturing lymphocytes have passed the positive selection test and will proceed to migrate into the medulla of the thymus. If binding fails, however, the cells will undergo apoptosis.

T-lymphocytes that make it to the medulla are now capable of binding MHC I or MHC II proteins. It is essential, however, that these cells do not bind to self-antigens. Therefore, a wide variety of tissue-specific antigens are expressed by medullary thymic

epithelial cells through activation of the autoimmune regulator (AIRE) gene. Dendritic cells will present variations of these self-antigens to the developing T-lymphocytes. If the T-lymphocytes bind to the self-antigen, then they will undergo apoptosis. Approximately 2% of T-lymphocytes do not bind to the self-antigen. These "approved' cells are then able to leave the thymus and carry out their functions in the periphery. From start to finish, thymic education takes approximately 2 weeks to complete.

3.2 The Neuromuscular Junction

3.2.1 Structure and function

The neuromuscular junction (NMJ) is the site of communication between motor nerves and muscle fibers. It is composed of two main structures: The axon terminal and motor end plate. These two structures are separated by a narrow space called the synaptic cleft.

3.2.2 Axon Terminal

Axonal terminals are specialized nerve endings whose purpose is to release the neurotransmitters of the presynaptic cell. The chemical means of communication between the motor nerve and the adjacent muscle is the neurotransmitter acetylcholine (ACh). Ach is synthesized from the coenzyme Acetyl CoA and ammonium salt choline by the transferase enzyme choline acetyltransferase (ChAT) within the cytoplasm of the nerve terminal. The ACh is then stored in small vesicles within the cytoplasm of the nerve terminal.

3.2.3 Motor End Plate

The motor end plate is a highly excitable region of the muscle fiber plasma membrane responsible for initiating action potentials across the muscle surface; this action ultimately results in muscle contraction. It is the part of the sarcolemma of muscle cell, which is in closest proximity to the synaptic end bulb. Components of the motor end plate include:

i. Synaptic Gutter: The invaginated membrane, which forms space for the synaptic end bulbs to reach close to the muscle fiber sarcolemma.

ii. Subneural Clefts: These are small folds of the muscle membrane present at the bottom of the synaptic gutter. They greatly increase the surface area at which the neurotransmitter can act.

iii. Increased number of mitochondria: Because the neuromuscular junction experiences high energy demands, the area of the muscle fiber surrounding the motor end plate shows a significant increase in the number of mitochondria.

3.2.4 Acetylcholine Receptors

In order to be effective, ACh must join with the surface of the motor end plate. This is accomplished through binding to specialized proteins called receptors. The primary receptor present in muscle for motor nerve-muscle communication, nicotinic acetylcholine receptors, or nAChRs, are receptor proteins that respond to the neurotransmitter acetylcholine and subsequently control muscle contraction. Densely packed, acetylcholine receptors are located in the subneural clefts of the motor end plate plasma membrane. The concentration of AChRs in the membrane opposite presynaptic release sites is about 10 000 per μm^2 on the surface of fold peaks. In its initial state, the AChR is in a closed state. Once two ACh molecules have bound to the α subunits on its surface, the receptor opens, allowing the passage of cations, including Na+ and K+, to cross the membrane into the muscle. This passage of cations induces a local depolarization of the muscle membrane. As more molecules of ACh activate more receptors, the net depolarization of the muscle membrane progresses until an action potential is generated sufficient to recruit the muscle fiber. When this effect occurs collectively across many muscle fibers, the result is muscle movement.

3.2.5 Neuromuscular Transmission: Sequence of Events

When a skeletal muscle movement is initiated (e.g. leg movement), the action potential travels down the motor nerve until it reaches the location of the muscle to activated. Once the impulse has reached the end of the nerve (i.e.axon terminal) it elicits a muscle action potential in the following four steps:

i. <u>Neuromuscular release of acetylcholine.</u> The motor nerve action potential arrives at the synaptic end bulbs, opening voltage-gated

channels. Following the electrochemical gradient, Ca^{2+} flows inward through the open channels. The entering Ca^{2+} in turn stimulates the synaptic vesicles to fuse with the synaptic end bulb plasma membrane and undergo exocytosis. During exocytosis, the synaptic vesicles release ACh into the synaptic cleft. The ACh then diffuses across the synaptic cleft between the motor neuron and the motor end plate.

ii. Activation of ACh receptors. ACh arriving at the motor end plate begins binding to sites on the ACh receptors on its surface. The binding of two molecules of ACh to the receptor opens a transmembrane ion channel within the ACh receptor. Once the channel is open, small cations, most importantly Na+ (inward) and K+ (outward), begin to flow across the membrane.

iii. Production of muscle action potential. The inflow of Na+ changes the membrane potential, triggering a muscle action potential. Normally, one nerve impulse elicits one muscle action potential. The muscle action potential then travels along the sarcolemma, causing the sarcoplasmic reticulum to release its stored Ca2+ back into the sarcoplasm and eliciting a muscle fiber contraction.

iv. Termination of ACh activity. The effect of ACh binding is very brief; it takes approximately 1 ms for the ACh to diffuse away and be rapidly broken down by the enzyme acetylcholinesterase (AChE). Attached to collagen fibers in the extracellular matrix of the synaptic cleft, AChE breaks down ACh into acetyl and choline, products reabsorbed back into the synaptic bulb and used to form additional ACh, thus continuing the cycle.

3.2.6 Safety Factor

Reliability of neuromuscular transmission is normally ensured by the release of more ACh than is required to initiate an action potential. The term safety factor is used to describe this excess.[23] Postsynaptic features influencing the response to ACh include acetylcholinesterase (AChE) and the density and distribution of ACh receptors.

Before it can bind to an AChR, each ACh molecule must traverse the AChE-rich membrane of the motor end plate. Under normal conditions, this happens sufficiently rapidly that most ACh molecules are bound to AChRs before they are broken down (hydrolyzed).

When AChE activity is substantially reduced, the safety factor is enhanced. The medication pyridostigmine bromide (Mestion) is an anti-cholinesterase that carbamylates (i.e. binds the negative ion cyanate to form a molecular complex[24]) about 30% of peripheral cholinesterase.[23] The carbamylated enzyme complex is unable to catalyze the breakdown of ACh, increasing the amount and duration of ACh exposure in the synaptic cleft. Eventually, AChE regenerates by natural hydrolysis and excess ACh levels revert to normal.

The effect of ACh released from the nerve is also influenced by the number of AChRs it can activate. Reductions in the density and distribution of AChRs are known to occur in MG. This leads to a reduction in the safety factor and impairment of neuromuscular transmission.

CHAPTER 4.

IMMUNOMETABOLISM

4.1 The glycolytic metabolic pathway (glycolysis)

Arguably the most important immunometabolic pathway, is essential to a number of immune processes.

Previous work has indicated that both activated macrophages and T-cells have a substantial appetite for glucose.[25,26] These studies and others have pointed to glycolysis as being essential to immune cell function. Metabolically, this is unexpected as glycolysis is not the most effective way to generate energy, in the form of ATP. Glycolysis generates 2 molecules of ATP from 1 molecule of glucose. Much more efficient, oxidative phosphorylation generates 36 ATP molecules from a single molecule of glucose. However, while the yield of glycolysis is less than that of OP, glycolysis can be rapidly activated via the induction of enzymes that are involved in this pathway. By contrast, initiating oxidative phosphorylation requires mitochondrial biogenesis, a much more complex and slower process. Accordingly, cells requiring rapidly available ATP will switch to glycolysis.

Equally important to rapid ATP synthesis, however, is the capacity of glycolysis to provide biosynthetic intermediates to support rapid cell growth. Activating signals such as growth factors strongly promote increased glucose uptake and glycolysis, which supplies ATP, supports the TCA cycle, and donates intermediates for the PPP, glycosylation reactions and synthesis of key biomass constituents, including serine, glycine, alanine, and acetyl-CoA for lipid synthesis.

Glycolysis is essential to the activation of several cell types. Enhanced glycolysis occurs in lipopolysaccharide (LPS)-activated macrophages and DCs,[27,28] in activated natural killer (NK) cells,[29] in activated effector T-cells[30] and in activated B-cells.[31] Following activation, effector T-cell subsets all show an increase in glycolysis, most notably T helper 17 (TH17) cells15, TH1 and TH2 cells[30] and activated effector CD8+ T-cells[32]

Increased kinase mTOR pathway activity is correlated with increased glycolysis. While it appears to correlate with the initial generation of peripherally-induced regulatory T (pTreg) cells, the effect on their long-term survival and lineage stability is unclear.[33–35]

As noted previously, glycolysis enables the immune cell to generate sufficient ATP and biosynthetic intermediates to carry out the cells particular effector functions. For macrophages this includes phagocytosis and inflammatory cytokine production, for DCs this includes antigen presentation[36] and for T-cells this includes the production of effector cytokines (such as IL-17 in the case of TH17 cells[37]). A similar role for glycolysis in immune cell reprogramming has been reported in TH17 cells; glycolysis is inhibited with 2-deoxyglucose converting TH17 cells into Treg cells.[37] In contrast, hyperactivation of mTOR pathway signalling results in increased glycolysis in peripheral Treg cells; paradoxically, this may limit their survival.[25,31–42]

These studies further emphasize the link between metabolism and the phenotype of an immune cell, with glycolysis, along with hypoxia-reactive transcription subunit (HIF1a) induction, leading to the evolution of a more inflammatory phenotype. While oxidative phosphorylation has been associated with a more anti-inflammatory cell phenotype,[43,44] recent studies have shown that human Treg cells can utilize glycolysis,[40,45] suggesting that glycolysis is not solely associated with inflammatory cell functions.

4.2 The TCA cycle (Citric acid cycle, Krebs cycle)

The TCA, or Krebs, cycle has been studied extensively in immune cells.

While there is a shift towards glycolysis and away from the TCA cycle in effector T-cells, the TCA cycle is very prominent in memory CD8+ T-cells.[46]

The TCA cycle is also expressed differentially in distinct macrophage sub-types. In M2 macrophages, there is an intact TCA cycle that is coupled to oxidative phosphorylation.[43] This allows the generation of intracellular signaling nucleotide

monosaccharide (UDP-GlcNAc) intermediates that are essential for the glycosylation of M2-associated receptors, such as the mannose receptor.[43]

M1 macrophages present a different scenario. In these cells, the TCA cycle functions atypically, allowing for an excess of citrate. The accumulated citrate is then transported from the mitochondria where it is utilized for the production of fatty acids, which in turn are used for membrane biogenesis.

This broken TCA cycle is also seen in activated DCs. Here, the production of citrate is especially vital for proper function, as DCs require substantial membrane production to support antigen presentation.[36] Pathogen degradation pathways are also fed by excess citrate. Crucial effector molecules, nitric acid and prostaglandins, are generated by macrophages that employ citrate in their production. Finally, the metabolic intermediate succcinate accumulates in M1 macrophages as a consequence of a broken TCA cycle. This accumulation has a direct impact on macrophage cytokine production.[47] The above cases show that alterations in the TCA cycle occurring in M1 macrophages lead to an accumulation of metabolites which can significantly affect their expression of immune functions.

4.3 The pentose phosphate pathway

NADPH has multiple functions in immune cells. First, it is used by the NADPH oxidase to generate reactive oxygen species (ROS) during the respiratory burst, the rapid release of reactive oxygen species (superoxide radical and hydrogen peroxide) employed to degrade pathogens. Conversely, NADPH also acts as a counter-balance in the generation of glutathione and other antioxidants. During an infection, macrophages and neutrophils probably require both of these NADPH-dependent functions. In the purge and cleanup of the immune response, the rapid production of ROS is useful initially to clear infectious agents, followed by induction of antioxidants to prevent post-clearance tissue damage.[48]

DCs also use NADPH and lipid synthesis to endorse endoplasmic reticulum synthesis, which is necessary for DC activation and cytokine secretion.[49,50]

4.4 Fatty acid oxidation and immune function

Fatty acid oxidation has key roles in the regulation of adaptive and innate immune responses. In contrast to aerobic glycolysis that is often observed in inflammatory and rapidly proliferating immune cells, a reliance on fatty acid oxidation has been observed in many immune cells that are not inflammatory in nature and exhibit increased cellular lifespans, including M2 macrophages, Treg cells and memory T-cells.

4.5 Fatty acid oxidation and macrophage function

Fatty acid oxidation regulates the inflammatory functions of macrophages[51,52] and also plays a key role in macrophage polarization. While glycolytic metabolism is essential for the activation of M1 macrophages,[53] M2 macrophages are specifically activated by fatty acid oxidation.[54]

4.6 Fatty acid oxidation and T-cell responses

Fatty acid oxidation also plays a contributing role in regulating T-cell responses. Previous work has observed that fatty acid oxidation effects regulate the balance between inflammatory effector T-cells and suppressive Treg cells. Fatty acid oxidation effects also promote sustained immune function through long-lived memory T-cells. The contribution of fatty acid oxidation regulation to the balance between effector T-cells and Treg cells are two-fold: a) Treg cells exhibit increase fatty acid oxidation relative to TH1, TH2 and TH17 cells and b) fatty acid oxidation promotes the generation of Treg cells while inhibiting effector T-cell polarization.[55] Further, effector T-cells have been shown to downregulate fatty acid oxidation during activation.[56]

Fatty acid oxidation has additional roles in the proliferation and ongoing support of long-lived memory CD8+ T-cells. Memory CD8+ T-cells, quiescent under steady-state conditions,[57] appear to require fatty acid oxidation to for rapid response to antigen stimulation.[58] Stimulation of memory CD8+ T-cells with IL-15 increases

their expression of the CPT1A gene and promotes fatty acid oxidation, resulting in increased cell survival.[38] The CPT1A gene, essential for fatty acid oxidation, provides instructions for making the enzyme carnitine palmitoyltransferase, a fundamental step in sphingolipid synthesis. Together, these observations indicate key roles for fatty acid oxidation both in the generation of tolerance-maintaining Treg cells as well as in the support of long-lived memory CD8+ T-cells.

4.7 Fatty acid synthesis

In contrast to fatty acid oxidation, which points toward the development and activity of non-inflammatory immune cells, fatty acid synthesis appears to positively regulate immune cells of both the innate and adaptive immune systems. Numerous studies suggest that inflammatory stimuli such as the membrane antigens (bacterial lipopolysaccharides) and pro-inflammatory cytokines trigger an increase in fatty acid synthesis in macrophages.[59,60] Notably, the observed increase in fatty acid synthesis was also crucial to the differentiation and inflammatory function of macrophages.[61] Taken together, these observations suggest that fatty acid synthesis is required for the promotion of inflammatory macrophage responses.

Similarly, fatty acid synthesis provides a link between innate and adaptive immunity through regulation of DC function. Toll-like receptor (TLR)-mediated DC activation has been observed to upregulate fatty acid synthesis. This increased fatty acid synthesis is essential for DC activation and the associated stimulation of CD8+ T-cell responses.[36]

Fatty acid synthesis is also key to the cell intrinsic function of T-cells and B-cells; synthesis of fatty acids and sterols has been shown to be necessary for cell proliferation once activation is initiated through antigen receptors.[62,63]

Overall, fatty acid oxidation and fatty acid synthesis appear to play opposing roles in the immune system; fatty acid oxidation is preferentially used by non-inflammatory and tolerogenic (i.e. induce tolerance of specific antigens) immune cells, whereas fatty acid synthesis is more characteristic of inflammatory responses within the

innate and adaptive immune systems. The mechanisms behind this distinction are unclear. While fatty acid metabolism may lead to these opposing immunological functions, the efficiency of lipid oxidation for energy generation coupled with the necessity of lipid synthesis for biosynthesis and cell growth suggest that pro-inflammatory and regulatory immune cells exhibit fundamental differences in the role of ATP generation. Further, it is possible that effector cells require fatty acid synthesis during rapid growth to allow membrane biogenesis, while, in contrast, the slow growth of memory cells occurs as a result of fatty acid oxidation.

Finally, there are additional reviews which comment in detail upon the metabolism of other lipid species in immune cell function, notably of cholesterol and sphingolipids.[42,64]

4.8 Amino acid metabolic pathways

The protein kinase mTOR links with other proteins and serves as a core component for two distinct protein complexes, mTOR complex 1 and mTOR complex 2, which regulate different cellular processes.[65] In particular, mTOR functions as a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy, and transcription.[65,66] The mTOR pathway has important roles in cellular metabolism, including in the sensing of amino acid levels to couple nutrient availability to cellular growth and proliferation. Accordingly, the availability and metabolism of amino plays a role in immune function.

4.8.1 Glutamine metabolism

Glutamine catabolism regulates numerous aspects of immune cell function. Glutamine metabolism is important for the generation of nitric oxide, exhibiting a role for glutamine in the cytotoxic functions of macrophages. Further, T-cell and B-cell responses are also regulated by glutamine metabolism. Glutamine usage increases markedly upon both T-cell and B-cell activation, and both populations require glutamine to respond to antigen receptor stimulation.[56,67,68]

4.8.2 Arginine metabolism

Arginine metabolism plays a key role in the inflammatory function of macrophages.[69] Macrophages use arginine in two distinct metabolic pathways, the nitric oxide synthesis pathway and the arginase pathway.

The arginine metabolism pathway has profound effects on the immune function of the cell. The inflammatory M1 macrophage phenotype is associated with a flux of arginine into the nitric oxide synthesis pathway. In contrast, arginine flux through the arginase pathway is associated with a more tolerant immune response, often associated with wound healing.[70] Curiously, arginase expression in macrophages also limits the inflammatory potential of effector T-cells.[71] This indicates a probable immunoregulatory role for arginine metabolism beyond macrophages. As a further example, arginine regulates the expression of T-cell receptor[72] components and promotes proliferation of human T-cells.[73]

CHAPTER 5.

MYASTHENIA GRAVIS

5.1 Epidemiology

The most common disorder affecting the neuromuscular junction, myasthenia gravis is also a rare disease. The incidence ranges from 1.7 to 21.3 per million, and the prevalence is between 15 and 179 per million inhabitants, depending on the location.[74,75] In Canada, a study from British Columbia, Canada, found the overall average anti-AChR seropositivity IR of 13.2 per million with an increasing incidence in the elderly.[76] Additionally, in 2013, there were 3611 prevalent cases in Ontario, and the crude prevalence rate was 32.0/100,000 population.[77] Based on epidemiological data, it has been estimated that the number of patients with MG in the United States was ~60,000 in 2003.[78] At observed rates, this number would be much higher at this time. The reported prevalence of the disease has increased in every decade since the 1950s.[79,80] By the 1990s the prevalence of the disease was at least four times higher than that of the 1950s.[79] The increase in the number of patients with MG can most likely be attributed to several factors, including improved recognition of the disease, the availability of diagnostic tests with higher sensitivity and specificity, longer life span in affected patients due to more effective treatment, and an increase in the population at risk due to aging.

Myasthenia gravis can present at any age, but there is a bimodal peak of incidence, with the first peak in the third decade (predominantly affecting women) and the second peak in the sixth and seventh decades (predominantly affecting men). It has been suggested that incidence falls after 70 years of age. It is also probable that MG is underdiagnosed in the elderly. A 2003 study reviewed the medical records of 3183 AChR seropositive individuals. This study concluded that myasthenia gravis in the UK is substantially underdiagnosed in people over 75 years.[81]

Additionally, MG is associated with a higher incidence of comorbid autoimmune disease. MG patients are at increased risk for complicating autoimmune diseases, most commonly autoimmune thyroid disease, systemic lupus erythematosus and rheumatoid arthritis.[82]

5.2 Clinical Description

5.2.1 Presentation

MG presents with a range of symptoms, including trouble speaking, facial paralysis, difficulty breathing due to muscle weakness, difficulty swallowing or chewing, fatigue, hoarse voice, or drooping of eyelids (ptosis). Because of the broad range of presentations, which can involve a variety of muscle groups, coupled with its relative rarity and the variable severity of weakness, MG can be difficult for the clinician to identify.

Evidence is accumulating that MG subtypes exist with clinical and immunological differences from the conventional forms of the disease. For example, patients who present predominantly with weakness in distal extremity muscles have been described.[83,84] These patients often have different electrophysiological and immunological findings alongside atypical limb presentations. An Austrian study has examined the prevalence of this specific presentation of MG.[85] Of 84 MG patients, 6 had either distal onset of disease or a predominance of weakness in distal muscles. This number, although small at 7%, is large enough to consider the presence of an additional MG subtype.

The sections below discuss specific clinical phenotypes:

5.2.2 Clinical subtypes

Several clinical subgroups of MG patients with anti-AChR antibodies have been identified, as described below.

5.2.2.1 Ocular Myasthenia (oMG)

In approximately 15% of MG patients, weakness is restricted to the ocular muscles.[86] The distinguishing features of oMG are ptosis, produced by weakened levator palpebrae, and double vision, the most common symptom of extraocular muscles (EOM). In nearly half of patients with MG, ocular manifestations will be the first symptoms of MG; almost all patients will develop double vision or ptosis, during the course of their illness.[87] Since this is a cardinal sign of MG, a diagnosis of MG may need to be questioned if ocular manifestations do not occur. Approximately

half of all patients develop weakness within 6 months of the onset of visual symptoms. After 2-3 years, ~6% of ocular patients will transition into generalized MG.[87]

5.2.2.2 Generalized Myasthenia (gMG)

In generalized disease, the weakness commonly affects ocular muscles, but it also involves a variable combination of bulbar, limb, and respiratory muscles. While many patients exhibit a widespread subclinical defect in neuromuscular transmission, at some point most MG patients will have weakness that involves one or more skeletal muscles to some degree. In general, patients have compromised limb strength, which leads to difficulties with daily activities such as walking, opening jars, shaving, or climbing stairs. Weakness of neck extensors or distal limb muscles may occur, producing foot drop or wrist drop, mimicking peripheral nerve involvement. Approximately 15% of patients with MG will develop prominent weakness of cranial nerve innervated muscles, leading to a clinical classification of this subgroup as "bulbar".[88] Grave weakness of the respiratory muscles, "myasthenic crisis" may occur in the context of severe, worsening generalized weakness. Crisis is potentially life-threatening and requires immediate medical intervention.

Patients with generalized MG can be divided into three subgroups according to the age of onset:

5.2.2.2.1 Early-onset form (EOMG) (age of onset <50 years).

Most early onset MG (EOMG) patients exhibit high levels of anti-AChR antibodies along with thymic follicular hyperplasia characterized by ectopic germinal centers (GCs). [89] Further, sex hormones may play a role in this form of the disease; more than 80% of patients with follicular hyperplasia are women.[90] Finally, patients in this subgroup may have other autoantibodies and are at risk of developing other autoimmune diseases, such as thyroiditis.[91]

5.2.2.2.2 Late-onset form (LOMG) (age of onset >50 years).

Usually generalized and severe, presenting with bulbar signs and frequent severe respiratory crises, [92] LOMG is also frequently associated with the presence of a thymoma.

5.2.2.2.3 Very late-onset form (VLOMG) (age of onset >60 years).

Recently, a form of MG that appears after 60 years of age has been described. Predominantly affecting males, VLOMG is distinct from LOMG, as patients do not present with thymoma.[93]

5.3 Diagnosis

The clinical diagnosis of MG is confirmed by bedside evaluations, electrodiagnostic studies, and serology for autoantibodies.

5.3.1 Bedside evaluations

On first suspicion of MG, an acetylcholinesterase inhibitor such as edrophonium (Tensilon), can be administered. If improvement occurs in the suspected weakened muscle, the test can be considered positive. Alternately, with ptosis, the ice pack test can be performed. An ice pack is placed over the patient's affected eye for 5 minutes. If the eyelid lifts normally, this also is considered a positive result for MG. In the proper contexts, these tests are very reliable.

5.3.2 Electrodiagnostic studies

Electrodiagnostic studies are an essential tool for the evaluation of possible MG. With repetitive nerve stimulation (RNS), a decremental response of the compound muscle action potential is identified in about three-quarters of patients.[94] Despite the absence of generalized weakness, patients with ocular myasthenia will also frequently demonstrate a decremental response. Single-fiber electromyography (SFEMG), where a single muscle fiber is stimulated and its responses measured, is currently the most sensitive test for detecting abnormalities consistent with MG.[95]

5.3.3 Serology

For the diagnosis of the AChR binding antibody, a standard radioimmunoassay methodology is used, with human AChR as the antigen. About 85% of patients with generalized MG have elevated titers of binding antibodies, 50% or less of those with ocular myasthenia will be positive.[96] Seropositivity increases with time and therefore repeat testing is beneficial to confirm the diagnosis. Although uncommon, AChR antibodies may be detected at low levels in other conditions (patients with thymoma, amyotrophic lateral sclerosis, and rheumatoid arthritis, as well as family members of patients with MG) when there is no clinical or electrophysiological evidence of MG. Otherwise, the AChR binding test is highly sensitive.

Some caution should be exercised if relying on serology alone. Approximately 10-20 % of patients are seronegative for AChR, and confirmation of the diagnosis relies on other diagnostic testing.[97]

5.4 Treatments

Like many autoimmune diseases, therapeutic management of MG presents a challenge for the clinician. Many treatments come with the risk of significant side effects, requiring the balancing of an improvement of MG symptoms with potential side effects. Presently, there is no cure for myasthenia gravis.

There are four basic therapies used to treat myasthenia gravis (MG):

5.4.1 Symptomatic treatments

Acetylcholinesterase inhibitors are often the first line of treatment due to their safety and ease of use. Acetylcholinesterase inhibitors, such as pyridostigmine bromide (Mestinon), are symptomatic treatments; they don't meaningfully affect the clinical course of MG.

Acetylcholinesterase inhibitors hinder the hydrolysis of acetylcholine in the neuromuscular junction.[98] As a result, the presence of neurotransmitter within the neuromuscular gap is prolonged, leading to a variable improvement in strength. While effective in a majority of cases, diplopia is particularly resistant to these medications in many patients.[87]

Very rarely, cholinergic crisis, a paradoxical weakening with excessive anticholinesterase medication, can occur. Requiring hospitalization, myasthenic crisis requires immediate attention and can be life-threatening.

5.4.2 Chronic immunomodulating treatments

Immunomodulators weaken or modulate the activity of the immune system. That, in turn, decreases the inflammatory response. Commonly used immunotherapeutic drugs in MG are prednisone, azathioprine, cyclosporine, and mycophenolate mofetil. In cases of refractory MG, where other immunomodulating treatments have failed, agents such as the monoclonal antibody rituximab and interleukin-2 inhibitor tacrolimus may be considered. While frequently quite effective, these medications are often very expensive.

5.4.3 Rapid immunomodulating treatments

Plasmapheresis and IVIG are rapid immunotherapies that work quickly but have a short duration of action. Plasmapheresis and IVIG are also immunomodulating. However, they are rapid acting, with benefits lasting only short term (weeks). Plasmapheresis (plasma exchange) directly removes acetylcholine receptor (AChR) antibodies from the circulation with clinical improvement corresponding roughly with the reduction in antibody levels.[99] IVIg is the infusion of pooled Ig immunoglobulin; its mechanism of action in MG is uncertain.

Plasmapheresis and IVIg are used primarily in four cases:

i. Myasthenic crisis
ii. Preoperatively before thymectomy or other surgery.
iii. As a "bridge" to slower acting immunotherapies.
iv. Periodically to maintain remission in patients with MG that is not well controlled.

5.4.4 Surgical treatment

Thymectomy is beneficial for patients with nonthymomatous, generalized acetylcholine receptor (AChR) antibody-associated MG. However, it generally takes months to years for the benefits of thymectomy to accrue. Typically, this option is most suited to younger patients and those in whom thymoma has been diagnosed.

5.5 Pathophysiology

For a disorder to fulfill the criteria for an autoimmune disease, the criteria of Witebsky's postulates must be met:[100] (1) direct evidence from transfer of disease-causing antibody or disease-causing T lymphocyte white blood cells; (2) indirect evidence based on reproduction of the autoimmune disease in experimental animals; (3) circumstantial evidence from clinical clues; (4) genetic evidence suggesting "clustering" with other autoimmune diseases.

MG is a prototypical disease in that it supports these criteria: (1) experimentally acquired Myasthenia Gravis (EAMG): Purified AChR antigens administered to an animal can induce a disorder with features similar to those of the human disorder; (2) administration of human or

animal autoantibodies into animal models reproduce the phenotype of patients with MG; (3) radiolabelled AChR immunoglobulin will bind to the neuromuscular junctions of patients and has further been determined to bind the autoantigen epitopes; (4) AChR antibodies can be detected in greater than 95% of patients; and (5) treatments that reduce the serum concentration of anti-AChR antibody, such as plasma exchange or intravenous IVIg, improve MG symptoms, especially fatigue.

AChR antibodies are heterogeneous, with a variation in immunoglobulin G (IgG) subclasses. Different subclasses of the IgG heavy chain (subclass 1 or 3 in human myasthenia) bind to various sites on the AChR.

5.5.1 Damage to ACh Receptors Alters Function

AChR antibodies, the source of MG pathology, impair neuromuscular transmission by three mechanisms: AChR Blocking, antigenic modulation, and complement mechanisms.

5.5.1.1 AChR Blocking

Anti-AChR antibodies may form physical barriers to ACh by blocking AChr binding sites or by obstructing ion channel function, both of which would be expected to produce particularly severe weakness. Antibodies of this type appear to play only a small part in MG pathology, however, as they appear to represent only a portion of AChR antibodies among patients. Animal models provide further evidence. Anti-cholinergic antibodies from human patients can cause acute, severe weakness in animals, suggesting that at low concentration these antibodies can create a functional block of AChRs and induce produce profound weakness.[101] Monoclonal antibodies specific to the ACh binding site of animal receptors can block ion channels, resulting in rapid onset of weakness.[102]

5.5.1.2 AChR Crosslinking

Antigenic modulation is the ability of an antibody to cross-link two receptor epitopes, triggering cellular signals which initiate accelerated endocytosis and degradation of those receptors.[103] Modelling reveals that immunoglobulins extracted from patients with MG accelerate the degradation rate of the *AChR in vivo* and in cultured muscle cells. [101] Autoantibodies do not always engage in crosslinking, however. It is thought that variations in the epitope location on the AChR surface may prevent cross-linking from occurring, as

antibody FAB regions fail to align with the epitopes of adjacent receptors. Further, as a consequence of complement initiated damage to the membrane, receptor structure may be altered, further inhibiting epitope alignment. Additionally, damage to the postsynaptic features resulting from complement injury could interfere with cross-linking by altering receptor structure.

5.5.1.3 Complement Mechanisms

Probably the most important mechanism by which antibodies produce weakness is complement-mediated remodelling of the motor end plate.[104] An area of significant interest, the contribution of the complement cascade to the functional pathophysiology of MG is evidenced by several observations.[105,106] Ultrastructural, light microscopic localization and electrophysiologic evidence suggests the postsynaptic surface in patients and EAMG animals contains complement component activation fragments and the membrane attack complex (MAC).[107] This has been experimentally confirmed using animal models. As proof of concept, complement activity in animals has been disrupted to demonstrate that the disruption of complement activity by various means protects animals from EAMG. Mice deficient in complement components are resistant to the development of weakness induced by EAMG.[108–110] This resistance to EAMG indicates that without activation of complement, AChR antibodies cannot significantly compromise neuromuscular transmission.

5.5.2 AChR Epitopes and Antibody Binding

Nicotinic ACh receptors are made up of five subunits, arranged symmetrically around a central pore.[111] AChR antibodies are polyclonal, binding to a heterogeneous set of epitopes on all five AChR subunits and representing all IgG subclasses.[112] The epitope and the type of heavy chain present within AChR antibodies determines their pathogenicity,[113] although not all AChR antibodies are pathogenic.[114] Because AChR autoantibodies may bind without cross-linking AChR or be of an undetermined subtype, it is very likely that this functional irregularity is why serum AChR antibodies correlate poorly with disease severity.

5.5.3 T-cells and Tolerance

Autoantibody production in MG is a T-cell dependent process. Specifically, a breakdown in tolerance towards self-antigens appears to be the primary malfunction pathogenic for MG. The thymus sensitizes lymphocytes to foreign antigens, while also removing autoreactive T-cells.

Immature T-cells first pass through the thymic cortex; those that recognize self-major histocompatibility complex (MHC) antigens then pass through to the medulla. During this stage in the thymic cortex, T-cells that would react towards self-antigens are removed. Malfunctions in this process may lead to autoimmunity. Once in the medulla, the T-cells differentiate into helper and suppressor cells which are eventually released into circulation. Healthy individuals usually have T-cells specific for autoantigens, including the muscle AChR, but their presence rarely leads to autoimmune diseases. The MHC complex is a mechanism in place to remove these "self-attacking" or autoreactive cells. MHC complex binds to autoreactive T-cells, preventing their proliferation. If the MHC complex binds poorly with the T-cell (i.e has low affinity), the autoreactive T-cell may survive. These low-affinity autoreactive T-cells may never become activated during life, and most often die. Because of the high number of somatic mutations, the large numbers of potentially auto-reactive T-cells require ongoing vigilance to keep self-reactive T-cell activity in check. Failure of these mechanisms is a likely cause of MG and autoimmune diseases in general.

Recently, additional interest has focused on T-cells that express the transcription factor Foxp-3. Referred to as T-regulatory cells or Tregs, they play a crucial role in the maintenance of peripheral tolerance towards self-antigens,[115] and impairment of their function has been demonstrated in patients with MG. How this loss of tolerance develops in MG is not well understood, but thymic abnormalities appear to be important in the pathogenesis of MG.[96,116,117]

5.5.4 B-Cells and Tolerance

Recently, B-cells have emerged as a novel therapeutic target[118] and a previously overlooked participant in the pathogenesis of MG.[119] Within a subgroup of MG patients, the thymus displays signs of ongoing immunostimulation. B-cells collect in the germinal centres and correlate positively with circulating AChR antibodies. This B-cell population is heterogenous, however, with very few cells expressing significant autoreactivity to AChRs. The removal of the thymus in these patients is associated with a reduction in AChR autoantibodies, however, detectable levels persist. This would seem to indicate that other, more peripheral sources of B-cells are present. The presence of AChR-specific B-cells in the circulation,[120,121] lymph nodes[122] and bone marrow[123] strongly suggest these sources are alternate immunological compartments responsible for the ongoing titers of AChR autoantibodies.

While post-thymectomy levels are strongly suggestive of peripheral B-cell involvement, identifying the specific cells involved has been inconclusive. No significant difference is observed between the populations of circulating memory B-cells and antibody-secreting B-cells in Mg and healthy patients. Regulatory B-cells show more promise as they vary in frequency as compared to healthy controls while correlating with disease severity. During naive B-cell maturation, to manage the emergence of autoreactive B-cells, processes are in place to eliminate them from the pool and prevent their proliferation. First, a B-cell tolerance checkpoint in the bone marrow removes developing B-cells that express autoreactive antibodies. At this point, the majority of autoreactive cells are intercepted and removed.[124] Additionally, a B-cell tolerance checkpoint in the periphery identifies autoreactive cells and removes them before they can enter the mature naive B-cell compartment. As with T-cells, if these safeguards malfunction, autoreactive cells may be allowed to proliferate. Many autoimmune diseases exhibit defective B-cell tolerance checkpoints. Indeed, dysfunctional B-cell tolerance mechanisms have been observed in AChR+ MG patients, evident as a naïve B-cell repertoire in MG which differs from that of individuals in which the B-cell checkpoints are functioning.

5.6 Biomarkers

Recent review papers have identified the need for improved and novel biomarkers for MG.[125,126]

Several agencies, including the Food and Drug Administration[127] National Institutes of Health (NIH),[128] and others[129] have identified biomarker discovery and validation as key research priorities.

As an orphan disease, MG has received comparatively little attention in the development of innovative diagnostic and treatment alternatives. There is a broad consensus among MG experts about the urgent need for robust biologic markers in MG in order to

1. Increase diagnostic accuracy, particularly in patients in whom the clinical signs are ambiguous, diagnosis is equivocal and laboratory tests are indeterminate.

2. Delineate the metabolic changes and potential biochemical mechanisms underlying the course, distribution, and severity of the disease in order to develop novel and more effective therapeutic modalities.

3. Accurately predict response to therapies to optimize and individualize treatment.

4. Provide an objective and quantitative assessment tool to be employed as an "end-point" in routine clinical management as well as clinical trials.

Currently, biomarkers for MG are primarily clinical and diagnostic in nature.[15] Further, diagnosis is usually determined on the basis of several biomarkers in combination; no universally accepted biomarkers exist for the prediction of therapeutic outcome, disease progression or severity.[37]

5.6.1 Bedside Assessments

The Tensilon (edrophonium) challenge test is useful in distinguishing myasthenic from cholinergic crisis. Edrophonium is a readily reversible acetylcholinesterase inhibitor which acts to prolong the action of acetylcholine. The sensitivity of this test is ~60 %.

Another bedside assessment, the ice pack test is useful in diagnosing ocular myasthenia. By applying an ice pack over the eye of a suspected MG patient, the action of acetylcholinesterase is inhibited resulting in temporary remission of eye droop (ptosis). A recent study evaluated the value of the ice test in diagnosing MG.[130] The test was positive in 48 (96%) patients and negative in two patients of the myasthenia group. In the control group, 6 (12%) patients had a false positive ice test and in the remaining 44 patients (88%), the ice pack test was negative. Contrary to anecdote, the ice pack test appears to have a high specificity and sensitivity in the differential diagnosis of myasthenic ptosis.

5.6.2 Electrophysiology

Electrophysiological testing is essential for diagnosing MG, and the most commonly used electrophysiological techniques are repetitive nerve stimulation (RNS) and single-fiber electromyography (SFEMG). In MG, nerve signal transduction decreases because of damaged acetylcholine receptors (AChRs). Low frequency (2-5 Hz) RNS results show a decreasing amplitude of compound muscle action potential (CMAP). With these low rates of motor nerve stimulation, RNS depletes the immediate stores of acetylcholine at the neuromuscular junction; decrements of >10% are typically regarded as abnormal.[131] In clinical practice, however, the degree of decrement varies greatly within individual patients. Recently, a retrospective chart review of 75 MG patients was undertaken to revisit the correlation between RNS and clinical characteristics.[94] Clinical characteristics were compared between patients with high jitter (>100 μs) and decrement (>10%), and patients with lower values to explore the correlations and

optimal thresholds of jitter and decrement for different clinical features. High jitter and decrement values revealed multiple correlations. More severe disease, as manifested by more frequent symptomatic bulbar and limb muscle weakness, more frequent ocular and limb muscle weakness on

examination, higher quantitative MG score, and generalized disease were all associated with raised jitter and decrement values. These finding suggest some predictive value may lie in electrophysiological testing as a biomarker of future disease-related decline.

SFEMG is the most sensitive diagnostic test for detecting abnormal neuromuscular transmission. In SFEMG, individual muscle fiber action potentials generated by the same motor neuron are recorded. The variability in time interval between the firing of one muscle fiber potential with relation to the other is termed the neuromuscular jitter.[132] SFEMG is ideally performed in a clinically weak muscle whenever possible. Typically, the extensor digitorum communis (EDC) is studied initially. If the findings are normal in the EDC, a facial muscle should be studied.[133] When a facial and a limb muscle are studied, SFEMG is over 97% sensitive for detecting MG[134]. Although highly sensitive in diagnosing MG, SFEMG has shown limited value as a biomarker for other clinical parameters such as prognosis or therapeutic response. Summary clinical utility parameters for RNS and SFEMG are listed in Table 5.1.

Test	Utility	Notes
RNS[a]	• Sensitivity and specificity of 50% and 97% respectively for ocular. • Sensitivity and specificity of 79% and 97% respectively for generalized.	
EMG[b]	• Sensitivity 86% to 92% and specificity 70% to 96% in facial muscles in ocular. • Sensitivity and specificity of 98% for generalized.	• Abnormal EMG may be seen in LEMS and ALS, inflammatory myopathies, or patients injected with Botox • Painful, impractical for serial measures, not widely available. • Requires skill to perform.
[A]Repetitive nerve stimulation [b]Electromyography		

Table 5.1 Clinical utility parameters for RNS and SFEMG

5.6.3 Immunologic/serologic

Autoantibodies against AChR proteins in the postsynaptic membrane are the most predictive biomarkers guiding diagnosis of MG and defining patient MG subtype. AChR antibodies can be detected with routine assays in ~70% of all patients with MG.[135–138] In another 5–10% of patients. As yet, no consistent correlation has been established between anti-AChR antibody serum titers and MG disease severity.

Antibodies against the α subunit of the AChR are more pathogenic than those against the β subunit; therefore, the AChR epitope pattern influences disease severity.[139] Although total AChR antibody concentration does not correlate well with symptom severity,[135–138] patients with ocular MG tend to have lower antibody titers compared with patients with generalized MG.[140–142] Fluctuations in AChR antibody concentration have been also reported to correspond with the severity of muscle weakness and to predict exacerbations in individual AChR+ patients.[143] The value of serial anti-AChR antibody level measurements in individual MG patients is remains unclear. Several studies have reported clinical improvement of response to reductions in AChR antibody titers correlating with immunomodulation and thymectomy.[144–149] In a prospective study of 60 MG patients, changes in AChR-binding antibody levels correlated

with changes in the quantitative clinical score in most patients, and correlations with clinical state were stronger for binding compared with blocking AChR antibodies.[145] Additionally, a larger study noted a strong correlation between change in AChR antibody titer and clinical status after treatment with prednisone or immunotherapy, and post-thymectomy.[146,147]

Anecdotally, fluctuating antibody levels can influence therapeutic decisions: an increase in antibody concentration is thought to indicate exacerbation of MG, whereas a stable or decreasing concentration could indicate stable disease. Functionally, however, it is not autoantibody concentrations that directly affect the severity of the disease, but rather reduced number of available receptors. The loss of receptors depends not only on total AChR-antibody concentration, but also on autoantibody epitope patterns and non-antibody factors.[135–138]

Anti-AChR-binding antibodies are not specific for MG; patients may be categorized 'falsely seronegative' due to immunosuppression or if the test is performed too early in the disease course.[150] Although uncommon, anti-AChR-binding antibodies may also be found in patients with autoimmune liver disease, systemic lupus, rheumatoid arthritis patients receiving penicillamine, in allogeneic bone marrow transplantation patients who develop graft-versus-host disease,[151] and in patients with thymoma without MG,[151] as well as in neuromyelitis optica.[152]

However, neither the presence nor the absolute concentration of antibodies precisely predicts disease class in all MG patients, nor does it accurately predict clinical disease course or therapeutic response in individual patients. Clinical utility parameters for AChR antibody testing are listed in Table 5.2.

Test		Utility	Notes
Serology	Anti-AChR[a]	• Sensitivity and specificity of 44%-66% and 95%-100% respectively, in ocular. • Sensitivity and specificity of 90% and 99%-100% respectively in generalized. • Detectable in 80% to 90% of patients. • Low in seronegative, high in seropositive patients.	• Seronegative result nonspecific for Anti-MuSK MG.
[a]Anti-acetylcholine receptor antibody			

Table 5.2 Clinical utility parameters for serology

5.6.4 Genetic

Since the first report in 1976,[153] several studies have reported evidence of association of antigens/alleles with MG. The human leukocyte antigen (HLA) locus is of particular interest as multiple genetic association studies have observed an association with MG (Table 5.3). Recently, the genotyping of single nucleotide polymorphisms (SNPs) of tumour necrosis factor α-induced protein 3 (TNFAIP3) genes in MG patients was undertaken. 215 adult MG patients were divided into subgroups according to their clinical features, age of onset, thymic pathology, and autoantibodies. For comparison, 235 healthy controls were also divided into subgroups and gender- and age-matched. The distribution of TNFAIP3 gene rs7749323∗A allele in late-onset MG (AChR+, without thymoma) patients was significantly higher than that of gender- and age-matched healthy controls. Multiple studies have reported an association of A1-B8-DR3-DQ2 with EOMG.[154–156] A northern Europe GWAS study of 600 patients also found interacting protein TNFAIP3, related to the HLA-B08 serotype gene, is associated with EOMG.[157] More often, genes have been associated with LOMG. HLA genes DRB1*15:01, DQB1*05:02, DRB1*16 and HLA-DQA1, all corresponding to MHC class II, are all associated with LOMG.[158–160] Additionally, non-HLA genes have demonstrated associations with MG. Primarily related to T-cell subtype and function, several of these are listed in Table 5.4.

	MHC class II		MHC class I			
	Predisposing	**Protective**	**Predisposing**	**Protective**	**Cell Signalling**	**References**
Genetic	*DQB1*05:02, DRB1*03, DRB1*04, DQB1*02, DQB1*03, DRB1*09, DRB1*15:01, DQB1*05:02, DRB1*16, DQA1*03:02/DQB1*03:03:02*	*DRB1*08, DRB1*13:01, DQA1*05:01*	*B*08, C*07:01*	–	*CTLA4, PTPN22, TNFRSF11A22 and TNFAIP3*	[158,160–169]

Table 5.3. Summary of HLA class I and II associated susceptible or protective alleles in neurological diseases. Adapted from: Immunology (2018) 153:399-414.[170]

Locus symbol, gene product	Variant or marker	Mechanism	References
Cathepsin L2 (CTSL2)	Association between rs4361859 with EO MG	Unknown	171
Cellular tyrosine phosphatase 22 (PTPN22)	Coding (Arg620Trp)	Trp allele impairs binding to Csk kinase	172–175
Cytotoxic T cell late antigen 4 (CTLA4)	Two SNPs in the promoter region	Abnormal alternative splicing	176,177
Galectin-1 (LGALS1)	Association with regulatory region (rs4820293, rs4820294)	Unknown	178
Fork head/winged-helix transcription factor 3 (FOXP3)	SNP in the intron region IVS9+459 (A/G, rs2280883)	Unknown	179
Interleukin receptor 2β (IL2Rβ)	Association with regulatory region (rs743777, rs228941)	Unknown	178
Interferon-γ (IFNG)	Noncoding SNP (+874A/T)	Putative NF-κB binding site	178
Interleukin-4 receptor a (IL4R)	Coding I75V	Reduced responsiveness to interleukin-4	180
Interleukin-10 (IL10)	5′ flanking sequence of the human IL-10 gene (rs45552637 (A/C), rs1800872 (T/C), and rs1800896 (A/G))	Correlated with IL-10 protein production *in vitro*	181,182
Muscle nicotinic acetylcholine receptor α-subunit (CHRNA1)	Upstream polymorphism (−478A/G)	Alters binding of IRF8	183,184
Muscle nicotinic acetylcholine receptor δ-subunits (CHRND)	Intronic microsatellite	Unknown	185
Tumor necrosis factor alpha (TNF)	Rs1800629(-308G<A)	Higher secretion of TNF-alpha	186–188
TNFAIP3-interacting protein 1 (TNIP1)	rs2233290(Pro151Ala)	Ubiquitin-dependent dysregulation of NF-kappaB signaling	189

Table 5.4. Non-HLA genes associated with MG. Source: Journal of Autoimmunity (2014) 52:146-153.[190]

5.6.5 Proteomic

To date, two studies have profiled the MG proteome. A proteome-wide search for potential serum protein diagnostic markers for MG was undertaken using surface-enhanced laser desorption/ionization (SELDI) time-of-flight mass spectrometry (TOFMS).[191] The proteomic spectra from 80 MG patients and 80 healthy individuals were generated by SELDI. The SELDI TOFMS analysis generated 101 peaks. Among them, 9 peaks were down-regulated, and 30 others were up-regulated in the MG sera compared with the controls. A decision tree model was then constructed. The model identified MG patients and healthy individuals with a sensitivity of 83.3% and a specificity of 87.5%, validating the method as a useful tool for the detection and identification of potential serum biomarkers that can diagnose MG.

In a subsequent study, profiling was conducted in an experimental myasthenia gravis setting.[192] Chronic EAMG was induced in seven-week-old rats by immunization with purified AChRs. Using mass spectroscopy and gel electrophoresis, the proteomic profile of rat tibialis anterior muscle was analyzed at different EAMG disease stages. In all, 22 differentially expressed proteins were identified. The majority of these proteins are involved in metabolic pathways (glycolysis and the citric acid cycle), while others are related to cellular-stress responses (e.g. glutathione S-transferase Yb3, 60 KDa heat shock protein), or are contractile proteins (myosin-4 and myozenin-1). Compared to control rats, a reduction of β-enolase and an increase of CAIII levels were detected in all EAMG disease stages. The study failed, however, to identify any changes in well-known NMJ-related protein between EAMG and control muscles.

5.6.6 Metabolomic

To date, only two studies have examined the serum metabolomic profile of myasthenia gravis.[193,194] Both study cohorts were composed of AChR seropositive patients. In 2012, Lu et al. published the first description of the metabolomic profile of myasthenia. An LC-MS chemical analysis was conducted on the serum samples from 42 MG patients and 16 healthy volunteers. The OPLS discriminant analysis (OPLS-DA) class prediction models of acquired metabolomic spectra accurately discriminated MG from healthy controls with 92.8% sensitivity, 83.3% specificity and 90% accuracy. Furthermore, these models also described differences in metabolic profiles between early- and late stage MG patients. Nine potential biomarkers were identified, including gammaaminobutyric acid and sphingosine 1-phosphate.

Two years later, in 2014, Sengupta et al. explored the utility of metabolomic biomarkers as a surrogate measure of treatment response to prednisone. 15 mild to moderate MG patients were recruited. Patients were AChR seropositive and immunosuppressive naïve. Samples were taken at baseline and after 12 weeks of prednisone treatment. Analysis was then performed using ultra-performance liquid chromatography coupled with electro-spray quadrupole time of flight mass spectrometry to obtain comparative metabolomic and lipidomic profiles. Untargeted metabolic profiling of serum showed a clear distinction between pre- and post- treatment groups. Metabolite profiling of chronic prednisone treatment revealed associations with proinflammatory, glycerophospholipid synthesis pathways. Upregulation was observed for membrane associated glycerophospholipids: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, 1, 2- diacyl-sn glycerol 3 phosphate and 1-Acyl-sn-glycero-3-phosphocholine. Arachidonic acid (AA) and AA derived proinflammatory eicosanoids such as 18-carboxy dinor leukotriene B4 and 15 hydroxyeicosatetraenoic acids were reduced. Additional changes in amino acid, carbohydrate, vitamin and lipid metabolism were also observed.

CHAPTER 6.

RHEUMATOID ARTHRITIS AS A COMPARATIVE DISEASE

Rheumatoid arthritis (RA) is a chronic inflammatory and systemic disease characterized by extensive synovial inflammation resulting in the erosion of articular cartilage and marginal bone leading to joint destruction.[195] Like MG, RA is an autoimmune disease; autoantibodies are produced against the Fc fragment of immunoglobulin (Ig) G molecules (rheumatoid factor) and citrullinated proteins and peptides (anti-citrullinated protein antibodies). As a humoral disease, RA shares many immunopathogenic and immunometabolic features with MG. This, along with its well-understood pathophysiology and widespread patient availability, make RA a useful comparative disease.

6.1 Pathogenesis of RA

The pathogenesis of RA is a gradual process; beginning with the development of autoimmunity, RA progresses with local inflammation and eventually stimulates bone destruction.[195,196]

The synovial membrane is the primary target of the immune response. The synovial membrane is connective tissue formed by two main layers: the synovial lining and the synovial sublining. In RA patients, the synovial membrane is characterized by cellular hyperplasia, increased vascularity, and the infiltration of inflammatory cells that invade, grow and destroy adjacent cartilage and bone. This influx of cells, combined with reduced cell death, increases oxygen demand, resulting in local hypoxia. The inadequate oxygenation which results promotes the inflammatory response.[197,198] This process supports further infiltration of inflammatory cells ($CD4^+$ T cells, B cells, plasma cells, NK cells, dendritic cells (DCs) and mast cells), production of inflammatory mediators, and further degradation of supporting tissues.[199–201]

Unlike MG, both the adaptive and the innate immune pathways are activated and contribute to the inflammatory process. In MG, only the adaptive immune pathway is stimulated, further activating the classical complement pathway. Where primary immunoprogramming and maturation takes place in the thymus in MG, the interactions among dendritic cells, T-cells and B-cells occur primarily in the lymph node in RA and generate both the autoimmune response and the activation of T-cells. The RA immune response is also an example of Type III hypersensitivity. Type III hypersensitivity occurs when there is accumulation of immune complexes (antigen-antibody complexes) that have not been adequately cleared by innate immune cells, giving rise to an inflammatory response and

attraction of leukocytes. It involves soluble antigens that are not bound to cell surfaces (as opposed to those in type II hypersensitivity, such as MG). A complex cytokine network promotes inflammation[202] and perpetuates the disease through positive feedback loops. These loops further promote the manifestation of systemic disorders.[203]

Genetic contributions to the pathogenesis of RA have also been observed. Transcription factor signal transducer and activator of transcription 4 (STAT4) play a key role in the interleukin (IL)-12 signalling in T-cells and natural killer (NK) cells, leading to the production of interferon (IFN)-γ and the differentiation of T helper (Th)1 and Th17 cells.[204] The STAT4 gene provides instructions for a protein that acts as a transcription factor, which means that it attaches (binds) to specific regions of DNA and helps control the activity of certain genes. The STAT4 protein is turned on (activated) by cytokines. When activated, the STAT4 protein increases the activity of genes that promote the maturation of naive T-cells into specialized T-cells, called Th1 cells, which suppress or regulate immune responses. Similarly, other candidate genes have immunomodulatory effects.[204,205]

Finally, the contribution of environmental factors to the pathogenesis of RA have been studied extensively. Smoking, infections, sex hormones, birth weight, alcohol intake and socioeconomic status can all modify the risk for RA.[206,207]

6.1.1 RA is an Energy-Intensive Disease

The widespread systemic effects mediated by pro-inflammatory cytokines in RA impact on metabolism and on lymphocyte metabolism in particular. In the presence of pathogens or products of inflamed tissues that provoke inflammation, macrophages and lymphocytes rapidly switch from resting to a highly active state and exhibit a pronounced increase in production of host defence factors resulting in enhanced phagocytosis and antigen presentation.[208] Mounting an inflammatory response is an energy-consuming process. Activation, growth, and proliferation of leukocytes all impose heavy metabolic demands. Activated macrophages exhibit a high hexokinase activity, the first enzyme involved in glycolysis and in the pentose phosphate pathway. Glycolysis and glutamine metabolism are also markedly increased during phagocytosis.[27] A shift towards high glycolysis is a property of inflammatory cells, whereas oxidative phosphorylation is more characteristic of anti-inflammatory cells. A shift towards aerobic glycolysis occurs in macrophages and DCs

acting through Toll-like receptor 4 (TLR4), and in inflammatory macrophages and in Th17 lymphocytes.[37,209] Cells limiting inflammation, such as regulatory T-cells (Treg),[210] anti-inflammatory macrophages and quiescent memory T-cells that carry the CD8 antigen, exhibit oxidative metabolism with more limited rates of glycolysis.[38] Glucose uptake and glycolysis are increased in Th17 cell β-oxidation (as well as Th2 and Th1 cells) compared with Treg cells, which in turn have increased membrane potential and oxidize lipids at a higher rate than other subsets of cells that carry the CD4 antigen.[211,212] Conversely, excess metabolism may prevent apoptosis, exacerbate cell function, and thus promote T-cell hyper-reactivity, leading to autoimmunity and inflammatory diseases.[213,214]

6.2 References

1. Willis T. De anima brutorum quae hominis vitalis ac sentitiva est : exercitationes duae. apud Joannem Balcu; Epub 1672.

2. Goldflam S. Ueber einen scheinbar heilbaren bulbärparalytischen Symptomencomplex mit Betheiligung der Extremitäten. Dtsch Z Nervenheilkd [online serial]. Springer-Verlag; 1893;4:312–352. Accessed at: http://link.springer.com/10.1007/BF01665294. Accessed June 18, 2018.

3. Erb W. Zur casuistik der bulbären lähmungen. Arch Psychiatr Nervenkr. 1879;9:325–350.

4. Jolly F. Ueber myasthenia gravis pseudoparalytica. Berl Klin Wschr. 1895;32:1–7.

5. Desmedt JE. The neuromuscular disorder in myasthenia gravis. New Concepts Mot Unit, Neuromuscul Disord Electromyogr Kinesiol. Karger Publishers; 1973. p. 241–304.

6. Walker MB. Treatment of myasthenia gravis with physostigmine. Lancet. Elsevier; 1934;223:1200–1201.

7. Dale HH, Feldberg W, Vogt M. Release of acetylcholine at voluntary motor nerve endings. J Physiol. Wiley Online Library; 1936;86:353–380.

8. Weigert C. II. Pathologisch-anatomischer Beitrag zur Erb'schen Krankheit (Myasthenia gravis). 1901.

9. Sauerbruch F. Technik der Thoraxchirurgie. J. Springer; 1911.

10. Castleman B, Norris EH. The pathology of the thymus in myasthenia gravis: A study of 35 cases. Medicine (Baltimore). LWW; 1949;28:27–58.

11. Lennon VA, Lindstrom JM, Seybold ME. Experimental autoimmune myasthenia gravis: cellular and humoral immune responses. Ann N Y Acad Sci. Wiley Online Library; 1976;274:283–299.

12. Patrick J, Lindstrom J. Autoimmune response to acetylcholine receptor. Science (80-). American Association for the Advancement of Science; 1973;180:871–872.

13. Fambrough DM, Drachman DB, Satyamurti S. Neuromuscular junction in myasthenia gravis: decreased acetylcholine receptors. Science (80-). American Association for the Advancement of Science; 1973;182:293–295.

14. Buckley C, Douek D, Newsom-Davis J, Vincent A, Willcox N. Mature, long-lived $CD4^+$ and $CD8^+$ T cells are generated by the thymoma in myasthenia gravis. Ann Neurol. 2001;50:64–72.

15. Sussulini A. Metabolomics: From Fundamentals to Clinical Applications. Springer; 2017.

16. Emwas A-HM. The Strengths and Weaknesses of NMR Spectroscopy and Mass Spectrometry with Particular Focus on Metabolomics Research. Humana Press, New York, NY; 2015. p. 161–193. Accessed at: http://link.springer.com/10.1007/978-1-4939-2377-9_13. Accessed June 17, 2018.

17. Zhao S, Luo X, Li L. Chemical Isotope Labeling LC-MS for High Coverage and Quantitative Profiling of the Hydroxyl Submetabolome in Metabolomics. Anal Chem [online serial]. American Chemical Society; 2016;88:10617–10623. Accessed at: http://pubs.acs.org/doi/abs/10.1021/acs.analchem.6b02967. Accessed March 27, 2017.

18. Zhou R, Tseng C-L, Huan T, Li L. IsoMS: Automated Processing of LC-MS Data Generated by a Chemical Isotope Labeling Metabolomics Platform. Anal Chem [online serial]. American Chemical Society; 2014;86:4675–4679. Accessed at: http://pubs.acs.org/doi/abs/10.1021/ac5009089. Accessed March 7, 2017.

19. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing [online]. J. R. Stat. Soc. Ser. B WileyRoyal Statistical Society; 1995.

p. 289–300. Accessed at: https://www.jstor.org/stable/2346101. Accessed June 17, 2018.

20. Benjamini Y, Hochberg Y. On the Adaptive Control of the False Discovery Rate in Multiple Testing With Independent Statistics. J Educ Behav Stat [online serial]. SAGE PublicationsSage CA: Los Angeles, CA; 2000;25:60–83. Accessed at: http://journals.sagepub.com/doi/10.3102/10769986025001060. Accessed June 17, 2018.

21. Pike N. Using false discovery rates for multiple comparisons in ecology and evolution. Accessed at: https://besjournals.onlinelibrary.wiley.com/doi/pdf/10.1111/j.2041-210X.2010.00061.x. Accessed June 17, 2018.

22. Dausset J. The major histocompatibility complex in man. Science [online serial]. 1981;213:1469–1474. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/6792704. Accessed June 18, 2018.

23. Wood SJ, Slater CR. Safety factor at the neuromuscular junction. Prog Neurobiol [online serial]. 2001;64:393–429. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/11275359. Accessed June 6, 2018.

24. Shi J, van Veelen PA, Mahler M, et al. Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. Autoimmun Rev [online serial]. Elsevier; 2014;13:225–230. Accessed at: https://www.sciencedirect.com/science/article/pii/S1568997213002012. Accessed June 11, 2018.

25. Newsholme P, Curi R, Gordon S, Newsholme EA. Metabolism of glucose, glutamine, long-chain fatty acids and ketone bodies by murine macrophages. Biochem J. ; 1986;239:121–125.

26. Alonso D, Nungester WJ. Comparative study of host resistance of guinea pigs and rats V. The effect of pneumococcal products on glycolysis and oxygen uptake by polymorphonuclear leucocytes. J Infect Dis. ; 1956;99:174–181.

27. Rodriguez-Prados JC. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. J Immunol. ; 2010;185:605–614.

28. Krawczyk CM. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood. ; 2010;115:4742–4749.

29. Donnelly RP. mTORC1-dependent metabolic reprogramming is a prerequisite for NK cell effector function. J Immunol. ; 2014;193:4477–4484.

30. Michalek RD, Gerriets VA, Jacobs SR, et al. Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CD4+ T Cell Subsets. J Immunol [online serial]. 2011;186:3299–3303. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/21317389. Accessed May 29, 2018.

31. Doughty CA. Antigen receptor-mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. Blood. ; 2006;107:4458–4465.

32. Gubser PM. Rapid effector function of memory CD8+ T cells requires an immediate-early glycolytic switch. Nat Immunol. ; 2013;14:1064–1072.

33. Shrestha S. Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. Nat Immunol. ; 2015;16:178–187.

34. Huynh A. Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability. Nat Immunol. ; 2015;16:188–196.

35. Wei J. Autophagy enforces functional integrity of regulatory T cells by coupling environmental cues and metabolic homeostasis. Nat Immunol. ; 2016;17:277–285.

36. Everts B. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. Nat Immunol. ; 2014;15:323–332.

37. Shi LZ. HIF1α-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. J Exp Med. ; 2011;208:1367–1376.

38. van der Windt GJW, Everts B, Chang C-H, et al. Mitochondrial Respiratory Capacity Is a Critical Regulator of CD8+ T Cell Memory Development. Immunity [online serial]. 2012;36:68–78.

Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/22206904. Accessed May 29, 2018.

39. Hamilton JA, Vairo G, Lingelbach SR. CSF-1 stimulates glucose uptake in murine bone marrow-derived macrophages. Biochem Biophys Res Commun. ; 1986;138:445–454.

40. De Rosa V, Galgani M, Porcellini A, et al. Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. Nat Immunol. 2015;16:1174–1184.

41. Michl J, Ohlbaum DJ, Silverstein SC. 2-Deoxyglucose selectively inhibits Fc and complement receptor-mediated phagocytosis in mouse peritoneal macrophages. I. Description of the inhibitory effect. J Exp Med. ; 1976;144:1465–1483.

42. Maceyka M, Spiegel S. Sphingolipid metabolites in inflammatory disease. Nature [online serial]. NIH Public Access; 2014;510:58–67. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/24899305. Accessed January 25, 2018.

43. Jha AK. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. Immunity. ; 2015;42:419–430.

44. Beier UH. Essential role of mitochondrial energy metabolism in Foxp3+ T-regulatory cell function and allograft survival. Faseb J. ; 2015;29:2315–2326.

45. Procaccini C. The proteomic landscape of human ex vivo regulatory and conventional T cells reveals specific metabolic requirements. Immunity. ; 2016;44:406–421.

46. O'Sullivan D. Memory CD8+ T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development. Immunity. ; 2014;41:75–88.

47. Tannahill GM. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. Nature. ; 2013;496:238–242.

48. Wientjes FB, Segal AW. NADPH oxidase and the respiratory burst. Semin Cell Biol [online serial]. Academic Press; 1995;6:357–365. Accessed at: https://www.sciencedirect.com/science/article/pii/S1043468205800066. Accessed June 14,

2018.

49. Rehman A, Hemmert KC, Ochi A, et al. Role of fatty-acid synthesis in dendritic cell generation and function. J Immunol [online serial]. NIH Public Access; 2013;190:4640–4649. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/23536633. Accessed June 14, 2018.

50. Blander JM. Regulation of the Cell Biology of Antigen Cross-Presentation. Annu Rev Immunol [online serial]. Annual Reviews ; 2018;36:717–753. Accessed at: http://www.annualreviews.org/doi/10.1146/annurev-immunol-041015-055523. Accessed June 14, 2018.

51. Remmerie A, Scott CL. Macrophages and lipid metabolism. Cell Immunol [online serial]. Academic Press; Epub 2018 Feb 2. Accessed at: https://www.sciencedirect.com/science/article/pii/S0008874918300327. Accessed June 14, 2018.

52. Shibata N, Glass CK. Regulation of macrophage function in inflammation and atherosclerosis. J Lipid Res [online serial]. American Society for Biochemistry and Molecular Biology; 2009;50 Suppl:S277-81. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/18987388. Accessed June 14, 2018.

53. Galván-Peña S, O'Neill LAJ. Metabolic reprograming in macrophage polarization. Front Immunol [online serial]. Frontiers Media SA; 2014;5:420. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/25228902. Accessed June 14, 2018.

54. Nomura M, Liu J, Rovira II, et al. Fatty acid oxidation in macrophage polarization. Nat Immunol [online serial]. Nature Publishing Group; 2016;17:216–217. Accessed at: http://www.nature.com/articles/ni.3366. Accessed June 14, 2018.

55. Michalek RD, Gerriets VA, Jacobs SR, et al. Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CD4+ T Cell Subsets. J Immunol. 2011;186:3299–3303.

56. Wang R, Dillon CP, Shi LZ, et al. The Transcription Factor Myc Controls Metabolic

Reprogramming upon T Lymphocyte Activation. Immunity [online serial]. 2011;35:871–882. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/22195744. Accessed June 14, 2018.

57. Bruno L, von Boehmer H, Kirberg J. Cell division in the compartment of naive and memory T lymphocytes. Eur J Immunol. ; 1996;26:3179–3184.

58. van der Windt GJW, O'Sullivan D, Everts B, et al. CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. Proc Natl Acad Sci [online serial]. 2013;110:14336–14341. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/23940348. Accessed May 29, 2018.

59. Feingold KR. Mechanisms of triglyceride accumulation in activated macrophages. J Leukoc Biol. ; 2012;92:829–839.

60. Posokhova EN, Khoshchenko OM, Chasovskikh MI, Pivovarova EN, Dushkin MI. Lipid synthesis in macrophages during inflammation in vivo: effect of agonists of peroxisome proliferator activated receptors α and γ and of retinoid X receptors. Biochem. ; 2008;73:296–304.

61. Ecker J. Induction of fatty acid synthesis is a key requirement for phagocytic differentiation of human monocytes. Proc Natl Acad Sci Usa. ; 2010;107:7817–7822.

62. Chen HW, Heiniger HJ, Kandutsch AA. Relationship between sterol synthesis and DNA-synthesis in phytohemagglutinin-stimulated mouse lymphocytes. Proc Natl Acad Sci Usa. ; 1975;72:1950–1954.

63. Dufort FJ. Glucose-dependent de novo lipogenesis in B lymphocytes: a requirement for atp-citrate lyase in lipopolysaccharide-induced differentiation. J Biol Chem. ; 2014;289:7011–7024.

64. Fessler MB. Regulation of adaptive immunity in health and disease by cholesterol metabolism. Curr Allergy Asthma Rep. ; 2015;15:48.

65. Lipton JO, Sahin M. The neurology of mTOR. Neuron [online serial]. NIH Public Access; 2014;84:275–291. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/25374355. Accessed June 16, 2018.

66. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev [online serial]. Cold Spring Harbor Laboratory Press; 2004;18:1926–1945. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/15314020. Accessed June 16, 2018.

67. Crawford J, Cohen HJ. The essential role of L-glutamine in lymphocyte differentiation in vitro. J Cell Physiol [online serial]. 1985;124:275–282. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/4044655. Accessed June 14, 2018.

68. Carr EL, Kelman A, Wu GS, et al. Glutamine Uptake and Metabolism Are Coordinately Regulated by ERK/MAPK during T Lymphocyte Activation. J Immunol [online serial]. 2010;185:1037–1044. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/20554958. Accessed June 14, 2018.

69. Rath M, Muller I, Kropf P, Closs EI, Munder M. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. Front Immunol. ; 2014;5:532.

70. Albina JE. Arginine metabolism in wounds. Am J Physiol. ; 1988;254:E459–E467.

71. Pesce JT. Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. P Lo S Pathog. ; 2009;5:e1000371.

72. Rodriguez PC. Regulation of T cell receptor CD3ζ chain expression by l-arginine. J Biol Chem. ; 2002;277:21123–21129.

73. Rodriguez PC, Quiceno DG, Ochoa AC. l-arginine availability regulates T-lymphocyte cell-cycle progression. Blood. ; 2007;109:1568–1573.

74. Somnier FE, Keiding N, Paulson OB. Epidemiology of myasthenia gravis in Denmark a longitudinal and comprehensive population survey. Arch Neurol. American Medical Association; 1991;48:733–739.

75. Carr AS, Cardwell CR, McCarron PO, McConville J. A systematic review of population based epidemiological studies in Myasthenia Gravis. BMC Neurol [online serial]. 2010;10:46. Accessed at: http://bmcneurol.biomedcentral.com/articles/10.1186/1471-2377-10-46. Accessed July 10, 2017.

76. Pakzad Z, Aziz T, Oger J. Increasing incidence of myasthenia gravis among elderly in British Columbia, Canada. Neurology [online serial]. 2011;76:1526–1528. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/21519005. Accessed June 1, 2017.

77. Breiner A, Widdifield J, Katzberg HD, Barnett C, Bril V, Tu K. Epidemiology of myasthenia gravis in Ontario, Canada. Neuromuscul Disord [online serial]. 2016;26:41–46. Accessed at: http://linkinghub.elsevier.com/retrieve/pii/S0960896615007622. Accessed March 7, 2017.

78. Phillips LH. The epidemiology of myasthenia gravis. Ann N Y Acad Sci. 2003;998:407–412.

79. Phillips II LH. The epidemiology of myasthenia gravis. Neurol Clin. 1994;12:263–271.

80. Phillips LH, Torner JC. Epidemiologic evidence for a changing natural history of myasthenia gravis. Neurology. 1996;47:1233–1238.

81. Vincent A, Clover L, Buckley C, Evans JG, Rothwell PM. Evidence of underdiagnosis of myasthenia gravis in older people. J Neurol Neurosurg Psychiatry. BMJ Publishing Group Ltd; 2003;74:1105–1108.

82. Nacu A, Andersen JB, Lisnic V, Owe JF, Gilhus NE. Complicating autoimmune diseases in myasthenia gravis: a review. Autoimmunity [online serial]. Taylor & Francis; 2015;48:362–368. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/25915571. Accessed June 23, 2018.

83. Uncini A, Di Guglielmo G, Di Muzio A. Distal myasthenia gravis and sensory neuronopathy with anti-50 kDa antibody mimicking sensory-motor neuropathy. J Neurol Neurosurg Psychiatry. 1997;63:414–415.

84. Nations SP, Wolfe GI, Amato AA. Distal myasthenia gravis. Neurology. 1999;52:632–634.

85. Werner P, Kiechl S, Löscher S. Distal myasthenia gravis: frequency and clinical course in a large prospective series. Acta Neurol Scand. 2003;108:209–210.

86. Bhanushali MJ, Wuu J, Benatar M. Treatment of ocular symptoms in myasthenia gravis. Neurology [online serial]. 2008;71:1335–1341. Accessed at: http://www.neurology.org/cgi/doi/10.1212/01.wnl.0000327669.75695.38. Accessed June 21,

2018.

87. Nair AG, Patil-Chhablani P, Venkatramani D V, Gandhi RA. Ocular myasthenia gravis: a review. Indian J Ophthalmol [online serial]. Wolters Kluwer -- Medknow Publications; 2014;62:985–991. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/25449931. Accessed June 21, 2018.

88. Basiri K, Ansari B, Okhovat AA. Life-threatening misdiagnosis of bulbar onset myasthenia gravis as a motor neuron disease: How much can one rely on exaggerated deep tendon reflexes. Adv Biomed Res [online serial]. Wolters Kluwer -- Medknow Publications; 2015;4:58. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/25802827. Accessed June 24, 2018.

89. Berrih-Aknin S, Ruhlmann N, Bismuth J, et al. CCL21 overexpressed on lymphatic vessels drives thymic hyperplasia in myasthenia. Ann Neurol [online serial]. 2009;66:521–531. Accessed at: http://doi.wiley.com/10.1002/ana.21628. Accessed June 23, 2018.

90. Eymard B, Berrih-Aknin S. Role of the thymus in the physiopathology of myasthenia. Rev Neurol (Paris). 1995;151:6–15.

91. Thorlacius S, Aarli JA, Riise T, Matre R, Johnsen HJ. Associated disorders in myasthenia gravis: autoimmune diseases and their relation to thymectomy. Acta Neurol Scand [online serial]. 1989;80:290–295. Accessed at: http://doi.wiley.com/10.1111/j.1600-0404.1989.tb03881.x. Accessed June 23, 2018.

92. Romi F, Aarli JA, Gilhus NE. Myasthenia gravis patients with ryanodine receptor antibodies have distinctive clinical features. Eur J Neurol [online serial]. 2007;14:617–620. Accessed at: http://doi.wiley.com/10.1111/j.1468-1331.2007.01785.x. Accessed June 23, 2018.

93. Aragones JM, Bolibar I, Bonfill X, et al. Myasthenia gravis: A higher than expected incidence in the elderly. Neurology [online serial]. 2003;60:1024–1026. Accessed at: http://www.neurology.org/cgi/doi/10.1212/01.WNL.0000050461.05432.C5. Accessed June 23, 2018.

94. Abraham A, Breiner A, Barnett C, Katzberg HD, Lovblom LE, Bril V. Electrophysiological testing is correlated with myasthenia gravis severity. Muscle Nerve. Wiley Online Library; 2017;56:445–

448.

95. Jing F, Cui F, Chen Z, Yang F, Ling LL, Huang X. Clinical and electrophysiological markers in myasthenia gravis patients. Eur Neurol [online serial]. Karger Publishers; 2015;74:22–27. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/26138989. Accessed March 26, 2017.

96. Engel A. Myasthenia Gravis and Myasthenic Disorders [online]. New York: Oxford University Press; 2012. Accessed at: http://login.ezproxy.library.ualberta.ca/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=nlebk&AN=454380&site=ehost-live&scope=site.

97. Romi F, Aarli JA, Gilhus NE. Seronegative myasthenia gravis: disease severity and prognosis. Eur J Neurol [online serial]. 2005;12:413–418. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/15885043. Accessed June 24, 2018.

98. Punga AR, Stålberg E. Acetylcholinesterase inhibitors in MG: to be or not to be? Muscle Nerve. Wiley Online Library; 2009;39:724–728.

99. Yeh J, Chiu H. Plasmapheresis in myasthenia gravis. Acta Neurol Scand. Wiley Online Library; 1999;99:147–151.

100. Rose NR, Bona C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). Immunol Today. Elsevier; 1993;14:426–430.

101. Toyka KV, Drachman DB, Pestronk A, Kao I. Myasthenia gravis: Passive transfer from man to mouse. Science (80-). 1975;190:397–399.

102. Ruff RL, Lennon VA. End-plate voltage-gated sodium channels are lost in clinical and experimental myasthenia gravis. Ann Neurol [online serial]. Wiley-Blackwell; 1998;43:370–379. Accessed at: http://doi.wiley.com/10.1002/ana.410430315. Accessed June 23, 2018.

103. Lindstrom J, Einarson B. Antigenic modulation and receptor loss in experimental autoimmune myasthenia gravis. Muscle Nerve. Wiley Online Library; 1979;2:173–179.

104. Kusner LL, Kaminski HJ, Soltys J. Effect of complement and its regulation on myasthenia gravis

pathogenesis. Expert Rev Clin Immunol [online serial]. England; 2008;4:43–52. Accessed at: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=prem&NEWS=N&AN=20477586.

105. Kusner LL, Kaminski HJ, Soltys J. Effect of complement and its regulation on myasthenia gravis pathogenesis. Expert Rev Clin Immunol [online serial]. NIH Public Access; 2008;4:43–52. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/20477586. Accessed March 26, 2018.

106. Tuzun E, Huda R, Christadoss P. Complement and cytokine based therapeutic strategies in myasthenia gravis. J Autoimmun [online serial]. England; 2011;37:136–143. Accessed at: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=med7&NEWS=N&AN=21636248.

107. Lennon VA, Seybold ME, Lindstrom JM, Cochrane C, Ulevitch R. Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis. J Exp Med. Rockefeller University Press; 1978;147:973–983.

108. Biesecker G, Gomez CM. Inhibition of acute passive transfer experimental autoimmune myasthenia gravis with Fab antibody to complement C6. J Immunol [online serial]. 1989;142:2654–2659. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/2703710. Accessed June 23, 2018.

109. Piddlesden SJ, Jiang S, Levin JL, Vincent A, Morgan BP. Soluble complement receptor 1 (sCR1) protects against experimental autoimmune myasthenia gravis. J Neuroimmunol [online serial]. 1996;71:173–177. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/8982117. Accessed June 23, 2018.

110. Lennon VA, Seybold ME, Lindstrom JM, Cochrane C, Ulevitch R. Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis. J Exp Med [online serial]. 1978;147:973–983. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/206648. Accessed June 23, 2018.

111. Albuquerque EX, Pereira EFR, Alkondon M, Rogers SW. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev [online serial]. NIH Public Access;

2009;89:73–120. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/19126755. Accessed June 23, 2018.

112. Liu Y, Wang W, Li J. Evaluation of Serum IgG Subclass Concentrations in Myasthenia Gravis Patients. Int J Neurosci [online serial]. Taylor & Francis; 2011;121:570–574. Accessed at: https://doi.org/10.3109/00207454.2011.596293.

113. Beasley SJ, Greer JM, Judith Greer CM. Autoantibodies and their potential roles in diseases of the nervous system. Accessed at: https://onlinelibrary.wiley.com/doi/pdf/10.1111/cen3.12269. Accessed June 23, 2018.

114. Zigmond MJ, Coyle JT, Rowland LP. Neurobiology of brain disorders : biological basis of neurological and psychiatric disorders [online]. Accessed at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/book/9780123982704#srch=myasthenia. Accessed March 31, 2018.

115. Kornete M, Piccirillo CA. Functional crosstalk between dendritic cells and Foxp3+ regulatory T cells in the maintenance of immune tolerance. Front Immunol. Frontiers; 2012;3:165.

116. Kaminski HJ. Myasthenia Gravis and Related Disorders [online]. Totowa, NJ: Humana Press; 2009. Accessed at: http://link.springer.com/10.1007/978-1-59745-156-7. Accessed March 31, 2018.

117. Cavalcante P, Le Panse R, Berrih-aknin S, et al. The thymus in myasthenia gravis: Site of “innate autoimmunity”? Muscle Nerve [online serial]. 2011;44:467–484. Accessed at: http://doi.wiley.com/10.1002/mus.22103. Accessed June 18, 2018.

118. Ragheb S, Lisak RP. B-cell-activating factor and autoimmune myasthenia gravis. Autoimmune Dis [online serial]. Hindawi Limited; 2011;2011:939520. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/22235365. Accessed June 24, 2018.

119. Yi JS, Guptill JT, Stathopoulos P, Nowak RJ, O’Connor KC. B cells in the pathophysiology of myasthenia gravis. Muscle Nerve [online serial]. Wiley-Blackwell; 2018;57:172–184. Accessed at: http://doi.wiley.com/10.1002/mus.25973. Accessed March 29, 2018.

120. Newsom-Davis J, Willcox N, Scadding G, Calder L, Vincent A. Anti-acetylcholine receptor antibody synthesis by cultured lymphocytes in myasthenia gravis: thymic and peripheral blood cell interactions. Ann N Y Acad Sci [online serial]. 1981;377:393–402. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/6978662. Accessed June 17, 2018.

121. Lisak RP, Laramore C, Levinson AI, Zweiman B, Moskovitz AR, Witte A. In vitro synthesis of antibodies to acetylcholine receptor by peripheral blood cells: role of suppressor T cells in normal subjects. Neurology [online serial]. 1984;34:802–805. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/6233499. Accessed June 19, 2018.

122. Fujii Y, Monden Y, Hashimoto J, Nakahara K, Kawashima Y. Acetylcholine receptor antibody-producing cells in thymus and lymph nodes in myasthenia gravis. Clin Immunol Immunopathol [online serial]. 1985;34:141–146. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/4038384. Accessed June 17, 2018.

123. Fujii Y, Monden Y, Hashimoto J, Nakahara K, Kawashima Y. Acetylcholine receptor antibody production by bone marrow cells in a patient with myasthenia gravis. Neurology. 1985;35:577–579.

124. Meffre E, Wardemann H. B-cell tolerance checkpoints in health and autoimmunity. Curr Opin Immunol. 2008;20:632–638.

125. Kaminski HJ, Kusner LL, Wolfe GI, et al. Biomarker development for myasthenia gravis. Ann N Y Acad Sci [online serial]. Wiley/Blackwell (10.1111); 2012;1275:101–106. Accessed at: http://doi.wiley.com/10.1111/j.1749-6632.2012.06787.x. Accessed March 19, 2018.

126. Benatar M, Sanders DB, Burns TM, et al. Recommendations for myasthenia gravis clinical trials. Muscle Nerve [online serial]. 2012;45:909–917. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/22581550. Accessed June 1, 2017.

127. Butterfield LH, Palucka AK, Britten CM, et al. Recommendations from the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers. Clin Cancer Res [online serial]. 2011;17:3064–3076. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/21558394. Accessed June 1, 2017.

128. Dickler HB, McCoy JP, Nussenblatt R, et al. The National Institutes of Health Center for Human Immunology, Autoimmunity, and Inflammation: history and progress. Ann N Y Acad Sci [online serial]. 2013;1285:133–147. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/23692568. Accessed June 1, 2017.

129. Kaminski HJ, Kusner LL, Wolfe GI, et al. Biomarker development for myasthenia gravis. Ann N Y Acad Sci [online serial]. 2012;1275:101–106. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/23278584. Accessed June 1, 2017.

130. Natarajan B, Saifudheen K, Gafoor VA, Jose J. Accuracy of the ice test in the diagnosis of myasthenic ptosis. Neurol India. Medknow; 2016;64:1169.

131. Lee HE, Kim YH, Kim SM, Shin HY. Clinical Significance of Repetitive Compound Muscle Action Potentials in Patients with Myasthenia Gravis: A Predictor for Cholinergic Side Effects of Acetylcholinesterase Inhibitors. J Clin Neurol [online serial]. Korean Neurological Association; 2016;12:482–488. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/27819419. Accessed June 23, 2018.

132. Bril V. Single Fiber Electromyography: Studies in Healthy and Diseased Muscle. Neurology. AAN Enterprises; 1995;45:1793.

133. AAEM Quality Assurance Committee. American Association of Electrodiagnostic Medicine. Practice parameter for repetitive nerve stimulation and single fiber EMG evaluation of adults with suspected myasthenia gravis or Lambert-Eaton myasthenic syndrome: summary statement. Muscle Nerve [online serial]. 2001;24:1236–1238. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/11494280. Accessed June 23, 2018.

134. Howard JF. Electrodiagnosis of disorders of neuromuscular transmission. Phys Med Rehabil Clin. Elsevier; 2013;24:169–192.

135. Querol L, Illa I. Myasthenia gravis and the neuromuscular junction. Curr Opin Neurol. LWW; 2013;26:459–465.

136. Gilhus NE. Myasthenia and the neuromuscular junction. Curr Opin Neurol. LWW; 2012;25:523–

529.

137. Verschuuren JJGM, Huijbers MG, Plomp JJ, et al. Pathophysiology of myasthenia gravis with antibodies to the acetylcholine receptor, muscle-specific kinase and low-density lipoprotein receptor-related protein 4. Autoimmun Rev. Elsevier; 2013;12:918–923.

138. Heldal AT, Eide GE, Romi F, Owe JF, Gilhus NE. Repeated acetylcholine receptor antibody-concentrations and association to clinical myasthenia gravis development. PLoS One. Public Library of Science; 2014;9:e114060.

139. Kordas G, Lagoumintzis G, Sideris S, Poulas K, Tzartos SJ. Direct proof of the in vivo pathogenic role of the AChR autoantibodies from myasthenia gravis patients. PLoS One. Public Library of Science; 2014;9:e108327.

140. Vincent A, Newsom-Davis J. Acetylcholine receptor antibody as a diagnostic test for myasthenia gravis: results in 153 validated cases and 2967 diagnostic assays. J Neurol Neurosurg Psychiatry [online serial]. 1985;48:1246–1252. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/4087000. Accessed June 23, 2018.

141. Cao M, Vincent A. Autoantibody Testing in the Diagnosis and Management of Autoimmune Disorders of Neuromuscular Transmission and Related Diseases. Myasthenia Gravis Relat Disord. Springer; 2018. p. 153–168.

142. Vincent A, Newsom-Davis J. Acetylcholine receptor antibody characteristics in myasthenia gravis. I. Patients with generalized myasthenia or disease restricted to ocular muscles. Clin Exp Immunol [online serial]. 1982;49:257–265. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/6813004. Accessed June 23, 2018.

143. Heldal ATAT, Eide GEGE, Romi F, Owe JFJF, Gilhus NENENE. Repeated acetylcholine receptor antibody-concentrations and association to clinical myasthenia gravis development. Saruhan-Direskeneli G, editor. PLoS One [online serial]. Public Library of Science; 2014;9:e114060. Accessed at: http://dx.plos.org/10.1371/journal.pone.0114060. Accessed March 26, 2017.

144. Tindall RSA. Humoral immunity in myasthenia gravis: Biochemical characterization of acquired

antireceptor antibodies and clinical correlations. Ann Neurol [online serial]. 1981;10:437–447. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/7305297. Accessed June 23, 2018.

145. Besinger UA, Toyka K V, Hömberg M, Heininger K, Hohlfeld R, Fateh-Moghadam A. Myasthenia gravis: long-term correlation of binding and bungarotoxin blocking antibodies against acetylcholine receptors with changes in disease severity. Neurology [online serial]. 1983;33:1316–1321. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/6684226. Accessed June 23, 2018.

146. Oosterhuis HJ, Limburg PC, Hummel-Tappel E, The TH. Anti-acetylcholine receptor antibodies in myasthenia gravis. Part 2. Clinical and serological follow-up of individual patients. J Neurol Sci [online serial]. 1983;58:371–385. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/6842265. Accessed June 23, 2018.

147. Oosterhuis HJ, Limburg PC, Hummel-Tappel E, Van den Burg W, The TH. Anti-acetylcholine receptor antibodies in myasthenia gravis. Part 3. The effect of thymectomy. J Neurol Sci [online serial]. 1985;69:335–343. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/4031948. Accessed June 23, 2018.

148. Tindall RS. Humoral immunity in myasthenia gravis: effect of steroids and thymectomy. Neurology [online serial]. 1980;30:554–557. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/7189263. Accessed June 23, 2018.

149. Kuks JBM, Oosterhuis HJGH, Limburg PC, The TH. Anti-acetylcholine receptor antibodies decrease after thymectomy in patients with myasthenia gravis. Clinical correlations. J Autoimmun. 1991;4:197–211.

150. Chan KH, Lachance DH, Harper CM, Lennon VA. Frequency of seronegativity in adult-acquired generalized myasthenia gravis. Muscle Nerve [online serial]. 2007;36:651–658. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/17654558. Accessed June 23, 2018.

151. Lefvert AK, Björkholm M. Antibodies against the acetylcholine receptor in hematologic disorders: implications for the development of myasthenia gravis after bone marrow grafting. N Engl J Med. 1987;317:170.

152. McKeon A, Lennon VA, Jacob A, et al. Coexistence of myasthenia gravis and serological markers of neurological autoimmunity in neuromyelitis optica. Muscle Nerve [online serial]. 2009;39:87–90. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/19086079. Accessed June 23, 2018.

153. Pirskanen R. Genetic associations between myasthenia gravis and the HL-A system. J Neurol Neurosurg Psychiatry. BMJ Publishing Group Ltd; 1976;39:23–33.

154. Vandiedonck C, Beaurain G, Giraud M, et al. Pleiotropic effects of the 8.1 HLA haplotype in patients with autoimmune myasthemia gravis and thymus hyperplasia. Proc Natl Acad Sci U S A. 2004;101:15464–15469.

155. Janer M, Cowland A, Picard J, et al. A susceptibility region for myasthenia gravis extending into the HLA-class I sector telomeric to HLA-C. Hum Immunol. Elsevier; 1999;60:909–917.

156. Giraud M, Beaurain G, Eymard B, Tranchant C, Gajdos P, Garchon HJ. Genetic control of autoantibody expression in autoimmune myasthenia gravis: role of the self-antigen and of HLA-linked loci. Genes Immun. Nature Publishing Group; 2004;5:398.

157. K. GP, Roman K, T. LA, et al. Risk for myasthenia gravis maps to a 151Pro→Ala change in TNIP1 and to human leukocyte antigen-B*08. Ann Neurol [online serial]. Wiley-Blackwell; 2012;72:927–935. Accessed at: https://doi.org/10.1002/ana.23691.

158. Testi M, Terracciano C, Guagnano A, et al. Association of HLA-DQB1* 05: 02 and DRB1* 16 alleles with late-onset, nonthymomatous, AChR-Ab-positive myasthenia gravis. Autoimmune Dis. Hindawi; 2012;2012.

159. Renton AE, Pliner HA, Provenzano C, et al. A genome-wide association study of myasthenia gravis. JAMA Neurol [online serial]. NIH Public Access; 2015;72:396–404. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/25643325. Accessed June 18, 2018.

160. Maniaol AH, Elsais A, Lorentzen ÅR, et al. Late onset myasthenia gravis is associated with HLA DRB1* 15: 01 in the Norwegian population. PLoS One. Public Library of Science; 2012;7:e36603.

161. Baggi F, Antozzi C, Andreetta F, et al. Identification of a novel HLA class II association with

DQB1* 0502 in an Italian myasthenic population. Ann N Y Acad Sci. Wiley Online Library; 1998;841:355–359.

162. Yi JS, Guptill JT, Stathopoulos P, Nowak RJ, O'Connor KC. B cells in the pathophysiology of myasthenia gravis. Muscle and Nerve. 2018;57:172–184.

163. Renton AE, Pliner HA, Provenzano C, et al. A Genome-Wide Association Study of Myasthenia Gravis. JAMA Neurol [online serial]. American Medical Association; 2015;72:396. Accessed at: http://archneur.jamanetwork.com/article.aspx?doi=10.1001/jamaneurol.2014.4103. Accessed March 26, 2017.

164. Rioux JD, Goyette P, Vyse TJ, et al. Mapping of multiple susceptibility variants within the MHC region for 7 immune-mediated diseases. Proc Natl Acad Sci U S A. 2009;106:18680–18685.

165. Zhu W-H, Lu J-H, Lin J, et al. HLA-DQA1* 03: 02/DQB1* 03: 03: 02 is strongly associated with susceptibility to childhood-onset ocular myasthenia gravis in Southern Han Chinese. J Neuroimmunol. Elsevier; 2012;247:81–85.

166. Gregersen PK, Kosoy R, Lee AT, et al. Risk for myasthenia gravis maps to a 151Pro→ Ala change in TNIP1 and to human leukocyte antigen-B* 08. Ann Neurol. Wiley Online Library; 2012;72:927–935.

167. Xie Y, Qu Y, Sun L, et al. Association between HLA-DRB1 and myasthenia gravis in a northern Han Chinese population. J Clin Neurosci. Elsevier; 2011;18:1524–1527.

168. Santos E, Bettencourt A, da Silva AM, et al. HLA and age of onset in myasthenia gravis. Neuromuscul Disord. Elsevier; 2017;27:650–654.

169. Fekih-Mrissa N, Klai S, Zaouali J, Gritli N, Mrissa R. Association of HLA-DR/DQ polymorphism with myasthenia gravis in Tunisian patients. Clin Neurol Neurosurg. Elsevier; 2013;115:32–36.

170. Misra MK, Damotte V, Hollenbach JA. The immunogenetics of neurological disease. Immunology [online serial]. Wiley/Blackwell (10.1111); 2018;153:399–414. Accessed at: http://doi.wiley.com/10.1111/imm.12869. Accessed June 22, 2018.

171. Viken MK, Dahlen Sollid H, Joner G, et al. Polymorphisms in the cathepsin L2 (CTSL2) gene show association with type 1 diabetes and early-onset myasthenia gravis. Hum Immunol [online serial]. 2007;68:748–755. Accessed at: http://linkinghub.elsevier.com/retrieve/pii/S0198885907001267. Accessed June 22, 2018.

172. Provenzano C, Ricciardi R, Scuderi F, et al. PTPN22 and myasthenia gravis: Replication in an Italian population and meta-analysis of literature data. Neuromuscul Disord [online serial]. 2012;22:131–138. Accessed at: http://linkinghub.elsevier.com/retrieve/pii/S0960896611013411. Accessed June 22, 2018.

173. Greve B, Hoffmann P, Illes Z, et al. The autoimmunity-related polymorphism PTPN22 1858C/T is associated with anti-titin antibody-positive myasthenia gravis. Hum Immunol [online serial]. 2009;70:540–542. Accessed at: http://linkinghub.elsevier.com/retrieve/pii/S0198885909001165. Accessed June 22, 2018.

174. Vandiedonck C, Capdevielle C, Giraud M, et al. Association of the PTPN22*R620W polymorphism with autoimmune myasthenia gravis. Ann Neurol [online serial]. 2006;59:404–407. Accessed at: http://doi.wiley.com/10.1002/ana.20751. Accessed June 22, 2018.

175. Lefvert AK, Zhao Y, Ramanujam R, Yu S, Pirskanen R, Hammarström L. PTPN22 R620W promotes production of anti-AChR autoantibodies and IL-2 in myasthenia gravis. J Neuroimmunol [online serial]. 2008;197:110–113. Accessed at: http://linkinghub.elsevier.com/retrieve/pii/S0165572808001008. Accessed June 22, 2018.

176. Wang X, Pirskanen R, Giscombe R, Lefvert AK. Two SNPs in the promoter region of the CTLA-4 gene affect binding of transcription factors and are associated with human myasthenia gravis. J Intern Med [online serial]. 2007;263:61–69. Accessed at: http://doi.wiley.com/10.1111/j.1365-2796.2007.01879.x. Accessed June 22, 2018.

177. Gu M, Kakoulidou M, Giscombe R, et al. Identification of CTLA-4 isoforms produced by alternative splicing and their association with myasthenia gravis. Clin Immunol [online serial]. 2008;128:374–381. Accessed at: http://linkinghub.elsevier.com/retrieve/pii/S1521661608006748. Accessed June 22, 2018.

178. Pál Z, Antal P, Millinghoffer A, et al. A novel galectin-1 and interleukin 2 receptor β haplotype is associated with autoimmune myasthenia gravis. J Neuroimmunol. 2010;229:107–111.

179. Zhang J, Chen Y, Jia G, et al. FOXP3 -3279 and IVS9+459 polymorphisms are associated with genetic susceptibility to myasthenia gravis. Neurosci Lett. 2013;534:274–278.

180. Pál Z, Varga Z, Semsei T, et al. Interleukin-4 receptor alpha polymorphisms in autoimmune myasthenia gravis in a Caucasian population. Hum Immunol. 2012;73:193–195.

181. Alseth EH, Nakkestad HL, Aarseth J, Gilhus NE, Skeie GO. Interleukin-10 promoter polymorphisms in myasthenia gravis. J Neuroimmunol. 2009;210:63–66.

182. Zagoriti Z, Georgitsi M, Giannakopoulou O, et al. Genetics of myasthenia gravis: a case-control association study in the Hellenic population. Clin Dev Immunol. Hindawi Publishing Corporation; 2012;2012.

183. Heckmann JM, Morrison KE, Emeryk-Szajewska B, et al. Human muscle acetylcholine receptor alpha-subunit gene (CHRNA1) association with autoimmune myasthenia gravis in Black, Mixed-Ancestry and Caucasian subjects. J Autoimmun. 1996;9:175–180.

184. Giraud M, Taubert R, Vandiedonck C, et al. An IRF8-binding promoter variant and AIRE control CHRNA1 promiscuous expression in thymus. Nature. 2007;448:934–937.

185. Giraud M, Eymard B, Tranchant C, Gajdos P, Garchon H-J. Association of the gene encoding the δ-subunit of the muscle acetylcholine receptor (CHRND) with acquired autoimmune myasthenia gravis. Genes Immun. 2004;5:80–83.

186. Skeie GO, Pandey JP, Aarli JA, Gilhus NE. TNFA and TNFB polymorphisms in myasthenia gravis. Arch Neurol. 1999;56:457–461.

187. Huang D, Pirskanen R, Matell G, Lefvert AK. Tumour necrosis factor-α polymorphism and secretion in myasthenia gravis. J Neuroimmunol. 1999;94:165–171.

188. Elahi MM, Asotra K, Matata BM, Mastana SS. Tumor necrosis factor alpha - 308 gene locus promoter polymorphism: An analysis of association with health and disease. Biochim Biophys

Acta - Mol Basis Dis. 2009;1792:163–172.

189. Gregersen PK, Kosoy R, Lee AT, et al. Risk for myasthenia gravis maps to a^{151}Pro→Ala change in TNIP1 and to human leukocyte antigen-B*08. Ann Neurol. 2012;72:927–935.

190. Avidan N, Miller A. Genetic basis of myasthenia gravis – A comprehensive review. J Autoimmun [online serial]. Academic Press; 2014;52:146–153. Accessed at: https://www.sciencedirect.com/science/article/pii/S0896841113001480. Accessed June 22, 2018.

191. Cheng C, Wu G, Yeung S-CJ, et al. Serum Protein Profiles in Myasthenia Gravis. Ann Thorac Surg. 2009;88:1118–1123.

192. Gomez AM, Vanheel A, Losen M, et al. Proteomic analysis of rat tibialis anterior muscles at different stages of experimental autoimmune myasthenia gravis. J Neuroimmunol. 2013;261:141–145.

193. Sengupta M, Cheema A, Kaminski HJ, Kusner LL. Serum metabolomic response of myasthenia gravis patients to chronic prednisone treatment. PLoS One. 2014;9.

194. Lu Y, Wang C, Chen Z, et al. Serum metabolomics for the diagnosis and classification of myasthenia gravis. Metabolomics [online serial]. Springer US; 2012;8:704–713. Accessed at: http://link.springer.com/10.1007/s11306-011-0364-6. Accessed June 1, 2017.

195. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. ; 2011;365:2205–2219.

196. Zordan P, Rigamonti E, Freudenberg K, Conti V, Azzoni E, Rovere-Querini P. Macrophages commit postnatal endothelium-derived progenitors to angiogenesis and restrict endothelial to mesenchymal transition during muscle regeneration. Cell Death Dis. ; 2014;5:e1031.

197. Jongbloed SL, Lebre MC, Fraser AR, Gracie JA, Sturrock RD, Tak PP. Enumeration and phenotypical analysis of distinct dendritic cell subsets in psoriatic arthritis and rheumatoid arthritis. Arthritis Res Ther. ; 2006;8:R15.

198. Mihaly SR, Ninomiya-Tsuji J, Morioka S. TAK1 control of cell death. Cell Death Differ. ; 2014;21:1667–1676.

199. Shalini S, Dorstyn L, Dawar S, Kumar S. Old, new and emerging functions of caspases. Cell Death Differ. ; 2015;22:526–539.

200. Yang L, Karin M. Roles of tumor suppressors in regulating tumor-associated inflammation. Cell Death Differ. ; 2014;21:1677–1686.

201. Reis AC, Alessandri AL, Athayde RM, Perez DA, Vago JP, Ávila T V. Induction of eosinophil apoptosis by hydrogen peroxide promotes the resolution of allergic inflammation. Cell Death Dis. ; 2015;6:e1632.

202. Través PG, Pardo V, Pimentel-Santillana M, et al. Pivotal role of protein tyrosine phosphatase 1B (PTP1B) in the macrophage response to pro-inflammatory and anti-inflammatory challenge. Cell Death Dis [online serial]. 2017;5:e1125–e1125. Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/24625984. Accessed June 18, 2018.

203. Brennan FM, McInnes IB. Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest. ; 2008;118:3537–3545.

204. Plenge RM, Seielstad M, Padyukov L, Lee AT, Remmers EF, Ding B. TRAF1-C5 as a risk locus for rheumatoid arthritis: a genomewide study. N Engl J Med. ; 2007;357:1199–1209.

205. Hu R, Chen ZF, Yan J, Li QF, Huang Y, Xu H. Complement C5a exacerbates acute lung injury induced through autophagy-mediated alveolar macrophage apoptosis. Cell Death Dis. ; 2014;5:e1330.

206. Cauwels A, Rogge E, Vandendriessche B, Shiva S, Brouckaert P. Extracellular ATP drives systemic inflammation, tissue damage and mortality. Cell Death Dis. ; 2014;5:e1102.

207. Pedersen M, Jacobsen S, Klarlund M, Pedersen B V, Wiik A, Wohlfahrt J. Environmental risk factors differ between rheumatoid arthritis with and without auto-antibodies against cyclic citrullinated peptides. Arthritis Res Ther. ; 2006;8:R133.

208. Hiebert PR, Wu D, Granville DJ. Granzyme B degrades extracellular matrix and contributes to delayed wound closure in apolipoprotein E knockout mice. Cell Death Differ. ; 2013;20:1404–1414.

209. Yin YW, Liao SQ, Zhang MJ, Liu Y, Li BH, Zhou Y. TLR4-mediated inflammation promotes foam cell formation of vascular smooth muscle cell by upregulating ACAT1 expression. Cell Death Dis. ; 2015;6:1659.

210. Dang E V, Barbi J, Yang HY, Jinasena D, Yu H, Zheng Y. Control of TH17/Treg balance by hypoxia-inducible factor 1. Cell. ; 2011;146:772–784.

211. Cencioni MT, Santini S, Ruocco G, Borsellino G, De Bardi M, Grasso MG. FAS-ligand regulates differential activation-induced cell death of human T-helper 1 and 17 cells in healthy donors and multiple sclerosis patients. Cell Death Dis. ; 2015;6:e1741.

212. Li W, Ren G, Huang Y, Su J, Han Y, Li J. Mesenchymal stem cells: a double-edged sword in regulating immune responses. Cell Death Differ. ; 2012;19:1505–1513.

213. Tanner DC, Campbell A, O'Banion KM, Noble M, Mayer-Pröschel M. cFLIP is critical for oligodendrocyte protection from inflammation. Cell Death Differ. ; 2015;22:1489–1501.

214. Ewald F, Annemann M, Pils MC, Plaza-Sirvent C, Neff F, Erck C. Constitutive expression of murine c-FLIPR causes autoimmunity in aged mice. Cell Death Dis. ; 2014;5:e1168.

CHAPTER 7.

METHODS

7.1.1 Clinical characteristics of study subjects

This study was conducted in accordance with the Declaration of Helsinki ethical principles for medical research involving human subjects and received ethics approval from the University of Alberta Research Ethics Board and operational approvals from Alberta Health Services. Each study patient provided his/her informed consent for the study.

We prospectively enrolled 50 seropositive MG, and 50 seropositive RA patients, and 50 healthy controls (HC). MG and RA serostatus was confirmed with antibody testing for either anti-AChR (MG) or rheumatoid factor (RA). For the purposes of this study, ocular and generalized MG subtypes were considered phenotypically identical and serum sample were collected from both. RA patients were diagnosed in accordance with the American Rheumatology Association 1987 criteria.[1] To exclude the confounds of race, only Caucasian patients were included in this study. There were no smokers and no statistically significant differences between all groups from time of last meal or BMI.

Clinical patients and healthy controls were enrolled in a prospective observational trial to obtain serum. MG and HC were collected within the same clinic. RA samples were collected in multiple clinics. Study subjects were age and gender matched, within the limitations of opportunistic sampling in the clinical setting. Further, patients had no history of any other autoimmune disease or thymoma. Finally, due to the nature of recruitment, patients were not required to fast. In total, 27 age and gender-matched samples were added to the cohort for a sum of 50. Batch effects were considered significant, however, and these samples were excluded, leaving a study cohort of 46 MG, 23 RA and 49 controls. Table 7.1 summarizes patient data.

Feature	Value
MG	**n= 46**
Gender (M/F, %)	61/39
Age (years)	59 ± 3.1 (Range: 19-93)
BMI	28.08 ± 0.56 (Range: 19.06-34.97)
Class (ocular/generalized, %)	24/76
MMT	4 ± 0.75 (Range: 0-23)
Age of onset (years)	52 ± 3.3 (Range: 15-86)
Duration since diagnosis (years)	7 ± 1.1 (Range: 0-46)
Myasthenia Gravis Medications	**(n/46, %)**
Pyridostigmine	35 (76)
Prednisone	19 (41)
Azathioprine	14 (30)
Mycophenolate mofetil	7 (15)
Tacrolimus	1 (2)
RA	**n= 23**
Gender (M/F, %))	71/29
Age (years)	56 ± 3.2 (Range: 24-84)
RF (% positive)	61.3
BMI	26.18 ± 0.83 (Range: 19.63-42.57)
Symptom Duration (years)	35 ± 4.05 (Range: 1-75)
Diagnosis Duration (years)	37 ± 4.02 (Range: 1-76)
DAS	4 ± 0.33 (Range: 1-8)
HAQ	1 ± 0.18 (Range: 0-4)
ESR	24.54 ± 5.02 (Range: 0-108)
CRP	13.62 ± 3.91 (Range: 0.2-86)
Controls	**n= 49**
Gender (M/F, %)	53/47
Age (years)	49 ± 2.7 (Range: 19-88)
BMI	27.47 ± 0.55 (Range: 18.07-36.58)

BMI= Body Mass index, MMT= manual muscle testing, RF= Rheumatoid factor, DAS=Disease activity score, HAQ= Health assessment questionnaire, ESR=Erythrocyte sedimentation rate , CRP= C-Reactive protein

Table 7.1. Demographic data and clinical profiles

24 ml blood samples were drawn from the anticubital vein using a 21 G needle and vacutainer® red top no additive tubes (Becton Dickenson Ref:366408). Collected samples were allowed to clot for 30 minutes, at which time serum was drawn using a 0.2 um syringe filter and dispensed into a sterile labelled 15 ml centrifuge tube. (Corning part # 431224). The blood samples were centrifuged at 2200g

for 10 minutes at 4°C within 45 min and the serum was immediately frozen on dry ice. Collected samples were stored at -80 °C until further use.

7.1.2 Metabolite Extraction and Labelling

The workflow for CIL LC-MS profiling of serum is illustrated in Figure 7.1

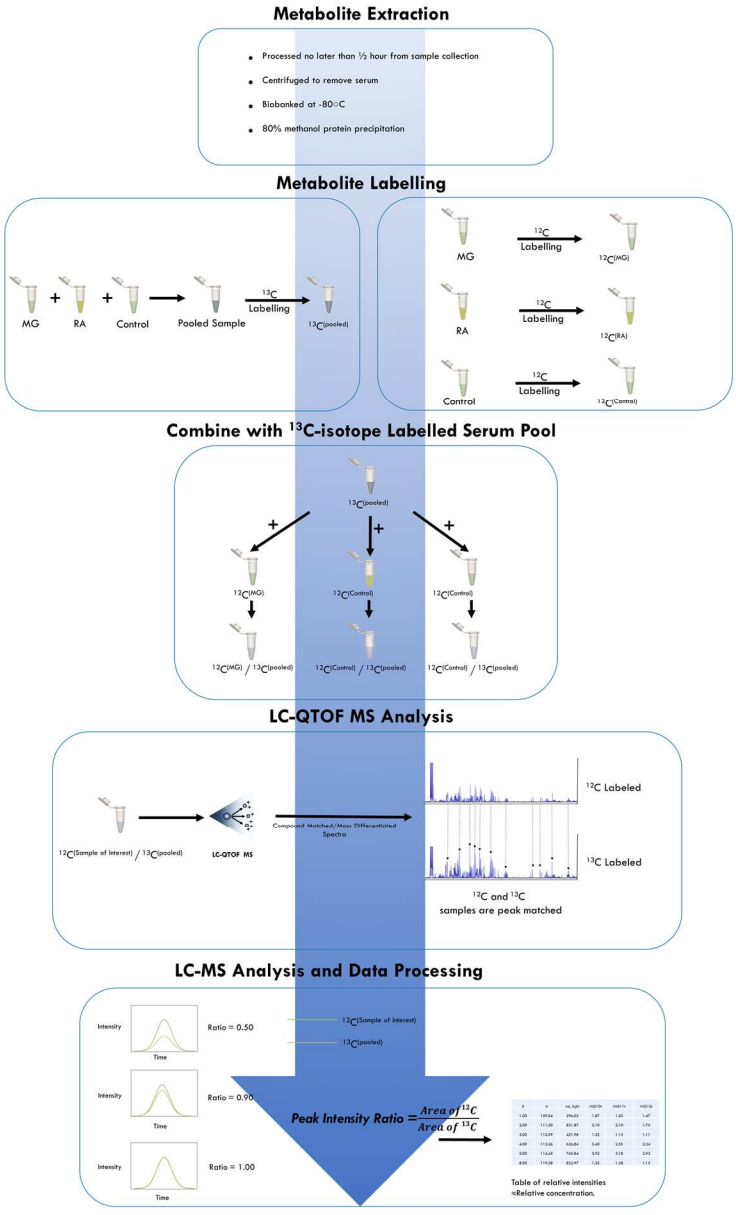


Figure 7.1 workflow for CIL LC-MS profiling

7.1.2.1 Dansylation Labelling

In brief, frozen serum samples were thawed on ice, vortexed to dissolve precipitates and then centrifuged at 14000 rpm for 10 min. 25 µL supernatant was transferred into an Eppendorf tube and was mixed with 75 µL cold methanol. The mixture was then incubated on ice for 15 min to precipitate any proteins. Next, the mixture was centrifuged at 14000 rpm for 15 min. 75 µL supernatant was taken and dried using a SpeedVac. The sample was reconstituted in 50 µL 1:1(v:v) Water/ACN solution. An aliquot of 25 µL serum solution was then mixed with 25 µL of 250 mM sodium carbonate/sodium. This bicarbonate buffer was further added into the sample to make a basic environment for the dansylation reaction. The solution was vortexed, spun down, and mixed with 25 µL of freshly prepared ^{12}C-dansyl chloride solution (18 mg/mL) (for light labelling) or ^{13}C-dansyl chloride solution (18 mg/mL) (for heavy labelling). After 45 min incubation at 40 °C, 5 µL of 250 mM NaOH was added to the reaction mixture to quench the excess dansyl chloride. The solution was then incubated at 40 °C for another 10 min. Finally, 25 µL formic acid (425 mM) in 1:1(v:v) ACN/H2O was added to acidify the solution.

7.1.2.2 DmPA (dimethylaminophenacyl) labelling

For DmPA labelling after protein precipitation, an aliquot of 20 µL reconstituted serum sample (described above) was mixed with 20 µL water and 5 µL 6 M HCl solution, then followed by adding 5 µL of saturated NaCl solution. The vial was vortexed, then spun down and the sample was extracted using 150 µL ethyl acetate. Each vial was further vortexed for 30s and then centrifuged at 8000 rpm for 5 min. The organic phase was transferred into another centrifuge vial with a screw cap and the pH was adjusted to 8 by adding 20 µL TEA solution (20 mg/mL in acetonitrile). Afterwards, the sample was dried down by using SpeedVac and then reconstituted in 30 µL TEA solution (10 mg/mL in acetonitrile). A solution of either ^{12}C-DmPA or ^{13}C-DmPA (10 mg/mL in acetonitrile) was added into the vial. The vial was again vortexed and spun down. Finally, the vial was incubated in an oven at 85◦C for 55 min.

7.1.2.3 LC−UV Quantification

Sample amount was normalized using a protocol previously described[2] based on LC−UV measurement of the total concentration of dansyl or DmPA labelled metabolites in a sample. A Waters ACQUITY UPLC system with a photodiode array (PDA) detector was used. 4 µL (dansyl-labelled) or 1 µL (DmPA-labelled) of each labelled serum sample was injected onto a Phenomenex Kinetex C18 column (50 mm × 2.1 mm, 1.7 µm particle size, 100 Å pore size) for a fast-step gradient run. Solvent A was 0.1% formic acid/5% ACN/water(v/v/v), and solvent B was 0.1% formic acid/ACN((v/v). The step gradient started with 0% B for 1 min and was increased to 95% within 0.01 min and held at 95% B for

1 min to ensure complete elution of all labelled metabolites. The flow rate was set at 0.45 mL/min. The total peak area of the labelled metabolites was measured at 338 nm and integrated using the Empower software (6.00.2154.003). Based on the quantification results, the ^{12}C - and ^{13}C -labelled samples were then mixed in equal amounts.

7.1.2.4 LC−QTOF-MS

Labelled serum samples were analyzed using a Bruker HD Impact quadrupole time-of-flight (QTOF) mass spectrometer (Billerica, MA, U.S.A.) with ESI linked to an Agilent 1100 series HPLC system (Palo Alto, CA, U.S.A.) along with an Agilent eclipse plus C18 column (100 mm × 2.1 mm, 1.8 μm particle size, 95 A pore size). LC Solvent A was 0.1% formic acid/5% ACN/water(v/v/v), and solvent B was 0.1% formic acid/ACN((v/v). The gradient elution profile was as follows: t = 0 min, 20% B; t = 3.5 min, 35% B; t = 18.0 min, 65% B; t = 24.0 min, 99% B; t = 28.0 min, 99% B. After each injection, the column was re-equilibrated with the initial mobile phase conditions for 15 min. The flow rate was set at 180 μL/min. Sample loading amount was optimized (data not included) and the same amount of each mixed sample was injected into the LC-MS system according to the LC-UV quantification result. The flow was loaded to the electrospray ionization (ESI) source of a Bruker maXis impact high-resolution quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker, Billerica, MA). All MS spectra were obtained in the positive ion mode.

7.1.2.5 Data Processing

After LC− QTOF-MS analysis, entire peak lists were exported from Bruker Data Analysis software with a signal-to-noise threshold of 3. An in-house IsoMS software was used for peak-pair picking, peak-pair filtering, and peak-pair intensity ratio calculations.[3] The program eliminates false-positive peaks such as dimers and common adducts. A zero-fill program developed in-house was used afterwards to fill in missing values in the CSV file by searching the raw data file for missed peaks. Finally, peak pairs were reconstructed, and their chromatographic peak ratios were determined using IsoMS-Quant.

7.1.3 Statistical Analysis

Data cleaning was performed on the provided intensity ratio data. Only those peak-pair features shared by more than 50% of the samples were retained for statistical analysis. Sample imputation was performed on those remaining features missing <50% data by k-nearest neighbors algorithm using the "Impute" package of the BioConductor package (www.Bioconductor.org) under R (https://cran.r-project.org/). All data was mean-centered and auto-scaled (unit variance) prior to analysis. Multivariate

statistical analysis including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) was carried out using SIMCA-P+ 14.1 (Umetrics, Umeå, Sweden). PLS-DA and OPLS-DA validation was performed using 999-permutation test built into the SIMCA-P+ 14.1 program. VIP values used in part to choose metabolites were calculated by PLS-DA. To calculate p-value, Student's t tests were performed using SPSS 25.0 (IBM Corp., Armonk, NY, USA). To estimate the false discovery rate, the multiple-testing-corrected p-value (q-value) was calculated using R (https://cran.r-project.org/) and BioConductor[4] with the package "qvalue".[5,6] For comparisons between two groups, the area under the curve (AUC), was calculated using Metaboanalyst 3.[7]

Strict selection criteria were then applied to the analyzed data. These criteria were: fold change > 1.5, *q*-value < 0.05, VIP >1. Only those metabolites meeting these criteria were selected as potential biomarkers.

CHAPTER 8.

SERUM METABOLOMIC PROFILING OF MYASTHENIA GRAVIS

8.1 Abstract

High-throughput, multiplexed, metabolomics profiling is rapidly becoming a new standard for biomarker discovery in the diagnosis, prognosis and therapy of disease. The present study applied a two-control, multi-label metabolomics profiling approach as a potential strategy for the identification of biomarkers unique to myasthenia gravis (MG). Metabolic analyses using acid- and dansyl-labelled serum from seropositive MG (n=46), rheumatoid arthritis (RA) (n=23) and healthy controls (HC) (n=49) were performed on samples from adult patients presenting to the University of Alberta Hospital neuromuscular and rheumatology clinics. Comparisons between patients with MG vs. HC, and RA vs. HC were made using univariate and multivariate statistics. Serum biomarker patterns were statistically significantly different between groups. PLS-DA and OPLS-DA models exhibited considerable distinction between all groups. Metabolites were then filtered to remove peak pairs common to both disease cohorts. Combined metabolite panels revealed clear separation between MG and HC for both library-matched (AUROC: 0.92 ± 0.03) and highest AUC patients (AUROC: 0.94 ± 0.05). In patients presenting to the clinic with seropositive MG, metabolomic profiling is capable of distinguishing patients with disease from those without. These results provide an important first step towards a potential biomarker for improving MG identification.

8.2 Introduction

Myasthenia gravis (MG) is a chronic autoimmune neuromuscular junction disorder characterized by the breakdown of normal communication between nerves and muscles, resulting in fluctuating weakness of the voluntary muscle groups. Rapid, accurate diagnosis of MG presents a challenge, particularly in the emergency room setting. Patients may not present with the typical symptoms, frequently mimicking other neurological conditions, such as stroke, or Gullain Barré Syndrome that can also produce facial and limb weakness like that seen in MG.

Biomarkers confirming MG are few and primarily diagnostic in nature. To date, no single biomarker has demonstrated reliable predictive power in MG. Serum antibodies are limited to diagnosis and differentiation of MG subtypes. In limited cases, such as muscle specific kinase (MuSK) antibodies, the response to therapy can be anticipated as refractory; this rarely informs the care of the patient, however. Electrophysiological studies are often used for the diagnosis of MG; these may be limited by factors related to accuracy, reproducibility and availability. Indeed, significant practice variation in the diagnosis of MG has been recognized, further illustrating the need for novel methods of diagnosis.

In the present study we aim to evaluate the potential of a novel, multiplexed, dual- control metabolomics-based approach to aid the diagnosis and management of MG. Metabolites represent the intermediary and final products of the metabolic pathways within an organism; therefore, it is possible to achieve unique insight by studying these compounds under any given physiological condition. In addition, profiling of the extended spectrum of chemically-labelled species could provide an expanded understanding of the overall immune response. In pilot studies, other groups have explored the feasibility of a metabolomic approach for differentiating MG patients from healthy controls. To the best of our knowledge, however, no metabolomic study of autoimmune disease has attempted to remove the confounding effects of common pathophysiology, such as generalized immune responses. The aim of the present study is to apply a novel humoral-disease control approach to accurately identify unique metabolomic serum biomarkers, which distinguish patients presenting with seropositive MG from a reference autoimmune disease i.e. Rheumatoid Arthritis (RA) and healthy controls.

8.3 Methods

8.3.1 See Section 7.1

8.4 Results

8.4.1 Multivariate modelling

As an initial data survey, principal component analysis was conducted on the different combinations of samples. Appendices 1 and 2 illustrate these plots; Appendix 3 describes the model R2 and Q2 values.

To further illustrate class separation, initial multivariate modelling of all three groups was performed using partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). Score plots are shown in Figures 8.1 & 8.2.

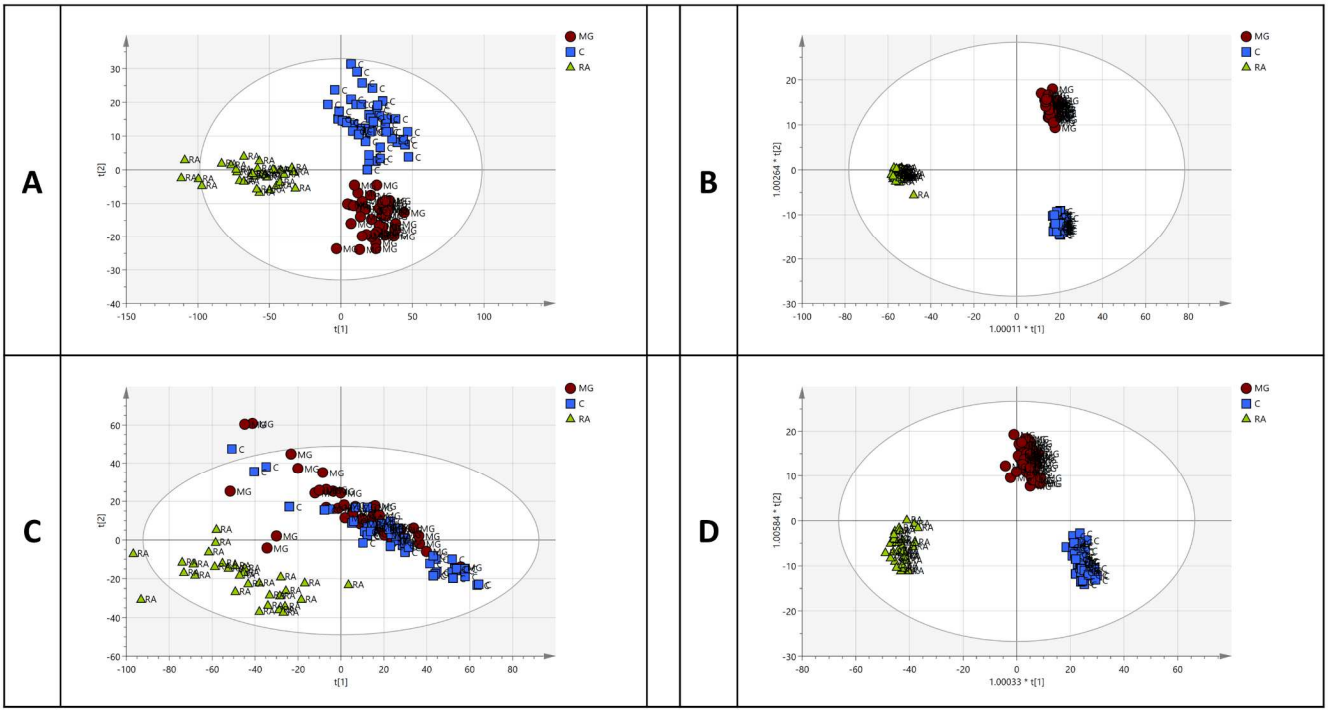


Figure 8.1 Score Plots for MG vs. RA vs. C

PLS-DA and OPLS-DA score plots for Acid-labelled (A, B) and Dansyl-labelled (C, D) MG vs. RA vs. C groups. PLS-DA plots are positioned as the first column, OPLS-DA as the second column.

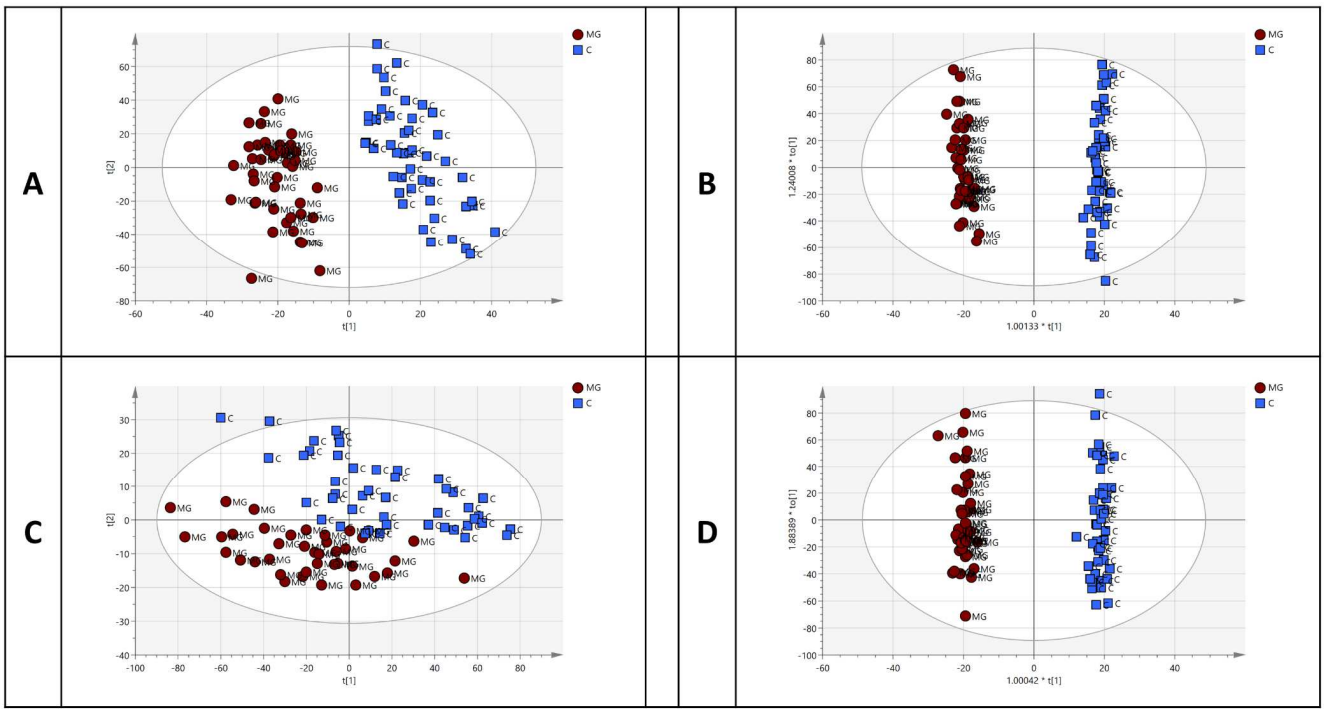


Figure 8.2 Score Plots for MG vs. C

PLS-DA and OPLS-DA score plots for Acid-labelled (A, B) and Dansyl-labelled (C, D) MG vs. C groups. PLS-DA plots are positioned as the first column, OPLS-DA as the second column.

The acid-labelled groups exhibited excellent discrimination of the three cohorts. The dansyl-labelled groups, while still good, showed less tight clustering and greater overlap of the MG and control groups for the PLS-DA model than the acid-labelled groups, indicating reduced intergroup variability for these samples. MG vs. C modelling revealed a significant difference between the healthy controls (squares) and the MG patients (circles), illustrating good class difference. This group separation was further validated by permutation testing as found in Appendix 4. The model performance indicators (the R2, Q2 values and permutation intercepts) are provided in Tables 8.1 and 8.2.

Dansyl						Intercepts	
	A	N	R2X(cum)	R2Y(cum)	Q2(cum)	R2	Q2
MG vs. RA vs. C	7	118	0.562	0.977	0.78	0.82	0.04
MG vs. C	5	95	0.477	0.982	0.781	0.95	0.29
RA vs. C	3	72	0.545	0.991	0.97	0.69	0.21
Acid	**A**	**N**	**R2X(cum)**	**R2Y(cum)**	**Q2(cum)**	**R2**	**Q2**
MG vs. RA vs. C	5	118	0.412	0.986	0.949	0.71	0.06
MG vs. C	3	95	0.302	0.98	0.944	0.79	0.33
RA vs. C	2	72	0.333	0.989	0.982	0.56	0.11

Table 8.1 PLS-DA model Scores

Dansyl						Intercepts	
	A	N	R2X(cum)	R2Y(cum)	Q2(cum)	R2	Q2
MG vs. RA vs. C	2+5+0	118	0.591	0.955	0.747	0.80	0.37
MG vs. C	1+5+0	95	0.506	0.991	0.678	0.96	0.36
RA vs. C	1+2+0	72	0.545	0.991	0.965	0.69	0.34
Acid	**A**	**N**	**R2X(cum)**	**R2Y(cum)**	**Q2(cum)**	**R2**	**Q2**
MG vs. RA vs. C	2+5+0	118	0.467	0.989	0.892	0.84	0.23
MG vs. C	1+3+0	95	0.353	0.991	0.942	0.92	0.24
RA vs. C	1+1+0	72	0.333	0.989	0.98	0.57	0.33

Table 8.2 OPLS-DA model Scores

8.4.2 Multivariate Model Diagnostic Power

To evaluate the diagnostic power of each PLS-DA model, the sensitivity (SE), specificity (SP), positive predictive power (PPP) and negative predictive power (NPP) were calculated. These are presented as Appendix 5. All models demonstrated excellent SE, SP, PPP, and NPP.

8.4.3 Metabolite Identification

The UMS LC-MS technique, provided broad coverage of both the carboxyl and phenol/amine serum submetabolomes for all three patient groups: MG, RA and HC. In total, the application of two labelling techniques revealed an overall total of 9954 $^{12}C/^{13}C$ carboxyl-labelled peak pairs (features) and 7458 phenol or amine features. After data processing, metabolites were matched to in-house standard libraries. A total of 160 individual metabolites were positively identified. Of these, 13 were matched to

MG, while 147 were matched to RA. For the remaining features, an accurate mass search of the Human Metabolome Database was undertaken, using the MycompoundID library.[8] A mass tolerance of 0.005 Da was set as a tolerance cutoff. This search revealed a further 401 putatively-matched metabolites, 19 for MG and 382 for RA. In total, 496 metabolites were either library-matched or putatively identified. Of note also, many metabolites contained carboxyl groups, as a majority of identified metabolites were acid-labelled. Further, benzenoids were heavily represented for both the library-matched and putative metabolites, with six present for each. This was followed by amino acids at five for putatively-matched compounds.

8.4.4 Discriminating Metabolites

Metabolites meeting the following criteria were subjected to further analysis: p-value ≤ 0.05, fold change ≥ 1.5, q-value ≤ 0.05 and VIP ≥ 1. Employing the strategy endorsed by Lindahl et.al[9], metabolites common to MG and RA were identified and removed from the analysis identifying those unique to MG. After simple filtering, a total of five library-matched metabolites were identified as unique to MG, and eight metabolites common to both MG and RA. Of the putatively identified metabolites, seven were unique to MG and 12 common to both groups. Of these, no dansyl-labelled metabolites were matched, either by standard library, or putatively. Of the library-matched, two metabolites were short-chain keto acids, while structural lipids dominated putative matches with five. A complete list of metabolites common to both MG and RA is presented in Appendix 6.

The total number of metabolites discovered is illustrated by Figure 8.3, and further detailed by the Venn diagrams and tables found in Appendix 7 and Appendix 8. Appendix 9 summarizes the chemical taxonomies and associated pathways for all identified common and unique metabolites.

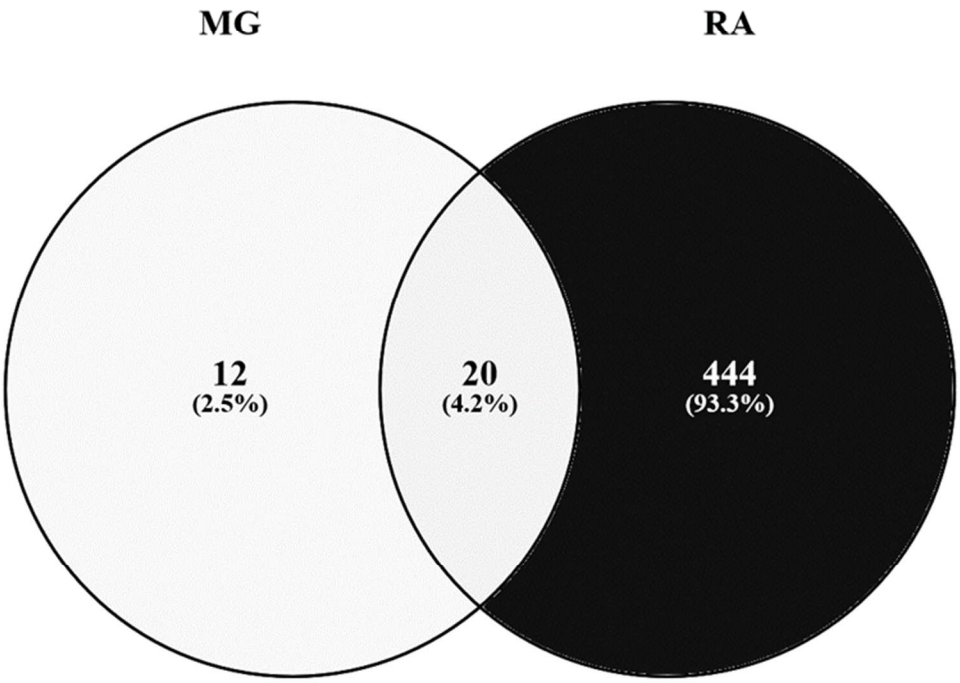


Figure 8.3 Venn chart illustrating numbers of significant metabolites identified by group

8.4.5 Receiver Operating Characteristic Curves

Metaboanalyst 3.0 was used to generate receiver operating characteristic (ROC) curves for differentiating MG and RA from healthy controls both for individual labelling strategies and combined. Classification models were built using the random forest method based on the five most predictive metabolites as presented in Tables 8.3, 8.4. Biomarker panels were composed of the four library-matched candidate biomarkers and the 5 candidate biomarkers with highest AUC overall. Terephthalic acid was excluded as a xenometabolite, and, therefore, of little predictive value.

HMDB	Name	Sample mz	Monoistopic mz	rt	AUC	q-value	VIP	Ratio	FC
HMDB00008	2-Hydroxybutyric acid	104.0472	104.0468	577.24	0.80	3.53E-08	1.78	2.08	2.08
HMDB00011	(R)-3-Hydroxybutyric acid	104.0464	104.0468	610.10	0.83	3.82E-08	0.58	1.60	1.60
HMDB00060	Acetoacetic acid	102.0299	102.0311	913.06	0.81	6.54E-07	2.43	1.75	1.75
HMDB00005	2-Ketobutyric acid	102.0299	102.0311	913.06	0.81	6.54E-07	2.43	1.75	1.75

*Terephthalic acid excluded as a xenometabolite.

Table 8.3 Library-matched metabolites

HMDB	Name	Sample mz	Monoistopic mz	rt	AUC	q-value	VIP	Ratio	FC
HMDB11489	LysoPE(0:0/20:5(5Z,8Z,11Z,14Z,17Z))	499.268	499.2699	1227.72	0.88	3.46E-09	2.27	0.42	-2.36
HMDB00328	12-Ketodeoxycholic acid	390.2757	390.277	2300.92	0.87	2.54E-08	3.15	0.45	-2.24
HMDB11496	LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	525.2857	525.2855	1132.3	0.85	4.67E-07	2.41	0.57	-1.76
HMDB11494	LysoPE(0:0/22:5(4Z,7Z,10Z,13Z,16Z))	527.2992	527.3012	1398.73	0.84	1.11E-07	2.32	0.56	-1.79
HMDB00139	Glyceric acid	106.0253	106.0266	645.02	0.83	1.51E-07	2.12	2.07	2.07

Table 8.4 Highest AUC metabolites

As illustrated in Figures 8.4 and 8.5, significant discrimination was evident in both combinations. To obviate any suggestion of overfitting, permutation tests of each ROC curve were undertaken. Using these permutation tests, we did not find any overfitting of the ROC results.

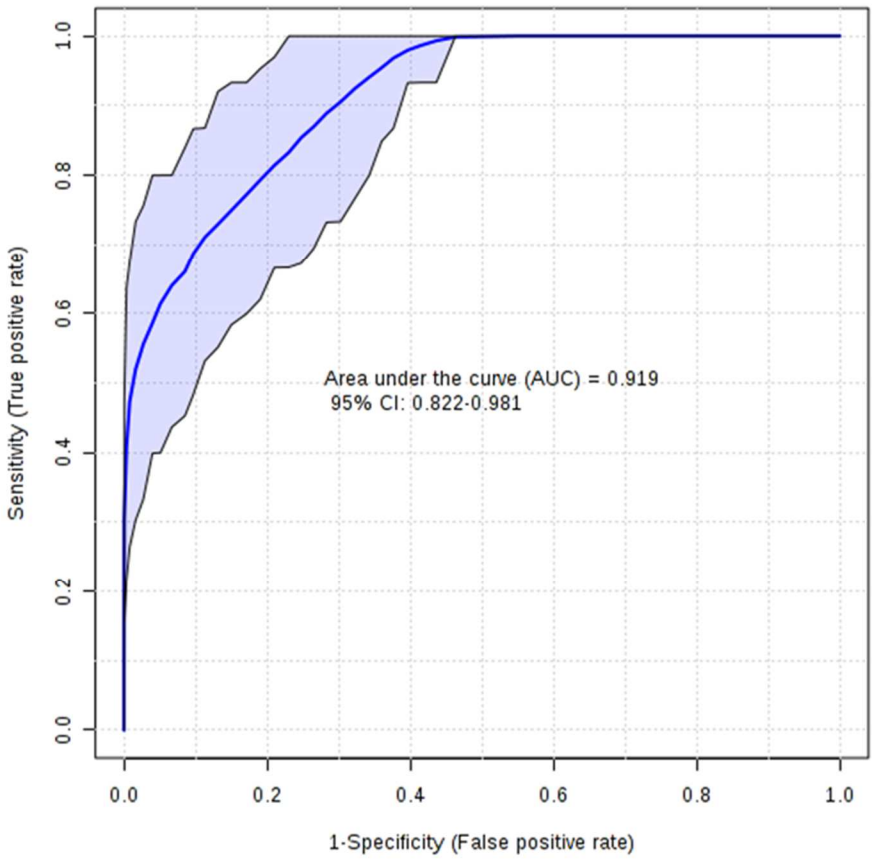


Figure 8.4 ROC curve permutation plots for the top 4 library-matched MG vs. C metabolites panel

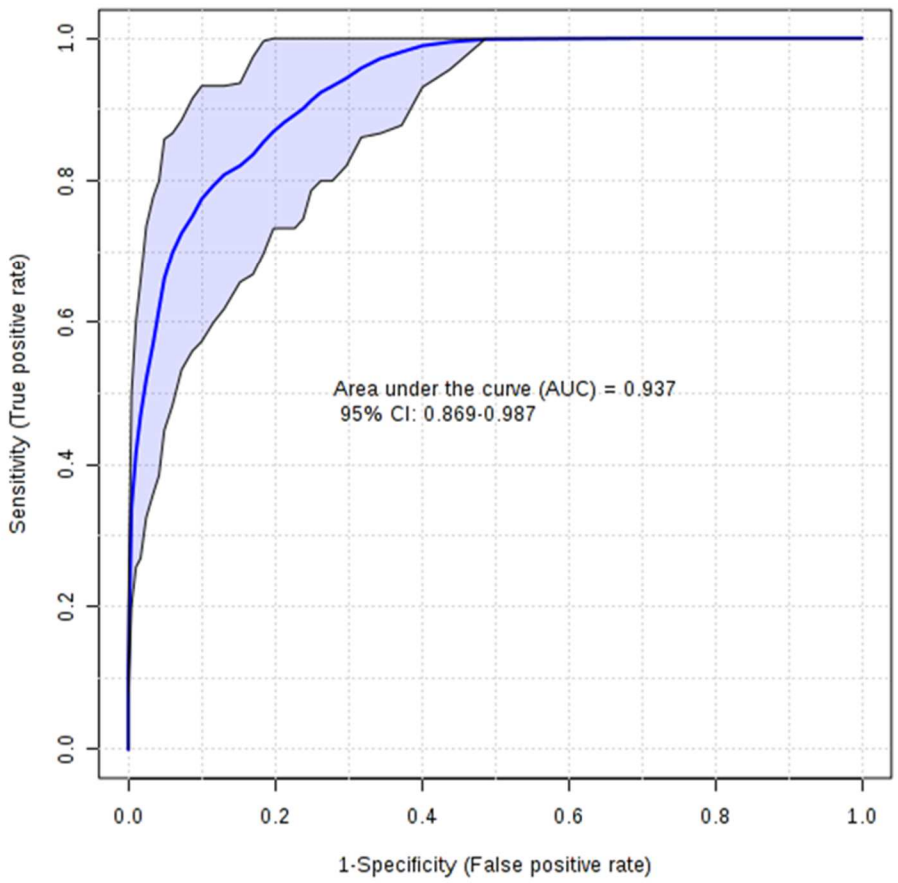


Figure 8.5 ROC curve permutation plots for the top 5 MG vs. C metabolites panel

Finally, to evaluate within-model prediction power, a cross-validation was performed for each of the diagnosis panels. Samples were randomly picked and labelled as "new data". Using metaboanalyst, these data were then input into the ROC models of each panel, and the results observed. If the output group information of these samples matches the true group information, the diagnosis model is considered validated by this small internal set of samples. As presented in Appendices 10 and 11, all results showed good predictive power.

8.5 Discussion

No biomarkers exist that reliably predict the clinical course or therapeutic response in MG. Present biomarkers are primarily diagnostic in nature; both Acetylcholine receptor (AChR) and anti-MuSK antibodies confirm the diagnosis of MG, but do not correlate with disease severity and/or clinical response.[10–12] Two recent reviews summarize the current state of biomarkers for MG, identifying the

need for metabolomic markers.[13,14] Previously, only two studies have explored the metabolomic basis of MG[15,16]; a clear need exists for further exploration of metabolomic analysis for the study of MG. In this study we explored the utility of metabolomics to identify metabolites differentially regulated in MG patients compared to healthy volunteers and patients with RA.

We first explored the relationships between the MG, RA and HC cohorts using multivariate analysis. PLS-DA and OPLS-DA modelling were used to reveal patterns of between-class variance for both all classes and class pairs for each acid- and dansyl- chemical-labelling strategy. Clear separation was observed for all groups. This is consistent with previous work which not only distinguished MG from healthy controls,[15] but also acted as a surrogate measure of disease severity in MG[16]; less severe MG patients were metabolomically distinct from the more severe. Within-class spread was also observed within our MG groups. This may reflect the presence of patients exhibiting significant refractory disease, or duration of disease, possibly representing metabolomically separate subgroups of AChR seropositive MG.

Next, we further investigated the potential of metabolic profiling as a diagnostic tool. Sera from MG and RA patients were observed to have overlapping metabolomic profiles when compared to healthy individuals. The 20 metabolites common to MG and RA were removed from the MG profile on the assumption that these did not represent compounds unique to MG, but rather to more generalized physiological states common to both diseases (such as inflammation). Analysis of these common compounds will be the topic of a parallel manuscript as we seek a better understanding of metabolic profile overlap in antibody-mediated disease. Filtering of common metabolites revealed a set of 12 unique metabolites specific to MG. We found a markedly different metabolic profile in MG patients vs. HC as compared to RA patients vs. HC: 6 metabolites were significantly up-regulated and 6 down-regulated in MG compared to the controls according to stringent AUC, p-values, q-values, VIP and fold-change criteria. Furthermore, the ROC curve analysis of multiple metabolite panels of MG reflected the excellent performance of the applied OPLS-DA modelling of metabolic profiles for the discrimination between studied diseases and controls, with cross-validated predictive scores >80%.

Since our study was focused on AChR seropositive MG, we placed the observed metabolic profile differentiating MG from HC within a biochemical context. Previous work by Lu et al. illustrated significant changes in amino acid, fatty acid, bile acid, structural lipid and oxidative phosphorylation pathways.[15] Of these, only bile acid metabolism change was observed in our study. Bile acid metabolites were previously observed to be largely downregulated compared to healthy subjects. This

was also confirmed by our study, where 12-Ketodeoxycholic acid was likewise observed to be downregulated. Along with their role in the absorption, transport, and metabolism of dietary fats and lipid-soluble vitamins, bile acids have also been implicated in cell signalling, glucose metabolism[17] and inflammation.[18] The reason for the observed reduction of bile acids in the MG group remains unclear. Pharmacological effects seem unlikely since while pyridostigmine is subject to metabolism during the first passage through the liver, its hepatotoxicity is quite low. It has been suggested that gut microbiota may reduce the production of bile acids while increasing inflammation,[19] although the broader implications for autoimmune disease are uncertain.

Several membrane glycerophospholipids (lysophospholipids) were also changed when compared to controls. This reflects the findings of an earlier paper that also observed perturbation of glycerophospholipid metabolism in response to prednisone treatment in MG patients.[16] Paradoxically, however, where it was previously observed that glycerophospholipids were upregulated in those patients taking prednisone, in our cohort glycerophospholipid downregulation was noted for all patients, including those on prednisone (n=19, fold-change mean = -2.71). Pathway-associated glycerolipids (Monoradylglycerolipids) were also found to be downregulated, with MG(0:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0) exhibiting similar negative fold change to measured glycerophospholipids. Arachidonic acid can be released from activated inflammatory cell membrane phospholipids by phospholipase D (PLA2).[20,21] This downregulation of lipids may reflect a higher activity of phospholipase A2 (PLA2), a family of enzymes present in the arachidonic acid pathway, which hydrolyze membrane glycerophospholipids to lysoPCs and fatty acids.[22]

A majority of identified metabolites have significant roles within energy production pathways. Glyceric acid is a natural three-carbon sugar acid obtained from the oxidation of glycerol. Several phosphate derivatives of glyceric acid, including 2-phosphoglyceric acid, 3-phosphoglyceric acid, 2,3-bisphosphoglyceric acid, and 1,3-bisphosphoglyceric acid, are important biochemical intermediates in glycolysis.[23] Many cells ranging from microbes to lymphocytes use aerobic glycolysis during rapid proliferation, which suggests it may play a fundamental role in supporting cell growth. Activated B- and T-cells also experience increases in aerobic glycolysis and oxygen consumption.[24–26]

In our cohort, upregulation of short-chain keto acids in MG patients compared to controls was observed. This could suggest increased activity in any of several metabolic pathways. 2-ketobutyric acid (a-Ketobutyrate) is involved in the synthesis of aspartic acid, glutamic acid, asparagine, glutamine, ornithine and proline. α-Ketobutyric acid is a product of the lysis of cystathionine. It is also one of the

degradation products of threonine, produced by the catabolism of the amino acid by threonine dehydratase. Additionally, 2-ketobutyric acid is produced by the degradation of homocysteine and the metabolism of methionine. Within the mitochondria, it can be converted into propionyl-CoA (and subsequently methylmalonyl CoA, which can be converted into succinyl CoA, a citric acid cycle intermediate), and thus enter the citric acid cycle. Ketone bodies (acetoacetic acid, and β-hydroxybutyric acid) are produced mainly in the mitochondrial matrix of liver cells, from acetyl-CoA when a scarcity of carbohydrates requires that energy must be obtained from the breaking down of fatty acids, in a process called ketogenesis. Ketone bodies are transported from the liver to other tissues, where acetoacetate and β-hydroxybutyrate can be reconverted to acetyl-CoA to produce energy. Some of the acetyl-CoA produced by fatty acid oxidation in liver mitochondria is converted to acetone, acetoacetate and β-hydroxybutyrate. β-hydroxy butyrate ((R)-3-Hydroxybutyric acid) is further converted to acetoacetate for energy. Both are elevated in uncontrolled diabetes mellitus (DMII); due to an absence of insulin, cells, metabolically starved, turn to gluconeogenesis and fat/protein catabolism for energy.

Upregulation of ketone bodies (3-OH-butyrate and acetoacetate) has been also observed in the blood of patients with MS.[27] Suggestive of an "energy shift", in conditions of low glucose or carbohydrate concentrations, ketone bodies are produced in mitochondria through fatty acid catabolism. Acetoacetic acid and β-hydroxybutyric acid cross the blood-brain barrier and can be used by cells as an energy source (i.e., converted to acetyl coenzyme A to participate in the citric acid cycle in mitochondria). Impaired glycolysis leading to reduced ATP synthesis may ultimately lead to cell death or degeneration, especially as the mitochondria generates most of the energy for neuronal cells.[28]

2-Hydroxybutyric acid (or α-Hydroxybutyrate) may be an early marker of DMII.[2930] Previously, high levels of 2--hydroxybutyrate were previously suggested to be an early marker for impaired glucose regulation.[31] This may arise due to increased lipid oxidation (as evidenced by an elevated NADH/NAD+ ratio) and oxidative stress because 2-hydroxybutyrate is produced from threonine and methionine catabolism as well as glutathione metabolism, in response to increased production of oxidizing species.[32] Further, accumulating evidence has shown that oxidative stress contributes toward the pathogenesis in MG,[33] neurodegenerative disease,[34] and inflammatory/autoimmune-mediated tissue damage.[35] Several studies have suggested the relationship between 2-hydroxybutyrate and disorders such as dihydrolipoyl dehydrogenase (E3) deficiency[36] and cerebral lactic acidosis.[37] Oxidative stress has also been implicated in the pathogenesis of MS.[38]

Finally, phthalates are xenoestrogenic compounds, environmental toxicants that mimic or induce endogenous hormones. Also known as endocrine disrupting chemicals (EDCs), these include terephthalic acid, which was found to be upregulated in MG. EDCSs can disrupt the immune-neuroendocrine network (INEN), the network joining the endocrine, immune and nervous systems.[39] Environmental stressors such as phthalate chemical exposure may result in cytokine-induced neurotoxicity by inducing oxidative stress,[40] and neuroinflammation.[41,42] Phthalates are also widely used as excipients for the enteric coating of pharmaceutical Tablets.[43] Increased terephthalic acid levels may also reflect high-frequency medication schedules, or the taking of large quantities of phthalate-coated medication contiguous with study blood sample collection.

There are multiple drawbacks in our study, which may affect our results. Use of acetylcholinesterase inhibitor and immunosuppressive in the MG patients can change the metabolic profile by introducing new metabolites, and possibly disrupting important immunopathogenic pathways. Larger studios with subgroup analysis is needed to discern the effect of medications. Our patients did not fast prior to sample collection, which can potentially introduce variability in the serum metabolome. Ideally, this would add some measure of consistency, but the nature of opportunist sampling of a rare disease precluded the inclusion of this criteria as logistically unwieldy.

8.6 Conclusion

This study successfully illustrated the potential of LC–MS-based serum metabolomics for rapid identification and distinguishing of MG sera from healthy subjects and a reference autoimmune disease. Metabolomics can play an important diagnostic and prognostic role in clinical medicine, although further work with larger samples remains to correlate the results of previous studies, and possibly those of other autoimmune diseases, to establish robust metabolomic models of MG.

8.7 References

1. Hutchinson, D. Classification criteria: The 1987 American Rheumatism Association revised criteria for the classification of rheumatoid arthritis. *CPD Rheumatol.* 1, 13–14 (1999).

2. Zhao, S., Luo, X. & Li, L. Chemical Isotope Labeling LC-MS for High Coverage and Quantitative Profiling of the Hydroxyl Submetabolome in Metabolomics. *Anal. Chem.* 88, 10617–10623 (2016).

3. Zhou, R., Tseng, C.-L., Huan, T. & Li, L. IsoMS: Automated Processing of LC-MS Data Generated by a Chemical Isotope Labeling Metabolomics Platform. *Anal. Chem.* 86, 4675–4679 (2014).

4. Gentleman, R. C. *et al.* Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5, R80 (2004).

5. Storey, J. D. The positive false discovery rate: a Bayesian interpretation and the q -value. *Ann. Stat.* 31, 2013–2035 (2003).

6. Bass JD, D. A. and R. D. qvalue: Q-value estimation for false discovery rate control. *R package version 2.10.0* (2015). Available at: http://github.com/jdstorey/qvalue.

7. Xia, J., Mandal, R., Sinelnikov, I. V., Broadhurst, D. & Wishart, D. S. MetaboAnalyst 2.0--a comprehensive server for metabolomic data analysis. *Nucleic Acids Res.* 40, W127–W133 (2012).

8. Huan, T. *et al.* MyCompoundID MS/MS Search: Metabolite Identification Using a Library of Predicted Fragment-Ion-Spectra of 383,830 Possible Human Metabolites. *Anal. Chem.* 87, 10619–10626 (2015).

9. Lindahl, A., Forshed, J. & Nordström, A. Overlap in serum metabolic profiles between non-related diseases: Implications for LC-MS metabolomics biomarker discovery. *Biochem. Biophys. Res. Commun.* 478, 1472–1477 (2016).

10. Meriggioli, M. N. & Sanders, D. B. Muscle autoantibodies in myasthenia gravis: beyond diagnosis? *Expert Rev. Clin. Immunol.* 8, 427–38 (2012).

11. Guptill, J. T. & Sanders, D. B. Update on muscle-specific tyrosine kinase antibody positive

myasthenia gravis. *Curr. Opin. Neurol.* 23, 530–535 (2010).

12. Howard, F. M., Lennon, V. A., Finley, J., Matsumoto, J. & Elveback, L. R. Clinical correlations of antibodies that bind, block, or modulate human acetylcholine receptors in myasthenia gravis. *Ann. N. Y. Acad. Sci.* 505, 526–38 (1987).

13. Kaminski, H. J. *et al.* Biomarker development for myasthenia gravis. *Ann. N. Y. Acad. Sci.* 1275, 101–106 (2012).

14. Benatar, M. *et al.* Recommendations for myasthenia gravis clinical trials. *Muscle Nerve* 45, 909–917 (2012).

15. Lu, Y. *et al.* Serum metabolomics for the diagnosis and classification of myasthenia gravis. *Metabolomics* 8, 704–713 (2012).

16. Sengupta, M. *et al.* Serum Metabolomic Response of Myasthenia Gravis Patients to Chronic Prednisone Treatment. *PLoS One* 9, e102635 (2014).

17. Nguyen, A. & Bouscarel, B. Bile acids and signal transduction: Role in glucose homeostasis. *Cell. Signal.* 20, 2180–2197 (2008).

18. Zhu, C., Fuchs, C. D., Halilbasic, E. & Trauner, M. Bile acids in regulation of inflammation and immunity: friend or foe? *Clin. Exp. Rheumatol.* 34, 25–31

19. Staley, C., Weingarden, A. R., Khoruts, A. & Sadowsky, M. J. Interaction of gut microbiota with bile acid metabolism and its influence on disease states. *Appl. Microbiol. Biotechnol.* 101, 47–64 (2017).

20. Astudillo, A. M., Balgoma, D., Balboa, M. A. & Balsinde, J. Dynamics of arachidonic acid mobilization by inflammatory cells. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1821, 249–256 (2012).

21. Lone, A. M. & Taskén, K. Proinflammatory and Immunoregulatory Roles of Eicosanoids in T Cells. *Front. Immunol.* 4, 130 (2013).

22. Cummings, B. S., McHowat, J. & Schnellmann, R. G. Phospholipase A2s in Cell Injury and Death. *J. Pharmacol. Exp. Ther.* 294, (2000).

23. Pollard, T. D. (Thomas D., Earnshaw, W. C., Lippincott-Schwartz, J., Johnson, G. T. & Preceded by: Pollard, T. D. (Thomas D. *Cell biology*. (Elsevier, 2016).

24. Loftus, R. M. & Finlay, D. K. Immunometabolism: Cellular Metabolism Turns Immune Regulator. *J. Biol. Chem.* 291, 1–10 (2016).

25. Lunt, S. Y. & Vander Heiden, M. G. Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation. *Annu. Rev. Cell Dev. Biol.* 27, 441–464 (2011).

26. Boothby, M. & Rickert, R. C. Metabolic Regulation of the Immune Humoral Response. *Immunity* 46, 743–755 (2017).

27. Cocco, E. *et al.* (1)H-NMR analysis provides a metabolomic profile of patients with multiple sclerosis. *Neurol. Neuroimmunol. neuroinflammation* 3, e185 (2016).

28. Lin, M. T. & Beal, M. F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* 443, 787–795 (2006).

29. Ferrannini, E. *et al.* Early metabolic markers of the development of dysglycemia and type 2 diabetes and their physiological significance. *Diabetes* 62, 1730–7 (2013).

30. Cobb, J. *et al.* α-Hydroxybutyric Acid Is a Selective Metabolite Biomarker of Impaired Glucose Tolerance. *Diabetes Care* 39, 988–95 (2016).

31. Gall, W. E. *et al.* α-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population. *PLoS One* 5, (2010).

32. Gall, W. E. *et al.* α-Hydroxybutyrate Is an Early Biomarker of Insulin Resistance and Glucose Intolerance in a Nondiabetic Population. *PLoS One* 5, e10883 (2010).

33. Adamczyk-Sowa, M. *et al.* Oxidative modifications of blood serum proteins in myasthenia gravis. *J. Neuroimmunol.* 305, 145–153 (2017).

34. Stuerenburg, H. J. The Roles of Carnosine in Aging of Skeletal Muscle and in Neuromuscular Diseases. *Biochem.* 65, 862–865 (2000).

35. Park, H., Bourla, A. B., Kastner, D. L., Colbert, R. A. & Siegel, R. M. Lighting the fires within: the cell biology of autoinflammatory diseases. *Nat. Rev. Immunol.* 12, 570–80 (2012).

36. Tomiko, K. *et al.* Studies of urinary organic acid profiles of a patient with dihydrolipoyl dehydrogenase deficiency. *Clin. Chim. Acta* 133, 133–140 (1983).

37. Hoffmann, G. F. *et al.* Physiology and pathophysiology of organic acids in cerebrospinal fluid. *J. Inherit. Metab. Dis.* 16, 648–669 (1993).

38. Lassmann, H., Van Horssen, J. & Mahad, D. Progressive multiple sclerosis: Pathology and pathogenesis. *Nat. Rev. Neurol.* 8, 647–656 (2012).

39. Manley, K., Han, W., Zelin, G. & Lawrence, D. A. Crosstalk between the immune, endocrine, and nervous systems in immunotoxicology. *Curr. Opin. Toxicol.* 10, 37–45 (2018).

40. Franken, C. *et al.* Phthalate-induced oxidative stress and association with asthma-related airway inflammation in adolescents. *Int. J. Hyg. Environ. Health* 220, 468–477 (2017).

41. Yegambaram, M., Manivannan, B., Beach, T. G. & Halden, R. U. Role of environmental contaminants in the etiology of Alzheimer's disease: a review. *Curr. Alzheimer Res.* 12, 116–46 (2015).

42. Win-Shwe, T.-T., Yanagisawa, R., Koike, E., Nitta, H. & Takano, H. Expression levels of neuroimmune biomarkers in hypothalamus of allergic mice after phthalate exposure. *J. Appl. Toxicol.* 33, 1070–1078 (2013).

43. *Excipient Applications in Formulation Design and Drug Delivery*. (Springer International Publishing, 2015). doi:10.1007/978-3-319-20206-8

CHAPTER 9.

METABOLOMIC PROFILE OVERLAP IN PROTOTYPICAL AUTOIMMUNE HUMORAL DISEASE. A COMPARISON OF MYASTHENIA GRAVIS AND RHEUMATOID ARTHRITIS

9.1 Abstract

Myasthenia gravis (MG) and rheumatoid arthritis are examples of antibody-mediated chronic, progressive autoimmune diseases. Phenotypically dissimilar, MG and RA share common immunological features. This study illustrates the metabolomic profile overlap found between these two diseases and describes the immunometabolomic significance. Metabolic analyses using acid- and dansyl-labelled serum from seropositive myasthenia gravis (n=46), rheumatoid arthritis (n=23) and healthy controls (n=49) were performed on samples from adult patients presenting to the University of Alberta Hospital neuromuscular and rheumatology clinics. Metabolites matching our criteria for significance were selected if they were present in both groups. Biochemical pathway analysis was then conducted to gain understanding of the principal pathways involved in antibody-mediated pathogenesis. We found 20 metabolites dysregulated in both MG and RA when compared to healthy controls. Most prominently, observed changes were related to pathways associated with phenylalanine metabolism, tyrosine metabolism, ubiquinone and other terpenoid-quinone biosynthesis, and pyruvate metabolism.

9.2 Introduction

Metabolomic analysis has significantly increased our understanding of autoimmune disease in recent years. In particular, the field of immunometabolism has not only revealed the major roles played by immune cells in metabolic homeostasis but also the impact of metabolic pathways on immune cell function.[1–4] Further, the metabolomic profiles of chronic inflammation,[5] metabolic disease[6] and the metabolic underpinnings of immune cell function,[7–9] all rooted in immunometabolism, have been documented for several autoimmune diseases.

Autoimmune diseases encompass a wide range of immunoresponses, many of which are non-specific. Cell signalling, immune cell proliferation, cellular debris from membrane attack complexes, fatty acid and glucose energy metabolism, and oxidative stress all affect the flux of metabolites present in humoral autoimmune disease.[10,11] Previous work has identified the unique metabolomic profiles of several autoimmune diseases.[12–15] However, how informative these profiles are remains in question. Do these differences truly represent the unique metabolomic signature of the disease in question, or are they the result of common pathophysiological responses common to all humoral disease? Conversely, these shared metabolites may be hypothesis generating, illuminating additional directions of inquiry that may enhance our understanding of autoimmune responses.

Recently, examples of metabolomic overlap have been published. Through subtractive merging of metabolomic profiles, a more accurate metabolome should emerge, representing the true metabolomic profile of each disease. The aim of the present study is to describe a specific metabolomic profile shared by humoral autoimmune diseases. By applying this subtractive approach, we hope to accurately identify metabolomic biomarkers shared by immunophenotypically similar humoral diseases, and profile their biochemical categories and functions.

9.3 Methods

9.3.1 See Section 7.1

9.4 Results

The UMS LC-MS technique employed in this study provided broad coverage of both the carboxyl and phenol/amine serum submetabolomes for all three patient groups: MG, RA and C. In total, the application of two labelling techniques revealed an overall total of 9954 C12/C13 carboxyl-labelled peak pairs (features) and 7458 phenol or amine features. After data processing, metabolites were matched to in-house standard libraries. A total of 160 individual metabolites were positively identified. Of these, 13 were matched to MG, while 147 were matched to RA. For the remaining features, an accurate mass search of the Human Metabolome Database was undertaken using the MycompoundID portal. A mass tolerance of 0.005 Da was set as a tolerance cutoff. This search revealed a further 401 putatively-matched metabolites, 19 for MG and 382 for RA. In total, 496 metabolites were either library-matched or putatively identified. Of note also, it was observed that many metabolites contained carboxyl groups, as a majority of identified metabolites were acid-labelled. Further, benzenoids were heavily represented for both the library-matched and putative metabolites, with 6 present for each. This was followed by amino acids at 5 for putatively-matched compounds.

9.5 Common Discriminating Metabolites

Employing the simple filtering strategy endorsed by Lindahl et al., metabolites common to MG and RA were identified. After filtering, a total of 5 library-matched metabolites were identified as unique to MG, 139 unique to RA and 8 metabolites common to both MG and RA. Of the putatively identified metabolites, 7 were unique to MG, 370 unique to RA and 12 common to both groups. A complete list of metabolites common to both MG and RA is presented in Table 9.1.

		MG			RA		
HMDB I.D.	**Name**	***p*-value**	***q*-value**	**FC**	***p*-value**	***q*-value**	**FC**
HMDB00339	2-Methylbutyrylglycine	7.23E-10	3.42E-08	1.70	5.61E-09	9.11E-09	1.78
HMDB02466	3-Hydroxybenzoic acid	8.06E-07	1.16E-05	2.09	1.09E-08	1.70E-08	2.04
HMDB00022	3-Methoxytyramine	1.55E-07	7.43E-06	1.55	1.50E-08	4.56E-09	1.77
HMDB00500	4-Hydroxybenzoic acid	8.06E-07	1.16E-05	2.09	1.09E-08	1.70E-08	2.05
HMDB00707	4-Hydroxyphenylpyruvic acid	1.96E-06	2.44E-05	-2.22	2.03E-03	1.18E-03	1.96
HMDB00503	7a-Hydroxy-3-oxo-5b-cholanoic acid	4.93E-10	2.54E-08	-2.24	1.35E-07	1.75E-07	2.05
HMDB28691	Alanyl-Leucine	1.61E-06	1.73E-05	1.62	6.31E-10	2.44E-10	2.00
HMDB00511	Capric acid	3.72E-08	8.79E-07	-2.28	2.10E-12	5.57E-12	2.41
HMDB00451	cis-4-Hydroxycyclohexylacetic acid	1.36E-11	2.31E-09	2.34	1.41E-08	2.14E-08	2.42
HMDB01311	D-Lactic acid	1.92E-10	1.37E-08	2.36	5.48E-08	3.48E-09	3.31
HMDB11162	L-beta-aspartyl-L-alanine	1.08E-12	2.60E-10	2.71	1.07E-11	5.87E-12	1.81
HMDB00158	L-Tyrosine	7.28E-11	7.78E-09	1.66	1.21E-05	2.24E-06	1.61
HMDB11487	LysoPE(0:0/20:4(5Z,8Z,11Z,14Z))	5.87E-07	8.94E-06	-2.17	1.51E-07	3.84E-08	1.44
HMDB00691	Malonic acid	3.21E-09	1.10E-07	2.21	1.31E-11	3.10E-11	4.76
HMDB00202	Methylmalonic acid	4.91E-11	5.90E-09	2.19	2.04E-09	3.52E-09	3.38
HMDB12271	O-Ureidohomoserine	2.68E-07	4.74E-06	-2.06	0.00E+00	0.00E+00	4.04
HMDB00220	Palmitic acid	2.74E-03	7.24E-03	-1.14	6.94E-14	2.23E-13	-4.35
HMDB00209	Phenylacetic acid	1.31E-09	5.48E-08	2.25	8.26E-06	7.67E-06	1.79
HMDB02107	Phthalic acid	1.77E-07	3.34E-06	1.60	5.83E-08	7.95E-08	1.81
HMDB00252	Sphingosine	5.07E-10	2.57E-08	-1.80	3.00E-17	5.00E-17	3.47

Table 9.1 List of significantly altered metabolites common to MG and RA serum samples meeting selection criteria (fold change > 1.5, p < 0.05, q < 0.05, VIP >1).

Appendix 12 illustrates group distribution of MG and RA metabolite profiles. Appendix 13 summarizes the chemical taxonomies and associated pathways for all identified common metabolites.

9.5.1 Biological functions of potential biomarkers

To place potential biomarker metabolites in a biological context, enrichment and pathway analyses were undertaken. Analysis was first undertaken to construct a broad view of the contribution selected metabolites had on known metabolic pathways. By requiring only metabolite names, this analysis simply evaluates the statistical contribution of selected grouped metabolites to known pathways without any a priori knowledge of actual sample metabolite abundance (i.e. concentration, upregulation/downregulation). Pathways most relevant to the group of interest are characterized by

both large − log (p) (i.e. low p-value) and high pathway impact values. This first look identified three significant pathway perturbations: Phenylalanine, Ubiquinone and other terpenoid-quinone biosynthesis, tyrosine metabolism. Metabolites contributing significantly to these pathways were phenylacetic acid, 4-Hydroxybenzoic acid, L-Tyrosine, 4-Hydroxybenzoic acid, 4-Hydroxyphenylpyruvic acid, and 3-Methoxytyramine.

To further explore the disease-specific metabolic relationships of the potential biomarkers, a more exhaustive pathway analysis was undertaken. Quantitative enrichment and pathway topology analyses further considered the extent of metabolite fold change and abundance in the list of significant entities, the inclusion of any metabolite in the list typically depending on a fixed arbitrary threshold (such as *p*-value). The concentration tables of MG vs. healthy control and RA vs. healthy control were individually analysed (Figures 9.1, 9.2). These analyses revealed similar pathway effects as metabolite contributions to phenylalanine metabolism, ubiquinone and other terpenoid-quinone biosynthesis, and pyruvate metabolism were significant. Metabolites correlated with these pathways were phenylacetic acid, 4-Hydroxybenzoic acid, L-Tyrosine, L-Lactic acid, 4-Hydroxyphenylpyruvic acid. Appendix 14 details the relative abundance of each metabolite for each study cohort as box and whisker plots.

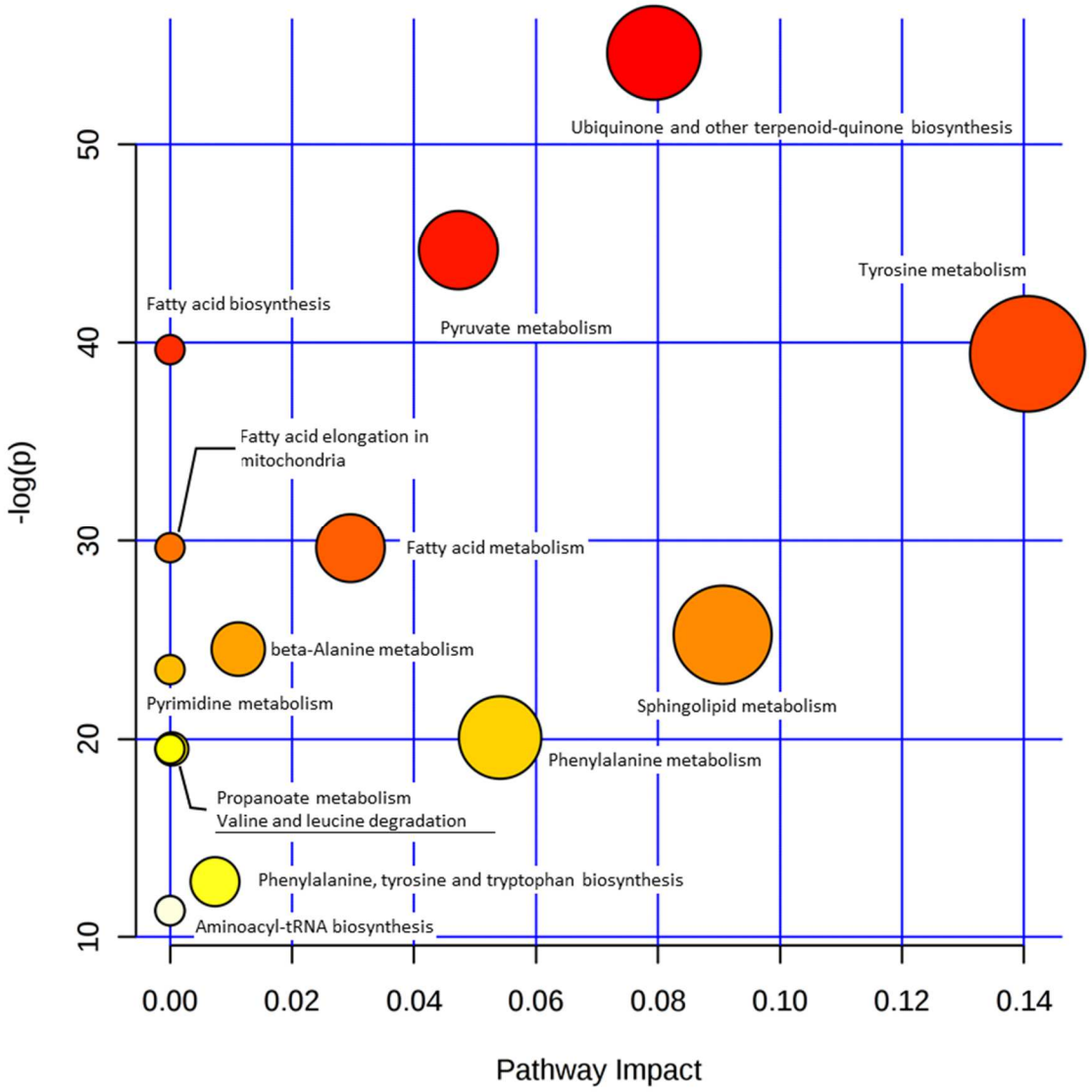


Figure 9.1 Overview of pathway analysis based on the concentration of common metabolites in the MG cohort. The node color and radius were determined by p-value and pathway impact value, respectively.

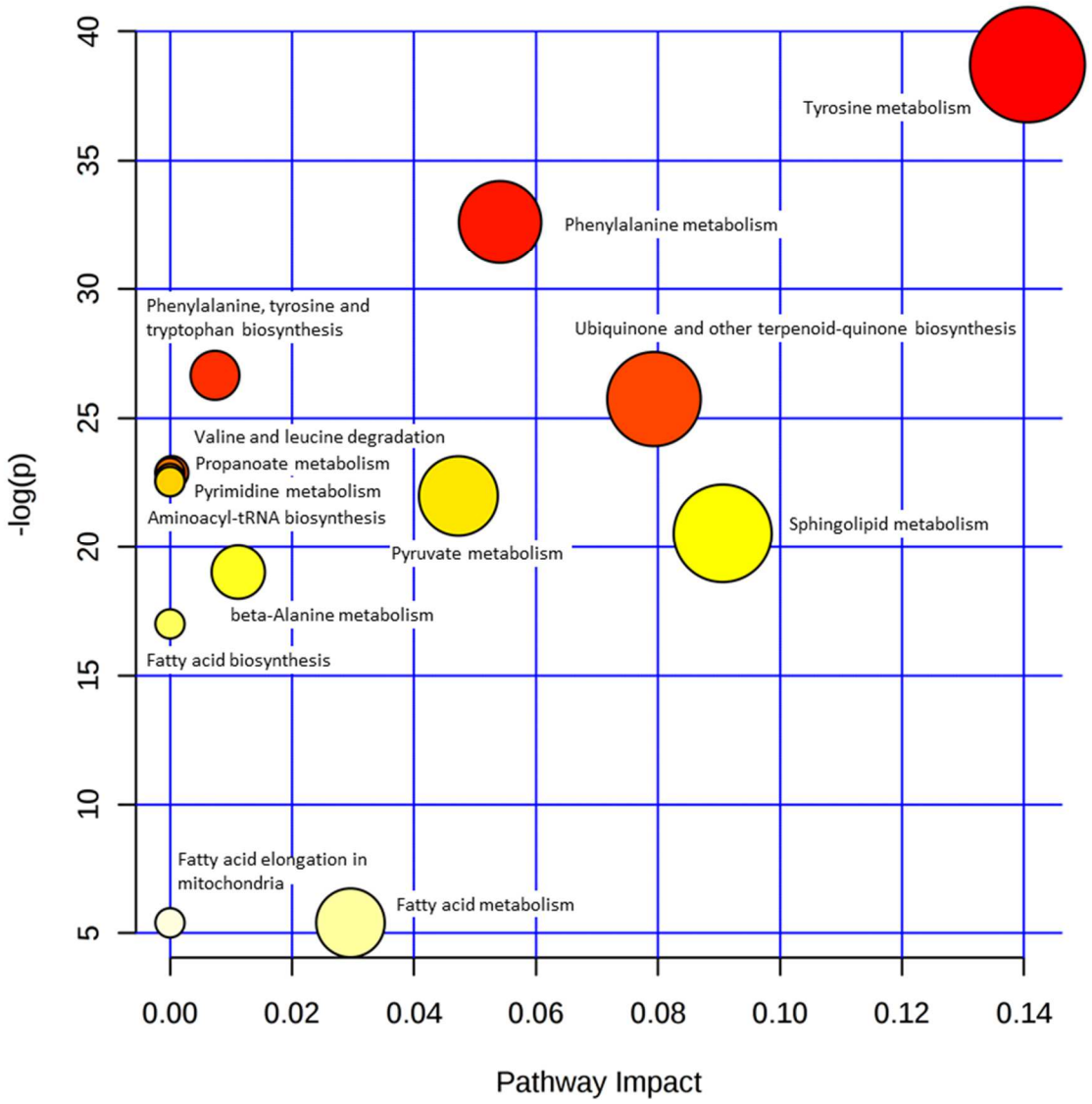


Figure 9.2 Overview of pathway analysis based on the concentration of common metabolites in the RA cohort. The node color and radius were determined by p-value and pathway impact value, respectively.

9.6 Discussion

There is emerging awareness of potential overlap between disease metabolomic profiles. For example, the metabolite profiles of rheumatoid diseases (Systemic lupus erythematosus (SLE), primary Sjögrens syndrome (pSS) and systemic sclerosis (SSc)) have been compared[18], while pathophysiologically disparate conditions such as pneumonia, congestive heart failure, lymphoma and healthy controls have also been investigated. [19] Driving this examination is the certainty that, in order to identify reliable biomarkers, the specificity of any particular metabolite to the disease or physiological parameter in question must be determined. Conversely, between-disease comparisons of metabolomic profiles may reveal common biochemical mechanisms that inform the systems biology of either disease, disease class or both. In this report we describe, for the first time, overlapping biomarker profiles for MG and RA, by LC-MS-based metabolomic analysis of serum.

Autoimmune diseases result from a complex interplay of metabolic pathways, molecular and cellular events and immunoreactive cycles which promote the emergence of autoreactivity, followed by ongoing self-sustaining tissue damage. Enormously complex, these mechanisms present a significant challenge in the elucidation of autoimmune pathophysiology. Factors essential to the autoimmune response include abnormalities in antigenic tolerance, regulatory T-cell (Treg) development, and immune-signalling thresholds. Lymphocyte activation and proliferation, in particular, underlies a great deal of the autoimmune response; immune cells may persist in a state of quiescence, roam as sentinels or become rapidly stimulated in a burst of activity. Accordingly, the cellular metabolic response contributes significantly to the immunometabolomic profile.

In the present study, we revealed a total of 20 significantly changed metabolites shared by MG and RA, many of which exhibit profound immunometabolomic properties.

9.7 Identified metabolites that contribute to energy metabolism

Central to the mobilization of the immune response is energy metabolism. A ubiquitous organic acid, lactic acid is a principal metabolic intermediate in most living organisms, the normal endpoint of glucose breakdown in tissue, or glycolysis. Glycolysis is crucial in both immunity and disease states, serving both anabolic and catabolic roles. Occurring mainly in the cell cytoplasm under hypoxic conditions or as a consequence of high flux of glycolysis in proliferating cells,[20] glycolysis enables the conversion of one glucose molecule to 2 pyruvate molecules, with subsequent production of lactate,

NAD+, and ATP. Lactate is produced in conditions where pyruvate production exceeds the rate of pyruvate oxidation and cytosolic NAD+/NADH is reduced.[21]

Immune processes have significant bioenergetic and biosynthetic demands, which are met by dynamic changes in energy metabolism. Quiescent T- and B- cells typically rely on anaerobic glycolysis and fatty acid oxidation for energy metabolism, preferentially metabolizing glucose to pyruvate, rather than lactic acid.[22] Once activated, however, lymphocytes proliferate, secrete cytokines, and can make antibodies. Activated B- and T- cells meet the bioenergetic demand for these processes by up-regulating aerobic glycolysis and fatty acid metabolism.[23] Upon antigen encounter, T-cells significantly enhance rates of glucose uptake, through increased expression of the cell-surface transporter Glut1, B-cells.[24] Extracellular lactate levels also strongly correlate with T- cell proliferation.[25] B-cells, dendritic cells and macrophages also experience increased glycolysis upon activation,[22] expressed as increased serum lactic acid, as glucose is preferentially metabolized to lactic acid as opposed to pyruvate. Glycolysis also plays an additional role in the initiation and maintenance of inflammation through cell signalling.[26] Finally, lactic acid is alternatively formed and accumulated in muscle under conditions of high energy demand, rapid fluctuations of the energy requirement and insufficient supply of O2.[27]

Lactic acid was upregulated in both the MG and RA patient groups. This shift in lactate levels is indicative of increased levels of glycolysis.

Elevated levels of lactic acid have been found in MG patients when compared to healthy controls.[28] Studies in humans have also revealed that patients with autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) display defects in metabolic pathways such as glycolysis.[29] The joints of RA patients are observed to be lactate rich.[30] The result of inflammation, infiltration of immune cells, and high synovial cell metabolic demand,[31] lactate accumulates in the synovial fluid. Similarly, a recent study carried out on the sera samples of RA patients has highlighted a metabolic signature of RA patients compared to healthy controls.[14] RA patients displayed decreased levels of amino acids (aa) (leucine, phenylalanine, pyroglutamate, serine, isoleucine, methionine, threonine, proline, and valine) and glucose alongside with increased levels of fatty acids such as palmitelaidate, oleate, trans9-octadecenoate,cis-5,8,11-eicosatrienoate, docosahexaenoate, 2-ketoisocaproateand 3-methyl-2-oxovalerate, and cholesterol. Along with perturbations in several bioenergetic pathways such as fatty acid and aa metabolism and other related metabolic pathways, this profile suggests that glycolysis plays a pivotal role in the metabolic pathogenesis of RA.

Similarly, SLE exhibits metabolic dysregulation in both humans and mice. Specifically, CD4+ T cells from SLE patients exhibit enhanced glycolysis and mitochondrial metabolism that correlate with their activation status.[32,33] In contrast, a metabolomics study on SLE sera revealed reduced glycolysis, Krebs cycle, fatty acid β oxidation, and aa metabolism in these patients compared to healthy controls. This dysregulation reflects a state of dampened energy generation, high oxidative stress, inflammation, and altered lipid profiles.[34]

Lactic acid dysregulation has also been described in MS, a chronic inflammatory disease, characterized by focal plaques of demyelination and tissue injury in the CNS. De Rosa et al.[35] recently observed that glycolysis is required for the expression of FOXP3 (forkhead box P3), a master regulator in the development and suppressive function of Treg cells. Impaired rates of glycolysis have been noted in MS patients, which in turn alters peripheral Treg-cell generation and functions.[35] Conversely, other authors have found increased levels of lactate in the cerebrospinal fluid[36] and serum of MS patients when compared to controls.[37]

Additionally, locally high concentrations of lactate have been found near many tumours due to the upregulation of lactate dehydrogenase.[38] These locally high concentrations of lactic acid are known to markedly impede the function of normal immune cells and will lead to a loss of T-cell function of human tumour-infiltrating lymphocytes.[39] Known as Warburg metabolism, lactic acid produced by tumours through aerobic glycolysis acts as an immunosuppressant and tumour promoter.[40] A positive correlation between LDH-A, high lactate levels, and tumour progression has been widely described in various tumours and is associated with disease progression and activity.[37] Interestingly, high levels of lactic acid have also been found in tissues proximal to thymic carcinoma.[41] It may be that lactic acid is an early biomarker of MG in younger patients who will later develop AChR-seropositive disease resulting from thymic tumours.

Coenzyme Q10, also known as ubiquinone or 4-Hydroxybenzoic acid, is a coenzyme ubiquitous in animals and most bacteria. Coenzyme Q10 is present in all respiring eukaryotic cells, primarily in the mitochondria. It is a component of the electron transport chain and participates in aerobic cellular respiration, which generates energy in the form of ATP.[42] Ninety-five percent of human metabolic energy is generated this way.[43] Upregulated in both RA and MG, circulating levels of Q10 have been positively correlated with serum cholesterol or triglycerides, gender (increased in men) and age, BMI and smoking; Q10 is negatively correlated with aerobic conditioning.[44] It is possible that the upregulation of Q10 observed in our study was the result of gender bias in favour of men, age or a

general lack of conditioning that might be expected from these factors in combination with decreased activity as might be seen in autoimmune diseases. Additionally, coenzyme Q10 is an important lipophilic antioxidant, preventing the generation of free radicals as well as oxidative modifications of proteins, lipids, and DNA. It might therefore be the case that a number of patients in this study consumed additional supplements of Q10.

Activated T-cells also rely on fatty- or amino- acid metabolism.[29,45] Methylmalonic acid is a malonic acid derivative, which is a vital intermediate in the metabolism of fat and protein. MMA in serum is derived from the hydrolysis of d-methylmalonyl-CoA (MMA-CoA), which is a metabolic intermediate in the conversion of propionic acid (the product of fatty acid and amino acid metabolism) to succinic acid.[46] Upregulated in both MG and RA, methylmalonic acid, in its coenzyme A-linked form methylmalonyl-CoA, is converted into succinyl-CoA by methylmalonyl-CoA mutase in a reaction that requires vitamin B12 as a cofactor. In this way, methylmalonic acid enters the Krebs cycle and is thus part of one of the anaplerotic reactions. If insufficient B12 is available, serum methylmalonic acid levels will increase. In this way, methylmalonic acid is a useful surrogate measure of B12 deficiency; methylmalonic acid is markedly elevated in the vast majority (>98%) of patients with clinical B12 deficiency.[47]

Malonic acid is also a fatty acid pathway participant. A dicarboxylic acid, malonic acid is the archetypal example of a competitive inhibitor: it acts against succinate dehydrogenase (complex II) in the respiratory electron transport chain.[48] Further, malonyl-CoA, a coenzyme A derivative of malonic acid, is an essential component of the fatty acid biosynthesis pathways for capric and palmitic acid, among others.[49] Malonic acid was upregulated in both MG and RA.

9.8 The contribution of fatty acids to immune pathways

The final product of fatty acid biosynthesis, palmitic acid (or palmitate), is a saturated fatty acid also found in plant oils. Palmitate is the precursor of stearate and longer-chain saturated fatty acids, as well as the monounsaturated acids, palmitoleate and oleate. Fatty acids are a primary energy source and an important component of membrane lipids.[50] They also serve as cellular signalling molecules that play an important role in the etiology of metabolic syndrome.[51]

It has been established that free fatty acids activate or inhibit certain cell types through TLR4.[52,53] Palmitate also activates CCL4 expression in human monocytic cells.[52,53] The use of fatty acids as fuel increases the risk of enhanced oxidative stress.[54] Reactive oxygen species (ROS) produced during the

reduction of molecular oxygen by the electron transport chain (ETC) forms superoxides that can cause damage to lipids, proteins and DNA[55] and can be toxic and pro-inflammatory.[56]

Preferred energy substrates vary based on immune cell state. FAO is the preferred energy source for low metabolic need or slow-to-activate cells for development and long-term survival. Lipid oxidation, for example, is important in the maintenance of quiescent memory T-cells[57–59]generation of both regulatory T-cells (Tregs)[60] and memory CD8+ T-cells[61,62], and M2 macrophage metabolism.[62]

Previous studies have revealed changes in palmitic acid expression in rheumatoid disease; however, results have been mixed. While palmitate has been observed as downregulated in RA[63] and systemic lupus erythematosus (SLE)[13] , Yan et al. noted positive correlations between C4 and two metabolites, glycerol and palmitic acid in patients with SLE.[64] Shin et al. also examined serum palmitic acid. The levels of myristic and palmitic acid were significantly higher in SLE as compared to controls. [65] This contrasts with our study, where serum levels of palmitic acid were downregulated for both MG and RA. These results indicate that the fatty acid metabolism was overall less activated in the control group than in the RA group and more activated in the control group than in the MG group.

The reasons for diminished levels of palmitic acid in our samples are unclear. It may be that dietary fatty acid intake or palmitic acid synthesis is unable to meet demand, resulting in reduced serum palmitate. For instance, it has been observed that innate immune cytokines, such as IL-1, can prevent fatty acid synthesis.[66]

Another fatty acid, capric acid, was found to be downregulated in MG and upregulated in RA. Capric acid is a member of the series of fatty acids found in oils and animal fats. A medium chain fatty acid, capric acid changes have been observed in lupus nephritis[67] and osteoarthritis.[68] Orally ingested medium chain fatty acids are very rapidly degraded by first-pass metabolism by being taken up in the liver, and quickly metabolized via coenzyme A intermediates through β-oxidation and the citric acid cycle to produce carbon dioxide, acetate and ketone bodies for use in energy metabolism.[69]

Sphingosine is an 18-carbon amino alcohol with a long unsaturated hydrocarbon chain; sphingosine and its derivative sphinganine form the major bases of the sphingolipids in mammals.[70] Sphingosine can be phosphorylated via kinases sphingosine kinase type 1 and type 2 to lead to the formation of sphingosine-1-phosphate (S1P), a potent signalling lipid. S1P controls numerous aspects of cell physiology, including cell survival and mammalian inflammatory responses including cyclooxygenase-2 induction (COX-2), and regulation of eicosanoids production.[71] In our samples, sphingosine was

downregulated in MG but upregulated in RA. This is consistent with previous studies, which demonstrated the increased presence of S1P in RA patients.[72] S1P in RA exhibits increased production of pro-inflammatory chemokines and cytokines, particularly tumour necrosis factor-alpha (TNF-α), a critical cytokine responsible for RA activity.[73] Sphingosine 1-phosphate (S1P) receptor antagonists, such as fingolimod (Gilenya™ / FTY720) or the more recently developed siponimod (BAF312) are thought to supress S1P activity, resulting from upregulated sphingosine, in RA. Consistent with our observations, fingolimod or siponimod therapy had no significant effect on antibody titers and disease severity in mice with experimental MG.[74] This suggests that sphingosine, or sphingosine kinase activity may be reduced in MG.

2-Methylbutyrylglycine is another amino acid, an acyl glycine, and is upregulated in both MG and RA. Acyl glycines are normally minor metabolites of fatty acids, and therefore play a role most closely related to energy production and cell structure maintenance. However, 2-Methylbutyrylglycine has been observed to induce lipid oxidative damage and decrease the antioxidant defenses in rat brain.[75]

The lysophospholipid LysoPE(0:0/20:4(5Z,8Z,11Z,14Z)) was found to be downregulated in MG and upregulated in RA. This reflects the findings of an earlier paper that also observed perturbation of glycerophospholipid metabolism in response to prednisone treatment in MG patients.[76] Paradoxically, however, where it was previously observed that glycerophospholipids were upregulated in those patients taking prednisone, in our cohort glycerophospholipid downregulation was noted for all patients, including those on prednisone (n=19, fold-change mean = -2.71). Fatty acids are strongly correlated with inflammation;[77] arachidonic acid can be released from activated inflammatory cell membrane phospholipids by phospholipase D (PLA2).[78,79] This downregulation of lipids may reflect a higher activity of phospholipase A2 (PLA2), a family of enzymes present in the arachidonic acid pathway, which hydrolyze membrane glycerophospholipids to lysoPCs and fatty acids.[80]

9.9 Tyrosine and related metabolites

L-Tyrosine and 3-Methoxytyramine were found to be upregulated in both MG and RA. L-Tyrosine is a non-essential amino acid, one of the 20 standard amino acids that are used by cells to synthesize proteins. L-Tyrosine is also a key precursor metabolite in the synthesis of catecholamines, the thyroid hormones triiodothyronine (T3) and thyroxine (T4), and the coenzyme Q10.[81] L-Tyrosine is also an element of fatty acid biosynthesis. The decomposition of L-tyrosine into acetoacetate further results in the liberation of acetyl-CoA, which can be used for fatty acid synthesis.[82]

3-Methoxytyramine is a dopamine metabolite. During DA synthesis, L-DOPA is produced from the amino acid tyrosine by tyrosine hydroxylase. After release and activation of its receptors, DA undergoes dilution by diffusion, but also becomes subject to metabolic degradation by catechol-o-methyl transferase (COMT). This process yields the major extracellular metabolite, 3-methoxytyramine (3-MT) which has been observed to exert significant neuromodulatory/neurotransmitter actions which may affect neuroimmune responses.[83] The dopaminergic system is highly involved in immunomodulation and inflammation within autoimmune disease, including lupus, rheumatoid arthritis and inflammatory bowel disease.[84] Changes in tyrosine expression may be relect this upregulation. Autoimmune thyroid disease is a common cormorbidity alongside other autoimmune diseases, including MG and RA.[85] Thyroid disease may disrupt thyroid hormone production, resulting in endocrine changes that lead to an excess of unmetabolized tyrosine. The tyrosine metabolite cis-4-Hydroxycyclohexylacetic acid was also upregulated in both MG and RA.

4-Hydroxyphenylpyruvic acid (4-HPPA) is a keto acid, part of the tyrosine catabolism pathway. It is a product of the enzyme (R)-4-hydroxyphenyllactate dehydrogenase and is formed during tyrosine metabolism. 4-HPPA was found to be downregulated in MG and upregulated in RA. The role of 4-HPPA in the current study is unclear.

9.10 Diet, gut microbiota and bile acids contribute to the immune response and homeostasis

A byproduct of xenobiotic metabolism, 3-Hydroxybenzoic acid is produced by the gut microflora as one of the three main metabolites formed from the catechin diet.[86] Upregulated in both groups, 3- and 4-Hydroxybenzoic acid is also a product of benzoate degradation. Converted to 2,5 Dihydroxybenzoate, it is a precursor to tyrosine metabolism.

Bile acids are increasingly recognized for their role in autoimmunity. Several metabolomics studies have identified perturbation of bile acids and their pathways in several diseases, including inflammatory bowel disease,[87] RA, ankylosing spondylitis, MG, Parkinson's, MS.[88] At the time of this manuscript, Johns Hopkins University is currently conducting a trial to assess the efficacy of bile acid supplementation to normalize blood bile acid levels, abnormal immune responses and the gut microbiome (ClinicalTrials.gov Identifier: NCT03423121). The secondary bile acid 7a-Hydroxy-3-oxo-5b-cholanoic acid was downregulated in MG but upregulated in RA.

Secondary bile acids are derived from the primary bile acids by the enzymatic action of intestinal bacteria through the process of deconjugation and dehydroxylation.[89] Complex mechanisms regulate

the biosynthesis of bile acids and the way bile acid receptors and their effectors affect cholesterol, glucose, fatty acid, and energy metabolism.[90,91]

Along with their role in the absorption, transport, and metabolism of dietary fats and lipid-soluble vitamins, bile acids have also been implicated in cell signalling, glucose metabolism[92] and inflammation.[93] The reason behind the observed reduction of bile acids in the MG group remains unclear. Pharmacological effects seem unlikely since while pyridostigmine is subject to metabolism during the first passage through the liver, its hepatotoxicity is quite low.[94] It has been suggested that gut microbiota may reduce the production of bile acids while increasing inflammation,[95] although the broader implications for autoimmune disease are uncertain.

9.11 Phthalates, environmental xenometabolites

Finally, phthalates are xenoestrogenic compounds, environmental toxicants that mimic or induce endogenous hormones. Also known as endocrine disrupting chemicals (EDCs), these include terephthalic acid, which was found to be upregulated. EDCSs can disrupt the immune-neuroendocrine network (INEN), the network joining the endocrine, immune and nervous systems.[96] Environmental stressors such as phthalate chemical exposure may result in cytokine-induced neurotoxicity by inducing oxidative stress[97] and neuroinflammation.[98,99] Phthalates are also widely used as excipients for the enteric coating of pharmaceutical tablets.[100] Increased terephthalic acid levels may also reflect high-frequency medication schedules, or the taking of large quantities of phthalate-coated medication contiguous with study blood sample collection.

9.12 Metabolites of unclear significance

Four identified metabolites were of undetermined significance. Phenylacetate (or phenylacetate) is a carboxylic acid ester that has been found in the biofluids of patients with nephritis and/or hepatitis, as well as patients with phenylketonuria (PKU). Phenylacetate is also produced endogenously as 2-Phenylethylamine is metabolized in the small intestine by monoamine oxidase B (MAO-B) and then aldehyde dehydrogenase (ALDH), which convert it to phenylacetic acid. 2-phenylethylamine is an "endogenous amphetamine" which may modulate central adrenergic functions.[101] The role of phenylacetate in the current study is unclear; however, the anti-inflammatory diclofenac is also a phenylacetate. While the metabolite profile for diclofenac has only been partially revealed, widespread use among RA patients indicates phenylacetate may be an as-yet unobserved metabolite. Alanyl-Leucine is a dipeptide composed of alanine and leucine and was upregulated in both MG and RA samples. It is the product of the incomplete breakdown of protein digestion or protein catabolism. The

amino acid O-Ureidohomoserine was found to be downregulated in MG and upregulated in RA. O-Ureidohomoserine is a participant in the canavanine biosynthesis pathway. It can be generated from the enzymatic reduction of canavaninosuccinate or enzymatic oxidation of L-canaline. Canavanine is a non-proteinogenic amino acid found in certain leguminous plants. It's been observed that mice fed L-canavanine develop a syndrome similar to systemic lupus erythematosus.[102] Finally, L-beta-aspartyl-l-alanine, a peptidomimetic, is the result of proteolytic breakdown product of larger proteins. Upregulated in both MG and RA, its role in this study is unknown.

9.13 Conclusion

In summary, 20 identified metabolites have emerged as shared biomarkers for the pathophysiology common to MG and RA. We have shown that many of these biomarkers are strongly related to immunological mechanisms disrupted in humoral autoimmune disease. In this regard, the further elucidation of shared metabolomic profiles may lead to an improved understanding of broader autoimmune disruptions and to novel immunometabolic therapies for autoimmune diseases.

9.14 References

1. de Candia, P. *et al.* Immunometabolism of human autoimmune diseases: from metabolites to extracellular vesicles. *FEBS Lett.* 591, 3119–3134 (2017).
2. Lee, Y. S., Wollam, J. & Olefsky, J. M. An Integrated View of Immunometabolism. *Cell* 172, 22–40 (2018).
3. O'Neill, L. A. J., Kishton, R. J. & Rathmell, J. A guide to immunometabolism for immunologists. *Nat. Rev. Immunol.* 16, 553–565 (2016).
4. Loftus, R. M. & Finlay, D. K. Immunometabolism: Cellular Metabolism Turns Immune Regulator. *J. Biol. Chem.* 291, 1–10 (2016).
5. Kominsky, D. J., Campbell, E. L. & Colgan, S. P. Metabolic shifts in immunity and inflammation. *J. Immunol.* 184, 4062–8 (2010).
6. Amersfoort, J. & Kuiper, J. T cell metabolism in metabolic disease-associated autoimmunity. *Immunobiology* 222, 925–936 (2017).
7. Pearce, E. L. & Pearce, E. J. Metabolic pathways in immune cell activation and quiescence. *Immunity* 38, 633–43 (2013).
8. Pearce, E. L., Poffenberger, M. C., Chang, C.-H. & Jones, R. G. Fueling Immunity: Insights into Metabolism and Lymphocyte Function. *Science (80-.).* 342, 1242454–1242454 (2013).
9. Olenchock, B. A., Rathmell, J. C. & Vander Heiden, M. G. Biochemical Underpinnings of Immune Cell Metabolic Phenotypes. *Immunity* 46, 703–713 (2017).

10. Kang, J., Zhu, L., Lu, J. & Zhang, X. Application of metabolomics in autoimmune diseases: Insight into biomarkers and pathology. *J. Neuroimmunol.* 279, 25–32 (2015).

11. Seeger, K. Metabolic changes in autoimmune diseases. *Curr. Drug Discov. Technol.* 6, 256–61 (2009).

12. Lu, Y. *et al.* Serum metabolomics for the diagnosis and classification of myasthenia gravis. *Metabolomics* 8, 704–713 (2012).

13. Yan, B. *et al.* Serum metabolomic profiling in patients with systemic lupus erythematosus by GC/MS. *Mod. Rheumatol.* 26, 914–922 (2016).

14. Zhou, J. *et al.* Exploration of the serum metabolite signature in patients with rheumatoid arthritis using gas chromatography–mass spectrometry. *J. Pharm. Biomed. Anal.* 127, 60–67 (2016).

15. Villoslada, P. *et al.* Metabolomic signatures associated with disease severity in multiple sclerosis. *Neurol. Neuroimmunol. NeuroInflammation* 4, (2017).

16. Zhao, S., Luo, X. & Li, L. Chemical Isotope Labeling LC-MS for High Coverage and Quantitative Profiling of the Hydroxyl Submetabolome in Metabolomics. *Anal. Chem.* 88, 10617–10623 (2016).

17. Guo, K. & Li, L. Differential ^{12}C-/ ^{13}C-Isotope Dansylation Labeling and Fast Liquid Chromatography/Mass Spectrometry for Absolute and Relative Quantification of the Metabolome. *Anal. Chem.* 81, 3919–3932 (2009).

18. Bengtsson, A. A. *et al.* Metabolic Profiling of Systemic Lupus Erythematosus and Comparison with Primary Sjögren's Syndrome and Systemic Sclerosis. *PLoS One* 11, e0159384 (2016).

19. Lindahl, A., Forshed, J. & Nordström, A. Overlap in serum metabolic profiles between non-related diseases: Implications for LC-MS metabolomics biomarker discovery. *Biochem. Biophys. Res. Commun.* 478, 1472–1477 (2016).

20. Bonuccelli, G. *et al.* Ketones and lactate "fuel" tumor growth and metastasis. *Cell Cycle* 9, 3506–3514 (2010).

21. Olenchock, B. A., Rathmell, J. C. & Vander Heiden, M. G. Biochemical Underpinnings of Immune Cell Metabolic Phenotypes. *Immunity* 46, 703–713 (2017).

22. Pearce, E. L. & Pearce, E. J. Metabolic pathways in immune cell activation and quiescence. *Immunity* 38, 633–43 (2013).

23. Buck, M. D., Sowell, R. T., Kaech, S. M. & Pearce, E. L. Metabolic Instruction of Immunity. *Cell* 169, 570–586 (2017).

24. Dimeloe, S., Burgener, A.-V., Grählert, J. & Hess, C. T-cell metabolism governing activation, proliferation and differentiation; a modular view. *Immunology* 150, 35–44 (2017).

25. Grist, J. T. *et al.* Extracellular Lactate: A Novel Measure of T Cell Proliferation. *J. Immunol.* 200, 1220–1226 (2018).

26. Seki, S. M. & Gaultier, A. Exploring Non-Metabolic Functions of Glycolytic Enzymes in Immunity. *Front. Immunol.* 8, 1549 (2017).

27. Westerblad, H., Allen, D. G. & Lännergren, J. Muscle Fatigue: Lactic Acid or Inorganic Phosphate the Major Cause? *Physiology* 17, 17–21 (2002).

28. Kolins, J. & Gilroy, J. Serum enzyme levels in patients with myasthenia gravis after aerobic and ischaemic exercise. *J. Neurol. Neurosurg. Psychiatry* 35, 34–40 (1972).

29. Yang, Z., Matteson, E. L., Goronzy, J. J. & Weyand, C. M. T-cell metabolism in autoimmune disease. *Arthritis Res. Ther.* 17, 29 (2015).

30. Haas, R. *et al.* Lactate Regulates Metabolic and Pro-inflammatory Circuits in Control of T Cell Migration and Effector Functions. *PLoS Biol.* 13, e1002202 (2015).

31. Haas, R. *et al.* Lactate Regulates Metabolic and Pro-inflammatory Circuits in Control of T Cell Migration and Effector Functions. *PLOS Biol.* 13, e1002202 (2015).

32. Mehta, M. M. & Chandel, N. S. Targeting metabolism for lupus therapy. *Sci. Transl. Med.* 7, (2015).

33. Yin, Y. *et al.* Normalization of CD4⁺ T cell metabolism reverses lupus. *Sci. Transl. Med.* 7, (2015).

34. Wu, T. *et al.* Metabolic disturbances associated with systemic lupus erythematosus. *PLoS One* 7, (2012).

35. De Rosa, V. *et al.* Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. *Nat. Immunol.* 16, 1174–1184 (2015).

36. Regenold, W. T., Phatak, P., Makley, M. J., Stone, R. D. & Kling, M. A. Cerebrospinal fluid evidence of increased extra-mitochondrial glucose metabolism implicates mitochondrial dysfunction in multiple sclerosis disease progression. *J. Neurol. Sci.* 275, 106–112 (2008).

37. Amorini, A. M. *et al.* Serum lactate as a novel potential biomarker in multiple sclerosis. *Biochim. Biophys. Acta - Mol. Basis Dis.* 1842, 1137–1143 (2014).

38. Walenta, S., Schroeder, T. & Mueller-Klieser, W. Lactate in solid malignant tumors: Potential basis of a metabolic classification in clinical oncology. *Curr. Med. Chem.* 11, 2195–2204 (2004).

39. Hirschhaeuser, F., Sattler, U. G. A. & Mueller-Klieser, W. Lactate: A metabolic key player in cancer. *Cancer Res.* 71, 6921–6925 (2011).

40. Choi, S. Y. C., Collins, C. C., Gout, P. W. & Wang, Y. Cancer-generated lactic acid: A regulatory, immunosuppressive metabolite? *J. Pathol.* 230, 350–355 (2013).

41. Yuan, Z.-Y. *et al.* Prognostic value of preoperative serum lactate dehydrogenase in thymic carcinoma. *J. Thorac. Dis.* 8, 2464 (2016).

42. Siemieniuk, E. & Skrzydlewska, E. [Coenzyme Q10: its biosynthesis and biological significance in animal organisms and in humans]. *Postepy Hig. Med. Dosw. (Online)* 59, 150–9 (2005).

43. Ernster, L. & Dallner, G. Biochemical, physiological and medical aspects of ubiquinone function. *Biochim. Biophys. Acta* 1271, 195–204 (1995).

44. Kaikkonen, J., Nyyssönen, K., Tuomainen, T.-P., Ristonmaa, U. & Salonen, J. T. Determinants of plasma coenzyme Q10 in humans. *FEBS Lett.* 443, 163–166 (1999).

45. Poffenberger, M. C. & Jones, R. G. Amino Acids Fuel T Cell-Mediated Inflammation. *Immunity* 40, 635–637 (2014).

46. Allen, R. H., Stabler, S. P., Savage, D. G. & Lindenbaum, J. Metabolic abnormalities in cobalamin (vitamin B12) and folate deficiency. *FASEB J.* 7, 1344–53 (1993).

47. Vashi, P., Edwin, P., Popiel, B., Lammersfeld, C. & Gupta, D. Methylmalonic Acid and Homocysteine as Indicators of Vitamin B-12 Deficiency in Cancer. *PLoS One* 11, e0147843 (2016).

48. Kim, Y. S. Malonate metabolism: biochemistry, molecular biology, physiology, and industrial application. *J. Biochem. Mol. Biol.* 35, 443–51 (2002).

49. Small Molecule Pathway Database. Available at: http://smpdb.ca/. (Accessed: 25th April 2018)

50. Wakil, S. J. & Abu-Elheiga, L. A. Fatty acid metabolism: target for metabolic syndrome. *J. Lipid Res.* 50 Suppl, S138-43 (2009).

51. Wakil, S. J. & Abu-Elheiga, L. A. Fatty acid metabolism: target for metabolic syndrome. *J. Lipid Res.* 50

Suppl, S138-43 (2009).

52. Shi, H. *et al.* TLR4 links innate immunity and fatty acid–induced insulin resistance. *J. Clin. Invest.* 116, 3015–3025 (2006).

53. Schaeffler, A. *et al.* Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-κB pathway in adipocytes links nutritional signalling with innate immunity. *Immunology* 126, 233–245 (2009).

54. Schönfeld, P. & Reiser, G. Why does brain metabolism not favor burning of fatty acids to provide energy? Reflections on disadvantages of the use of free fatty acids as fuel for brain. *J. Cereb. Blood Flow Metab.* 33, 1493–9 (2013).

55. Schieber, M. & Chandel, N. S. ROS function in redox signaling and oxidative stress. *Curr. Biol.* 24, R453-62 (2014).

56. Guzik, T. J., Korbut, R. & Adamek-Guzik, T. Nitric oxide and superoxide in inflammation and immune regulation. *J. Physiol. Pharmacol.* 54, 469–87 (2003).

57. Pan, Y. *et al.* Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. *Nature* 543, 252–256 (2017).

58. van der Windt, G. J. W. *et al.* CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. *Proc. Natl. Acad. Sci.* 110, 14336–14341 (2013).

59. van der Windt, G. J. W. *et al.* Mitochondrial Respiratory Capacity Is a Critical Regulator of CD8+ T Cell Memory Development. *Immunity* 36, 68–78 (2012).

60. Michalek, R. D. *et al.* Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CD4+ T Cell Subsets. *J. Immunol.* 186, 3299–3303 (2011).

61. Hart, I. K. Acquired neuromyotonia: A new autoantibody-mediated neuronal potassium channelopathy. *Am. J. Med. Sci.* 319, 209–216 (2000).

62. Araki, K. *et al.* mTOR regulates memory CD8 T-cell differentiation. *Nature* 460, 108–112 (2009).

63. Rodríguez-Carrio, J., Alperi-López, M., López, P., Ballina-García, F. J. & Suárez, A. Non-Esterified Fatty Acids Profiling in Rheumatoid Arthritis: Associations with Clinical Features and Th1 Response. *PLoS One* 11, e0159573 (2016).

64. Yan, B. *et al.* Serum metabolomic profiling in patients with systemic lupus erythematosus by GC/MS. *Mod. Rheumatol.* 26, 914–922 (2016).

65. Shin, T. H. *et al.* Analysis of the free fatty acid metabolome in the plasma of patients with systemic lupus erythematosus and fever. *Metabolomics* 14, 14 (2018).

66. Matsuki, T., Horai, R., Sudo, K. & Iwakura, Y. IL-1 Plays an Important Role in Lipid Metabolism by Regulating Insulin Levels under Physiological Conditions. *J. Exp. Med.* 198, 877–888 (2003).

67. Li, J. *et al.* Metabolic profiling reveals new serum biomarkers of lupus nephritis. *Lupus* 26, 1166–1173 (2017).

68. Kim, S. *et al.* Metabolite profiles of synovial fluid change with the radiographic severity of knee osteoarthritis. *Jt. Bone Spine* 84, 605–610 (2017).

69. Schönfeld, P. & Wojtczak, L. Short- and medium-chain fatty acids in energy metabolism: the cellular perspective. *J. Lipid Res.* 57, 943–54 (2016).

70. Maceyka, M. & Spiegel, S. Sphingolipid metabolites in inflammatory disease. *Nature* 510, 58–67 (2014).

71. Maceyka, M., Harikumar, K. B., Milstien, S. & Spiegel, S. Sphingosine-1-phosphate signaling and its role in disease. *Trends Cell Biol.* 22, 50–60 (2012).

72. Jaigirdar, S. A. *et al.* Sphingosine-1-phosphate promotes the persistence of activated CD4 T cells in inflamed sites. *Front. Immunol.* 8, (2017).

73. Zhao, C. *et al.* Specific and overlapping sphingosine-1-phosphate receptor functions in human synoviocytes: Impact of TNF-α. *J. Lipid Res.* 49, 2323–2337 (2008).

74. Pelz, A. *et al.* S1P receptor antagonists fingolimod and siponimod do not improve the outcome of experimental autoimmune myasthenia gravis mice after disease onset. *Eur. J. Immunol.* 48, 498–508 (2018).

75. Knebel, L. A. *et al.* 2-Methylbutyrylglycine induces lipid oxidative damage and decreases the antioxidant defenses in rat brain. *Brain Res.* 1478, 74–82 (2012).

76. Sengupta, M. *et al.* Serum Metabolomic Response of Myasthenia Gravis Patients to Chronic Prednisone Treatment. *PLoS One* 9, e102635 (2014).

77. Young, S. P. *et al.* The Impact of Inflammation on Metabolomic Profiles in Patients With Arthritis. *Arthritis Rheum.* 65, 2015–2023 (2013).

78. Astudillo, A. M., Balgoma, D., Balboa, M. A. & Balsinde, J. Dynamics of arachidonic acid mobilization by inflammatory cells. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1821, 249–256 (2012).

79. Lone, A. M. & Taskén, K. Proinflammatory and Immunoregulatory Roles of Eicosanoids in T Cells. *Front. Immunol.* 4, 130 (2013).

80. Cummings, B. S., McHowat, J. & Schnellmann, R. G. Phospholipase A2s in Cell Injury and Death. *J. Pharmacol. Exp. Ther.* 294, (2000).

81. Wu, G. *Amino Acids*. (CRC Press, 2013). doi:10.1201/b14661

82. Foster, D. W. Malonyl-CoA: the regulator of fatty acid synthesis and oxidation. *J. Clin. Invest.* 122, 1958–9 (2012).

83. Sotnikova, T. D. *et al.* The dopamine metabolite 3-methoxytyramine is a neuromodulator. *PLoS One* 5, e13452 (2010).

84. Pacheco, R., Contreras, F. & Zouali, M. The dopaminergic system in autoimmune diseases. *Front. Immunol.* 5, (2014).

85. Jenkins, R. C. & Weetman, A. P. Disease associations with autoimmune thyroid disease. *Thyroid* 12, 977–988 (2002).

86. Lafay, S. & Gil-Izquierdo, A. Bioavailability of phenolic acids. *Phytochem. Rev.* 7, 301–311 (2008).

87. Moco, S. *et al.* Systems biology approaches for inflammatory bowel disease: Emphasis on gut microbial metabolism. *Inflamm. Bowel Dis.* 20, 2104–2114 (2014).

88. Bhargava, P. & Calabresi, P. A. Metabolomics in multiple sclerosis. *Mult. Scler.* 22, 451–460 (2016).

89. Masubuchi, N. *et al.* Oxidative stress markers, secondary bile acids and sulfated bile acids classify the clinical liver injury type: Promising diagnostic biomarkers for cholestasis. *Chem. Biol. Interact.* 255, 83–91 (2016).

90. Staels, B. & Fonseca, V. A. Bile acids and metabolic regulation: mechanisms and clinical responses to bile acid sequestration. *Diabetes Care* 32 Suppl 2, S237-45 (2009).

91. Chiang, J. Y. L. Bile acid metabolism and signaling. *Compr. Physiol.* 3, 1191–212 (2013).

92. Nguyen, A. & Bouscarel, B. Bile acids and signal transduction: Role in glucose homeostasis. *Cell. Signal.* 20, 2180–2197 (2008).

93. Zhu, C., Fuchs, C. D., Halilbasic, E. & Trauner, M. Bile acids in regulation of inflammation and immunity:

friend or foe? *Clin. Exp. Rheumatol.* 34, 25–31

94. Zhao, B., Moochhala, S. M., Lu, J., Tan, D. & Hoon Lai, M. Determination of pyridostigmine bromide and its metabolites in biological samples. *J Pharm Pharm. Sci* 9, 71–81 (2006).

95. Staley, C., Weingarden, A. R., Khoruts, A. & Sadowsky, M. J. Interaction of gut microbiota with bile acid metabolism and its influence on disease states. *Appl. Microbiol. Biotechnol.* 101, 47–64 (2017).

96. Manley, K., Han, W., Zelin, G. & Lawrence, D. A. Crosstalk between the immune, endocrine, and nervous systems in immunotoxicology. *Curr. Opin. Toxicol.* 10, 37–45 (2018).

97. Franken, C. *et al.* Phthalate-induced oxidative stress and association with asthma-related airway inflammation in adolescents. *Int. J. Hyg. Environ. Health* 220, 468–477 (2017).

98. Yegambaram, M., Manivannan, B., Beach, T. G. & Halden, R. U. Role of environmental contaminants in the etiology of Alzheimer's disease: a review. *Curr. Alzheimer Res.* 12, 116–46 (2015).

99. Win-Shwe, T.-T., Yanagisawa, R., Koike, E., Nitta, H. & Takano, H. Expression levels of neuroimmune biomarkers in hypothalamus of allergic mice after phthalate exposure. *J. Appl. Toxicol.* 33, 1070–1078 (2013).

100. *Excipient Applications in Formulation Design and Drug Delivery*. (Springer International Publishing, 2015). doi:10.1007/978-3-319-20206-8

101. Sabelli, H. C. *et al.* Urinary phenyl acetate: a diagnostic test for depression? *Science* 220, 1187–8 (1983).

102. Akaogi, J. *et al.* Role of non-protein amino acid l-canavanine in autoimmunity. *Autoimmun. Rev.* 5, 429–435 (2006).

CHAPTER 10.

CLINICAL AND METABOLOMIC CORRELATIONS IN MYASTHENIA GRAVIS

10.1 Abstract

Ideally, high value research is translational. In the case of metabolomics, this often means relating observed biomarker data to real world clinical practice parameters. The present study sought to summarize the correlations found between a two-control, multi-label metabolomics profiling of biomarkers unique to myasthenia gravis (MG), and matched clinical and laboratory measures. Independent T-tests and linear regression models were used for comparisons. Clinical correlations revealed negative associations between treatment efficacy, MG class and disease duration. A positive correlation was observed for prednisone benefit and early or late onset MG. T-testing further revealed differences in mestinon response between MG classes. Age of onset also differed MG class and gender. Multiple regression also produced predictive models of therapy response based on clinical and laboratory factors. Biomarker analysis revealed predictive models for MG age of onset and manual muscle testing employing analyl-leucine and pthalic acid as factors. Response to therapy also produced valid models, with the identified metabolites Alanyl-Leucine, MG(0:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0), LysoPE(0:0/22:5(4Z,7Z,10Z,13Z,16Z)), Malonic acid, 3-Methoxytyramine, Terephthalic acid all explaining variance in several models. These results demonstrate the potential for metabolomic profiling to reveal useful clinical biomarkers.

10.2 Introduction

Biomarkers are important tools in the diagnosis and management of disease. Useful not only for diagnosis, biomarkers should ideally provide information about disease state and prognosis as well as therapeutic effectiveness. At present, very few biomarkers exist which consistently correlate with clinical measures in myasthenia gravis (MG).

Biomarkers presently available for MG are primarily diagnostic in nature; acetylcholine receptor (AChR) antibodies confirm the diagnosis of AChR-seropositive MG, but correlate poorly with disease severity and clinical response. [1–3] Biomarkers are important in clinical trials as well, where robust biomarkers reflect the underlying disease process in a sensitive and reliable manner. The Myasthenia Gravis Foundation of America Task Force evaluated several potential biomarkers observed in MG, including serum antibody levels and single-fibre EMG, and concluded that none fulfilled the criteria for meaningful surrogate clinical research endpoints.

Metabolomic biomarkers may serve as rapidly translatable and cost-effective alternatives to less easily deployed tests such as genomic analysis. Further, metabolomic biomarkers reflect the current state of

disease and, as such, offer a snapshot of underpinning pathophysiology. The goal of the present study is to examine metabolomic biomarkers which correlate with clinical measures specific to the AChR-seropositive myasthenia gravis population.

10.3 Methods

10.3.1 See Section 7.1

10.3.2 Clinical Data

The demographic data of each patient was collected upon enrolment. Date of birth, gender, height, weight and BMI were noted. MG classification (ocular or generalized) and manual muscle testing (MMT) scores were also collected. Using a brief survey, data including MG serostatus, age of symptom onset, duration of disease, perceived response to previously taken therapies and lifestyle measures were noted. Information regarding previous therapies employed an ordinal scale with 0=no effect on MG symptoms, 1=some positive effect on MG symptoms and 2=significant positive effect on MG symptoms. Where possible, patient recollections were confirmed with notes from the patient record.

10.4 Results

The UMS LC-MS technique employed in this study provided broad coverage of both the carboxyl and phenol/amine serum submetabolomes for MG. In total, the application of two labelling techniques revealed an overall total of 9954 C12/C13 carboxyl-labelled peak pairs (features) and 7458 phenol or amine features. After data processing, metabolites were matched to in-house standard libraries or putatively identified using an accurate mass search using the MycompoundID portal. A total of 12 compounds were library- or putatively-matched matched to MG. Chemical species data for the 12 MG metabolites is listed in Table 10.1.

Library-matched				
HMDB	**Name**	**Sample mz**	**Monoistopic mz**	**Regulation**
HMDB00011	(R)-3-Hydroxybutyric acid	104.05	104.05	Upregulated
HMDB00008	2-Hydroxybutyric acid	104.05	104.05	Upregulated
HMDB00005	2-Ketobutyric acid	102.03	102.03	Upregulated
HMDB00060	Acetoacetic acid	102.03	102.03	Upregulated
HMDB02428	Terephthalic acid	327.11	327.11	Upregulated
Putative				
HMDB	**Name**	**Sample mz**	**Monoistopic mz**	**Regulation**
HMDB00328	12-Ketodeoxycholic acid	390.28	390.28	Downregulated
HMDB00139	Glyceric acid	106.03	106.03	Upregulated
HMDB11477	LysoPE(0:0/18:2(9Z,12Z))	477.28	477.29	Downregulated
HMDB11489	LysoPE(0:0/20:5(5Z,8Z,11Z,14Z,17Z))	499.27	499.27	Downregulated
HMDB11494	LysoPE(0:0/22:5(4Z,7Z,10Z,13Z,16Z))	527.30	527.30	Downregulated
HMDB11496	LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z, 19Z))	525.29	525.29	Downregulated
HMDB11555	MG(0:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)	404.29	404.29	Downregulated

Table 10.1 Library-matched and putative MG metabolites

10.4.1 Clinical Correlations

Independent sample T-tests were conducted to compare the therapeutic benefit of MG medications between groups for demographic and laboratory measures. Three comparisons were meaningful. A significant difference exists between the means of generalized and ocular MG when compared for mestinon benefit (mean difference = 0.44, $p<0.01$); ocular patients respond less often. Furthermore, age of onset displayed mean differences when compared by class (generalized/ocular) and gender. A mean difference of 15.44 years, $p= 0.03$ for gender and 15.18 years, $p=0.05$ by class. The data for these T-test is presented in Tables 10.2 and 10.3.

MG Class					
	Class	**N (Generalized)**	**N (Ocular)**	**df**	***P*-value**
Mestinon	Generalized	31	9	38	0.006
Prednisione	Generalized	25	4	27	0.574
Imuran	Generalized	25	5	28	0.189
Duration of Disease					
	Disease duration	**N (≤2 years)**	**N (> 2years)**	**df**	***P*-value**
Mestinon	2 years or less	7	33	38	0.516
Prednisione	2 years or less	5	24	27	0.521
Imuran	2 years or less	5	25	28	0.853
IVIG	2 years or less	7	24	29	0.262

Table 10.2 Independent T-testing comparing therapeutic benefit with MG class and Duration of disease

Gender				
	N (Male)	**N (Female)**	**df**	***P*-value**
Mestinon	25	15	38	0.717
Prednisione	21	8	27	0.384
Imuran	19	11	28	0.626
CellCept	9	5	12	0.519
IVIG	19	12	29	0.633
Plasmapheresis	8	5	11	0.453
Age of onset				
	N (Early onset)	**N (Late onset)**	**df**	***P*-value**
Mestinon	14	26	38	0.3
Prednisione	10	19	27	0.05
Imuran	11	19	28	0.63
CellCept	7	7	12	0.27
IVIG	12	19	29	0.56
Plasmapheresis	7	6	11	0.3
MG class				
	N (Generalized)	**N (Ocular)**	**df**	***P*-value**
MMT	33	11	42	0.394
Age of onset	33	11	42	0.049
Gender				
	N (Male)	**N (Female)**	**df**	***P*-value**
MMT	28	16	42	0.669
Age of onset	28	16	42	0.025

Table 10.3 Independent T-testing comparing therapeutic benefit with gender and age of onset, MMT with MG class and gender

Several multiple regression models were constructed to assess the accuracy of demographic and laboratory measures to correctly predict therapeutic response to MG medications. Models were chosen based on a cutoff of adjusted $R^2 \geq 0.70$. Two models exhibited moderate predictability with significance better than p=0.05. In the first model, disease duration was found to be predictive of prednisone response. The total variance explained by the model as a whole was 50.2%, $p< .001$. Multiple factors predicted plasmapheresis response, in the second model. Early or Late disease onset, MMT, disease duration ≤, 2 years or > 2 years, Gender, Age, and age of onset together explained 56.1%, p=0.05 of the variance in the model. a third model for mestinon was significant, however lacked predictive power. A total of 16.0% of the model's variance could be explained by the influence of MG class (generalized/ocular) All other models for Imuran, cellcept and IVIg were non-predictive and possessed p >0.05. Model summaries are found in Table 10.4.

Gender		
	Adjusted R^2	***P*-value**
Mestinon	0.023	0.70
Prednisone	0.008	0.38
Imuran	0.027	0.63
CellCept	0.045	0.52
IVIg	0.026	0.63
Plasmapheresis	0.034	0.45
MMT		
	Adjusted R^2	***P*-value**
Mestinon	0.025	0.86
Prednisone	0.050	0.13
Imuran	0.035	0.86
CellCept	0.083	0.93
IVIg	0.026	0.62
Plasmapeheresis	0.053	0.54

Table. 10.4 Regression models for therapeutic benefit comparing gender and MMT scores

Regression models were also constructed to explore the contribution of gender and MMT to explained variance in therapeutic response (Table 10.5) None of these models proved predictive.

	Adjusted R^2	*P*-value	
Mestinon	0.160	<0.01	Class
Prednisone	0.502	<0.01	Disease duration
Imuran	0.166	0.07	Age, disease duration, Class, Early or Late MG
CellCept	0.246	0.25	Age, MMT, Class, disease duration, gender, Early or Late MG
IVIg	0.166	0.80	Age, MMT, Class, disease duration, gender, Early or Late MG
Plasmapheresis	0.561	0.05	Early or Late MG, MMT, disease duration recode, gender, Age

Table. 10.5 Regression models for therapeutic benefit comparing factor panels

10.4.2 Biomarker Predictability

To explore for potentially useful biomarkers, the relationships between significantly altered metabolites and demographic and laboratory measures were explored. Multiple regression models were constructed describing the relationship between measured metabolites and clinical and laboratory observations. The models for age of MG onset (R^2= 0.739, p= <0.01), early or late onset MG (R^2= 0.700, p= <0.01) and MMT (R^2= 0.725, p= <0.01) described significant predictive relationships. Models for MG class (generalized/ocular), duration of disease and disease duration ≤, 2 years or > 2 years were failed to achieve the R^2=0.70 threshold. Of those metabolites previously identified by chemical library, only analyl-leucine and pthalic acid explained variance in any model.

To explore the usefulness of measured metabolites to predict the effectiveness of MG therapies, multiple regression models were constructed for each. The models for Mestinon (R2= 0.701, p= <0.01), Imuran (R2= 0.739, p= <0.01), Cellcept (R2= 0.779, p= <0.01), IVIg (R2= 0.749, p= <0.01), plasmapheresis (R2= 0.705, p= <0.01) all exhibited predictive scores. The model for Prednisone was valid as well but did not meet the required R^2=0.70 threshold. The identified metabolites Alanyl-Leucine, MG(0:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0), LysoPE(0:0/22:5(4Z,7Z,10Z,13Z,16Z)), Malonic acid, 3-Methoxytyramine, Terephthalic acid explained variance in several models. A summary is detailed in Table 10.6.

Clinical and laboratory factors	Adjusted R^2	*P*-value	Metabolomic factors
Age of Onset	0.739	<0.01	Acid:(8955, 16993, 9145, 5590, 19549, 1224, 2640))
Class (Generalized, Ocular)	0.271	<0.01	Acid:(1286)
Duration of Disease	0.133	<0.01	Acid:(19110)
Early or Late MG	0.700	<0.01	Acid:(1343)
MMT	0.725	<0.01	Acid:(12046, 644, 1224, 14387, 19236, 8091), Dansyl:(12828), phthalic acid, alanyl-Leucine
≤ 2 years, > 2 years	0.273	<0.01	Acid:(22137, 6833), dansyl:(10171)
Therapeutic benefit	**Adjusted R^2**	***P*-value**	
Mestinon	0.701	<0.01	Acid:(2244, 1317, 2610), dansyl:(4565)
Prednisone	0.453	<0.01	Acid:(17947), MG(0:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)
Imuran	0.739	<0.01	Acid:(9884, 9405), dansyl:(5349), lysoPE(0:0/22:5(4Z,7Z,10Z,13Z,16Z)), malonic acid, alanyl-Leucine
CellCept	0.779	<0.01	Acid:(1343), Methoxytyramine
IVIg	0.749	<0.01	Acid:(5628, 8091, 4739), dansyl:(5349), alanyl-Leucine
Plasmapheresis	0.705	<0.01	Acid:(2401), terephthalic acid

Table 10.6 Regression models for Clinical and laboratory factors and therapeutic benefit comparing metabolomic factors

10.5 Discussion

The purpose of this study was to explore the clinical, laboratory and metabolomic biomarker correlations of MG. Although other studies have done so previously, to the best of our knowledge, this is the most comprehensive to date. We first performed a broad correlative analysis to identify potentially significant relationships. In our group, moderately reduced effectiveness of Mestinon was reported in the ocular group. This is line with observations from previous studies that suggest pure oMG may respond less favorably to mestinon therapy.[6] Disease duration and prednisone efficacy were also negatively correlated. Because prednisone is a first-line therapy, this finding suggests that patients begin to habituate to increasing doses over time, require upward titration. Indeed, this has been observed in other studies where disease duration was significantly longer in a high prednisone dose MG group.[7] Finally, a difference in prednisone benefit between early and late onset MG further establishes the theory that these represent two different instantiations of MG.[8]

Independent T-testing further supports the correlative observation that Mestinon efficacy in oMG is less than that in generalized; mean perceived effectiveness was 1.56 as compared to 2 for generalized. Additionally, late onset disease was more common in males than females, which is consistent and well-documented.[8] Finally, ocular MG patients were much older than those with generalized MG in our study. This, again, is consistent with previous studies.[9]

Demographic and laboratory data were largely nonpredictive of MG therapy benefit. However, disease duration was found to be moderately predictive of response to prednisone. This again, is supported by the note above, that disease duration and prednisone dose were correlated. A composite model of the factors early or late disease onset, MMT, disease duration ≤, 2 years or > 2 years, gender, age, and age of onset was also predictive, for plasmapheresis. This multifactorial approach may also be of value when assessing correlations of therapy with other demographic measures.

Of the most interest, however, was the exploration of the utility of acquired metabolomic data in the prediction of MG disease types, onset and therapeutic efficacy. To this end, the results were quite promising. Potential biomarkers described predictive relationships with age of MG onset, early or late onset and MMT. To our knowledge, this has not been done previously. Furthermore, predictive models were successfully described for MG therapies using panels of metabolites. While many metabolites remain unidentified, those that were included lipids, a dipeptide, carboxylic acid, benzoid and phthalate.

In our models, MG(0:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0) was predictive of positive benefit from prednisone. MG(0:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0) is a monoacylglycerol lipid. In the previous report by Sengupta et al.[10], several lipids were upregulated in response to prednisone indicating correlation. The significance of other compounds remains unclear. Further studies are required studies to confirm our results and provide metabolites of additional chemical classes to help build a more complete predictive model.

10.6 References

1. Meriggioli, M. N. & Sanders, D. B. Muscle autoantibodies in myasthenia gravis: beyond diagnosis? *Expert Rev. Clin. Immunol.* 8, 427–38 (2012).

2. Guptill, J. T. & Sanders, D. B. Update on muscle-specific tyrosine kinase antibody positive myasthenia gravis. *Curr. Opin. Neurol.* 23, 530–535 (2010).

3. Howard, F. M., Lennon, V. A., Finley, J., Matsumoto, J. & Elveback, L. R. Clinical correlations of antibodies that bind, block, or modulate human acetylcholine receptors in myasthenia gravis. *Ann. N. Y. Acad. Sci.* 505, 526–38 (1987).

4. Zhao, S., Luo, X. & Li, L. Chemical Isotope Labeling LC-MS for High Coverage and Quantitative Profiling of the Hydroxyl Submetabolome in Metabolomics. *Anal. Chem.* 88, 10617–10623 (2016).

5. Guo, K. & Li, L. Differential ^{12}C-/ ^{13}C-Isotope Dansylation Labeling and Fast Liquid Chromatography/Mass Spectrometry for Absolute and Relative Quantification of the Metabolome. *Anal. Chem.* 81, 3919–3932 (2009).

6. Bhanushali, M. J., Wuu, J. & Benatar, M. Treatment of ocular symptoms in myasthenia gravis. *Neurology* 71, 1335–1341 (2008).

7. Imai, T. *et al.* Oral corticosteroid dosing regimen and long-term prognosis in generalised myasthenia gravis: a multicentre cross-sectional study in Japan. *J. Neurol. Neurosurg. Psychiatry* 89, 513–517 (2018).

8. Živković, S. A., Clemens, P. R. & Lacomis, D. Characteristics of late-onset myasthenia gravis. *J. Neurol.* 259, 2167–2171 (2012).

9. Nair, A. G., Patil-Chhablani, P., Venkatramani, D. V & Gandhi, R. A. Ocular myasthenia gravis: a review. *Indian J. Ophthalmol.* 62, 985–91 (2014).

10. Sengupta, M. *et al.* Serum Metabolomic Response of Myasthenia Gravis Patients to Chronic Prednisone Treatment. *PLoS One* 9, e102635 (2014).

11 SUMMARY

Overall, this work has provided novel information about the application of metabolomics in the study of myasthenia gravis. Additionally, it's hoped that the research conducted will provide new insights into the pathogenesis of autoimmune disease like myasthenia gravis as well as opening new avenues for research into alternate biomarkers of disease. The hypotheses generated from this research will hopefully encourage others to pursue similar work in that it might be reproduced and fulfil the promise of translational medicine.

REFERENCES

AAEM Quality Assurance Committee. American Association of Electrodiagnostic Medicine, 2001. Practice parameter for repetitive nerve stimulation and single fiber EMG evaluation of adults with suspected myasthenia gravis or Lambert-Eaton myasthenic syndrome: summary statement. Muscle & nerve, 24(9), pp.1236–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11494280 [Accessed June 23, 2018].

Abraham, A. et al., 2017. Electrophysiological testing is correlated with myasthenia gravis severity. Muscle & nerve, 56(3), pp.445–448.

Adamczyk-Sowa, M. et al., 2017. Oxidative modifications of blood serum proteins in myasthenia gravis. Journal of neuroimmunology, 305, pp.145–153. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28284335 [Accessed April 11, 2018].

Akaogi, J. et al., 2006. Role of non-protein amino acid l-canavanine in autoimmunity. Autoimmunity Reviews, 5(6), pp.429–435. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16890899 [Accessed April 16, 2018].

Albina, J.E., 1988. Arginine metabolism in wounds. Am. J. Physiol., 254, pp.E459–E467.

Albuquerque, E.X. et al., 2009. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiological reviews, 89(1), pp.73–120. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19126755 [Accessed June 23, 2018].

Allen, R.H. et al., 1993. Metabolic abnormalities in cobalamin (vitamin B12) and folate deficiency. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 7(14), pp.1344–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7901104 [Accessed April 24, 2018].

Alonso, D. & Nungester, W.J., 1956. Comparative study of host resistance of guinea pigs and rats V. The effect of pneumococcal products on glycolysis and oxygen uptake by polymorphonuclear leucocytes. J. Infect. Dis., 99, pp.174–181.

Alseth, E.H. et al., 2009. Interleukin-10 promoter polymorphisms in myasthenia gravis. Journal of Neuroimmunology, 210(1–2), pp.63–66.

Amersfoort, J. & Kuiper, J., 2017. T cell metabolism in metabolic disease-associated autoimmunity. Immunobiology, 222(10), pp.925–936. Available at: https://www.sciencedirect.com/science/article/pii/S0171298517300542 [Accessed June 1, 2018].

Amorini, A.M. et al., 2014. Serum lactate as a novel potential biomarker in multiple sclerosis. Biochimica et Biophysica Acta - Molecular Basis of Disease, 1842(7), pp.1137–1143.

Anon, Small Molecule Pathway Database. Available at: http://smpdb.ca/ [Accessed April 25, 2018].

Aragones, J.M. et al., 2003. Myasthenia gravis: A higher than expected incidence in the elderly. Neurology, 60(6), pp.1024–1026. Available at: http://www.neurology.org/cgi/doi/10.1212/01.WNL.0000050461.05432.C5 [Accessed June 23, 2018].

Araki, K. et al., 2009. mTOR regulates memory CD8 T-cell differentiation. Nature, 460(7251), pp.108–112. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19543266 [Accessed May 29, 2018].

Astudillo, A.M. et al., 2012. Dynamics of arachidonic acid mobilization by inflammatory cells. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1821(2), pp.249–256. Available at: https://www.sciencedirect.com/science/article/pii/S1388198111002496?via%3Dihub [Accessed April 11, 2018].

Avidan, N. & Miller, A., 2014. Genetic basis of myasthenia gravis – A comprehensive review. Journal of Autoimmunity, 52, pp.146–153. Available at: https://www.sciencedirect.com/science/article/pii/S0896841113001480 [Accessed June 22, 2018].

Baggi, F. et al., 1998. Identification of a novel HLA class II association with DQB1* 0502 in an Italian myasthenic population. Annals of the New York Academy of Sciences, 841(1), pp.355–359.

Basiri, K., Ansari, B. & Okhovat, A.A., 2015. Life-threatening misdiagnosis of bulbar onset myasthenia gravis as a motor neuron disease: How much can one rely on exaggerated deep tendon reflexes. Advanced biomedical research, 4, p.58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25802827 [Accessed June 24, 2018].

Bass JD, D.A. and R.D., 2015. qvalue: Q-value estimation for false discovery rate control. R package version 2.10.0. Available at: http://github.com/jdstorey/qvalue.

Beasley, S.J., Greer, J.M. & Judith Greer, C.M., Autoantibodies and their potential roles in diseases of the nervous system. Available at: https://onlinelibrary.wiley.com/doi/pdf/10.1111/cen3.12269 [Accessed June 23, 2018].

Beier, U.H., 2015. Essential role of mitochondrial energy metabolism in Foxp3+ T-regulatory cell function and allograft survival. Faseb J., 29, pp.2315–2326.

Benatar, M. et al., 2012. Recommendations for myasthenia gravis clinical trials. Muscle & Nerve, 45(6), pp.909–917. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22581550 [Accessed June 1, 2017].

Bengtsson, A.A. et al., 2016. Metabolic Profiling of Systemic Lupus Erythematosus and Comparison with Primary Sjögren's Syndrome and Systemic Sclerosis K. H. Kim, ed. PLOS ONE, 11(7), p.e0159384. Available at: http://dx.plos.org/10.1371/journal.pone.0159384 [Accessed March 17, 2018].

Benjamini, Y. & Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological), 57, pp.289–300. Available at: https://www.jstor.org/stable/2346101 [Accessed June 17, 2018].

Benjamini, Y. & Hochberg, Y., 2000. On the Adaptive Control of the False Discovery Rate in Multiple Testing With Independent Statistics. Journal of Educational and Behavioral Statistics, 25(1), pp.60–83. Available at: http://journals.sagepub.com/doi/10.3102/10769986025001060 [Accessed June 17, 2018].

Berrih-Aknin, S. et al., 2009. CCL21 overexpressed on lymphatic vessels drives thymic hyperplasia in myasthenia. Annals of Neurology, 66(4), pp.521–531. Available at: http://doi.wiley.com/10.1002/ana.21628 [Accessed June 23, 2018].

Besinger, U.A. et al., 1983. Myasthenia gravis: long-term correlation of binding and bungarotoxin blocking antibodies against acetylcholine receptors with changes in disease severity. Neurology,

33(10), pp.1316–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6684226 [Accessed June 23, 2018].

Bhanushali, M.J., Wuu, J. & Benatar, M., 2008. Treatment of ocular symptoms in myasthenia gravis. Neurology, 71(17), pp.1335–1341. Available at: http://www.neurology.org/cgi/doi/10.1212/01.wnl.0000327669.75695.38 [Accessed June 21, 2018].

Bhargava, P. & Calabresi, P.A., 2016. Metabolomics in multiple sclerosis. Multiple Sclerosis, 22(4), pp.451–460.

Biesecker, G. & Gomez, C.M., 1989. Inhibition of acute passive transfer experimental autoimmune myasthenia gravis with Fab antibody to complement C6. Journal of immunology (Baltimore, Md. : 1950), 142(8), pp.2654–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2703710 [Accessed June 23, 2018].

Blander, J.M., 2018. Regulation of the Cell Biology of Antigen Cross-Presentation. Annual Review of Immunology, 36(1), pp.717–753. Available at: http://www.annualreviews.org/doi/10.1146/annurev-immunol-041015-055523 [Accessed June 14, 2018].

Bonuccelli, G. et al., 2010. Ketones and lactate “fuel” tumor growth and metastasis. Cell Cycle, 9(17), pp.3506–3514. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20818174 [Accessed May 29, 2018].

Boothby, M. & Rickert, R.C., 2017. Metabolic Regulation of the Immune Humoral Response. Immunity, 46(5), pp.743–755. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28514675 [Accessed March 30, 2018].

Breiner, A. et al., 2016. Epidemiology of myasthenia gravis in Ontario, Canada. Neuromuscular Disorders, 26(1), pp.41–46. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0960896615007622 [Accessed March 7, 2017].

Brennan, F.M. & McInnes, I.B., 2008. Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest, 118, pp.3537–3545.

Bril, V., 1995. Single Fiber Electromyography: Studies in Healthy and Diseased Muscle. Neurology, 45(9), p.1793.

Bruno, L., von Boehmer, H. & Kirberg, J., 1996. Cell division in the compartment of naive and memory T lymphocytes. Eur. J. Immunol., 26, pp.3179–3184.

Buck, M.D. et al., 2017. Metabolic Instruction of Immunity. Cell, 169(4), pp.570–586. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28475890 [Accessed May 29, 2018].

Buckley, C. et al., 2001. Mature, long-lived CD4+ and CD8+ T cells are generated by the thymoma in myasthenia gravis. Annals of Neurology, 50(1), pp.64–72.

Butterfield, L.H. et al., 2011. Recommendations from the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers. Clinical Cancer Research, 17(10), pp.3064–3076. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21558394 [Accessed June 1, 2017].

de Candia, P. et al., 2017. Immunometabolism of human autoimmune diseases: from metabolites to extracellular vesicles. FEBS Letters, 591(19), pp.3119–3134. Available at: http://doi.wiley.com/10.1002/1873-3468.12733 [Accessed March 19, 2018].

Cao, M. & Vincent, A., 2018. Autoantibody Testing in the Diagnosis and Management of Autoimmune Disorders of Neuromuscular Transmission and Related Diseases. In Myasthenia Gravis and Related Disorders. Springer, pp. 153–168.

Carr, A.S. et al., 2010. A systematic review of population based epidemiological studies in Myasthenia Gravis. BMC Neurology, 10(1), p.46. Available at: http://bmcneurol.biomedcentral.com/articles/10.1186/1471-2377-10-46 [Accessed July 10, 2017].

Carr, E.L. et al., 2010. Glutamine Uptake and Metabolism Are Coordinately Regulated by ERK/MAPK during T Lymphocyte Activation. The Journal of Immunology, 185(2), pp.1037–1044. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20554958 [Accessed June 14, 2018].

Castleman, B. & Norris, E.H., 1949. The pathology of the thymus in myasthenia gravis: A study of 35 cases. Medicine, 28(1), pp.27–58.

Cauwels, A. et al., 2014. Extracellular ATP drives systemic inflammation, tissue damage and mortality. Cell Death Dis, 5, p.e1102.

Cavalcante, P. et al., 2011. The thymus in myasthenia gravis: Site of “innate autoimmunity”? Muscle & Nerve, 44(4), pp.467–484. Available at: http://doi.wiley.com/10.1002/mus.22103 [Accessed June 18, 2018].

Cencioni, M.T. et al., 2015. FAS-ligand regulates differential activation-induced cell death of human T-helper 1 and 17 cells in healthy donors and multiple sclerosis patients. Cell Death Dis, 6, p.e1741.

Chan, K.H. et al., 2007. Frequency of seronegativity in adult-acquired generalized myasthenia gravis. Muscle & Nerve, 36(5), pp.651–658. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17654558 [Accessed June 23, 2018].

Chen, H.W., Heiniger, H.J. & Kandutsch, A.A., 1975. Relationship between sterol synthesis and DNA-synthesis in phytohemagglutinin-stimulated mouse lymphocytes. Proc. Natl Acad. Sci. Usa, 72, pp.1950–1954.

Cheng, C. et al., 2009. Serum Protein Profiles in Myasthenia Gravis. Annals of Thoracic Surgery, 88(4), pp.1118–1123.

Chiang, J.Y.L., 2013. Bile acid metabolism and signaling. Comprehensive Physiology, 3(3), pp.1191–212. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23897684 [Accessed June 2, 2018].

Choi, S.Y.C. et al., 2013. Cancer-generated lactic acid: A regulatory, immunosuppressive metabolite? Journal of Pathology, 230(4), pp.350–355.

Cobb, J. et al., 2016. α-Hydroxybutyric Acid Is a Selective Metabolite Biomarker of Impaired Glucose Tolerance. Diabetes care, 39(6), pp.988–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27208342 [Accessed March 19, 2018].

Cocco, E. et al., 2016. (1)H-NMR analysis provides a metabolomic profile of patients with multiple sclerosis. Neurology(R) neuroimmunology & neuroinflammation, 3(1), p.e185. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26740964 [Accessed April 11, 2018].

Crawford, J. & Cohen, H.J., 1985. The essential role of L-glutamine in lymphocyte differentiation in vitro. Journal of Cellular Physiology, 124(2), pp.275–282. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4044655 [Accessed June 14, 2018].

Cummings, B.S., McHowat, J. & Schnellmann, R.G., 2000. Phospholipase A2s in Cell Injury and Death. Journal of Pharmacology and Experimental Therapeutics, 294(3).

Dale, H.H., Feldberg, W. & Vogt, M., 1936. Release of acetylcholine at voluntary motor nerve endings. The Journal of physiology, 86(4), pp.353–380.

Dang, E. V et al., 2011. Control of TH17/Treg balance by hypoxia-inducible factor 1. Cell, 146, pp.772–784.

Dausset, J., 1981. The major histocompatibility complex in man. Science (New York, N.Y.), 213(4515), pp.1469–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6792704 [Accessed June 18, 2018].

Desmedt, J.E., 1973. The neuromuscular disorder in myasthenia gravis. In New Concepts of the Motor Unit, Neuromuscular Disorders, Electromyographic Kinesiology. Karger Publishers, pp. 241–304.

Dickler, H.B. et al., 2013. The National Institutes of Health Center for Human Immunology, Autoimmunity, and Inflammation: history and progress. Annals of the New York Academy of Sciences, 1285(1), pp.133–147. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23692568 [Accessed June 1, 2017].

Dimeloe, S. et al., 2017. T-cell metabolism governing activation, proliferation and differentiation; a modular view. Immunology, 150(1), pp.35–44. Available at: http://doi.wiley.com/10.1111/imm.12655 [Accessed April 16, 2018].

Donnelly, R.P., 2014. mTORC1-dependent metabolic reprogramming is a prerequisite for NK cell effector function. J. Immunol., 193, pp.4477–4484.

Doughty, C.A., 2006. Antigen receptor-mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. Blood, 107, pp.4458–4465.

Dufort, F.J., 2014. Glucose-dependent de novo lipogenesis in B lymphocytes: a requirement for atp-citrate lyase in lipopolysaccharide-induced differentiation. J. Biol. Chem., 289, pp.7011–7024.

Ecker, J., 2010. Induction of fatty acid synthesis is a key requirement for phagocytic differentiation of human monocytes. Proc. Natl Acad. Sci. Usa, 107, pp.7817–7822.

Elahi, M.M. et al., 2009. Tumor necrosis factor alpha - 308 gene locus promoter polymorphism: An analysis of association with health and disease. Biochimica et Biophysica Acta - Molecular Basis of Disease, 1792(3), pp.163–172.

Emwas, A.-H.M., 2015. The Strengths and Weaknesses of NMR Spectroscopy and Mass Spectrometry with Particular Focus on Metabolomics Research. In Humana Press, New York, NY, pp. 161–193. Available at: http://link.springer.com/10.1007/978-1-4939-2377-9_13 [Accessed June 17, 2018].

Engel, A., 2012. Myasthenia Gravis and Myasthenic Disorders, New York: Oxford University Press. Available at: http://login.ezproxy.library.ualberta.ca/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=nlebk&AN=454380&site=ehost-live&scope=site.

Erb, W., 1879. Zur casuistik der bulbären lähmungen. Archiv für Psychiatrie und Nervenkrankheiten, 9(2), pp.325–350.

Ernster, L. & Dallner, G., 1995. Biochemical, physiological and medical aspects of ubiquinone function. Biochimica et biophysica acta, 1271(1), pp.195–204. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7599208 [Accessed April 16, 2018].

Everts, B., 2014. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. Nat. Immunol., 15, pp.323–332.

Ewald, F. et al., 2014. Constitutive expression of murine c-FLIPR causes autoimmunity in aged mice. Cell Death Dis, 5, p.e1168.

Eymard, B. & Berrih-Aknin, S., 1995. Role of the thymus in the physiopathology of myasthenia. Revue neurologique, 151(1), pp.6–15.

Fambrough, D.M., Drachman, D.B. & Satyamurti, S., 1973. Neuromuscular junction in myasthenia gravis: decreased acetylcholine receptors. Science, 182(4109), pp.293–295.

Feingold, K.R., 2012. Mechanisms of triglyceride accumulation in activated macrophages. J. Leukoc. Biol., 92, pp.829–839.

Fekih-Mrissa, N. et al., 2013. Association of HLA-DR/DQ polymorphism with myasthenia gravis in Tunisian patients. Clinical neurology and neurosurgery, 115(1), pp.32–36.

Ferrannini, E. et al., 2013. Early metabolic markers of the development of dysglycemia and type 2 diabetes and their physiological significance. Diabetes, 62(5), pp.1730–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23160532 [Accessed March 19, 2018].

Fessler, M.B., 2015. Regulation of adaptive immunity in health and disease by cholesterol metabolism. Curr. Allergy Asthma Rep., 15, p.48.

Foster, D.W., 2012. Malonyl-CoA: the regulator of fatty acid synthesis and oxidation. The Journal of clinical investigation, 122(6), pp.1958–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22833869 [Accessed April 16, 2018].

Franken, C. et al., 2017. Phthalate-induced oxidative stress and association with asthma-related airway inflammation in adolescents. International Journal of Hygiene and Environmental Health, 220(2), pp.468–477. Available at: https://www.sciencedirect.com/science/article/pii/S143846391630219X?via%3Dihub [Accessed April 11, 2018].

Fujii, Y. et al., 1985. Acetylcholine receptor antibody-producing cells in thymus and lymph nodes in myasthenia gravis. Clinical immunology and immunopathology, 34(1), pp.141–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4038384 [Accessed June 17, 2018].

Fujii, Y. et al., 1985. Acetylcholine receptor antibody production by bone marrow cells in a patient with myasthenia gravis. Neurology, 35(4), pp.577–579.

Gall, W.E. et al., 2010. α-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population. PLoS ONE, 5(5).

Gall, W.E. et al., 2010. α-Hydroxybutyrate Is an Early Biomarker of Insulin Resistance and Glucose Intolerance in a Nondiabetic Population M. Federici, ed. PLoS ONE, 5(5), p.e10883. Available at: http://dx.plos.org/10.1371/journal.pone.0010883 [Accessed April 11, 2018].

Galván-Peña, S. & O'Neill, L.A.J., 2014. Metabolic reprograming in macrophage polarization. Frontiers in immunology, 5, p.420. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25228902 [Accessed June 14, 2018].

Gentleman, R.C. et al., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biology, 5(10), p.R80. Available at: http://genomebiology.biomedcentral.com/articles/10.1186/gb-2004-5-10-r80 [Accessed March 7, 2017].

Gilhus, N.E., 2012. Myasthenia and the neuromuscular junction. Current opinion in neurology, 25(5), pp.523–529.

Giraud, M. et al., 2007. An IRF8-binding promoter variant and AIRE control CHRNA1 promiscuous expression in thymus. Nature, 448(7156), pp.934–937.

Giraud, M. et al., 2004. Association of the gene encoding the δ-subunit of the muscle acetylcholine receptor (CHRND) with acquired autoimmune myasthenia gravis. Genes and Immunity, 5(1), pp.80–83.

Giraud, M. et al., 2004. Genetic control of autoantibody expression in autoimmune myasthenia gravis: role of the self-antigen and of HLA-linked loci. Genes and immunity, 5(5), p.398.

Goldflam, S., 1893. Ueber einen scheinbar heilbaren bulbärparalytischen Symptomencomplex mit Betheiligung der Extremitäten. Deutsche Zeitschrift für Nervenheilkunde, 4(3–4), pp.312–352. Available at: http://link.springer.com/10.1007/BF01665294 [Accessed June 18, 2018].

Gomez, A.M. et al., 2013. Proteomic analysis of rat tibialis anterior muscles at different stages of experimental autoimmune myasthenia gravis. Journal of Neuroimmunology, 261(1–2), pp.141–145.

Gregersen, P.K. et al., 2012. Risk for myasthenia gravis maps to a 151Pro→ Ala change in TNIP1 and to human leukocyte antigen-B* 08. Annals of neurology, 72(6), pp.927–935.

Gregersen, P.K. et al., 2012. Risk for myasthenia gravis maps to a151Pro→Ala change in TNIP1 and to human leukocyte antigen-B*08. Annals of Neurology, 72(6), pp.927–935.

Greve, B. et al., 2009. The autoimmunity-related polymorphism PTPN22 1858C/T is associated with anti-titin antibody-positive myasthenia gravis. Human Immunology, 70(7), pp.540–542. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0198885909001165 [Accessed June 22, 2018].

Grist, J.T. et al., 2018. Extracellular Lactate: A Novel Measure of T Cell Proliferation. Journal of immunology (Baltimore, Md. : 1950), 200(3), pp.1220–1226. Available at: http://www.ncbi.nlm.nih.gov/pubmed/29288205 [Accessed April 24, 2018].

Gu, M. et al., 2008. Identification of CTLA-4 isoforms produced by alternative splicing and their association with myasthenia gravis. Clinical Immunology, 128(3), pp.374–381. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1521661608006748 [Accessed June 22, 2018].

Gubser, P.M., 2013. Rapid effector function of memory CD8+ T cells requires an immediate-early glycolytic switch. Nat. Immunol., 14, pp.1064–1072.

Guptill, J.T. & Sanders, D.B., 2010. Update on muscle-specific tyrosine kinase antibody positive myasthenia gravis. Current Opinion in Neurology, 23(5), pp.530–535. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20613516 [Accessed April 9, 2018].

Guzik, T.J., Korbut, R. & Adamek-Guzik, T., 2003. Nitric oxide and superoxide in inflammation and immune regulation. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society, 54(4), pp.469–87. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14726604 [Accessed May 30, 2018].

Haas, R., Smith, J., Rocher-Ros, V., Nadkarni, S., Montero-Melendez, T., D'Acquisto, F., Bland, E.J., et al., 2015. Lactate Regulates Metabolic and Pro-inflammatory Circuits in Control of T Cell Migration and Effector Functions. PLoS biology, 13(7), p.e1002202. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26181372 [Accessed April 15, 2018].

Haas, R., Smith, J., Rocher-Ros, V., Nadkarni, S., Montero-Melendez, T., D'Acquisto, F., Bland, E.J., et al., 2015. Lactate Regulates Metabolic and Pro-inflammatory Circuits in Control of T Cell Migration

and Effector Functions P. Marrack, ed. PLOS Biology, 13(7), p.e1002202. Available at: http://dx.plos.org/10.1371/journal.pbio.1002202 [Accessed May 29, 2018].

Hamilton, J.A., Vairo, G. & Lingelbach, S.R., 1986. CSF-1 stimulates glucose uptake in murine bone marrow-derived macrophages. Biochem. Biophys. Res. Commun., 138, pp.445–454.

Hart, I.K., 2000. Acquired neuromyotonia: A new autoantibody-mediated neuronal potassium channelopathy. American Journal of the Medical Sciences, 319(4), pp.209–216.

Hay, N. & Sonenberg, N., 2004. Upstream and downstream of mTOR. Genes & development, 18(16), pp.1926–45. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15314020 [Accessed June 16, 2018].

Heckmann, J.M. et al., 1996. Human muscle acetylcholine receptor alpha-subunit gene (CHRNA1) association with autoimmune myasthenia gravis in Black, Mixed-Ancestry and Caucasian subjects. Journal of Autoimmunity, 9(2), pp.175–180.

Heldal, A.T. et al., 2014. Repeated acetylcholine receptor antibody-concentrations and association to clinical myasthenia gravis development. PLoS One, 9(12), p.e114060.

Heldal, A.T.A.T. et al., 2014. Repeated acetylcholine receptor antibody-concentrations and association to clinical myasthenia gravis development G. Saruhan-Direskeneli, ed. PLoS ONE, 9(12), p.e114060. Available at: http://dx.plos.org/10.1371/journal.pone.0114060 [Accessed March 26, 2017].

Hiebert, P.R., Wu, D. & Granville, D.J., 2013. Granzyme B degrades extracellular matrix and contributes to delayed wound closure in apolipoprotein E knockout mice. Cell Death Differ, 20, pp.1404–1414.

Hirschhaeuser, F., Sattler, U.G.A. & Mueller-Klieser, W., 2011. Lactate: A metabolic key player in cancer. Cancer Research, 71(22), pp.6921–6925.

Hoffmann, G.F. et al., 1993. Physiology and pathophysiology of organic acids in cerebrospinal fluid. Journal of Inherited Metabolic Disease, 16(4), pp.648–669.

Howard, F.M. et al., 1987. Clinical correlations of antibodies that bind, block, or modulate human acetylcholine receptors in myasthenia gravis. Annals of the New York Academy of Sciences, 505, pp.526–38.

Howard, J.F., 2013. Electrodiagnosis of disorders of neuromuscular transmission. Physical Medicine and Rehabilitation Clinics, 24(1), pp.169–192.

Hu, R. et al., 2014. Complement C5a exacerbates acute lung injury induced through autophagy-mediated alveolar macrophage apoptosis. Cell Death Dis, 5, p.e1330.

Huan, T. et al., 2015. MyCompoundID MS/MS Search: Metabolite Identification Using a Library of Predicted Fragment-Ion-Spectra of 383,830 Possible Human Metabolites. Analytical Chemistry, 87(20), pp.10619–10626. Available at: http://pubs.acs.org/doi/10.1021/acs.analchem.5b03126 [Accessed June 12, 2017].

Huang, D. et al., 1999. Tumour necrosis factor-α polymorphism and secretion in myasthenia gravis. Journal of Neuroimmunology, 94(1–2), pp.165–171.

Hutchinson, D., 1999. Classification criteria: The 1987 American Rheumatism Association revised criteria for the classification of rheumatoid arthritis. CPD Rheumatology, 1(1), pp.13–14.

Huynh, A., 2015. Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability. Nat. Immunol., 16, pp.188–196.

Imai, T. et al., 2018. Oral corticosteroid dosing regimen and long-term prognosis in generalised myasthenia gravis: a multicentre cross-sectional study in Japan. Journal of neurology, neurosurgery, and psychiatry, 89(5), pp.513–517. Available at: http://www.ncbi.nlm.nih.gov/pubmed/29175893 [Accessed June 21, 2018].

Jaigirdar, S.A. et al., 2017. Sphingosine-1-phosphate promotes the persistence of activated CD4 T cells in inflamed sites. Frontiers in Immunology, 8(NOV).

Janer, M. et al., 1999. A susceptibility region for myasthenia gravis extending into the HLA-class I sector telomeric to HLA-C. Human immunology, 60(9), pp.909–917.

Jenkins, R.C. & Weetman, A.P., 2002. Disease associations with autoimmune thyroid disease. Thyroid, 12(11), pp.977–988.

Jha, A.K., 2015. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. Immunity, 42, pp.419–430.

Jing, F. et al., 2015. Clinical and electrophysiological markers in myasthenia gravis patients. European Neurology, 74(1–2), pp.22–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26138989 [Accessed March 26, 2017].

Jolly, F., 1895. Ueber myasthenia gravis pseudoparalytica. Berl Klin Wschr, 32, pp.1–7.

Jongbloed, S.L. et al., 2006. Enumeration and phenotypical analysis of distinct dendritic cell subsets in psoriatic arthritis and rheumatoid arthritis. Arthritis Res Ther, 8, p.R15.

K., G.P. et al., 2012. Risk for myasthenia gravis maps to a 151Pro→Ala change in TNIP1 and to human leukocyte antigen-B*08. Annals of Neurology, 72(6), pp.927–935. Available at: https://doi.org/10.1002/ana.23691.

Kaikkonen, J. et al., 1999. Determinants of plasma coenzyme Q10 in humans. FEBS Letters, 443(2), pp.163–166. Available at: https://www.sciencedirect.com/science/article/pii/S0014579398017128 [Accessed April 16, 2018].

Kaminski, H.J. et al., 2012a. Biomarker development for myasthenia gravis. Annals of the New York Academy of Sciences, 1275(1), pp.101–106. Available at: http://doi.wiley.com/10.1111/j.1749-6632.2012.06787.x [Accessed March 19, 2018].

Kaminski, H.J. et al., 2012b. Biomarker development for myasthenia gravis. Annals of the New York Academy of Sciences, 1275(1), pp.101–106. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23278584 [Accessed June 1, 2017].

Kaminski, H.J. et al., 2012c. Biomarker development for myasthenia gravis. Annals of the New York Academy of Sciences, 1275(1), pp.101–106. Available at: http://doi.wiley.com/10.1111/j.1749-6632.2012.06787.x [Accessed March 11, 2018].

Kaminski, H.J., 2009. Myasthenia Gravis and Related Disorders, Totowa, NJ: Humana Press. Available at: http://link.springer.com/10.1007/978-1-59745-156-7 [Accessed March 31, 2018].

Kang, J. et al., 2015. Application of metabolomics in autoimmune diseases: Insight into biomarkers and pathology. Journal of Neuroimmunology, 279, pp.25–32. Available at: http://www.sciencedirect.com/science/article/pii/S016557281500003X?via%3Dihub [Accessed January 22, 2018].

Kim, S. et al., 2017. Metabolite profiles of synovial fluid change with the radiographic severity of knee osteoarthritis. Joint Bone Spine, 84(5), pp.605–610. Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S1297319X16301130 [Accessed April 16, 2018].

Kim, Y.S., 2002. Malonate metabolism: biochemistry, molecular biology, physiology, and industrial application. Journal of biochemistry and molecular biology, 35(5), pp.443–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12359084 [Accessed April 17, 2018].

Knebel, L.A. et al., 2012. 2-Methylbutyrylglycine induces lipid oxidative damage and decreases the antioxidant defenses in rat brain. Brain Research, 1478, pp.74–82.

Kolins, J. & Gilroy, J., 1972. Serum enzyme levels in patients with myasthenia gravis after aerobic and ischaemic exercise. Journal of neurology, neurosurgery, and psychiatry, 35(1), pp.34–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5026009 [Accessed April 14, 2018].

Kominsky, D.J., Campbell, E.L. & Colgan, S.P., 2010. Metabolic shifts in immunity and inflammation. Journal of immunology (Baltimore, Md. : 1950), 184(8), pp.4062–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20368286 [Accessed April 22, 2018].

Kordas, G. et al., 2014. Direct proof of the in vivo pathogenic role of the AChR autoantibodies from myasthenia gravis patients. PLoS One, 9(9), p.e108327.

Kornete, M. & Piccirillo, C.A., 2012. Functional crosstalk between dendritic cells and Foxp3+ regulatory T cells in the maintenance of immune tolerance. Frontiers in immunology, 3, p.165.

Krawczyk, C.M., 2010. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood, 115, pp.4742–4749.

Kuks, J.B.M. et al., 1991. Anti-acetylcholine receptor antibodies decrease after thymectomy in patients with myasthenia gravis. Clinical correlations. Journal of Autoimmunity, 4(2), pp.197–211.

Kusner, L.L., Kaminski, H.J. & Soltys, J., 2008a. Effect of complement and its regulation on myasthenia gravis pathogenesis. Expert review of clinical immunology, 4(1), pp.43–52. Available at: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=prem&NEWS=N&AN=20477586.

Kusner, L.L., Kaminski, H.J. & Soltys, J., 2008b. Effect of complement and its regulation on myasthenia gravis pathogenesis. Expert review of clinical immunology, 4(1), pp.43–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20477586 [Accessed March 26, 2018].

Lafay, S. & Gil-Izquierdo, A., 2008. Bioavailability of phenolic acids. Phytochemistry Reviews, 7(2), pp.301–311. Available at: http://link.springer.com/10.1007/s11101-007-9077-x [Accessed April 16, 2018].

Lassmann, H., Van Horssen, J. & Mahad, D., 2012. Progressive multiple sclerosis: Pathology and pathogenesis. Nature Reviews Neurology, 8(11), pp.647–656.

Lee, H.E. et al., 2016. Clinical Significance of Repetitive Compound Muscle Action Potentials in Patients with Myasthenia Gravis: A Predictor for Cholinergic Side Effects of Acetylcholinesterase Inhibitors. Journal of clinical neurology (Seoul, Korea), 12(4), pp.482–488. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27819419 [Accessed June 23, 2018].

Lee, Y.S., Wollam, J. & Olefsky, J.M., 2018. An Integrated View of Immunometabolism. Cell, 172(1–2), pp.22–40. Available at: https://www.sciencedirect.com/science/article/pii/S0092867417315052 [Accessed March 19, 2018].

Lefvert, A.K. et al., 2008. PTPN22 R620W promotes production of anti-AChR autoantibodies and IL-2 in myasthenia gravis. Journal of Neuroimmunology, 197(2), pp.110–113. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0165572808001008 [Accessed June 22, 2018].

Lefvert, A.K. & Björkholm, M., 1987. Antibodies against the acetylcholine receptor in hematologic disorders: implications for the development of myasthenia gravis after bone marrow grafting. The New England journal of medicine, 317(3), p.170.

Lennon, V.A. et al., 1978. Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis. Journal of Experimental Medicine, 147(4), pp.973–983.

Lennon, V.A. et al., 1978. Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis. The Journal of experimental medicine, 147(4), pp.973–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/206648 [Accessed June 23, 2018].

Lennon, V.A., Lindstrom, J.M. & Seybold, M.E., 1976. Experimental autoimmune myasthenia gravis: cellular and humoral immune responses. Annals of the New York Academy of Sciences, 274(1), pp.283–299.

Li, J. et al., 2017. Metabolic profiling reveals new serum biomarkers of lupus nephritis. Lupus, 26(11), pp.1166–1173. Available at: http://journals.sagepub.com/doi/10.1177/0961203317694256 [Accessed April 16, 2018].

Li, W. et al., 2012. Mesenchymal stem cells: a double-edged sword in regulating immune responses. Cell Death Differ, 19, pp.1505–1513.

Lin, M.T. & Beal, M.F., 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature, 443(7113), pp.787–795. Available at: http://www.nature.com/articles/nature05292 [Accessed April 11, 2018].

Lindahl, A., Forshed, J. & Nordström, A., 2016a. Overlap in serum metabolic profiles between non-related diseases: Implications for LC-MS metabolomics biomarker discovery. Biochemical and Biophysical Research Communications, 478(3), pp.1472–1477. Available at: https://www.sciencedirect.com/science/article/pii/S0006291X1631419X?via%3Dihub [Accessed April 12, 2018].

Lindahl, A., Forshed, J. & Nordström, A., 2016b. Overlap in serum metabolic profiles between non-related diseases: Implications for LC-MS metabolomics biomarker discovery. Biochemical and

Biophysical Research Communications, 478(3), pp.1472–1477. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0006291X1631419X.

Lindstrom, J. & Einarson, B., 1979. Antigenic modulation and receptor loss in experimental autoimmune myasthenia gravis. Muscle & nerve, 2(3), pp.173–179.

Lipton, J.O. & Sahin, M., 2014. The neurology of mTOR. Neuron, 84(2), pp.275–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25374355 [Accessed June 16, 2018].

Lisak, R.P. et al., 1984. In vitro synthesis of antibodies to acetylcholine receptor by peripheral blood cells: role of suppressor T cells in normal subjects. Neurology, 34(6), pp.802–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6233499 [Accessed June 19, 2018].

Liu, Y., Wang, W. & Li, J., 2011. Evaluation of Serum IgG Subclass Concentrations in Myasthenia Gravis Patients. International Journal of Neuroscience, 121(10), pp.570–574. Available at: https://doi.org/10.3109/00207454.2011.596293.

Loftus, R.M. & Finlay, D.K., 2016. Immunometabolism: Cellular Metabolism Turns Immune Regulator. The Journal of biological chemistry, 291(1), pp.1–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26534957 [Accessed April 9, 2018].

Lone, A.M. & Taskén, K., 2013. Proinflammatory and Immunoregulatory Roles of Eicosanoids in T Cells. Frontiers in Immunology, 4, p.130. Available at: http://journal.frontiersin.org/article/10.3389/fimmu.2013.00130/abstract [Accessed April 11, 2018].

Lu, Y. et al., 2012. Serum metabolomics for the diagnosis and classification of myasthenia gravis. Metabolomics, 8(4), pp.704–713. Available at: http://link.springer.com/10.1007/s11306-011-0364-6 [Accessed June 1, 2017].

Lunt, S.Y. & Vander Heiden, M.G., 2011. Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation. Annual Review of Cell and Developmental Biology, 27(1), pp.441–464. Available at: http://www.annualreviews.org/doi/10.1146/annurev-cellbio-092910-154237 [Accessed April 9, 2018].

Maceyka, M. et al., 2012. Sphingosine-1-phosphate signaling and its role in disease. Trends in cell biology, 22(1), pp.50–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22001186 [Accessed April 16, 2018].

Maceyka, M. & Spiegel, S., 2014. Sphingolipid metabolites in inflammatory disease. Nature, 510(7503), pp.58–67. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24899305 [Accessed January 25, 2018].

Maniaol, A.H. et al., 2012. Late onset myasthenia gravis is associated with HLA DRB1* 15: 01 in the Norwegian population. PLoS One, 7(5), p.e36603.

Manley, K. et al., 2018. Crosstalk between the immune, endocrine, and nervous systems in immunotoxicology. Current Opinion in Toxicology, 10, pp.37–45. Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S2468202017301171 [Accessed March 19, 2018].

Masubuchi, N. et al., 2016. Oxidative stress markers, secondary bile acids and sulfated bile acids classify the clinical liver injury type: Promising diagnostic biomarkers for cholestasis. Chemico-Biological Interactions, 255, pp.83–91. Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0009279715300442 [Accessed April 16, 2018].

Matsuki, T. et al., 2003. IL-1 Plays an Important Role in Lipid Metabolism by Regulating Insulin Levels under Physiological Conditions. The Journal of Experimental Medicine, 198(6), pp.877–888. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12975454 [Accessed May 29, 2018].

McInnes, I.B. & Schett, G., 2011. The pathogenesis of rheumatoid arthritis. N Engl J Med, 365, pp.2205–2219.

McKeon, A. et al., 2009. Coexistence of myasthenia gravis and serological markers of neurological autoimmunity in neuromyelitis optica. Muscle & Nerve, 39(1), pp.87–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19086079 [Accessed June 23, 2018].

Meffre, E. & Wardemann, H., 2008. B-cell tolerance checkpoints in health and autoimmunity. Current Opinion in Immunology, 20(6), pp.632–638.

Mehta, M.M. & Chandel, N.S., 2015. Targeting metabolism for lupus therapy. Science Translational Medicine, 7(274).

Meriggioli, M.N. & Sanders, D.B., 2012. Muscle autoantibodies in myasthenia gravis: beyond diagnosis? Expert review of clinical immunology, 8(5), pp.427–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22882218 [Accessed April 9, 2018].

Michalek, R.D. et al., 2011a. Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CD4+ T Cell Subsets. The Journal of Immunology, 186(6), pp.3299–3303. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21317389 [Accessed May 29, 2018].

Michalek, R.D. et al., 2011b. Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CD4+ T Cell Subsets. The Journal of Immunology, 186(6), pp.3299–3303.

Michl, J., Ohlbaum, D.J. & Silverstein, S.C., 1976. 2-Deoxyglucose selectively inhibits Fc and complement receptor-mediated phagocytosis in mouse peritoneal macrophages. I. Description of the inhibitory effect. J. Exp. Med., 144, pp.1465–1483.

Mihaly, S.R., Ninomiya-Tsuji, J. & Morioka, S., 2014. TAK1 control of cell death. Cell Death Differ, 21, pp.1667–1676.

Misra, M.K., Damotte, V. & Hollenbach, J.A., 2018. The immunogenetics of neurological disease. Immunology, 153(4), pp.399–414. Available at: http://doi.wiley.com/10.1111/imm.12869 [Accessed June 22, 2018].

Moco, S. et al., 2014. Systems biology approaches for inflammatory bowel disease: Emphasis on gut microbial metabolism. Inflammatory Bowel Diseases, 20(11), pp.2104–2114.

Nacu, A. et al., 2015. Complicating autoimmune diseases in myasthenia gravis: a review. Autoimmunity, 48(6), pp.362–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25915571 [Accessed June 23, 2018].

Nair, A.G. et al., 2014. Ocular myasthenia gravis: a review. Indian journal of ophthalmology, 62(10), pp.985–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25449931 [Accessed June 21, 2018].

Narang, A.S. & Boddu, S.H.S. eds., 2015. Excipient Applications in Formulation Design and Drug Delivery, Cham: Springer International Publishing. Available at: http://link.springer.com/10.1007/978-3-319-20206-8 [Accessed March 19, 2018].

Natarajan, B. et al., 2016. Accuracy of the ice test in the diagnosis of myasthenic ptosis. Neurology India, 64(6), p.1169.

Nations, S.P., Wolfe, G.I. & Amato, A.A., 1999. Distal myasthenia gravis. Neurology, 52, pp.632–634.

Newsholme, P. et al., 1986. Metabolism of glucose, glutamine, long-chain fatty acids and ketone bodies by murine macrophages. Biochem. J., 239, pp.121–125.

Newsom-Davis, J. et al., 1981. Anti-acetylcholine receptor antibody synthesis by cultured lymphocytes in myasthenia gravis: thymic and peripheral blood cell interactions. Annals of the New York Academy of Sciences, 377, pp.393–402. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6978662 [Accessed June 17, 2018].

Nguyen, A. & Bouscarel, B., 2008. Bile acids and signal transduction: Role in glucose homeostasis. Cellular Signalling, 20(12), pp.2180–2197. Available at: https://www.sciencedirect.com/science/article/pii/S089865680800185X [Accessed March 20, 2018].

Nomura, M. et al., 2016. Fatty acid oxidation in macrophage polarization. Nature Immunology, 17(3), pp.216–217. Available at: http://www.nature.com/articles/ni.3366 [Accessed June 14, 2018].

O'Neill, L.A.J., Kishton, R.J. & Rathmell, J., 2016. A guide to immunometabolism for immunologists. Nature Reviews Immunology, 16(9), pp.553–565.

O'Sullivan, D., 2014. Memory CD8+ T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development. Immunity, 41, pp.75–88.

Olenchock, B.A., Rathmell, J.C. & Vander Heiden, M.G., 2017. Biochemical Underpinnings of Immune Cell Metabolic Phenotypes. Immunity, 46(5), pp.703–713. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28514672 [Accessed May 29, 2018].

Olenchock, B.A., Rathmell, J.C. & Vander Heiden, M.G., 2017. Biochemical Underpinnings of Immune Cell Metabolic Phenotypes. Immunity, 46(5), pp.703–713. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28514672 [Accessed May 29, 2018].

Oosterhuis, H.J. et al., 1983. Anti-acetylcholine receptor antibodies in myasthenia gravis. Part 2. Clinical and serological follow-up of individual patients. Journal of the neurological sciences, 58(3), pp.371–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6842265 [Accessed June 23, 2018].

Oosterhuis, H.J. et al., 1985. Anti-acetylcholine receptor antibodies in myasthenia gravis. Part 3. The effect of thymectomy. Journal of the neurological sciences, 69(3), pp.335–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4031948 [Accessed June 23, 2018].

Pacheco, R., Contreras, F. & Zouali, M., 2014. The dopaminergic system in autoimmune diseases. Frontiers in Immunology, 5(MAR).

Pakzad, Z., Aziz, T. & Oger, J., 2011. Increasing incidence of myasthenia gravis among elderly in British Columbia, Canada. Neurology, 76(17), pp.1526–1528. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21519005 [Accessed June 1, 2017].

Pál, Z. et al., 2010. A novel galectin-1 and interleukin 2 receptor β haplotype is associated with autoimmune myasthenia gravis. Journal of Neuroimmunology, 229(1–2), pp.107–111.

Pál, Z. et al., 2012. Interleukin-4 receptor alpha polymorphisms in autoimmune myasthenia gravis in a Caucasian population. Human Immunology, 73(2), pp.193–195.

Pan, Y. et al., 2017. Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. Nature, 543(7644), pp.252–256. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28219080 [Accessed May 29, 2018].

Park, H. et al., 2012. Lighting the fires within: the cell biology of autoinflammatory diseases. Nature reviews. Immunology, 12(8), pp.570–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22828911 [Accessed April 11, 2018].

Patrick, J. & Lindstrom, J., 1973. Autoimmune response to acetylcholine receptor. Science, 180(4088), pp.871–872.

Pearce, E.L. et al., 2013. Fueling Immunity: Insights into Metabolism and Lymphocyte Function. Science, 342(6155), pp.1242454–1242454. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24115444 [Accessed May 29, 2018].

Pearce, E.L. & Pearce, E.J., 2013a. Metabolic pathways in immune cell activation and quiescence. Immunity, 38(4), pp.633–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23601682 [Accessed May 29, 2018].

Pearce, E.L. & Pearce, E.J., 2013b. Metabolic pathways in immune cell activation and quiescence. Immunity, 38(4), pp.633–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23601682 [Accessed April 16, 2018].

Pedersen, M. et al., 2006. Environmental risk factors differ between rheumatoid arthritis with and without auto-antibodies against cyclic citrullinated peptides. Arthritis Res Ther, 8, p.R133.

Pelz, A. et al., 2018. S1P receptor antagonists fingolimod and siponimod do not improve the outcome of experimental autoimmune myasthenia gravis mice after disease onset. European Journal of Immunology, 48(3), pp.498–508.

Pesce, J.T., 2009. Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. P Lo S Pathog., 5, p.e1000371.

Phillips II, L.H., 1994. The epidemiology of myasthenia gravis. Neurologic Clinics, 12(2), pp.263–271.

Phillips, L.H., 2003. The epidemiology of myasthenia gravis. Ann N Y Acad Sci, 998, pp.407–412.

Phillips, L.H. & Torner, J.C., 1996. Epidemiologic evidence for a changing natural history of myasthenia gravis. Neurology, 47, pp.1233–1238.

Piddlesden, S.J. et al., 1996. Soluble complement receptor 1 (sCR1) protects against experimental autoimmune myasthenia gravis. Journal of neuroimmunology, 71(1–2), pp.173–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8982117 [Accessed June 23, 2018].

Pike, N., Using false discovery rates for multiple comparisons in ecology and evolution. Available at: https://besjournals.onlinelibrary.wiley.com/doi/pdf/10.1111/j.2041-210X.2010.00061.x [Accessed June 17, 2018].

Pirskanen, R., 1976. Genetic associations between myasthenia gravis and the HL-A system. Journal of Neurology, Neurosurgery & Psychiatry, 39(1), pp.23–33.

Plenge, R.M. et al., 2007. TRAF1-C5 as a risk locus for rheumatoid arthritis: a genomewide study. N Engl J Med, 357, pp.1199–1209.

Poffenberger, M.C. & Jones, R.G., 2014. Amino Acids Fuel T Cell-Mediated Inflammation. Immunity, 40(5), pp.635–637. Available at: https://www.sciencedirect.com/science/article/pii/S1074761314001551 [Accessed April 24, 2018].

Pollard, T.D. (Thomas D. et al., 2016. Cell biology 3rd ed., Elsevier.

Posokhova, E.N. et al., 2008. Lipid synthesis in macrophages during inflammation in vivo: effect of agonists of peroxisome proliferator activated receptors α and γ and of retinoid X receptors. Biochem. (Mosc), 73, pp.296–304.

Procaccini, C., 2016. The proteomic landscape of human ex vivo regulatory and conventional T cells reveals specific metabolic requirements. Immunity, 44, pp.406–421.

Provenzano, C. et al., 2012. PTPN22 and myasthenia gravis: Replication in an Italian population and meta-analysis of literature data. Neuromuscular Disorders, 22(2), pp.131–138. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0960896611013411 [Accessed June 22, 2018].

Punga, A.R. & Stålberg, E., 2009. Acetylcholinesterase inhibitors in MG: to be or not to be? Muscle & nerve, 39(6), pp.724–728.

Querol, L. & Illa, I., 2013. Myasthenia gravis and the neuromuscular junction. Current opinion in neurology, 26(5), pp.459–465.

Ragheb, S. & Lisak, R.P., 2011. B-cell-activating factor and autoimmune myasthenia gravis. Autoimmune diseases, 2011, p.939520. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22235365 [Accessed June 24, 2018].

Rath, M. et al., 2014. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. Front. Immunol., 5, p.532.

Regenold, W.T. et al., 2008. Cerebrospinal fluid evidence of increased extra-mitochondrial glucose metabolism implicates mitochondrial dysfunction in multiple sclerosis disease progression. Journal of the Neurological Sciences, 275(1–2), pp.106–112.

Rehman, A. et al., 2013. Role of fatty-acid synthesis in dendritic cell generation and function. Journal of immunology (Baltimore, Md. : 1950), 190(9), pp.4640–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23536633 [Accessed June 14, 2018].

Reis, A.C. et al., 2015. Induction of eosinophil apoptosis by hydrogen peroxide promotes the resolution of allergic inflammation. Cell Death Dis, 6, p.e1632.

Remmerie, A. & Scott, C.L., 2018. Macrophages and lipid metabolism. Cellular Immunology. Available at: https://www.sciencedirect.com/science/article/pii/S0008874918300327 [Accessed June 14, 2018].

Renton, A.E. et al., 2015. A genome-wide association study of myasthenia gravis. JAMA neurology, 72(4), pp.396–404. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25643325 [Accessed June 18, 2018].

Renton, A.E. et al., 2015. A Genome-Wide Association Study of Myasthenia Gravis. JAMA Neurology, 72(4), p.396. Available at: http://archneur.jamanetwork.com/article.aspx?doi=10.1001/jamaneurol.2014.4103 [Accessed March 26, 2017].

Rioux, J.D. et al., 2009. Mapping of multiple susceptibility variants within the MHC region for 7 immune-mediated diseases. Proceedings of the National Academy of Sciences of the United States of America, 106(44), pp.18680–18685.

Rodríguez-Carrio, J. et al., 2016. Non-Esterified Fatty Acids Profiling in Rheumatoid Arthritis: Associations with Clinical Features and Th1 Response O. Gualillo, ed. PLOS ONE, 11(8), p.e0159573. Available at: http://dx.plos.org/10.1371/journal.pone.0159573 [Accessed January 20, 2018].

Rodriguez-Prados, J.C., 2010. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. J. Immunol., 185, pp.605–614.

Rodriguez, P.C., 2002. Regulation of T cell receptor CD3ζ chain expression by l-arginine. J. Biol. Chem., 277, pp.21123–21129.

Rodriguez, P.C., Quiceno, D.G. & Ochoa, A.C., 2007. l-arginine availability regulates T-lymphocyte cell-cycle progression. Blood, 109, pp.1568–1573.

Romi, F., Aarli, J.A. & Gilhus, N.E., 2007. Myasthenia gravis patients with ryanodine receptor antibodies have distinctive clinical features. European Journal of Neurology, 14(6), pp.617–620. Available at: http://doi.wiley.com/10.1111/j.1468-1331.2007.01785.x [Accessed June 23, 2018].

Romi, F., Aarli, J.A. & Gilhus, N.E., 2005. Seronegative myasthenia gravis: disease severity and prognosis. European Journal of Neurology, 12(6), pp.413–418. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15885043 [Accessed June 24, 2018].

De Rosa, V. et al., 2015. Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. Nature Immunology, 16(11), pp.1174–1184.

Rose, N.R. & Bona, C., 1993. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). Immunology today, 14(9), pp.426–430.

Ruff, R.L. & Lennon, V.A., 1998. End-plate voltage-gated sodium channels are lost in clinical and experimental myasthenia gravis. Annals of Neurology, 43(3), pp.370–379. Available at: http://doi.wiley.com/10.1002/ana.410430315 [Accessed June 23, 2018].

Sabelli, H.C. et al., 1983. Urinary phenyl acetate: a diagnostic test for depression? Science (New York, N.Y.), 220(4602), pp.1187–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6857245 [Accessed April 16, 2018].

Santos, E. et al., 2017. HLA and age of onset in myasthenia gravis. Neuromuscular Disorders, 27(7), pp.650–654.

Sauerbruch, F., 1911. Technik der Thoraxchirurgie, J. Springer.

Schaeffler, A. et al., 2009. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-κB pathway in adipocytes links nutritional signalling with innate immunity. Immunology, 126(2), pp.233–245. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18624726 [Accessed May 7, 2018].

Schieber, M. & Chandel, N.S., 2014. ROS function in redox signaling and oxidative stress. Current biology : CB, 24(10), pp.R453-62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24845678 [Accessed May 30, 2018].

Schönfeld, P. & Reiser, G., 2013. Why does brain metabolism not favor burning of fatty acids to provide energy? Reflections on disadvantages of the use of free fatty acids as fuel for brain. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism, 33(10), pp.1493–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23921897 [Accessed May 30, 2018].

Schönfeld, P. & Wojtczak, L., 2016. Short- and medium-chain fatty acids in energy metabolism: the cellular perspective. Journal of lipid research, 57(6), pp.943–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27080715 [Accessed April 16, 2018].

Seeger, K., 2009. Metabolic changes in autoimmune diseases. Current drug discovery technologies, 6(4), pp.256–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20025593 [Accessed June 1, 2017].

Seki, S.M. & Gaultier, A., 2017. Exploring Non-Metabolic Functions of Glycolytic Enzymes in Immunity. Frontiers in Immunology, 8, p.1549. Available at: http://journal.frontiersin.org/article/10.3389/fimmu.2017.01549/full [Accessed April 16, 2018].

Sengupta, M. et al., 2014. Serum metabolomic response of myasthenia gravis patients to chronic prednisone treatment. PLoS ONE, 9(7).

Sengupta, M. et al., 2014. Serum Metabolomic Response of Myasthenia Gravis Patients to Chronic Prednisone Treatment G. Saruhan-Direskeneli, ed. PLoS ONE, 9(7), p.e102635. Available at: http://dx.plos.org/10.1371/journal.pone.0102635 [Accessed June 1, 2017].

Shalini, S. et al., 2015. Old, new and emerging functions of caspases. Cell Death Differ, 22, pp.526–539.

Shi, H. et al., 2006. TLR4 links innate immunity and fatty acid–induced insulin resistance. Journal of Clinical Investigation, 116(11), pp.3015–3025. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17053832 [Accessed May 7, 2018].

Shi, J. et al., 2014. Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. Autoimmunity Reviews, 13(3), pp.225–230. Available at: https://www.sciencedirect.com/science/article/pii/S1568997213002012 [Accessed June 11, 2018].

Shi, L.Z., 2011. HIF1α-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. J. Exp. Med., 208, pp.1367–1376.

Shibata, N. & Glass, C.K., 2009. Regulation of macrophage function in inflammation and atherosclerosis. Journal of lipid research, 50 Suppl(Suppl), pp.S277-81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18987388 [Accessed June 14, 2018].

Shin, T.H. et al., 2018. Analysis of the free fatty acid metabolome in the plasma of patients with systemic lupus erythematosus and fever. Metabolomics, 14(1), p.14. Available at: http://link.springer.com/10.1007/s11306-017-1308-6 [Accessed April 16, 2018].

Shrestha, S., 2015. Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. Nat. Immunol., 16, pp.178–187.

Siemieniuk, E. & Skrzydlewska, E., 2005. [Coenzyme Q10: its biosynthesis and biological significance in animal organisms and in humans]. Postepy higieny i medycyny doswiadczalnej (Online), 59, pp.150–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15928598 [Accessed April 14, 2018].

Skeie, G.O. et al., 1999. TNFA and TNFB polymorphisms in myasthenia gravis. Archives of Neurology, 56(4), pp.457–461.

Somnier, F.E., Keiding, N. & Paulson, O.B., 1991. Epidemiology of myasthenia gravis in Denmark a longitudinal and comprehensive population survey. Archives of neurology, 48(7), pp.733–739.

Sotnikova, T.D. et al., 2010. The dopamine metabolite 3-methoxytyramine is a neuromodulator. PloS one, 5(10), p.e13452. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20976142 [Accessed April 16, 2018].

Staels, B. & Fonseca, V.A., 2009. Bile acids and metabolic regulation: mechanisms and clinical responses to bile acid sequestration. Diabetes care, 32 Suppl 2(Suppl 2), pp.S237-45. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19875558 [Accessed June 2, 2018].

Staley, C. et al., 2017. Interaction of gut microbiota with bile acid metabolism and its influence on disease states. Applied Microbiology and Biotechnology, 101(1), pp.47–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27888332 [Accessed March 20, 2018].

Storey, J.D., 2003. The positive false discovery rate: a Bayesian interpretation and the q -value. The Annals of Statistics, 31(6), pp.2013–2035. Available at: http://projecteuclid.org/euclid.aos/1074290335 [Accessed April 18, 2018].

Stuerenburg, H.J., 2000. The Roles of Carnosine in Aging of Skeletal Muscle and in Neuromuscular Diseases. Biochemistry (Moscow), 65(7), pp.862–865.

Sussulini, A., 2017. Metabolomics: From Fundamentals to Clinical Applications, Springer.

Tannahill, G.M., 2013. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. Nature, 496, pp.238–242.

Tanner, D.C. et al., 2015. cFLIP is critical for oligodendrocyte protection from inflammation. Cell Death Differ, 22, pp.1489–1501.

Testi, M. et al., 2012. Association of HLA-DQB1* 05: 02 and DRB1* 16 alleles with late-onset, nonthymomatous, AChR-Ab-positive myasthenia gravis. Autoimmune diseases, 2012.

Thorlacius, S. et al., 1989. Associated disorders in myasthenia gravis: autoimmune diseases and their relation to thymectomy. Acta Neurologica Scandinavica, 80(4), pp.290–295. Available at: http://doi.wiley.com/10.1111/j.1600-0404.1989.tb03881.x [Accessed June 23, 2018].

Tindall, R.S., 1980. Humoral immunity in myasthenia gravis: effect of steroids and thymectomy. Neurology, 30(5), pp.554–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7189263 [Accessed June 23, 2018].

Tindall, R.S.A., 1981. Humoral immunity in myasthenia gravis: Biochemical characterization of acquired antireceptor antibodies and clinical correlations. Annals of Neurology, 10(5), pp.437–447. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7305297 [Accessed June 23, 2018].

Tomiko, K. et al., 1983. Studies of urinary organic acid profiles of a patient with dihydrolipoyl dehydrogenase deficiency. Clinica Chimica Acta, 133(2), pp.133–140.

Toyka, K.V. et al., 1975. Myasthenia gravis: Passive transfer from man to mouse. Science, 190(4212), pp.397–399.

Través, P.G. et al., 2017. Pivotal role of protein tyrosine phosphatase 1B (PTP1B) in the macrophage response to pro-inflammatory and anti-inflammatory challenge. Cell Death & Disease, 5(3), pp.e1125–e1125. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24625984 [Accessed June 18, 2018].

Tuzun, E., Huda, R. & Christadoss, P., 2011. Complement and cytokine based therapeutic strategies in myasthenia gravis. Journal of autoimmunity, 37(2), pp.136–143. Available at: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=med7&NEWS=N&AN=21636248.

Uncini, A., Di Guglielmo, G. & Di Muzio, A., 1997. Distal myasthenia gravis and sensory neuronopathy with anti-50 kDa antibody mimicking sensory-motor neuropathy. J Neurol Neurosurg Psychiatry, 63, pp.414–415.

van der Windt, G.J.W. et al., 2012. Mitochondrial Respiratory Capacity Is a Critical Regulator of CD8+ T Cell Memory Development. Immunity, 36(1), pp.68–78. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22206904 [Accessed May 29, 2018].

Vandiedonck, C. et al., 2006. Association of the PTPN22*R620W polymorphism with autoimmune myasthenia gravis. Annals of Neurology, 59(2), pp.404–407. Available at: http://doi.wiley.com/10.1002/ana.20751 [Accessed June 22, 2018].

Vandiedonck, C. et al., 2004. Pleiotropic effects of the 8.1 HLA haplotype in patients with autoimmune myasthemia gravis and thymus hyperplasia. Proceedings of the National Academy of Sciences of the United States of America, 101(43), pp.15464–15469.

Vashi, P. et al., 2016. Methylmalonic Acid and Homocysteine as Indicators of Vitamin B-12 Deficiency in Cancer. PloS one, 11(1), p.e0147843. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26807790 [Accessed April 16, 2018].

Verschuuren, J.J.G.M. et al., 2013. Pathophysiology of myasthenia gravis with antibodies to the acetylcholine receptor, muscle-specific kinase and low-density lipoprotein receptor-related protein 4. Autoimmunity reviews, 12(9), pp.918–923.

Viken, M.K. et al., 2007. Polymorphisms in the cathepsin L2 (CTSL2) gene show association with type 1 diabetes and early-onset myasthenia gravis. Human Immunology, 68(9), pp.748–755. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0198885907001267 [Accessed June 22, 2018].

Villoslada, P. et al., 2017. Metabolomic signatures associated with disease severity in multiple sclerosis. Neurology: Neuroimmunology and NeuroInflammation, 4(2).

Vincent, A. et al., 2003. Evidence of underdiagnosis of myasthenia gravis in older people. Journal of Neurology, Neurosurgery & Psychiatry, 74(8), pp.1105–1108.

Vincent, A. & Newsom-Davis, J., 1985. Acetylcholine receptor antibody as a diagnostic test for myasthenia gravis: results in 153 validated cases and 2967 diagnostic assays. Journal of neurology, neurosurgery, and psychiatry, 48(12), pp.1246–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4087000 [Accessed June 23, 2018].

Vincent, A. & Newsom-Davis, J., 1982. Acetylcholine receptor antibody characteristics in myasthenia gravis. I. Patients with generalized myasthenia or disease restricted to ocular muscles. Clinical and

experimental immunology, 49(2), pp.257–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6813004 [Accessed June 23, 2018].

Wakil, S.J. & Abu-Elheiga, L.A., 2009a. Fatty acid metabolism: target for metabolic syndrome. Journal of lipid research, 50 Suppl(Supplement), pp.S138-43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19047759 [Accessed May 30, 2018].

Wakil, S.J. & Abu-Elheiga, L.A., 2009b. Fatty acid metabolism: target for metabolic syndrome. Journal of lipid research, 50 Suppl(Supplement), pp.S138-43.

Walenta, S., Schroeder, T. & Mueller-Klieser, W., 2004. Lactate in solid malignant tumors: Potential basis of a metabolic classification in clinical oncology. Current Medicinal Chemistry, 11(16), pp.2195–2204.

Walker, M.B., 1934. Treatment of myasthenia gravis with physostigmine. The Lancet, 223(5779), pp.1200–1201.

Wang, R. et al., 2011. The Transcription Factor Myc Controls Metabolic Reprogramming upon T Lymphocyte Activation. Immunity, 35(6), pp.871–882. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22195744 [Accessed June 14, 2018].

Wang, X. et al., 2007. Two SNPs in the promoter region of the CTLA-4 gene affect binding of transcription factors and are associated with human myasthenia gravis. Journal of Internal Medicine, 263(1), pp.61–69. Available at: http://doi.wiley.com/10.1111/j.1365-2796.2007.01879.x [Accessed June 22, 2018].

Wei, J., 2016. Autophagy enforces functional integrity of regulatory T cells by coupling environmental cues and metabolic homeostasis. Nat. Immunol., 17, pp.277–285.

Weigert, C., 1901. II. Pathologisch-anatomischer Beitrag zur Erb'schen Krankheit (Myasthenia gravis),

Werner, P., Kiechl, S. & Löscher, S., 2003. Distal myasthenia gravis: frequency and clinical course in a large prospective series. Acta Neurol Scand, 108, pp.209–210.

Westerblad, H., Allen, D.G. & Lännergren, J., 2002. Muscle Fatigue: Lactic Acid or Inorganic Phosphate the Major Cause? Physiology, 17(1), pp.17–21. Available at: http://www.physiology.org/doi/10.1152/physiologyonline.2002.17.1.17 [Accessed April 24, 2018].

Wientjes, F.B. & Segal, A.W., 1995. NADPH oxidase and the respiratory burst. Seminars in Cell Biology, 6(6), pp.357–365. Available at: https://www.sciencedirect.com/science/article/pii/S1043468205800066 [Accessed June 14, 2018].

Willis, T., 1672. De anima brutorum quae hominis vitalis ac sentitiva est : exercitationes duae.

Win-Shwe, T.-T. et al., 2013. Expression levels of neuroimmune biomarkers in hypothalamus of allergic mice after phthalate exposure. Journal of Applied Toxicology, 33(10), pp.1070–1078. Available at: http://doi.wiley.com/10.1002/jat.2835 [Accessed April 11, 2018].

van der Windt, G.J.W. et al., 2013. CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. Proceedings of the National Academy of Sciences, 110(35), pp.14336–14341. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23940348 [Accessed May 29, 2018].

Wood, S.J. & Slater, C.R., 2001. Safety factor at the neuromuscular junction. Progress in neurobiology, 64(4), pp.393–429. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11275359 [Accessed June 6, 2018].

Wu, G., 2013. Amino Acids, CRC Press. Available at: https://www.taylorfrancis.com/books/9781439861905 [Accessed April 16, 2018].

Wu, T. et al., 2012. Metabolic disturbances associated with systemic lupus erythematosus. PLoS ONE, 7(6).

Xia, J. et al., 2012. MetaboAnalyst 2.0--a comprehensive server for metabolomic data analysis. Nucleic Acids Research, 40(W1), pp.W127–W133. Available at: https://academic.oup.com/nar/article-lookup/doi/10.1093/nar/gks374 [Accessed March 7, 2017].

Xie, Y. et al., 2011. Association between HLA-DRB1 and myasthenia gravis in a northern Han Chinese population. Journal of Clinical Neuroscience, 18(11), pp.1524–1527.

Yan, B. et al., 2016. Serum metabolomic profiling in patients with systemic lupus erythematosus by GC/MS. Modern Rheumatology, 26(6), pp.914–922.

Yan, B. et al., 2016. Serum metabolomic profiling in patients with systemic lupus erythematosus by GC/MS. Modern Rheumatology, 26(6), pp.914–922. Available at: https://www.tandfonline.com/doi/full/10.3109/14397595.2016.1158895 [Accessed April 16, 2018].

Yang, L. & Karin, M., 2014. Roles of tumor suppressors in regulating tumor-associated inflammation. Cell Death Differ, 21, pp.1677–1686.

Yang, Z. et al., 2015. T-cell metabolism in autoimmune disease. Arthritis research & therapy, 17(1), p.29. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25890351 [Accessed April 24, 2018].

Yegambaram, M. et al., 2015. Role of environmental contaminants in the etiology of Alzheimer's disease: a review. Current Alzheimer research, 12(2), pp.116–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25654508 [Accessed April 11, 2018].

Yeh, J. & Chiu, H., 1999. Plasmapheresis in myasthenia gravis. Acta neurologica scandinavica, 99(3), pp.147–151.

Yi, J.S. et al., 2018. B cells in the pathophysiology of myasthenia gravis. Muscle & Nerve, 57(2), pp.172–184. Available at: http://doi.wiley.com/10.1002/mus.25973 [Accessed March 29, 2018].

Yi, J.S. et al., 2018. B cells in the pathophysiology of myasthenia gravis. Muscle and Nerve, 57(2), pp.172–184.

Yin, Y. et al., 2015. Normalization of CD4⁺ T cell metabolism reverses lupus. Science Translational Medicine, 7(274).

Yin, Y.W. et al., 2015. TLR4-mediated inflammation promotes foam cell formation of vascular smooth muscle cell by upregulating ACAT1 expression. Cell Death Dis, 6, p.1659.

Young, S.P. et al., 2013. The Impact of Inflammation on Metabolomic Profiles in Patients With Arthritis. Arthritis & Rheumatism, 65(8), pp.2015–2023. Available at: http://doi.wiley.com/10.1002/art.38021 [Accessed February 4, 2018].

Yuan, Z.-Y. et al., 2016. Prognostic value of preoperative serum lactate dehydrogenase in thymic carcinoma. Journal of Thoracic Disease, 8(9), p.2464. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27746998 [Accessed April 16, 2018].

Zagoriti, Z. et al., 2012. Genetics of myasthenia gravis: a case-control association study in the Hellenic population. Clinical and Developmental Immunology, 2012.

Zhang, J. et al., 2013. FOXP3 -3279 and IVS9+459 polymorphisms are associated with genetic susceptibility to myasthenia gravis. Neuroscience Letters, 534(1), pp.274–278.

Zhao, B. et al., 2006. Determination of pyridostigmine bromide and its metabolites in biological samples. J Pharm Pharmaceut Sci (www.cspsCanada.org), 9(1), pp.71–81. Available at: https://sites.ualberta.ca/~csps/JPPS9(1)/Zhao.B/Review.pdf [Accessed February 19, 2018].

Zhao, C. et al., 2008. Specific and overlapping sphingosine-1-phosphate receptor functions in human synoviocytes: Impact of TNF-α. Journal of Lipid Research, 49(11), pp.2323–2337.

Zhao, S., Luo, X. & Li, L., 2016. Chemical Isotope Labeling LC-MS for High Coverage and Quantitative Profiling of the Hydroxyl Submetabolome in Metabolomics. Analytical Chemistry, 88(21), pp.10617–10623. Available at: http://pubs.acs.org/doi/abs/10.1021/acs.analchem.6b02967 [Accessed March 27, 2017].

Zhou, J. et al., 2016. Exploration of the serum metabolite signature in patients with rheumatoid arthritis using gas chromatography–mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis, 127, pp.60–67. Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0731708516300668 [Accessed March 5, 2018].

Zhou, R. et al., 2014. IsoMS: Automated Processing of LC-MS Data Generated by a Chemical Isotope Labeling Metabolomics Platform. Analytical Chemistry, 86(10), pp.4675–4679. Available at: http://pubs.acs.org/doi/abs/10.1021/ac5009089 [Accessed March 7, 2017].

Zhu, C. et al., Bile acids in regulation of inflammation and immunity: friend or foe? Clinical and experimental rheumatology, 34(4 Suppl 98), pp.25–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27586800 [Accessed March 20, 2018].

Zhu, W.-H. et al., 2012. HLA-DQA1* 03: 02/DQB1* 03: 03: 02 is strongly associated with susceptibility to childhood-onset ocular myasthenia gravis in Southern Han Chinese. Journal of neuroimmunology, 247(1), pp.81–85.

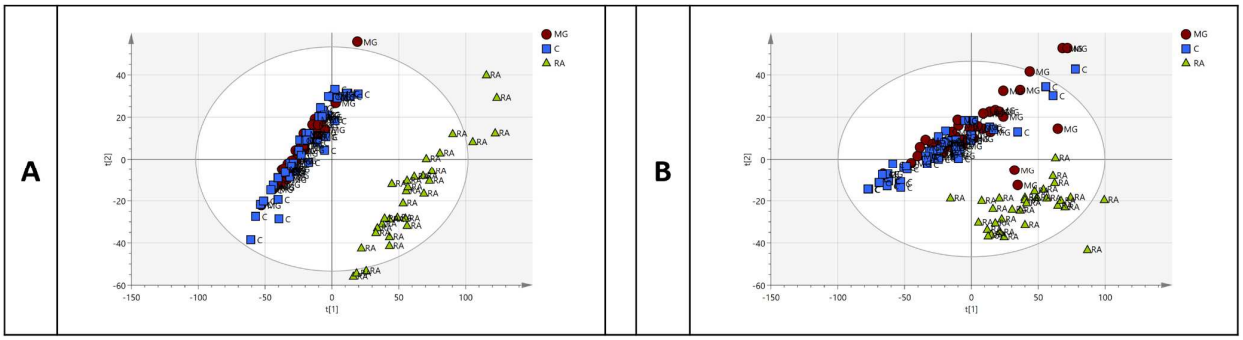
Zigmond, M.J., Coyle, J.T. & Rowland, L.P., Neurobiology of brain disorders : biological basis of neurological and psychiatric disorders, Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/book/9780123982704#srch=myasthenia [Accessed March 31, 2018].

Živković, S.A., Clemens, P.R. & Lacomis, D., 2012. Characteristics of late-onset myasthenia gravis. Journal of Neurology, 259(10), pp.2167–2171. Available at: http://link.springer.com/10.1007/s00415-012-6478-6 [Accessed June 21, 2018].

Zordan, P. et al., 2014. Macrophages commit postnatal endothelium-derived progenitors to angiogenesis and restrict endothelial to mesenchymal transition during muscle regeneration. Cell Death Dis, 5, p.e1031.

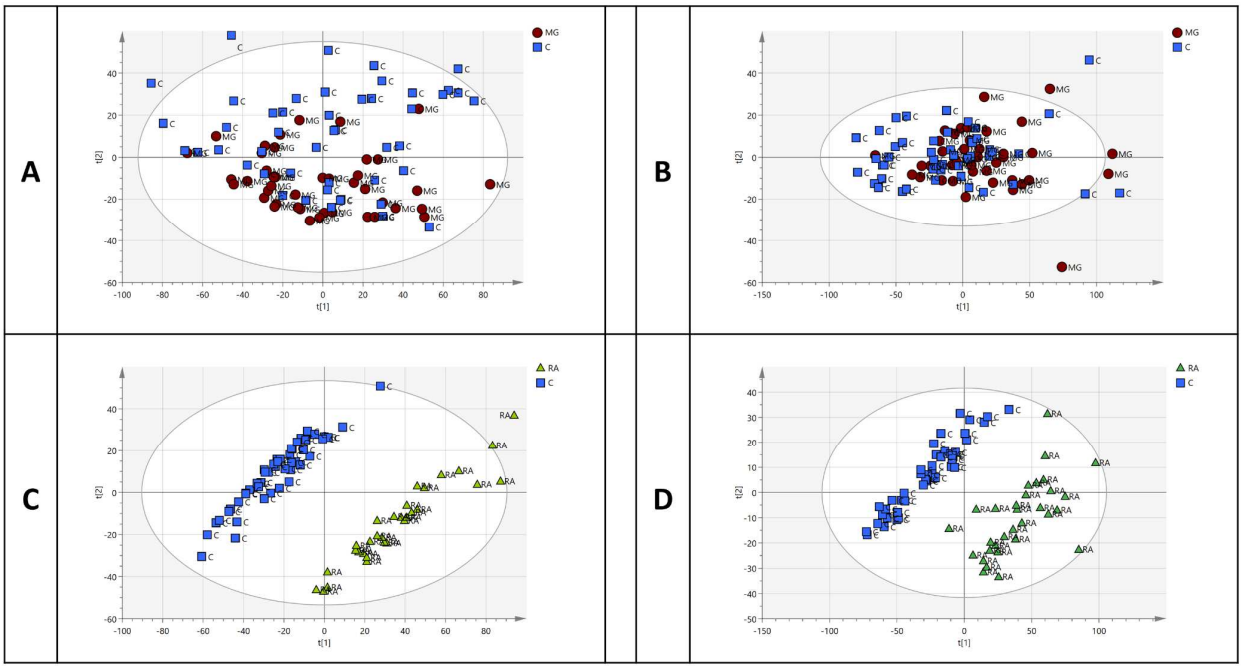
APPENDICES

Appendix 1. Score plots for MG vs. RA vs. C.



PCA score plots for Acid-labelled (A) and Dansyl-labelled (B) MG vs. RA vs. C groups.

Appendix 2. Score plots for MG vs. C and RA vs. C.

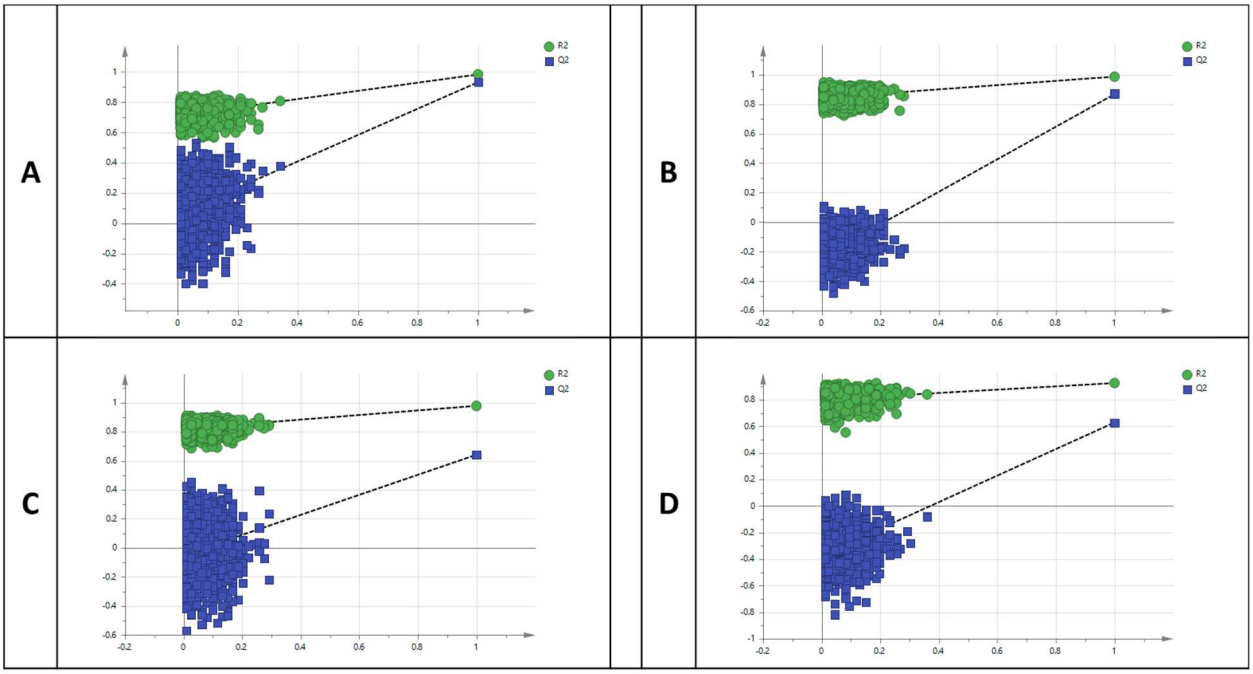


PCA score plots for Acid-labelled (A) and Dansyl-labelled (B) MG vs. C groups and Acid-labelled (A) and Dansyl-labelled (B) RA vs. C.

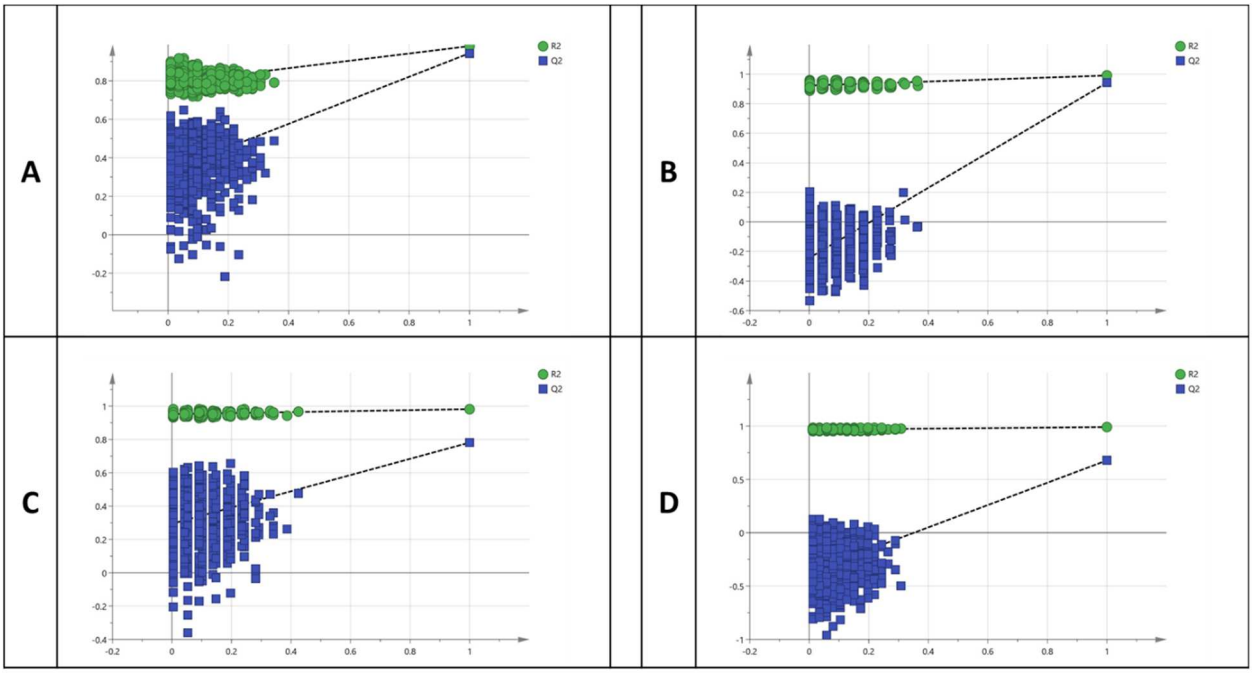
Appendix 3. Group PCA model scores.

Dansyl	A	N	R2X(cum)	Q2(cum)
MG vs. RA vs. C	10	118	0.637	0.499
MG vs. C	8	95	0.607	0.452
RA vs. C	5	72	0.601	0.504
Acid	**A**	**N**	**R2X(cum)**	**Q2(cum)**
MG vs. RA vs. C	12	118	0.548	0.336
MG vs. C	9	95	0.496	0.317
RA vs. C	7	72	0.485	0.327

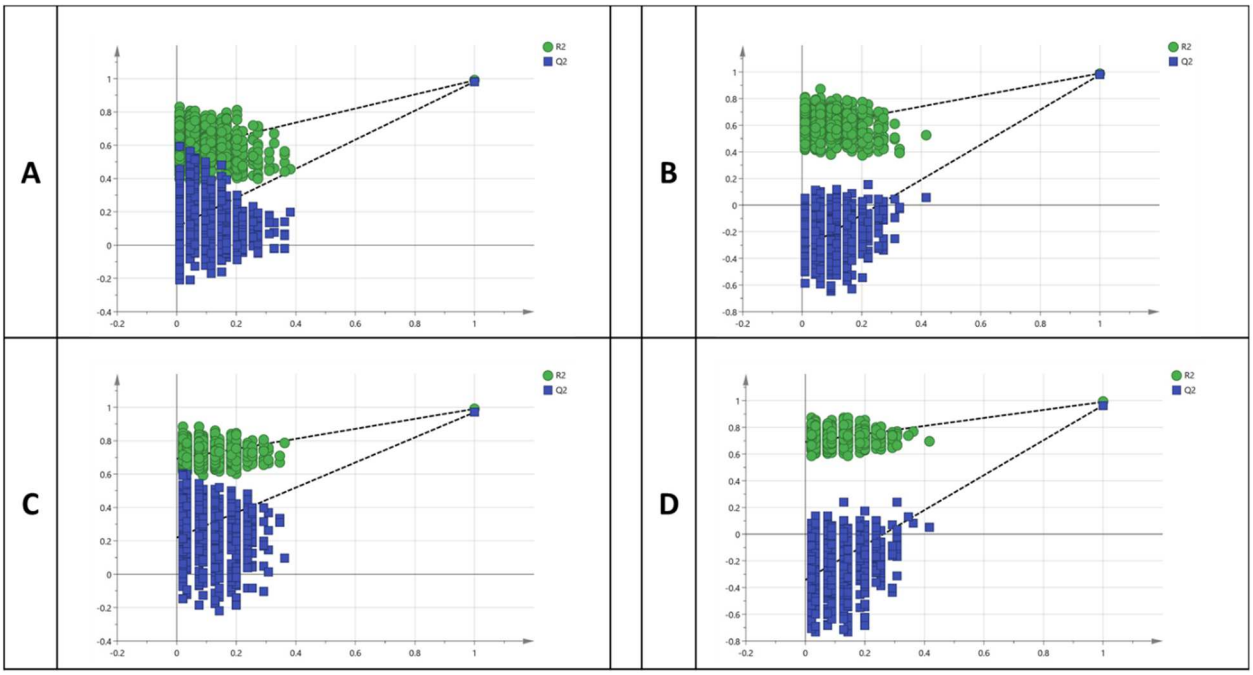
Appendix 4. Permutation plots for MG vs. RA vs. C, mg vs. C and RA vs. C.



PLS-DA and OPLS-DA permutation plots for Acid-labelled (A, B) and Dansyl-labelled (C, D) MG vs. RA vs. C groups.



PLS-DA and OPLS-DA permutation plots for Acid-labelled (A, B) and Dansyl-labelled (C, D) MG vs. C groups



PLS-DA and OPLS-DA permutation plots for Acid-labelled (A, B) and Dansyl-labelled (C, D) RA vs. C groups.

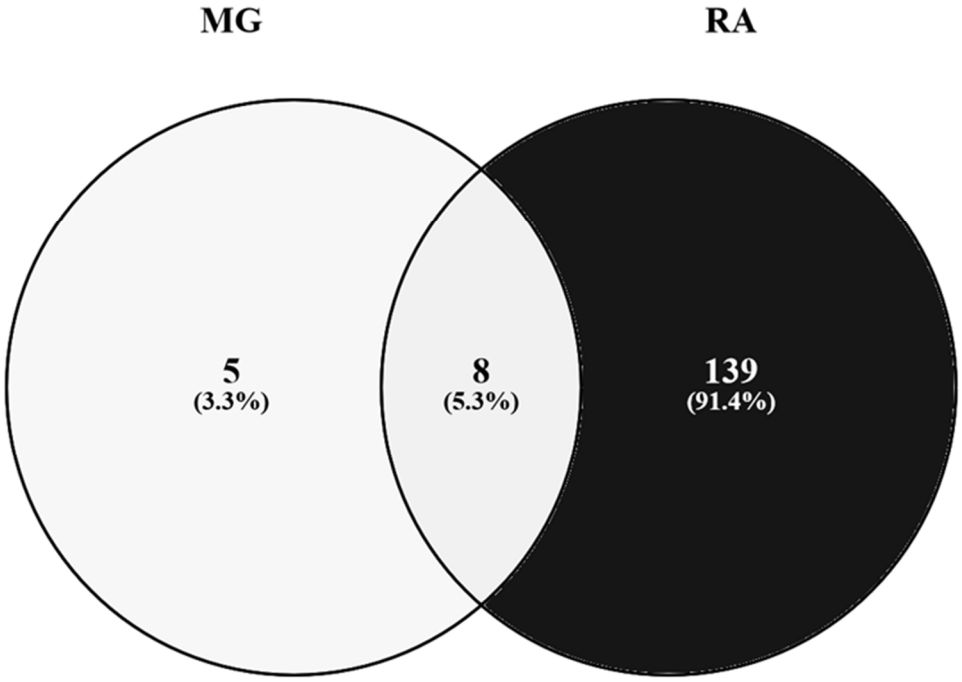
Appendix 5. Contingency table results for PLS-DA models.

	Dansyl				Acid			
	MG	Control	RA	Control	MG	Control	RA	Control
OPLS-DA Positive	45	0	31	0	42	4	31	0
OPLS-DA Negative	2	46	0	49	1	48	0	49
Sensitivity*	0.96 *(0.85-0.99)*		1.00 *(0.89-1.00)*		0.98 *(0.88-1.00)*		1.00 *(0.89-1.00)*	
Specificity*	1.00 *(0.92-1.00)*		1.00 *(0.93-1.00)*		0.92 *(0.81-0.98)*		1.00 *(0.93-1.00)*	
Positive Predictive Value*	1.00		1.00		0.91 *(0.80-0.96)*		1.00	
Negative Predictive Value*	0.96 *(0.86-0.99)*		1.00		0.98 (0.87-1.00)		1.00	

Appendix 6. Venn categorized significant metabolites.

Metabolites Common to MG and RA Groups								
HMDB	Name	AUC	ttp	Q_Value	VIP	Ratio	Fold_Change	Regulation
HMDB02466	3-Hydroxybenzoic acid	0.82	8.06E-07	1.16E-05	2.54	2.09	2.09	Upregulated
HMDB00503	7a-Hydroxy-3-oxo-5b-cholanoic acid	0.87	4.93E-10	2.54E-08	3.14	0.45	-2.24	Downregulated
HMDB28691	Alanyl-Leucine	0.81	1.61E-06	1.73E-05	1.41	1.62	1.62	Upregulated
HMDB00511	Capric acid	0.80	3.72E-08	8.79E-07	2.31	0.44	-2.28	Downregulated
HMDB01311	D-Lactic acid	0.81	1.92E-10	1.37E-08	2.79	2.36	2.36	Upregulated
HMDB00202	Methylmalonic acid	0.80	4.91E-11	5.90E-09	2.35	2.19	2.19	Upregulated
HMDB00209	Phenylacetic acid	0.81	1.31E-09	5.48E-08	2.49	2.25	2.25	Upregulated
HMDB02107	Phthalic acid	0.80	1.77E-07	3.34E-06	2.30	1.60	1.60	Upregulated
HMDB00339	2-Methylbutyrylglycine	0.84	7.23E-10	3.42E-08	2.4	1.7	1.7	Upregulated
HMDB00022	3-Methoxytyramine	0.81	1.55E-07	7.43E-06	1.75	1.5	1.5	Upregulated
HMDB00500	4-Hydroxybenzoic acid	0.83	8.06E-07	1.16E-05	2.55	2.09	2.09	Upregulated
HMDB00707	4-Hydroxyphenylpyruvic acid	0.81	1.96E-06	2.44E-05	2.18	0.45	-2.22	Downregulated
HMDB00451	cis-4-Hydroxycyclohexylacetic acid	0.83	1.36E-11	2.31E-09	2.71	2.34	2.34	Upregulated
HMDB11162	L-beta-aspartyl-L-alanine	0.88	1.08E-12	2.60E-10	3.17	2.71	2.71	Upregulated
HMDB00158	L-Tyrosine	0.86	7.28E-11	7.78E-09	2.91	1.66	1.66	Upregulated
HMDB11487	LysoPE(0:0/20:4(5Z,8Z,11Z,14Z))	0.86	5.87E-07	8.94E-06	2.6	0.46	-2.17	Downregulated
HMDB00691	Malonic acid	0.81	3.21E-09	1.10E-07	2.09	2.21	2.21	Upregulated
HMDB12271	O-Ureidohomoserine	0.82	2.68E-07	4.74E-06	1.67	0.49	-2.06	Downregulated
HMDB00220	Palmitic acid	0.82	2.74E-03	7.24E-03	1.14	0.88	-1.14	Downregulated
HMDB00252	Sphingosine	0.87	5.07E-10	2.57E-08	2.27	0.56	-1.8	Downregulated
Metabolites Exclusive to MG Group								
HMDB	Name	AUC	ttp	Q_Value	VIP	Ratio	Fold_Change	Regulation
HMDB00011	(R)-3-Hydroxybutyric acid	0.83	8.62E-10	3.82E-08	1.00	1.60	1.60	Upregulated
HMDB00008	2-Hydroxybutyric acid	0.80	7.58E-10	3.53E-08	1.78	2.08	2.08	Upregulated
HMDB00005	2-Ketobutyric acid	0.81	2.63E-08	6.54E-07	2.43	1.75	1.75	Upregulated
HMDB00060	Acetoacetic acid	0.81	2.63E-08	6.54E-07	2.43	1.75	1.75	Upregulated
HMDB02428	Terephthalic acid	0.80	1.71E-07	3.29E-06	2.27	1.53	1.53	Upregulated
HMDB00328	12-Ketodeoxycholic acid	0.87	4.93E-10	2.54E-08	3.15	0.45	-2.24	Downregulated
HMDB00139	Glyceric acid	0.83	4.93E-09	1.51E-07	2.12	2.07	2.07	Upregulated
HMDB11477	LysoPE(0:0/18:2(9Z,12Z))	0.8	2.39E-06	2.85E-05	1.77	0.68	-1.46	Downregulated
HMDB11489	LysoPE(0:0/20:5(5Z,8Z,11Z,14Z,17Z))	0.88	2.34E-11	3.46E-09	2.27	0.42	-2.36	Downregulated
HMDB11494	LysoPE(0:0/22:5(4Z,7Z,10Z,13Z,16Z))	0.84	3.30E-09	1.11E-07	2.32	0.56	-1.79	Downregulated
HMDB11496	LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	0.85	1.83E-08	4.67E-07	2.41	0.57	-1.76	Downregulated
HMDB11555	MG(0:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)	0.81	3.28E-06	3.72E-05	2.57	0.6	-1.66	Downregulated

Appendix 7. Venn charts illustrating group distribution of library-matched (a) and putatively-matched (b) metabolites.



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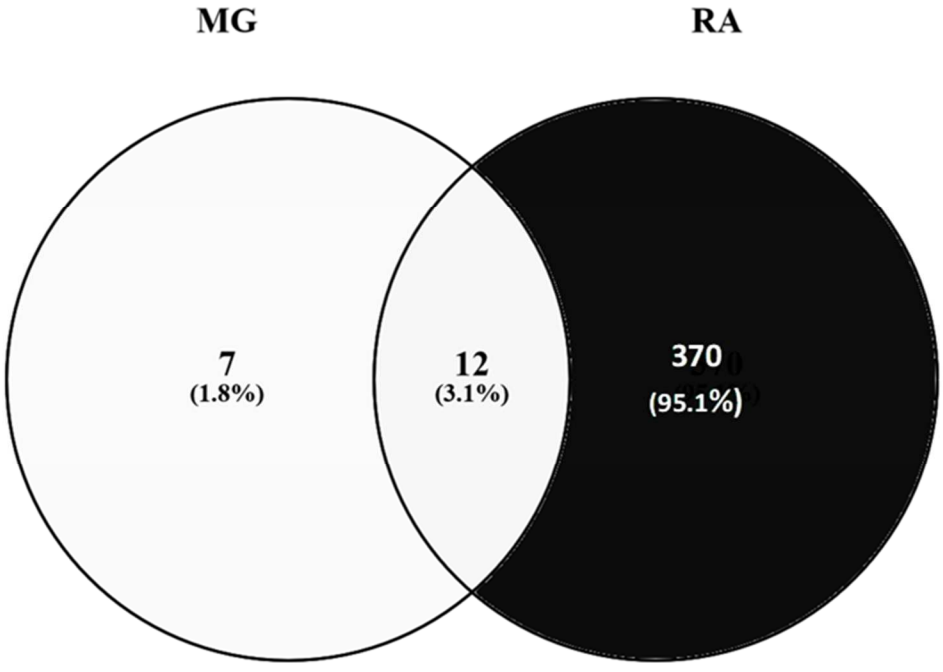

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Appendix 8. Library-matched (a) and putatively-matched (b), venn-categorized significant metabolites.

Metabolites Common to MG and RA Groups											
HMDB	Name	Sample_mz	Monoistopic_mz	rt	AUC	ttp	Q_Value	VIP	Ratio	Fold_Change	Regulation
HMDB02466	3-Hydroxybenzoic acid	138.03	138.03	1101.57	0.82	8.06E-07	1.16E-05	2.54	2.09	2.09	Upregulated
HMDB00503	7a-Hydroxy-3-oxo-5b-cholanoic acid	390.28	390.28	2300.92	0.87	4.93E-10	2.54E-08	3.14	0.45	-2.24	Downregulated
HMDB28691	Alanyl-Leucine	202.13	202.13	456.69	0.81	1.61E-06	1.73E-05	1.41	1.62	1.62	Upregulated
HMDB00511	Capric acid	180.04	180.04	1024.22	0.80	3.72E-08	8.79E-07	2.31	0.44	-2.28	Downregulated
HMDB01311	D-Lactic acid	90.03	90.03	465.90	0.81	1.92E-10	1.37E-08	2.79	2.36	2.36	Upregulated
HMDB00202	Methylmalonic acid	118.03	118.03	820.26	0.80	4.91E-11	5.90E-09	2.35	2.19	2.19	Upregulated
HMDB00209	Phenylacetic acid	136.05	136.05	1008.02	0.81	1.31E-09	5.48E-08	2.49	2.25	2.25	Upregulated
HMDB02107	Phthalic acid	327.11	327.11	1496.43	0.80	1.77E-07	3.34E-06	2.30	1.60	1.60	Upregulated

A

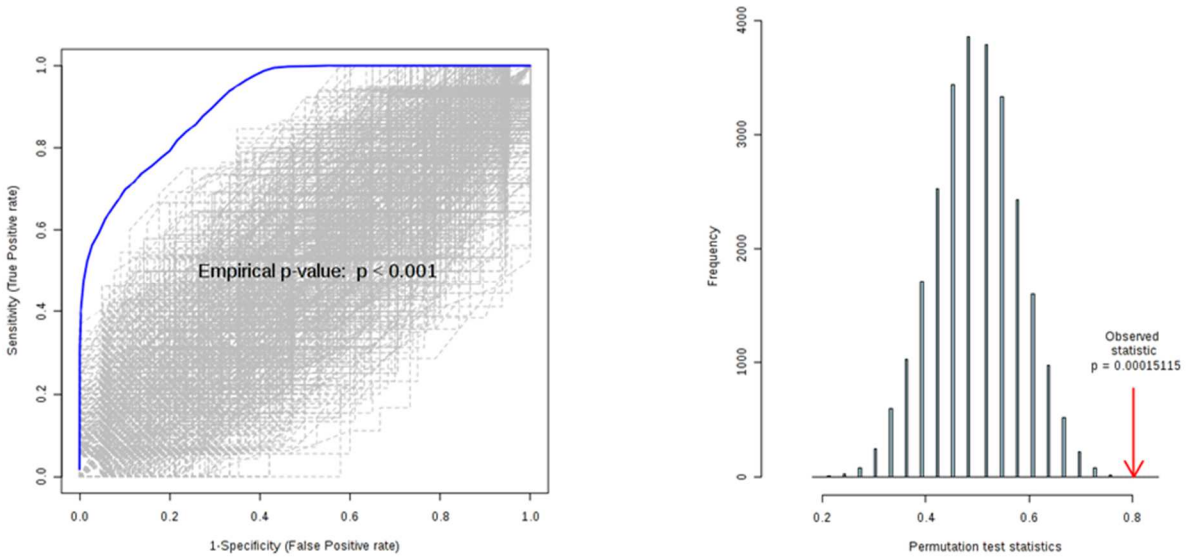
Metabolites Common to MG and RA Groups											
HMDB	Name	Sample_mz	Monoistopic_mz	rt	AUC	ttp	Q_Value	VIP	Ratio	Fold_Change	Regulation
HMDB00339	2-Methylbutyrylglycine	159.09	159.09	1055.07	0.84	7.23E-10	3.42E-08	2.4	1.70	1.7	Upregulated
HMDB00022	3-Methoxytyramine	167.09	167.09	184.96	0.81	1.55E-07	7.43E-06	1.75	1.50	1.5	Upregulated
HMDB00500	4-Hydroxybenzoic acid	138.03	138.03	1101.57	0.83	8.06E-07	1.16E-05	2.55	2.09	2.09	Upregulated
HMDB00707	4-Hydroxyphenylpyruvic acid	180.04	180.04	1281.68	0.81	1.96E-06	2.44E-05	2.18	0.45	-2.22	Downregulated
HMDB00451	cis-4-Hydroxycyclohexylacetic acid	158.09	158.09	1463.38	0.83	1.36E-11	2.31E-09	2.71	2.34	2.34	Upregulated
HMDB11162	L-beta-aspartyl-L-alanine	204.07	204.07	1149.66	0.88	1.08E-12	2.60E-10	3.17	2.71	2.71	Upregulated
HMDB00158	L-Tyrosine	181.07	181.07	1062.93	0.86	7.28E-11	7.78E-09	2.91	1.66	1.66	Upregulated
HMDB11487	LysoPE(0:0/20:4(5Z,8Z,11Z,14Z))	501.28	501.29	1308.68	0.86	5.87E-07	8.94E-06	2.6	0.46	-2.17	Downregulated
HMDB00691	Malonic acid	104.01	104.01	760.69	0.81	3.21E-09	1.10E-07	2.09	2.21	2.21	Upregulated
HMDB12271	O-Ureidohomoserine	177.08	177.07	1109.08	0.82	2.68E-07	4.74E-06	1.67	0.49	-2.06	Downregulated
HMDB00220	Palmitic acid	256.24	256.24	1391.38	0.82	2.74E-03	7.24E-03	1.14	0.88	-1.14	Downregulated
HMDB00252	Sphingosine	299.28	299.28	1830.72	0.87	5.07E-10	2.57E-08	2.27	0.56	-1.8	Downregulated
Metabolites Exclusive to MG Group											
HMDB	Name	Sample_mz	Monoistopic_mz	rt	AUC	ttp	Q_Value	VIP	Ratio	Fold_Change	Regulation
HMDB00328	12-Ketodeoxycholic acid	390.28	390.28	2300.92	0.87	4.93E-10	2.54E-08	3.15	0.45	-2.24	Downregulated
HMDB00139	Glyceric acid	106.03	106.03	645.02	0.83	4.93E-09	1.51E-07	2.12	2.07	2.07	Upregulated
HMDB11477	LysoPE(0:0/18:2(9Z,12Z))	477.28	477.29	1302.48	0.8	2.39E-06	2.85E-05	1.77	0.68	-1.46	Downregulated
HMDB11489	LysoPE(0:0/20:5(5Z,8Z,11Z,14Z,17Z))	499.27	499.27	1227.72	0.88	2.34E-11	3.46E-09	2.27	0.42	-2.36	Downregulated
HMDB11494	LysoPE(0:0/22:5(4Z,7Z,10Z,13Z,16Z))	527.30	527.30	1398.73	0.84	3.30E-09	1.11E-07	2.32	0.56	-1.79	Downregulated
HMDB11496	LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	525.29	525.29	1132.30	0.85	1.83E-08	4.67E-07	2.41	0.57	-1.76	Downregulated
HMDB11555	MG(0:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)	404.29	404.29	2069.61	0.81	3.28E-06	3.72E-05	2.57	0.60	-1.66	Downregulated

B

Appendix 9. Chemical class and pathway associations of MG-specific metabolites.

HMDB	Name	Labelling	Library	Chemical Class	Pathways (KEGG, SMPDB)
HMDB00005	2-Ketobutyric acid	Acid	Standard	Short-chain keto acid	Selenoamino Acid Metabolism
					Homocysteine Degradation
					Methionine Metabolism
					Glycine and Serine Metabolism
					Threonine and 2-Oxobutanoate Degradation
HMDB00008	2-Hydroxybutyric acid	Acid	Standard	Alpha hydroxy acid	Propanoate metabolism
HMDB00011	(R)-3-Hydroxybutyric acid	Acid	Standard	Beta hydroxy acid	Ketone Body Metabolism
					Butanoate metabolism
					cAMP signaling pathway
HMDB00060	Acetoacetic acid	Acid	Standard	Short-chain keto acid	Butyrate Metabolism
					Ketone Body Metabolism
					Phenylalanine and Tyrosine Metabolism
					Valine, Leucine and Isoleucine Degradation
					Tyrosine Metabolism
					Fatty Acid Biosynthesis
HMDB02428	Terephthalic acid	Acid	Standard	Benzenoid	Polycyclic aromatic hydrocarbon degradation
					Microbial metabolism in diverse environments
					Degradation of aromatic compounds
					ABC transporters
HMDB00139	Glyceric acid	Acid	Putative	Organooxygen compounds	Glycerolipid Metabolism
					Glycine and Serine Metabolism
HMDB00328	12-Ketodeoxycholic acid	Acid	Putative	Bile acids	Secondary bile acid biosynthesis
HMDB11477	LysoPE(0:0/18:2(9Z,12Z))	Acid	Putative	Glycerophospholipids	Glycerophospholipid metabolism
HMDB11489	LysoPE(0:0/20:5(5Z,8Z,11Z,14Z,17Z))	Acid	Putative	Glycerophospholipids	Glycerophospholipid metabolism
HMDB11494	LysoPE(0:0/22:5(4Z,7Z,10Z,13Z,16Z))	Acid	Putative	Glycerophospholipids	Glycerophospholipid metabolism
HMDB11496	LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Acid	Putative	Glycerophospholipids	Glycerophospholipid metabolism
HMDB11555	MG(0:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)	Acid	Putative	Glycerolipids	No metabolic pathways indexed

Appendix 10. ROC curve permutation plots for the top 4 library-matched MG vs. C metabolites panel (a) and the top 5 MG vs. C metabolites panel (b).



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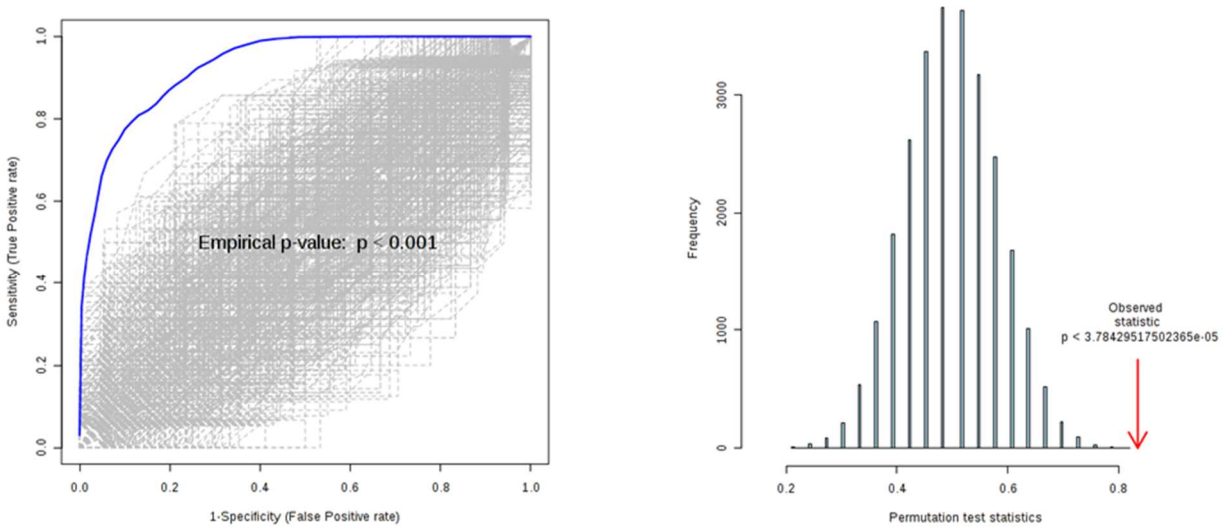

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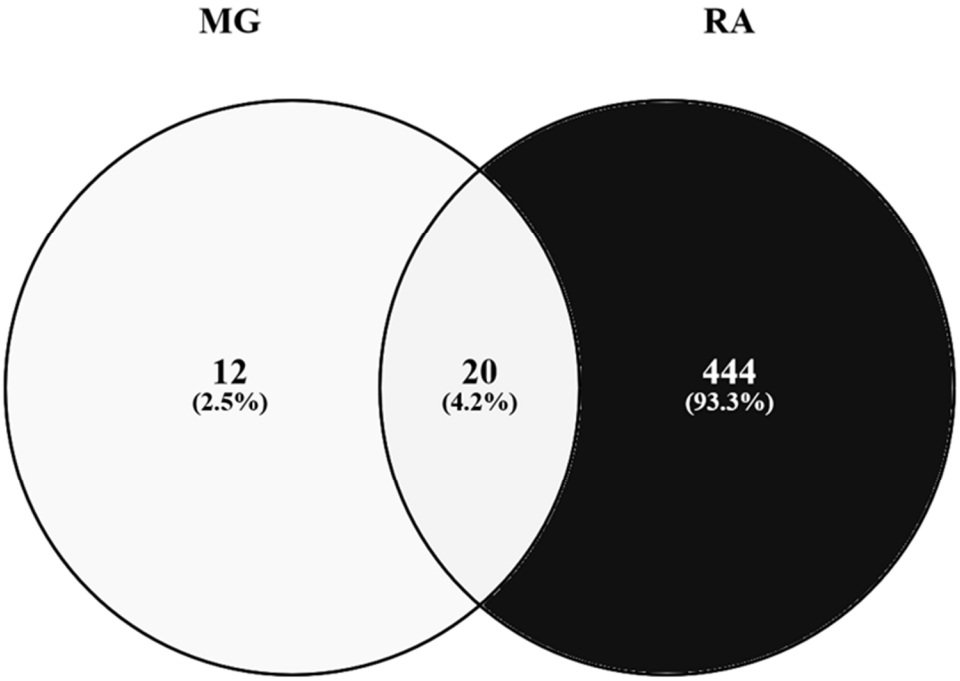
Appendix 11. Biomarker panel cross-validation. Listed numbers represent the cumulative probabilities that predicted group assignment is correct.

	MG	
	Top 5 Unique	**Top 4 Library-Matched**
Mean	0.83	0.89
Median	0.84	0.93
SD	0.12	0.14

Appendix 12. Venn chart illustrating group distribution of MG and RA metabolite profiles.



Appendix 13. Chemical taxonomies and associated pathways for all identified common metabolites.

HMDB	Name	Library	Chemical Class	Metabolic Pathways
HMDB00022	3-Methoxytyramine	Putative	Benzenoids	Tyrosine Metabolism
HMDB00158	L-Tyrosine	Putative	Amino acids	Catecholamine Biosynthesis
				Phenylalanine and Tyrosine Metabolism
				Tyrosine Metabolism
				Thyroid hormone synthesis
HMDB00220	Palmitic acid	Putative	Fatty acid/Fatty Acyl	Fatty Acid Elongation In Mitochondria
				Fatty acid Metabolism
				Glycerolipid Metabolism
				Bile Acid Biosynthesis
				Fatty Acid Biosynthesis
HMDB00252	Sphingosine	Putative	Amines	Sphingolipid Metabolism
HMDB00339	2-Methylbutyrylglycine	Putative	Amino acids	No metabolic pathways indexed
HMDB00451	cis-4-Hydroxycyclohexylacetic acid	Putative	Organooxygen compounds	No metabolic pathways indexed
HMDB00500	4-Hydroxybenzoic acid	Putative	Benzenoids	Ubiquinone Biosynthesis
HMDB00691	Malonic acid	Putative	Organic acids and derivatives	Fatty Acid Biosynthesis
HMDB00707	4-Hydroxyphenylpyruvic acid	Putative	Benzenoids	Phenylalanine and Tyrosine Metabolism
	4-Hydroxyphenylpyruvic acid			Tyrosine Metabolism
HMDB11162	L-beta-aspartyl-L-alanine	Putative	Peptidomimetics	No metabolic pathways indexed
HMDB11487	LysoPE(0:0/20:4(5Z,8Z,11Z,14Z))	Putative	Glycerophospholipids	Glycerophospholipid metabolism
HMDB12271	O-Ureidohomoserine	Putative	Amino acids	canavanine biosynthesis pathway
HMDB00202	Methylmalonic acid	Standard	Dicarboxylic acids and derivatives	Vitamin K Metabolism
				Valine, Leucine and Isoleucine Degradation
				Propanoate Metabolism
HMDB00209	Phenylacetic acid	Standard	Benzenoids	Phenylacetate Metabolism
HMDB00503	7a-Hydroxy-3-oxo-5b-cholanoic acid	Standard	Bile acids	Secondary bile acid biosynthesis
HMDB00511	Capric acid	Standard	Fatty acid/Fatty Acyl	Fatty Acid Biosynthesis
HMDB01311	D-Lactic acid	Standard	Alpha hydroxy acids and derivatives	Pyruvaldehyde Degradation
				Pyruvate Metabolism
HMDB02107	Phthalic acid	Standard	Benzenoids	Polycyclic aromatic hydrocarbon degradation
				Microbial metabolism in diverse environments
				Degradation of aromatic compounds
				ABC transporters
HMDB02466	3-Hydroxybenzoic acid	Standard	Benzenoids	Benzoate degradation
				Phenylalanine, tyrosine and tryptophan biosynthesis
				Toluene degradation
				Polycyclic aromatic hydrocarbon degradation
				Microbial metabolism in diverse environments
				Degradation of aromatic compounds
HMDB28691	Alanyl-Leucine	Standard	Dipeptide	Amino acid degradation

Appendix 14. The peak ratios of common metabolites for each cohort shown as box-and-whisker plots.