University of Alberta

Depression, Cardiovascular Disease and Amino Acids

by

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Dedication and Acknowledgements

I would like to sincerely thank all those who helped me succeed and improve over the years. I would like to thank firstly Dr. Glen Baker, a person I believe exemplifies being the right person in the right place in life in a way very rarely seen. I would also like to sincerely thank Dr. Gabor Gyenes for his patience, help, guidance and understanding over the years. Thank you also to Dr. Esther Fujiwara for taking the extra time to listen to my concerns. Thank you to Dr. Bryan Kolb for getting me started in the very beginning.

Abstract

Major depression (MD) is one of the most prominent medical conditions worldwide in terms of societal cost and morbidity. MD has been associated with increased risk for cardiovascular disease (CVD) and conversely CVD has been associated with increased MD symptoms. Previous studies have shown that serum levels of nitric oxide (NO) were reduced in MD patients and in patients with CVD. We measured serum levels of arginine, the precursor amino acid of NO and found that they were reduced in MD subjects compared to healthy controls (HCs). Similarly, levels of citrulline, an amino acid formed during the formation of NO from arginine, were reduced. These results suggest that reduced levels of arginine may be contributing to the reduced NO observed in MD subjects. Dysfunction of the arteries has been identified as a precursor of CVD risk and has been proposed to be related to decreased NO. We hypothesized that endothelium function would be impaired in MD patients compared to HCs. Endothelial function was examined but no differences were observed between the two groups. It may be the case that despite risk of CVD being increased in MD patients, impaired endothelial dysfunction may not be observable. A variety of other amino acids proposed to be important in neurotransmission and the etiology of MD were measured in a population of unmedicated MD subjects and HCs matched for age, sex, dietary intake and lipid profiles; smokers and obese subjects were not included (similar conditions applied

in the two studies mentioned above). Cysteine and histidine levels were elevated in the MD group. When male MD subjects were compared to their corresponding controls, the decreases in levels of arginine and citrulline were present, as was the increase in levels of cysteine, but levels of taurine, aspartate, glutamine and tryptophan were also significantly lower in MD subjects. In contrast, when female MD subjects were compared with their corresponding HCs, the differences were not significant. These results emphasize the importance of studying both male and female subjects and their corresponding controls when conducting such biomarker studies.

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LIST OF ABBREVIATIONS

88GP	1988 Grid Position
A	Label of Apolipoprotein-A Series: the First Molecule in the Series
ADMA	Asymmetric Dimethylarginine
B	Label of Apoliprotein-B Series: the Second Molecule Series After the A Series
BDI-II	Beck Depression Inventory II
BH_4	Tetrahydrobiopterin
BMI	Body Mass Index
C	Capsular (C-Reactive Protein)
CANMAT	Canadian Network for Mood and Anxiety Treatments
CBS	Cystathionine Beta-Lyase
cm	centimetre
CV	Cardiovascular
CVD	Cardiovascular Disease
DALY	Disability-Adjusted Life Years
DDAH	Dimethylarginine Dimethylaminohydrolase
df	Degrees of Freedom
DOPA	Dihydroxyphenyalanine
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders Four, Text Revision

ECG	Electrocardiogram
ECT	Electroconvulsive Therapy
EDRF	Endothelial-Dependent Relaxation Factor
EDHF	Endothelial-Hyperpolarizing Factor
eNOS	Endothelial Nitric Oxide Synthase
ENRICHD	Enhancing Recovery in Coronary Heart Disease Trial
FATE	Firefighters and Their Endothelium
FMD	Flow-Mediated Dilatation
GABA	Gamma-Aminobutyric Acid
H_2S	Dihydrogen Sulfide
HC	Healthy Control
HDL-C	High-Density Lipoprotein Cholesterol
HPA	Hypothalamic Pituitary Adrenal
HRV	Heart Rate Variability
kcal/kg/day	kilocalorie per kilogram per day
kcal/kg/hour	kilocalorie per kilogram per hour
kg/m^2	kg per metre squared
LDL-C	Low-Density Lipoprotein Cholesterol
LNAA	Large Neutral Amino Acid
MAOI	Monoamine Oxidase Inhibitor
MD	Major Depression

mg	milligram
MHz	Megahertz
MI	Myocardial Infarction
MIND-IT	Myocardial Infarction and Depression Intervention Trial
ml/min	millilitre per minute
mm	millimetre
mm Hg	millimetre of mercury
mmol\L	millimole per Litre
n	Number
NA	Not Applicable
NADPH	Nicotinamide Adenine Dinculeotide Phosphate, Reduced
HNE	4-Hydroxy-2-Nonenal
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOx	Nitric Oxide Metabolites
p	Probability
R	Pearson Product-Moment Correlation Coefficient
SNRI	Serotonin Norepinephrine Reuptake Inhibitor

SPSS	Statistical Package for the Social Sciences
SSRI	Selective Serotonin Reuptake Inhibitor
TCA	Tricyclic Antidepressant
THB	Tetrahydrobiopterin
US	Ultrasound
µmol/L	micromole per litre

CHAPTER 1

General Introduction

1.1. Major Depression

The Diagnostic and Statistical Manual of Mental Disorders, version Five (DSM-5), specifies that an episode of major depression (MD) is defined as being two weeks or more of depressed mood, anhedonia, and five of a set of additional symptoms (American Psychiatric Association, 2013). The five additional symptoms of MD include disrupted eating patterns, an increase or decrease in body weight, either insomnia or hypersomnia, reduced physical energy, inability to concentrate or indecisiveness, and feelings of worthlessness as well as inappropriate or excessive guilt.

MD is one of the most costly medical disorders in the world today in terms of morbidity (Raffaitin et al., 2011). The prevalence of MD has been estimated to be approximately 8-12% in the general population worldwide (Andrade et al., 2003). There are significant economic, social, occupational, and quality of life losses associated with MD, and by the year 2020 MD is expected to be among the top three medical conditions in terms of Disability-Adjusted Life Years (DALY) lost to morbidity (Rifel et al., 2010).

MD is associated with several comorbid diseases. Some prominent comorbid conditions that have been related to MD include anxiety disorders, diabetes, obesity and, notably, increased risk for cardiovascular disease (CVD) (Vieweg et al., 2010). It has been found that symptoms of MD contribute to comorbid illness, and vice versa. For example, the occurrence

of CVD in MD patients is significantly higher than among physically and mentally healthy populations (Carney and Freedland, 2008; Le Melledo et al., 2004). Patients who have suffered a myocardial infarction (MI), death of part of the heart muscle, have a far higher rate of MD symptoms than CVD-free populations (Anderson, 2007). These findings suggest that a strong mechanistic link exists between MD and related comorbid conditions. However, an underlying understanding of how CVD risk is increased in MD patients is currently incomplete.

1.2. Brain Alterations in Major Depression and Cardiovascular Disease

The symptoms of MD have direct relationships to changes in brain neurochemistry and anatomy (Mitchell and Baker 2010; Grieve et al., 2013). These changes are further associated with CVD risk, as demonstrated by previous studies showing increases in CVD and CVD risk factors among MD patients (Carney and Freedland, 2008). Because of the observed relationship between MD and CVD, it is important to note that there are specific MD-related changes in brain anatomy and function that contribute to risk factors for CVD. These risk factors are mentioned later in this thesis.

MD is found to be associated with an overall decrease in gray matter volume as observed using magnetic resonance imaging (Grieve et al., 2013). As well, changes in activity in various brain regions have been observed in MD patients. In imaging studies, changes in activity of the

orbital prefrontal cortex, anterior cingulate cortex, and amygdala have been observed using functional magnetic resonance imaging (Ho et al., 2013).

Changes observed in higher brain areas in MD may be responsible for the physiological changes related to CVD observed in MD patients. For example, a subset of MD patients are known to have elevated glucocorticoid levels (cortisol), the release of which is controlled by the hypothalamus and anterior pituitary gland (Brown et al., 2004). Elevated cortisol release has been associated with CVD morbidity and mortality (Violanti et al., 2009).

There is also a subset of MD patients who demonstrate changes in autonomic nervous system activity controlled by lower brain areas (Berger et al., 2011; Geraldes et al., 2013). For example, physiological changes that influence blood pressure, risk of CVD, and CV health include deficiencies in the baroreflex response and possibly heart rate variability (HRV) (Kemp et al., 2012).

1.3. Nitric Oxide

Nitric oxide (NO) is an extremely important messenger molecule that has a large number of functions in different mammalian cells. NO has roles outside the cardiovascular (CV) system including involvement in the immune response, platelet function and neuronal signaling (Chrapko et al., 2004; Laranjinha et al., 2012). NO is synthesized from the semi-essential amino acid L-arginine and molecular oxygen (Pratt and Cornely, 2004).

The enzyme nitric oxide synthase (NOS) catalyzes the production of NO using the coenzyme nicotinamide adenine dinucleotide phosphate (NADPH) (Arimura et al., 2001). NOS exists as three different isoforms, namely endothelial, neuronal, and cytokine-inducible NOS (Berkowitz et al., 2003). NO has a large number of important functions in the brain, the vascular endothelium, and other organs. Endothelial NOS is the primary enzyme responsible for synthesizing NO in the vascular endothelium and subsequently will be the isoform considered exclusively in this thesis as it is by far the most relevant to the research described herein.

NO is produced in the inner medial/endothelial cell lining of the arteries from the semi-essential amino acid L-arginine using the enzyme endothelial NOS with tetrahydrobiopterin and NADPH as cofactors (Ghofrani et al., 2006). NO and the amino acid L-citrulline are produced as end-products (Chrapko et al., 2006). L-Citrulline can then be recycled into L-arginine by the action of aminosuccinate synthase followed by aminosuccinate lyase via the urea cycle (Romero et al., 2006) (Figure 1-1). After being produced in the media/endothelium layer of the artery by endothelial NOS, NO diffuses to the smooth muscle cell layer of the artery (Yetik-Anacak and Catravas, 2006). Once NO diffuses to the smooth muscle layer, the result is relaxation of the medial layer of the artery and an increase in the diameter of the lumen, allowing for increased blood flow.

In terms of arterial function, it is also relevant to note that NO may also be produced in platelets using endothelial NOS. NO production in platelets involves the same mechanism as in the endothelium (Radomski et al., 1990). In platelets, adequate NO production is necessary to prevent activation of platelet clotting factors (Chrapko et al., 2006). In the absence of adequate NO production, platelets are activated and aggregate, an action that normally occurs when blood vessels are damaged (van Hinsbergh, 2012). When unnecessary adhesion of platelets occurs, thrombosis, an impairment of blood flow due to platelet aggregation, can occur, leading to impairment of blood flow through affected arteries (Furie and Furie, 2008).

1.4. Ultrasonography for Observing the Effects of Nitric Oxide Production in the Cardiovascular System

Ultrasonography enables observation of flow-mediated dilatation (FMD) and it can indirectly assess the function of the vascular endothelium, which is dependent on the production and availability of NO. FMD is an expression of an artery's ability to increase its diameter (dilatation) calculated by determining artery width during a resting condition and then dilating the artery following the mechanical obstruction of the vessel, causing a large amount of endogenous NO production and release. The change in arterial diameter between the two conditions is measured in millimeters and reported as a percentage change.

Celermajer et al. (1992) were the first to use a non-invasive ultrasonography technique to observe FMD. Ultrasonography to assess FMD is typically performed on the brachial artery near the elbow joint in humans for the sake of convenience and accessibility.

To receive useful data from the brachial artery using ultrasound, the changes in sound wave amplitude resulting from the Doppler Effect are used to determine the makeup of the target structure. The Doppler Effect is, in brief, the change in the frequency of a wave (in the case of ultrasonography, sound waves above the human audible range) dependent on the conditions that wave has been exposed to. In the case of medical ultrasonography, the sound waves used are altered as a result of reflection off a target surface in the subject being analyzed. The returning waves are then recorded to determine the shape and motion of the area being imaged.

Sound waves produced by the ultrasound machine are able to determine the velocity of a fluid (blood) very accurately, enabling analysis of the subject's arterial condition from the velocity of the blood. The reflection velocity and change in amplitude of the sound waves are then used as raw data to produce an image using computer software (Anderson, 1999).

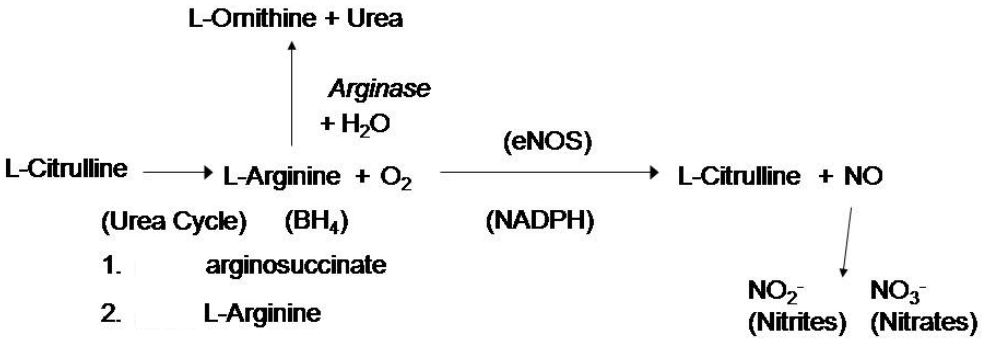

FIGURE 1-1. PATHWAYS OF NITRIC OXIDE PRODUCTION
Beginning with L-citrulline, L-arginine can be produced via intermediaries in the urea cycle. The enzyme argininosuccinate synthetase converts L-citrulline into argininosuccinate. Argininosuccinate is then converted to L-arginine by argininosuccinate lyase. L-Arginine can then be used to produce nitric oxide (NO). The enzyme endothelial nitric oxide synthase (eNOS) as well as the coenzyme nicotinamide adenine dinucleotide phosphate [reduced (NADPH)] converts L-arginine into L-citrulline and NO in the presence of oxygen and the co-factor tetrahydrobiopterin (BH_4). NO then reacts with oxygen in the blood stream to form nitrites (NO_2^-) and nitrates (NO_3^-).

At the beginning of the FMD ultrasonography procedure, the patient rests in a supine position. An initial measurement of the width of the target artery is taken. A pressure cuff is inflated over the upper arm for a short period of time causing local ischemia, stimulating a release of NO. Ischemia is followed by a second measurement of brachial artery diameter (Celermajer et al., 1992). The expansion of the diameter of the artery, hence the increased blood flow in response to the pressure cuff stimulus, is referred to as reactive hyperemia.

To determine the maximum possible extent of brachial artery dilation, a compound such as nitroglycerin capable of releasing NO into the artery is administered sublingually after a repetition of the supine rest and baseline assessment procedure outlined above. The maximum extent of NO-dependent (endogenous NO production-independent) vasodilation can then be observed directly and compared to dilatation produced by endogenous NO production (Anderson et al., 1995).

FMD observed by ultrasonography has been shown to have a high correlation with other endothelial investigation methods. In 1995, Anderson et al. determined that FMD results in healthy volunteers are closely correlated with those taken using invasive methods such as coronary artery dilatation using acetylcholine injection. A recently identified limitation of the FMD technique is standardization of endogenous NO-dependent vasodilatation using a pressure cuff (Stoner et

al., 2012). As well, a review by Ter Avest et al. (2007) suggests that active use of FMD to predict future CVD may not be practical as of yet due to data variability between subjects. More recent papers such as that of Nakamura et al. (2012) have demonstrated that combining FMD with other CVD imaging techniques allow early signs of CVD to be detected.

1.5. Alternate and Complementary Vasodilators to Nitric Oxide

NO is referred to as an endothelium-dependent relaxation factor (EDRF) due to its ability to cause arterial dilatation. It is important to note that in addition to NO, several other EDRFs have been discovered. These EDRFs may supplement or replace the action of NO under certain conditions or in certain arteries (Wang, 2009). There are several molecules that have been shown to be capable of acting as EDRFs. Some examples include dihydrogen sulfide (H_2S), substance P, prostacyclin, and hydrogen peroxide (Barton, 2010; Qi et al., 2011).

Despite the well-documented effects of impaired NO production in MD patients, no publications appear to deal directly with the issue of altered production or release of alternative EDRFs on endothelial function among MD patients. It is possible that these alternate endothelial dilators compensate for NO production, allowing endothelial dilation to continue when NO production is impaired.

1.6. Risk Factors for Cardiovascular Disease

CVD risk factors are categorized into modifiable and non-modifiable risk factors. Non-modifiable risk factors include sex, age, and hereditary factors that modulate the development of atherosclerosis (Negi and Anand, 2010). Modifiable factors influencing CVD risk include high low-density lipoprotein cholesterol levels (LDL-C) (Meyers et al., 2004), smoking (Pryor et al., 1995), hypertension (Jamil et al., 2013), and diabetes mellitus (Vieweg et al., 2010). Other factors that may enhance or influence modifiable risk factors include low physical activity levels (particularly a lack of aerobic activity) (Franco et al., 2011), as well as poor health maintenance behaviours that negatively influence CV health (Beydoun and Wang, 2010). It is also a possibility that high serum cortisol from chronic stress may influence risk for CVD, although measuring such stress is difficult to do consistently (Violanti et al., 2009).

The major mechanism of CVD is atherosclerosis, stiffening of the arteries resulting in a loss of arterial function (Nicholson et al., 2006). The arteries (particularly the medium and large arteries) comprise the vulnerable site for the development of atherosclerosis and they consist of an outer connective tissue layer (adventitia), an inner smooth muscle layer (media), and an inner endothelial cell layer (intima) while the interior of the artery, the lumen, is the site where blood flow occurs (Feletou and Vanhoutte, 2009) (Figure 1-2). The effects of atherosclerosis are particularly serious

when they affect the coronary arteries that provide blood to the heart muscle itself. Narrowing of the lumen due to atherosclerosis or a failure of the lumen to increase in diameter adequately in response to physiological needs due to atherosclerosis are the primary pathophysiological mechanisms of CVD that will be considered, as they are the most relevant to the comorbid diseases associated with MD. Atherosclerosis develops from a deficiency in EDRFs that modulate the elasticity of the arteries (Feletou and Vanhoutte, 2009).

1.6.1. Development of Atherosclerosis

Atherosclerosis occurs as a result of the build-up of oxidized cholesterol crystals, causing formation of plaques or atheroma, chronic activation of the inflammatory response, and the calcification of outer base regions of cholesterol plaques over long periods of time (Kampoli et al., 2009). Atherosclerosis proceeds relatively slowly as a chronic disease that remains asymptomatic for long periods (Ross, 1993). The functional diameter of the lumen is decreased in affected areteries and the result is restricted blood flow and inhibition of vasodilation (Parthasarathy et al., 2008). When atherosclerosis begins to significantly affect the coronary arteries, the probability of a myocardial infarction (MI) or stroke increases dramatically (Pizzi et al., 2008).

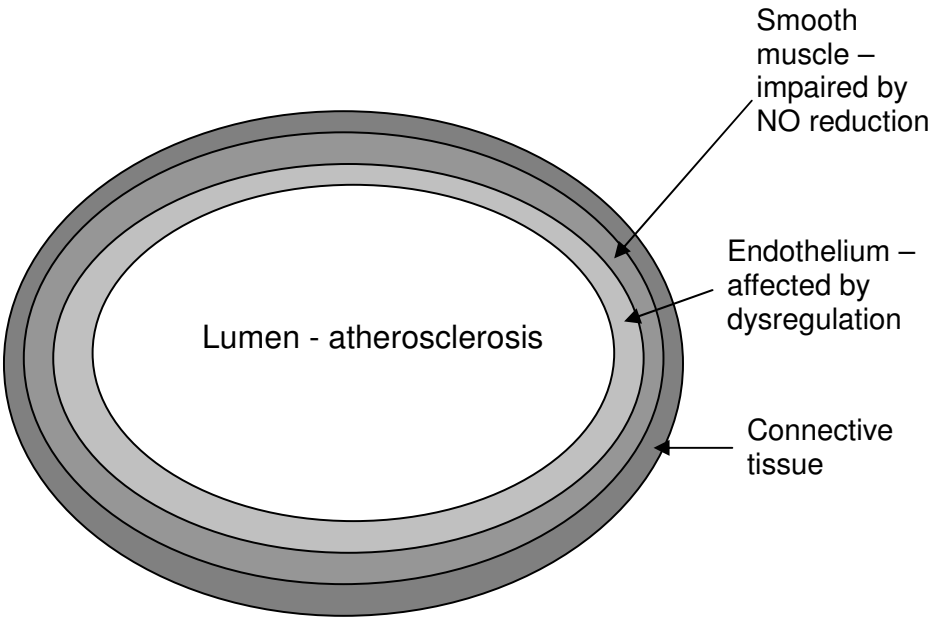

FIGURE 1-2. MAJOR ARTERIAL COMPONENTS VULNERABLE TO CARDIOVASCULAR DISEASE

The vascular endothelium is the layer of endothelial cells comprising the innermost layer of the arteries. NO is produced in the inner layer of endothelial cells and is responsible for regulating the dilation of the middle smooth muscle layer responsible for arterial dilation. Many physiological factors can damage the vascular endothelium and impair its function, contributing to CV risk. The lumen, or interior of the artery, is narrowed due to atherosclerosis (Joynt et al., 2003) and impairment of smooth muscle dilation due to endothelium dysregulation (Arimura et al., 2001).

LDL-C is one of the main constituents of atherosclerotic plaques, and high levels of LDL-C are a major contributor to the development of CVD. LDL-C particles available in the blood stream become oxidized and attach themselves to the endothelium, decreasing the functional diameter of the lumen (Martin et al., 2012). Blood-borne oxidation sources will oxidize LDL-C (Rizvi, 2009). The build-up of oxidized LDL-C then contributes to increased inflammatory activation (Witztum and Steinberg, 2001).

The presence of oxidized LDL-C influences the release of factors that control the recruitment of inflammatory cells onto the endothelium (Badimon and Vilahur, 2012). Once the vascular endothelium is irritated by the presence of oxidized LDL-C, increased attraction and adhesion of leukocytes is promoted along with elevated release of inflammatory signaling molecules (Leonarduzzi et al., 2012). Macrophages (white blood cells) begin to accumulate at the site of atheroma formation. Macrophages then develop into foam cells after ingesting large quantities of oxidized LDL-C particles (Westhorpe et al., 2012). Foam cells make up a large portion of an atheroma once they begin to accumulate.

LDL-C is synthesized by the liver and is also derived heavily from triglyceride intake (Shanes, 2012). Triglycerides are consumed in the diet and initially converted into very-low density lipoproteins. These lipoproteins then lose triglyceride molecules by the action of lipoprotein lipase, forming LDL-C as a result (Meyers et al., 2004). The function of

LDL-C is to provide cholesterol for steroid production, membrane synthesis and cell proliferation (Sakamoto and Rosenberg, 2011).

The largest component of LDL-C is apolipoprotein-B, only one molecule of which is present per LDL-C particle (Fogelstrand and Boren, 2012). Apoliprotein-B is responsible for allowing fatty acids to remain soluble in aqueous solutions and for acting as a ligand at LDL-C receptors, allowing cholesterol delivery to cells (Sakamoto and Rosenberg, 2011). Apolipoprotein B's ability to bind to endothelial LDL-C receptors on arterial walls is essential for the formation of atherosclerotic plaques. When NO production decreases and proinflammatory signaling increases, the adherence of platelets to the vascular endothelium is promoted and atheroma develop (Kaplan and Jackson, 2011). Once platelets are bound to the endothelium, they also play a role in the promotion of inflammatory processes, further exacerbating atheroma formation (Kaplan and Jackson, 2011).

Chronologically, calcification is the final process that occurs during atherosclerosis. However, there is some newer evidence that the process of calcification occurs continually during plaque development, despite previous suggestions (Li, 2011). Calcification of blood vessels greatly reduces blood vessel elasticity and is associated with an increase in MI and stroke risk (Karwowski et al., 2012).

The smooth muscle cells of the arterial wall produce bone-associated proteins that serve as starting sites for the calcification process (London, 2011). The change to active mineralization at sites within the artery occurs due to an increase in production of reactive oxygen species and physical injury to the arteries due to atheroma (Shao et al., 2010).

1.6.2. Nitric Oxide Deficiencies in Patients with Major Depression and Cardiovascular Risk

MD patients have previously been shown to demonstrate decreased blood serum levels of NO, as revealed by determining blood serum concentrations of NO metabolites (NOx) (Chrapko et al., 2004; Garcia et al., 2011; Ikenouchi-Sugita et al., 2009). Low blood serum NOx, and by inference NO, has been correlated with an increased risk of CVD (Lichtman et al., 2008). For example, low blood serum NOx levels have been associated with increased inflammation, damaging the endothelium (Douglas et al., 2004), increased platelet reactivity (Musselman et al., 2000), promotion of dyslipidemia (Musselman et al., 2003), hypercortisolemia, and upregulation or downregulation of factors that influence eNOS. The result is the development of atherosclerosis.

The direct mechanisms leading to decreased NO production in MD patients are not well understood, although some likely mechanisms linking NO production and MD are well known. Studies on blood serum samples from MD patients have shown several notable differences compared to

healthy controls (HCs). Many of the elevated blood serum factors seen in MD patients are responsible for regulating different aspects of NO production and/or the development of atherosclerosis.

Asymmetric dimethylarginine (ADMA) (Figure 1-3A) is a metabolic by-product of protein modification that acts as a competitive inhibitor of NO synthesis (Vallance et al., 1992). ADMA has been shown to be elevated in the blood serum of MD patients (Selley, 2004). ADMA acts as a competitive inhibitor of eNOS, inhibiting the production of NO by binding at the site on eNOS where L-arginine (Figure 1-3B) is modified into L-citrulline (Figure 1-3C) (Boger, 2004). The reactive aldehyde 4-hydroxy-2-nonenal (HNE) produced as a result of lipid peroxidation is suggested to be the source of increased ADMA in MD patients. When HNE concentrations are in excess, the enzyme dimethylarginine dimethylaminohydrolase (DDAH) is prevented from metabolizing ADMA into L-citrulline and methylamine (Ogawa et al., 1989).

Another factor that may indirectly alter NO production in MD patients is the catabolic enzyme arginase. Arginase has been found to be elevated in blood serum of MD patients, suggesting an as-of-yet undiscovered mechanism that systemically impairs NO production in MD patients (Elgun and Kumbasar, 2000). Arginase is found in two forms, arginase I and arginase II (Pinto et al., 2012). Arginase II is found in human platelets, where it may play a role in inhibiting platelet NO

production in MD patients if the concentration of arginase II is greater than normal (Rodrigues Pereira et al., 2010). Arginase catalyzes the hydrolysis of L-arginine into L-ornithine (Figure 1-3D) and urea (Berkowitz et al., 2003), and the activity of arginase in human patients is estimated by measuring blood serum L-ornithine levels (Elgun and Kumbasar, 2000).

There is also evidence that inflammatory markers are increased in MD patients in the absence of other medical illness, indicating elevated inflammatory activity (Krishnadas and Cavanagh, 2012; Chang et al., 2012; Brietzke et al., 2009; Anisman, 2009). In MD patients, the general inflammatory signaling molecule C-reactive protein has been observed to be elevated in blood serum (Chang et al., 2012). The severity of depressive

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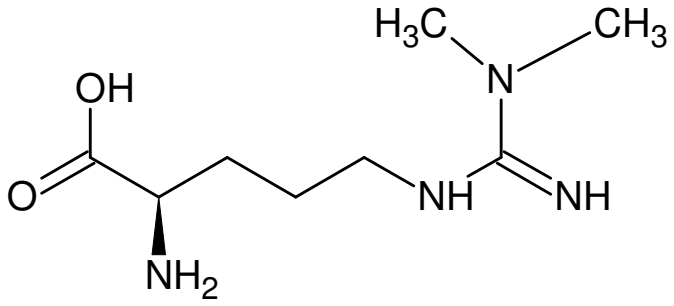

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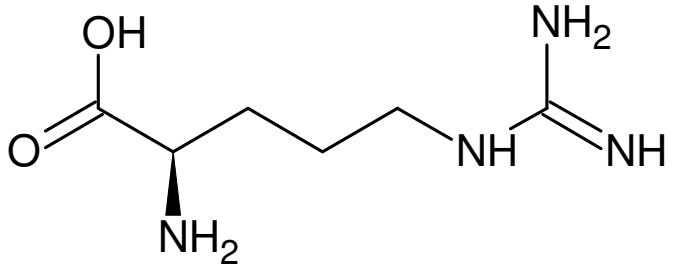

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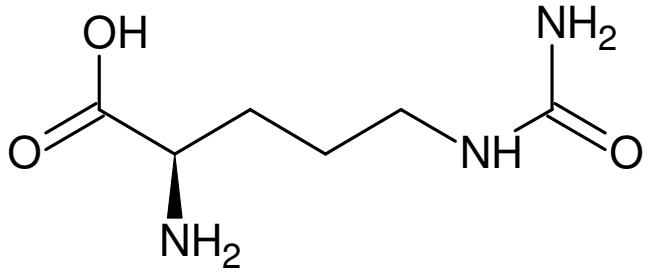

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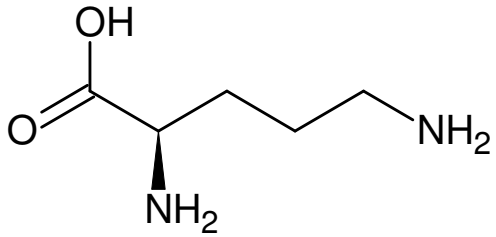

FIGURE 1-3. STRUCTURES OF ASYMMETRIC DIMETHYLARGININE, L-ARGININE, L-CITRULLINE, AND L-ORNITHINE (Modified from Pratt and Cornely, 2004).

A. Asymmetric Dimethylarginine
B. L-Arginine
C. L-Citrulline
D. L-Ornithine

symptoms may also be tied to the degree of inflammatory activation observed. A prospective study examined levels of C-reactive protein and other pro-inflammatory cytokines, finding that higher levels of these mediators were correlated with more severe depression symptoms in MD patients (van den Biggelaar et al., 2007). As mentioned previously, inflammatory activation is a core component of atherosclerosis. With over-activation of inflammation, promotion of increased atherosclerotic plaque formation follows. As well, patients treated with proinflammatory cytokines have been shown to develop MD symptoms over the period of treatment (Krishnadas and Cavanagh, 2012). MD is also a common occurrence in disease states that include inflammation such as arthritis, diabetes, and CVD (Misener et al., 2008).

Cortisol is a glucocorticoid associated with response to stressful stimuli and its secretion is often dysregulated in MD patients (Violanti et al., 2009). The release of cortisol is controlled by the hypothalamic-pituitary-adrenal (HPA) axis. Hyperactivity of the HPA axis is one of the proposed mechanisms linking CVD to MD symptoms (Jokinen and Nordstrom, 2009) and elevated blood serum levels of glucocorticoids and HPA axis activation are often observed in MD patients (Jokinen and Nordstrom, 2009). Most importantly, glucocorticoids have been found to affect the vascular actions of NO generated from the endothelium and influence the regulation of eNOS expression, contributing to arteriosclerosis (Toda and Nakanishi-

Toda, 2011). There is also some evidence that diabetes (Mezuk et al., 2013) and hypertension (Davidson et al., 2000) and more likely to develop among MD patients.

Both acute and chronic stresses have been observed to cause changes in endothelium function (Toda and Nakanishi-Toda, 2011). Elevated levels of glucocorticoids promote the development of atherosclerosis by means of increased vasoconstriction, platelet activation, and heart rate elevation (Joynt et al., 2003). Release of inflammatory markers, such as endothelin-1, and glucocorticoids, promote down-regulation of eNOS and deleterious effects on endothelium function (Liu et al., 2009).

Dysfunction of the autonomic nervous system is a common finding among MD patients and it is also associated with increased glucocorticoid release (Friedman, 2007). HRV is a description of the time variability between heart beats. An adequate amount of HRV is necessary for normal cardiac function, and decreased HRV is associated with morbidity and mortality following MI (Bigger et al., 1992). Decreased HRV has been observed among MD patients, and it is another factor leading to increased CVD risk (Tonhajzerova et al., 2012; Nemeroff and Goldschmidt-Clermont, 2012).

In a sample of 20 MD adolescent females, Tonhajzerova et al. (2012) showed that HRV complexity at rest and in response to physiological stress was lowered in MD patients. Interestingly, a recent paper by Gordon et al.

(2012) demonstrated that MD patients exhibit altered HRV and that MD patients with primarily cognitive MD symptoms demonstrate poorer heart rate reactivity (change in HRV) in response to increased CV demand. In contrast, patients with primarily somatic MD symptoms have deficiencies in systolic blood pressure, returning to normal following increased heart rate demand.

Several groups (de Jonge et al., 2006; Martens et al., 2012; Schiffer et al., 2009; Carney et al., 2012; Kemp et al., 2012) have suggested that chronic hypervigilance to threats may play a role in reducing the ability of MD patients to modulate HRV. Kemp et al. (2012) found that among MD patients, those with comorbid generalized anxiety disorder have the greatest reduction in HRV, supporting the idea that long term hypervigilance is important in altering HRV over extended periods of time.

1.6.3. Diabetes, Major Depression, and Cardiovascular Risk

The development of CVD has been linked to diabetes, and CVD ultimately accounts for almost 70% of morbidity and mortality among patients with diabetes (Khardori and Nguyen, 2012). It is also important to note that among patients with type 2 diabetes, the prevalence of MD is almost twice that of the general population (Abrahamian et al., 2012). Diabetes among MD patients is associated with a two- to five-fold increased risk of all-cause mortality as well (as opposed to MD patients who are not suffering from diabetes) (Silva et al., 2012).

A recent review by the Canadian Network for Mood and Anxiety Treatments (CANMAT) demonstrated that consensus exists supporting the idea that patients with mood disorders, including MD, should be routinely screened for risk factors contributing to diabetes symptomatology such as weight gain and hyperglycemia (McIntyre et al., 2012). However, the relationship between MD and diabetes is likely mediated by many factors that are not fully understood at the moment (Silva et al., 2012). Diabetes contributes to CVD by forming glycation endproducts in the blood stream that are directly capable of altering protein function and altering NO production, eventually contributing to increased CVD risk (Ding and Triggle, 2010). High blood serum glucose levels can also have epigenetic effects that lead to gene regulation changes by means of altered histone methylation (Brasacchio et al., 2009). Some deleterious effects of altered methylation include uncoupling of eNOS and the production of free radicals (Ding and Triggle, 2010).

1.7. Major Studies Demonstrating an Association Between Cardiovascular Risk and Major Depression

Many longitudinal and cohort studies on MD patients have observed that risk of CV morbidity and mortality are increased in these subjects. Increased CVD risk among MD patients has been identified independently of conventional CV risk factors (Lichtman et al., 2008). Historically, these findings can be traced back as far as Malzberg et al. (1937) who observed

increased cardiac death among institutionalized MD patients. Several decades later, the relationship between MD and CVD was further substantiated with large clinical studies examining CV events in a cohort that was highly susceptible to MD symptoms based on individual traits (Parkes, 1964).

In the 1990s, many groups began publishing findings showing that risk factors for MD were associated with CVD and MI. For example, Anda et al. (1993) showed that reported feelings of hopelessness were associated with both fatal and non-fatal MI in a cohort of both male and female adults. A study examining perceived stress followed by CV events observed that middle-aged adults with higher reported stress were more likely to suffer mortality from MI (Rosengren et al., 1991).

Later on, one of the first modern, well-controlled studies (published by Barefoot and Schroll, 1996 in *Circulation*) demonstrated a link between MD and MI directly. Using a longitudinal method, these researchers demonstrated that patients with moderate to severe MD had 69% greater odds of CV death than non-MD patients. Patients with even mild MD symptoms had 38% greater odds of dying due to CVD than non-MD patients.

Following the discoveries linking MD to CVD in the 1990's and earlier, a large multi-national, multicentre study was conducted by Rosengren et al. (2004). This study, named the INTERHEART study,

included 12,461 patients with acute MI from 52 different countries and compared their history of psychosocial stressors and other deleterious events to 14,637 HCs who were age-, sex- and site-matched (Rosengren et al., 2004). The subjects included patients from the world's major cultural and ethnic groups, and the researchers reported statistically higher rates of psychosocial stress (associated with MD) in patients with acute MI than in the healthy control (HC) group. Among those who had recently suffered an MI, factors linked to the development of MD were found to be among the greatest risk factors predicting a future MI.

1.8. Major Depression Following Myocardial Infarction

In addition to patients with MD being at increased risk for CVD, patients with CVD are also more likely to develop MD symptoms (Nemeroff and Goldschmidt-Clermont, 2012). For example, approximately 20% of patients who have suffered a MI have been shown to develop MD the following year (Strik et al., 2004; Frasure-Smith et al., 1993). The development of MD symptoms following a MI has been related to a worse prognosis over time (Meurs et al., 2012). Even dysthymia following an MI are associated with an increase in mortality and morbidity and recurrent MI (van Melle et al., 2004).

In cases where MD patients exhibit CVD and are at risk of MI, treatment with conventional antidepressants may decrease the severity of CVD symptoms and improve outcome. For example, the ongoing

Myocardial Infarction and Depression Intervention Trial (MIND-IT) involves examination of outcomes among patients treated with antidepressants following MI. When administering the antidepressant mirtazapine in a double-blind placebo controlled study, Honig et al. (2007) demonstrated that mirtazapine does not present any notable increase in CV risk while reducing reported MD symptoms in many patients. As well, it is known that a lack of response to antidepressant treatment may be associated with an increased risk of MI, suggesting that the antidepressant response may serve as a factor in future CV risk (de Jonge et al., 2007).

While there is promise that antidepressant treatments might decrease the risk of recurrent MI by reducing MD symptoms, it is also important to note that in publications using physically healthy MD patients receiving antidepressant medications, impaired endothelial function (assessed by FMD) has been demonstrated. Both Rajagopalan et al. (2001) and later Broadley et al. (2002) demonstrated impaired endothelial function in MD patients receiving antidepressants. However, when an unmedicated depressed population was examined by Garcia et al. (2011) and by Zhuo et al. (2011), no significant difference between MD groups and matched HCs was seen. The implication of these findings is that antidepressant treatment may be in some way responsible for impairment of endothelial function in MD patients, although several other explanations may exist (see Chapter 5).

1.9. Major Depression Symptoms and Cardiovascular Risk

The severity of MD symptoms has been correlated with an increase in CVD morbidity and mortality (Lepine and Briley, 2011). Previously, in a sample of 361,662 middle-aged men (Gump et al., 2005), it was demonstrated that higher scores on the Center for Epidemiologic Studies Depression Scale over the course of six years (with reported over 90% attendance for follow-up visits) led to an increase in CVD mortality as well as all-cause mortality. More recently in Australia, a study using the Composite International Diagnostic Interview 3.0 to diagnose MD among 8,820 subjects demonstrated that with increasing MD severity, an increase in CV-related disease occurs (Murphy et al., 2012).

Some symptoms of MD may be responsible for a greater share of CVD risk than others. For example, nearly 20 years ago Anda et al. (1993) studied a group of 2,832 adults aged 45-77 years with no evidence of CVD at the beginning of the study. Over the course of follow-up (a mean latency of 12.4 years), subjects with greater reported helplessness (and more severe MD symptoms in general) showed a greater risk of CVD-related morbidity and mortality.

Recent work by Stewart et al. (2012) demonstrates that the affective symptoms of MD may be closely associated with faster advancement of atheroma. They investigated a population of MD patients without CVD or observable arterial calcification at the beginning of the study and showed

that the strongest predictors of arterial calcification and advancement of atheroma were related to depressed mood and negative affect, including perceived sense of failure.

Many of the publications dealing with the issue of specific MD symptoms and CVD risk were based on studies conducted in patients already suffering from CVD. For example, Bekke-Hansen et al. (2012) have shown that increased risk of mortality in post-MI patients is related to somatic symptoms. Using subjects from the Enhancing Recovery in Coronary Heart Disease (ENRICHD) trial for analysis, they found that insomnia, fatigability and loss of appetite were strongly correlated with all-cause mortality in their sample. Similar results were found earlier by de Jonge et al. (2006), who observed that somatic-related MD symptoms were correlated with CV death. Earlier, Barefoot et al. (2000) demonstrated that lack of somatic symptoms of MD significantly predicted survival in patients with coronary artery disease.

Based on the range of studies investigating specific MD symptoms and CVD-related morbidity and mortality, it may be the case that affective MD symptoms are indicative of CVD risk in MD patients prior to CVD, while somatic symptoms are relevant to the chances of MI recurring and CVD-related death post-CVD.

1.10. Antidepressants and Cardiovascular Risk

It has long been recognized that certain antidepressant medications such as tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) can increase the risk of CVD morbidity and mortality (Carney, 2008). Antidepressants have been shown to have several CV-related effects including changes in autonomic function, reduction in heart rate variability, hypotension, hypertension, changes in platelet function, and arrhythmia (Taylor, 2008). For example, TCAs act as cardiac sodium and potassium channel antagonists that alter myocardial repolarization as seen by electrocardiogram, an effect that is dose-dependent (Thanacoody and Thomas, 2005).

The CV risk caused by MAOI administration may be manifested through interactions with particular kinds of food, although orthostatic hypotension and acute hypertension may also be caused directly by acute MAOI use (Robinson and Amsterdam, 2008). MAOIs are capable of producing hypertensive reactions when eaten with a sufficient quantity of foods rich in the sympathomimetic amine tyramine (Ngo et al., 2010). This adverse effect is the result of irreversible inhibition of MAO-A, the enzyme responsible for catabolism of tyramine in the gut. When MAO-A is inhibited irreversibly, tyramine enters the circulation and causes release of large amounts of noradrenaline, which in turn is not catabolized because it too is a substrate for MAO-A (Finberg and Gillman, 2011). Hypertensive

reactions produced by MAOIs interacting with tyramine can lead to severe morbidity, including stroke and cardiac arrhythmia in some cases. However, the CVD effects of particular MAOIs vary, with antidepressants such as the reversible inhibitors of MAO-A like moclobemide being less likely to produce a hypertensive crisis after tyramine ingestion (Lotufo-Neto et al., 1999).

More modern antidepressants than TCAs and MAOIs may also increase CVD risk. For example, Le Melledo et al. (2009) demonstrated that the selective serotonin reuptake inhibitor (SSRI) paroxetine increased blood serum LDL-C levels. However, this side effect of the SSRI class may not necessarily be harmful when given to MD patients with comorbid CVD by reducing morbidity caused by MD symptoms. For example, upon reviewing a one-year follow-up after treating CVD patients with comorbid MD using the SSRI escitalopram, Hanash et al. (2012) demonstrated no differences between the electrocardiograms of the escitalopram-treated group and a HC group. Another review (Taylor, 2008) also suggests that SSRIs are relatively safe in patients with established coronary artery disease while treating MD symptoms.

After conducting a review of available studies employing antidepressants in MD patients suffering from CVD, Taylor et al. (2008) concluded that the antidepressants sertraline, fluoxetine, citalopram, buproprion, and mirtazapine were relatively safe in terms of treating MD

symptoms among patients with CVD. However, a more recent review (Weeke et al., 2012) demonstrated that adverse CV events following a hospital stay for CVD were associated with TCA and SSRI use while the serotonin/norepinephrine reuptake inhibitor (SNRI) venlafaxine was far less likely to have the same effect. Similarly, a study performed by Tully et al. (2012) showed that following coronary bypass graft surgery, patients given venlafaxine or duloxetine (another SNRI) were not statistically more likely to die from all-cause mortality following treatment than MD patients who did not receive an antidepressant.

1.11. Overview of Research Project

As previously mentioned, MD patients have significantly decreased blood serum levels of NOx when compared to matched HCs (Garcia et al., 2011; Ikenouchi-Sugita et al., 2009; Chrapko et al., 2004). As low blood serum NO has also been linked to CVD (Anderson, 2007), it is assumed that a mechanism impairing NO production in MD patients may contribute to increased risk of CVD. The activity of platelet eNOS has been shown to be reduced in MD patients (Chrapko et al., 2006). This reduction in eNOS activity may be related to a decrease in availability of eNOS's substrate, L-arginine, but to our knowledge this has not been investigated in MD patients.

Decreased brachial FMD is indicative of developing atherosclerosis and arteriosclerosis and is associated with future CV events such as MI

(Anderson, 2007). FMD was recently shown to not be decreased in MD patients (Garcia et al., 2011), despite decreased NO blood serum levels being well documented (Chrapko et al., 2004; Ikenouchi-Sugita et al., 2009). As NO is a surrogate indicator of endothelial health (Chrapko et al., 2006), decreased NO levels suggest that endothelial function will be impaired in physically healthy MD patients.

The study on the amino acids arginine and citrulline was expanded to also include and investigation of serum levels of other amino acids that have also been implicated in MD (see Chapter 4). The amino acids of interest included gamma-aminobutryic acid (GABA), phenylalanine, tyrosine, tryptophan, glutamate, glutamine, aspartate, asparagine, glycine, alanine, taurine, serine, ornithine, histidine, valine, methionine, leucine, and isoleucine.

For decades researchers have been measuring levels of these amino acids in serum or plasma from MD patients in the hope that these levels might reflect what is happening in the brain and/or be useful biomarkers in MD subjects. Overall, the results have been variable and disappointing, likely because very small numbers of subjects have been studied or because of problems with study design. Patients have often been on medication, and patients and corresponding controls have not been well matched. In the study described in this thesis, we have attempted to overcome many such limitations in comparing MD subjects and HCs. Before describing those

studies in detail, I will give a brief overview of the relevant literature in this area. Although the literature comparing MD subjects and controls is too extensive to summarize, Table 1-1 shows results obtained in those studies in which the MD subjects were medication-free.

1.11.1. Gamma-Aminobutyric Acid

GABA is the major inhibitory neurotransmitter in the mammalian brain. Dysregulation of GABA in the brain has been reported in MD patients using imaging methods (Zhao et al., 2012; Song et al., 2012). Correspondingly, serum GABA levels have been reported to be decreased in MD patients (Xu et al., 2012; Kalueff and Nutt, 2007). Petty et al. (1992) reported that while MD patients had lower mean serum GABA levels than controls, only 40% actually demonstrated lower GABA serum levels than HCs and suggested there may be two MD patient populations: one with low serum GABA levels and one with normal serum levels of GABA (Petty et al., 1995). SSRI antidepressants have been reported (using magnetic resonance spectroscopy measurements) to increase brain levels of GABA in human brain (Sanacora et al., 2002). In animal studies, SSRIs have been reported to be potent at elevating brain levels of allopregnanolone, a neurosteroid that is a strong positive neuromodulator at GABA-A receptors (Pinna et al., 2009), and the MAOI antidepressant phenelzine causes a marked elevation of brain GABA levels (Baker et al., 1991).

TABLE 1-1. SERUM AMINO ACID LEVELS IN UNMEDICATED MAJOR DEPRESSION PATIENTS COMPARED TO HEALTHY CONTROLS

PUBLICATION	PATIENTS	CONTROLS	AMINO ACIDS IN MAJOR DEPRESSION COMPARED TO HEALTHY CONTROLS
Kim et al., 1982	8 MD unmedicated, 29 MD treated (endogenous MD), 9 MD unmedicated, 18 MD treated (neurotic MD)	34 comparison HCs	**higher:** glutamate in treated and untreated MD combined (not in untreated MD alone)
Russ et al., 1990	16 MD inpatients, unmedicated	9 comparison HCs	**lower:** tryptophan to five large neutral amino acids (tyrosine, phenylalanine, leucine, isoleucine, valine)
Petty et al., 1992	58 MD unmedicated	51 comparison HCs	**lower:** GABA (subset of MD patients)
Altamura et al., 1993	15 with mood disorders	19 comparison HCs	**higher**: glutamate
Altamura et al., 1995	25 unmedicated MD patients	22 comparison HCs	**higher:** taurine, serine/glycine ratio **lower:** glycine
Maes et al., 1995	123 MD subjects	50 comparison HCs	**higher:** serine
Mauri et al., 1998	29 MD outpatients	28 comparison HCs	**higher:** (in platelets), aspartate, serine and lysine (plasma) glutamate, taurine, lysine **lower:** ratio of tryptophan to five large neutral amino acids (tyrosine, phenylalanine, leucine, isoleucine, valine)
Maes, et al., 1998	treatment resistant MD, after 5 weeks of medication	15 comparison HCs	**lower:** aspartate, glutamate, taurine **higher:** glutamine (after 5 weeks of medication)
Sa et al., 2012	60 MD patients	110 comparison HCs	**lower:** serotonin/tryptophan ratio, tyrosine, tryptophan
Xu et al., 2012	treatment-naïve MD	25 comparison HCs	**lower:** lysine, tryptophan, GABA
Pinto et al., 2012	19 unmedicated MD patients	19 comparison HCs	**lower:** arginine
Fu et al., 2012	15 unmedicated MD patients, later 7 MD patients 2 months after antidepressant treatment	14 comparison HCs	**lower:** aspartate, glycine (not after antidepressant treatment)

An issue related to the analysis of serum GABA levels among MD patients is the interaction of GABA with the blood-brain barrier. GABA is not able to penetrate the blood-brain barrier (Smith, 2000) and peripheral serum levels of GABA in MD patients may not directly influence GABA activity in the brain as a result. While it is not possible for GABA to cross the blood-brain barrier, GABA from the brain enters the blood stream and is therefore observable in blood serum, although levels are many times lower in serum than in brain (Ferkany et al., 1979).

1.11.2. Glutamate, Glutamine, Aspartate, Asparagine and Glycine

Glutamate is the most abundant excitatory neurotransmitter in the brain. In imaging studies utilizing magnetic resonance spectroscopy (MRS) there is detailed evidence suggesting that glutamate levels are altered in the brains of MD patients, particularly in the prefrontal cortex (Salvadore et al., 2012). For example, levels of combined glutamate and glutamine in the medial and dorsal prefrontal cortices have been shown to be decreased in MD subjects, and the resulting deficit in neurotransmission has been related to the symptoms of MD (Hasler et al., 2007).

Despite imaging results generally showing decreased levels of glutamate in cortical regions in brain (Auer et al., 2000; Pfleiderer et al., 2003), studies of glutamate serum levels have reported varying findings. Glutamate serum levels have been reported to be significantly higher (Altamura et al., 1993; Kim et al., 1982), not significantly different from

(Altamura et al., 1995), and significantly lower (Maes et al., 1998) in MD than HCs. The last group of researchers demonstrated significantly decreased serum glutamate only among treatment-resistant MD patients compared to HCs. A more recent study by Mitani et al. (2006) reported significantly higher serum levels of glutamate in MD patients than in HCs and showed a positive correlation between serum glutamate levels and severity of MD. However, in this study 17 out of 23 MD patients received unreported antidepressants of various classes.

It is of interest that although neuroimaging studies suggest decreased glutamate levels in certain brain regions in depressed subjects, studies in animal models of depression suggest the opposite (Paul and Skolnick, 2003), and administration of the NMDA glutamate receptor antagonist ketamine to MD patients has been reported to produce rapid antidepressant effects (Zarate et al., 2006, 2010; Mitchell and Baker, 2010).

Glutamine is a metabolite of glutamate and serum levels of glutamine have been reported to be lower in treatment-resistant MD subjects relative to HCs (Maes et al., 1998). Pharmacological induction of glutamine deficiency in the prefrontal cortex of male mice increases depressive-like behaviours, and infusion of glutamine can reverse these changes (Lee et al., 2007, 2012), and activity of glutamine synthetase, the enzyme that converts glutamate to glutamine, has reported to be reduced in MD subjects (Choudary et al., 2005).

Like glutamate, aspartate is an excitatory amino acid. Serum levels of aspartate have been reported to be lower than control values in unmedicated MD subjects (Maes et al., 1998; Fu et al., 2012). Asparagine is a metabolite of aspartate (similar to the relationship of glutamine to glutamate). In higher brain regions, glycine is a potent co-agonist at the NMDA glutamate receptor, and serum levels of glycine have been reported to be reduced in MD subjects relative to controls (Altamura et al., 1995; Fu et al., 2012).

1.11.3. Tryptophan

Tryptophan is the precursor amino acid for serotonin (Figure 1-4) and levels of this amino acid have been reported to be decreased in serum samples from MD patients (Sa et al., 2012). Tryptophan present in serum is critical for the production of serotonin (5-hydroxytryptamine, 5-HT) in the brain, as free tryptophan in blood is able to pass through the blood-brain barrier actively and become available for production of serotonin. Low serum tryptophan has been suggested to be indicative of negative effects on mood due to inferred losses in serotonin production (Toker et al., 2010; Russo et al., 2003). Causing acute serum tryptophan depletion results in only a modest lowering of mood in most subjects and a temporary reappearance of depressed mood in newly recovered depressed patients on antidepressants (Delgado et al., 1999; Young, 2013). The ratio of plasma levels of tryptophan to those of five large neutral amino acids (LNAAs)

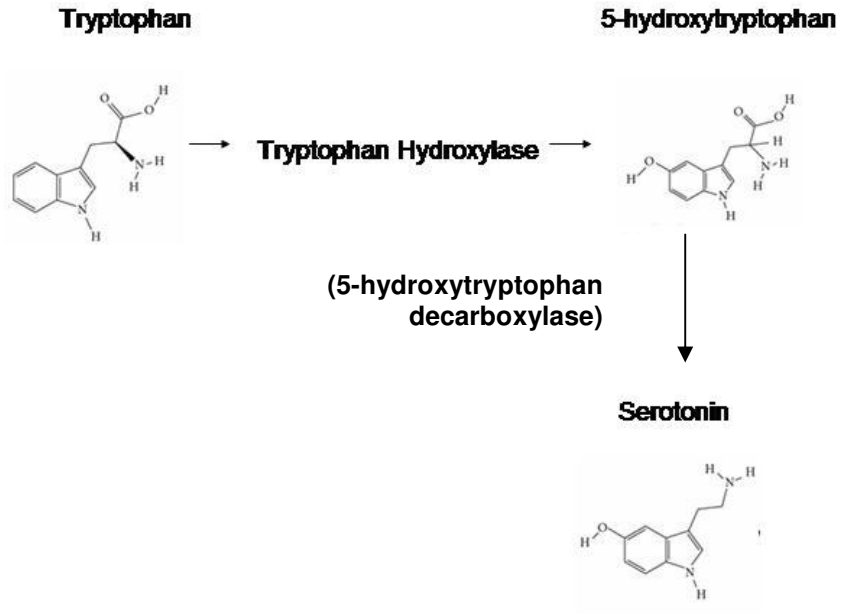

FIGURE 1-4. TRYPTOPHAN AND SEROTONIN PRODUCTION

The amino acid tryptophan is converted into 5-hydroxytryptophan by the enzyme tryptophan hydroxylase. 5-Hydroxytryptophan is then converted into the neurotransmitter serotonin by 5-hydroxytryptophan decarboxylase. Diagram derived from Park et al. (2011).

with which it shares a transporter has been reported to be lower in unmedicated MD patients compared to HCs (Russ et al., 1990; Mauri et al., 1998).

1.11.4. Tyrosine

Tyrosine, which is produced from phenylalanine, is necessary in the production of dopamine and norepinephrine, first being metabolized into levodopa by the enzyme tyrosine hydroxylase (Bosier et al., 2012; Figure 1-5).

In the thyroid, triiodothyronine and thyroxine are derived from tyrosine (Deng et al., 2012) and both of these substances have significant ability to affect mood (Garlow et al., 2012). Serum tyrosine has been reported to be lower in MD patients compared to HCs (Russ et al., 1990; Sa et al., 2012), and administering tyrosine to MD patients has been reported to improve mood (Gelenberg et al., 1982).

1.11.5. Serine, Alanine, Lysine, Taurine, Methionine, Cysteine, Histidine and Ornithine

Serine is related metabolically to glycine, and higher serum serine/glycine ratios and higher serine serum levels have been reported in MD subjects compared to HCs (Altamura et al., 1995; Maes et al., 1995). D-Serine is a strong co-agonist at the NMDA receptor (Labrie and Roder, 2010; Labrie et al., 2012) and has been reported to have both antipsychotic and antidepressant properties in animal studies (Labrie et al., 2010;

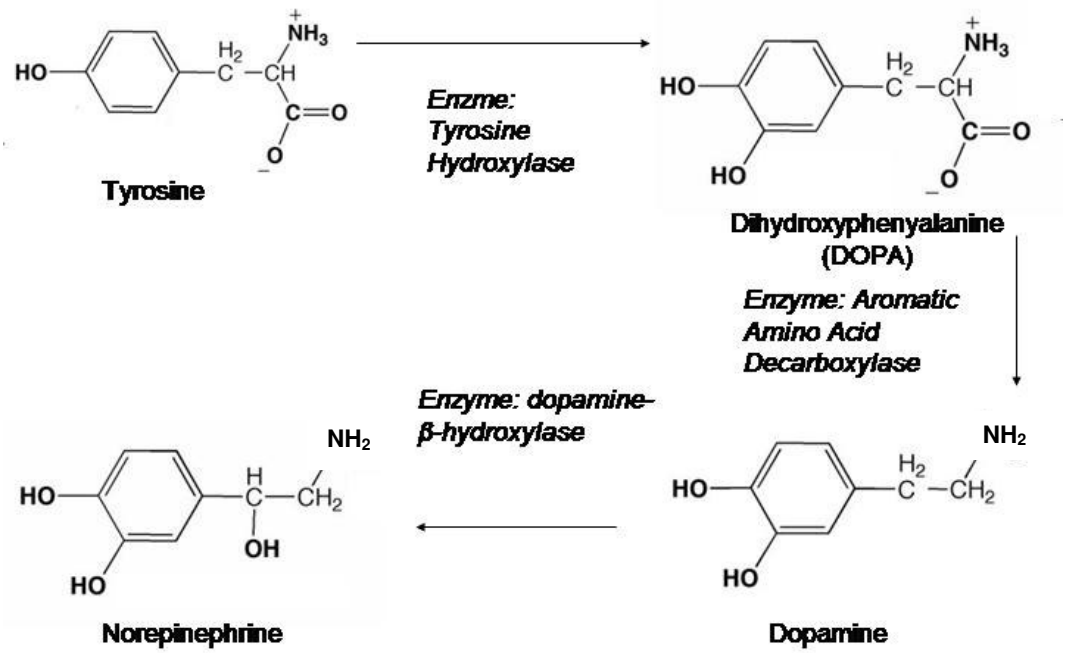

FIGURE 1-5. TYROSINE INVOLVEMENT IN CATECHOLAMINE PRODUCTION

The amino acid tyrosine is converted to 3,4-dihydroxyphenyalanine (DOPA) by the enzyme tyrosine hydroxylase. DOPA is then converted to dopamine by aromatic amino acid decarboxylase. Dopamine may then be converted to norepinephrine by dopamine-β-hydroxylase. Derived from Teraishi et al. (2012).

Malkesman et al., 2012). Alanine is a weak co-agonist at the NMDA receptor, and its brain levels are increased markedly by the antidepressant phenelzine (Tanay et al., 2001). Lysine has been reported to have anxiolytic actions in laboratory animals and humans (Smriga and Torii, 2003; Smriga et al., 2002, 2004). Taurine is a sulfur-containing amino acid involved in numerous physiological functions (Huxtable, 2000), including as a trophic factor in development of the CNS and as a neuroprotector against glutamate-induced neurotoxicity (Wu and Prentice, 2010). Taurine levels in serum and platelets of MD patients have been reported to be higher than in controls (Altamura et al., 1995; Mauri et al., 1998; Maes et al., 1998).

Methionine is converted in the body to S-adenosylmethionine (SAM), which contributes methyl groups to many metabolic reactions in the body. SAM has also been proposed to have antidepressant properties (Popakostas, 2003). Ornithine is related metabolically to arginine (see Figure 1-1) and is also a precursor for polyamines and glutamate. The MAO-inhibiting antidepressant phenelzine causes a marked increase in brain levels of ornithine in rats (MacKenzie et al., 2008). N-Acetylcysteine, which is metabolized in the body to cysteine, has been reported to decrease depressive symptoms in bipolar disorder and is thought to exert this activity through modulation of NMDA glutamate receptors and/or formation of the potent antioxidant glutathione (Berk et al., 2008). Histidine has been

reported to have antidepressant activity in the forced swim test in lab animals (Lamberti et al., 1998). Histidine is the amino acid precursor of histamine, an amine that has been proposed to be hypofunctional in MD (Kano et al., 2004), although Altamura et al. (1995) did not find decreased plasma levels of histidine in MD patients and Shan et al. (2013) reported an unaltered histamine system in postmortem cortical tissue from depressed subjects.

1.12. Alteration of Serum Amino Acid Levels With Antidepressant Medications

Maes et al. (1998) reported that serum levels of glutamate were significantly reduced in MD patients who were treatment-resistant at the beginning of the study. However, after MD patients had been given the antidepressant trazodone (in addition to supplementary medications including fluoxetine and pindolol) for 5-weeks, serum levels of glutamate were significantly higher than at baseline. This would seem to fit in well with neuroimaging studies showing reduced cortical levels of glutamate in MD, but as mentioned above, the NMDA glutamate receptor antagonist ketamine produces rapid antidepressant effects in MD patients.

Kucukibrahimoglu et al. (2009) showed that among patients with MD treated with the SSRI antidepressants citalopram or fluoxetine, blood serum levels of GABA were increased after successful treatment. Esel et al. (2008) showed a similar increase in blood serum levels of GABA in MD

patients after a successful course of electroconvulsive therapy (ECT). A similar study by Palmio et al. (2005) had previously demonstrated that tryptophan serum levels are also increased following ECT. Ille et al. (2007) suggested that specifically tailored amino acid treatments as adjuncts to antidepressants may improve MD symptoms while offering a better side-effect profile.

Lima et al. (2003) reported that a positive correlation exists between lymphocyte levels of taurine and depression severity on the Hamilton depression rating scale. As well, prior to treatment, MD patients in the Lima et al. (2003) study had increased taurine, aspartate, and glutamine lymphocyte levels relative to controls. The authors found that after the antidepressant mirtazapine was administered for six weeks, the lymphocyte amino acid levels were no longer significantly different from those of HCs.

The ratio of tryptophan to the LNAAs (tyrosine, phenylalanine, leucine, isoleucine and valine) has been used as a marker of antidepressant effectiveness (Porter et al., 2005). Tryptophan is transported across the blood-brain barrier by a specific carrier for neutral amino acids, but it must compete with the LNAAs to do so. As a result, entry of tryptophan into the brain to form serotonin depends on the ratio of tryptophan to competing LNAAs (Fernstrom and Wurtman, 1971). When patients with a low ratio of tryptophan to LNAAs are treated with antidepressants, the ratio of tryptophan to LNAAs has been reported to increase (Porter et al., 2005).

1.13. Objectives and Hypothesis

The first two objectives of the studies presented in this thesis were to investigate, directly and indirectly respectively, NO-related deficits in MD patients linked to CVD risk. Although researchers in our laboratories had previously demonstrated decreased serum levels of NO_x (nitrate plus nitrite) and decreased platelet eNOS in MD patients compared to HCs (Chrapko et al., 2004, 2006), the serum levels of L-arginine (the precursor of NO) had not been investigated in these two populations. Thus, one of the objectives of the present research was to investigate serum levels of the amino acids L-arginine and L-citrulline (a precursor of L-arginine and also the product of the action of eNOS on L-arginine), in medication-free, non-smoking, physically healthy MD patients compared to matched HCs. This study formed the basis of chapter 2 of this thesis. In the third study reported in this thesis (chapter 4), we studied in well matched MD and HC subjects (see chapter 2) serum levels of a variety of other amino acids that have been proposed to be involved in the etiology and/or pharmacotherapy of depression. Based on some previous reports in the literature, we hypothesized that serum levels of GABA and tryptophan (or the L-tryptophan/LNAA ratio) in the MD subjects would be lower than values in HCs while serum levels of L-glutamate, glycine and taurine would be higher.

In the second study related to NO, brachial artery ultrasonography was used to study FMD in unmedicated MD patients. A deficiency in FMD as observed by ultrasonography was hypothesized in MD patients based on previous research demonstrating decreased NO production among MD patients not receiving antidepressants (Chrapko et al., 2004; Garcia et al., 2011; Ikenouchi-Sugita et al., 2009). The study formed the basis for chapter 3 of this thesis.

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CHAPTER 2

DECREASED SERUM L-ARGININE AND L-CITRULLINE LEVELS IN MAJOR DEPRESSION

Part of the the content of this chapter is represented in a manuscript submitted for publication. The authors are Scott L. Hess, Stephen C. Newman, Gabor Gyenes, Ross Tsuyuki, Glen B. Baker, Rahima Bhanji, and Jean-Michel Le Mellédo. The author of this thesis played a major role in the study, including research visits, data collection, data analysis, and writing of the manuscript.

2.1. Abstract

OBJECTIVE: It has been suggested that decreased nitric (NO) production by the vascular endothelium and platelets may contribute to the consistently observed increased risk of developing cardiovascular disease (CVD) in physically healthy patients suffering from major depression (MD). The biological mechanisms leading to decreased NO production in MD patients remain to be fully explained. L-Arginine is an amino acid needed for the production of NO, and we have hypothesized that L-arginine availability may be responsible for impaired endothelial NO production in patients with MD. L-Citrulline is an end product of the reaction forming NO and plasma levels of L-citrulline should also be decreased when NO production is lowered.

METHODS: Serum levels of L-arginine and L-citrulline were measured, using a Biochrom 30 amino acid analyser, in unmedicated physically healthy patients with MD (n = 35) and healthy controls (n = 36) after a 12 hour fast.

RESULTS: L-Arginine was significantly lower in MD patients than in healthy controls (HCs): 73.54 ± 21.53 µmol/L and 84.89 ± 25.16 µmol/L respectively ($p = 0.04$). L-Citrulline levels were also significantly lower in MD patients (31.58 ± 6.05 µmol/L) than HCs (35.19 ± 6.85 µmol/L $df = 65$, $t = -2.27$, $p = 0.03$).

CONCLUSION: Decreased availability of L-arginine in physically healthy MD patients may contribute to decreased NO production by the endothelium and platelets. Our findings demonstrate a possible underlying mechanism causing decreased NO production in MD patients. It remains to be determined whether therapeutic normalization of L-arginine levels in MD patients would lead to a decrease in the CV risk associated with MD.

2.2. Introduction

Physically healthy patients suffering from major depression (MD) have been found to be at increased risk of developing cardiovascular disease (CVD) independent of conventional CVD risk factors (Lichtman et al., 2008). However, the exact mechanisms leading to increased CVD risk in MD patients remain unknown. A decrease in nitric oxide (NO) production by the vascular endothelium and platelets in MD patients is suspected to contribute to the causal association between MD and CVD. Lowered NO production in MD patients is thought to contribute to impaired endothelium function as observed by ultrasonography (Rajagopalan et al., 2001). However, these results are disputed based on more recent work showing decreased NO levels, but not impaired endothelium function, in MD patients (Garcia et al., 2011).

NO is a gas synthesized from L-arginine (a conditionally essential amino acid) and oxygen by endothelial NO synthase (eNOS) (McLeod et al., 2001). The end products of NO production via eNOS include both NO

and the amino acid L-citrulline. Although it is possible to measure NO *in vivo* in humans using a variety of methods (Siervo et al., 2011), it is technically complex and has not been used routinely in human research. In blood, NO is rapidly reduced by hemoglobin to form methemoglobin or oxidized to form the aqueous anions nitrite (NO_2^-) and nitrate (NO_3^-), classically referred to together as NO metabolites (NOx) (Chrapko et al., 2004). Measuring plasma NOx levels in blood samples using an *in vitro* method is a valid alternative. As a result, measuring plasma NOx levels has been widely accepted as a surrogate measurement of endothelial NO production (Moncada and Higgs, 2006).

Low vascular NO leads to endothelial dysfunction and has been shown to be a contributor to the development of CVD including hypertension, coronary artery disease, and heart failure (Anderson, 2007). Decreased endothelial production of NO has also been associated with numerous CV risk factors, including hypercholesterolemia, hypertension, diabetes, hyperhomocysteinemia, and increased inflammation (Landmesser et al., 2000; Holven et al., 2001; Guven et al., 2012; Verma et al., 2002). As well, inadequate endothelial NO production allows for more oxidation of low-density lipoproteins, accelerating arterial plaque formation (Ignarro, 1989). Adequate endothelial NO production is necessary for CV health, including maintenance of endothelial smooth muscle functions and controlling platelet adhesion activity (Javanmard et al., 2010).

Previously, our laboratory has demonstrated that the plasma concentrations of NOx are markedly reduced in physically healthy non-medicated MD patients (Chrapko et al., 2004), a finding replicated by us (Chrapko et al., 2006) and others (Ikenouchi-Sugita et al., 2009; Selley, 2004). In order to refine our understanding of decreased endothelial NO production in MD patients, we investigated serum levels of L-arginine and L-citrulline in unmedicated physically healthy MD sufferers and HC subjects.

2.3. Methods

This study was approved by the University of Alberta Health Research Ethics Board. All participants provided informed consent before entering the study. Thirty-six HCs were included in the study and they consisted of sixteen females and twenty males. Thirty-five MD subjects participated and they consisted of fifteen females and twenty males. There were no significant differences in age between the two groups [25.97 ± 8.47 years and 27.06 ± 9.43 years respectively ($p = 0.61$)].

All subjects were assessed for Axis-I disorders using the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders Version Four Text Revision (DSM-IV-TR) Axis-I Disorders. The HC group consisted of subjects without any current Axis-I disorders or history. MD patients had to meet DSM-IV-TR criteria for a current unipolar major depressive episode as a primary disorder. MD patients with

comorbid anxiety disorders were included because of the high comorbidity of MD and anxiety disorders (Rapaport, 2001), while other Axis-I disorders (schizophrenia, bipolar disorder, etc) were exclusion criteria. MD patients with comorbid anxiety disorders included five cases of generalized anxiety disorders and two cases each of phobia, panic disorder, obsessive-compulsive disorder, and social phobia. One MD patient had a history of alcohol dependence and that person had been abstinent from alcohol use for approximately three years prior to the beginning of the study.

The presence of conventional risk factors such as a Body-Mass Index (BMI) higher than 30 kg/m^2, an abnormal lipid profile such as an elevated low-density lipoprotein cholesterol (LDL-C), elevated glucose levels, high blood pressure as well as many factors associated with increased CVD risk were exclusion criteria. We assessed physical activity levels using a survey system, and it was important to control for this factor as it has been shown that either acute or chronic aerobic exercise significantly increases plasma levels of NOx (Maeda et al., 2001; Sessa et al., 1994).

Patients with a BMI over 30 kg/m^2 were excluded since a high BMI indicating obesity may contribute to development of atherosclerosis and CVD risk (Rizvi, 2009). Patients with high LDL-C serum levels (over 3.00 mmol\L) were also excluded as increased LDL-C concentration is a major contributor to CVD and a direct cause of endothelial dysfunction

(Laclaustra et al., 2008). Current smoking and past chronic smoking were also exclusion criteria. Smoking was defined as consumption of more than five cigarettes per calendar week. Indeed, even light current smoking has been shown to significantly impair endothelial NO production (Barua et al., 2002). Chronic smoking has been shown to significantly decrease endothelial NO production (Barua et al., 2002) and chronic smoking is still associated with decreased endothelial NO production up to at least a year after smoking discontinuation (Johnson et al., 2010). A family history of CVD events before age fifty with potential for genetic causation was also used as an exclusion criterion.

None of the subjects were receiving or had received antidepressant treatment within at least 4 weeks of beginning the study (the requirement was increased to 6 weeks in the case of fluoxetine due to its long half-life). All MD subjects had either discontinued any medication voluntarily for 4 or more weeks prior to being screened for the study (n = 16) or had never taken antidepressant medications previously (n = 19). Three subjects had previously received citalopram; three had taken buproprion, two sertraline, two venlafaxine, one fluoxetine, and one paroxetine while four additional subjects who were aware that they had taken antidepressants were unable to recall their prescription history accurately.

The severity of depressive symptomatology in each subject was assessed using the self-administered Beck Depression Inventory II (BDI-II).

The 7-Day Physical Activity Recall Interview (Blair et al., 1985) as well as a semi-quantitative food frequency questionnaire (88GP, Brigham and Women's Hospital: Department of Nutrition, Harvard University, Boston, MA, USA, 1988) were used to assess physical activity and diet respectively. Activity on the 7-Day Physical Activity Recall Interview was rated as moderate (4 kcal/kg/hour), hard (6 kcal/kg/hour), or very hard (10 kcal/kg/hour). BMI was determined by measuring subjects' weight and height. Blood pressure measurements and an electrocardiogram (ECG) were performed on all subjects.

Blood sampling was performed after 12-16 hours fasting. Centrifuging and analyses of total fasting cholesterol, LDL and high-density lipoprotein (HDL) cholesterol, triglycerides, and glucose were conducted by the University of Alberta Hospital Core Lab. Blood samples were taken during the follicular phase of the menstrual cycle in female subjects to ensure that hormonal variations did not account for variations in L-arginine levels.

A Biochrom 30 Amino Acid Analyser was used by the University of Alberta Hospital Core Lab to determine serum levels of L-arginine and L-citrulline. Briefly, a flow chromatography colorimetric procedure was used involving binding of ninhydrin to amino acids followed by analysis with a photometer (Moore et al., 1958). Results were displayed using a chart recorder, and custom software determined the amount of L-arginine and L-

citrulline. The limit of amino acid detection sensitivity was 9 picomoles per litre.

Analyses were conducted using two-tailed independent t-tests for each factor. A p-value of less than 0.05 was considered statistically significant for all statistical tests. To detect a difference of of 0.5 standard deviations between group means, independent t-tests were used with a probability of type-I error set at 0.05. A Pearson's R was conducted to investigate correlations between L-arginine levels and BDI-II scores. All analyses were conducted using GraphPad Prism 4.0 for Windows (La Jolla, CA, USA).

2.4. Results

HC subjects had a mean BDI-II score of 2.22 ± 3.84 while MD patients scored 28.42 ± 7.82 ($p < 0.0001$). Age, BMI (the patients were of normal weight, i.e. not obese), lipid and triglyceride levels, fasting glucose levels, systolic blood pressure, physical activity, and average metabolism (as measured by kcal/day consumed) were not significantly different between MD patients and HCs (Table 2-1). L-Arginine levels were significantly lower in MD patients than in HCs (73.54 ± 21.53 µmol/L and 84.89 ± 25.16 µmol/L respectively, $p = 0.04$) (Figure 2-1). Additionally, L-citrulline was significantly decreased in MD patients (31.58 ± 6.05) compared to HC subjects (35.19 ± 6.85) ($p = 0.03$) (Figure 2-2). Both MD patients (23.23 ± 4.03) and HC subjects (23.81 ± 3.58) were of normal

weight with similar BMI values. No statistically significant correlation was found

TABLE 2-1. PHYSICAL CHARACTERISTICS OF SUBJECTS ENROLLED

CHARACTERISTIC	MD ± SD	HC ± SD	n MD	n HC	t	df	p-value
Age (years)	27.06 ± 9.43	25.97 ± 8.47	35	36	0.51	69	0.61
BMI (kg/m^2)	23.75 ± 4.03	23.53 ± 3.55	30	35	0.24	63	0.81
LDL-C (mmol/L)	2.78 ± 1.16	2.75 ± 0.62	30	36	1.39	64	0.89
HDL-C (mmol/L)	1.37 ± 0.39	1.21 ± 0.34	30	36	1.82	64	0.074
Resting Systolic Blood Pressure (mm Hg)	111.45 ± 14.67	107.11 ± 9.30	31	36	1.47	65	0.15
Resting Diastolic Blood Pressure (mm Hg)	66.90 ± 8.70	62.94 ± 7.00	31	36	2.06	65	0.043
Physical Activity (kcal/kg/day)	43.16 ± 9.93	42.81 ± 9.73	29	36	0.14	63	0.89
Triglycerides (mmol/L)	1.13 ± 0.72	1.06 ± 0.63	30	36	0.42	64	0.67

Results are expressed as means ± SD.

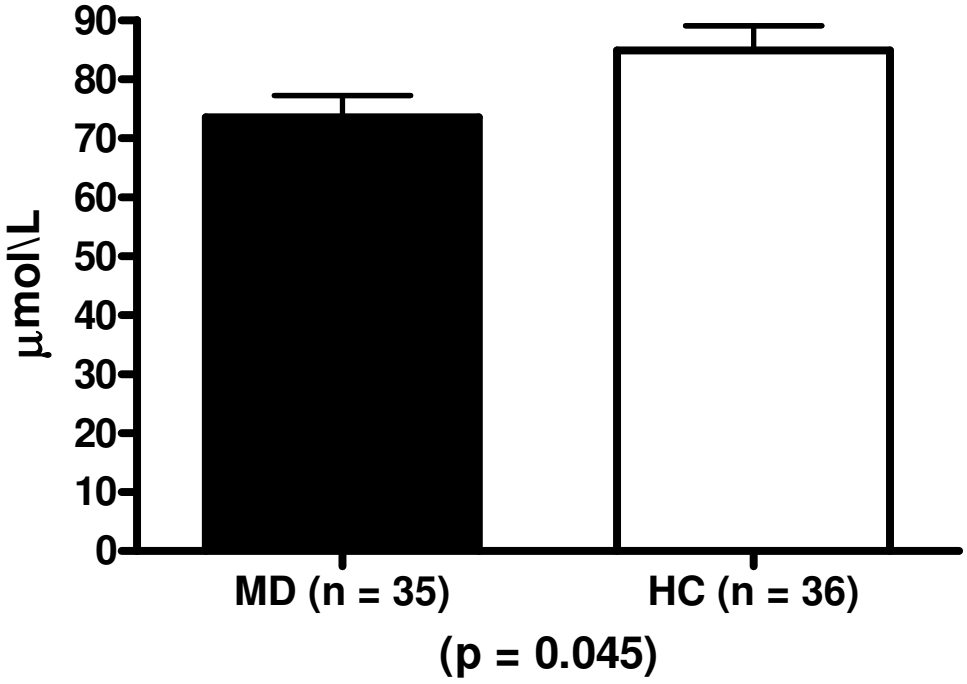

FIGURE 2-1. BLOOD SERUM LEVELS OF L-ARGININE

Blood serum levels of L-arginine (as measured in µmol/L) were found to be significantly decreased in MD patients compared to HCs ($p = 0.045$). The error bars represent Standard Error of the Mean.

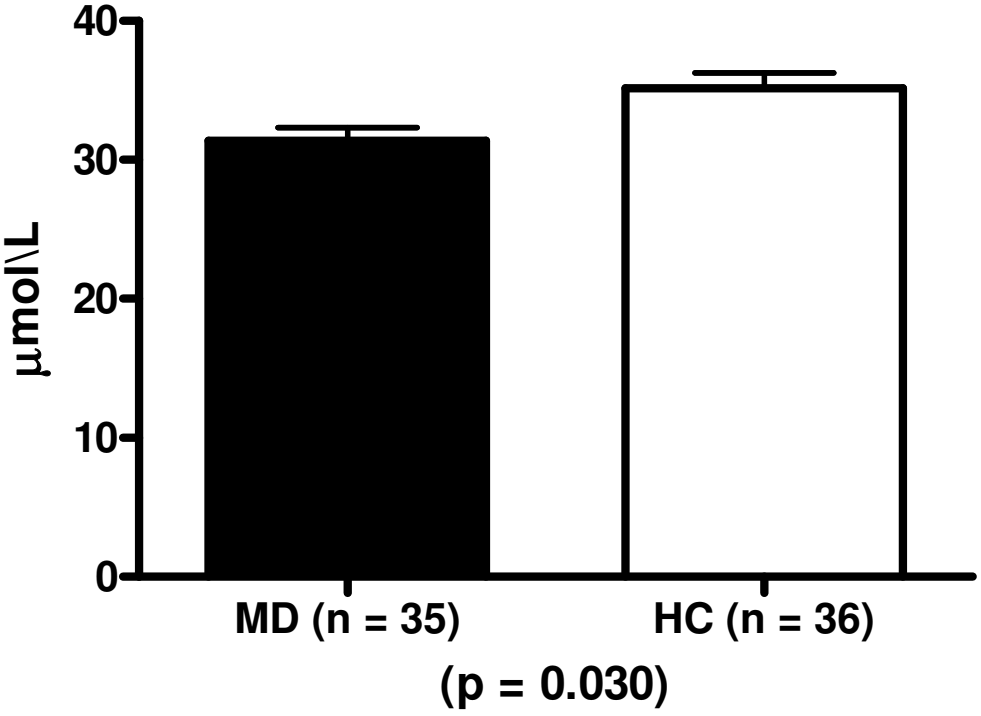

FIGURE 2-2. BLOOD SERUM LEVELS OF L-CITRULLINE

Blood serum levels of L-citrulline (as measured in µmol/L) were found to be significantly decreased in major depression patients compared to healthy controls ($p = 0.030$). The error bars represent Standard Error of the Mean.

between BDI-II scores and L-arginine levels in MD patients ($r = -0.24$, $p = 0.20$). Additionally, no significant correlation was found between BDI-II scores and L-citrulline levels in MD patients ($r = -0.16$, $p = 0.41$).

2.5. Discussion

We demonstrated that the plasma levels of the amino acids L-arginine and L-citrulline are decreased in unmedicated MD patients compared to HCs. These results suggest that a lack of available substrate for eNOS may be responsible for the consistent findings of decreased plasma NOx in physically healthy MD patients, a surrogate measurement of endothelial NO production (Chrapko et al., 2006; Chrapko et al., 2004; Ikenouchi-Sugita et al., 2009; Selley, 2004). Lower L-citrulline levels are also suggestive of decreased production of NO by the endothelium in MD patients, as L-citrulline is an end product of NO synthesis (Guerreiro et al., 2009). However, due to the fact that L-citrulline can be converted to L-arginine via the citrulline NO-pathway as well as the urea cycle, we cannot exclude the possibility that decreased L-citrulline contributes to decreased L-arginine levels in physically healthy MD patients (Erez et al., 2011).

Our results are supported by a recent publication by Pinto et al. (2012) who found that L-arginine plasma levels were decreased in a very small sample of physically healthy unmedicated MD patients compared to HCs (five in each group). Although the authors did not control for several factors (including diet), it is interesting that the decrease in L-arginine

observed in their small MD sample is comparable in magnitude to the one we found in our larger sample.

As MD has been associated with changes in appetite and diet (Van Citters et al., 2010), we controlled for diet in our study using a validated questionnaire in order to assess whether an alteration in the diet of MD patients could be responsible for decreased L-arginine serum concentrations in MD patients. We did not detect a difference between MD patients and HCs in their dietary intake of amino acids, which suggests that this exogenous factor is unlikely to be the cause of decreased L-arginine and L-citrulline levels in MD patients.

There are many biological factors that may influence L-arginine levels. Tetrahydrobiopterin (THB) is a co-factor necessary in the reaction forming NO from L-arginine by eNOS and may play a role in determining L-arginine levels (Maier et al., 2000). However, we did not detect a decrease in plasma levels of THB in MD patients when compared to HCs in a previous investigation (Chrapko et al., 2004). Furthermore, a decrease in THB would be expected to be associated with an increased availability of L-arginine and subsequent increased plasma L-arginine levels due to inhibition of eNOS (Chrapko et al., 2004).

Asymmetric dimethylarginine (ADMA) is another potential endogenous factor affecting L-arginine levels. High ADMA levels are associated with increased CV risk and poor overall CV health (Valkonen et

al., 2001). ADMA competitively inhibits eNOS and prevents endothelial NO production (Cooke and Ghebremariam, 2011). Higher levels of plasma ADMA have been observed in physically healthy MD patients when compared to matched HCs (Selley, 2004). Increased ADMA would be expected to be associated with higher L-arginine levels as eNOS is inhibited by ADMA and L-arginine is not consumed as a result. On the contrary, we found that L-arginine levels were decreased in MD patients.

Low L-arginine levels in MD patients may be explained by increased activity of the enzyme arginase. For example, Elgun and Kumbasar (2000) found indirect evidence of increased arginase activity in the serum of MD patients. Indeed, it has been suggested that the enzyme arginase may compete for L-arginine with eNOS, reducing endothelial NO production (Katusic, 2007). NO would be reduced as a result of high arginase levels, as eNOS would not have an adequate amount of substrate to produce NO (Saghatelian et al., 2004; Jin and Loscalzo, 2010). However, arginase measurements are indirect and deduced from ornithine levels in Elgun and Kumbasar's paper (Elgun and Kumbasar, 2000), leading to some degree of uncertainty as to whether high arginase is consistently found in MD patients. We did not find any significant differences in serum levels of ornithine between MD subjects and HCs (see chapter 4 in this thesis).

Previously, our laboratory determined that platelet eNOS activity is significantly decreased in MD patients (Chrapko et al., 2004). Those results are consistent with the current findings of decreased L-arginine availability in MD patients, as a reduction in eNOS activity may be a result of a decreased availability of the substrate for eNOS, L-arginine. A classical observation in enzymology is that when the amount of a substrate is significantly decreased, the activity of that substrate's enzyme is reduced as a result (Saghatelian et al., 2004). Our previous results of decreased eNOS in platelets associated with our current results of low L-arginine plasma levels are consistent with recently published findings by Pinto et al. (2012) who observed a decreased L-arginine influx in platelets in a small sample of MD patients (Pinto et al., 2012).

Intracellular L-arginine is responsible for NO production in the endothelium, and it may be questioned whether L-arginine serum levels reflect intracellular L-arginine. However, it has been suggested that serum levels of L-arginine do reflect intracellular L-arginine (Loscalzo, 2001) and that exogenous administration of L-arginine induces a functional increase in endothelial NO production.

A strength of our study is that we matched MD subjects and HCs for diet and non-modifiable and modifiable CV risk factors (lipid levels, blood glucose, blood pressure, BMI, physical activity level and current smoking and past chronic smoking) which have been shown to be associated with

decreased blood plasma NOx levels (Pearson et al., 2003). Results showing low L-arginine levels may indicate that supplementary L-arginine could be helpful beneficial for those patients with poor endothelial function. For example, Lekakis et al. (2002) have shown that, in hypertensive patients, oral L-arginine can improve endothelial dysfunction measured with flow mediated dilation via ultrasonography. Additionally, Kamada et al. (2001) performed an intravenous infusion of L-arginine to a patient who presented with a rare metabolic disorder preventing amino acid synthesis, including production of L-arginine. L-Arginine infusion induced an increase in NOx plasma levels and improved endothelial function (measured using flow mediated dilation) to parity with control subjects despite the initial vasodilation and plasma NOx level of the patient being approximately 70% that of HC subjects (Kamada et al., 2001).

Future investigations should also assess the impact of antidepressant medications on L-arginine and L-citrulline levels in HCs and MD patients. Indeed, we have previously observed that treatment with the SSRI antidepressant paroxetine normalized serum NOx levels in physically healthy MD patients (Chrapko et al., 2006), and van Zyl et al. (2009) have suggested that citalopram increases serum NOx levels in MD patients with coronary artery disease. Furthermore, a recent double-blind placebo controlled investigation has shown that the SSRI sertraline, but not placebo, improved NO-derived endothelial function in MD patients with coronary

artery disease as measured by flow mediated dilatation of the brachial artery (Pizzi et al., 2009).

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CHAPTER 3

Flow-Mediated Vasodilatation in Unmedicated Physically Healthy Patients with Major Depression

The author of this thesis played a major role in the study, including organizing research visits, data collection, data analysis, and writing of this chapter.

3.1. Abstract

BACKGROUND: Physically healthy patients suffering from major depression (MD) have an increased risk of developing cardiovascular disease (CVD). Vascular endothelial dysfunction, measured using flow-mediated dilatation (FMD) has been found to contribute to the development of CVD (Anderson, 2007). Numerous investigations have found that plasma levels of NO metabolites (NOx), an indirect measurement of endothelial NO production, are decreased in physically healthy MD patients. We sought to examine endothelial function in the brachial artery using FMD in a population of unmedicated physically healthy patients with MD.

METHODS: FMD involves determining brachial artery diameter via ultrasound before and after a stimulus condition that causes vasodilatation. Our sample consisted of 19 healthy controls (HCs) and 21 MD patients. Any known CV risk factors, with the exception of MD, were exclusion criteria.

RESULTS: FMD expressed as a percentage change in brachial artery diameter did not differ significantly between the MD and HC groups (0.82% ± 4.08% and 2.63% ± 4.91%, $p = 0.20$).

CONCLUSIONS: The lack of a significant difference in FMD between MD and HC groups is surprising considering the consistent results of decreased plasma NOx levels previously reported in physically healthy

MD patients. This may be accounted for by a compensatory mechanism. As our sample was relatively young, it is possible that MD is only progressively associated with endothelial dysfunction over time.

3.2. Introduction

Physically healthy patients suffering from major depression (MD) have been found to be at increased risk of developing cardiovascular disease (CVD) (Lichtman et al., 2008). However, the causes of increased CVD risk in MD patients remain unknown. Vascular endothelial dysfunction, associated with decreased endothelial nitric oxide (NO) production, has been found to contribute to the development of CVD (Anderson, 2007). Endothelial dysfunction has also been observed to predate symptoms of CVD by many years and it has been found to be predictive of future CVD (Schachinger et al., 2000). Endothelial dysfunction related to decreased NO production has also been shown to promote atherosclerosis through vasoconstriction, inflammation, platelet adhesion, and collagen breakdown (Vogel, 1999).

NO is the molecule responsible for regulation of endothelial function through modulation of vascular smooth muscle tone (Furchgott and Zawadzki, 1980). NO is produced by endothelial cells in the vasculature in response to increased shear stress due to an increase in local blood pressure caused by ischemia (Pyke and Tschakovsky, 2005). In endothelial cells, NO is synthesized from L-arginine (a conditionally

essential amino acid) and oxygen by endothelial NO synthase (eNOS) (McLeod et al., 2001).

Previously, our laboratory determined that the plasma concentration of NO metabolites (NOx), an indirect measurement of endothelial NO production, is markedly reduced in physically healthy non-medicated MD patients (Chrapko et al., 2004), a finding that was replicated by ourselves (Chrapko et al., 2006) and others (Selley, 2004; Arslan and Uzun, 2008; Garcia et al., 2011; Yapislar et al., 2011). Despite the compelling nature of these previous findings, NOx plasma levels are only indirect evidence of impaired endothelial function in MD patients.

Brachial artery flow-mediated dilatation (FMD) measured by an ultrasound (US) technique is the most commonly used assessment of endothelial function, i.e. NO-dependent vasodilatation. Typically, endothelium-dependent FMD is measured first in response to a hyperemic shear stress stimulus after brachial flow occlusion using a pressure cuff. After a rest period a smooth muscle dilator and NO-provider, such as nitroglycerin, is administered and dilatation is measured again, providing an assessment of endothelial-independent vasomotion (Corretti et al., 2002). Previous studies have suggested that FMD is significantly decreased in MD patients compared to HCs. However, there were many confounding factors in these previous studies. MD patients were taking a variety of psychotrophic medications capable of affecting endothelium function

(Rajagopalan et al., 2001; Broadley et al., 2002), or in the case of Wagner et al. (2009) there was an older population of only post-menopausal women with diabetes whose MD symptoms were in remission at the time FMD data were collected.

Our objective was to determine, using the FMD technique, whether endothelial-dependent FMD is impaired in unmedicated and physically healthy MD patients without any CV risk factors that could be associated with endothelial dysfunction.

3.3. Methods

Forty-one subjects with no known or suspected CVD participated in the study over the course of three and a half years. Nineteen healthy controls (HCs) were included in the study and they consisted of 10 females and 9 males. Twenty-two MD subjects participated and they consisted of 9 females and 13 males. There were no significant age differences between the groups of HC subjects and MD patients (23.53 ± 5.25 years and 30.41 ± 10.64 years respectively, $p = 0.14$). MD subjects had a mean episode length of 122.47 weeks, with the shortest recorded episode being 2 weeks and the longest being 520 weeks. The study was approved by the University of Alberta Health Research Ethics Board. All participants provided informed consent before entering the study.

All subjects were assessed for Axis-I disorders using the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental

Disorders Four, Text Revision (DSM-IV-TR) Axis-I Disorders. The HC group consisted of subjects without any history of Axis-I disorders. MD subjects had to currently meet DSM-IV-TR criteria exclusively for unipolar MD, and MD patients with any psychiatric comorbidity other than anxiety disorders were excluded. MD patients with comorbid anxiety disorders were included because of the high prevalence of comorbidity between MD and anxiety disorders (Rapaport, 2001). Six MD patients suffered from comorbid anxiety disorders.

Exclusion criteria for both groups included any illness or treatment known to influence endothelial function such as a Body Mass Index (BMI) > 30 kg/m^2, dyslipidemia, and high blood pressure as well as any family history of premature CVD (in the event that inherited CV abnormalities would influence our results). No current or past smokers were allowed in the study, as even light smoking has been shown to significantly impair endothelial function (Barua et al., 2002). None of the subjects were receiving or had received antidepressant treatment within at least 4 weeks of beginning the study (6 weeks in the case of fluoxetine because of its long half-life). All MD subjects had either discontinued medication voluntarily for 4 or more weeks prior to being screened for the study (n = 9) or had never taken antidepressant medications previously (n = 8). As well, subjects with conditions presenting a potential health danger (for example

low blood pressure in the context of nitroglycerin administration or suicide risk) were also excluded.

The severity of depressive symptomatology in each subject was assessed using the self-administered Beck Depression Inventory II (BDI-II). The 7-Day Physical Activity Recall Interview (Blair et al., 1985) as well as a semi-quantitative food frequency questionnaire (Brigham and Women's Hospital: Department of Nutrition, Harvard University, Boston, MA, USA, 1988) were used to assess physical activity and diet respectively to ensure that these variables did not significantly influence FMD. BMI was determined and blood pressure measurements and an electrocardiogram (ECG) were performed on all subjects.

Blood sampling was performed prior to US, and subjects were asked to fast for 12-16 hours beforehand. Blood analyses of both cholesterol types, i.e. Low Density Lipoprotein Cholesterol (LDL-C) and High Density Lipoprotein Cholesterol (HDL-C), and triglycerides were conducted by the University of Alberta Hospital Core Lab. Subjects were also asked to abstain from consuming trans-fats, vitamin C, and caffeine for >12 hours prior to the US procedure to minimize sources of variability in US measurements. The US procedure was conducted during the follicular phase for female subjects, as female menstrual cycle-related hormonal changes influence endothelial function (Majmudar et al., 2000).

During the US procedure, subjects lay in a supine position and ECG leads were placed on the subject to continuously monitor diastole and systole in order to control the diastolic timing of our US measurements. Doppler US using frequencies between 9 and 12 MHz was performed using a Phillips iU-22 US machine. ECG initiation was followed by a 10 minute rest period. After determining blood flow velocity and blood pressure, the resting diameter of the brachial artery was determined by measuring the inter-intima distance. All brachial artery diameters were determined by calculating the mean of five measurements of the inter-intima distance using the iU-22 imaging software.

All US procedures were carried out between 8 am and 11 am in a room kept at a standard temperature of approximately 20º Celsius. At the beginning of the US procedure, baseline brachial artery diameter was determined. Following baseline measurements, a pressure cuff was inflated on the subject's arm 2-3 cm proximal from the antecubital fossa to a pressure of 50 mm Hg above systolic pressure. Brachial artery diameter was measured 5 minutes after release of the cuff to determine the extent of endothelium-dependent artery dilatation.

Subjects were given a 10-minute rest after the reactive hyperemia described above, and this was followed by a second measurement of arterial diameter. After baseline measurements were taken, a spray of 0.4 mg sublingual nitroglycerin was administered to determine the extent of

endothelium-independent vasodilatation. After a 4-minute delay, brachial artery diameter was measured again after nitroglycerin administration.

The value of FMD was determined as a function of the percent change in arterial dilatation after reactive hyperemia. FMD was determined by subtracting the brachial artery diameter during reactive hyperemia from baseline artery diameter. Similarly, nitroglycerin-mediated dilatation was determined by subtracting the nitroglycerin condition from baseline artery diameter. Either result was then divided by the baseline diameter to calculate the extent of FMD or nitroglycerin-mediated dilatation expressed as a percentage change in brachial artery diameter. Reactive hyperemia brachial artery flow velocity was calculated by means of the standard formula already described elsewhere (Celermajer et al., 1992) using Doppler measurements to ascertain blood flow in ml/min.

Analyses were conducted using two-tailed independent t-tests. Pearson's R was used to determine correlations. A p-value of less than 0.05 was considered statistically significant for all statistical tests and was used as a probability level of type I error. All analyses were conducted using SPSS 16.0 (SPSS, Inc, Chicago, Ill, USA).

3.4. Results

Age, BMI, levels of lipids and triglycerides, 12-hour fasting glucose levels, systolic and diastolic blood pressure, physical activity (measured in total hours of moderate, hard, and very hard exercise per week), and

average metabolism (as measured by approximate number of kcal/day consumed) were not significantly different between MD and HCs (Table 3-1). No statistically significant correlation was found between BDI-II scores and the percentage change in brachial artery diameter in MD patients ($r = 0.055$, $p = 0.74$).

Mean baseline brachial artery diameter prior to reactive hyperemia was 3.69 mm ± 0.76 mm in MD patients and 3.51 mm ± 0.70 mm in HCs ($df = 39$, $t = 1.13$, $p = 0.97$). The percentage change in brachial artery diameter ± standard deviation induced by reactive hyperemia did not differ in MD patients compared to HCs (0.82% ± 4.08% and 2.63% ± 4.91% respectively, $p = 0.20$, Figure 3-1). There were no differences in reactive hyperemic velocity between MD (3.92 ml/min ± 2.88 ml/min) and HC groups (4.29 ml/min ± 2.12 ml/min, $p = 0.50$). The MD and HC groups did not differ in nitroglycerin-mediated dilatation (12.99% ± 7.39% and 17.70% ± 8.28%, $p = 0.10$).

3.5. Discussion

Our study found no differences in FMD between HCs and MD patients. These findings suggest that physically healthy, relatively young, unmedicated, and currently depressed MD patients do not exhibit decreased endothelial function when compared to HCs. Although there is evidence for deficient endothelial NO production reflected by reduced

TABLE 3-1. BASELINE CHARACTERISTICS OF THE SUBJECTS

Characteristic	Mean MD	MD SD	Mean HC	HC SD	p Value	t-value
Age (years)	30.41	10.64	23.53	5.25	0.14	2.56
BMI (kg/m^2)	23.26	4.02	21.97	3.00	0.29	1.08
Beck Depression Inventory	25.56	9.61	1.47	1.83	$p < 0.0001$	10.72
Physical Activity (kcal/kg/day)	43.49	11.71	41.86	8.04	0.64	0.47
Triglycerides (mmol\L)	1.04	.64	.87	.35	0.32	1.01
LDL-C (mmol\L)	2.82	.96	2.67	.61	0.58	0.55
HDL-C (mmol\L)	1.44	0.38	1.19	.32	0.50	2.00
Fasting Glucose (mmol\L)	4.82	.35	4.69	.43	0.38	0.90
Resting Systolic Blood Pressure (mm Hg)	107.94	12.09	104.84	9.29	0.39	0.88
Resting Diastolic Blood Pressure (mm Hg)	64.94	8.98	61.47	5.62	0.16	1.42
Mean Brachial Artery Diameter (mm)	.37	0.075	0.34	0.06	0.26	1.13
Apolipoprotein A1 (mmol\L)	1.55	.24	1.47	.24	0.45	0.77
Apolipoprotein B100 (mmol\L)	.84	.26	.71	.18	0.22	1.27
Hamilton D Score	14.63	7.80	.42	.77	$p < 0.0001$	7.92
Episode Length (weeks)	122.20	163.16	NA	NA	NA	NA

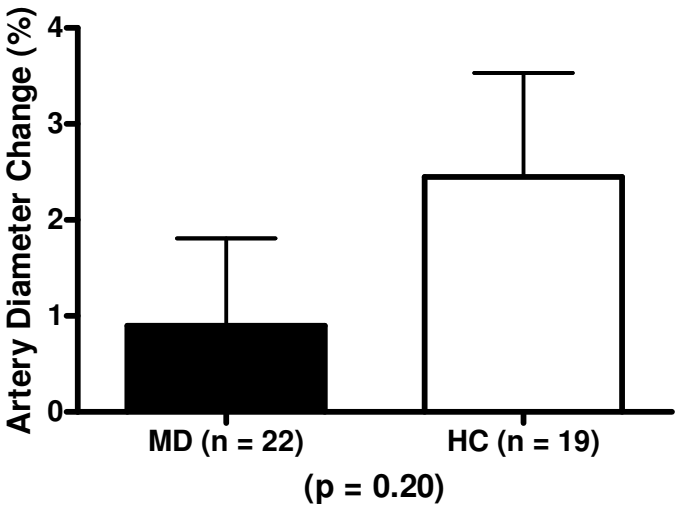

FIGURE 3-1. FLOW MEDIATED DILATATION

Flow mediated dilatation was not significantly different between the MD patients compared to HCs as measured by percentage change in artery diameter following reactive hyperemia (p = 0.20). Error bars represent Standard Error of the Mean.

plasma NOx in physically healthy MD patients (Chrapko et al., 2004; Chrapko et al., 2006; Garcia et al., 2011), we did not find that this translated into endothelial dysfunction measured by FMD in MD patients. These results are supported by the only investigation (Garcia et al., 2011) that concomitantly measured NOx plasma levels and FMD in a similar population of young and physically healthy unmedicated MD patients. Garcia et al. (2011) also found that although NOx plasma levels were decreased in MD patients, this did not result in lower FMD. Indeed, they found no difference in FMD between MD patients and matched HCs.

The study by Garcia et al. (2011) had a larger sample size (50 subjects in both MD and HC groups) and similar methodology, i.e. they matched HC and MD patients by age and gender and smoking status and excluded MD patients who were taking psychotrophic medication and who had comorbid CVD. We excluded patients with a history of smoking and we controlled for phase of the menstrual cycle.

Our results, along with those of Garcia et al. (2011), contradict previous results in MD patients without comorbid CVD. Rajagopalan et al. (2001) were early investigators of brachial artery FMD in MD patients. They excluded MD patients with conventional risk factors for CVD including obesity, diabetes, hypertension, hyperlipidemia, smoking, a family history of CVD, and hyperhomocysteinemia. Subjects were also close in age, with the HC group having a mean age of 31 ± 7 years and the

MD group having a mean age of 29 ± 10. However, the MD group was receiving various medications, as stated by the authors: "Patients were receiving medications, including antidepressants, at the time of the study" Rajagopalan et al. (2001) observed that FMD in MD patients was significantly decreased compared to HCs. However, the decrease in brachial artery diameter observed may be attributable to the various undocumented medications that were taken by their MD patients.

Broadley et al. (2002) measured brachial FMD in two independent samples of MD patients without CVD. In this study patients had a mean age of 39 in the MD group and 35 in the HC group. The authors excluded for traditional CVD risk factors including smoking, high cholesterol, hyperhomocysteinemia, hypertension, and diabetes. In a subsequent investigation (Broadley et al., 2006), MD patients were 40.1 years old and HCs 39.5 years old. Again, the authors excluded MD patients and HC subjects with CV risk factors such as smoking, high cholesterol, hyperhomocysteinemia and diabetes. In both cases, MD patients were taking various antidepressants. In the first publication (Broadley et al., 2002), MD patients had been taking various undocumented antidepressant medications for a minimum of 3 months and were in remission at the time FMD data was collected. In the second study, MD patients were depressed and were also taking various undocumented antidepressants. In both studies, brachial FMD was decreased in MD patients compared to HCs.

The medications used in all three studies mentioned above included a wide range of psychotropics including SSRIs, TCAs, MAOIs, norepinephrine reuptake inhibitors, and lithium. These medications have a wide variety of mechanisms and potential effects on the vascular endothelium, and they may alter FMD significantly. For example, we and others (Musselman et al., 2000; Le Melledo et al., 2009; van Zyl et al., 2009) have previously demonstrated that SSRI treatment alters plasma NOx levels in MD patients.

Another investigation examined brachial FMD in postmenopausal female patients with MD who were unmedicated while in remission with no known or suspected CVD (Wagner et al., 2006). FMD was found to be decreased in women with a history of MD compared to women who had never had an episode of MD. This study was well controlled and excluded any patient with angina, documented coronary artery disease, diabetes, current smoking, heart failure, or any other CV history. However, this study was performed in a very different population compared to ours. Subjects were only female, and much older (postmenopausal between 45 and 80 years old) and their MD was in remission at the time of the study.

Although both the current study and that of Garcia et al. (2011) found no difference between a well controlled population of MD and HC subjects in FMD, it should be noted that both our work and that of Garcia et al. (2011) used a relatively young sample. The mean ages of the MD and

HC groups in the Garcia et al. (2011) study was 22.60 ± 4.60 and 23.40 ± 4.80 years respectively. It is possible that endothelial dysfunction is due to duration of MD symptoms or antidepressant use rather than acute MD.

Secondary factors related to MD could contribute to increased CV risk over time. MD is often associated with poor health habits, including a diet that enhances CV risk, smoking, and lower adherence to aerobic exercise routines (Van Citters et al., 2010). It has been suggested that patients with MD have a greater risk of obesity (Cizza, 2011). A study by Yary et al. (2010) showed a link between severe MD and a diet high in cholesterol when a MD group of 120 patients was compared to a matched sample HC group. These chronic deleterious health habits could lead to increased CV risk over time, escalating with aging.

However, in the current study, MD patients with overtly negative health behaviours (for example, smoking, or a BMI over 30 kg/m^2) were excluded in order to examine the direct causal link between MD and CV risk. The results of our study and those of Garcia et al. (2011) may be due in part to the purposeful selection of a particularly healthy sample of MD patients.

Anderson et al. (2011) demonstrated that reactive hyperemia velocity and not FMD was correlated with CV risk in males, as shown in the Firefighters and Their Endothelium (FATE) Study. The FATE Study demonstrates that impaired FMD may not be as important in CVD as

previously thought. These results, in addition to our own, may indicate that impaired FMD may not necessarily be readily observable in a population at high risk for CVD. However, unlike the authors of the FATE study, we did not observe a difference in reactive hyperemia velocity between MD patients and HCs.

The consistent findings of low plasma NOx levels in MD associated with normal FMD in physically healthy patients with MD may suggest that a compensatory mechanism takes the place of NO in order to ensure endothelial dilatation continues to occur. For example, Wang (2009) demonstrated that H_2S is another molecule capable of causing dilatation of the vascular endothelium. The production of other EDRFs including prostaglandins, substance P, hydrogen peroxide and others might be upregulated by decreased endothelial NO production in MD patients (Barton, 2010; Qi et al., 2011). Such physiological changes could account for the lack of a difference in FMD when a group of young physically healthy MD patients is compared to a HC group. Whether these compensatory mechanisms continue to allow vasodilatation or deteriorate over long periods of time remains to be determined.

3.6. Conclusions

Our results suggest that FMD is not decreased in young, unmedicated, physically healthy MD patients without modifiable CV risk factors. Although NOx has been consistently found to be reduced in

physically healthy MD patients, decreased NOx plasma levels may not translate into an observable loss of endothelial function. The lack of decreased FMD when young MD patients are compared to young HCs may be due to the existence of yet unexplored compensatory mechanisms. Further research could prospectively observe how FMD evolves with age over long periods of time, controlling for health habits and medications.

3.7. Acknowledgements

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CHAPTER 4

SERUM LEVELS OF A VARIETY OF AMINO ACIDS IN PATIENTS WITH MAJOR DEPRESSION

The author of this thesis played a major role in the study, including research visits, data collection and analysis and writing of the chapter.

4.1. Abstract

INTRODUCTION: Several amino acids play an important role in brain function and mood including gamma-aminobutryic acid (GABA), glutamate, tryptophan, and tyrosine, and levels of some of these have been reported to be altered in patients with Major Depression (MD), although there is a great deal of controversy in the literature. In many cases, these studies were done in subjects in whom factors such as medication and sex were not taken into consideration. In the study described in this chapter, we have investigated levels of these amino acids and several others in serum from unmedicated, non-smoking MD patients and healthy controls matched for age, sex, lipid profile, exercise, and weight.

METHODS: MD patients (n = 35) not currently receiving antidepressant treatments and HCs (n = 36) were recruited. Blood serum levels of amino acids were measured, using an amino acid analyser or high performance liquid chromatography, after a 12-16 hour fast.

RESULTS: In the comparison of all MD subjects and all HCs, serum levels of arginine and citrulline (as described in chapter 2) were decreased in MD subjects relative to HCs and levels of cysteine ($p = 0.020$) and histidine ($p = 0.038$) were increased. When male MD subjects were compared to their corresponding controls, decreases in levels of arginine and citrulline were present, as was an increase in levels of cysteine, but levels of taurine ($p = 0.040$), aspartate ($p = 0.031$), glutamine ($p = 0.048$) and tryptophan ($p =$

0.029) were also significantly lower in the MD subjects. In contrast, when female MD subjects were compared with their corresponding HCs, the differences (as mentioned above in the male MD subjects) were no longer significant. Further statistical analysis indicated that in some cases the differences were due to differences in levels between male and female HCs and in some cases due to differences between levels in male and female MD subjects.

CONCLUSION: Considerable controversy exists in reported results of serum levels of amino acids in patients with MD. In the present comprehensive study, when dietary considerations, lipid profiles, obesity, age, exercise, smoking and sex-matching of HC and MD groups were taken into account, only a small number of amino acids were different between MD subjects and matched HCs. Significant differences between MD patients and HCs in serum levels of arginine, citrulline (lower in MDs), histidine and cysteine (higher in MDs) were noted. There was a marked difference between males and females when compared to their corresponding HCs, with differences in levels of the amino acid arginine, citrulline, taurine, glutamine, aspartate and cysteine evident in males but not females. This study emphasizes the importance of controlling for the above factors, particularly sex of the subjects when comparing serum levels of potential biomarkers such as amino acids.

4.2. Introduction

There are over 500 known amino acids, although most are artificial or not biologically relevant (Thompson et al., 1969). The structures of amino acids include an amine group, a carboxylic group and a defining functional group that may vary greatly (Bell et al., 2001). However, among the proteins that typically make up life, there are only 22 amino acids and these amino acids are coded for by deoxyribonucleic acid, with some exceptions.

Several related amino acids have important biological functions and prominent examples are arginine, citrulline (both involved in the production of NO) (Romero et al., 2006), GABA (a major inhibitory neurotransmitter) (Cooper et al., 1996; Szabo et al. 2009), glutamate (a major excitatory neurotransmitter) (Schoepfer et al., 1994; Coyle, 2009), glutamine (a precursor and metabolite of glutamate), tryptophan and tyrosine, precursors of the biogenic amine neurotransmitters 5-HT and catecholamines (dopamine and noradrenaline) respectively (Cooper et al., 1996), alanine and glycine (involved in metabolic interconversions with other amino acids as well as acting as coagonists at NMDA glutamate receptors) (Hassel and Dingledine, 2006), taurine (a proposed neuroprotective amino acid that acts as a trophic factor in CNS development, helps maintain integrity of membranes and regulates calcium transport and homeostasis) (Wu and Prentice, 2010) and ornithine (an amino acid involved in formation of

glutamate, citrulline, arginine and polyamines and implicated in a number of neurodegenerative disorders) (Wood, 2006; MacKenzie et al., 2007). Valine, leucine, isoleucine and histidine, termed large neutral amino acids (LNAAs), are involved in promoting healing of tissues and bone and in regulating metabolism and compete with tryptophan for transport into the CNS. Methionine reacts with adenosine triphosphate (ATP) to form S-adenosylmethionine (SAM), an important methyl donor in the body which has also been reported to have antidepressant properties in humans when administered as a supplement (Papakostas, 2009). Lysine, like most other amino acids, is a building block for protein but is also involved in Ca^{2+} absorption and is converted in the body to acetyl-CoA, the precursor for acetylcholine. Lysine has been reported to act through 5-HT receptors to reduce anxiety (Smriga and Torri, 2003; Smriga et al., 2002, 2004; Lakhan and Vieira, 2010). Cysteine is a powerful antioxidant that is metabolized in the body to glutathione, an important member of the body's toxic waste disposal system. N-Acetylcysteine, which is converted in the body to cysteine and then to glutathione, has been proposed as a possible efficacious drug in the treatment of addictions, schizophrenia, bipolar disorder and Alzheimer's disease (Dean et al., 2011). Serine is an amino acid converted to glycine metabolically and to the methyl donor SAM. Although aspartic acid is an excitatory amino acid in the brain (and like glutamate is interconverted metabolically to its corresponding amide,

asparagine), there is a paucity of information available about its possible involvement in mental illness. However, aspartate is converted in neurons to N-acetylaspartate, a neurochemical that appears to link lipid synthesis and energy production in the CNS (Moffett et al., 2007) and is used as a neuronal marker in neuroimaging studies.

For many decades, there have been studies reported in which serum levels of many amino acids have been investigated in the hope that they may reflect what is happening in the brain and/or be useful biomarkers for the diagnosis of MD and/or predicting best treatment options. Despite the numerous studies, overall the results have been disappointing and often contradictory. Reasons for the disparity in results include small numbers of subjects, not taking into account gender of the subjects or medications they were on, and generally poor matching of patients and HCs (age, smoking status, comorbidity of other disorders, exercise, etc.). In the study on the serum levels of the amino acids reported in this thesis, many of these factors were taken into account when designing the project.

4.3. Objective

The objective of the study reported in this chapter was to compare serum amino acid levels in a relatively large number of MD subjects and HCs matched for age and sex. The MD subjects were medication-free and factors such as lipid profiles, exercise and smoking were taken into account.

4.4. Methods

This study was approved by the University of Alberta Health Research Ethics Board. All participants provided informed consent before entering the study. Thirty-seven HCs were included in the study and they consisted of 16 females and 21 males. Thirty-five MD subjects participated and they consisted of 15 females and 20 males. The diagnostic criteria used and the factors controlled for and the blood sampling technique are summarized in the Methods section of Chapter 2.

Serum levels of all the amino acids except tryptophan were analyzed by the University of Alberta Hospital Core Laboratory using a Biochrom 30 Amino Acid Analyser following derivatization of the amino acids with ninhydrin (Moore et al., 1958).

A modification of the procedure of Grant et al. (2006) was used for analysis of tryptophan levels. Water and 50 µl serum samples were added to 150 µl of ice-cold methanol and the mixture was vortexed thoroughly and left on ice for 10 minutes. The samples were centrifuged for 4 minutes at 12,000 g at 4 ºC. The supernatant was retained, and a 5 µl aliquot and 5 µl of derivatizing reagent (2 mg N-isobutyryl-L-cysteine, 1 mg o-phthaldialdehyde dissolved in 0.1 ml methanol followed by addition of 0.9 ml 0.1 M sodium borate buffer) were mixed and held in the injection loop for 5 minutes prior to injection onto a Waters 2695 Alliance HPLC. Separation was carried out on a Symmetry C18 column (4.6 mm x 150 mm

x 3.5 mm) (Waters) coupled with a guard column of the same stationary phase. The column heater was set at 30 ºC and the sample cooler was held at 4 ºC. The flow rate of the mobile phase through the column was 0.5 ml/min with a gradient to provide adequate separation. Mobile phase A consisted of 850 ml of 0.04 M sodium phosphate buffer and 150 ml methanol, pH 6.2. Mobile phase B consisted of 670 ml of 0.04 M sodium phosphate buffer, 555 ml methanol and 30 ml tetrahydrofuran, pH 6.2. Initial conditions were 12% A, 88% B at 0.5 ml/minutes and final conditions were 100% B at approximately 20 minutes. The run time was 60 minutes for column washout and equilibrium. A Waters 474 fluorescence detector with an excitation wavelength of 344 nm and emission wavelength of 433 nm was employed in this assay.

4.5. Statistical Analyses

4.5.1. Analysis One

For all collected data (MD = 35, HCs = 37) represented in each of the observed variables of interest, the null hypothesis that data in the vectors MD and HCs were independent random samples from normal distributions with equal means and equal but unknown variances was tested using a two-tailed Student's t-test, with a critical p-value of 0.05. Before testing each test data vector was corrected for age using least-squares regression. Additionally, the correlation between each of the observed variables was calculated using pairwise Pearson's linear correlation

coefficient. The results are presented in the form of a Spring Embedded correlation plot. Here a network of "nodes" and "spring-edges" are constructed such that each node represents each of the tested variables and the spring constant of each edge is proportional to the correlation coefficient between two connected variables. The size of each node is proportional to significance of that variable and colour represents both the level of significance and whether the mean MD value was greater or lower than mean control value (Red = $p<0.05$ and MD>Control; Orange = $p<0.2$ and MD>Control; Dark Blue = $p<0.05$ and Control>MD; Light Blue = $p<0.2$ and Control>MD; Green = $p> 0.2$). Edges were only included in the network if the correlation was significant at a $p<0.05$ level. The correlation coefficient between two variables is labeled next to the corresponding edge. Once the network is constructed it is allowed to "relax". That is, the connected spring-edges compete against each other to pull the nodes in a given direction based on the spring constant (the higher the correlation, the stiffer the spring, and hence the more power organizing the clustering of the node). Once relaxed (i.e. the model is in a low energy configuration) the spring embedded plot can be viewed as a simple multivariate cluster analysis. Nodes clustered close to each other can be considered to be highly correlated in a multivariate sense. Networks are constructed using the graph visualization software – Graphviz (www.graphviz .org) using the *neato* virtual physics model (Ellson et al., 2004).

4.5.2. Analysis Two

Data were then split by gender and the above analysis repeated.

4.6. Results

Note: Most amino acids contain a chiral centre (examples of exceptions: GABA, glycine, taurine) and thus D- and L-isomers are possible. In humans, the D-isomers do not exist or are in very low concentrations in the case of almost all amino acids (D-serine is an example of an exception). Therefore in the rest of the thesis, where amino acids are mentioned it is assumed that I am referring to the L-isomer (or that no chiral centre is present in the molecule, e.g. GABA).

A summary of the amino acid levels in MD subjects and HCs is given in Table 4-1. The serum levels of the amino acids arginine and citrulline were lower in the MD subjects than in the HCs, as described in Chapter 2. As indicated in Figure 4-1, there were also differences in levels of histidine (increased) and cysteine (increased) in the MD subjects compared to HCs. A comparison of male and female subjects (Figures 4-2 and 4-3) in the MD and HC groups indicated that the differences in arginine, citrulline, and cysteine were apparent when comparing male MD and HC groups, but did not show up in the comparison of female MD subjects and HCs. In addition, significant decreases in levels of taurine, glutamine, tryptophan and aspartate were apparent in the comparison of male MD subjects and HCs, but not in the corresponding comparison of female MD

subjects and HCs. A further comparison showed no significant differences in levels of arginine, citrulline, cysteine, histidine, or aspartate between male and female HCs or between male and female MD subjects, i.e. these differences were only evident in male MDs compared to their HCs. However, with tryptophan, the differences appear to be due to significantly different levels of tryptophan between male and female HCs. A different effect is seen with taurine and glutamine, where HC levels are not significantly different between controls but levels of these amino acids are lower in male MD subjects than in female MD subjects

Age, BMI, lipid and triglyceride levels, 12-hour fasting glucose levels, systolic blood pressure, physical activity and average metabolism (as measured by kcal/day consumed) were not significantly different between MD patients and HCs (Table 2-1 in Chapter 2). Serum level ratios of tryptophan/LNAAs were not significantly different between MD and HC groups.

TABLE 4-1. SERUM AMINO ACID LEVELS

AMINO ACID	MD	± SD	HC	± SD
Taurine	79.12	36.49	88.55	55.69
Aspartic Acid	8.87	4.89	10.52	9.16
Threonine	135.41	28.46	134.33	28.68
Serine	119.50	22.04	119.76	31.42
Asparagine	44.58	10.64	45.27	14.53
Glutamic Acid	46.41	20.23	44.30	23.13
Glutamine	553.71	75.68	563.64	99.93
Glycine	277.09	74.79	271.09	74.54
Alanine	393.56	95.1	388.91	78.56
Gamma-Aminobutyric Acid	20.12	7.97	21.12	9.36
Valine	221.94	43.67	220.76	55.31
Cysteine	42.67	12.16	38.83	13.67
Methionine	24.85	6.09	25.03	4.90
Isoleucine	58.62	16.46	60.58	17.68
Tyrosine	62.32	20.9	59.58	11.40
Phenylalanine	53.00	9.95	56.70	12.86
Ornithine	59.73	23.03	72.76	23.81
Lysine	145.97	59.24	169.58	45.83
Histidine	118.53	59.63	89.21	22.42
Leucine	128.41	24.78	125.33	26.73
Tryptophan	71.58	2.10	65.95	3.25

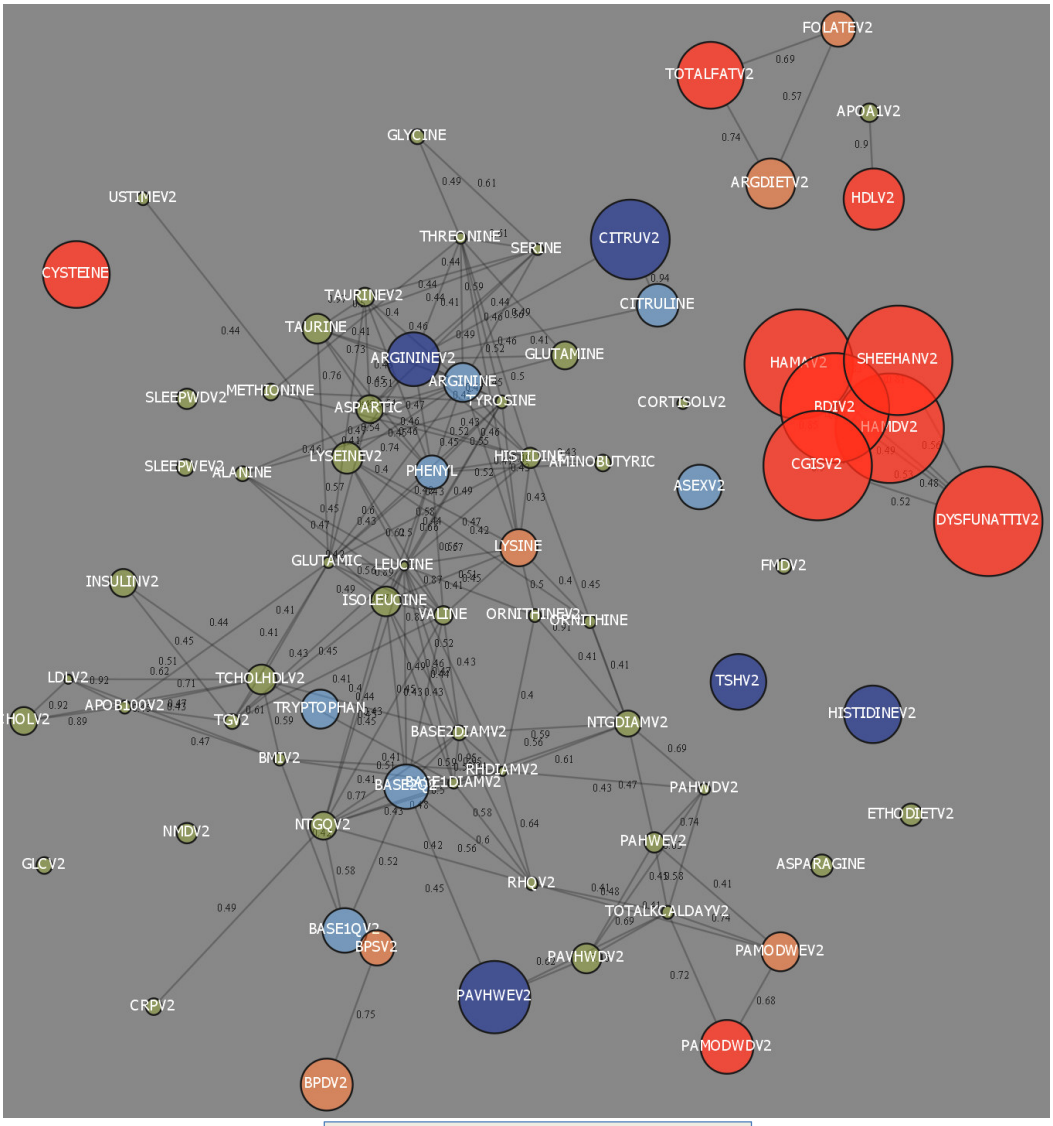
Blood serum amino acid levels (µmol/L) in MD subjects and HCs. Results are expressed as means ± SD.

FIGURE 4-1 (NEXT PAGE). SPRING PLOT AND DATA SET FOR ALL SUBJECTS (MD VS HCs).

CGISV2: Clinical Global Impression, BDIV2: Beck Depression Inventory, SHEEHANV2: Sheehan disability Scale, HAMDV2: Hamilton D Depression Scale, HAMAV2: Hamilton A anxiety Scale, DYSFUNATTIV2: Dysfunctional Attitude Score, CITRUV2: blood serum L-citrulline levels, PAVHWEV2: hard weekday physical activity, TOTALFATV2: total dietary fat intake, CYSTEINE: blood serum L-cysteine levels, HDLV2: high-density lipoprotein cholesterol, HISTIDINEV2: histidine, TSHV2: thyroid-stimulating hormone, ARGININEV2: blood serum L-arginine, PAMODWDV2: moderate physical activity on week days. Case refers to MD patients.

Variable	MD n	HC n	MD missing	HC missing	t-test	Mean Sign
CGIS	35	37	5	0	1.16E-18	1
BDI	35	37	6	0	1.8E-14	1
SHEEHAN	35	37	6	0	7.37E-14	1
HAMD	35	37	6	0	1.8E-11	1
HAMA	35	37	7	0	2.16E-10	1
DYSFUNATTI	35	37	5	1	2.22E-05	1
CITRU	35	37	4	1	0.007752	-1
PAVHWE	35	37	6	0	0.014177	-1
CYSTEINE	35	37	0	1	0.020474	1
HDL	35	37	5	1	0.027915	1
HISTIDINE	35	37	5	3	0.037518	1
TSH	35	37	8	6	0.040854	-1
ARGININE	35	37	0	1	0.047739	-1
PAMODWD	35	37	6	0	0.04925	1

FIGURE 4-1.



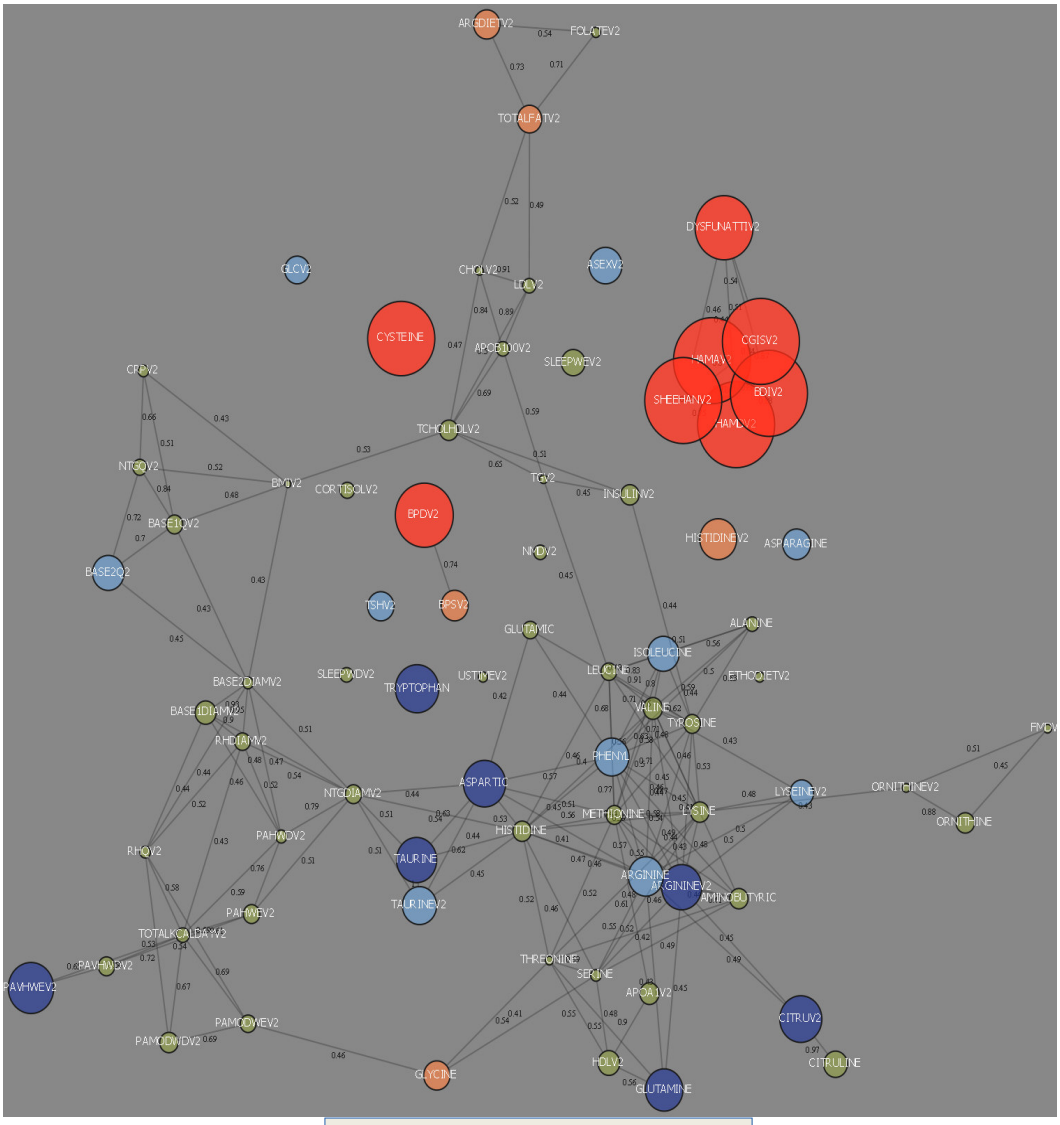
Spring Plot
Complete Data Set
(continuous variables only)
RED = significant increase in Cases ($p<0.05$)
ORANGE = non-significant increase in Cases $p<0.2$
Dark BLUE = significant decrease in Cases ($p<0.05$)
Light BLUE = non-significant decrease in Cases $p<0.2$
Green = no change
Size of node ≈ significance (ttest)
Line = correlation coefficient (Pearson's)

FIGURE 4-2. SPRING PLOT AND DATA SET FOR MALE SUBJECTS (DEPRESSED VS HCs)

Abbreviations are defined in Figure 4-1. BPD: diastolic blood pressure, PAVHWE: hard physical activity week ends, TRYPTOPHAN: blood serum levels of tryptophan, ASPARTIC: aspartic acid (aspartate), CITRU: blood serum L-citrulline, TAURINE: blood serum taurine levels, GLUTAMINE: blood serum glutamine. Case refers to MD patients.

Variable	MD n	HC n	MD missing	HC missing	t-test	Mean Sign
CGIS	20	21	4	0	8.02E-09	1
BDI	20	21	4	0	3.1E-08	1
SHEEHAN	20	21	5	0	6.89E-07	1
HAMD	20	21	5	0	1.64E-05	1
HAMA	20	21	6	0	2.89E-05	1
CYSTEINE	20	21	0	1	0.002868	1
PAVHWE	20	21	5	0	0.022507	-1
TRYPTOPHAN	20	21	0	6	0.028962	-1
ASPARTIC	20	21	0	1	0.03129	-1
CITRU	20	21	3	1	0.034345	-1
ARGININE	20	21	0	1	0.036491	-1
TAURINE	20	21	0	1	0.040004	-1
GLUTAMINE	20	21	0	1	0.048318	-1

FIGURE 4-2.



Spring Plot
Complete Data Set
(continuous variables only)
RED = significant increase in Cases ($p<0.05$)
ORANGE = non-significant increase in Cases $p<0.2$
Dark BLUE = significant decrease in Cases ($p<0.05$)
Light BLUE = non-significant decrease in Cases $p<0.2$
Green = no change
Size of node ≈ significance (ttest)
Line = correlation coefficient (Pearson's)

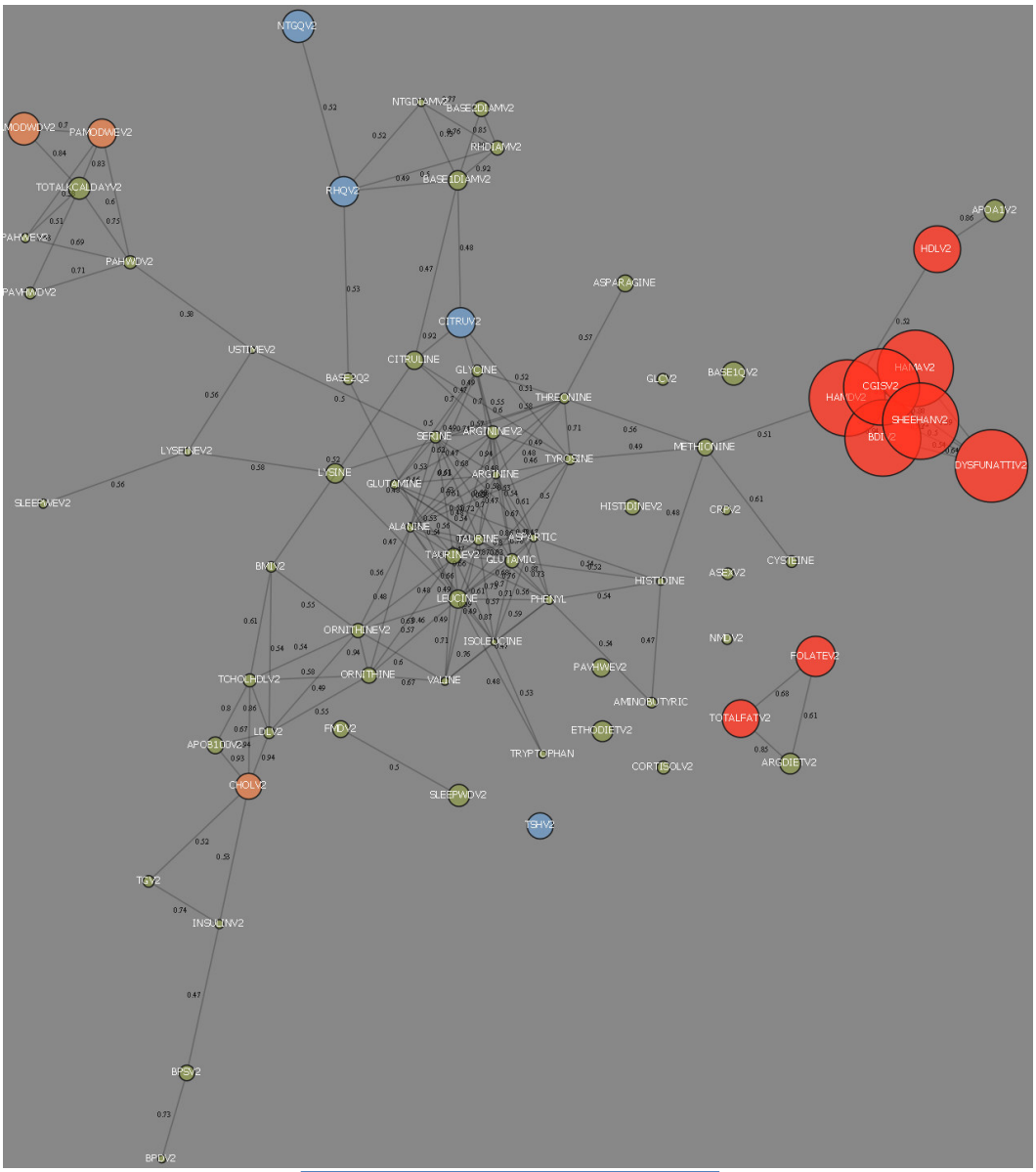
FIGURE 4-3. SPRING PLOT AND DATA SET FOR FEMALE SUBJECTS (DEPRESSED VS HCs)

Abbreviations are defined in Figure 4-1 with the addition of FOLATE: dietary levels of folate. Case refers to MD patients.

Variable	MD n	HC n	MD missing	HC missing	t-test	Mean Sign
CGIS	15	16	1	0	7.62E-10	1
SHEEHAN	15	16	1	0	5.15E-08	1
HAMD	15	16	1	0	3.82E-07	1
BDI	15	16	2	0	5.92E-07	1
HAMA	15	16	1	0	3.24E-06	1
DYSFUNATTI	15	16	1	0	0.001479	1

No significant differences in amino acid levels between female MD subjects and female controls were observed.

FIGURE 4-3.



Spring Plot
Complete Data Set
(continuous variables only)
RED = significant increase in Cases (p<0.05)
ORANGE = non-significant increase in Cases p<0.2
Dark BLUE = significant decrease in Cases (p<0.05)
Light BLUE = non-significant decrease in Cases p<0.2
Green = no change
Size of node ≈ significance (ttest)
Line = correlation coefficient (Pearson's)

4.7. Discussion

4.7.1. Major Depression and Blood Serum Amino Acids

Although numerous studies on serum levels of amino acids in MD subjects are reported in the literature, there are many inconsistencies among those findings (e.g. Xu et al., 2012; Maes et al., 1998; Fu et al., 2012; Pinto et al., 2012) (see also Table 1-1 in Chapter 1). Several studies examining serum amino acid levels among MD patients purport to have identified an amino acid or ratio of amino acids to one another as biomarkers for MD that can be simply and easily measured and correlated with psychiatric symptoms. However, the lack of consistency among findings of different studies makes such a conclusion difficult to confirm.

Major depressive disorder occurs more commonly in women than men (Kessler et al., 1993; Weissman et al., 1996; Nolen-Hoeksema et al., 1999). A reciprocal relationship between obesity and depression has been reported, with obesity found to increase risk of depression and depression reported to be predictive of developing obesity (Luppino et al., 2011). Several comprehensive studies have shown that tobacco smoking is a risk factor for depression (e.g. Pasco et al., 2008; Flensberg-Madsen et al., 2010) and can also affect NO levels (see Chapter 2). Although the benefits of exercise in depression are difficult to assess, a growing body of evidence supports the efficacy of exercise as an adjunct treatment of depression (Craft et al., 2004).

My results indicate that in this study in which the above factors were taken into consideration, serum levels of arginine and citrulline were decreased (Chapter 2), while levels of cysteine and histidine were increased in the overall group of MD subjects relative to HCs. A further comparison of the results in male and female groups indicated that the significant differences in levels of arginine, citrulline, and cysteine showed up only in the male MD group versus their HC group. An analysis of the male group also indicated that the male MD subjects also showed decreases in levels of taurine, glutamine, tryptophan and aspartate compared to their HCs; these differences were not present when the female MD subjects were compared to their corresponding HCs.

The relevance of the decreased levels of arginine and citrulline are discussed in Chapter 2 of this thesis. The increased serum levels of histidine and cysteine found in the MD group in the study reported in this thesis are interesting. These amino acids have not been investigated extensively in the literature, but the current results suggest that further investigation is warranted. Histidine and histamine H1 receptor agonists have been reported in preclinical studies to have antidepressant effects (Lamberti et al., 1998). However, other researchers (Ito, 2000; Kumar et al., 2007; Haas et al., 2008) have reported that histidine, histamine and H1 receptor agonists can produce anxiety in animal models while Shan et al. (2013) reported no significant changes in the histaminergic system in postmortem brain tissue

from MD subjects compared to controls. The increase in levels of the antioxidant amino acid cysteine in MD patients may be a reaction to the oxidative stress that appears to occur in depression and a number of other psychiatric disorders (Dean et al., 2010; Scapagnini et al., 2012). Cysteine is converted in the body to the universal antioxidant glutathione (Holdiness, 1991; Lavoie et al., 2008) and is involved in the regulation of neuronal intra- and extracelluar exchange of glutamate (Himi et al, 2003; Janaky et al., 2007), an amino acid whose activity has been proposed to be altered in MD (Zarate et al., 2010; Mitchell and Baker, 2010; Sanacora et al., 2012).

Maes et al. (1998), comparing treatment-resistant MD patients and HCs, found lower taurine serum levels in MD patients, similar to our findings in male MD patients, but Altamura et al. (1995) reported higher taurine levels in MD subjects compared to HCs. Maes et al. (1998) did not find a significant difference between MD and HC groups with regard to glutamine levels (we found a decrease in male MD patients), but after 5 weeks of antidepressant treatment, glutamine levels in their study were higher in the MD patients than prior to the 5-week treatment program. Activity of glutamine synthetase, the enzyme involved in synthesis of glutamine, has been reported to be decreased in MD (Choudhary et al., 2005), and glutamine deficiency in prefrontal cortex of male mice increases depressive-like behaviours (Lee et al, 2012). As indicated above, I found lower tryptophan levels in MD subjects than in HCs, but only in the male

group. Xu et al., (2012) also reported lower tryptophan levels in unmedicated MD patients compared to HCs, but did not compare male and female groups.

As mentioned ealier in this chapter, the differences in tryptophan levels are a result of differences in levels between male and female HCs while with taurine and glutamine the difference is between female and male MD subjects.

The findings reported in this chapter emphasize particularly the importance of taking into account sex of the subjects when doing studies on serum acids as possible biomarkers in MD. With regard to these possible male-female differences, these should take into account not only comparisons between MD subject themselves but also between the male and female HC groups used.

4.8. Acknowledgements

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4.9. References

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CHAPTER 5

General Discussion

5.1 Overview of Results

The initial studies described in this thesis were conducted in order to further investigate neurochemical/physiological abnormalities in MD patients that might be linked to CVD risk. Initially this involved studying serum levels of the amino acids arginine and citrulline in MD subjects, and it was shown that serum levels of both of these amino acids involved in the synthetic pathway for NO were reduced in MD subjects compared to HCs. Because of the strong links between MD and CVD and the possible link involving NO, a second messenger that is involved in regulation of the vascular endothelium, we decided to conduct a brachial FMD study using MD subjects and HCs since this is an indirect measurement of the function of the vascular endothelium, which in turn is dependent on the production and availability of NO. However, FMD did not differ significantly between the MD and HC groups.

In each of the investigations mentioned above, physically healthy, unmedicated, non-smoking MD patients were studied and the groups were matched for age and sex. The serum samples from those patients participating in the study on arginine and citrulline were further analyzed for a number of other amino acids that have been reported in the literature to be possible biomarkers in MD. However, most of those studies did not include patients whose cardiovascular risk factors were as well controlled for as in our studies. Other than the decreases in levels of arginine and

citrulline the only changes observed in serum amino acid levels in the overall comparison of MD patients and HCs were increases in histidine levels and cysteine levels in MD subjects. A further analysis broken down into male and female MD subject groups compared to corresponding HCs showed that these changes observed in arginine, citrulline and cysteine levels in the total group were attributable to differences in the male subjects compared to their corresponding controls. In addition, further statistical analysis showed decreases in serum levels of taurine, tryptophan, aspartate and glutamine that were detectable in male but not in female MD subjects.

5.2. Serum Levels of L-Arginine and L-Citrulline in Major Depression

Previously, nitric oxide metabolites (NOx) had been shown to be significantly lower in an unmedicated group of MD patients compared to HCs (Chrapko et al., 2004). The lower L-arginine and L-citrulline levels in the present study may indicate that abnormalities in metabolism of these amino acids are responsible for lower NO production. Pinto et al. (2012) have also demonstrated significantly decreased L-arginine serum levels in MD patients. Lower NOx in blood serum suggests dysregulation in L-arginine availability. Because L-citrulline is an end product of NO production (produced by the action of NOS on arginine), significantly lower serum levels of L-citrulline may be an indicator of decreased NO production resulting from low L-arginine.

The deficiency of a substrate is well known to cause a corresponding reduction in the activity of its enzyme (Saghatelian et al., 2004), and Chrapko et al. (2006) have demonstrated reduced eNOS activity in MD. In addition to showing that serum levels of L-arginine are decreased significantly in a MD patient group compared to HCs, Pinto et al. (2012) demonstrated significantly decreased NOx in blood serum of MD patients. These results match previous work done by Chrapko et al. (2004) and Ikenouchi-Sugita et al. (2009) who also demonstrated significantly decreased NOx among MD patients when compared to HCs. Papers by Garcia et al. (2011) as well as Zhuo et al. (2011) showed significantly decreased NOx in MD patients. Both Garcia et al. (2011) and Zhuo et al. (2011) also demonstrated no significant difference between MD and HC subjects in FMD in MD patients.

These results suggest that the L-arginine pathway involving NO production plays a role in MD and the high risk for CVD in MD. While there are several suggested mechanisms which might cause a reduction of L-arginine levels in blood serum (increased activity of arginase, decreased tetrahydrobiopterin, increased asymmetric dimethylarginine), it remains unclear as to why blood serum levels of L-arginine are reduced in MD patients.

5.3. Flow Mediated Dilatation in Major Depression Subjects

The third chapter presented in this thesis represents an investigation of FMD in unmedicated (at the time of study participation) and physically healthy MD patients. Our results suggest that although the risk of CVD has been shown to be increased in MD patients (Vieweg et al., 2010), there is not necessarily an observable change in endothelium function as measured by FMD of the brachial artery. The endothelial dysfunction observed in other studies may therefore be due to traditional CVD risks or even antidepressant use, and not MD specifically. No endothelial dysfunction was observed in this study when MD patients and HCs were well-matched for other CVD risk factors, including Body Mass Index (BMI), smoking, and several other factors that modulate CVD risk.

Rajagopalan et al. (2001), Broadley et al. (2002, 2006) and Wagner et al. (2009) have previously reported decreased FMD in MD patients, compared to HCs. As well, a meta-review by Cooper et al. (2011) concluded that MD symptoms and symptom severity lead to impaired FMD. However, all of these studies involved MD patients who were receiving various medications or who had CV risk factors (post menopausal status, diabetes, erectile dysfunction, etc). In the study by Cooper et al. (2011), no account was taken of the fact that the papers used in the review all utilized patients receiving medication or with confounding medical conditions.

Rajagopalan et al. (2001) found that the change in artery diameter after reactive hyperemia was approximately 4% in the MD group and 9% in the HC group, resulting in a statistically significant difference between the two groups. These results were demonstrated in a sample of MD patients having a brachial artery thickness of 3.60 ± 0.01 mm and HCs having a mean brachial artery thickness of 3.67 ± 0.01 mm, indicating that the subjects had similar physical properties between groups. However, there are several factors that may have contributed to decreased FMD in MD patients. For example, the authors used a sample that included 11 females for every 4 males in their study. Having a sample that was primarily female as well as the use of unspecified antidepressant medications among the subjects may have influenced the results in this study of Rajagopalan et al. (2001).

The next publication to demonstrate impaired FMD in MD patients was that of Broadley et al. (2002), who used a sample that was well balanced for demographics and without extraneous factors such as CVD risk markers or disease. However, in that study, it is again possible that the impairments in FMD were a result of the multiple medications that were used by the MD patients. Medications used in that study included SNRIs, SSRIs, and supplemental medications such as lithium. Two patients were reported to not be compliant with their medication regimes throughout the

study, raising the question of exactly how much variance existed in medication regimes in that particular MD patient population.

Another study demonstrating impaired FMD in MD patients was conducted by Broadley et al. (2006). The authors lowered cortisol levels using metyrapone, a competitive inhibitor of the conversion of 11-deoxycortisol to cortisol by 11-beta-hydroxylase, in an attempt to improve FMD following reactive hyperemia. The MD patients in the study had a history of recurrent MD comprising at least two episodes of at least moderate severity. The MD patients were not receiving medication throughout the study. These authors showed that at baseline, a sample of 30 MD patients, when compared to 36 matched HC subjects, showed significantly impaired FMD, at -1.27% compared to 4.37% respectively. Broadley et al. (2006) excluded all subjects with non-modifiable CV risk factors including a history of CV disease as well as modifiable risk factors such as smoking, evidence of diabetes mellitus and high plasma cholesterol. Once metyrapone, a cortisol synthesis inhibitor, was administered and lowered cortisol levels, FMD was no longer significantly lower in MD patients compared to HCs.

Broadley et al. (2006) actually excluded for medication use among the MD patients and they included a sample with a mean age of 40.1 ± 1.88 years for the MD group and 39.5 ± 1.70 for the HC group. However, in the MD group, patients were 27% male while 47% of the HC group was male.

Gender differences may have influenced the FMD results. However, subjects were matched for BMI, baseline brachial artery diameter (in mm) as well as cholesterol levels and blood pressure.

Recent studies investigating an unmedicated MD population showed no significant difference in FMD between a group of MD and HCs (Garcia et al., 2011; Zhuo et al., 2011). Similar to publications such as that of Chrapko et al. (2004), Garcia et al. (2011) found decreased NOx in MD patients despite a lack of difference in FMD between MD and HC groups. Garcia et al. (2011) also used a relatively large sample of 50 MDs and 50 HCs, and both groups consisted of 32% male subjects. In contrast to Broadley et al. (2006), Garcia et al. (2011) recruited a sample with a mean age of 22.6 ± 4.60 for the MD group and 23.4 ± 4.80 for the HC group, approximately 20 years less than the group used by Broadley et al. (2006).

A potential issue with the publication by Garcia et al. (2011) is that smokers were allowed into both the MD and HC groups. Including smokers may have reduced the mean FMD observed in the HC group. Both HC and MD groups smoked the same mean number of cigarettes per day (2.77 ± 2.61 for the MD group and 2.72 ± 2.27 for the HC group), with the MD group consisting of 18% smokers and the HC group consisting of 24% smokers. The larger proportion of HC patients who smoked could have decreased the mean FMD in the HC group compared to the MD group. While Garcia et al. (2011) had similar findings to the FMD study in Chapter

3 of this thesis, the results in Chapter 3 confirm the findings of non-significant FMD differences between MD patients and a matched sample of HC controls using a cleaner and better balanced sample.

While there are several factors that could explain the lack of difference between MD and HC groups in FMD (sex imbalance, the presence of smokers, etc) in the paper published by Garcia et al. (2011), Zhuo et al. (2011) found similar results to those of Garcia et al. (2011). Zhuo et al. (2011) demonstrated both a lack of significantly decreased FMD in MD patients and a significant decrease in NO (as measured by NOx from blood serum). Zhuo et al. (2011) utilized a sample of antidepressant-naïve MD patients with a mean age of 31.6 years and a sex ratio of 15 males to 9 females. As with Broadley et al. (2006) and Garcia et al. (2011), the subjects were not receiving medication at the time of the study. The fact that the gender imbalance favours males in the study of Zhuo et al. (2011) while the imbalance in Garcia et al. (2011) study favours females may indicate that gender is not related to the FMD results seen in both papers as both have similar results. Also, the results in Chapter 3 of this thesis were derived from groups that were evenly matched for male and female subjects in the MD and HC groups while demonstrating the same statistical results of Zhuo et al. (2011) and Garcia et al. (2011). In conclusion, despite the findings of previous work on FMD in MD patients, all previous

publications showing a decrease in FMD among MD patients failed to control for a number of important factors.

It is possible that extended episodes of MD may lead to endothelial dysfunction, demonstrating an interaction with age. The MD sample recruited in my study may have been too young to exhibit deterioration in FMD function, having a mean age of 28.51 ± 10.18 years. Previous publications appear to also support the idea of deterioration in endothelium function over time. For example, Broadley et al. (2006), with a mean age of 40.1 ± 1.88 in the MD patient group, demonstrated a significant decrease in FMD in MD patients when compared to HCs. In contrast, Garcia et al. (2011) had a mean age of 22.6 ± 4.60 years in the MD group while Zhuo et al. (2011) had a mean age of 31.6 ± 4.30. Both Garcia et al. (2011) and Zhuo et al. (2011) showed no significant difference between MD and HC groups in FMD.

There is also evidence that long-term MD symptoms may contribute to higher BMI, poorer lifestyle choices (Beydoun and Wang, 2010) and poor medication compliance (Kruisdijk et al., 2012). As a result, it may be the case that lifestyle factors associated with MD induce degradation of endothelial function over time (Yary et al., 2010). For example, continuing a health-promoting behaviour such as aerobic exercise may be advantageous for decreasing CVD risk over time. Previous findings have

shown that aerobic exercise training over a period as short as 8 weeks can also increase blood plasma levels of NOx significantly (Maeda et al., 2001).

5.4. Additional Serum Amino Acids in Major Depression

Chapter 4 of this thesis deals with the possibility of using serum levels of a number of physiologically important amino acids other than arginine and citrulline as potential biomarkers for MD. There is a great deal of controversy in the literature about this topic, and our results support the view that in well controlled studies serum levels of many of these other amino acids are not useful biomarkers of MD. Our findings of the marked differences when male and female subjects were compared with their corresponding HCs emphasize the importance of doing male-female comparisons when conducting studies on MD and of comparing amino acid levels not only in the MD subject groups but in their corresponding HCs. It has been well documented that rates of MD in females are approximately twice those in males (Nolen-Hoeksema, 2001; Weissman et al., 1993), but there are very few studies comparing markers between males and females.

The findings on cysteine in the study on other serum amino acids in MD are very interesting since N-acetylcysteine, which is converted in the body to cysteine and then the neuroprotectant glutathione, has been reported to have antidepressant properties (Berk et al. 2008), and future studies on MD subjects should include not only take measurement of cysteine but of glutathione as well. There is limited clinical information

available with regard to the involvement of histidine in MD, but it has been reported to have antidepressant-like activity in the forced swim test in rats (Lamberti et al, 1998) and it is a precursor of histamine, an amine that has been proposed to be hypofunctional in MD (Kano et al, 2004). The deficiency of glutamine in the male MD subjects relative to their HCs is interesting since Maes et al. (1990) reported low serum glutamine levels in treatment-resistant MD patients, and the enzyme that converts glutamate to glutamine (glutamine synthetase) has been reported to be reduced in MD subjects (Chaudhary et al., 2005). Studies with glutamine in mice suggest that glutamine deficiency can result in an analogue of MD symptoms (Lee et al., 2007, 2012). The decreased serum tryptophan levels in the male MD subjects is also of interest since tryptophan is the precursor for serotonin, a biogenic amine which is generally agreed to be functionally deficient in MD (Delgado, 2006). Aspartate levels have been reported by other groups to be lower in unmedicated MD subjects than in controls (Maes et al., 1998; Fu et a., 1992). Taurine is of interest because of its reported trophic and neuroprotective effects (Wu and Prentice, 2012), but previous studies on levels of this amino acid in serum and platelets have reported higher levels in MD subjects than in controls (Altamura et al., 1995; Mauri et al., 1998; Maes et al., 1998).

5.5. Future Directions

Although there is no evidence from the current study that endothelium function is impaired in MD patients compared to a HC sample, in the future it may be valuable to examine a variety of factors related to FMD and MD. For example, a longitudinal study might examine the effects of lifestyle factors that could lead to degradation of endothelial function (and thus, impaired FMD) over time. Such a longitudinal study would take several years or decades and should compare the FMD of a chronic MD-suffering population and a HC population.

Additionally, the mystery of why FMD is not decreased in MD patients relative to a HC sample remains, but there are several possible explanations. As mentioned previously, the lack of a difference between MD and HC subjects might be explained by age, in that deterioration of the endothelium is more pronounced in MD patients over time as opposed to healthy individuals who have never suffered from MD. Therefore, a longitudinal study examining FMD in MD patients may reveal how the relationship between MD and endothelial dysfunction develops over time.

Another important area is that of the status of alternate vasodilators in MD patients. There are a variety of mechanisms capable of dilating the endothelium and it is possible that several of these may be upregulated in response to the downregulation of NO production. These include the endothelial relaxing factors (EDRFs) other than NO and the endothelial

hyperpolarizing factors (EDHFs). EDRFs that may take the place of NO in MD patients include H_2S, neuropeptides and many other potential molecules that have not as of yet been thoroughly investigated. Using the example of H_2S, it has been found that H_2S is produced in the endothelium alongside NO using L-cysteine as opposed to L-arginine by the enzyme cystathionine γ-lyase (CBS) (Wang, 2009). Unlike NOS, which has been shown to be reduced in MD patients compared to matched HCs (Chrapko et al., 2004), no known papers have been published on variations in CBS activity or H_2S synthesis in the vascular endothelium of MD patients.

As observed in the study in chapter 2, levels of L-arginine and L-citrulline are decreased in MD patients. Further studies on factors affecting the availability of arginine are now warranted. It is interesting that citrulline is both a precursor and a metabolite of arginine, and future studies on MD should include investigations on the activity of arginosuccinate synthetase and arginosuccinate lyase, the enzymes responsible for the synthesis of arginine from citrulline and arginase, another enzyme responsible for the catabolism of arginine. However, as indicated earlier in this thesis, blood serum levels of L-ornithine are used as an indicator of arginase activity in humans (Elgun and Kumbasar, 2000), and I did not find any differences in L-ornithine serum levels between the MD and HC groups.

The dramatic differences between male and female MD subjects relative to their corresponding HCs also indicate that future comprehensive

studies on male versus female MD subjects and their corresponding matched controls with regard to serum levels of amino acids are definitely warranted.

5.6. References

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