Endogenous markers of nitric oxide in the Flinders sensitive line (FSL) rat, a genetic animal model of depression

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Abstract

The rising number of the population that present with major depressive disorder has intensified the need to identify and elucidate new biological markers for the diagnosis and treatment of depression. Depression presents with evidence of changes in the nitric oxide (NO) pathway. In this study, levels of various endogenous markers of the NO cascade, viz. nitrite (NO_2^-), asymmetrical dimethylarginine (ADMA) and arginase II activity, were investigated in the Flinders Sensitive Line (FSL) rat, a genetic animal model of depression.

The aim of the current study was to determine if there are differences between these markers in the plasma of the FSL rat compared to its healthy control, the (Flinders Resistant Line) FRL rat, with the possibility of considering their use as biomarkers of depression. Nitrite was chosen as metabolite over nitrate (NO_3^-) because the dietary intake of nitrite and/or nitrate does not significantly affect nitrite (NO_2^-) levels in plasma. Although this is of no significance if applied to rats, it is an important factor to be considered when doing clinical studies.

For neurochemical determination of nitrite a sensitive fluorometric reversed phase high-performance liquid chromatographic (HPLC) assay was developed to analyze nitrite in human and rat plasma. Derivatization of sample nitrite was performed with 2,3-diaminonaphthalene (DAN) followed by the quantification of the stable and highly fluorescent product, 2,3-naphthotriazole (NAT).

Determination of arginase II activity was performed by measuring L-arginine and L-ornithine concentrations in the plasma, while ADMA was measured simultaneously with L-arginine and L-ornithine using liquid chromatography/tandem mass spectrometry, or LC/MS/MS.

Plasma nitrite levels of FSL rats were significantly decreased compared to plasma nitrite levels in the FRL rat, but neither the levels of ADMA nor arginase II activity showed a significant difference between the FSL and FRL rat groups. From these results it is concluded that in accordance with previous studies, the NO pathway plays an important role in the pathophysiology of depression, as depicted in the differences found between plasma nitrite levels in the FSL rat compared to its healthy control.

Keywords: *nitric oxide pathway; nitrite; arginase II activity; ADMA; L-arginine; L-ornithine; Flinders Sensitive Line rat (FSL); depression;*

Opsomming

Na aanleiding van die toenemende aantal van die populasie wat met major depressie presenteer, word dit al hoe belangriker om nuwe neurobiologiese teikens te identifiseer vir hulp met die diagnose en behandeling van depressiewe pasiënte. Veranderinge in die stikstofoksied (NO) weg is een van die fisiologiese eienskappe kenmerkend van depressie. Die huidige studie behels die bepaling van die vlakke van nitriet (NO_2), asimmetriese dimetielarginien (ADMA) en die aktiwiteit van die arginase II ensiem as endogene merkers van stikstofoksied in ’n genetiese dieremodel van depressie, die sensitiewe lyn Flindersrot (FSL).

Die doel van hierdie studie behels die bepaling en vergelyking van die bogenoemde merkers in die plasma van die FSL rot met sy gesonde kontrole, die resistente lyn Flindersrot (FRL), met die moontlikheid om genoemde merkers as biomerkers vir depressie te gebruik. Aangesien die nitriet en/of nitraat inhoud in ons daaglikse dieet nie die plasma nitriet vlakke beinvloed nie, is daar besluit om nitriet (NO_2^-) eerder as nitraat (NO_3^-) in die plasma te bepaal. Alhoewel dit nie van toepassing is indien rotte gebruik word nie, is dit ‘n belangrike faktor wanneer kliniese studies oorweeg word.

Neurochemiese bepaling van nitriet is deur middel van die ontwikkeling van 'n sensitiewe fluorometriese omgekeerde fase hoëdoeltreffendheid-vloeistofchromatografiese (HDVC) metode, vir nitriet in menslike sowel as rotplasma, bepaal. Die nitriet is met 2,3-diaminonaftaleen (DAN) gederivatiseer en daarna is die stabiele en hoogs fluoresserende produk, 2,3-naftotriasool (NAT) gekwantifiseer.

Deur plasma arginien- en ornitien konsentrasies te bepaal, is die arginase II aktiwiteit vasgestel.. ADMA kon ook gelyktydig saam met die arginien en ornitien bepaal word deur gebruik te maak van ‘n vloeistof chromatografie gekoppelde massa spektrometrie (LC/MS/MS) metode.

Nitriet vlakke in die plasma van die FSL rot was statisties betekenisvol verminder in vergelyking met die van die FRL rot, maar ADMA en arginase II aktiwiteit het geen statistiese verskil getoon tussen die twee groepe rotte nie. Na aanleiding van die verskille wat in die nitrietvlakke in hierdie

studie waargeneem is en wat in ooreenstemming met ander studies is, is daar bevestig dat die stikstofoksiedweg wel ‘n belangrike rol in die patofisiologie van depressie speel.

Sleutelwoorde: *stikstofoksiedweg; nitriet; arginase II aktiwiteit; ADMA; arginien; ornitien; sensitiewe lyn Flindersrot (FSL); depressie*

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A 1st place was earned in the Young Scientist Competition

List of abbreviations

Abbreviation	Explanation
5-HT	Serotonin
Ach	Acetylcholine
ACN	Acetonitrile
ACTH	Adrenocorticotropic hormone
AD	Antidepressant
ADMA	Asymmetrical dimethylarginine
AII	Arginase II
AL	Arginino succinate lyase
AS	Arginino succinate synthethase
APA	American Psychiatric Association
ATL	Analytical technology laboratory
AUC	Area under the curve
Ca^{2+}	Calcium
CAD	Collision gas
CAT	Cationic amino acid transporter
CE	Collision energy
CEP	Collision cell entrance potential
cGMP	Cyclic guanosine 3′,5′-monophosphate
cNOS	Constitutive nitric oxide synthase
Conc.	Concentration
CRH	Corticotrophin releasing hormone
CUR	Curtain gas
CVD	Cardiovascular disease
CXP	Collision cell exit potential
DA	Dopamine

DAN	2,3-diaminonaphtalene
DD	Double distilled
DDAH	Dimethylarginine dimethylaminohydrolase
Dl	Dorsolateral
dlPFC	Dorsolateral prefrontal cortex
DNA	Deoxyribonucleic acid
DNRI	Dopamine noradrenalin reuptake inhibitors
DP	Declustering potential
DSM-IV	Diagnostic and statistical manual of mental disorders
eNOS	Endothelial nitric oxide synthase
EP	Entrance potential
FP	Focusing potential
FRL	Flinders resistant line
FSL	Flinders sensitive line
GABA	γ-aminobutyric acid
GC	Gass chromatography
GPX	Glutathione peroxidase
GS1	Nebulizer gas
GS2	Heater gas
GTP	Guanosine 5′-triphosphate
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HCOOH	Formic acid
HO.	Hydroxyl radical
HPA	Hypothalamic–pituitary–adrenal
HPLC	High performance liquid chromatography
iNOS	Inducible nitric oxide synthase
IS	Ion spray voltage
K^+	Potassium

KH_2PO_4	Potassium dihydrogen orthophosphate
LC	Liquid chromatography
LC/MS/MS	Tandem liquid chromatography mass spectrometry
LPS	Lipopolysaccharide
LTD	Long term depression
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitors
MDA	Malondialdehyde
MDD	Major depressive disorder
MDE	Episodic major depression
MeOH	Methanol
MRM	Multiple reactions monitoring
Ms	Millisecond
MS	Mass spectrometry
Na	Sodium
NA	Noradrenalin
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NAT	2,3-Naphthotriazole
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
NO_x	Nitrite and nitrate
NPY	Neuropeptied Y
O_2^-	Superoxide
OS	Oxidative stress
PDE	Phosphodiesterase

PFC	Prefrontal cortex
REM	Rapid eye movement
RNA	Ribonucleic acid
RNOS	Reactive nitrogen oxide species
ROS	Reactive oxygen species
SDMA	Symmetrical dimethylarginine
sGC	Soluble guanylyl cyclase
SNRI	Serotonin noradrenalin reuptake inhibitors
SPE	Solid phase extraction
SSRI	Selective serotonin reuptake inhibitors
TB	Tuberculosis
TEM	Source temperature
TRIS	Hydroxymethyl aminomethane
Vm	Ventromedial
vmPFC	Ventromedial prefrontal cortex

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INTRODUCTION

Chapter 1

1.1 Project Motivation and Problem Statement

Major Depressive Disorder (MDD) is a serious and debilitating neuropsychiatric disorder affecting approximately 121 million people worldwide (Rosenzweig-Lipson *et al.*, 2007). MDD may be related to the normal emotions of sadness and bereavement, but it does not remit when the external cause of these emotions dissipates, and it is disproportionate to their cause (Belmaker *et al.*, 2008). As many as 10% of adults experience a clinically diagnosable episode of this affective disorder at some time in their lives. Depression is probably the most common psychiatric diagnosis in primary care populations, with MDD diagnosable in 6 – 9% of all such patients. While clinical depression is self-limiting in about 25 – 55% of affected persons, positive response rates to therapy range from 60% to 90% for those who do not recover spontaneously. It is estimated that up to 15% of major depression cases eventuate in suicide. Given that 50 – 60% of people seeking help for clinical depression are treated exclusively in the primary care sector, its accurate detection is an important task for primary care physicians (Coulehan *et al.*, 1989). Only 25 – 45% of people on antidepressant therapy go into remission (Thase, 2001), while 30% of patients show no response to any of the current available antidepressants (Skolnick, 1999, Rosenzweig-Lipson *et al.*, 2007).

In some cases depression has no external cause such as stress, panic or guilt. Heritability has also been investigated as a cause of depression. It has been determined that the heritability of depression is about 37% (Maes, 1995). Similarly, another study found that MDD is 36% heritable for females and 18% for males (Jang *et al.*, 2004).

The monoamines as neurotransmitters are believed to be involved in the pathogenesis of several mental disorders. It is now well accepted that noradrenalin (NA), serotonin (5-HT) and/or dopamine (DA) are involved in mental depression, hence the monoamine theory of depression. Drugs used to treat MDD acutely increase monoamine availability either through the inhibition of presynaptic reuptake, the inhibition of metabolism through the enzyme monoamine oxidase (MAO) or by blocking the α_2 auto- and hetero-receptors on the monoaminergic neuron (Mongeau *et al.*, 1997). This acute increase in the amount of the monoamines at the synapse has been found to induce long-term adaptive changes in the monoamine systems that end up in the desensitization of the inhibitory auto- and hetero-receptors including the presynaptic α_2 and 5-HT_{1B} receptors and the

somatodendritic 5-HT_{1A} receptors located in certain brain regions (Elhwuegi, 2004). The desensitization of these inhibitory receptors would result in higher central monoaminergic activity that coincides with the appearance of the therapeutic response.

Other theories include the effect of stress, the role of the hypothalamic-pituitary-adrenal axis and growth factors. When stress occurs, it is perceived by the cortex of the brain and transmitted to the hypothalamus, where corticotropin-releasing hormone (CRH) is released to act on pituitary receptors. This stimulus results in the secretion of corticotropin, stimulation of corticotropin receptors in the adrenal cortex, and release of cortisol into the blood. Hypothalamic cortisol receptors respond by decreasing CRH production to maintain homeostasis. There is considerable evidence that cortisol and its central releasing factor, CRH, are involved in depression (Merali *et al.*, 2004). Patients with depression may have elevated cortisol levels in plasma (Burke *et al.*, 2005), elevated CRH levels in cerebrospinal fluid, and increased levels of CRH messenger RNA and peptide in limbic brain regions. In studies using dexamethasone to evaluate the sensitivity of the hypothalamus to feedback signals for the shutdown of CRH release, the normal cortisol-suppression response is absent in about half of the most severely depressed patients (Carroll *et al.*, 2007).

A NO hypothesis of depression was postulated some 14 years ago (Harvey, 1996). More recent, relative to the previously mentioned study, evidence regarding the possible role of degenerative phenomena in depression (MacQueen et al, 2003), and considering the role of NO in neurodegeneration (Calabrese *et al*., 2007), has flagged the NO pathway as an attractive option for novel drug development. NO is rapidly inactivated by hemoglobin or oxidized to form several forms of nitrogen dioxide such as nitrates and nitrites (NO_x). This metabolism of NO makes it difficult to measure NO release into the blood and thus the determination of NO is done by measuring the levels of these oxidative metabolites, in different biological mediums (Ikenouchi-Sugita *et al.*, 2009). Duport & Garthwaite (2005) reported that exposure of the hippocampus to three times higher than physiological concentrations of NO caused extensive neural damage that was not reversible with NOS-inhibitors. NO overproduction occurs due to the persistent stimulation of neuronal NOS (Guix *et al.*, 2005), an event that may follow chronic stress and as such underlie the suppression of hippocampal neurogenesis observed in depression (Zhou *et al*., 2007).

Although the possible link between depression and NO has been extensively investigated, there is no conclusion to what the standard basal levels of NO is, (Grau *et al.*, 2007), and whether the nitrite levels is elevated or decreased in the plasma (Chrapko *et al.*, 2004, Herken *et al.*, 2007, Kim *et al.*, 2006). A wide range of nitrite concentrations in plasma, erythrocytes and whole blood has been observed. Published concentrations for circulating nitrite in healthy humans range from “non detectable” to 26 µM (Grau *et al.*, 2007).

The determination of nitrite in biological fluids represents a considerable analytical challenge. Methodological problems often arise from pre-analytical sample preparation, sample contamination due to the ubiquity of nitrite, and from lack of selectivity and sensitivity. These analytical difficulties may be a plausible explanation for reported highly diverging concentrations of nitrite in the human circulation. Although these factors can influence the measured levels significantly, precautionary measures can be taken to prevent such contamination. Another important variable exists which in most cases is overseen in the determination of nitrite levels. Large amounts of NO are reported to be generated in the gastric lumen after oral ingestion of inorganic NO_x, suggesting a possible physiological role of NO_x in the diet. However, high nitrate intake leads to increased nitrate and nitrite concentrations in urine and saliva with corresponding increases in plasma nitrate levels but does not change plasma nitrite concentration (Pannala *et al.*, 2003), suggesting that, at least in humans, selectively measuring plasma nitrite may represent a viable surrogate marker of endogenous NO synthesis. Nevertheless, most investigators include other putative NO markers in their analysis in order to increase the validity of their findings, e.g. N-methyl-D-aspartate (NMDA) receptor density, arginine levels, cGMP levels or NOS activity. ADMA and arginase II activity has also been included in some earlier studies. ADMA acts as an endogenous inhibitor of NOS, which in turn is responsible for the conversion of L-arginine to NO and L-citrulline (Nonaka *et al.*, 2005). Arginase II (AII) also forms part of the NO-pathway where it is responsible for the conversion of L-arginine to L-ornithine through hydrolysis (Zimmermann *et al.*, 2006).

In this project, we have investigated evidence for differences in selected markers of the NO pathway in plasma of the Flinders sensitive line (FSL) rat, a genetic rodent model of depression, and its healthy control, the Flinders resistant line (FRL) rat. Critical to this study was the development of an in-house HPLC-method for the analysis of nitrite concentration in plasma, as well as a LC/MS/MS method for the simultaneous determination of ADMA and arginase II activity in plasma.

Although a number of methods exist to determine NO_x in biological samples (El Menyawi *et al.*, 1998, Ellis *et al.*, 1998, Gapper *et al.*, 2004, Jedlicková *et al.*, 2002, Romitelli *et al.*, 2007, Smith *et al.*, 2002) only a few of these methods are indeed applicable to human biological fluids, notably plasma, serum, blood or urine. Most of the analytical methods originally developed to be used for the quantification of nitrite in fluids such as drinking and surface water could not be adopted for blood or plasma, mainly because of their complexity and their relatively low content of nitrite (Grau *et al.*, 2007).

Arginase II activity has been established by measuring (a) a decrease in L-arginine concentration and (b) an increase in L-ornithine concentration (Geyer *et al.*, 1971). After measuring L-arginine and L-ornithine levels, plasma arginase II activity can be expressed as units per liter, where 1 U represents 1 mol/l L-ornithine formed per minute (Prins *et al.*, 2000).

The measurement of the ADMA concentrations in plasma has been of interest, since its association with NO metabolism was first discovered in 1992 (Valtonen *et al.*, 2005). A LC/MS/MS method was chosen because it offered better sensitivity, selectivity and, very importantly, simultaneous determination of ADMA, SDMA, L-arginine and L-ornithine.

In this study, a genetic rodent model of depression, the FSL rat, a selectively bred genetic model of depression together with its healthy FRL control rat, was used. The model has demonstrated good face and some construct and predictive validity for major depression (Overstreet *et al.*, 2005). From these data, new strategies may be devised from which more effective antidepressant treatments can be developed. The analysis of tissue from animal models provide a useful means of investigating illness cause and effect as opposed to using the post-mortem brains of suicide victims.

1.2 FSL rat as a genetic rodent model of depression

The use of a validated genetic animal model of depression makes an investigation into the neuropathology of depression possible without the need to create a new animal model or necessitate the need for using humans. Moreover, since depression is recognized as a heritable illness, especially manifested as an inability to cope with environmental stressors (Jang *et al.*, 2004), increases the validity of using such a model in pre-clinical research.

The FSL rat model of depression is used because it resembles depressed individuals in terms of a number of key behavioural, neurochemical, and pharmacological features that are evident in MDD, for example reduced appetite, psychomotor function, sleep (elevated REM sleep, reduced REM sleep latency), immune abnormalities and increased stress sensitivity and anxiety (Overstreet *et al.*, 2005). Based on various theoretical neurobiological models, Table 1 describes the similarities between the FSL rat model and depressed human subjects.

Table 1: Comparison of theoretical models in human and FSL rats

Theoretical model	Identification in humans	Similarity in FSL rats
Cholinergic model	ACh over activity or super sensitivity	Greater sensitivity to cholinergic agonists
Seretonergic model	Reduced 5-HT_{1A} sensitivity	Reduced 5-HT sensitivity
Noradrenergic model	-	-

Dopaminergic model	Reduced DA transporter	Reduced transporter
GABAergic model	-	-
Neuropeptied Y model	Reduced NPY levels	Reduced NPY levels
HPA-axis model	-	-
Circadian rhythm model	Phase advance in some	Phase advance
Neurotrophin model	Increase with AD treatment	Increase with AD treatment

(Overstreet *et al.*, 2005)

1.3 Project Aims

The current project aims to contribute to the research done on the NO-pathway and its importance in the pathophysiology of major depressive disorder and thereby leading to the development of more effective pharmacotherapy.

The project aspires to:

- Develop and optimize an analytical method for the analysis of the NO_2^- in samples of rodent plasma.
- Develop and optimize an analytical method for the simultaneous analysis of ADMA and arginase II activity in rodent plasma.
- Investigate the differences, if any, in NO_2^- levels, ADMA levels and arginase II activity in FSL rats compared to their healthy control, the FRL rat.

1.4 Project Layout

The project consists of three main components involving the analyses of nitrite, ADMA levels and arginase II activity in the plasma of FSL and FRL rats. This will be performed using two in-house developed assay methods in rat plasma. Nitrite will be determined as an oxidative metabolite of NO using high performance liquid chromatography (HPLC) and fluorescence detection. ADMA, an endogenous inhibitor of NOS, will be determined by liquid chromatography tandem mass spectrometry (LC/MS/MS), with arginase II activity determined through the conversion of L-arginine to L-ornithine using LC/MS/MS.

A total amount of 15 FSL rats and 15 FRL rats will be used for the analyses. Approval of the study protocol was granted by the Animal Ethics Committee of the North-West University (Ethics approval number NWU0003207S2)

Table 2: Biological analysis and methods

Biological analysis	Substance measured	Method
Nitric Oxide pathway analysis		
NO_x determination	Nitrite (NO_2^-)	HPLC : fluorescence detection
ADMA	ADMA	LC/MS/MS
Arginase II activity	L-arginine and L-ornithine	LC/MS/MS

1.5 Expected Results

The FSL rat is widely recognized as an animal model of depression. It has extensive face validity when compared to the current theories underlying the neuropathology and pathophysiology of depression. The levels of endogenous markers of the NO pathway in the FSL rat will be compared to those in the healthy FRL control rat.

1.5.1 Nitrite (NO_2^-)

Measurement of nitrite, one of the stable oxidation products of NO, provides a useful tool to study NO *in vivo, in vitro* and in cell cultures (Li *et al.*, 2000). In humans with depression, levels of nitrite show both an increase (Kim *et al.*, 2006) and a decrease (Chrapko *et al.*, 2004) compared to healthy controls, making it essential that further studies in both human and animals be performed to obtain clarity on the matter, especially if nitrite is to be used as a biomarker. However, since the majority of studies show a decrease in NO_x in depression (Chrapko *et al.*, 2004, Selley, 2004, Ikenouchi-Sugita *et al.*, 2009), I expect to find a significant decrease in nitrite levels in the "depressed" FSL rat.

1.5.2 Asymmetrical dimethylarginine (ADMA)

A highly significant negative correlation is detected between the plasma concentrations of ADMA and that of NO while it was also found that ADMA levels are higher in depressed patients than that

observed in healthy controls (Selley, 2004). Thus, I expect to find increased ADMA levels in the FSL rat, compared to the FRL rat.

1.5.3 Arginase II activity

L-arginine in the brain is utilized by arginase II (EC 3.5.3.1) to synthesize L-ornithine (Swamy *et al.*, 2005). Since the levels of arginase II activity are increased in patients with depression (Elgün *et al.*, 2000) I propose that arginase II will be elevated in the plasma of the FSL rat.

LITERATURE REVIEW

Chapter 2

This chapter will discuss the relevant topics concerning major depressive disorder (MDD), including epidemiology, symptoms and co-morbidities, aetiology as well as a comprehensive overview of the neurobiological and pharmacological aspects of the disorder.

In the introduction, I will discuss definitions, history, prevalence and statistics on depression. Then the aetiology, symptomatology, diagnosis and the most recently believed hypotheses will be presented. A brief discussion of the treatment of the disorder will follow which will include the discovery of anti-depressant drugs, a brief classification, and the proposed mechanisms of action of antidepressants. Lastly, it is a well known fact that currently available antidepressants have significant shortfalls in efficacy and onset of action. Ongoing research aimed at improving our understanding of the underlying neurobiology of major depressive disorder, as well as the development of new strategies or targets for the treatment of this disorder will be discussed.

2.1 Major Depressive Disorder (MDD)

Major depressive disorder (MDD), also called major depression, is defined as a mental disorder by the American Psychiatric Association (APA) (Gruenberg *et al.,* 2005). MDD is characterized by sustained depression of mood, anhedonia, sleep and appetite disturbances, and feelings of worthlessness, guilt, and hopelessness. Diagnostic criteria for a major depressive episode (DSM-IV) include a depressed mood, a marked reduction of interest or pleasure in virtually all activities, or both, lasting for at least 2 weeks. In addition, 3 or more of the following must be present: gain or loss of weight, increased or decreased sleep, increased or decreased level of psychomotor activity, fatigue, feelings of guilt or worthlessness, diminished ability to concentrate, and recurring thoughts of death or suicide.

2.1.1 History

In France, in the 1930s, research on phenothiazine compounds was undertaken to investigate their possible use in the treatment of psychosis. An important event in the discovery of drugs for mental illness occurred in 1952, when the antidepressant effects of chlorpromazine were first described.

In 1957, 400 000 patients were given the antituberculosis drug, iproniazid, for depression with significant results. Later it was discovered that iproniazid acts as a monoamine oxidase inhibitor

(MAOI). Soon other MAOIs were also tested and found to be clinically effective antidepressants. The first tricyclic antidepressant, imipramine, was brought onto the market in the late 1950s as Tofranil ®. A year later amitriptyline followed (Wrobel, 2007).

In 1987, the first selective serotonin reuptake inhibitor (SSRI), fluoxetine, entered the market. More than 20 years later, the mechanism by which this popular class of antidepressant, as well as other antidepressant drugs work, remains to be fully elucidated. Since then, many novel hypotheses have been postulated to try and explain the aetiology of depression and the mechanism of action of antidepressants. (ANON. 2009. A short history of SSRI's. www.propeller.com 17 Oct 2009.)

Although there has been an improvement in the treatment of depression since the first tricyclic antidepressants and MAOIs were introduced, particularly with regard to acceptability and side effect profiles, treatment of depression remains unsatisfactory (Thase *et al.,* 2005). Thus, the need for more effective treatment is of utmost importance, as is the need for research to better understand the disease and its pathophysiology.

2.1.2 Prevalence

MDD is characterized by a combination of symptoms that interfere with a person's ability to work, sleep, study, eat, enjoy once-pleasurable activities, and otherwise function normally. An episode of major depression may occur only once in a person's lifetime, but it is more likely to recur throughout a person's life *(Hashimoto, 2009)*.

Prevalence is estimated at between 10% and 25% for women and between 5% and 12% for men. The greatest risk for major depression occurs between the ages of 18 - 44 years of age, with a lower risk for persons 65 and over (Leahy *et al.*, 2000).

MDD is therefore not limited to a specific gender, race, age, socio-economic standing or past life experience, although socio-economic conditions and trauma can play an important role in triggering the disorder. Several studies have been done on the prevalence of depression and include specific studies which focus on age and ethnicity.

A study done in China aimed to determine the prevalence and causes among inpatients of general hospitals (Zhong *et al.*, 2010). 513 patients were randomly selected from 1598 patients in two different hospitals. The prevalence of all current depressive disorders and MDD was found to be 16.2% and 9.4% respectively. The causes for depression included divorce, being widowed, separation, low family income, chronic diseases, lack of medical insurance, dwelling in rural area, suffering from severe illness and multiple hospitalization history.

Another study was recently done in Australia on the elderly community. Five practices agreed to submit patients which were all 60 years of age and older. Their study suggests that, at any given point in time, around 8% of older Australians are experiencing clinically significant depressive symptoms, while nearly 2% may be experiencing a major depressive episode (Pirkis *et al.*, 2009).

Another example of the current high prevalence rate of depression was demonstrated by a study done on Mongolian woman after childbirth. A total of 1044 women who had delivered healthy babies were screened for depression. The prevalence of depression in this sample was 9.1%. Variables that could be significantly and independently associated with a risk of developing maternal depression included economic factors, physical abuse of the mother, dissatisfied with the pregnancy, concerned about her baby's behavior and concerns regarding her own health problems (Pollock *et al.*, 2009).

2.1.3 Aetiology

The exact aetiology of MDD is unknown, although a number of hypotheses exist which contributes to the understanding of the cause and more specifically the pathophysiology of this disorder. These hypotheses can roughly be divided into three groups of factors i.e. biological-, physiological-, and personal factors.

Table 3: Several hypotheses explaining the aetiology of depression

Factor	Hypothesis	Explanation
Biological	Serotonin **(Van de Kar, 1989)**	Decrease synaptic release of 5HT Increase in the density of 5-HT_2 receptors
	GABA **(Lloyd *et al.*, 1989)**	Decrease in GABA levels in plasma Decrease in $GABA_B$ receptors GABA release is diminished in hippocampus
	HPA-axis **(Holsboer, 2000)**	Corticosteroid receptor signaling impaired Altered regulation of ACTH and cortical secretory activity Increased production and secretion of CRH

	ACh **(Dilsaver, 1986)**	Increase in ACh levels Supersensitivity of cholinergic systems
Physiological	Genetic **(Hankin, 2006)**	Up to 80% chance of heritability
Personal (Hankin, 2006)	Life events	Experiencing negative life events and stressors
	Personality	Neuroticism (negative emotionality)
	Cognitive	Negative inferential styles about causes, consequences, and the self Dysfunctional attitudes The tendency to ruminate in response to depressed mood Self-criticism

2.1.4 Symptomatology

Major depression can be divided into two groups according to the different symptoms that occur during the depressive episode as well as the length of the depressive episode. This can then be further divided into four subtypes which indicate the different symptoms experienced by the patient.

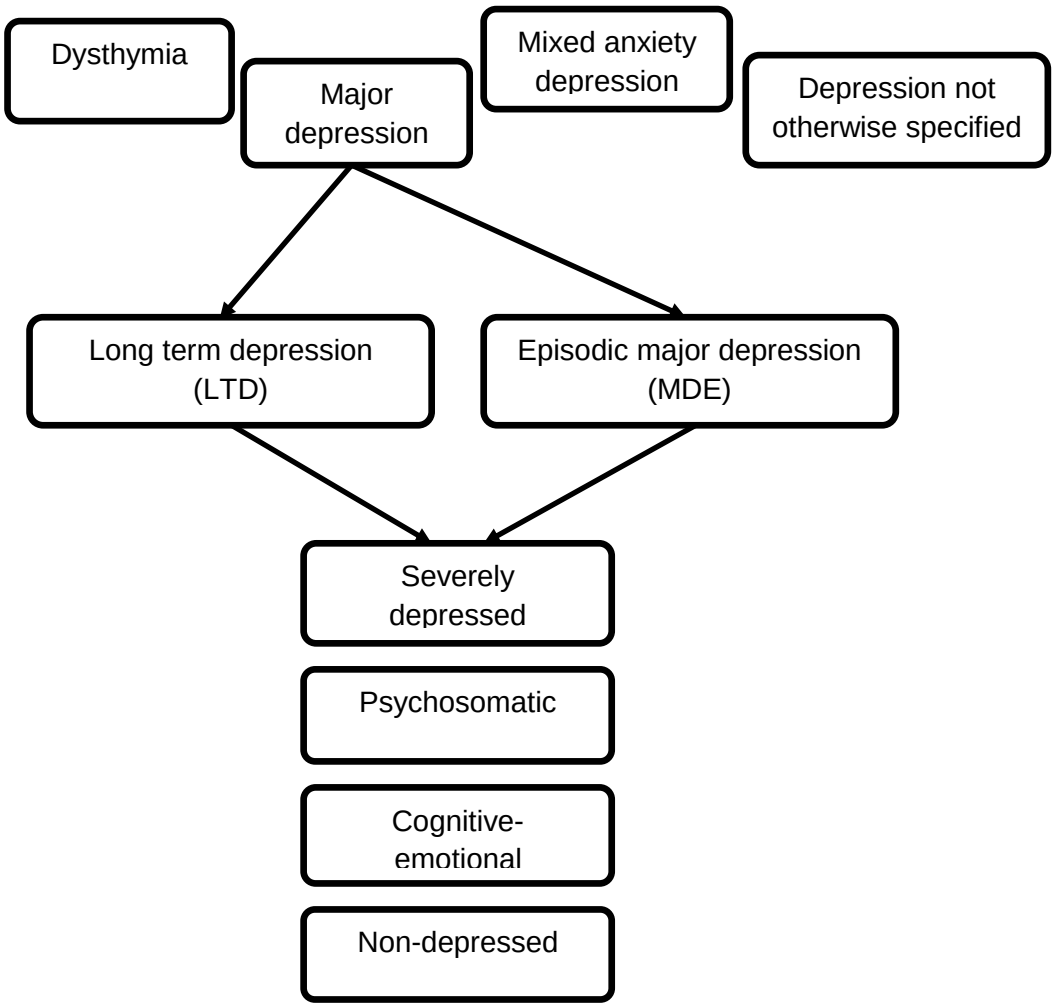


Figure 1: Sub classification of depression according to symptomatology. (Angst *et al.*, 2009, Carragher *et al.*, 2009)

Following the DSM-IV criterion, four classes of depressed patients are identified according to the symptom or group of symptoms which are experienced. As shown in Table 4 the four groups are as follow: 1) severely depressed patients who highly endorse each of the depressive criteria, 2) psychosomatic patients who have high probabilities of experiencing appetite and sleep disturbances, psychomotor complaints and impaired concentration / indecision, 3) cognitive-emotional patients who have high probabilities of experiencing feelings of worthlessness / excessive guilt, impaired concentration / indecision and death / thoughts of suicide and 4) non-depressed patients who have depressive symptomatology but display low endorsement rates in DSM-IV criteria.

Table 4: Symptoms of Major Depressive Disorder in four sub classification areas.

(Carragher *et al.*, 2009)

DSM-IV criterion	**Probability of endorsing each criterion**			
	Severely depressed	Psycho-somatic	Cognitive–emotional	Non-depressed
Appetite/weight change	**0.876**	**0.609**	0.442	0.257
Sleep disturbance	**0.984**	**0.857**	0.390	0.168
Psychomotor difficulties	**0.815**	0.394	0.312	0.040
Fatigue	**0.889**	**0.704**	0.350	0.081
Excessive guilt	**0.927**	0.294	**0.814**	0.025
Indecision	**0.959**	**0.702**	**0.670**	0.142
Suicidal	**0.692**	0.237	**0.504**	0.102

2.1.5 Treatment of depression

Depression is difficult to treat, which may be the result of many influencing factors such as patients' adherence to treatment, genetic differences as in the case of metabolizing enzymes and different causes of depression such as environmental factors or physiological differences in neurochemical substances. It has been recognized that one third of patients treated for major depression do not respond satisfactorily to their first exposure to antidepressant pharmacotherapy. Furthermore, a considerable proportion of cases have a poor prognosis in follow-up observations, with as much as 30% still suffering from major depression 2 years after the onset of this disorder despite multiple interventions (Souery *et al.*, 1999).

Treatment emphasizes four major considerations that are required to ensure optimal treatment of depression and to minimize side effects experienced by the patient. These areas include general support, psychotherapy, pharmacological treatment and in some resistant cases electron convulsive therapy.

Initial support consists of the physician who sees the patient weekly / biweekly to provide support and education and to monitor progress. Psychotherapy includes cognitive behavioral therapy alone or in a group. According to Beers (2006:18) pharmacological treatment is the most common approach and includes a number of different drug classes:

- TCA's (Tricyclic antidepressants e.g. amitriptyline)
- SSRI's (Selective serotonin re-uptake inhibitors e.g. fluoxetine)
- 5HT-blockers (Serotonin blocker e.g. mirtazapine)
- SNRI's (Serotonin noradrenalin re-uptake inhibitor e.g. venlafaxine)
- DNRI's (Dopamine noradrenaline re-uptake inhibitors e.g. bupropion)
- MAOI's (Monoamine oxidase inhibitors e.g. tranylcypromine)

In some cases it may be useful to use combinations of pharmacological treatments. Although a number of drug options are available for the treatment of depression, current anti-depressant treatment does not satisfy all clinical needs. Problems like delayed onset of action, ineffectiveness in refractory patients and those with treatment resistant depression, inadequate reduction of cognitive deficits caused by depression, weak symptomatic pain treatment, as well as troublesome side effect profile (sexual dysfunction, gastrointestinal events, weight gain and cardiovascular side-effects) still exist (Rosenzweig-Lipson *et al.*, 2007). Electroconvulsive therapy is not usually seen or applied as a treatment and should only be used as a last resort.

2.1.6 Neuroanatomy and neurochemistry of depression

Numerous studies have sought to identify the key brain areas involved in the pathogenesis of depression. The involvement of the prefrontal cortex (PFC) has been a major focus in most of these studies and is widely recognized as the area of specific change in the case of depression. The connection of the PFC to other regions of the brain is also thought to be involved in the pathogenesis of depression. There are two main brain neuroanatomic circuits believed to be involved in mood regulation. Firstly, a limbic-thalamic-cortical circuit – which includes the amygdala, mediodorsal nucleus of the thalamus and medial and ventrolateral prefrontal cortex (as indicated by the dotted lines). Secondly, a limbic-striatal-pallidal-thalamic cortical circuit, which includes the striatum, ventral pallidum and regions of the previously mentioned circuit (as indicated by the dashed line) (Soares *et al.*, 1997) (Figure 2, page 16).

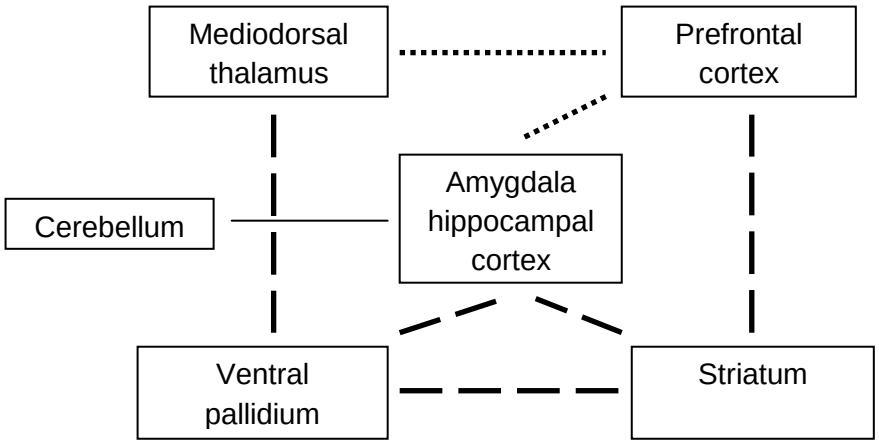


Figure 2: The two main brain circuits involved in the neuroanatomy of depression. (Soares *et al.*, 1997).

Perhaps the most widely accepted division of the prefrontal cortex, based on anatomical connectivity and functional specialization, implicated in depression is between the dorsolateral (dl) and (vm) ventromedial sectors. The vmPFC includes the ventral portion of the medial prefrontal cortex (below the level of the genu of the corpus callosum) and medial portion of the orbital surface (approximately the medial one-third of the orbitofrontal cortex in each hemisphere) (as indicated with the circles in Figure 3A, page 17). Targets of vmPFC projections include the hypothalamus and periaqueductal gray, which mediate the visceral autonomic activity associated with emotion, and the ventral striatum, which signals reward and motivational value. In addition, the vmPFC has dense reciprocal connections with the amygdala, which is involved in threat detection and fear conditioning (Soares *et al.*, 1997).

By contrast, the dlPFC, which includes portions of the middle and superior frontal gyri on the lateral surface of the frontal lobes (as indicated with the squares in Figure 3B, page 17), receives input from specific sensory cortices, and has dense interconnections with premotor areas, the frontal eye fields, and lateral parietal cortex. The distinct patterns of connectivity in these two regions of PFC suggest disparate functionality. The dlPFC has primarily been associated with “cognitive” or “executive” functions, whereas the vmPFC is largely ascribed “emotional” or “affective” functions. Functional imaging studies associate depression with opposite patterns of activity in these areas: hypo activity in dlPFC but hyperactivity in vmPFC (Koenigs *et al.*, 2009).

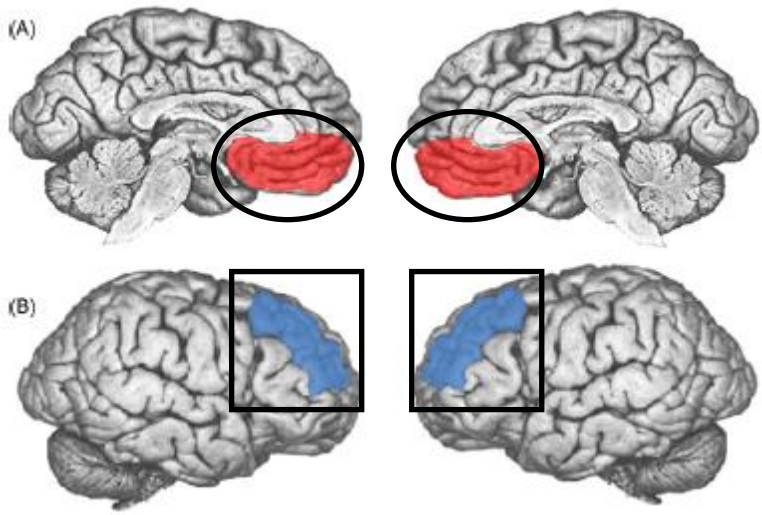


Figure 3: Regions of the prefrontal cortex, suggested to be involved in the pathogenesis of depression, (A) vmPFC and (B) dlPFC. (Koenigs *et al.*, 2009).

2.2 The role of the nitric oxide (NO) pathway in depression

2.2.1 Background

Nitric oxide (NO) has earned the reputation of being a signaling mediator with many diverse and often opposing biological activities. The diversity in response to this simple diatomic molecule comes from the enormous variety of chemical reactions and biological properties associated with it. In the past few years, the importance of steady-state NO concentrations have emerged as a key determinant of its biological function.

Nitric oxide was identified as a biological intercellular messenger just over 20 years ago, and its presence and potential importance in the nervous system was immediately noted. With the cloning of NO synthase and of the physiological NO receptor, soluble guanylyl cyclase (sGC), a variety of histochemical methods quickly led to a rather complete picture of where NO is produced and acts in the nervous system. The cerebellar cortex contains very high levels of NO synthase and NO production there appears to regulate functional hyperemia. Another area in which NO neurons have received a great deal of attention is the forebrain. A population of NO synthase interneurons is present in the striatum, and similar cells are also present throughout the cortex (Vincent, 2010).

NO is an endogenous gas and is thermodynamically unstable and tends to react with other molecules resulting in the oxidation, nitrosylation or nitration of proteins (Guix *et al.*, 2005). Nitric oxide (NO) is a simple molecule, consisting of one oxygen atom bound to one nitrogen atom. It is a remarkably stable, free radical and has been kept in the gas phase for at least 40 years without evidence of decomposition (Beckman, 1996).

NO acts as a short-lived intercellular messenger. The physiological implications are of major importance and have so far implicated nitric oxide in the regulation of blood pressure, platelet adhesion, neutrophil aggregation, as well as synaptic plasticity in the brain (Danson *et al.*, 2005, Ischiropoulos *et al.*, 2005). NO, depending on its concentration, have different physiological functions. This can be divided into direct and indirect effects of NO where the concentration of NO produced endogenously or supplied exogenously is considered. Direct effects occur at low NO concentrations (<1μM); whereas, indirect effects of NO involving the formation of reactive oxygen species (RNOS) become significant at higher local concentrations of NO (>1μM). Indirect effects can be further subdivided into nitrosation, oxidation, and nitration chemistry (Thomas *et al.*, 2008).

NO and its secondary oxidants are major cytotoxic agents produced by activated macrophages and neutrophils (Beckman, 1996). Cytotoxic activated macrophages synthesize NO from a terminal guanidino nitrogen atom of L-arginine which is converted to L-citrulline without loss of the guanidino carbon atom. The nitric oxide gas causes the same pattern of cytotoxicity in L10 hepatoma cells as is induced by cytotoxic activated macrophages including iron loss as well as inhibition of DNA synthesis, mitochondrial respiration, and aconitase activity (Hibbs *et al.*, 1988). NO may also exert some cytoprotective properties. This conflict of function is dependent on the concentration of NO, where lower concentration ranges of NO leads to NO being cytoprotective (Joshi *et al.*, 1999). To further determine the function of NO, the cell type should also be considered. NO protect cells *in vivo* such as hepatocytes from TNFα, thymocytes from INFγ, ovarian follicles from atretic degeneration, and lymphocytes (Wink *et al.*, 1998). In contradiction to the above metioned function, NO may also participate in apoptosis of some cortical neurons, neurons in the substantia nigra, chondrocytes, some thymocytes, macrophages, (Shimaoka *et al.*, 1995) and pancreatic RIN cells (Le *et al.*, 1995).

NO in endothelium has important vasodilator properties, participates in the modulation of vascular tone, and inhibits a number of pro-atherogenic processes, such as the oxidation of low-density lipoprotein and the proliferation of smooth muscle cells (Ignarro, 1989). In brain cells, L-arginine is supplied by protein breakdown or extracted from the blood through cationic amino acid transporters (CATs). L-arginine can also be recycled from L-citrulline produced by NOS activity, through argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) activities, and metabolized by arginase II (Braissant *et al.*, 1999). The NO production capacity is dependent on the intracellular L-arginine concentration. L-arginine is also utilized in the synthesis of proteins and creatine, and is metabolized by arginase II to L-ornithine and urea. Precise cellular responses are differentially regulated by specific NO concentrations (Thomas *et al.*, 2008).

NO is synthesized by NO synthases (NOS), of which there are three isoforms, type I (neuronal nitric oxide synthase, nNOS), type II (inducible nitric oxide synthase, iNOS), and type III (endothelium nitric oxide synthase, eNOS). Type I and III form a class of NOS that are referred to

as the constitutive form (cNOS). cNOS is continuously present in the cell and can be activated by calcium influx that results in calcium binding to the calmodulin receptor to activate the enzyme. The second class is the inducible form or sometimes referred to as iNOS or NOS-2. In some cells, iNOS is expressed after exposure to specific combinations of cytokines. Cell types containing cNOS generate low fluxes of NO for short periods of time; thus, direct effects of NO are the predominant chemistry and indirect effects are limited. However, in the presence of iNOS, production of NO is much greater and indirect effects such as nitrosation, nitration, and oxidation reactions occur (Wink *et al.*, 1998).

Endothelium-derived NO exhibits vasodilator properties that are responsible for modulating vascular tone, inhibiting platelet adhesion to the vascular wall and inhibits a number of bio-metabolic processes, such as the oxidation of low-density lipoproteins and the proliferation of smooth muscle cells (Okamura *et al.*, 1994). In the brain, NO is synthesized by neuronal and endothelial NOS, and as such has multiple functions in brain circuits, including neuroplasticity, neuroprotection and neurotoxicity, as well as behavior (Kim *et al.*, 2006, Yermolaieva *et al.,* 2000).

Most of the described effects of NO are due to the activation of soluble guanylate cyclase (sGC), which converts guanosine 5′-triphosphate (GTP) to the important intracellular messenger cyclic guanosine 3′,5′-monophosphate (cGMP) (Fossier *et al.*, 1999).

It has been previously demonstrated that NO, induced by N-methyl-D-aspartate (NMDA) receptor stimulation, activates the p21 (ras) pathway of signal transduction with a cascade involving extracellular signal-regulated kinases and phosphoinositide 3-kinase (Denninger *et al.*, 1999). These pathways are known to be involved in transmission of signals to the cell nuclei and may therefore form a basis for generation of long-lasting neuronal responses to NO. Other enzymes that constitute cellular targets for NO are cyclooxygenases, ribonucleotide reductase, some mitochondrial enzymes, and NOS itself. NO can also nitrosylate proteins and damage the DNA (Heiberg *et al.*, 2002).

NO also participates in the regulation of neurotransmission in the central nervous system and is known to influence cerebral monoaminergic activity, including the activity of serotonin (Kim *et al.*, 2006) as well as dopamine (Rettori *et al.*, 2002). Due to the physiochemical properties of NO, it is an ideal mediator of nonsynaptic interactions and while monoaminergic systems participate mainly in nonsynaptic interactions, NO may have an important role in the regulation of monoaminergic systems (Kiss, 2000).

The serotonergic system is widely distributed in the brain and a decrease occurs in the efficacy of 5-HT to modulate cholinergic synaptic transmission in the presence of NO or NOS activity (Fossier *et al.*, 1999). NOS activity also increases in areas where the 5HT depletion is higher, without detectable changes in nNOS concentration (Tagliaferro *et al.*, 2001).

NO generated by iNOS in glial cells or nNOS, participates in the cascade of events leading to the degeneration of dopamine containing neurons. Dopamine autoxidation is also associated with formation of oxidants, such as $O_2^{\bullet -}$, H_2O_2, and semiquinones which causes oxidative stress and neurodegeneration mainly caused by peroxynitrite (Rettori *et al.*, 2002).

2.2.2 The NO signaling pathway

NO is produced and released by many different types of cells in multicellular organisms, and is important for intercellular communication.

It is clear that NO acts as a neuromodulator and participate in several sub-cellular processes, such as cellular memory and neuronal toxicity. Nitrergic pathways may also play an important role in the degenerative pathology of the hippocampus and cognitive deficits which is characteristic of affective disorders and it is also suggested that the NO signaling pathway is involved in these disorders. The NO-pathway is therefore a potential target for antidepressant drug action in acute therapy as well as in prophylaxis (Wegener *et al.*, 2008).

Several meganisms are responsible for regulating the synthesis of NO. In neurons NO production is regulated by second messengers and their related protein kinases. NO by itself is able to elicit negative feedback on the activity of NOS, which attenuate its own rate of synthesis. Furthermore, NO modulates the release of neurotransmitters and alters the sensitivity of receptors that are coupled to stimulation of its synthesis (Hu *et al.*, 1996). L-arginine, an amino-acid, is the substrate used by the NOS enzymes to produce NO. Other regulators of nitric oxide synthesis include the factors involved in NOS enzymatic activity, such as molecular oxygen, NADPH, tetrahydrobiopterin, flavin mononucleotide, flavin adenine dinucleotide, calcium/calmodulin and heme (Krumenacker *et al.*, 2004).

In brief, L-arginine is converted to N-hydroxy-L-arginine, which is further converted to NO and citrulline by NOS (Figure 4) (Wegener *et al.*, 2008).

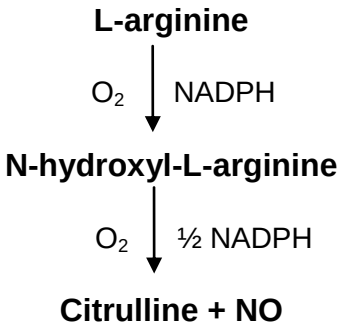


Figure 4: Basic synthesis of NO

L-arginine can also be metabolized by arginase I to urea in the liver or by arginase II to L-ornithine in the brain and of importance in the current study. L-ornithine may further be converted to citrulline by ornithine transcarbamylase (de Bono *et al.*, 2007). NO is further degraded in a few steps. Firstly, during spontaneous aerobic metabolism, oxygen (O_2) reacts with NO to yield peroxynitrite ($ONOO^-$). Peroxynitrite is a highly unstable molecule which is rapidly converted to more stable metabolites nitrite (NO_2^-) and nitrate (NO_3^-) also known collectively as NO_x (Figure 5) (Kelm, 1999:).

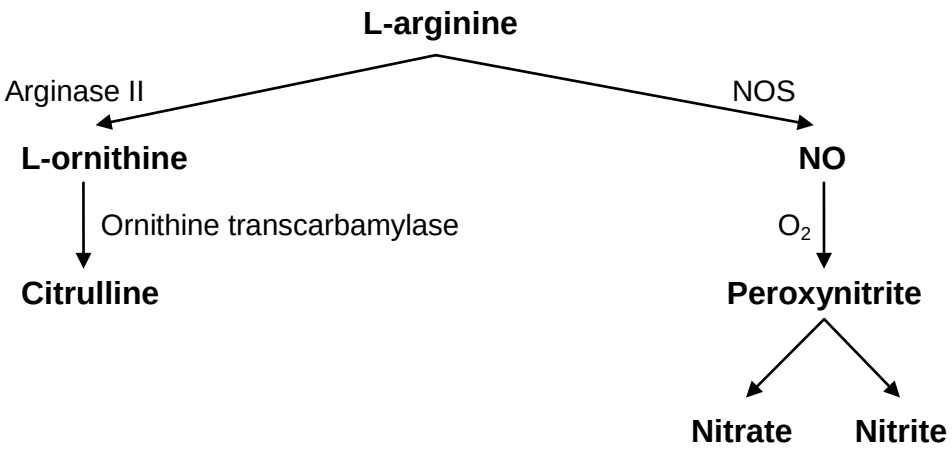


Figure 5: Degradation of L-arginine and NO

Secondly, NO activates soluble- and membrane-bound guanylate cyclases, which catalyzes the synthesis of cyclic guanylate monophosphate (cGMP), subsequently activating cGMP-kinase. This enzyme, by activation of K^+-channels and subsequent Ca^{2+}-channel inhibition, evokes a reduction of intracellular Ca^{2+} levels, finally resulting in vasodilatation. The downstream effects of NO are limited by phosphodiesterase (PDE)-induced degradation of cGMP (Figure 6) (Vincent, 2010).

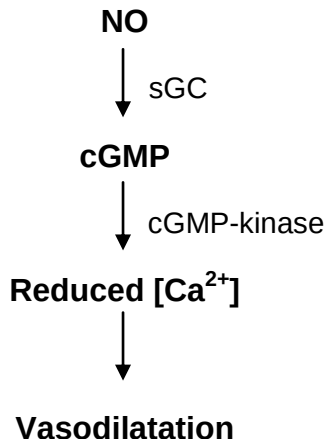


Figure 6: cGMP degradation of NO

2.2.3 Oxidative stress

Oxygen is essential for most life forms, but it is also inherently toxic due to its biotransformation into reactive oxygen species (ROS) (Hermes-Lima et al., 2002). Under normal physiological conditions in the human body, a balance exists between oxidative and antioxidative systems. Oxidative stress occurs when the balance between these two systems becomes disturbed, leading to increased generation of oxidative species and a reactive increase in antioxidative activity. Oxidative stress (OS) is the term used to describe adverse effects occurring when the generation of reactive oxygen species (ROS) in a system exceeds the system's ability to neutralize and eliminate them; excess ROS can damage a cell's lipids, protein or DNA. ROS is a collective term that describes the chemical species that are formed upon incomplete reduction of oxygen and includes the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\bullet}$) (Pitocco *et al.*, 2009). ROS, such as free radicals and peroxides, represent a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms. When the rate of ROS formation is excessive, it can overwhelm the antioxidant capacity of organisms, creating oxidative stress. Organisms are able to adapt to chronic situations of high exposure to ROS by increasing the expression of antioxidant enzymes and many other defense/response mechanisms, including the repair of oxidative damage. To date over 100 genes have been identified that are activated upon exposure of mammalian cells to ROS (Hermes-Lima *et al.*, 2002).

Major depression is characterized by significantly lower plasma concentrations of a number of key antioxidants, such as vitamin E, zinc and coenzyme Q10, and a lowered total antioxidant status (Maes *et al.*, 2010, Sheh *et al.*, 2007). Lowered antioxidant enzyme activity, for example glutathione peroxidase (GPX), is another characteristic of depression, which may impair protection against ROS, causing damage to fatty acids, proteins and DNA by oxidative stress (Abd el-gawad *et al.*, 2001, Maes *et al.*, 2010). Increased ROS in depression is demonstrated by increased levels of plasma peroxides and xanthine oxidase. Damage caused by OS is shown by increased levels of malondialdehyde (MDA), a by-product of polyunsaturated fatty acid peroxidation and arachidonic acid, and increased 8-hydroxy-2-deoxyguanosine, which indicates oxidative DNA damage (Maes *et al.*, 2010).

Free radical interactions will influence NO signaling. One of the consequences of ROS generation is to reduce NO concentrations. NO has been shown to possess either antioxidant or pro-oxidant properties. This difference in functionality is concentration-dependant and concludes that low NO levels are protective and high levels generated during pathological conditions being damaging. The mechanism by which low concentrations of NO is protective may involve diminished metal-catalyzed lipid peroxidation while mitochondrial dysfunction may be involved in the potentiation of oxidative stress seen with higher NO concentrations (Joshi *et al.*, 1999).

ROS generation antagonizes NO signaling and in some cases results in converting a cell-cycle arrest profile to a cell survival profile. The resulting reactive nitrogen species that are generated from these reactions can also have biological effects and increase oxidative and nitrosative stress responses (Thomas *et al.*, 2008). The determinants of oxidative stress are regulated by an individual's unique hereditary factors, as well as his/her environment and characteristic lifestyle. Unfortunately, under the present day life-style conditions many people run an abnormally high level of oxidative stress that could increase their probability of early incidence of decline in optimum body functions (Møller *et al.*, 1996).

Oxidative stress is also caused by the damaging effects of NO where NO increases cell damage through the formation of highly reactive peroxynitrite. Many psychiatric disorders, including major depression, are associated with oxidative stress. A higher production of oxygen free radicals has been observed in patients with depression and anxiety, allowing a link to be established between oxidative stress and alterations in behavior (Bouayed *et al.*, 2004). Additionally, rodent models of depression also support an oxidative stress model of depression (Túnez *et al.*, 2010).

2.3 Peripheral markers of the NO pathway, and relevance for major depression

NO is a free radical capable of reacting with a variety of molecules in biological fluids to produce oxidation products such as nitrite and nitrate. Furthermore these reactions also lead to the formation of nitrosyl (NO-heme) species and modification of thiols and amines to produce S- and N-nitroso products (Saijo *et al.*, 2010). Nitrite and nitrate have been used extensively as markers of NO in the diagnosis of a variety of diseases such as inflammation and depression, and is largely used because of their ease of determination using a variety of assays, including the widely available Griess reaction that can measure the combined concentrations of both anions (NO_x) in body fluids (Guevara *et al.*, 1998).

The availability of biomarkers for psychiatric disorders especially MDD is somewhat limited and diagnosis is currently based on the description of symptoms by the patient and cannot be supported by more objective measures. The identification of biomarkers for MDD could aid in the diagnosis and in predicting treatment response which would assist the psychiatrist in selecting appropriate treatments for individual patients, limiting unnecessary delays and exposure to adverse effects (Belzeaux *et al.*, 2010). Biomarkers could also support drug discovery in the search for new medicines. Moreover, diagnostic biomarkers could aid the study of the neurobiology of disease, which is still mostly unexplained for psychiatric disorders (Carboni *et al.*, 2010).

Since the biological half-life of authentic NO as a result of consumption by erythrocytes is 2 ms (Thomas *et al.*, 2001), making it nearly impossible to measure accurately, surrogate markers may be especially important as a valid endpoint in clinical and biological research into the NO pathway. However, their relevance and validity for the disorder in question needs to be ascertained and confirmed before being used as such.

Substances that occur in the NO- pathway and which may act as surrogate markers for NO include asymmetrical dimethylarginine (ADMA), arginase II (AII) and nitrite (NO_2^-) (Figure 7).

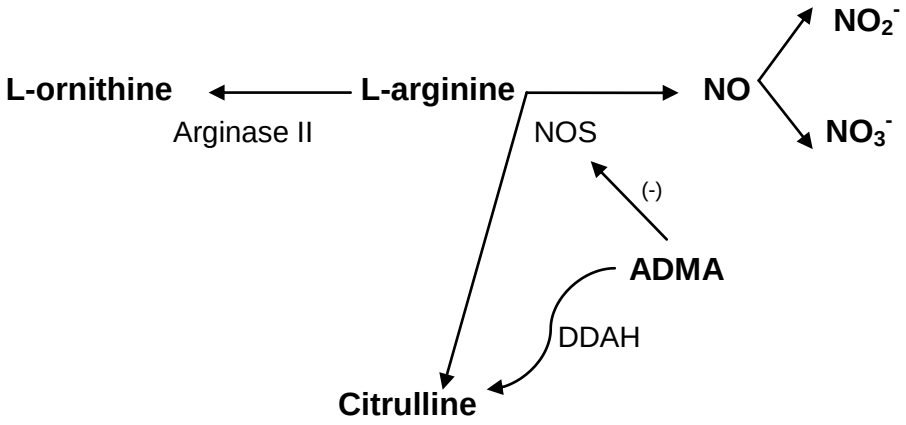


Figure 7: ADMA, arginase II and NO_2^- as they occur in the nitric oxide pathway (Tran *et al.*, 2003, Wiesinger, 2001).

2.3.1 Asymmetrical dimethylarginine (ADMA)

ADMA is synthesized by modification after genetic translation of RNA to proteins occurs, involving the addition of methyl groups to L-arginine residues in proteins by enzymes collectively called L-arginine methyltransferases, and is eliminated from the body both by urinary excretion and enzyme degradation in the kidney, liver, pancreas and endothelium (Pitocco *et al.*, 2009). ADMA presents in plasma where it acts as an inhibitor of NOS; more specifically it inhibits the production of NO from endothelial NOS (eNOS) (Nonaka *et al.*, 2005). The accumulation of ADMA is prevented through hydrolysis caused by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). DDAH then metabolizes ADMA to L-citrulline and methylamine. It has been proposed that DDAH regulates NOS activity by controlling the concentration of ADMA (Tran *et al.*, 2003).

ADMA has been shown to play an important role in the pathogenesis of a number of diseases including diabetes mellitus, cardiovascular risk factors such as hypertension and hypercholesterolemia, myocardial infarction and chronic kidney disease (Pitocco *et al.*, 2009, Turkes *et al.*, 2009, Sufi *et al.*, 2010, Tain *et al.*, 2010). There is growing evidence that depression is a risk factor for CVD and that nitric oxide is involved in neurotransmission that plays an important

role in the control of cerebral blood flow. This link caused some authors to speculate that ADMA may be involved in the pathology of depression (Selley, 2004). In a clinical study done in depressed patients by the above mentioned author, they also determined that there is a strong negative correlation between the levels of NO and ADMA. This negative association between the plasma concentrations of ADMA and NO suggest that ADMA inhibits NOS in major depression. Previous studies done on ADMA levels in depressed patients, showed conflicting results. In depressed patients with a CVD risk, elevated levels of ADMA were found (Ko *et al.,* 2010). A clinical study conducted on depressed individuals with no mentioned co-morbid diseases ADMA levels were also elevated in depressed patients (Selley, 2004) but another study done in depressed patients with no other cardiovascular co-morbid diseases, showed no significant change in ADMA levels (Taylor *et al.*, 2006). A study done on rats also found an increase in plasma and hepatic ADMA levels which were associated with oxidative stress in young bile-duct-ligation rats. Because oxidative stress is involved in depression as described previously in 2.2.2 this also shows that ADMA might be involved in the pathophysiology of MDD (Tain *et al.*, 2010, Selley, 2004).

2.3.2 Arginase II (AII)

L-arginine is metabolized by arginase II and yields L-ornithine (Zimmermann *et al.*, 2006). Arginase exists in two isoforms the body. Liver type arginase (arginase I) exists almost exclusively in the liver, and catalyzes the last step of the urea cycle. The other isoform of arginase, arginase II (AII) is encoded by a separate nuclear gene than arginase I, and differs from arginase I in catalytic, molecular and immunological properties and is expressed in extra-hepatic tissues, such as kidney, small intestine and most importantly for this study in the brain. It is responsible for the hydrolysis of L-arginine to L-ornithine in the NO-pathway in the brain. L-arginine can not be solely synthesized in the central nervous system, since the mitochondrial urea cycle enzymes, carbamoylphosphate synthetase and ornithine transcarbamylase, are not expressed in this region. L-arginine synthesis in brain cells depends on protein breakdown and on specific transporters to import L-arginine from the blood or transfer it from cell to cell. As shown in figure 8, L-arginine can also be recycled from citrulline generated by NOS activity, in a reaction catalyzed by the cytosolic argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) (Braissant *et al.*, 1999).

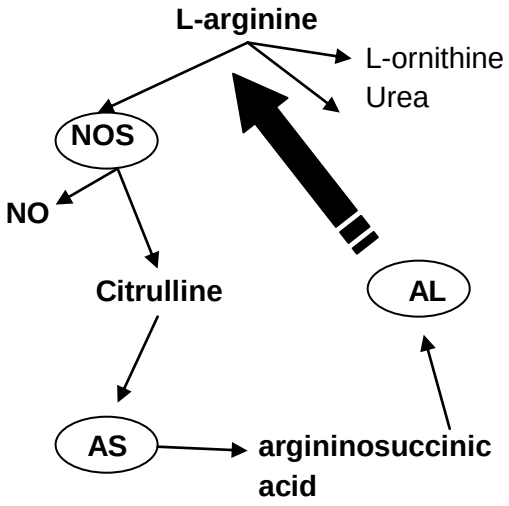


Figure 8: Recycling of L-arginine from citrulline (Braissant *et al.*, 1999)

Arginase II is found in most structures of the rat brain, both in neurons and glial cells. The regions containing the highest levels of arginase II include the ventral striatum, nucleus accumbens, islands of Calleja pyriform cortex and its transition towards the amygdala nuclei (Braissant *et al.*, 1999).

Recently, arginase activity as well as NOS was found to be induced in murine macrophage-like RAW 264.7 by bacterial lipopolysaccharide (LPS). The induced arginase was shown to be arginase II by immunochemical analysis (Gotoh *et al.*, 1996), and suggest that arginase II plays a role in down-regulation of NO synthesis by decreasing the L-arginine availability for the NOS reaction. Despite the fact that there might be some activity of arginase I in the central nervous system, arginase activity in the brain seems predominantly due to the mitochondrial arginase II. It was proposed that arginase II acts in combination with L-arginine biosynthetic pathways in order to modulate L-arginine levels (Braissant *et al.*, 1999). Arginase II also plays a role in the generation of L-ornithine for further synthesis of proline and glutamate (Wiesinger, 2001), and thus contributes indirectly to glutamatergic-directed signaling as well. The co-induction of arginase II and NOS (Wang *et al.*, 1995), the induction of arginase II by suppressors of nitric oxide synthesis (Corraliza *et al.*, 1995), the inhibition of arginase II by hydroxy-L arginine, a NO biosynthesis intermediate (Daghigh *et al.*, 1994), and the identification of compounds that co-regulate the expression of both arginase II and NOS (Palacios *et al.*, 1993) support the theory that arginase II may be essential in the regulation of NOS by modulating local L-arginine concentrations (Elgün *et al.*, 2000). Furthermore, some other studies have also showed that arginase II expression did indeed diminish the amount of NO formed when all other aspects of the experimental study were held constant (Cederbaum *et al.*, 2004).

2.3.3 Nitrite (NO_2^-)

As has been described in more detail earlier, L-arginine is metabolized via NOS to produce L-citrulline and NO (Zimmermann *et al.*, 2006). Nitrite is widely present in a number of tissues at concentrations that are either comparable (brain and heart), slightly lower (liver, kidney and lung) or higher (aorta) than in plasma (Kleinbongard *et al.*, 2003). Nitrite plays an important physiological role in tissues as a NO donor but also as a signaling molecule on its own that regulates vital cell functions, including gene expression. Particular focus has been on the cytoprotection that nitrite exerts on cellular necrosis and apoptosis (Jensen, 2009).

As previously said, NO is thermodynamically unstable and is rapidly inactivated by hemoglobin or oxidized to form several nitrogen dioxides such as nitrates and nitrites (NO_2^- and NO_3^- or collectively referred to as NO_x). Therefore NO levels in biological fluids can be indirectly measured by quantification of these stable oxidative metabolites (Chrapko *et al.*, 2004).

Nitrite is endogenously produced as an oxidative metabolite of nitric oxide, but it also functions as a NO donor that can be activated by a number of cellular proteins under hypoxic conditions. The nitrite reductase activity of deoxyhemoglobin is a major mechanism of NO generation from nitrite. The hemoglobin nitrite reductase activity depends on heme O_2 affinity and redox potential. Nitrite reduction to NO provides cytoprotection in tissues during ischemia–reperfusion events by inhibiting mitochondrial respiration and limiting the formation of reactive oxygen species (Jensen, 2009).

NO is regenerated from nitrite by acidic disproportionation (Zweier *et al.*, 1999) and by enzymatic reduction via xanthine oxidoreductase, (Millar *et al.*, 1998) mitochondrial enzymes, (Kozlov *et al.*, 1999) or deoxygenated hemoglobin, myoglobin and neuroglobin (Castello *et al.*, 2006). Recently, eNOS was also found capable of reducing nitrite to NO where there is a total reduction of oxygen (Gautier *et al.*, 2006). The mechanisms that reduce nitrite to NO are all favored by low oxygen tension and/or low pH, and the concept has emerged that endogenous nitrite constitutes a reservoir of NO activity that is activated and plays an important role under hypoxic conditions (Vatassery *et al.*, 2004, Jensen, 2009).

A study was done on suicidal depressive patients to determine NO levels, the data showed that plasma NO_x levels among suicidal depressive patients are higher than among non-suicidal depressive patients and normal controls. However, other studies have found no differences in plasma NO_x levels in non-suicidal depressive patients versus normal controls (Kim *et al.*, 2006). This latter result is inconsistent with studies done on humans presenting with major depression, where depression was associated with a significant decrease in levels of NO_x (Chrapko *et al.*, 2004, Selley, 2004). Other studies again have described an increase in NO_x in major depressed

patients (Kim *et al.*, 2006), emphasizing the great disparity in these findings. These differences in results may be due to a number of factors including contamination of samples, method of preparation etc. which will be explained in Chapter 3.

Due to the fact that contamination may affect nitrite quantification, regeneration of NO from nitrite and the numerous physiological contributors of nitrite, nitrite should probably not be recommended as a biomarker for depression. However, the importance of nitrite and its role in the NO-pathway with regard to the pathophysiology of depression should not be underestimated and is still worthwhile investigating.

CHROMATOGRAPHY

Chapter 3

This chapter describes the chromatographical methods used in the analyses of nitrite, ADMA and arginase II, including the high-performance liquid chromatography (HPLC) and LC/MS/MS methods. All the methods were developed in the Analytical Technology Laboratory (ATL) at Potchefstroom Campus of the North-West University, Potchefstroom, NW, South Africa.

3.1 Nitrite determination

3.1.1 Chromatographic information

The plasma nitrite levels of FSL and FRL rats were determined by using an Agilent 1100 series HPLC system. Fluorescence detection was performed by a Shimadzu RF-551 detector with excitation and emission set at 363 nM and 425 nM respectively. The range of detection was set at 2-fold and sensitivity at “LOW”, which was connected to an Eclipse XDB-C18 column, 4.6 x 150 mm, 5 μM (Agilent). Chemstation Rev. A.06.02 data acquisition and analysis software was used for calculating peak areas. The mobile phase was set at a flow rate of 1,2 ml/min and contained a 15 mM sodium phosphate buffer (pH=7.5) and HPLC-grade methanol adjusted with orthophosphoric acid.

3.1.2 Method and materials

Although nitrite alone does not fluoresce, the reaction with 2,3 diaminonaphtalene (DAN) to give 2,3 naphthotriazole (NAT) fluoresces intensely (Li *et al.*, 2000) and this derivative will be used to quantify sample nitrite.

Total plasma NO_x (nitrite and nitrate) concentration was initially thought to be of importance, and thus considered for analysis in this study. However, earlier studies (Pannala *et al.*, 2003) have found that plasma nitrate is influenced by the patient’s diet while that of nitrite remains unaffected. Consequently, although this may not necessarily apply to rats because their diet is controlled, for the purposes of this study only nitrite levels were determined in the plasma using a fluorometric,

isocratic reversed-phase liquid chromatography method with pre-column derivatization of nitrite with DAN.

Nitrite interacts with DAN, a relatively non-fluorescent compound, which adds a fluorophore to the nitrite molecule producing a highly fluorescent and stable aromatic compound, NAT. NAT allows the determination of nitrite by fluorometric detection following prior HPLC separation of DAN and NAT. NAT formation can then be used as a direct indicator of sample nitrite due to their stoichiometric (1:1) relationship.

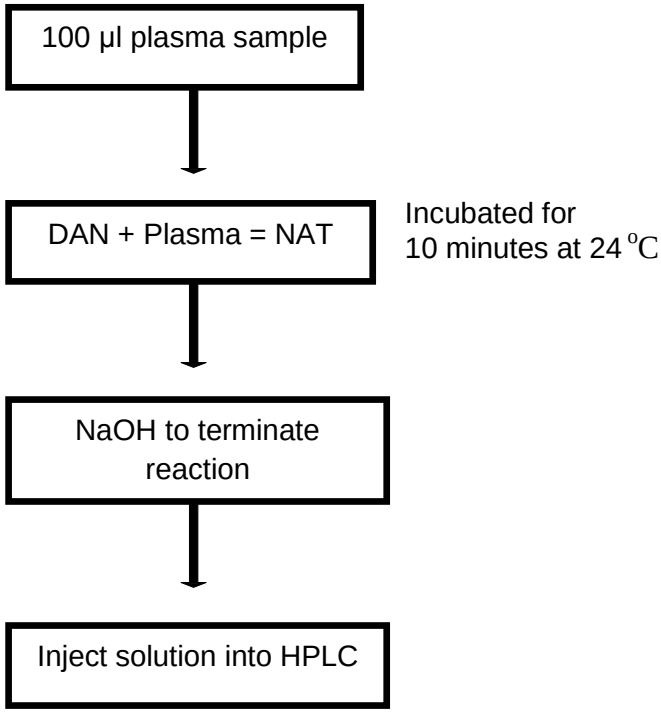
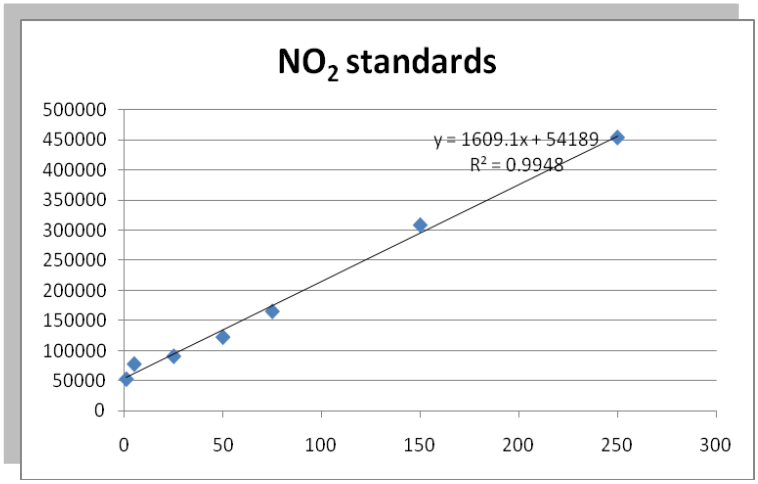


Figure 9: Nitrite determination through the reaction of nitrite with DAN to yield NAT under acidic conditions and then terminated by the addition of NaOH before injected into the HPLC (Li *et al*., 2000).

A number of methods can be used to determine nitrite in plasma samples, all with their own advantages and disadvantages. The HPLC-fluorescence method is however a popular and accurate way to analyze biological samples (Jedlicková *et al.*, 2002), provided that important precautions are taken throughout the entire assay to prevent sample contamination (Grau *et al.*, 2007). The main assay interferences are nitrite contamination from extraneous sources. Environmental nitrite contamination affects nitrite standard curves by causing falsely high NAT peak AUC values especially at lower standard concentrations (1-10 ng/ml). This can be avoided by preventing or minimizing any form of contamination as will be explained later in this chapter.

This method of nitrite analysis is more sensitive than other commonly used methods, including Griess assays and LC/MS/MS determinations (Bryan *et al.*, 2007). Fluorometric detection assures

that sample components are fully separated with less risk of errors and interferences (Tsikas, 2007). After adapting the method as previously described by Hui Li and colleagues (Li *et al.*, 2000), the assay proved to be valid, repeatable and highly sensitive to analyze nitrite in rat plasma samples.



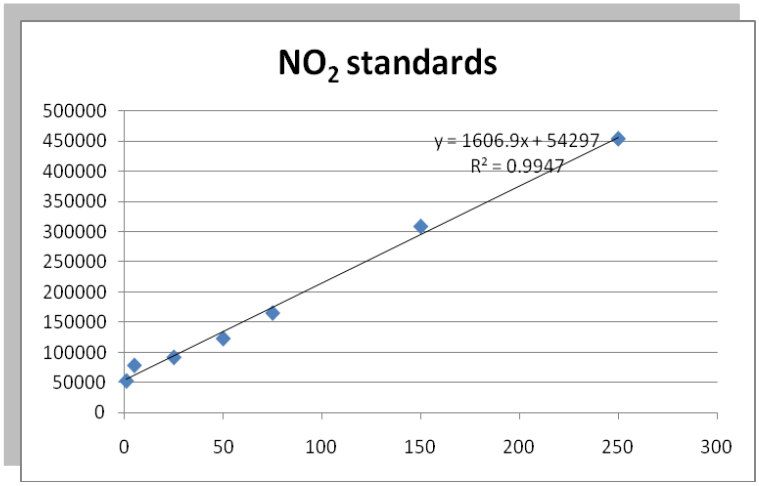


Figure 10: Two sets of standards injected into the HPLC on different days and different times, proving that the method is repeatable.

3.1.3 NO_x chromatogram and chemical reaction scheme

Figure 11 displays an example of the resultant chromatogram after total nitrite from rat plasma was derivatized with DAN. The product of DAN and nitrite is 2,3-Naphthotriazole (NAT).

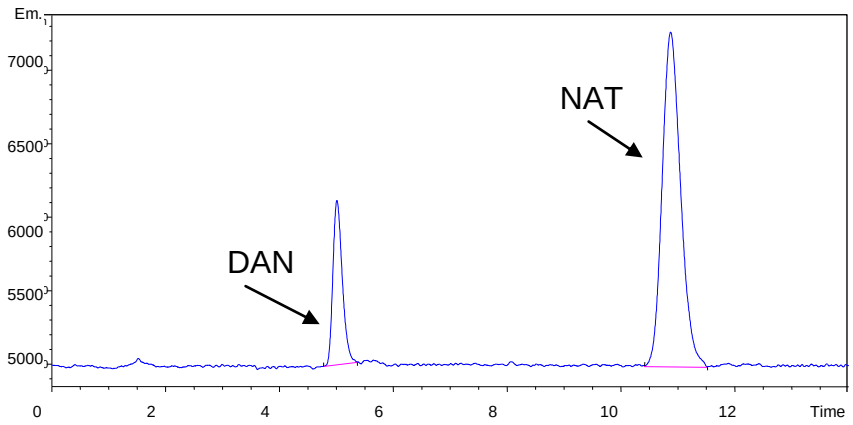


Figure 11: HPLC chromatogram representing a plasma sample derivatized with 2,3-diaminonaphtalene (DAN) to yield NAT (2,3-Naphthotriazole)

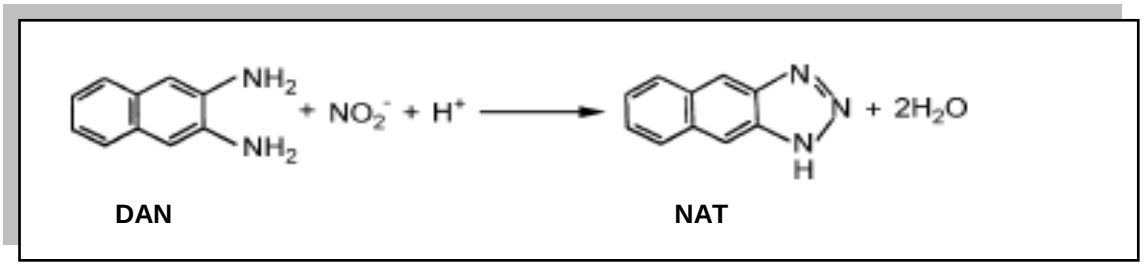


Figure 12: Reaction of nitrite with 2,3-diaminonaphthalene (DAN) to yield 2,3-naphthotriazole (NAT) under acidic conditions.

3.1.4 Chemicals and reagents

Chemicals were of analytical grade or higher purity and stored at the specified conditions. All aqueous solutions were prepared using HPLC-grade water. Chemicals used for the analysis of nitrite are listed in Table 5.

Table 5: List of reagents and chemicals used for quantification of NO_2

Function	Chemical	Supplier
Derivatization agent	2,3-Diaminonaphtalene (DAN)	Sigma- Aldrich
pH adjustment	Hydrochloric acid (HCl)	Saarchem
Reaction products and Standards	Sodium nitrite (NaNO2)	Sigma- Aldrich
Buffer	Hydroxymethyl aminomethane (TRIS)	Saarchem

Protein precipitator	Acetonitrile (ACN)	Sigma- Aldrich
Mobile phase	Potassium Dihydrogen Orthophosphate (KH_2PO_4) Sodium hydroxide (NaOH) Methanol (MeOH)	Saarchem
Stop solution	Sodium Hydroxide (NaOH)	Saarchem

3.1.5 Important experimental changes made on the original method

The initial method (Li *et al*., 2000) was adapted to optimize the results obtained.

Table 6: Changes made to optimize method for nitrite determination

Initially	Changes made	Reason
Samples were filtered to remove proteins	Proteins were precipitated using ACN	Filters contain high levels of nitrite
Plain DD-water was used for mixing	Added a TRIS buffer to DD-water	TRIS prevents contamination with nitrite from the water
DAN was mixed with HCl	Three drops of methanol was added to the DAN solution	Improves the solubility of DAN to yield a clear solution
Excitation 375 nM emission 415 nM	Excitation 363 nM emission 425 nM	Better detection

3.1.6 Standard solutions

A series of dilutions was made to obtain a concentration range of 1 ng/ml – 250 ng/ml nitrite, as illustrated in table 7.

The stock solution was made up to a concentration of 100 µg/ml by adding 7,5 mg of $NaNO_2$ to 50 ml of the 25 mM TRIS solution. From the stock solution 100 µl was extracted and added to 10 ml of

the 25 mM TRIS solution to obtain a concentration of 1 µg/ml. From this 1 µg/ml solution the rest of the standard solutions were diluted as demonstrated in the table below.

Table 7: Dilutions made for standard solutions

	Concentration of NO_2^-	Dilution (make up to 2 ml)	+	TRIS Buffer	=	Total Volume
	(ng/ml)	µl		µl		(ml)
1	1	2	+	1998	=	2
2	5	10	+	1990	=	2
3	25	50	+	1950	=	2
4	50	100	+	1900l	=	2
5	75	150	+	1850	=	2
6	150	300	+	1700	=	2
7	250	500	+	1500	=	2

3.1.7 Working concentration range of standard solutions

The working range of standard solutions contained 1 – 250 ng/ml NO_2^-. The percentage of sodium $NaNO_2$ were compensated for to ensure a final solution containing 100 % pure NO_2^-.

Table 8: Components of $NaNO_2$

Reagent	Molecular weight (MW)
$NaNO_2$	69.00 (100%)
Na^+	23.0 (33.33%)
NO_2^-	46.0 (66.67%)

The values of the areas under the curve (AUC), as shown in Table 9, represent standard solutions in ng/ml or nM concentrations. The regression curve as seen in Figure 10 of NO_2^-, displayed a positive, almost perfect linearity of 0.994.

Table 9: Nitrite concentrations and AUCs of standard solutions.

ng/ml	nM	AUC
1	0.0217	53404
5	0.1087	78326
25	0.5435	91292
50	1.0870	122961
75	1.6304	165626
150	3.2609	308677
250	5.4348	453712

3.1.8 Experimental precautions

3.1.8.1 Effect of environmental contamination on NO_2^- standard curve

Determining nitrite levels in any biological fluid gives rise to a number of analytical challenges. Methodological problems occur because of the numerous ways that the sample can be contaminated, the ubiquity of nitrite, and from a lack of selectivity and sensitivity. These analytical difficulties may explain the uncertainty found in the literature concerning basal levels of nitrite in biological fluids, especially plasma.

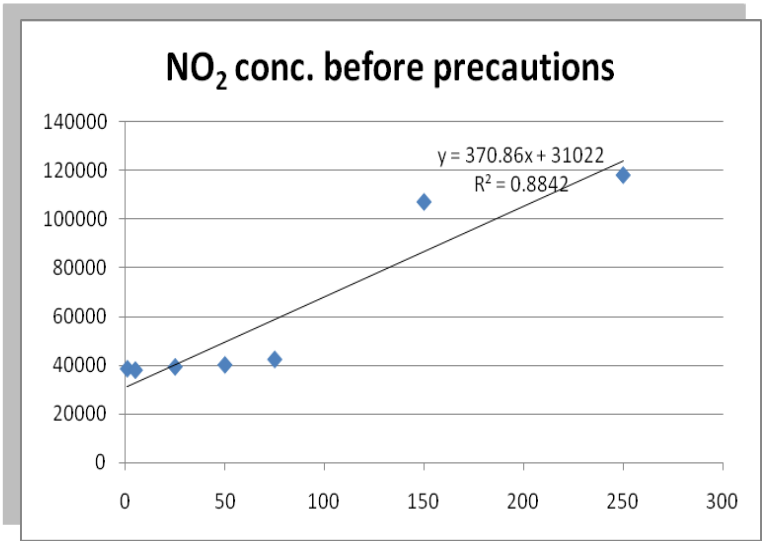
The major source of nitrite contamination has been reported to include plastics such as pipette tips and eppendorfs used to transfer solution. Soft glass ware and ultra filtration units for protein precipitation were found to contain considerable amounts of nitrite even after extensive washing procedures which may contribute to artificially elevated nitrite concentrations in the samples (Grau *et al.*, 2007). This may also be as a result of the nitrite concentration found in double distilled water (DD-water) 2.806 ng/mol or 61 pmol/ml (Li *et al.*, 2000). Before and while doing the nitrite assay, all glass and plastic apparatus were rinsed three times with the TRIS buffer before it was used.

3.1.8.2 Effect of experimental contamination on NO_2^- standard curve

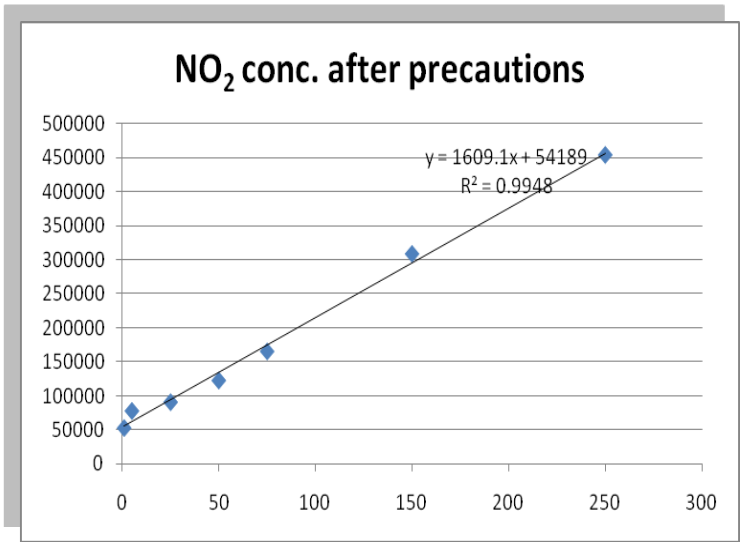
Whole blood and erythrocytes represent problematic biological fluids and are rarely used for quantitative analyses, because nitrite is unstable in these fluids. Plasma however, is the most frequently analyzed, with the least amount of complications. When using plasma, proteins should be precipitated. A method often used is filtering plasma through filters (Li *et al.*, 2000). It is recommended to avoid the use of soft glass, ultra filtration cartridges or vacuum blood sampling tubes demonstrating high inherent nitrite concentrations. Furthermore precipitation is sometimes done by the addition of an acid such as $HClO_4$. This also results in a rapid and abundant loss of nitrite due to nitrosation reactions and in some conditions due to reduction of nitrite to NO (Grau *et al.*, 2007). Therefore we used ACN to precipitate the protein from the plasma samples. The influence of contamination on the NO_2^- standard concentration curve is depicted in Table 10 and Figure 13.

Table 10: The influence of contamination on the NO_2^- standard concentration curve

Conc. NO_2^- (ng/ml)	AUC (before precautions)	AUC (after precautions)	Accuracy increase (%)
1	38469	53404	27.9
5	37877	78326	51.6
25	39214	91292	57
50	40025	122961	67.4
75	42311	165626	74.5
150	107213	308677	65.3
250	118246	453712	73.9



(a)



(b)

Figure 13: a) Standard concentration curve of NO_2^- before precautions taken against contamination. b) Standard concentration curve of NO_2^- after precautions taken against contamination.

In Figure 13a, the effect of contamination can be observed at especially the lower NO_2^- standard concentrations (1 to 75 ng/ml). As can be observed in Figure 13b, contamination could be

drastically reduced with the necessary precautions being taken throughout sample preparation. These precautions included flushing all pipette tips, eppendorfs and glass apparatus with TRIS buffer, and precipitation of proteins with ACN.

3.1.9 Determination of NO_2^- levels in rat plasma

FSL and FRL rats (200 +/- 20g) were sacrificed by decapitation and trunk blood collected in 5ml heparin tubes. The blood was immediately centrifuged at 2 800 xg for 15 minutes to separate the plasma from the red blood cells. The plasma was collected and stored at -80 ^{o}C until the day of analysis.

Before analysis the plasma samples were removed from the freezer and were left at room temperature to thaw. 100 µl ACN was then added to 100 µl of the plasma sample and centrifuged at 10 000 xg for 5 minutes to precipitate the proteins, before injecting it into the HPLC apparatus.

For analysis by HPLC with fluorometric detection, 100 µl of the prepared plasma were incubated at 24 ^{o}C with 10 µl of a 316 µM DAN in 0,62 M HCl solution. After 10 minutes 5 µl of a 2,8 M NaOH was added to stop the reaction and was injected into the HPLC apparatus at a flow rate of 1,2 ml/min.

3.2 Determination of ADMA and arginase II activity

3.2.1 Chromatographic information

The plasma ADMA levels and arginase II activity of FSL and FRL rats were determined using an Agilent 1100 series HPLC with a binary gradient pump, autosampler and vacuum degasser coupled to an Applied Biosystems API 2000 triple quadruple mass spectrometer. Data were analyzed using Analyst 1.4 data acquisition and analysis software (AB Sciex SA (Pty) Ltd, Johannesburg). A Luna 3 µm HILIC 200 Å column, 100 x 2 mm, (Phenomenex, Torrance, CA) was connected to the system. The mobile phase contained A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile

With a flow rate of 250µl/min and injection volume set at 10µl the gradient was as seen in Table 11.

Table 11: Gradient of LC/MS/MS for L-arginine, L-ornithine and ADMA determination

Time (minutes)	0.1% formic acid in water	0.1% formic acid in acetonitrile
0	5	95

1	5	95
5	35	65
10	35	65
10.4	5	95
15	5	95

Atmospheric pressure electron ionisation (Turbo ion spray source), positive ion mode, multiple reactions monitoring (MRM) scan with quantifier and qualifier ion pairs, set as described in Table 13.

Table 12: Molecular weight of quantified components.

Component	Molecular weight
ADMA:	202.8/46.1, 202.8/171.9
L-ornithine	132.89/70.0, 132.89/115.80
L-arginine:	174.92/70.10, 174.92/60.10

The system was set up as follows:

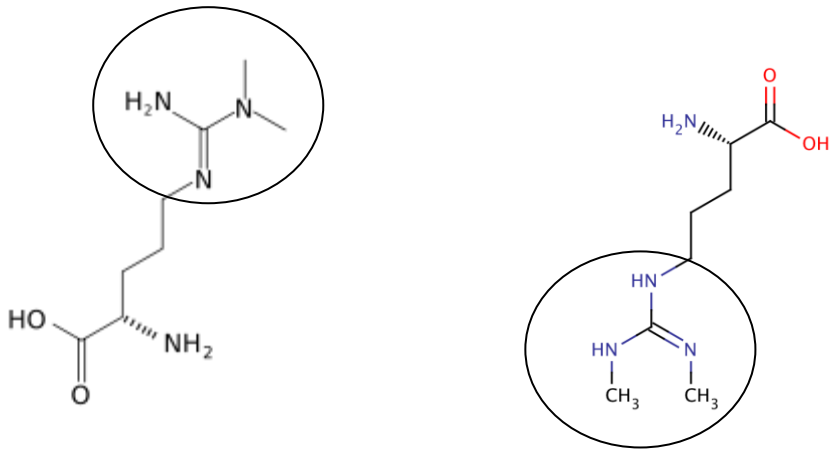
Table 13: Mass spectrometer settings

Component	Setting
Declustering potential (DP)	21 V
Focusing potential (FP)	370 V
Entrance potential (EP)	3.5 V
Collision cell entrance potential (CEP)	14 V
Collision energy (CE)	37 V
Collision cell exit potential (CXP)	4 V
Ion spray voltage (IS)	5500 V
Source temperature (TEM)	250 °C

Curtain gas (CUR)	20 psi
Nebulizer gas (GS1)	20 psi
Heater gas (GS2)	20 psi
Collision gas (CAD)	5 psi

Only two experimental methods were found that describe the determination of arginase II. The first, published in 1952 by Francis P. Chinard, provided minimum detail of the analytical procedure, while a method by Swamy was found to be a very lengthy and complicated process (Swamy *et al.*, 2005). Therefore it was decided to determine arginase II activity by measuring the precursor L-arginine and the product L-ornithine. Arginase II activity can then be characterised by the changes found in L-arginine and L-ornithine concentrations, where a decrease in L-arginine concentration and an increase in L-ornithine concentration is an indication of increased arginase II activity (Geyer *et al.,* 1971).

Because asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are both present in the plasma, the concern was that these two compounds might overlap during quantification. While the aim was to determine ADMA levels, the structure of these two molecules was thoroughly investigated, after which the conclusion was made that the ion which is of importance for the quantification of these two molecules differs and that ADMA could be quantified separately (Figure 14).



ADMA **SDMA**

Figure 14: Structural differences of ADMA and SDMA.

ADMA, L-arginine and L-ornithine were measured simultaneously using a very simple sample preparation which consists of protein precipitation of the plasma sample before it was injected into the LC/MS/MS.

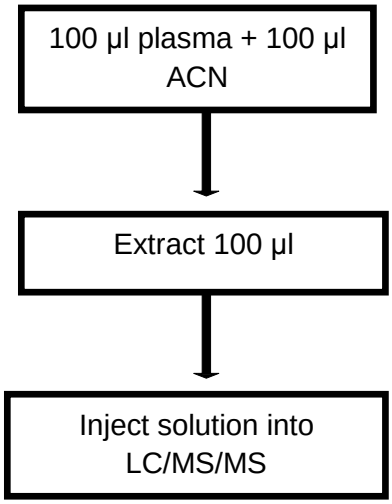
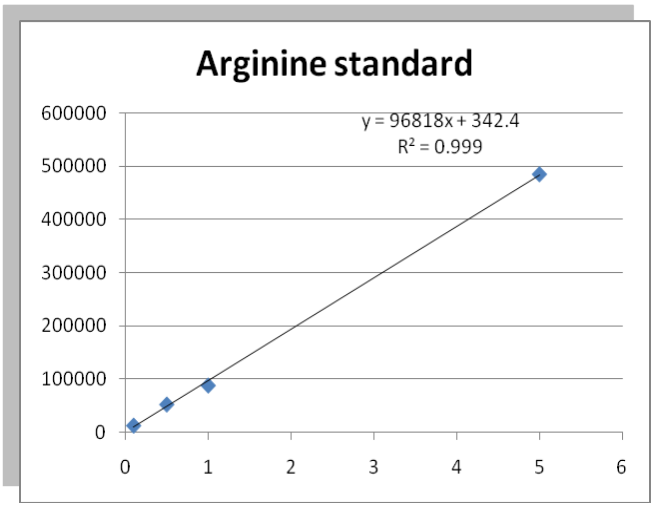


Figure 15: Determination of ADMA, L-arginine and L-ornithine using a LC/MS/MS.

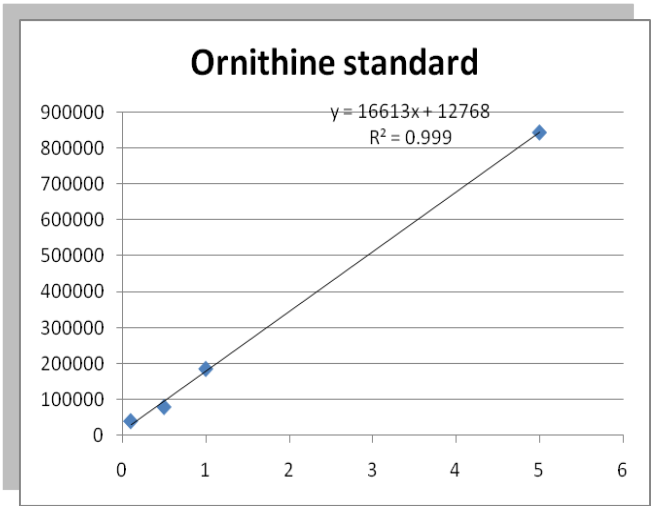
Over the last 15 years various methods for determining ADMA concentrations in plasma have been used. The most widely used assays are based on solid-phase extraction (SPE) of basic plasma components, followed by derivatization and chromatographic analysis by means of HPLC with fluorescence detection. Methods using mass spectrometry (MS) including gas chromatography (GC) and liquid chromatography (LC) have been developed more recently and enable more specific quantification of compounds of interest. An ELISA method is also available as a rapid tool for determining ADMA in plasma. Mass spectrometry is however the most specific analytical tool available to analysts measuring low levels of compounds in complex matrices, and this technique has provided reasonably consistent results for the quantification of ADMA (Horowitz *et al.*, 2007).

A set of standards including ADMA, L-arginine and L-ornithine in one solution were injected as first step and as part of the validation process into the LC/MS/MS to determine if the method is effective and repeatable. For each of the analytes, three sets of standards were injected into the LC/MS/MS and a linearity curve was set up to determine the regression values for each analyte. Each curve showed an acceptable regression value of between 0.996 and 1. The limit of detection for each analyte was found to be the following: ADMA = 0.02 µg/ml, L-arginine = 0.1 µg/ml and L-ornithine = 0.1 µg/ml.

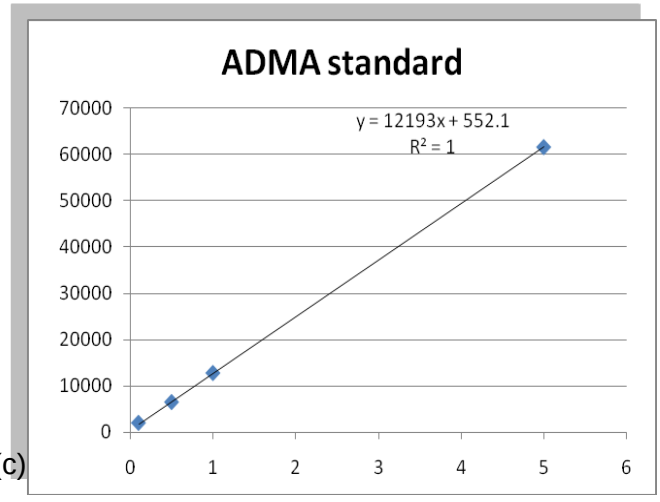
The method was found to be reliable and repeatable for the analysis of ADMA, L-arginine and L-ornithine in rat plasma samples.



(a)



(b)



(c)

Figure 16: (a) L-arginine (b) L-ornithine and (c) ADMA standard curve with regression values of 0.999; 0.999 and 1 respectively.

3.2.2 Chromatogram of L-ornithine, L-arginine, ADMA and SDMA

Figure 17 displays an example of the resultant chromatogram of the analysis of plasma samples. On this chromatogram it is also clearly illustrated that SDMA is quantified separately from ADMA.

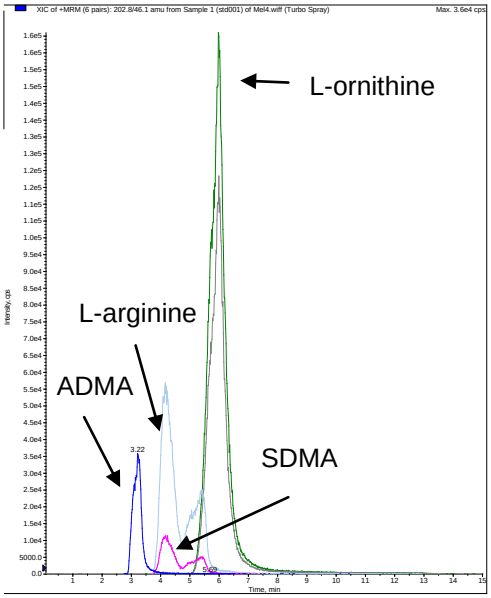


Figure 17: HPLC chromatogram representing the different peaks for ADMA, SDMA, L-arginine and L-ornithine following injection into the LC/MS/MS

3.2.3 Chemicals and reagents

Chemicals were of analytical grade or higher purity and stored at the specified conditions. All aqueous solutions were prepared using HPLC-grade water. Chemicals used for the analysis of ADMA, L-arginine and L-ornithine are listed in Table 14.

Table 14: List of reagents and chemicals used for quantification of ADMA, L-arginine and L-ornithine

Function	Chemical	Supplier
Reaction products and Standards	L-arginine (Arg) L-ornithine (Orn) N^G,N^G-dimethyl-l-arginine (ADMA)	Sigma- Aldrich
Protein precipitator	Acetonitrile (ACN)	Sigma- Aldrich
Mobile phase	Acetonitrile (ACN) Formic Acid (HCOOH) HPLC-grade water	Saarchem

3.2.4 Standard solutions

A series of dilutions for L-arginine, L-ornithine and ADMA, similar to the dilutions for nitrite, was made to obtain a concentration range of 1 ng/ml – 250 ng/ml. These concentrations were too low to quantify on the LC/MS/MS. According to the literature levels of L-arginine, L-ornithine and ADMA is in the micromolar (µM) range and thus it was decided to dilute a different series of standard solutions (Kondziella *et al.*, 2007 Sasaki *et al.*, 2004, Albsmeier *et al.*, 2004, Nonaka *et al.*, 2005).

One stock solution was prepared containing all three of the reaction products to be quantified. 1 mg equivalent of each reagent was added to 50 ml DD-water thus yielding a 20 µg/ml solution. From the stock solution the rest of the standard solutions were diluted as demonstrated in the table below. After the first test run, a 40 µg/ml standard which was obtained by adding 0.4 mg of each to 10 ml of water was added. The percentages of all three reagents were compensated for to ensure a final solution containing 100 % pure ADMA, L-arginine and L-ornithine.

Table 15: Dilutions made for standard solutions containing ADMA, L-arginine and L-ornithine

	Concentration	Dilution (make up to 2 ml)	+	DD-water	=	Total Volume
	(µg/ml)	(µl)		(µl)		(ml)
1	0,1	10	+	1990	=	2
2	0,5	50	+	1950	=	2
3	1	100	+	1900	=	2
4	5	500	+	1500	=	2
5	10	1000	+	1000	=	2
6	20	2000	+	0	=	2

3.2.5 Working concentration range of standard solutions

The working range of standard solutions contained 0.1 – 5 µg/ml ADMA, L-arginine and L-ornithine. After consulting the literature it was decided that only the lowest 4 concentrations will be used, because levels in rat plasma does not exceed 5 µg/ml.

Table 16: Components of L-arginine, L-ornithine and ADMA.

Reagent	Components	Molecular weight (MW)
L-arginine HCl		210.66 (100%)
	L-arginine	174.21 (82.69%)
	HCl	36.45 (17.31%)
L-ornithine HCl		168.62 (100%)
	L-ornithine	132.17 (78.38%)
	HCl	36.45 (21.62%)
ADMA $(HCl)_2$		275.18 (100%)
	ADMA	202.28 (73.5%)
	$(HCl)_2$	72.9 (26.5%)

The values of the areas under the curve (AUC) as shown in Table 17 represent standard solutions in µg/ml or µM concentrations. The regression curve as depicted in Figure 16 of these three reagents, displayed a linearity of 0.999.

Table 17: Concentrations and AUCs of standard solutions

µg/ml	µM	AUC
L-arginine		
0.1	0.57	13183
0.5	2.87	52950
1	5.74	88567
5	28.70	485667

µg/ml	µM	AUC
L-ornithine		
0.1	0.76	39700
0.5	3.78	79033
1	7.57	185167
5	37.83	843667

µg/ml	µM	AUC
ADMA		
0.1	0.49	1960
0.5	2.47	6453
1	4.94	12733
5	24.72	61533

3.2.6 Determination of arginase II activity and ADMA levels in rat plasma

FSL and FRL rats (200 +/- 20g) were sacrificed by decapitation and trunk blood collected in 5ml heparin tubes. The blood was immediately centrifuged at 2800 xg for 15 minutes to separate the plasma from the red blood cells where after the plasma was collected and stored at -80 ^{o}C until the day of analysis.

On the day of analysis the plasma samples were removed from the freezer and left at room temperature to thaw. 100 µl ACN was then added to 100 µl of the plasma sample and centrifuged at 10 000 xg for 5 minutes to precipitate the proteins, before injecting it into the LC/MS/MS apparatus.

3.3 Statistical information

Experimental data was analyzed using a number of statistical tests.

3.3.1 Grubbs test

The Grubbs' test, named after Frank E. Grubbs (Grubbs, 1969, Stefansky, 1972), is based on the assumption of normality. Grubbs' test detects one outlier at a time. This outlier is excluded from the dataset and the test is iterated until no outliers are detected. While the Grubbs test is only suitable for sample sizes exceeding six, this was an effective way to identify outliers in this study comprising of at least 15 data points.

The Grubbs' test was used to identify outliers within each group of FSL and FRL group of rats. It was also used to identify outliers when the standard series' were determined, to identify outliers between the different runs each day.

3.3.2 Student's T- Test

The Student's t-test assesses whether the means of two groups are statistically different from each other. This analysis is appropriate whenever you want to compare the means of two groups.

In this study, we compared data from the FSL rat group to that of the FRL rat group. Thus, we used Student's t-tests to determine the difference in the means of:

- L-arginine concentration in plasma
- L-ornithine concentration in plasma
- ADMA concentration in plasma
- Nitrite concentration in plasma

3.3.3 Pearson correlation

The correlation is one of the most common and most useful statistical techniques. A correlation is a single number that describes whether there is a relationship as well as the degree of relationship between two variables.

In this study we used Pearson correlation to determine if there is a relationship between any of the possible three markers analyzed in FSL and FRL group of rats, including nitrite, ADMA, L-arginine and L-ornithine.

3.3.4 Statistical analysis software

Statistical analysis was performed using GraphPad Prism® (Graphpad software, version 5 for Windows®San Diego, USA).

RESULTS

Chapter 4

This chapter will present all the data collected during the study, and their subsequent statistical analysis. It also explains the significance of the data and highlights the purpose and importance thereof within the context of the present study.

4.1 Plasma nitrite (NO_2^-) concentration in FRL versus FSL rats

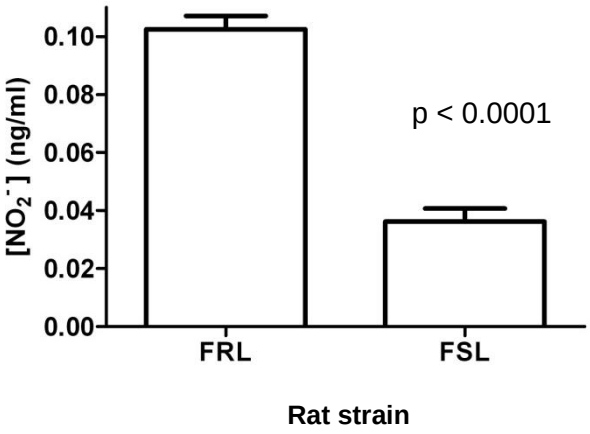


Figure 18: Plasma nitrite concentrations in FRL (n=15; 0.102 ± 0.00464 ng/ml) and FSL (n=14; 0.036 ± 0.00452 ng/ml) rat groups (T-test; p < 0.0001)

Figure 18 shows the comparison between the mean values of the plasma nitrite concentration in the FRL and FSL rat groups. From this comparison it is clear that there is a significant decrease in the plasma nitrite concentration in FSL rats compared to the FRL rats (0.036 ± 0.00452 vs. 0.102 ± 0.00464 ng/ml)

4.2 Plasma arginase II (AII) activity in FRL versus FSL rats

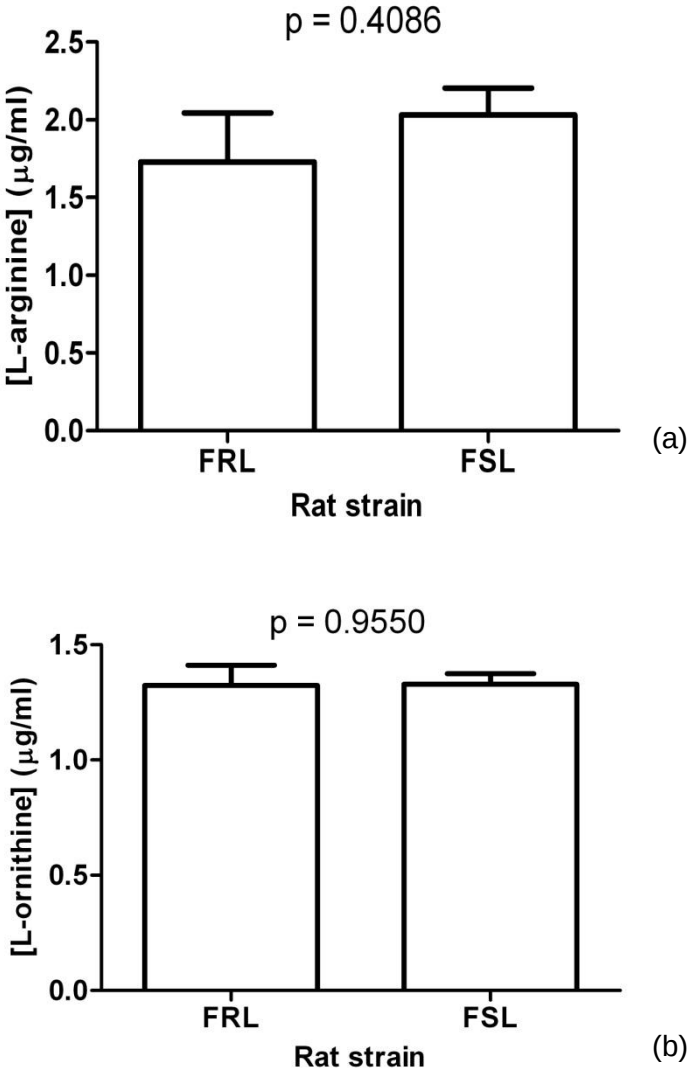


Figure 19: Plasma concentrations of a) L-arginine in FRL (n=15) versus FSL (n=14) rats (1.729 ± 0.3154 vs. 2.031 ± 0.1732 µg/ml) and b) L-ornithine in FRL (n=15) versus FSL (n=14) rats (1.323 ± 0.08776 vs. 1.328 ± 0.04675 µg/ml) (T-test p = 0.4086 and p = 0.9550 respectively).

From Figure 19a and b it is clear that there is no statistically significant difference in the mean values of either L-arginine or L-ornithine concentrations.

There is thus no statistically significant difference between arginase II activities in FSL rats compared to the FRL control rat.

4.3 Plasma asymmetric dimethylarginine (ADMA) concentration in FRL versus FSL rats

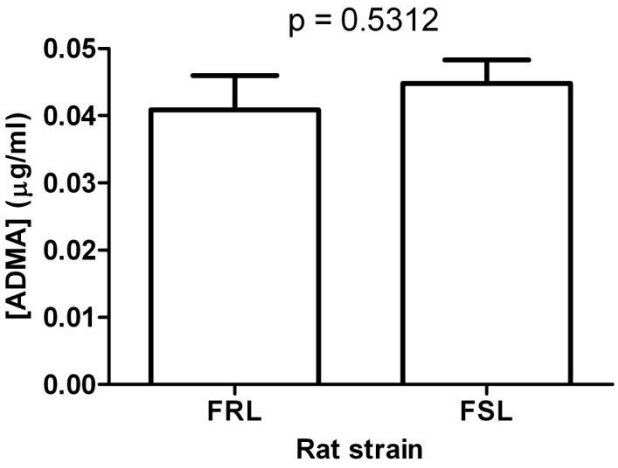


Figure 20: Plasma ADMA concentration in FRL (n=15) versus FSL (n=14) rats (0.0408 ± 0.005104 vs. 0.0448 ± 0.003524 µg/ml respectively (T-test, p = 0.5312).

From Figure 20 it is clear that there is no statistically significant difference in the mean values of ADMA concentrations between FRL and FSL rats.

4.4 Correlations

Pearson correlations were drawn between all three markers. None of the markers showed a significant correlation ($p < 0.05$).

Table 18: Pearson correlations between ADMA, L-arginine and L-ornithine.

Compared concentration	p-value
ADMA and nitrite	0.925
ADMA and L-arginine	0.108
ADMA and L-ornithine	0.420
L-ornithine and nitrite	0.417
L-ornithine and L-arginine	0.112
L-arginine and nitrite	0.316

DISCUSSION

Chapter 5

As previously discussed, the NO-pathway is of high importance in the pathophysiology of depression, and in the current study three steps in this pathway were investigated and determined.

5.1 Nitrite

Plasma nitrite was chosen because 1) nitrite in plasma was found not to be influenced by the diet of the subject, even after a high NO meal is consumed (Pannala *et al.*, 2003) and 2) nitrite measurements in plasma are the most sensitive and reliable manner to determine the levels in biological fluids (Grau *et al.*, 2007). Results from previous studies are highly inconsistent and some authors claim a decrease (Chrapko *et al.*, 2004, Vandel *et al.*, 1997, Selley, 2004) and others, an increase (Herken *et al.*, 2007, Kim *et al.*, 2006) in the plasma nitrite levels of depressed subjects compared to their healthy controls.

We found that there was a significant decrease in the nitrite levels of the FSL rat compared to its healthy control the FRL rat. This is consistent with results from clinical studies done by other authors who also found a decrease in plasma nitrite levels in depressed patients (Chrapko *et al.*, 2004, Vandel *et al.*, 1997, Selley, 2004) although the numerical values of the mean concentrations were found to be lower in the current study than they were in most of the other studies done on rats (Kleinbongard *et al.*, 2003). This might be due to a number of reasons especially the presence of contamination factors, which may give rise to false higher nitrite levels (see section 4.2.6). This phenomenon of contamination that poses as a big problem when determining NO_x levels, were also described in detail by previous authors and should be considered in future studies (Grau *et al.*, 2007). It is therefore of utmost importance to know exactly what measures were taken to prevent contamination before comparing nitrite levels from one laboratory with the results of another laboratory.

Nitrite showed a significant decrease in the FSL rat, a genetic animal model of depression. Regarding the results of this study and taking all previous data which show inconsistent results (Chrapko *et al.*, 2004, Ikenouchi-Sugita *et al.*, 2009, Kim *et al.*, 2006, Selley, 2004) as well as the many contamination factors (Grau *et al.*, 2007), into consideration, nitrite may be useful as biomarker only if all contributing factors such as contamination and diversity of mechanisms is

accounted for. Firstly, the multiple physiological and pathophysiological mechanisms by which NO operate; make it a very diverse molecule. Some studies imply that NO has cytotoxic properties and is the cause of numerous diseases and degenerative states, whereas other reports suggest that NO prevents injurious conditions from developing and promotes homeostatic events (Wink *et al.*, 1998). Secondly the factors which could contribute to the contamination of the sample to be quantified during sample preparation and storage is of great concern, (Grau *et al.*, 2007) and lastly the reduction of nitrite to NO (Gautier *et al.*, 2006) might also pose as a problem when quantifying and establishing nitrite levels in biological samples.

5.2 Arginase II activity

Arginase II (AII) also plays a pivotal role in the NO-pathway and is readily found in the central nervous system especially the brain. Arginase II is the enzyme responsible for the conversion from L-arginine to L-ornithine (Wiesinger, 2001). According to results from previous studies it was expected that arginase II levels would show an increase in depressed subjects compared to their healthy controls (Elgün *et al.*, 2000).

Arginase II activity was measured by determining the conversion of L-arginine to L-ornithine. Since arginase II converts L-arginine to L-ornithine, arginase II activity is determined by measuring the difference in the precursor (L-arginine) and product (L-ornithine). Thus a decrease in L-arginine concentration or an increase in L-ornithine concentration will indicate an increase in arginase II activity and visa versa (Geyer *et al.*, 1971).

We found no significant change in arginase II activity after comparing the mean values of the FSL rat to its healthy control the FRL rat. This is inconsistent with previous results where an increase in arginase II activity was observed in depressed subjects (Elgün *et al.*, 2000). This might be because depression is associated with the L-arginine-NO-pathway (Kulkarni *et al.*, 2007) and arginase II activity is not directly involved in this pathway, but has its effect on the conversion of L-arginine to L-ornithine (Wiesinger, 2001). Although an increase was observed by the above mentioned author, this was the only article according to our knowledge that addressed the role of arginase II activity in depressed subjects. Thus it is now clear that although L-arginine plays a role in the pathophysiology of depression (Rosa *et al.*, 2003) arginase II activity shows no significant difference between FSL and FRL rats in this animal model of depression.

Although arginase II levels in the FSL and FRL rats did not significantly differ from each other, it is not suggested that arginase II should be excluded in further clinical research. The possibility still

remain that human and FRL/FSL-rat neurobiology may be different or that other neurobiological subtypes exist in rats compared to humans.

5.3 ADMA

ADMA is an endogenous inhibitor of NOS, which is the enzyme responsible for converting L-arginine to NO. Previous studies showed an increase in plasma ADMA levels in depressed subjects compared to their healthy controls as well as a negative correlation between the levels of ADMA and NO (Selley, 2004).

We found no statistical difference after comparing mean plasma ADMA values of FSL rats to their healthy control the FRL rats. This is in agreement with data from a clinical study done in depressed men and woman (Taylor *et al.,* 2006)., although inconsistent with two other clinical studies where an increase in ADMA levels were found in depressed patients (Selley, 2004) and in depressed patients with an increased risk for CVD who also presented with increased ADMA levels. (Ko *et al.,* 2010).

It has already been established that ADMA plays a pivotal role in a number of cardiovascular diseases such as endothelial dysfunction, vasoconstriction, elevation of blood pressure, and aggravation of experimental atherosclerosis (Böger *et al.*, 2009) as well as in diabetes (Krzyzanowska *et al.*, 2008). The lack of a difference in ADMA levels in FSL (depressed) rats may be due to the fact that the ADMA levels are rather an indication of the higher CVD risk and not of depression as such.

Selly et al. also reported a strong negative correlation between NO and ADMA levels (Selley, 2004) but, we found no statistical correlation between these two markers. The fact that nitrite levels were decreased in this study but ADMA levels showed no significant change may further be an indication that there is no relationship between ADMA and depression, and that previously published data showing a correlation between ADMA and NO (Selley, 2004) may be due to a possible cardiovascular disease risk of the depressed patients.

5.4 Relevance of these data for the role of the NO-pathway in major depressive disorder

The NO-pathway plays a pivotal role in the pathophysiology of depression through a variety of mechanisims including oxidative stress through the formation of ROS and RNOS specifically peroxynitrite and the involvement of changes in the levels of L-arginine, NOS activity, NO and

other metabolites of NO (Herken *et al.*, 2007, Maes *et al.*, 2010, Mantovani *et al.*, 2003, van Amsterdam *et al.*, 1999, Wang *et al.*, 2008).

The rising number of patients presenting with depression and the current problem that 30% of patients show no response to any treatment given (Rosenzweig-Lipson *et al.*, 2007), emphasizes the fact that research done on depression is of high importance. The urgency to elucidate new markers for diagnosing MDD also emphasizes the importance of the current research.

As plasma nitrite levels were significantly lower in FSL rats versus their FRL control, these data confirm an imbalance in NO signaling in depression, specifically a reduction in NOS activity with lower NO production in these patients. Most studies in humans with depression have found a decrease in plasma NO_x (Chrapko *et al.*, 2004, Ikenouchi-Sugita *et al.*, 2009, Selley, 2004), although some studies have not agreed (Kim *et al.*, 2006:1091.). Moreover, animal studies have demonstrated that NOS inhibitors are antidepressant (Ulak *et al.*, 2008), while known antidepressants also inhibit NOS (Wegener *et al.*, 2008). Thus, the latter would suggest that depression is more associated with an increase in NOS activity and an increase in NO production. The above data, plus our data from FSL rats, highlights the varied data that has been reported with respect to nitrite, suggesting that nitrite may not be useful as a peripheral marker of depression if all influencing factors is not taken into consideration

In this study nitrite confirmed the involvement of the NO-pathway in an animal model of depression, but other components of the NO-pathway such as the NMDA-receptor cascade, proposed to be inhibited by selective serotonin reuptake inhibitors (SSRI's) such as escitalopram (Zomkowski *et al.*, 2010) may be a better option as a biomarker of depression.

Another possible reason for the lack of differences in the NO pathway between FSL and FRL rats may be that the current study was performed in stress-naive rats and as has recently been described by Wegener et al., 2010, it is possible that the NO cascade may react differently in the presence of stress in these animals.

CONCLUSION

Chapter 6

6.1 Successful analytical method development

Two validated analytical chromatography methods were developed during this study, viz. to quantify nitrite, asymmetrical dimethylarginine (ADMA), L-ornithine and L-arginine in rat plasma. Both of the analytical methods produce reliable and reproducible results and can therefore be considered for future studies.

The HPLC-method for plasma nitrite determination with fluorescence detection offers a number of benefits regarding 1) easy sample preparation, 2) relatively short retention time and 3) fast and highly sensitive detection of nitrite in plasma of rats. This same method will now be used in future studies to obtain plasma nitrite levels in human plasma.

The LC/MS/MS method for L-arginine, L-ornithine and ADMA determination has the advantage of being simple, fast and reliable. This method can also be used to quantify other L-arginine analogues, for example symmetric dimethylarginine (SDMA) or any other amino acid in rat plasma or for that matter in human plasma samples as well.

It is of importance however to always consider the risk that contamination plays in determining the plasma levels of these substances, and especially when determining nitrite concentrations in biological fluids.

6.2 NO-pathway in major depressive disorder

Levels of ADMA and arginase II activity showed no significant difference between FSL and FRL rats, while nitrite levels were decreased in the FSL ("depressed") rats. Although nitrite as biomarker is dependent on a number of influencing factors such as contamination it might be possible to use it as a prospective biomarker of depression. The difference in the nitrite plasma levels between FSL and FRL rats strongly suggests the involvement of the NO-pathway in the pathophysiology of depression. Thus considering the previously mentioned fact, components other than ADMA and arginase II which also form part of the NO-pathway may contribute to the search for better pharmacotherapy for MDD.

6.3 Future studies and recommendations

To further elucidate the results and the understanding of the NO-pathway especially with regard to MDD, it is of importance to expand on the current study, by including the following suggestions.

6.3.1 Post-stress behavioral and neurochemical study

This study only determined basal levels of the three possible markers in stress-naive rats. It may be useful to determine the levels of nitrite after a stressor has been applied to both the FSL and FRL rats, as well as including a behavioral study such as the forced swim test to correlate with the nitrite values found in the neurochemical study (Wegener *et al.*, 2010).

6.3.2 Neurochemical determination of peroxynitrite

Peroxynitrite is one of the causes of cell damage in the brain (Thomas *et al.*, 2008). Because the pathophysiology of depression is linked to the NO-pathway through neurodegeneration and oxidative stress, it may be useful to determine if there is a difference in the peroxynitrite levels between the two rat groups. According to van Zyl, J. 1994, determination of peroxynitrite is possible in *in vitro* studies but may be difficult to determine *in vivo* because of its short half life. This may be overcome by immediately alkalizing the biological fluid because peroxynitrite is more stable in an alkaline solution or to determine an alternative for peroxynitrite quantification.

6.3.3 Prospective clinical study

FSL rats present as a useful animal model of depression because of its close resemblance to the depressed patient (Overstreet *et al.*, 2005). A clinical study however, would be necessary to further prove the relevance to humans and whether nitrite can be used as a non-invasive biomarker of depression. Clinical studies should however focus on minimizing nitrite contamination of the samples during sample preparation and storage (Grau *et al.*, 2007) as well as considering the influence of the diet of the patient (Pannala *et al.*, 2003) before comparing results.

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