LC-MS/MS ANALYSIS OF PLASMA HOMOCYSTEINE AND INVESTIGATION ON THE ASSOCIATION OF HOMOCYSTEINE LEVELS WITH DIABETIC PERIPHERAL NEUROPATHY

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by

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To my beloved parents, Lim Ka Seong and Lee Foon Fung,

my brother and my sisters

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LIST OF ABBREVIATIONS, NOMENCLATURE AND SYMBOLS

ALT	Alanine transaminase
ANOVA	Analysis of variance
API	Atmospheric pressure ionisation
AST	Aspartate aminotransferase
BMI	Body mass index
C8	Carbon 8
C18	Carbon 18
°C	Celsius
CMIA	Chemiluminescence Immunoassay
CV	Coefficient of Variation
CI	Confidence interval
DPN	Diabetic peripheral neuropathy
DM	Diabetis mellitus
DTT	Dithiothreitol
eV	Electronvolt
ESI	Electrospray ionisation
FA	Folic Acid
FBS	Fasting blood glucose
FPIA	Fluorescence polarization immunoassay
GC-MS	Gas chromatography mass spectrometry
GGT	Gamma-glutamyl transferase
g	G-force
HbA1c	Glycosylated hemoglobin
Hcy	Homocysteine

HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
Hz	Hertz
IS	Internal standard
k	Kilo
LC-CN	Liquid chromatography cyano bonded phase column
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
l or L	Litre
L/hr	Litre per hour
mbar	Milibar
mg	Milligram
ml	Mililitre
min	Minutes
M	Molar
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
m/z	Mass per charge
µ	Micro
ng	Nanogram
nmol	Nanomole
NIS	Neuropathy impairment score
NMRR	National Medical Research Register

NNT	Number needed to treat
%	Percentage
p	Pico
pg	Picogram
pmol	Picomole
Q1	First quadrupole
Q3	Third quadrupole
QST	Quantitative sensory testing
rSAHHase	S-adenosyl homocysteine hydrolase
SAH	S-adenosyl homocysteine
SD	Standard deviation
SEM	Standard error of mean
TCEP	Tris(2-carboxyethyl)phosphine
TCSS	Toronto clinical scoring system
TSS	Total symptom score
Tukey's HSD	Tukey's Honestly Significant Different
UV	Ultraviolet
V	Volt
vs	Versus
v/v	Volume/volume percent
WHO	World Health Organisation

LIST OF APPENDICES

ANALISIS LC-MS/MS HOMOSISTEIN PLASMA DAN PENYELIDIKAN TENTANG KAITAN PARAS HOMOSISTEIN DENGAN NEUROPATI PERIFERAL DALAM KALANGAN PESAKIT DIABETES

ABSTRAK

Suatu kaedah LC/MS-MS untuk mengukur paras homosistein plasma dari sampel manusia telah dihasilkan. Kaedah ini melibatkan penyediaan sampel yang mudah dan telah menunjukkan kepersisan, kespesifikan, kesensitifan dan kejituan yang baik. Dengan itu, kaedah ini boleh digunakan untuk memeriksa aras homosistein sampel klinikal dalam bilangan yang besar. Di samping itu, kaedah ini juga dibandingkan dengan kaedah imunoasai yang digunakan oleh makmal patologi komersial untuk pengukuran paras homosistein. Kedua-dua kaedah ini menunjukkan kejituan yang baik dan min perbezaan yang rendah antara satu sama lain. Dua puluh enam peratus daripada 260 pesakit kencing manis dengan neuropati peripheral yang direkrut mempunyai paras homosistein yang tinggi. Terdapat lebih ramai lelaki daripada perempuan di dalam kumpulan hiperhomosistein (homosistein $\geq$15μM). Paras homosistein adalah berkaitan secara langsung dengan paras kreatinina pesakit kajian ini, manakala paras vitamin B12 dan asid folik adalah berhubungan songsang dengan paras homosistein pesakit. Kajian ini telah mendapati bahawa paras homosistein tidak berkorelasi dengan keterukan neuropati kencing manis. Walau bagaimanapun, umur, jantina dan jangka masa pesakit menghidapi kencing manis adalah berhubungan langsung dengan ketenatan neuropati. Golongan pesakit yang berumur lebih tinggi dan jantina lelaki mempunyai min skor gangguan neuropati yang tinggi. Pesakit yang dijangkiti penyakit kencing manis untuk tempoh yang lebih panjang juga mempunyai min skor gangguan neuropati yang lebih tinggi.

LC-MS/MS ANALYSIS OF PLASMA HOMOCYSTEINE AND INVESTIGATION ON THE ASSOCIATION OF HOMOCYSTEINE LEVELS WITH DIABETIC PERIPHERAL NEUROPATHY

ABSTRACT

A simple LC-MS/MS method was developed to measure homocysteine level in human plasma collected from diabetic patients. This method which involves simple sample preparation showed good precision, specificity, sensitivity and accuracy. Thus, it can be used to screen a vast number of clinical samples. This method's results were compared with commercial laboratory results derived from the immunoassay method. Both methods showed good accuracy with low mean difference. Of the 260 patients recruited with peripheral neuropathy, 26% showed hyperhomocysteinemia (homocysteine ≥15μM). The male gender was more prominent in the hyperhomocysteine group. Homocysteine level was directly associated with creatinine, while homocysteine level was inversely correlated with the vitamin status (B12 and folic acid). No correlation was observed between homocysteine level and severity of neuropathy in the present study. However, age, gender and duration of diabetes were associated with neuropathy severity. Higher age group patient and the male gender recorded higher means in neuropathy impairment score. A higher mean neuropathy impairment score was also observed with longer duration of diabetes.

CHAPTER 1

LITERATURE REVIEW

1.1 Diabetes Mellitus

Diabetes mellitus is a chronic metabolic disease characterised by high blood glucose levels resulting from defects in insulin secretion, insulin action, or both. Diabetes mellitus is diagnosed as having fasting plasma glucose level of more than 7.0mmol/l, HbA1c≥6.5% or two-hour plasma glucose of more than 11.1mmol/l during an oral glucose tolerance test (OGTT) (American Diabetes Association, 2014).

Diabetes can be classified into four clinical categories. Type 1 diabetes mellitus is characterised by the body's inability to produce insulin due to an autoimmune destruction of the pancreatic beta cells, whereas Type 2 diabetes is a non-insulin dependent illness, and it occurs due to beta cells secretory defects on the background of insulin resistance. There are other specific types of diabetes due to other causes, such as genetic defects in beta cells function, genetic defects in insulin action, and diseases of the exocrine pancreas. Gestational diabetes mellitus (GDM) is also one of the categories of diabetes (American Diabetes Association, 2014).

The prevalence of diabetes is rising worldwide, threatening to reach pandemic levels by 2030. In Malaysia, the fourth National Health and Morbidity Survey reported that the prevalence of type 2 diabetes mellitus for adults aged 18 years and above was 15.2% (Feisul *et al.*, 2014; Mustapha *et al.*, 2014). This figure is projected to rise to 21.6% by year 2020 (Feisul and Azmi, 2013).

Diabetes mellitus is a major public health concern as it causes other medical comorbidities, disabilities and premature mortality. Direct and indirect effects of hyperglycemia on human vascular tissue are the major causes of morbidity and mortality in Type 1 and 2 diabetic patients. Complications of diabetes mellitus include nephropathy, retinopathy, coronary arterial diseases, peripheral arterial diseases, stroke and neurodegenerative disorder like Alzheimer Disease. (Laakso, 2011; Forbes and Copper, 2013).

Diabetes Mellitus is a metabolic disorder. Metabolic damage as a result of hyperglycemia has a direct effect on nerves causing nerve damage leading to painful neuropathy. The condition is also exacerbated by decreased blood flow to the nerves due to microvascular complications of diabetes. The anatomy of the peripheral nervous system which is covered by perineurium, endoneurium is penetrated by only a few transperineurial arterioles. Thus, the vascular supply to the peripheral nervous system is sparse and there will be a lack of autoregulation and blood flow in the peripheral nerve (Smith, 1977; Yagihashi et al., 2011). A noticeable structural change in diabetic nerves due to thickened and multilayered basement membranes, cell debris of pericytes and endothelial cell disruption are shown in endoneurial microvessels. In addition, impaired blood supply in diabetic nerves is seen in epineurial microvessels innervation (Grover-Johnson *et al.*, 1981; Tesfaye *et al.*, 1994; Yagihashi *et al.*, 2011). Poor management of the condition can lead to gangrenous injuries and limb amputation. Thus, diabetic neuropathy is the most common and debilitating complication of diabetes.

1.2 Complications of Diabetes Mellitus

1.2.1 Types of Complications

Atherosclerosis results in chronic inflammation to the arterial wall of the coronary and peripheral vascular system. The metabolic abnormalities of diabetes such as hyperglycemia, increased free fatty acids, and insulin resistance contribute to vascular dysfunction. These occur by decreasing the bioavailability of nitric oxide (NO), increasing the oxidative stress, disturbances of intracellular signal transduction, and activation of receptors for advanced glycation end products (AGE). All these abnormalities will lead to atherosclerosis and increase the risk of adverse cardiovascular events in patients with diabetes and atherosclerosis (Creager and Lüscher, 2003).

Cardiovascular disease (CVD) is the leading cause of death in patients with Type 2 diabetes. People with diabetes, particularly Type 2 diabetes will have greater risk factors for CVD, which include age, dyslipidemia, hyperglycemia, hypertension, obesity, and tobacco use. These factors will cause an injury to the vascular endothelium and lead to macrovasculopathy and CVD in Type 2 diabetic patient (Beckman *et al.*, 2002). According to Haffner *et al.* (1998), there is a 5 fold higher risk of people with diabetes developing a myocardial infarction (MI) and a 2 fold increase risk of recurrent MI compared to people who had an MI previously but do not have diabetes history. After an occurrence of MI in people with diabetes, they have a growing risk for congestive heart failure and death (Malmberg *et al.*, 2000).

Furthermore, people with diabetes have an increased incidence of independent risk factor for stroke. The presence of diabetes increases the risk of

intracranial and extracranial atherosclerosis and thus affects the cerebrovascular circulation. People with both complications of diabetes and stroke have more neurological deficits and disability, and also a higher incidence of stroke recurrence. Hyperglycemia occurs to be a significant predictor of fatal and non-fatal stroke, and death from stroke (Sasaki *et al.*, 1995). In addition, there is an association between elevated blood levels of chronic inflammatory markers and higher risk of stroke in people with diabetes (Engstrom *et al.*, 2003).

Peripheral Arterial Disease (PAD) is characterised by occlusion of lower extremity arteries, causing intermittent claudication and pain that appears reproducibly with walking, resulting in functional impairment and disability (Vogt *et al.*, 1994; Schainfeld, 2001; McDermott *et al.*, 2004). The determination of PAD in diabetes has been difficult as most incidents occur asymptomatically, no uniform screening modalities are available and its pain perception is blunted due to peripheral neuropathy. Diabetes and smoking are the strongest risk factors for PAD. Others include elevated C-reactive protein level, apolipoprotein B, lipoprotein a, fibrinogen, glycation hemoglobin, homocysteine, and plasma viscosity, duration and severity of diabetes, hypertension, dyslipidemia and history of CVD (American Diabetes Association, 2003; Al-Delaimy *et al.*, 2004; Wattanakit *et al.*, 2005). There is a strong association between diabetes with femoral-popliteal and tibial PAD. People with diabetes and PAD are at a higher risk of presenting an ischemic ulcer, gangrene and lower-extremities amputation. PAD is also a marker for systemic vascular disease; contributing to an increased risk of myocardial infarction (MI), stroke and death (American Diabetes Association, 2003).

Diabetic nephropathy, one of the complications of diabetes, is preceded by microalbuminuria which is defined by albumin excretion of 30-299 mg/24 hours. This will typically progress to overt albuminuria and then to diabetic nephropathy. Comparing with the European populations, the risks of impaired fasting glucose and of impaired glucose tolerance in citizens of South-East Asian origin are higher (Gray *et al.*, 2010). Furthermore, the prevalence of any type of chronic kidney disease and its rate of progression, including diabetic nephropathy, is significantly higher in citizens of Asian origin, as observed both in the UK (Lightstone *et al.*, 1995) and in Canada (Barbour *et al.*, 2010). This result is presumably due to the different genetics and lifestyle. The characteristics of diabetic nephropathy include thickening of glomerular basement membrane and glomerular hyperfiltration, which will lead to mesangial extracellular matrix expansion which increases urinary albumin excretion and progresses to glomerular and tubular sclerosis and renal failure (Van Dijk and Berl, 2004; Bloomgarden, 2005; Fowler, 2008). The risk factors for diabetic nephropathy are hyperglycemia, longer duration of diabetes, age, dyslipidemia, hypertension and obesity (Ismail *et al.*, 1999; Timothy and Peter, 2000; Kramer *et al*., 2005).

Diabetic retinopathy affects the peripheral retina, macula, or both and it is the leading cause of visual disability and blindness. The prevalence of diabetic retinopathy increases with prolonged duration of diabetes (Donald *et al.*, 2003). Few pathological mechanisms that will lead to diabetic retinopathy include existence of aldose reductase, osmotic stress, glycoprotein injuries effect, hyperglycemia induced cellular injury and production of growth factors (Fowler, 2008). The onset of histological marker of diabetic retinopathy is the loss of pericytes, which will then

interfere with capillary constriction, new capillary generation and vessels protection against continuous exposure to noxious molecules (Cade, 2008).

1.2.2 Common Mechanism of Complications in Diabetes Mellitus

The pathophysiology of vascular disease in diabetes involves abnormalities in endothelial, vascular smooth muscle cells, and platelet formation. The common mediator in endothelial cells dysfunction is derangement of nitric oxide (NO) bioavailability. The presence of NO is vital as it stimulates vasodilatation and mediates inflammation (Wallace, 2005). NO also inhibits vascular smooth muscle cell migration and proliferation, and limits platelet activation. However, there are some mechanisms that lead to the loss of function of NO, including hyperglycemia, insulin resistance, and free-fatty acid production. Hyperglycemia blocks the function of endothelial nitric oxide synthase (NOS) and enables the production of reactive oxygen species, which will impair the endothelial vasodilation (Funk *et al.*, 2012). Hyperglycemia also increases the production of advanced glycation end-product (AGE), which inhibits NO production, impairs vasodilatory response in diabetes and causes an overproduction of vasoconstrictors, such as endothelin-1 (Rask-Madsen and King, 2007; Linden *et al*., 2008). Endothelin-1 induced oxidative stress, promotes inflammation and caused endothelial dysfunction (Touyz *et al.*, 2004).

Insulin stimulates endothelial cells NO vasodilatation and hence, improves the NOS activity. In the case of Type 2 diabetes mellitus, insulin signal transduction via the phosphatidylinositol-3 kinase (PI-3Ks) pathway is impaired, and insulin is less able to activate NOS and produce NO (Dresner *et al.*, 1999; Inoguchi *et al.*, 2000). Insulin resistance is associated with elevations of free fatty acids (FFA). The

existence of FFA activates protein kinase C (PKC), inhibits (PI-3Ks), and produces reactive oxygen species. This will decrease NOS activity. In response to endothelial injury, oxidized lipids will be accumulated. Angiotensin II will promote the oxidation, and then the monocytes will infiltrate the arterial wall and differentiate into macrophages, which accumulate oxidized lipid to form foam cells. Foam cells stimulate macrophage proliferation and attract T-lymphocytes. T-lymphocytes will then induce smooth muscle cell proliferation and collagen accumulation in the arterial wall. The rupture of the lipid-rich atherosclerotic lesions leads to acute vascular infarction (Boyle *et al.*, 2007).

Diabetes causes functional abnormalities in Vascular Smooth Muscle cells (VSMC), including reductions in (PI-3Ks), increase in oxidative stress and up regulations of PKC, receptors for AGE, and nuclear factor-kB (NF-kB). These changes promote atherosclerosis lesions and may increase VSMC apoptosis and tissue factor production (American Diabetes Association, 2003). Patients with diabetes have increased platelet aggregation due to oxidative stress, caused by platelet uptake of glucose in endothelial cells. Platelets in diabetic patients have increased glycoprotein Ib and IIb/IIIa receptors expression, which is involved in thrombosis. Abnormalities in platelet promote atherosclerosis, plaque disruption and atherothrombosis (American Diabetes Association, 2003).

1.3 Neuropathic Pain

1.3.1 Introduction

Neuropathic pain is caused by abnormal physiology of peripheral or central nervous system. Unlike physiologic pain, neuropathic pain does not arise from the

activation of primary afferent neurons (nociceptors) and is not related to the inflammatory response in the peripheral nervous system after tissue damage or inflammation (Dworkin *et al.,* 2003). Neuropathic pain and physiologic pain are different in many ways. Physiologic pain serves to warn and protect the individual against injury and usually subsides with time, while neuropathic pain serves no useful purpose and it is usually sustained and chronic. Neuropathic pain mechanism is complex, multifactorial, and evolves over time (Beydoun and Backonja, 2003; Dworkin *et al.*, 2003).

1.3.2 Pathophysiology of Neuropathic Pain

Neuropathic pain results from cellular changes that occur in both the peripheral and central nervous systems, leading to sensitisation to pain signal transmission. Figure 1.1 showed pathophysiological mechanisms of neuropathic pain. In the primary afferent pathways connection with the spinal cord dorsal horn, nociceptive C-fibres terminate at spinothalamic projection neurons in upper laminae, whilst non-nociceptive myelinated A-fibres project to deeper laminae. Ongoing discharges of peripheral afferent fibres that release excitatory aminoacids and neuropeptides within dorsal horn of the spinal cord lead to postsynaptic changes of second-order nociceptive neurons. These changes include the expression of voltage-gated sodium channels or the phosphorylation of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid (AMPA) receptors (Hains *et al.,* 2004; Ultenius *et al.,* 2006). Neuronal hyperexcitability that enables low-threshold mechanosensitive Aβ and Aδ afferent fibres is induced to activate second-order nociceptive neurons. The second-order projection neuron is a wide dynamic range (WDR) type. It receives a direct synaptic input from the nociceptive terminals

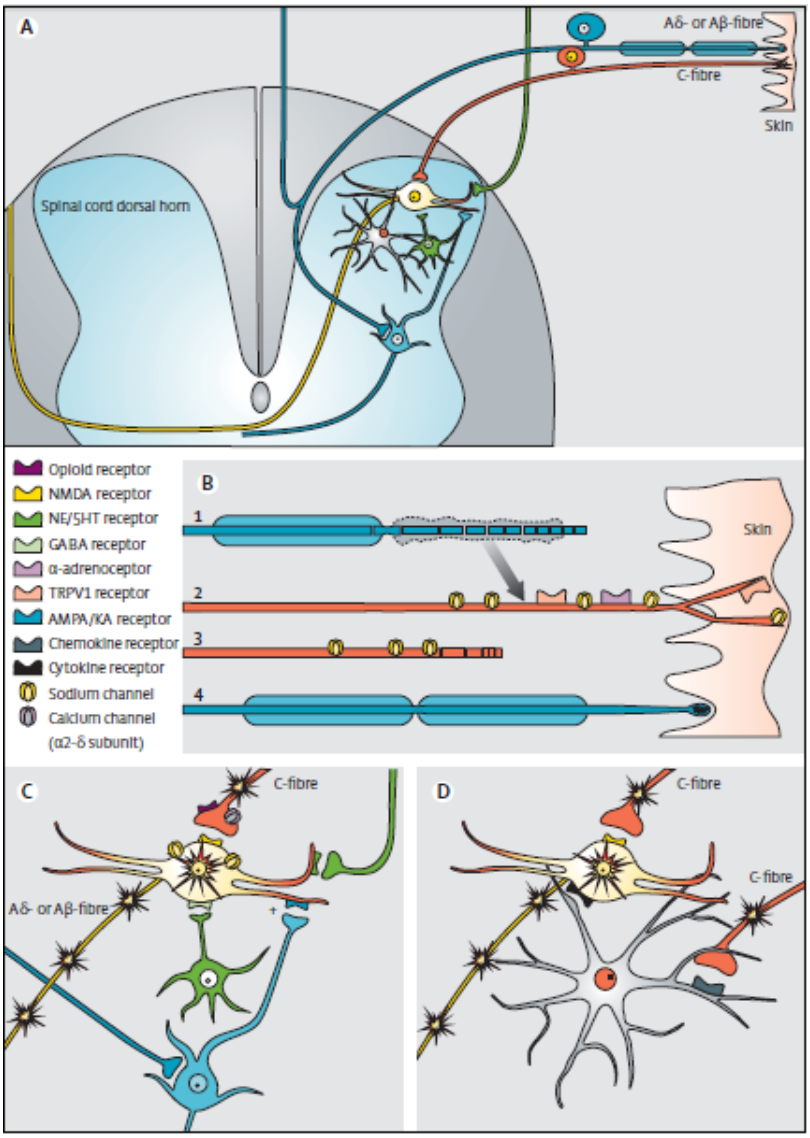

Figure 1.1 Pathophysiological mechanisms of neuropathic pain (Baron *et al.*, 2010).

A. Primary afferent pathways and their connections in the spinal cord dorsal horn. C-fibres (red); spinothalamic projection neurons (yellow); A-fibres (blue); microglia (grey); GABAergic interneurons (green).

B. Peripheral changes at primary afferent neurons after a partial nerve lesion, leading to peripheral sensitisation. Damaged and degenerated axon (axon 1 and 3); Axon are connected with peripheral end organ (axon 2 and 4).
C. Spontaneous activity in C-nociceptors induces secondary changes in central sensory processing. Second-order nociceptive neuron (star in yellow neuron); inhibitory interneurons (green).
D. Peripheral nerve injury. Spinal cord glial cell (grey).
(AMPA= α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid, GABA=gamma-aminobutyric acid, KA=kainite, NMDA=N-methyl-D-aspartate, TRPV1=transient receptor potential V1, NE=norepinephrine, 5HT=5-hydroxytryptamine)

and also multisynaptic input from myelinated A-fibres. Futhermore, interaction with microglia facilitates synaptic transmission. GABAergic interneurons normally exert inhibitory synaptic input on the WDR neuron. After a peripheral nerve lesion, loss of inhibitory GABAergic interneurons in the spinal cord occurs (Moore *et al.*, 2002). Mechanical and thermal hyperalgesia will be attenuated in the prevention of interneurons cell death, indicate disinhibition will lead to neuropathic pain (Scholz *et al.*, 2005).

Central mechanism of neuropathic pain is initiated by tachykinin and neurotransmitter released from peripheral nociceptors (Beydoun and Backonja, 2003). Meanwhile, spontaneous activity in C-nociceptors induces secondary changes in central sensory processing, leading to spinal cord hyperexcitability. This changes causes input from mechanoreceptive A-fibres to be perceived as pain. Several presynaptic molecular structures (opioid receptors and calcium channels) and postsynaptic molecular structures (glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid /kainite receptors, sodium/5-hydroxytryptamine receptors, gamma-aminobutyric acid receptors and sodium channels) are involved in central sensitization (Baron *et al.*, 2010). Besides, increased intracellular calcium due to prolonged binding of tachykinin and neurotransmitter to neural receptors had activated the N-methyl-D-aspartate (NMDA) receptors. This changes lead to a lower activation threshold, increased response to stimuli and enlarged receptive field of spinal dorsal horn neurons (Siddal and Cousins, 1997; Beydoun and Backonja, 2003). Moreover, between neurons ephaptic conduction, neuronal reorganisation and central inhibition may occur in central neuropathic pain (Attal, 2000; Bridges *et al.*, 2001). Inhibitory interneurons and descending modulatory control systems are dysfunctional

after nerve lesions, leading to disinhibition of spinal cord dorsal horn neurons and lead to central sensitization (Baron *et al.*, 2010). The clinical correlates include allodynia, secondary hyperalgesia, and sympathetically maintained pain. A list of central neuropathic pain syndromes includes compressive myelopathy from spinal stenosis, HIV myelopathy, multiple sclerosis pain, Parkinson's disease pain, poststroke pain, spinal cord injury pain and syringomyelia (Dworkin, 2002; Dworkin *et al.*, 2003).

Peripheral neuropathic pain mechanism is initiated by release of chemicals from damaged cells and inflammatory cells (Siddall and Cousins, 1997). This chemicals act to sensitize nociceptors. As a result, sodium and calcium channels are altered and the threshold for depolarisation is lowered, leading to peripheral sensitization (Beydoun and Backonja, 2003). Meanwhile, peripheral changes at primary afferent neurons (nociceptive C-fibers, non-nociceptive myelinated A-fibers) after partial nerve lesion will lead to peripheral sensitization. The lesion triggers the expression of sodium channels on damaged C-fibers, increases expression of sodium channels in dorsal root ganglia and around the terminal injury site of injured axons, and increases expression of α2δ calcium channels (Baron, 2006; Cohen and Mao, 2014). Furthermore, there will be collateral sprouting of primary afferent neurons, recruitment of nearby uninjured nociceptors and coupling between the sympathetic nervous system and sensory nervous system occur in peripheral neuropathic pain (Bridges *et al.*, 2001). Nerve growth factor that are associated with Wallerian degeneration are released in the vicinity of spared fibers, triggering channel and receptor expression (sodium channels, TRPV1 receptors, adrenoceptors) on uninjured fibers (Baron, 2006). Wallerian degeneration decreases the size of the cell

body and the axon diameter, and cause neuronal death. These changes decrease intraepidermal nociceptors density and lead to loss of sensation or increased pain (Cohen and Mao, 2014). Clinical correlates of peripheral neuropathic pain include hyperalgesia, burning pain, dysesthesias and paresthesias. There are few types of peripheral neuropathic pain syndromes, which comprise of alcoholic polyneuropathy, chemotherapy-induced neuropathy, complex regional pain syndrome, diabetic peripheral neuropathy, entrapment neuropathies, HIV sensory neuropathy, neuropathy secondary to tumor infiltration, phantom limb pain, postherpetic neuralgia, postmastectomy pain, radiculopathy, and trigeminal neuralgia.

1.4 Diabetic Neuropathy

1.4.1 Diabetic Peripheral Neuropathy (DPN)

DPN is defined as pain arising as a direct consequence of abnormalities in the peripheral somatosensory system among diabetes mellitus patients (Treed *et al.*, 2008; Tesfaye *et al.*, 2010). DPN occurs in up to 50% of diabetic patients and causes sensory, motor and autonomic dysfunction (Boulton *et al.*, 2005). DPN is a heterogeneous disorder which includes mononeuropathy, polyneuropathy, plexopathy and radiculopathy. Neuropathies can be classified as symmetrical form which is primarily sensory and autonomic; or asymmetrical form which is sensory, motor, or both, as well as affecting the individual cranial or peripheral nerves (Simmons and Feldman, 2002). The most common form of DPN is distal symmetrical form, which is known as diabetic sensorimotor peripheral neuropathy or distal symmetric diabetic peripheral neuropathy.

DPN begins with signs and symptoms in the feet, indicating that the most distal nerve fibers are affected first. It then proceeds to affect more proximal part of the lower limbs and then distal part of the upper limbs. It is often described as stocking-glove distribution neuropathy (Said, 2007). It usually presents with sensory disturbance in toes or feet, and a common complaint of tingling or pricking sensation. Vibration sense at the toes is affected most frequently (Llewelyn, 2003). However, in some patients with more severe loss of sensation, it may present with symptoms in the hands (Boulton, 2005). DPN may or may not be accompanied with clinically significant symptomatic autonomic neuropathy (Llewelyn, 2003; Vinik *et al.*, 2003). In addition, significant motor symptoms usually occur after autonomic neuropathy (Vinik *et al.*, 2013).

1.4.2 Other types of diabetic neuropathy

Autonomic neuropathy is a type of neuropathy frequently occurring in diabetic patients. This includes cardiovascular disturbance and postural hypotension. It is usually underdiagnosed and presents abnormalities such as resting tachycardia, exercise intolerance, resting heart rate variability, orthostasis and increased risk of silent myocardial ischemia and mortality (Said, 2007).

Focal and multifocal neuropathy is a less common neuropathy compared to DPN. It occurs mainly in patients with type 2 diabetes, and is usually seen after 50 years old. Focal neuropathies include cranial neuropathy, limb and truncal neuropathy and proximal diabetic neuropathy of the lower limbs (Said, 2007). Multifocal diabetic neuropathy is seen in a small proportion of diabetic patients. It involves roots and nerves of the lower limbs, trunk and upper extremities over

several weeks or months, sometimes with a relapsing course. The distal parts of the lower limbs are involved, and proximal deficits also occur in most patients (Said *et al.*, 2003).

1.4.3 Mechanism of diabetic neuropathy

Long-term hyperglycemia causes downstream metabolic cascades of increased polyol flux, deposition of advanced glycation end-products (AGE), enhanced expression of AGE receptor, and increased oxidative stress. These mechanisms applied to endoneurial microvessels and neural tissues results in changes of peripheral nerves. Polyol pathway activation due to hyperglycemia showed endoneurial reduction of PKC (α-isoform) activity and an increase in PKC (β-isoform) in epineurial artery, leading to neuropathy (Yamagishi *et al.*, 2003). Overexpression of human aldose reductase in transgenic mice increased polyol flux and developed peripheral nerve dysfunction (Yagihashi *et al*., 1996). In both human and animal diabetic nerves, AGE deposition cause injurious and direct toxicity to nerve tissues (Sugimoto *et al*., 2008). Furthermore, enhanced AGE receptor in the endothelial cells of transgenic mice showed delay nerve conduction velocity (Wada and Yagihashi, 2005). Free radicals formation due to hyperglycemia showed oxidative-stress tissues injury through mitochondrial changes, including release of cytochrome C, caspase-3 activation, altered biogenesis and fission (Leinninger *et al.*, 2006). Oxidative stress is also generated via activation of NADPH oxidase when AGE binds with AGE receptors. Then, lκBα-nuclear factor-(NF)-κB activate gene related cell death or survival and result in the nerve conduction delay, pain, degeneration of neuronal cells and demyelination of Schwann cells (Yagihashi *et al*., 2011). On the other hand, nerve tissues in diabetes undergo pro-inflammatory

process and develop neuropathy by inducing the release of cytokines and suppression of neurotrophins and migration of macrophages. The development of peripheral nerve pathology is also promoted by the presence of cellular components of bone marrow and ischemia (Yagihashi *et al.*, 2011).

1.4.4 Diagnostic methods for DPN

In order to fully classify neuropathy, the assessment such as clinical symptoms, clinical signs, electrodiagnostic studies, quantitative sensory testing and autonomic function testing is needed. Nerve biopsy is not necessary (American Diabetes Association, 1988; Cornblath, 2004). People with diabetes should examine their feet annually to identify high-risk conditions. There are several different tests to screen and assess DPN, including reflex testing, superficial pain testing, light touch perception, vibration testing, sympathetic skin response, quantitative sensory testing and nerve conduction testing.

For reflex testing, it is common to test all reflexes especially ankle reflex as it is most sensitive to early DPN. The examination is performed by gently striking with a reflex hammer, the foot dorsiflexes and Achilles tendon, when the patient is sitting or kneeling (Cornblath, 2004). It can be repeated with reinforcement if no reflex is observed. Results are scored according to the neuropathy composite score.

In superficial pain testing, pain sensation is tested with a sterile safety pin and the patient is asked if they feel it at all, and whether the feeling is sharp or dull. The site of testing may include the dorsum of the great toe or the plantar aspect of the

distal first, third and fifth toe of each foot (Cornblath, 2004). Results are scored accordingly.

The best calibrated device to test for light touch perception is the Semmes-Weinstein 10-g monofilament. This instrument provides a specific force that is 10 times the log of the force in milligrams exerted at the tip of the filament. Patients with diabetes or Hansen's disease with neuropathic ulcer could not sense the 10-g filament (Birke and Sims, 1986; Lee *et al.*, 2003). Patients without neuropathy should be able to sense a 0.4-g monofilament. The inability to sense monofilament of 1g and above is considered consistent with neuropathy (Tanenberg, 2009).

The sensitivity to vibration in the feet is checked using a 128Hz tuning fork. In the early stages of DPN, patients will show a deficit in the vibration perception of the great toes. As the condition progresses, the deficit will expand to metatarsal-phalangeal joints, dorsum of the foot, ankle and the mid-tibial region (Tanenberg, 2009).

Symphathetic skin measurement by quantitative sudomotor axon reflex testing (QSART) checks the changes in the skin potential. Increase of sweat production, which is a sign of axonal excitability, is seen in diabetic neuropathy (Illigens and Gibbons, 2009). On the other hand, quantitative sensory testing diagnoses the differentiation of relative deficit between small and large diameter axons and between peripheral neuropathy and mononeuropathy (Cornblath, 2004).

Nerve conduction test is used to assess the presence and severity of peripheral nerve injury in diabetic patients. This test can be performed with surface or needle electrodes. Surface techniques are widely used, easier to perform, and more comfortable. Nerve conduction study results show amplitudes, distal latency of compound muscle action and sensory potentials, conduction velocity of fastest conducting fibers and minimal F-wave latencies. Results of nerve conduction study do not always correlate well with symptoms and signs as some electro diagnostic abnormalities reflect metabolic changes that are not associated with symptoms (Vinik *et al.*, 2003; Cornblath, 2004).

1.4.5 Composite Score of Neuropathy

Various clinical composite scores have been developed and used to quantify the severity of neuropathy. Each composite system has its advantage and disadvantage. There are few instruments that are developed to evaluate at least 3 prominent domains affected by DPN, namely motor, sensory and autonomic. The Complete Neuropathy Assessment Symptom and Change score, the Neuropathy Impairment Score, Lower Limb Function test, Toronto Clinical Scoring System, Total Neuropathy Score, Total Symptom Score and the Michigan Diabetic Neuropathy Score are frequently used for neuropathy diagnosis in clinical trials, with and without the use of standard electrophysiology (Cornblath *et al.*, 1999; Ziegler *et al.*, 1999; Bril *et al.*, 2002; Dyck *et al.*, 2002).

1.4.6 Treatment

Four systematically active classes of drugs, namely calcium channel modifying anticonvulsants (e.g., pregabalin, gabapentin), sodium channel blockers

(e.g., carbamazepine, oxcarbazepine), tricyclic antidepressants (e.g., duloxetine, venlafaxine) and opiates are helpful in managing DPN symptoms (Baron, 2006).

Tricyclic antidepressants (TCA), calcium channel α2-δ ligands, and Serotonin-Norepinephrine Reuptake Inhibitors (SNRI) are the first line regimen for DPN. The actions of these agents are through the inhibition of the reuptake of norepinephrine and serotonin (Edwards *et al.,* 2008). Amitriptyline, desipramine, imipramine, clomipramine, and nortriptyline are effective TCA in relieving DPN symptoms. Amitriptyline and imipramine, which are serotonin inhibitors, have a number needed to treat (NNT) of 2.1 to obtain one patient with 50% pain reduction (Finnerup *et al.*, 2005). Desipramine, which is a noradrenaline reuptake inhibitor, has a NNT of 2.5 to obtain one patient with 50% pain reduction (Finnerup *et al*., 2005). However, amitriptyline shows significant side effects, including dry mouth, sedation and blurred vision (Edwards *et al.*, 2008). Generally, secondary amines (e.g. nortriptyline, desipramine) are tolerated better than tertiary amines (e.g. amitriptyline, imipramine) (Dworkin *et al.*, 2007). In patients with other complications such as cardiac arrhythmias, heart failure, orthostatic hypotension, urinary retention or glaucoma, caution should be taken when using TCA (Simmons *et al*., 2002). Selective serotonin reuptake inhibitors (SSRI) such as fluoxetine, paroxetine, citalopram and tramadol, have largely replaced TCA due to better tolerability. Furthermore, duloxetine and venlafaxine which are selective noradrenaline reuptake inhibitors (SNRI), alleviates DPN better than SSRI. Generally, duloxetine is tolerated better than venlafaxine as venlafaxine consumption increases the risk of cardiac changes (Rowbotham *et al*., 2004). A trial revealed the NNT of 5.1 after taking duloxetine (Wernicke *et al.*, 2006). On the other hand, gabapentin (α2-δ ligands)

works by binding at the site of voltage gated calcium channels, producing analgesia, decreasing calcium influx and reducing DPN. The NNT for gabapentin is 3.9 (Finnerup *et al.*, 2005). Moreover, it is well tolerated and more effective in relieving DPN, while common side effects include dizziness, ataxia, sedation, edema and weight gain (Dworkin *et al*., 2007).

The second line of DPN treatments include tramadol and opiates. Tramadol is a weak agonist that inhibits serotonin reuptake. It acts on monoaminergic receptors indirectly and opioid mechanisms directly (Onal *et al.*, 2007). In addition, it also acts centrally in blocking pain perception (St. Onge and Miller, 2008). The NNT to achieve 50 percent pain reduction after taking tramadol is 3.8 (Hollingshead *et al.*, 2006). Trial result showed that tramadol relieves DPN significantly over placebo (Sindrup *et al.*, 1999). In the study conducted by Freeman and coworkers (2007), tramadol/acetaminophen combination was shown to be effective in relieving DPN. Despite its efficacy, tramadol has side effects of nausea, constipation, headache and dyspepsia (Edwards *et al.*, 2008). Opiates, which include morphine and oxycodon, are effective in alleviating DPN. The use of opiates was evaluated in a 2006 Cochrane review. Clinical study showed a modest pain reduction of 20% to 30% in a portion of patients taking opiates (Eisenberg *et al.*, 2006; McNicol *et al*., 2013). In another design, oxycodon treatment was effective at a maximum dose of 80mg/day (Watson *et al.*, 2003). However, the side effects of opiates include constipation, urinary retention, impaired cognitive and immune function (Edwards *et al.*, 2008).

Furthermore, third line treatments include anti-epileptics, selective serotonin reuptake inhibitors (SSRIs), N-methyl- D-aspartate (NMDA) receptor blockers,

mexiletine and topical capsaicin. Anti-epileptics drugs include carbamazepine, lamotrigine, valproate, topiramate and pregabalin. Carbamazepine, lamotrigine and valproate act as sodium channel blockers peripherally, whereas topiramate acts as sodium channel and Gaba receptor blocker. Pregabalin blocks pain perception by acting at the GABA receptor (Huizinga and Petlier, 2007). The NNT for topiramate is 7.4 and thus this drug is not highly efficacious to treat DPN. For gabapentin, the NNT is 3.9 with few side effects such as dizziness and sedation. Pregabalin causes less sedation than gabapentin, with a NNT of 4.2 (Finnerup *et al.*, 2005; Huizinga and Petlier, 2007). On the other hand, mexilitine (anti-arrythmia medication) has shown a greater than 50% reduction in pain scores and it benefits those patients with burning pain, heat sensation and formication (Stracke *et al.,* 1992; Edwards *et al.*, 2008). The NMDA receptor blocker, dextromethorphan, produces a dose-dependent decrease in DPN treatment. Side effects of NMDA blockers include sedation, dry mouth and gastrointestinal distress (Edwards *et al.*, 2008). Besides, topical medication such as capsaicin cream stimulates C fibers to release and then deplete substance P in order to cause the skin to be insensitive to pain. The NNT for capsaicin is 6.4 at 4 weeks of treatment (Mason *et al.*, 2004). In addition, topical lidocaine which blocks neuronal sodium channel has been revealed to have a NNT for 50% pain reduction of 4.4 (Meier *et al*., 2003; Lindsay *et al.*, 2010).

1.5 Homocysteine

1.5.1 Introduction

Methionine and cysteine are sulfur containing amino acids, which play a crucial role in cells as a substrate for protein synthesis, as a methyl donor and for the synthesis of sulfur-containing compounds (intracellular tripeptide and gluthathione).

During the metabolism of methionine to cysteine, homocysteine being the intermediary metabolite is formed. Homocysteine is not present in food but is generated from methionine (Mudd, 1979; Singla *et al.*, 2014). The transformation of homocysteine through the remethylation and transsulfuration pathway is shown in Figure 1.2. Homocysteine is remethylated back to methionine or converted to cysteine by the transsulfuration pathway. In the remethylation pathway, homocysteine receives a methyl group through either the methionine synthase dependent route or through the betaine:homocysteine methyltransferase dependent route. In the transsulfuration pathway, cysteine is synthesised from homocysteine, which represents the loss of methionine carbon skeleton (Hoffer, 2004; Brosnan and Brosnan, 2006).

1.5.2 Factors that affect Homocysteine metabolic pathway

In the remethylation pathway, the methionine dependent route requires the cooperation of water and soluble vitamins such as folate, cobalamin and riboflavin. Meanwhile, the betaine:homocysteine methyltransferase dependent route requires betaine as a methyl donor (Hoffer, 2004; Rafii *et al.*, 2009). On the other hand, the transsulfuration pathway requires enzyme cystathionine-β-synthase which catalyses homocysteine and serine to form cysthathionine and enzyme cystathionine-γ-lyase which metabolises cystathionine to form cysteine (Brosnan and Brosnan, 2006; Rafii *et al.*, 2009). Hyperhomocysteinemia is defined as homocysteine level of above 15 µmol/L (Refsum *et al.*, 1998; Ganguly and Alam, 2015). It is caused by nutrient status deficiency, presence of nitric oxide, genetic defects such as cystathionine-β-synthase deficiency and methyltetrahydrofolate reductase deficiency; or certain diseases including renal disease and diabetes (Fowkers *et al.*, 2000; Monsen and

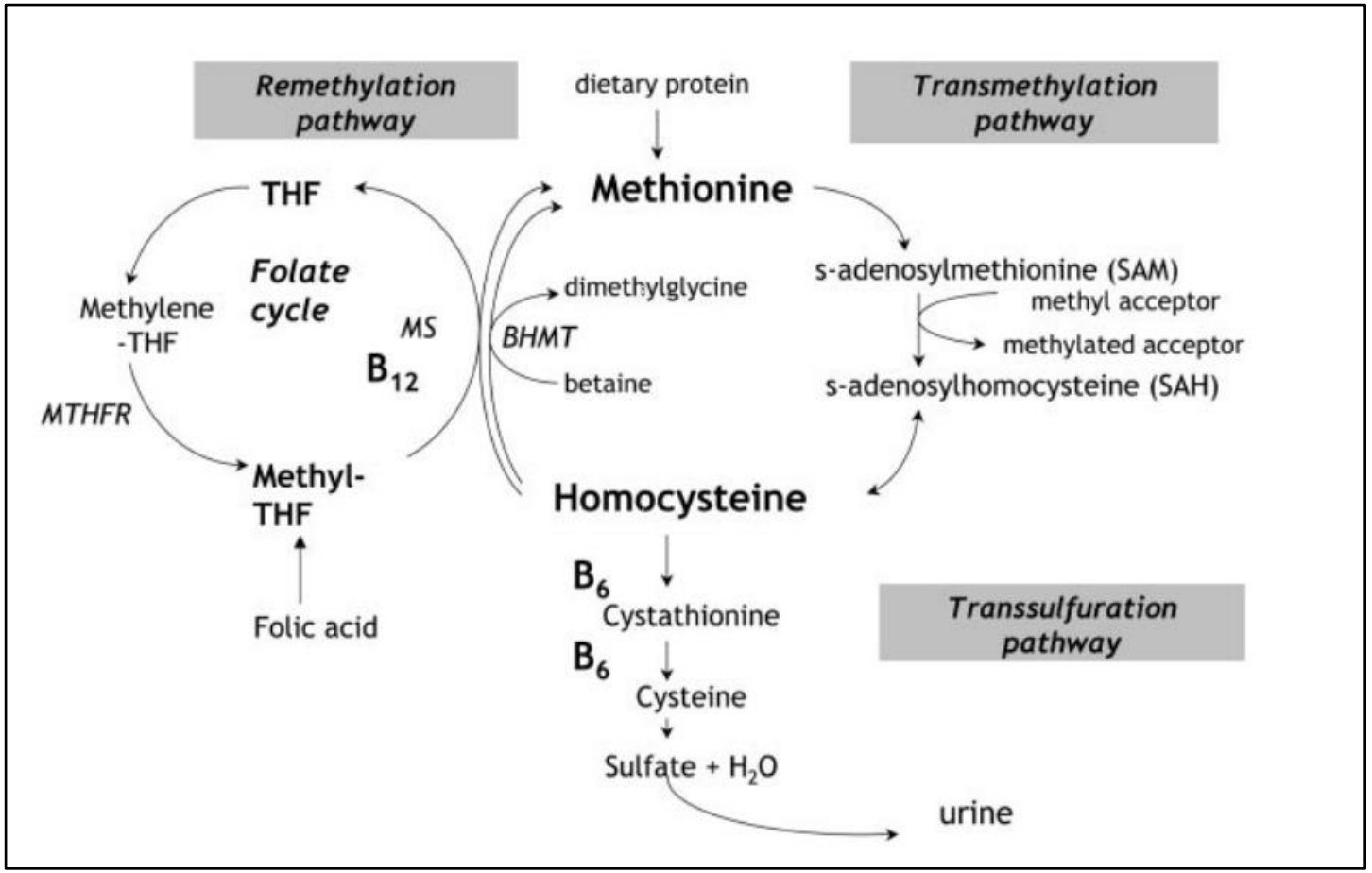

Figure 1.2 Homocysteine formation, remethylation and transsulfuration pathways (Smolders *et al.*, 2005). THF, Tetrahydrofolate; MS, Methionine Synthase; BHMT, Betaine-homocysteine Methyltransferase; CBS, cystathionine-β-synthase; B6, vitamin B6; B12, vitamin B12.

Ueland, 2003; Shelnutt *et al.*, 2004; Picker and Levy, 2004; Guldener, 2006).

1.5.3 Complications of High Homocysteine

Dysregulation in the metabolism of homocysteine is implicated in adverse clinical outcomes, including high risk of stroke, cardiovascular diseases, Alzheimer's disease, cognitive impairment in the elderly, dementia, osteoporosis, peripheral neuropathy, and neural tube defects in pregnant woman. (Diaz-Arrastia, 2000; Ambrosch *et al.*, 2001; Faraci, 2003; Selhub, 2008).

The presence of high homocysteine exaggerates the prevalence of DPN and exacerbates preexisting diabetic neuropathy. Hyperhomocysteinemia has been reported in clinical studies as an independent risk factor associated with diabetic neuropathy (Ambrosch *et al.*, 2001; Li *et al.*, 2011; Gonzalez *et al.*, 2012). Homocysteine promotes free radical formation, impairs vasodilating factors in the vascular wall, and injures endothelial cells (Basu *et al.*, 2014; Baszczuk and Kopczynski, 2014). This in turn promotes thrombosis and arteriosclerosis development in the coronary arteries, cerebral arteries, arteries in kidneys and peripheral nerves (Brattstrom *et al.*, 1990; Suwaidi *et al.*, 2000; Asfar and Safar, 2007). In addition, elevated plasma homocysteine enhances the excitotoxicity and oxidative-stress injury of neuronal cells, causing neuropathy (Mattson and Shea, 2003; Luo *et al.*, 2014).

1.5.4 Analysis methods to determine Homocysteine

Plasma or serum homocysteine exist in two forms, the oxidised form and the reduced form. In order to measure homocysteine, serum or plasma homocysteine is

treated with reductant to reduce them to total homocysteine. There are various methods in analysing total homocysteine, including enzyme and immunoassay, high performance liquid chromatography with UV, fluorescence or electrochemical detection, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography tandem mass spectrometry (Fiskerstrand *et al.*, 1993; Ducros *et al.*, 1999; Ubbink, 2000; Shinohara *et al.*, 2001; Cole *et al.*, 2004; Iciek *et al.*, 2004; Windelberg *et al.*, 2005; Kuhn *et al.*, 2006; Weaving *et al.*, 2006; Rafii *et al.*, 2007).

1.6 Scope of Study

Diabetes mellitus which is a long term metabolic disorder, is common with additional complications such as DPN. The interest in finding the potential risk factors of DPN leads us to conduct this research study with the following objectives:

A) To study the risk factors of diabetic peripheral neuropathy (DPN) by correlating the demographic variables and biochemistry profile with the neuropathy composite scores.

B) To develop and validate a quantitative LC-MS/MS method to measure human plasma homocysteine.

C) To study the association between homocysteine and the development of DPN.