METABOLOMICS IN ALZHEIMER’S DISEASE

A thesis submitted to The University of Manchester for the degree of
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SCHOOL OF MEDICINE
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<thead>
<tr>
<th>Abbr.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADAS-Cog</td>
<td>Alzheimer’s disease assessment scale – Cognitive subscale</td>
</tr>
<tr>
<td>ADRDA</td>
<td>Alzheimer’s disease and related disorders Association</td>
</tr>
<tr>
<td>APA</td>
<td>American Psychiatric Association</td>
</tr>
<tr>
<td>APoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>BMI</td>
<td>Body to Mass Index</td>
</tr>
<tr>
<td>BPSD</td>
<td>Behavioural and psychological symptoms of dementia</td>
</tr>
<tr>
<td>CAMCOG</td>
<td>The cognitive section of the Cambridge mental disorders of the elderly examination</td>
</tr>
<tr>
<td>CE-MS</td>
<td>Capillary Electrophoresis Mass Spectrometry</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical ionisation</td>
</tr>
<tr>
<td>CDR</td>
<td>Clinical Dementia Rating</td>
</tr>
<tr>
<td>C-trap</td>
<td>Curved trap</td>
</tr>
<tr>
<td>CV</td>
<td>Canonical variate</td>
</tr>
<tr>
<td>CVA</td>
<td>Canonical variate analysis</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DFA</td>
<td>Discriminant function analysis</td>
</tr>
<tr>
<td>DIMS</td>
<td>Direct injection mass spectrometry</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSM-IV</td>
<td>Diagnostic and statistical manual-IV</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EI</td>
<td>Electron impact</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>ESI (+)</td>
<td>Positive electrospray ionisation</td>
</tr>
<tr>
<td>ESI (-)</td>
<td>Negative electrospray ionisation</td>
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<tr>
<td>Fig</td>
<td>Figure</td>
</tr>
<tr>
<td>FDR</td>
<td>False Discovery Rate</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>GC-ToF-MS</td>
<td>Gas chromatography-Time-of-Flight mass spectrometry</td>
</tr>
<tr>
<td>GDS</td>
<td>Geriatric depression scale</td>
</tr>
<tr>
<td>HMDB</td>
<td>Human Metabolome Database</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HUSERMET</td>
<td>Human Serum Metabolome</td>
</tr>
<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
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<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LTQ</td>
<td>Linear trap quadrupole</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MMSE</td>
<td>Mini mental state examination</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<td>--------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Mann-Whitney Test</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>n</td>
<td>Number</td>
</tr>
<tr>
<td>NINCDS</td>
<td>National Institute of Neurological and Communicative Disorders and Stroke</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NMDA</td>
<td>n-methyl-D-aspartate</td>
</tr>
<tr>
<td>NPI</td>
<td>Neuropsychiatric Inventory</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
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<tr>
<td>PC</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>pM</td>
<td>Picomolar</td>
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<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>QA</td>
<td>Quality assurance</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>S</td>
<td>Second</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>s/n</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>ToF</td>
<td>Time-of-Flight</td>
</tr>
<tr>
<td>ToF-MS</td>
<td>Time-of-Flight-Mass spectrometry</td>
</tr>
</tbody>
</table>
\( t_R \) Retention time

UPLC Ultra performance liquid chromatography

UPLC-LTQ/Orbitrap-MS Ultra performance liquid chromatography (coupled to a) Linear Trap Quadrupole/Orbitrap mass spectrometry

UPLC-MS Ultra performance liquid chromatography mass spectrometry

WHO World Health Organisation

WSR Wilcoxon signed-rank

\( \mu l \) microlitre

\( \circ C \) degrees Celsius

\( \% \) per cent
Metabolites are a potentially useful source of detecting and identifying disease specific biomarkers. This thesis investigates the possibility of using metabolomics applications to detect Alzheimer’s disease associated metabolite peaks in patients and to detect longitudinal changes of the disease.

Serum samples and clinical data were collected from 60 healthy controls and 60 Alzheimer’s disease patients (60 at baseline and 60 at 12 month follow-up). The metabolic fingerprinting of serum samples using the FT-IR lacked discriminatory power to discriminate Alzheimer’s disease and non-disease samples due to the similar magnitude of biological and analytical variation. The metabolic profiling of serum samples using the GC-ToF-MS did not reveal any significantly altered metabolite peaks between the Alzheimer’s disease and non-disease groups. Metabolic profiling of serum samples using the UPLC-LTQ/Orbitrap-MS operated in the positive ionisation mode did not reveal any significantly altered metabolite peaks between the disease and non-disease groups. Up to twelve metabolite peaks were significantly altered in the Alzheimer’s disease baseline and follow-up samples, indicating a potential association with disease progression. Metabolic profiling of serum samples using the UPLC-LTQ/Orbitrap-MS operated in the negative ionisation mode did not reveal any significantly altered metabolite peaks between Alzheimer’s disease and non-disease groups. Three metabolite peaks were significantly altered in the Alzheimer’s disease baseline and follow-up samples, indicating a potential association with disease progression. Metabolic profiling of serum samples with the UPLC-LTQ/Orbitrap-MS may potentially be used to detect disease and disease progression associated metabolite peaks. The metabolite peaks require identification followed by a validation experiment.
Declaration

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Above all none of the work would not have been possible without the dedication of the Alzheimer’s disease patients, their carers and controls who volunteered for the studies.
Dedication

In the name of Allah the most Merciful the most Gracious.

All glory belongs to Allah the Almighty, the all Knowing and Seeing He alone can guide. I thank Allah subhana wa ta’ala for bestowing his mercy on me, and for guiding me. I send blessings and salutations to His Messenger and his Ahl.

I dedicate this thesis to Professor Nitin Purandare who sadly passed away, too early. I dedicate this thesis to all those who have supported me throughout the PhD, especially to my first teachers, my parents; to my children Maham and Ibraheem, and to my wife, Kiran.

Finally in the words of Allama Iqbal’s Jawab-e-Shiqwa:
1 INTRODUCTION

1.1 A REVIEW OF THE LITERATURE ON ALZHEIMER’S DISEASE

This section of the introduction reviews the literature on Alzheimer’s disease by focusing on the pathology, the clinical aspects and search to identify biomarkers of the disease.

Over 100 years ago the German psychiatrist Alois Alzheimer described the case of Auguste D., recording an ante-mortem history of impaired memory, problems of speech, paranoia and delusional ideation (Alzheimer et al., 1907; English translation Clin Anatomy 1995). The post-mortem revealed an atrophied brain, and for the first time neurofibrillary tangles were detected and described. The disorder became better known as Alzheimer’s disease, and is the most common cause of dementia in the elderly population of the world. Since the early finding by Alois Alzheimer the cause(s) for the disease remain unclear and at present the disease can only be definitively diagnosed post mortem. The identification of a biological marker(s) of Alzheimer’s disease would have utility in helping to diagnose the disease and to monitor and measure disease associated changes. The biological marker(s) could potentially lead to the development of new drug treatments and an early and accurate diagnosis of Alzheimer’s disease.

1.1.1 Alzheimer’s disease epidemiology and risk factors

In the developed world, Alzheimer's disease accounts for 50–60% of all dementia cases. Vascular, lewy body and frontal lobe, are some of the other causes of dementia. The prevalence of dementia increases with age, rising from 1% in the 60-
64 to between 24 and 33% in those 85 years or older (worldwide) (Blennow et al., 2006, Ferri et al., 2005). In 2001, an estimated 24.3 million people had dementia, this is expected to double every two decades as life expectancy also increases; rising to 81.1 million in 2040 (Ferri et al., 2005). The countries and regions of the world which are the most affected are western Europe, the USA and China. By 2040 these countries and region will be home to 55.7% of the total affected population worldwide (Figure 1.1) (Ferri et al., 2005).

![Figure 1.1 Estimates of the prevalence of dementia in the developed and developing countries.](image)

In 2001 global dementia levels were estimated at 24.3 million, with one case of dementia being diagnosed every 7 seconds. With rate of prevalence said to double every two decades, the greater toll will continue in the developing countries (e.g. India and China) where dementia cases will be 71% of all cases in 2040 (Ferri et al., 2005).

The incidence of the rate of new Alzheimer’s disease cases is well established at 1% of those aged 65 to 70, rising to 6% to 8% in those 85 years or more. The duration
of illness varies between 2 – 10 years (Wolfson et al., 2001), and is reflected in the overall prevalence rates in the 85 years or more, between 10% to 30% (Mayeux, 2003).

Aging is the primary risk factor in Alzheimer’s disease. However other risks factors include decreased reserve brain capacity, gross brain shrinkage, low mental achievement in early life, and minimal level mentally taxing occupations, followed by latter life reduced mental and physical activity. Head injury has also been cited as a risk factor (Mayeux, 2003).

Hypercholesterolaemia, hypertension, atherosclerosis, coronary heart disease, smoking, obesity, and diabetes, are all linked to Alzheimer’s disease dementia, as these are all considered to be vascular risk factors, affecting the effective supply of blood (Luchsinger and Mayeux, 2004, Shobab et al., 2005). Evidence suggests that countering risks that predispose an individual to dementia include benefits derived through diet alteration, that is increasing an intake of homocysteine-related vitamins (vitamin B12 and folate); antioxidants, such as vitamin C and E; unsaturated fatty acids (Blennow et al., 2006, Luchsinger and Mayeux, 2004). In a large population-based twin study, heritability for sporadic Alzheimer’s disease was high (79%) with the same genetic factors being as influential, irrespective of sex, and non-genetic risk factors (e.g. environment) (Gatz et al., 2006).
1.1.2 Alzheimer’s disease pathology

Alzheimer’s disease pathology is characterised by the formation and accumulation of misfolded proteins (plaques and tangles), in the brain.

**Amyloid β**

The formation of extracellular plaques is described by the amyloid cascade (also Aβ-protein) theory of plaque causing pathology (Figure 1.2) (Hardy and Higgins, 1992), (Van Dam and De Deyn, 2006). Plaques which are extracellular formations, arise when the amyloid precursor protein (β-APP) is cleaved by the beta-amyloid cleaving enzyme (BACE) to form primarily Aβ-42 type aggregations (Oddo et al., 2003a, Oddo et al., 2003b).

The presenilin proteins (PS1 and PS2) are critical in the enzymatic cleavage of the APP, and subsequent release of β-APP. Specific mutations in the presenilin genes result in familial Alzheimer’s disease (FAD), through an increase in APP cleavage, which causes an increase of β-amyloid (Mudher and Lovestone, 2002).

FAD research has shown that the allele apolipoprotein E type 4 (APOE ε 4) increases the risk of late onset Alzheimer’s disease (Mattson, 2004). The development of transgenic gene knockout animal models such as the APP_{swedish mutation} and PS1 and PS2 mutations have elucidated the molecular mechanisms underlying plaque pathology (Mattson, 2004, Mudher and Lovestone, 2002, Oddo et al., 2003a). The amyloid cascade theory is thought to be relevant to genetically inherited or predisposed people, whereby a ready mutation must exist (Mudher and Lovestone, 2002). The theory does not however explain the cause of sporadic
Alzheimer’s disease. Plaques are also common in non-demented individuals (Mudher and Lovestone, 2002).

The sortilin related receptor 1 (SORL1), is a neuronal sorting receptor which controls APP processing. SORL1 works by directing APP into the recycling pathway, and thus away from enzymatic cleaving by BACE and the presenilin proteins. As BACE and the presenilin proteins cannot act on APP, this results in a reduction of Aβ production. However, where an under expression of SORL1 does occur, there then occurs a direct transit of APP to BACE, which results in Aβ production. Because there is no known reduction in SORL1 in FAD, SORL1 may be the first gene that is linked to sporadic Alzheimer’s disease (Rogaeva et al., 2007). Levels of SORL1 have been shown to be reduced in the brains of Alzheimer’s disease patients (Rogaeva et al., 2007) and in the brains of individuals with mild cognitive impairment (Sager et al., 2007).

Clusterin is a chaperone protein involved in the production of Aβ. A recent genome-wide association study in patients with Alzheimer’s disease found that clusterin was associated with the severity and progression of Alzheimer’s disease (Thambisetty et al., 2010).

The identification of specific genes which are thought to confer the risk of Alzheimer’s disease is important. However, there remain problems with the identification of a specific risk gene is that a single gene will only confer a low level of risk (Hardy et al., 2004). Genome wide array studies have attempted to overcome
this by analysing the genome for risk associated genes. However, the role of underlying disease changes varies from one individual to another.

**Figure 1.2 Amyloid cascade hypothesis**

The hypothesis proposes that there is a primary imbalance between Aβ production and its subsequent clearance, with increased Aβ production in familial disease and decreased Aβ clearance in sporadic disease. Aβ oligomers may inhibit hippocampal function and impair the synaptic function, as well as leading to inflammation and oxidative stress caused by the aggregation and depositing of Aβ. These processes combine to impair neuronal and synaptic function with resulting neurotransmitter deficits and cognitive symptoms. Tau pathology with tangle formation is regarded as a downstream event, but could contribute to neuronal dysfunction and cognitive symptoms.
**Tau**

The second major hallmark of Alzheimer’s disease related changes in the brain are intracellular formations called neurofibrillary tangles (NFTs). NFTs are primarily composed of paired helical filaments (PHF). The major component of the NFTs is the protein tau, a microtubule associated protein (MAP) (Grundke-Iqbal et al., 1986), which binds with microtubulin to provide structural stability to a cell. Dissociation of the tau protein from the microtubulin leads to unbounded tau protein aggregation (Su et al., 1996). The reason for the aggregation is explained by the tau hypothesis (Su et al., 1996). Under normal conditions, tau which is a soluble protein undergoes phosphorylation and dephosphorylation, thus forming insoluble aggregates. An imbalance in this dynamic results in increased levels of abnormally hyperphosphorylated tau (P-tau 181, P-tau 199, P-tau 231, P-tau 396, P-tau 404), which in turn sequesters normal tau and other MAPs (MAP1 and MAP2) (Blennow et al., 2007). Hyperphosphorylated tau aggregates into PHF, and tangle formation. Parallel to the process of tangle formation is the disassembly of microtubules. The combined effect of tangle formation and disassembly of microtubules is that they compromise normal neuronal and synaptic function (Blennow et al., 2006). According to the amyloid cascade hypothesis it is the increase in concentration levels of Aβ that trigger the changes in tau thus leading to the formation of NFTs.

Alim et al., 2004 have shown that the protein α-synuclein (aberrant forms of which are core components of lewy body based pathologies, known as synucleinopathies), like tau is involved in microtubule assembly, serving as a binding for the tublin
protein. This binding ability however is lost when α-synuclein becomes mutated, resulting in tubulin aggregation (as well as α-synuclein). As microtubule function is necessary for normal neuronal and synaptic function, dysfunction of the microtubules may be central in neurodegeneration (Alim et al., 2004). The number of neurofibrillary tangles is a pathological marker of Alzheimer’s disease severity.

**Animal models & post mortem studies**

Support from the transgenic gene knockout animal model (AM) the P301L human tau mutation transgenic mouse model has been shown to produce tangles (Van Dam and De Deyn, 2006). This is in accordance with the view that the core symptoms have a neurobiological basis, with the aggregation of amyloid plaques and neurofibrillary tangles, both developing independently of one another, and with differing patterns of distribution (Braak et al., 1999, Van Dam and De Deyn, 2006). Following systematic research on post mortem brains of Alzheimer’s disease patients, Braak and Braak, have shown how the disease may progress topographically with respect to time. The brain regions involved are the medial temporal cortex, hippocampus, and entorhinal cortex, anterior cingulate gyrus (as well as disruption of the neocortex) - whilst other areas are unaffected - prominently cerebral and cerebellar cortex. This distribution or topographically predictive nature of aggregation is thought to be a five to six stage process whereby the first three stages are preclinical, with symptomatic or clinically diagnosable symptoms becoming prominent from stages III onwards. The symptoms of amnesia, are thought to be due to the hippocampus being affected and thus producing early changes in memory, and ultimately the progression to the final stage, where the neocortex is affected (Braak and Braak, 1991, Braak et al., 1999).
Moreover, it has been recently indicated that in the majority of cases, tangle formation precedes amyloid deposits (Braak and Del Tredici, 2004, Schonheit et al., 2004). This is contrary to the amyloid cascade theory, which explains tangle formation as a result of amyloid plaque formation (Hardy and Higgins, 1992). Support for this view is found in the novel creation of the “3xTgAD APP\textsuperscript{Swedish mutation},” “PS1” & “P301L” animal models (Oddo et al., 2003a, Van Dam and De Deyn, 2006) which have shown amyloid deposition, as an event which precedes tangle formation, and thereby revealing a temporal and spatial distribution of tau-pathology following amyloid deposition. Combination of the APP\textsuperscript{Swedish mutation} and “P301L” models have yielded both sets of pathologies; with amyloid considered the more pathogenic feature of the two, and therefore more liable to cause dementia.

1.1.3 Other aetiological hypotheses

**Oxidative Stress**

Oxidative stress is known to induce damage to various biological macromolecules in an uncontrolled manner and is considered to be a hallmark of neurodegenerative diseases. One consideration is that the plaques and tangles rather than being critical in the initiation or pathology of the disease are potentially acting as an antioxidant defence, that is a protective action. Therefore the subsequent appearance of Aβ deposits and tau hyperphosphorylation is a consequence of this defence (Smith et al., 2002). Nunomura and colleagues have shown in animal model study that oxidative damage precedes the pathological changes associated with Alzheimer’s disease (Nunomura et al., 2001).
**Inflammation**

Brain regions which are affected by Alzheimer’s disease are known to contain increased neuroinflammatory mediators (cytokines and microglia) through increased inflammatory cascades (Wyss-Coray and Mucke 2002). Whether this is a natural response to control inflammation or an out of control immune process is unknown. Cyclooxygenase (COX) a key mediator of the inflammatory cascade is targeted by non-steroidal anti-inflammatory drugs (NSAIDs), affecting COX levels. The use of NSAIDs does not reduce the risk or delay the onset of Alzheimer’s disease (Van Gool et al., 2001). Though this has largely negated the role of NSAIDs, there is some evidence that independent of COX inhibition, NSAIDs can reduce Aβ deposits in animal models of Alzheimer’s disease (Weggen et al., 2001). Microglia activation is thought to be an early event in the Alzheimer’s disease pathogenesis and may be critical in synaptic disruption and thus early memory impairment (Cagnin et al., 2001). The Alzheimer’s disease Anti-inflammatory prevention Trial (ADAPT) looked into the role of NSAIDs in people vulnerable to dementia, using COX-1 and COX-2 drugs. The trial was cancelled due to cardiovascular risks (ADAPT, 2006). Overall COX-1 targeting NSAIDs are thought to be a better option than COX-2 inhibitors (McGeer and McGeer, 2007).

**Cholinergic hypothesis**

The cholinergic hypothesis of Alzheimer’s disease suggests that destruction of the cholinergic pathway in the basal forebrain results in a reduction of cholinergic neurons, which release the neurotransmitter acetylcholine (Bartus, 2000, Terry and
Buccafusco, 2003). These neurons project to the hippocampus and neocortex, which are implicated in both memory disturbance and cognitive symptoms (Bartus, 2000). ACh is degraded by the enzyme acetylcholinesterase (AChE). Levels of this enzyme are reduced in moderate and severe Alzheimer’s disease patients, compared to mild patients; cholinesterase inhibition improves neurotransmitter function and provides relief of Alzheimer’s disease symptoms. (Terry and Buccafusco, 2003).

**Cholesterol metabolism**

The role of lipid/cholesterol metabolism and Alzheimer’s disease pathogenesis is gaining acceptability (Shobab et al., 2005). Cholesterol is known to affect the activity of enzymes involved in the metabolism of APP in the production of Aβ. Statins which are cholesterol-lowering drugs are also associated with a lower risk of developing a dementing illness (Jick et al., 2000). Apolipoprotein (APOE) is involved in the transporting of cholesterol, and APOE ε4 allele is a universally accepted marker which increases Alzheimer’s disease risk (Corder et al., 1993). APOE ε4 is also associated with lowering the age of onset for Alzheimer’s disease (Shobab et al., 2005, Jarvik et al., 1995). APOE ε4 not only confers a genetic risk for Alzheimer’s disease to an individual, it is also linked to the production and aggregation of both amyloid and tau (Reiman et al., 2009 and Holtzman et al., 2000). Specifically the ε4 allele is associated with increased amyloid burden and dysfunction of cholinergic neurotransmission. Individuals who inherit two ε4 alleles are thought seven times at higher risk of developing Alzheimer’s disease than those who have the E3 allele (Corder et al., 1993). High cholesterol levels during an individuals mid-life is considered a risk factor for Alzheimer’s disease, and studies have shown that the use of cholesterol lowering drugs, statins, may reduce the risk
(Kivipelto et al., 2001), however a recent trial has shown that the use of statins does not impact on improvement in recorded cognitive impairment (Jones et al., 2008).
1.2 DIAGNOSIS & CLINICAL SYMPTOMS OF ALZHEIMER'S DISEASE

1.2.1 Classification and Diagnostic criteria

The classification and the criteria used to diagnose dementia and Alzheimer’s disease is set out in the Diagnostic and Statistical Manual of Mental Disorders (4th ed, text revision, DSM-IV-TR) (APA) and the International Statistical Classification of Diseases and Health–related Problems, 10th revision (ICD-10) (WHO, 2007).

The DSM-IV-TR (APA) and ICD-10 classifies dementia as memory impairment with one or more impairment(s) in other cognitive domains.

The ICD-10 defines Alzheimer’s disease as “a primary degenerative cerebral disease of unknown etiology with characteristic neuropathological and neurochemical features. The disorder is usually insidious in onset and develops slowly but steadily over a period of several years.” (WHO) The DSM-IV-TR does not use the term Alzheimer’s disease, and instead uses dementia of the Alzheimer’s type (DAT). DAT is a manifestation of “early deficits in recent memory followed by the development of aphasia, apraxia, and agnosia after several years (APA).

The ICD-10, classifies Alzheimer’s disease as early and late onset. There is also as a classification for mixed type, this is where Alzheimer’s disease may not be exclusively responsible for the dementia (WHO). The DSM-IV-TR, also classifies the DAT as early onset (65 years or below) and late onset (65 years or above) (APA). The DM-IV-TR further categorizes DAT of the two groups based on the
presence or absence of predominant non-cognitive symptom. These symptoms are delirium, delusions and depressed mood, or an uncomplicated DAT (APA).

The National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer Disease and Related Disorders Association (NINCDS-ADRDA) criteria (McKhann et al., 1984), are another criteria which is used in the clinical diagnosis of Alzheimer’s disease. This NINCDS-ADRDA criteria is regarded as the gold standard for diagnostic purposes. Patients are categorised as having probable, possible or definite (post-mortem confirmation) Alzheimer’s disease.

For research purposes the accepted diagnosis of Alzheimer’s disease is usually based on the DSM-IV-TR and the NINCDS-ADRDA criteria. The application of the criteria is a two-step process, whereby in the first step an initial identification of a dementia is made, followed by step 2, where the criteria is applied using the clinical features of the Alzheimer's disease phenotype (Dubois et al., 2007). Knopman and colleagues reported that over 90% of patients diagnosed with Alzheimer’s disease ante-mortem using the current criteria were found to have Alzheimer's disease pathology post mortem (Knopman et al., 2001).

1.2.2 Clinical symptoms and the course of the illness

The main cognitive symptoms of Alzheimer’s disease are amnesia, aphasia, apraxia, agnosia, and executive dysfunction. Over the course of the illness these symptoms become more pronounced, owing to an increase in disease severity. There are also non-cognitive symptoms that are prominently manifested during the course of the disease. These are the behavioural and psychological symptoms. Symptoms such as
agitation, apathy, depression, hallucinations and delusions occur at some stage in most patients. They cause significant distress to both patients and their carers and lead to early institutionalization of patients. An additional impact of Alzheimer’s disease is on the day to day activity of patients (activities of daily living). These symptoms result in adverse effects of quality of life, impact on caregiver burden (Gonzalez-Salvador et al., 2000), and cost of care (Murman and Colenda, 2005).

Overall evaluation of dementia therefore falls into six broad categories, historical, cognitive motor, behavioural, functional, & medical (see Table 1.1). Symptoms are graded as mild, moderate and severe, and are assessed clinically sometimes using scales. The scales are used to assess and monitor symptoms and measure a patient’s response to medication.
<table>
<thead>
<tr>
<th>DOMAINS</th>
<th>PRE-ASSESSMENT MEASURES</th>
<th>POST ASSESSMENT INSTRUMENTS</th>
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<tr>
<td>Historical</td>
<td>Investigation of initial concerns?</td>
<td><strong>Dementia Staging:</strong> Clinical</td>
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<td>Dementia Rating (CDR) &amp;</td>
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<td>Cognitive</td>
<td>Language, attention, learning &amp; memory</td>
<td><strong>Cognitive Evaluation:</strong></td>
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<td>Alzheimer’s Disease Assessment Scale – Cognitive (ADAS-Cog),</td>
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<td>MMSE, and other batteries</td>
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<td>Behavioural</td>
<td>Affective disturbances: apathy, depression, anxiety, agitation,</td>
<td><strong>Behavioural Disturbances:</strong> NPI</td>
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<td>and the BEHAVE-AD</td>
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<tr>
<td>Functional</td>
<td>Activities of daily living (ADL)</td>
<td><strong>Functioning:</strong> ADL</td>
</tr>
<tr>
<td>Motor</td>
<td>Activities of daily living (ADL)</td>
<td><strong>Functioning:</strong> ADL</td>
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<tr>
<td>Medical</td>
<td>Dysfunction of type: hyper- or hypokinetic &amp; neurological signs</td>
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<td>Identification of risk factors</td>
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**Table 1.1 Evaluating dementia**

The evaluation of dementia falls into six broad categories, historical, cognitive motor, behavioural, functional, & medical and are used to capture a full a picture of the extent of dementia both physically and mentally (Burns & Winblad 2006).
1.2.3 Pharmacotherapy

Current treatments for Alzheimer’s disease are used to reduce the cognitive decline. The central role of these drugs is to stabilize and thus minimise disruption of two key neurotransmitters, acetylcholine (ACh) (the cholinergic hypothesis of Alzheimer’s disease), and glutamate. AChE inhibition is used to protect the cholinergic neurons and glutamate (Klafki et al., 2006).

The three compounds which work on the basis of AChE inhibition are the cholinergic drugs, donepezil, rivastigmine and galantamine. All three compounds are efficacious in reversing and improving memory and global cognition, in mild to moderately demented patients (Birks, 2006). The second key transmitter targeted is glutamate, the primary excitatory neurotransmitter in the brain. The interaction of glutamate with the N-methyl-D-aspartate (NMDA) receptor is important in the workings of memory and learning. In Alzheimer’s disease an increase in glutamate activity results in NMDA receptor being excessively activated which may lead to neurodegeneration (Klafki et al., 2006). Consequently memantine, an NMDA antagonist, is used to counter the loss or damage of NMDA receptors due to excess glutamate excitation in Alzheimer’s disease patients. Targeted primarily at moderate to severely demented patients, memantine is considered to overall reduce burden of care on the carer, as well as clinically reversing and improving memory and global cognition, reducing behavioural disturbances, and improvement in the quality of life (Areosa et al., 2005, Wilcock, 2003).
A number of treatments are also used to alleviate neuropsychiatric symptoms, including anti-depressants and anti-psychotics. Anti-psychotics are largely used to treat agitation, aggression and psychosis.

Pharmacotherapy for Alzheimer’s disease remains limited to AChEIs and Memantine. The so-called pipeline for new drugs to emerge is very limited. And of the drug which has made it to clinics, Vioxx, has been classed as “toxic” (Imbimbo, 2009). Biomarkers can readily bridge the gap between novel pipeline drugs and clinical efficacy of these drugs. Biomarkers are extremely useful in the measurement of efficacy of drugs, which can be measured during clinical trials. Biomarkers are also important in helping to reveal novel therapeutic areas which may not have been previously considered.
1.2.4 Behavioural and Psychological Symptoms of Dementia

Non-cognitive or behavioural symptoms are as important in Alzheimer’s disease as the cognitive symptoms. These symptoms are prevalent in up to 98% of patients (Steinberg et al., 2006). The non-cognitive symptoms of dementia are not well defined (Zaudig, 1996). The recognition that memory and cognitive impairments are fundamental to dementia, had for a period of time resulted in more research emphasis being placed on the cognitive functions (cognitive paradigm), and less attention on the non-cognitive symptoms of dementia, in particular behavioural disturbances (Zaudig, 2000). The cognitive paradigm of dementia finds its roots in the 1900-1930s (Berrios, 1986). The DSM-IV-TR lists the cognitive impairments of Alzheimer’s disease, but it does not reflect the prevalence and the range of the non-cognitive symptoms, thus reflecting the psychiatric symptoms and the behavioural disturbances reported in studies (see below) (Lyketsos, 2007). Therefore the extent of both functional disability and the impact on the quality of life for an individual and carers is not adequately recorded (McKeith and Cummings, 2005).

The behavioural and psychiatric problems have always been thought to be concomitant and secondary to the core cognitive impairment in dementia (see table 2). This relegation of importance of these secondary features in dementia was only explicitly corrected following on from early works in the 1980s. These studies though lacking latter day use of standardised and reproducible methodology, such as validated scales in assessment of the symptoms, relied firstly on psychiatric symptomatology based descriptive which were subjective (Burns and Levy 1992).
### Behavioural features of major dementias

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<tr>
<td><strong>Alzheimer’s disease</strong></td>
<td>Apathy, agitation, depression, anxiety, irritability; delusions and hallucinations are less common</td>
</tr>
<tr>
<td><strong>Dementia with Lewy bodies</strong></td>
<td>Visual hallucinations, delusions, depression, REM sleep behaviour disorder</td>
</tr>
<tr>
<td><strong>Vascular dementia</strong></td>
<td>Apathy, depression, delusions</td>
</tr>
<tr>
<td><strong>Dementia associated with Parkinson’s disease</strong></td>
<td>Visual hallucinations, delusions, depression, REM sleep behaviour disorder</td>
</tr>
<tr>
<td><strong>Frontotemporal dementia</strong></td>
<td>Apathy, disinhibition, elation, repetitive behaviours, appetite or eating changes</td>
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</table>

**Table 1.2 Behavioural features of major dementias**

The importance of recognising the neuropsychiatric behaviours which are prominent in Alzheimer’s disease is that they become appropriate targets for treatment, drug or otherwise.

Cohen-Mansfield et al., 1989, identified three sub-groups of agitation (aggressive behaviour, physically non-aggressive behaviour and verbally agitated behaviour) (Cohen-Mansfield et al., 1989). Burns et al., 1990, described four sets of non-cognitive disorders in Alzheimer’s disease patients. These included thought content...
(including delusions) (Burns et al., 1990a), perception (including hallucinations) (Burns et al., 1990b), mood (including depression) (Burns et al., 1990c) and behaviour (including aggression) (Burns et al., 1990d).

The behavioural and psychological symptoms of dementia (BPSD), “is a term used to describe a heterogeneous range of psychological reactions, psychiatric symptoms, and behaviours occurring in people with dementia of any aetiology” (Finkel et al., 2000). Thus BPSD is an umbrella term that catalogues and characterises the non-cognitive or neuropsychiatric symptoms (Cummings, 1987) of dementias including Alzheimer’s disease.

Even though in all forms of dementia it is the cognitive decline which is the defining component of the disease, neuropsychiatric assessments are employed to identify behavioural features of dementia, these behavioural features of dementias are well documented (Table 1.2).

1.3 SUB-TYPES OF ALZHEIMER’S DISEASE

1.3.1 Behavioural features of sub-types

Recent research on clustering of specific behavioural and psychological symptoms suggests that there may be three or more sub-types in Alzheimer’s disease. The notion of sub-types or sub-clinical entity of senile dementia, based on behavioural disturbances finds its roots in 1863 with Kahlbaum (Berrios, 1986). Subtypes are identified by cataloguing certain symptoms and behaviours which bear a close relationship to psychiatric disorders. Though what is unclear in this approach is the
exact extent and nature of the relationship between the observable behavioural and psychological symptoms and the psychiatric disorders they resemble (Reid et al., 1978).

Subtypes have been demonstrated in early studies on the basis of qualitative differences based on psychological data and neuropathological data (Jorm 1984). Jorm 1984, considered subtypes derived through psychological data, concluding that the age of onset (early and late onset) in Alzheimer’s disease is a single type. The variations in the intensity of the symptoms in this type of Alzheimer’s disease could be explained by genetic susceptibility. Furthermore an early age of onset was thought to be responsible for a more severe disorder, thus explaining symptoms characteristic of latter Alzheimer’s disease stage (Jorm 1984). However, where the subtypes are derived through neuropathological data, there is uniform presentation of Alzheimer’s disease pathology, despite differences in severity, and this therefore does not indicate a subtype (Jorm 1984).

1.3.2 Depression of Alzheimer’s disease

The extent of symptoms of depression in Alzheimer’s disease has been reported as over 50%, of which 23% was major depression (Migliorelli et al., 1995). Burns et al., 1990 and Cache county study (Lyketsos et al., 2000) reported over a 20% prevalence of depression in patients. Diagnosing depression in Alzheimer’s disease is difficult as there is difficulty in discriminating apathy symptoms from depressive symptoms. Over 50% of Alzheimer’s patients with depression were co-morbid apathy symptoms (Lyketsos et al., 2001). The expression of depressive symptoms is thought to change as Alzheimer’s disease progresses in patients (Lee and Lyketsos,
2003). Olin and colleagues report that knowledge regarding the natural history of depression in Alzheimer’s disease is limited (Olin et al., 2002). Devan and colleagues, reported that depression symptoms were prevalent over 6 months, with symptoms fluctuating over time (Devanand et al., 1997).

1.3.3 Psychotic symptoms in Alzheimer’s disease

Psychotic symptoms have been reported in a number of studies. The prevalence rate of psychotic symptoms is said to be between 30 and 40% (Wragg and Jeste, 1989). Alzheimer’s disease with psychosis (AD+P) is a combination of symptoms, which include delusions (persecution, infidelity, and abandonment), misidentification of individuals, hallucinations (Ballard et al., 1995, Jeste and Finkel, 2000). AD+P may be associated with a rapid decline, but this is not conclusive (Cook et al., 2003).

In recent years there has been a concerted effort to identify common neuropsychiatric features in dementia patients, principally in Alzheimer’s disease patients as they form the largest group. The initial approach to identifying neuropsychiatric symptoms was based on targeting of symptoms (Burns et al., 1990a, Burns et al., 1990b, Burns et al., 1990c, Burns et al., 1990d). The Neuropsychiatric Inventory (NPI) (Cummings et al., 1994) has been used extensively to identify and measure neuropsychiatric symptoms in Alzheimer’s disease, revealing clusters of symptoms. This has led to what is termed the “syndromic approach” (Lyketsos et al., 2001).

The Cache County study was the first US population based study of Alzheimer’s disease associated neuropsychiatric disturbances, employing a cross-sectional
design, and using latent class analysis (cluster analysis) to identify subgroups of patients based on individual patient symptom profile. 198 people with Alzheimer’s disease were classified into three groups, the largest of which (n=117 (40%)) had no neuropsychiatric symptoms, or had a mono-symptomatic disturbance (n=35) 19%), the second group were predominantly affective (n=55 (28%)), whilst the third group had a psychotic syndrome (n= (26) 13%). The drawback of the study was that neither severity of symptoms nor frequency was reported and a longitudinal study would have shed light on the stability of these symptoms. Schreizer and colleagues employed a cross-sectional design, and using the Behavioural Pathology in Alzheimer’s Disease Rating Scale (BEHAVE-AD) and Cohen-Mansfield Agitation Inventory (CMAI), to identify clusters (Schreiner et al., 2005). In this study (n=69) factor analysis (principle component analysis (PCA)) was used to create symptom clusters, from which three factors of behavioural pathology were identified, agitation (35%), affectivity (26%), and diurnal rhythm disturbances (10.3%). This was the first study to use the BEHAVE-AD sub-scores to discern subtypes, and took into account medication and severity of symptoms. Hollingworth et al., 2006, in 1,120 people and using the NPI, PCA pointed to the NPI symptoms falling into 4 components: behavioural dyscontrol, psychosis, mood and agitation. With each component there were at least two symptoms that remained consistent, with high factor loadings. The drawback of the study is as noted by the authors that stability of behavioural disturbances over time, and instead they were represented as a accumulation of symptoms over the course of the disease (Hollingworth et al., 2006). The largest study to date has involved the European Alzheimer’s Disease Consortium (EADC) with data from 2354 outpatients. The Maastricht Study of Behaviour in Dementia (the MAASBED study) showed that 95% of patients
developed one or more behavioural problem in the course of dementia (Aalten et al., 2005). Using the NPI, Aalten and colleagues (2007) (in press) identified four neuropsychiatric sub-syndromes (Aalten and colleagues, 2007a). The most common sub-syndrome was apathy (65%), hyperactivity (64%), affective (59%) and psychosis (38%). Although this study refers to apathy as a syndrome in its own right rather, this is different to previous studies which group apathy and depression as a mood sub-type. Further the sub-type apathy has a higher factor loading for appetite and eating abnormalities than it does for apathy (0.705 and 0.629 respectively). Nonetheless the extent of similarity between depression and apathy manifestation is thought to potentially mask the extent of these behaviours, Robert et al., 2002 have posited the notion of an apathy inventory to counter this possibility. A second study by the EADC group (Aalten and Colleagues, 2007b) (in press) looked at the stability of the four neuropsychiatric sub-syndromes in a total population of 2808 and found the four components to be stable, across dementias.

These studies agree that where neuropsychiatric symptoms are identified, whether through cluster analysis or factor analysis, and depending on the sample number, it seems three to four sub-syndromes are identifiable. The evidence for four sub-types is stronger as the values for each principal component factor are over 1 in the Hollingworth et al., 2006 study, and both Aalten et al., 2007a,b studies. Hollingworth et al., also add that using PCA, the components give more interpretable results (Hollingworth et al., 2006).
What these studies reveal is the existence of a significant presence of definable BPSD/neuropsychiatric features. This shift therefore highlights the non-cognitive heterogeneity of Alzheimer’s disease.

1.4 THE NEUROBIOLOGY OF SUB-TYPES OF ALZHEIMER’S DISEASE

Cognitive deficits and abnormalities at the neurobiological level have been identified with scanning procedures (e.g. positron emission tomography (PET)) (Blennow et al., 2006). The behavioural manifestations of symptoms are also thought to have a neurobiological basis (Ritchie and Lovestone, 2002, Shinosaki et al., 2000, Forstl and Fischer, 1994). And recently the Activities of Daily Living (ADL) has been correlated with increased pathologic burden in the frontal lobe (Marshall et al., 2006).

1.4.1 Apathy as a sub-type

Biological correlates of BPSD can be implied, when one notes the primary symptoms that load into the sub-types. Imaging studies have revealed that apathy in demented patients is associated with the hypoperfusion of the frontal and cingulate areas (Craig et al., 1996, Benoit et al., 2002)).

1.4.2 Depression as a sub-type

Hirono and colleagues, showed that hypometabolism and hypoferfusion in the frontal and cingulated gyrus was correlated with the NPI depression score in Alzheimer’s disease (Hirono et al., 1998). Increased degeneration of the locus...
coeruleus is associated with major depression, and a reduction of cortical serotonin reuptake sites (Zubenko 2000). Research into the genetic influences contributing to depression in Alzheimer’s disease is largely equivocal, candidates include APOE. APOE ε4 has been reported to be associated with depression in Alzheimer’s patients (Krishnan et al., 1996). The serotonin (hydroxytryptamine, 5-HT) transporter genes have been shown to be a risk factor of depression in Alzheimer’s patients (Mossner et al., 2000). However Zill et al., in a study of 222 patients showed that polymorphisms of the serotonin transporter was not a factor in the prevalence of depression in Alzheimer’s patients (Zill et al., 2000).

### 1.4.3 Psychosis as a sub-type

Alzheimer’s disease with psychotic symptoms (AD+P) has been associated with genetic changes in a number of studies. Genetic influences on the catechol-O-methyltransferase (COMT), involved in dopamine metabolism, have been associated with psychosis (Sweet et al., 2005). Holmes et al., also suggested that genetic variation of dopamine receptors, may be linked to AD+P (Holmes et al., 2001). Furthermore, genetic polymorphisms of the hydroxytryptamine receptors, 5-HT2A and 5-HT2C have also been linked to the prevalence of psychotic symptoms identified a genetic influence for psychotic symptoms in Alzheimer’s disease (Holmes et al., 1998). APOE4 ε4 allele carriers were at greater risk of developing psychotic symptoms (Zdanys et al., 2007). More certain is the that specific behaviours in the psychotic mood sub-syndrome, e.g. delusional misidentification are associated with right frontal and temporal atrophy (Forstl et al., 1994), whilst aggression in Alzheimer’s disease has been associated with hypoperfusion in the left anterior temporal cortex (Hirono et al., 2000). Aberrant motor behaviours are
associated with frontal lobe function, as is disinhibition and euphoria and are more commonly associated with frontotemporal dementias (Hollingworth et al., 2006). Sleep disturbances are also notable in Alzheimer’s disease. The circadian system which controls the sleep/wake cycle is reliant on the supercharismatic nucleus, deterioration of which leads to a disruption of sleep, both in the elderly and those who suffer from Alzheimer’s disease (Yesavage et al., 2003).

1.5 ALZHEIMER’S DISEASE AND THE NEED FOR BIOMARKERS

For Alzheimer’s disease there is a clear and present challenge to diagnose the disease as early as possible. This section of the introduction details the current scope of research related to Alzheimer’s disease biomarker discovery.

The diagnosis of Alzheimer’s disease is based on a criteria which has large-scale consensus (McKhann et al., DSM-IV-TR, ICD-10), and will usually take more than one clinic visit. The criteria is based a patient presenting to have Alzheimer’s disease, in the absence of any other neurodegenerative disorder. However the levels of accurate diagnosis outside of specialist memory clinics, that is in primary care is considered lower with approximately 33% of mild dementia cases failing to be accurately diagnosed (Lopponen et al., 2003).

Advances in Alzheimer’s disease research have allowed treatments to be translated into potential novel treatments which specifically aim to reduce the disease pathology and to modify disease progression. These have included anti-\(\text{A} \beta\) drugs, e.g. \(\text{A} \beta\) immunotherapies, secretase inhibitors and \(\text{A} \beta\) aggregation inhibitors. For any disease modifying drug to be effective in the early stages of the disease there is a
need to identify such patients possibly at earliest stage of the disease, where the pathology caused by neuronal damage has not manifested as dementia (Blennow et al., 2010). One way of achieving this goal is to detect and identify biomarkers for Alzheimer’s disease. A biomarker of any disease would ideally allow for the measurement of the pathological process associated with a disease, whether or not it was linked to any direct pathology. Therefore a biomarker could be used to determine whether the disease is or is not present and be part of the clinical diagnosis process, and whether any potential therapies for the treatment of the disease are effective. In Alzheimer’s disease a biomarker could be based in the cerebrospinal fluid, or a blood-based gene, protein or metabolite, a single entity or a platform of entities combining to provide an accurate diagnosis, in combination with clinical assessments (Hampel, 2010).

The existence of sub-types of Alzheimer’s disease may shed light on different aetiologies and thus the mechanisms responsible for the disease. In Alzheimer’s disease research there is a need to identify whether there are specific biological bases for these symptoms. This would provide therapeutic targets for pharmacological developments (Van Dam and De Deyn, 2006). Pharmacological treatments need to be tailored to target specific or multiple symptoms and as such sub-type identification with specific identification of CNS changes or aberrations are needed. Attempts at identifying biomarkers as early diagnostic material for diagnosis, have had limited success. There are a set of criteria for what a biomarker should be as set out by The Ronald and Nancy Reagan Research Institute of the Alzheimer’s Association and the National Institute on Aging Working Group (1998). This includes, the acquisition of appropriate biological material has to be non-invasive
and efficient, the levels for sensitivity should be in excess of 80% accuracy, with specificity in excess of 80%, with identification in patient sample also set at 80%

The primary sources of potential experimental biomarkers of Alzheimer’s disease are blood (serum and plasma), and the cerebrospinal fluid (CSF), and brain imaging techniques.

1.5.1 CSF Biomarkers of Alzheimer’s disease
The CSF is in direct contact with the central nervous system, and any biochemical changes that take place in the brain can potentially be monitored in the CSF, as there is no interference by the blood brain barrier (BBB). CSF can be accessed from patients through a lumbar puncture procedure (Hampel et al., 2004). CSF biomarkers include total tau (T-tau), phosphorylated tau (P-tau), and the 42 amino acid isoform of \( \beta \)-amyloid (A\( \beta \) 42) (Blennow et al., 2007).

1.5.2 Tau protein in CSF
T-tau found in CSF has been studied for well over a decade as a possible biomarker for Alzheimer’s disease. A meta-analysis by Sunderland et al., revealed that over 2200 Alzheimer’s patients had been studied, with 1000 age-matched controls (Sunderland et al., 2003). Consistently CSF T-tau has been significantly elevated (approximately three-fold increase) in Alzheimer’s disease patients. However, the sensitivity and specificity levels tend to vary. The sensitivity levels range between
40% and 80%, and specificity varies between 65% and 85%. There is also a link between T-tau and age related increases in non-demented persons (Hampel et al., 2004). Though CSF levels of T-tau are highly sensitive for Alzheimer’s disease, they do not possess a high level of specificity against other dementias (Andreasen et al., 2003).

1.5.3 Phosphorylated tau protein in CSF

There are numerous phosphorylated forms of tau, (P-tau 181, P-tau 199, P-tau 231, P-tau 396, P-tau 404) (Hampel et al., 2003). Buerger et al., using P-tau231 were able to discriminate with 90% sensitivity and specificity of 80%, between Alzheimer’s and non-Alzheimer’s patients (Buerger et al., 2002). P-tau 181 has been shown to be robust discriminating, biomarker between Alzheimer’s disease and other dementias (Schonknecht et al., 2003). Sub-groups of Alzheimer’s disease based on CSF proteins have been identified, where following analysis and measurement of the CSF forms of P-tau levels have been shown to cluster indicating different clinical profiles at 88% and 86% sensitivity and specificity (Iqbal et al., 2005).

1.5.4 β-Amyloid in CSF

The Aβ1-42 is a pathogenic protein, and is found in the CSF. The relative reduction in the CSF of Aβ1-42 in Alzheimer’s patients is thought to be due to the build up of the amyloid proteins. This is suggestive of a link between extracellular amyloid plaques and CSF Aβ1-42 (Hampel et al., 2003). Over the past decade close to 900 patients have been studied with 500 controls and the findings have been consistent in the levels of Aβ1-42 between the two sets of populations. These are decreased levels
of Aβ1-42 in the Alzheimer’s population, with a sensitivity range of 78% to 100% and a specificity range of 47% to 80% (Hampel et al., 2003). The issue with Aβ is that the levels found in CSF do not always correspond with levels found in plasma (Solfrizzi et al., 2006). The levels of Aβ are lower in plasma than corresponding concentrations in CSF (Scheuner et al., 1996). Furthermore plasma Aβ levels are thought to inadequate as a diagnostic measure. Plasma Aβ1-42 levels were found to be similar in both Alzheimer’s patients and controls in a cross-sectional studies (Mehta et al., 2000). A longitudinal study suggested that higher plasma Aβ a risk factor for developing Alzheimer’s disease is not thought to be useful (Mayeux et al., 2003, Mayeux et al., 1999). Plasma Aβ do not have the sensitivity or specificity compared to CSF Aβ (Solfrizzi et al., 2006).

1.5.5 β-Amyloid antibodies

In both CSF and blood, naturally occurring antibodies against Aβ have been found at lower levels in Alzheimer patients compared to healthy controls (Du et al., 2001). Following on from work in animal models, active and passive immunization with β-amyloid antibodies, resulted in a reduction of plaques (Schenk et al., 1999). Active immunization trials in humans with β-amyloid antibodies resulted in side effects (subacute meningoencephalitis) (Orgogozo et al., 2003). However a sub-group of patients who it is thought possessed auto-antibodies against developed antibodies to Aβ have a neuroprotective effect, with improved performance on neuropsychological tests (Hock et al., 2003).
1.5.6 Other potential biomarkers

The concentration of plasma total homocysteine and APOE ε4, are additional biomarkers of risk factors rather than diagnostic biomarkers. Both plasma total homocysteine and APOE ε4 lack sensitivity and specificity, and so are insufficient to discriminate between Alzheimer’s disease patients and controls, or other neurodegenerative diseases and dementias (Shaw et al., 2007). Increased homocysteine levels are considered a strong, independent risk factor in the development of Alzheimer’s disease, with high homocysteine levels almost doubling the risk of Alzheimer’s disease (Seshadri et al., 2002). APOE ε4 which is a plasma protein is involved in the transport of cholesterol, and repair of damaged nervous system (Rachakonda et al., 2004). APOE ε4 is a well-established risk factor for Alzheimer’s disease detected in up to 50% of patients, and implicated in aggregation and of Aβ (Farrer et al., 1997). Engelborghs et al., studied 50 autopsy-confirmed Alzheimer’s disease patients, to see if there was a link between the levels of CSF Aβ1-42, T-tau and P-tau with APOE ε4, as well as looking at the tangle and plaque burden (Engelborghs et al., 2007). They found no association between the CSF biomarkers and APOE ε4, and nor were the CSF biomarker levels associated with spread and extent of neurofibrillary tangles and amyloid plaques.

Hye et al., in a recent study, identified plasma based proteins, in 511 patients. They identified complement factor H (CFH) precursor and α-2-macroglobulin (α-2M) (this latter biomarker is highly abundant in plasma) with 56% sensitivity and 80% specificity, and these biomarkers were further associated with disease severity (Hye et al., 2006). With proteome-based plasma biomarkers, any analysis would require the plasma to be depleted of albumin and immunoglobulins (IgG), thus the
The possibility that potential biomarkers have to be discarded at the outset is problematical and real. Zhang et al., 2004, extracted serum proteins to identify potential biomarkers. In their study they identified 36 proteins whose levels were elevated compared to non-disease samples (Zhang et al., 2004). The two major proteins that were identified were haptoglobin and haemoglobin, these are acute response proteins. In addition α -2M, APOE ε4 and complement pathway involved proteins, biomarkers of inflammation (C3 and C4), levels were also elevated (Hampel et al., 2004).

Ray and colleagues analysed 259 plasma protein samples and found a pattern of 18 proteins in the plasma which could classify samples from Alzheimer’s disease and controls with almost 90% accuracy. The same plasma proteins could also predict the patients with mild cognitive impairment who would later develop AD (Ray et al., 2007). Using the same methodology and the same panel of 18 proteins the findings could not be replicated in an independent cohort (Soares et al., 2009).

A number of genome-wide association studies have investigated the CLU gene, which encodes for the protein clusterin and is involved in Alzheimer disease (Harold et al., 2009; Lambert et al., 2009) The protein clusterin, also known as ApoE J, has been suggested to be involved in the pathogenesis of Alzheimer’s disease as levels of plasma clusterin have been reported its possible use as a biomarker of the disease higher levels in rapidly progressing Alzheimer disease than in more slowly progressing disease, further plasma clusterin concentration was correlated with a decline in MMSE score in over 576 MCI and Alzheimer’s disease patients as well as the rate of progression of the disease (Thambisetty et al., 2010). Increased levels of
clusterin measured in the plasma were significantly also associated with baseline prevalence and severity of Alzheimer’s disease over a three year period (Schrijvers et al., 2011).

**Imaging as a biomarker (MRI & PET)**

Magnetic Resonance Imaging (MRI) has been used to reveal atrophy of the medial temporal lobe area in Alzheimer’s patients as compared to healthy controls, with greater than 85% sensitivity and specificity (Waldemar et al 2007), and depending on the severity, can be found in up to 96% of the Alzheimer’s disease cases (de Leon et al., 1997). The Alzheimer’s Disease Neuroimaging Initiative (ADNI), as part of a multicentre study are currently measuring hippocampus volumes and measuring cortical thickness to differentiate disease and non-disease brains (adni.loni.ucla.edu). Kloppel and colleagues used a computer aided learning technique to analyse MRI scans to differentiate with 89% sensitivity and specificity disease and non-disease MRI scans, based following post mortem confirmation (Kloppel et al., 2008).

Neuronal activity is impaired in Alzheimer’s disease, in the temporal parietal regions and in the posterior cingulate, positron emission tomography (PET), has been used to measure glucose metabolism in neurons, using the tracer flurodeoxyglucose (FDG), in patients with Alzheimer’s disease and healthy controls (Coleman, 2005 and Herholz, 2003).
1.6 SECTION SUMMARY - ALZHEIMER’S DISEASE

For over a century researchers have made significant advances in the understanding of Alzheimer’s disease. The first part of the literature review in this thesis has shown that the research into Alzheimer’s disease has fallen into three distinct areas (Section 1.1 – 1.5).

The first is the neuropathology of the disease which through a variety of hypotheses is thought to result in the two key features of change in the brains of patients: plaques and tangles (Section 1.1.2 – 1.1.3). What causes these plaques and tangles has been linked to a variety of processes including the accumulation of proteins, the role of a number of genes, and oxidative stress and inflammation in this process. However, to date there is no evidence that can explain the neuropathological basis of Alzheimer’s disease.

The second area of research has focused on the need to better understand and define the clinical aspects of Alzheimer’s disease (Section 1.2), including the identification and characterisation of the behavioural and psychological symptoms of dementia and the sub-types of the disease (Section 1.3). This process has been an important avenue of research as it has enabled clinicians to treat the variety of symptoms that are manifested through the course of the disease e.g. depression and psychosis (Section 1.2.3 – 1.2.4). The treatment of these psychiatric symptoms has a significant impact on the care-givers of patients by reducing the burden of day-to-day care (Section 1.2.3).
The third area of research of Alzheimer’s disease has focused on the need to link the pathology to the symptoms by identifying biomarkers of Alzheimer’s disease. One of the primary reasons for identifying robust biomarkers of disease is that they can be used not only to better understand the biological causes of the disease but also to generate new drugs and thereby reduce the suffering of patients.

The need to identify biomarkers of Alzheimer’s disease is of great importance as to date the diagnosis of the disease can only be confirmed post mortem, and to date there are no robust and reliable biomarkers that can be used to identify the high sensitivity and specificity levels of the currently used clinical diagnosis gold standard (Section 1.5).

1.7 A REVIEW OF THE LITERATURE ON METABOLOMICS

Biomarkers are important in the diagnosis of human diseases (Frank and Hargreaves, 2003). A recent proteomic study, identified serum protein patterns to identify cancer from non-cancer samples (Petricoin et al., 2002). Golub et al., 1999, using transcriptomic approach were able to classify cancer types on the basis of the genes (Golub et al., 1999). Alzheimer’s disease is a multi system disorder that is not linked to any one specific gene or protein. Any change in an individual’s genes and proteins can result in changes of metabolites and their concentrations. Measuring metabolites as well as changes of metabolite levels, is an ideal method to gauge the effect of genes and proteins in any disease.

Metabolites are small biological molecules, that can be found both intra- and extracellularly. The molecular mass of metabolites is usually below 1500 daltons
(Da), and common examples of metabolites include alkaloids (derivatives of amino acids), glycoproteins (hormones), oligosaccharides (sugars) and lipids (fats) (Breitling et al., 2006). Metabolomics is the identification and quantification of metabolites in cells, tissues, and biological fluids (Fiehn, 2001), whilst metabolome is the name given to all the metabolites likely to be found in any biological sample (Oliver et al 1998). Metabolites found in and outside of a cell are the direct result of a number of cellular processes; i) the gene, the code or DNA (deoxyribonucleic acid) (polymers of 4 different nucleotides) is (ii) transcribed to mRNA (messenger ribonucleic acid), which in turn is translated to (iii) form proteins (compositions of 22 amino acids); the enzymatic breakdown of the proteins results in the production of (iv) metabolites (Wishart et al., 2009).

“Omics” is the name given to the study of each of these processes. Therefore the study of the gene is termed (i) genomics, (including the draft mapping of the entire genome project, International Human Genome Sequencing Consortium (http://genome.wellcome.ac.uk)), (ii) transcriptomics is the study of mRNA transcripts (i.e. the expression of genes) (iii) proteomics is the identification and measurement of proteins and their interaction with one another (Human Proteome initiative (http://www.expasy.org/sprot /hpi/)). This is followed by (iv) metabolomics, which is a downstream to the preceding “omics” (Kell, 2006b). The experimental data derived from these four “omics” is combined in systems biology, in order to better understand the dynamics of a cell.
Metabolomics which is the full repertoire of metabolites is downstream from the genome and numbers far fewer candidates than its “omics” counterparts, and is considered closer to the phenotype of a disease.

1.7.1 The metabolome

As reported earlier, recent research has shown the potential of using a genome and protein to detect known and unknown biomarkers of Alzheimer’s disease (section 1.5.6). The potential of the use of the so-called “omics” technology and specifically the use of metabolomics as one such “omics” technology is reviewed below.

In genomics and transcriptomics, the targets for analysis are four different nucleotides (adenine cytosine guanine and thymine); whilst in proteomics the targets for analysis are 22 amino acids. Despite the similarity in chemical structures, the sheer quantities of potential targets make it difficult to analyse the entire chemical complement in a biological sample (Weckworth and Morgenthal 2005). As such the samples are either reduced in size through removal of proteins (deproteination), or specific chemicals are targeted. The chemical heterogeneity of the metabolome, makes its identification and measurement a challenge. This is because there can
exist in the same biological sample, inorganic compounds, carbohydrates, amino and non-amino organic acids, alcohols, ketones, hydrophobic lipids as well as other complex products (Villas-Boas et al., 2005). The human metabolome is constantly changing. The biochemical changes that occur due to the metabolic reactions (metabolic flux), are one of the reasons for the variation. The metabolome is further influenced by sex, age, diet, diurnal changes, disease, drugs and lifestyle. All these factors contribute in making the analysis of the metabolome a challenge.

1.7.2 Metabolomics and disease

According to Harrigan and Goodacre, 2003, there are 500 estimated diseases which are reflected in the change of metabolism. Metabolomics potentially can be used to identify disease biomarkers, which can distinguish disease from non-disease metabolome, and metabolome changes which may be due to drug treatments. Metabolomics can be applied to meet two important aspects of disease research. The first is in identifying disease biomarkers, to predict accurately disease diagnosis, to sub-classify diseases, elucidate disease aetiology and mechanisms; and secondly, to measure the effect of medication on the metabolome.

Blood which is home to proteins and metabolites is used in routine clinical tests. Most blood tests either analysis the plasma or serum constituent of blood. Plasma is the liquid component of blood, containing the protein fibrinogen, which is a clotting factor, and when the clotting factors are removed the liquid is termed serum. Serum like plasma contains proteins, electrolytes and moreover is home to a large number of metabolites, and is a complex aqueous solution, containing dissolved gases, hormones, metabolic waste and nutrients in the body, and is approximately 95%
water. In a biological sample such as human serum there are possibly between 1000 and 10000 metabolites, depending on their concentrations (Kell, 2006a, O'Hagan et al., 2007). Duarte and colleagues, have manually reconstructed a global human metabolic map, (homo sapiens Recon 1), and identified 2766 metabolites (Duarte et al., 2007). The total number of metabolites is thought to be dependent on the number of enzymatic reactions, and not all substrates are known as their concentrations may not be sufficient to allow them to be detected using current technologies, and more importantly may be of an unknown chemical identity (Kell, 2006a, Kell, 2006b).
<table>
<thead>
<tr>
<th>Metabolite Groups</th>
<th>Examples of Individual metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>alanine, cysteine, glutamic acid, tryptophan</td>
</tr>
<tr>
<td>Lipids &amp; Fatty Acids</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Organic Acids</td>
<td>succinic acid, uric acid, malonic acid, lactic acid</td>
</tr>
<tr>
<td>Sugars</td>
<td>sucrose, fructose, galactose, glucose-6-phosphate</td>
</tr>
<tr>
<td>Others</td>
<td>creatinine, urea, noradrenaline</td>
</tr>
</tbody>
</table>

Table 1.3 Metabolite groups and examples of metabolites

The Human Metabolome project (HMP) has to date identified 4000 out of a total of 6800 metabolites in the serum, information on these metabolites is held on the HMP (www.metabolomics.ca)

1.7.3 Measuring the metabolome

Current analytical platforms lack the technical ability to permit the analysis of the entire metabolome. The scale of heterogeneity of the metabolome is such that, to capture the entire complement of metabolome constituents, (the detection and quantification) cannot be achieved by a single analytical platform or apparatus. Metabolomics experiments, like any large scale experiment where a large number of samples are being analysed have to be robust. The elimination of errors in
experimentation, or control of errors require that experiments are undertaken in a carefully and controlled manner.

In a metabolomic experiment, the analysis of samples must be performed in an unbiased manner, using a robust and reproducible method, whilst maintaining high levels of sensitivity and accuracy (Dunn et al., 2005, Goodacre, 2005b, Kell, 2006b). Robustness (or repeatability) and reproducibility are key features of any omics experiment (Dunn et al., 2005, Goodacre, 2005b, Kell, 2006b). Repeatability implies that the same operator of an analytical platform in the same laboratory, whilst performing analysis will do so using the same settings. Reproducibility is built on the ability to practice robust repeatability. The concept of reproducibility, will allow the same experiment to be replicated in another laboratory by a different operator with similar measurements as the original experiment. Repeatability and reproducibility must be applied from the outset of any experiment, from the collection of any sample through to the analysis of all output data (Dunn et al., 2005, Goodacre, 2005b, Kell, 2006b).

There are a number of key steps that are critical in determining the metabolome of any biological sample. These are (i) stopping all the metabolic activity of a sample (this also termed quenching); (ii) analysis of the sample; (iii) handling the larger amounts of data generated, followed by; (iv) statistical analysis and modelling of the metabolome data. The metabolomic approach aims to identify and quantify the full metabolic repertoire (both known and unknown), and having undergone extraction, the sample undergoes a separation process, followed by detection of the metabolites
based on their mass. The other main analytical techniques are summarised in Table 1.4.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>metabolomics / metabonomics</td>
<td>when the entire metabolome is analysed and where the aim is to seek the biological origin and relevance of metabolites.</td>
</tr>
<tr>
<td>metabolic fingerprinting</td>
<td>when the goal is to fingerprint the effect of drug or disease on metabolite groups and not specific metabolites</td>
</tr>
<tr>
<td>metabolite target analysis</td>
<td>when specific metabolites belonging to a metabolite group(s) are targeted; or metabolites involved in specific enzyme reactions are targeted</td>
</tr>
<tr>
<td>metabolic profiling</td>
<td>when specific groups of metabolites are targeted within a given range (e.g. lipids)</td>
</tr>
</tbody>
</table>

Table 1.4  Main analytical techniques applied in metabolomics
There are four general applications or methodologies that are employed in metabolomics research, that attempt to attain the research goals for any given disease.

1.8 APPLICATIONS OF METABOLOMICS TO IDENTIFY AND DISEASE BIOMARKERS

The application of metabolomics experiments has been used to analyse the metabolome and identify disease biomarkers in a number of different diseases. Analysis of the metabolome has been used in cardiovascular disease (Brindle. et al.,
2002), Type 2 diabetes (Wang et al., 2005), and obesity (Hochberg, 2006), ovarian cancer (Denkert et al., 2006), prostate cancer (Sreekumar et al., 2009), and neurodegenerative disorders, including Huntingdon’s disease (Underwood et al., 2006), Parkinson’s disease (Bogdanov et al., 2008).

1.8.1 Metabolomics analytical techniques

Currently, there is no single analytical technique which can be used to analyse the entire complex nature of the metabolome content in any given biological sample. There are a number of techniques, which are available to researchers (Dunn et al., 2005; Lenz and Wilson et al., 2007) each of which may be employed for experimental purposes to analyse the same samples, in order to overcome the limitation in technologies (Crockford et al., 2006; Lenz et al., 2007).

Mass spectrometry (MS), nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FT-IR) are the most frequently used to detect the mass of metabolites. Both FT-IR and NMR techniques are able to directly analyse samples without resorting to sample extraction or chromatographic separation. NMR and FT-IR reveal only a “metabolic fingerprint”, as they do not provide specific metabolite information and detect smaller number of metabolites (Holmes et al., 2006). Nonetheless the proton nuclear magnetic resonance ($^{1}$H NMR) has been used predominantly for metabolite profiling, based on the magnetic properties of the metabolites and producing and helping to produce a spectra (peaks) from which the structure and thus identity of the metabolite (Holmes et al., 2006a, Holmes et al., 2006b), can be identified. This approach enables rapid measurement of metabolites usually in minutes, with no prior sample derivatization or separation (Clayton et al.,
2006), and indicates the overall impact of metabolic processes in a sample (Fiehn, 2002). NMR allows samples to be analysed in a non-destructive manner, and the information obtained regarding metabolites, allows both the identification and measurement of metabolites (Griffin, 2003). The disadvantage in the use of NMR is that, there is a lack of sensitivity as compared to a mass spectrometer, and also the NMR technique, produces many signals when detecting metabolites, thus it is difficult to distinguish the individual metabolites of the sample as they have not undergone chromatographic separation (Weckworth, 2003).

**FT-IR analysis of the metabolome**

The Fourier transform infrared spectroscopy (FT-IR) which works on the principle of excitation of molecules of a sample by infrared beam, results in an infrared absorbance spectrum, and has not been widely used in human metabolome analysis (Dunn et al., 2005) (Figure 1.4). The FT-IR is a method used to detect and investigate chemical changes. The infrared light region of the electromagnetic radiation spectrum occurs between a frequency region of 0.7 and 500µm. That is it lies between the microwaves and visible regions of the electromagnetic spectrum. FT-IR is an infrared spectroscopy method, in which infrared light when passed through a sample is in part absorbed and transmitted through the sample. When the sample molecules absorb the infrared light, there occur different types of vibrations of chemical bonds between the atoms of the molecules. Each molecule has a functional group(s) that are certain atoms that are responsible for the characteristic chemical reactions that the molecule can undergo; these specific reactions are the basis for classification of these groups. The manner in which these atoms are connected or chemical bonds vary depending on the individual atoms involved in the
molecule. Therefore different chemical bonds of molecules absorb infrared light at specific frequencies that are needed for vibrational movement. And the frequency at which vibration occurs is determined by the strength of the chemical bonds and the mass of the atoms. At specific frequencies, the nature of the vibration will be bending, asymmetric stretch, symmetric stretch, scissor, rocking or wagging. The infrared frequency ranges at which the vibrations and types of vibrations take place can be seen in Table 1.5.

<table>
<thead>
<tr>
<th>Infrared wavelength (cm(^{-1}))</th>
<th>Functional group</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2850-3100</td>
<td>(CH(_2))(_n)CH(_3)</td>
<td>Alkyl group (found in almost everything)</td>
</tr>
<tr>
<td>1680-1640</td>
<td>C=C</td>
<td>Alkenyl group (found in alkenes)</td>
</tr>
<tr>
<td>3500-3200</td>
<td>OH</td>
<td>Hydroxyl group (found in alcohols and carboxylic acids)</td>
</tr>
<tr>
<td>1760-1665</td>
<td>C=O</td>
<td>Carbonyl group (found in aldehydes, ketones, carboxylic acids, amides, esters)</td>
</tr>
<tr>
<td>3500-3300</td>
<td>N-H</td>
<td>Amines and amides</td>
</tr>
</tbody>
</table>

Table 1.5 Example of functional groups detected by FT-IR.

The infrared frequency ranges at which the vibrations and types of vibrations take place varies across the infrared wavelength range 1640- 3500 cm\(^{-1}\).
The FT-IR

Figure 1.4  A photograph of an FT-IR machine

The 96-well silican plate in the foreground is spotted with the sample under analysis.

Figure 1.5.  The FT-IR and sample analysis.
A FT-IR has four key components, the source, the interferometer, the sample and the detector. The FT-IR works by exciting the chemical bonds found within a biofluid and recording the vibrations. interference pattern of the light intensity from the light reflected of the stationary mirror and that from the moveable mirror.
The FT-IR is made of has four key components, the source, the interferometer, the sample and the detector. As the name suggests the source is from which the infrared beam is emitted, and enters the interferometer. The interferometer is an optical device, which importantly allows all the infrared frequencies to be measured simultaneously. The interferometer achieves this because it contains a beam splitter and two plane mirrors, one of which is in a fixed position whilst the other is able to move. The incoming infrared beam is split into two optical beams (the beamsplitter). One of these optical beams is directed on to the fixed mirror, and therefore travels a fixed length, whilst the second beam is directed on to the moveable mirror (which is constantly moving a few millimetres), and therefore has a variable optical length to travel (depending on which position the light was reflected of the mirror). The two reflected beams are then recombined by the beam splitter and then passed through the sample and the non-absorbed light travels to the detector. Even though the two beams are re-combined, there is a time-dependent change or interference of the infrared light pattern at the detector. The resultant signal that is produced from the interference of the light is termed an interferogram. The interferogram encodes the interference pattern of the light intensity from the light reflected of the stationary mirror and that from the moveable mirror. That is, all the data points which are a direct function of the moving mirror position are captured as a single signal, and this one signal contains information regarding the entire infrared frequency which is emitted from the source. This allows for all the infrared frequencies to be measured all at the same time, between seconds and minutes (Figure 1.5).
The interferogram owing to its complexity cannot be interpreted directly. In order to identify the biochemical variation in the samples, an infrared frequency spectrum has to be generated. This would show the signal intensity at each individual frequency, thereby indicating the presence of chemically functional groups. This is achieved by employing the Fourier transformation mathematical technique, which splits the interferogram into infrared frequencies. This operation is performed by the computer software provided by the FT-IR manufacturers.

**Mass spectrometry analysis of the metabolome**

The increase in the strength of mass spectrometry, allows for the increased mass measurement of smaller ions, specifically ions with decreasing molecular weights (Makarov et al., 2006). Metabolomics experiment use mass spectrometry, because it is able to sensitively detect metabolites, and provide information on both the molecular formula and the chemical structure of the metabolites, by producing a mass spectra, that enable identification of metabolites (Smith, 1988). Mass spectrometers work on the basic principle that to analyse a sample constituents, or analytes, if they can be converted into ions, and then accelerated through an electric field, following which the ions, are separated on the basis of their mass to charge ratio, that is, the ratio of molecular mass to the absolute molecular charge number (m/z) as the ion passes through a magnetic field. The separated and subsequently detected ions are recorded by a sensitive detector, as peaks of a mass spectrum as well as their relative abundance. Mass spectrometers contain three key sections. The first is the source section, which generates ions in a gaseous form which are directed and projected on to the second key section of the instrument, called the mass analyser. The second section is where the mass of the ions are analysed by
exploiting the mass to charge ratios of the individual gaseous ions. These mass to charge ratios are then in the same order recorded by the third key section of the mass spectrometer, the mass detector (Figure 1.5).

Mass Spectrometry

![Mass Spectrometry Diagram]

**Figure 1.5 The functional aspects of a mass spectrometer**

A typical MS contains three basic components: the ion source, which splits the sample molecules into the constituent ions, a mass analyzer which by applying an electromagnetic field is able to order ions on the basis of their respective masses. And finally a detector, which measures the quantity of the ions present. A spectral shows the mass of each fragment.

MS as the name implies measures the mass of a molecule from which potentially the chemical structure can be discerned. To do this the mass spectrometer has to electrically charge, or ionize the chemical compounds, and thus generate charged molecules or molecular fragments whose mass to charge ration (m/z) can be measured when the ionized samples are accelerated through an electromagnetic field,
which fragments the molecules and allows the detection of the differently charged ion species, or the sample molecules.

Mass spectrometry techniques allow analytes to be detected rapidly by way of analytes being carried by a flowing gas stream. Mass spectrometry produce an output of data which is for each separated analyte which has been eluted. The relative response of the detected analyte is measured as a strength of the signal against time. Individual spectra of each peak can be used to identify analytes using libraries if spectra which can be used in an automated manner to identify unknown peaks.

### 1.8.1.1.1 Electrospray ionisation

Electrospray ionisation (ESI) is a method of generating ions without causing fragmentation of the ions. ESI techniques have been used to analyse biological molecules extensively (Gaskell, 1997). In ESI, separated analytes are transported in liquid solution into a capillary. The flow of liquid is aided by an inert gas which passes in the same direction as the liquid. The capillary is either positively charged or negatively charged, depending on the type of ionisation required (positive or negative ionisation mode). The ions emerge as droplets from the column, and are carried by the inert gas towards the counter electrode, which is oppositely charged to the capillary. The gap between the capillary and the counter creates an electrode potential. The sprayed ions which are highly charged, and as they emerge from the capillary and until they reach the counter emerge as gas-ions. These ions are then introduced in to the MS where ions separated on the basis of their mass to charge ratio.
ESI is the most commonly used ionisation method in metabolomics experiments and is appropriate for targeting amino acids, organic acids, sugars, steroids, fatty acids, phospholipids (Allwood and Goodacre, 2010). ESI has advantages which include, as it can ably ionise across a large mass range, with good levels of sensitivity, preserves the ionised metabolites, and therefore does not cause further fragmentation of the analytes (Gaskell, 1997). ESI does have disadvantages, these include formation of adducts, metabolites are not ionised, i.e. ionisation is suppressed when there is a high concentration of other metabolites (King et al., 2000).

Because ESI works in either positive or negative mode, samples have to be tested in both modes. This allows for a greater comprehensive number of ions to be generated. The ionisation mode, allows for greater analysis of the metabolome in a given sample, by exploiting the amenability of compounds to ionise either in the positive or negative mode. In order to profile by detection the largest number of metabolites this ESI seems a suitable approach. Compounds depending on their structures have an affinity for ionisation either in the positive or negative state.

### 1.8.1.1.2 Time of flight

Samples that need to undergo MS analysis have to be in a gaseous state, thus the sample inlet of a MS is maintained at very high temperatures (up to 400°C). The entry of a sample in to the ionization chamber results in the fragmentation of the sample molecules. The ionization chamber contains electrons that are forced to accelerate when a high voltage is applied. When the sample molecules enter the ionization chamber, they are forced into collisions with the electrons. This results in
the fragmentation of the sample molecules. Consequently each of the fragmented species becomes ionized or charged. These charged ions are thus transported through the mass analyzer owing to the influence of the accelerating voltage. Because of the possible range of masses of all the fragments, the accelerating voltage also has to vary. The fragments are then projected to the detector.

In GC-ToF-MS, the charged particles (ions) that are needed for the mass analysis are induced by a process termed electron impact ionization (EI). The gaseous molecules which exit the GC are forced into collisions with a high-energy electron beam (70eV). When a molecule is struck by an electron, there results a removal of an electron from the molecule, thus creating a charged molecule. The ions are accelerated and travel a controlled corridor of the mass analyzer. The mass analyzer can be a time of flight (ToF), quadrupole, quadrupole ion trap, linear quadrupole ion trap (LTQ) (This will be discussed in further detail in the UPLC section).

The mass analyzer uses + and – voltages to control the path of the ions, and these ions then travel down the path based on their mass to charge ratio (m/z). In EI ionization only singly charged ions are produced, therefore the charge (z) is one, and the path of the ion is dependent on the mass (light or heavy). The passage of the ions is based on their mass to charge ratio (m/z). In the ToF mass analyzer, the ions of different m/z are dispersed over the duration of their passage or flight along the path of a known length after acceleration in an electric field, thus if all the ions start at the same time and point, the lighter ions travel faster than the heavier ions and so arrive earlier at the detector. The ionized molecules are introduced into the mass analyzer in a pulsed manner.
ToF based mass spectroscopy have fast acquisition rates and have high resolution capacity. The speed of the spectra which is generated is in microseconds. In normal situations, the GC chromatograms tend to have narrow peaks and require fast scanning detectors. ToF instruments allow for fast acquisition, therefore it complements the fast separation achieved by GC. ToF MS is known to have high resolution with at least 10,000 at full width half maxima (FWHM) peak height. This means that the ToF based mass spectroscopy can acquire accurate mass data, which can in turn be used to acquire accurate data pertaining to the mass of the ions. This data can then be used for corroboration of the chemical formula and quantity. It is these properties that endear the GC-ToF-MS for metabolomic profiling of sample compounds, their relative quantities and their subsequent identification (Williamson and Bartlett 2007)

1.8.1.1.3 Hybrid LTQ/Orbitrap mass analyser

The hybrid LTQ Orbitrap mass analyser is a hybrid MS, which is connected to an ESI source, ions from the ESI source are projected into a linear ion trap and via an Orbitrap are detected. The linear trap is a mass anlayser and the Orbitrap is a mass detector (Makarov et al., 2006 and Hu et al., 2005). Ions which are in a gaseous form are created by way of an ion source (in this context this could be the ToF ionisation technique or ESI). The ions are projected and then guided through pressure and electrode potential created by a series of guide and transport poles. The ions enter a third chamber which is termed the storage or linear ion trap, which contains a gas. Whilst in this storage chamber the ions speed is reduced as the ions try to pass through the gas. In doing so the ions are collected. The collected ions are
released in small packets onwards to the Orbitrap. The ions are accelerated and are forced through on to the Orbitrap. The Orbitrap obtains optimal performance when only a small number of ions are required for detection or mass analysis. Information regarding the ion is then collected and amplified.

The key advantage of the Orbitrap over other MS systems is that it can detect the mass of ions in a non-destructive manner. This means that ions can be removed from the trap using a highly powered vacuum pressure and there is no issue of competing fragments when the next ion is ready to be detected.

1.8.2 Metabolomics and chromatography

In chromatographic analysis, the sample which has to be analysed is mixed with either a gas, if it is to undergo gas chromatography (GC) separation, or a liquid for liquid chromatography (LC) separation. This gas or liquid, (the mobile phase) is then passed through a coated column (the stationary phase). The rate at which the sample constituents pass through the column depends upon the degree of affinity they have for the stationary phase. The movement through the column therefore results in separation of the sample, into the constituent parts. Retention time, (the time it takes for a peak to be eluted), is used to identify components of a sample, by comparing with standards. Retention is due to the extent of interaction of sample components with the column (Smith, 1988). Both GC and LC are coupled to sensitive mass detectors (mass spectrometer (MS)) (GC-MS and LC-MS).

The coupling of a mass spectrometer to such analytical instruments are usually referred to as hyphenated techniques or analytical platforms. The most commonly
used hyphenated techniques are GC- and LC-MS (Dunn et al., 2008 and Villas-Boas et al., 2005). The use of GC-MS is considered more appropriate for complex biological samples (Kenny et al., 2005). The combination of chromatography and mass detection enables both the identification and quantification of metabolites in a sample, but they are also considered destructive. NMR and FT-IR though lacking the sensitivity of the chromatographic and MS approach are considered high-throughput (1 minute or less) (Dunn et al., 2005).

**GC-MS**

Gas chromatography (GC) combined with mass spectrometry (MS) is an effective method in the chemical analysis of biological samples (Figure 1.6). Coupling of GC and MS provides a non-biased approach for detecting polar compounds. In metabolite profiling, a non-biased design approach can be used to potentially reveal known and unknown chemicals in a biological solution.

GC has been commonly and routinely used in drug testing analysis of food and drug compounds. It is an effective tool in identifying food contaminants. Separation of samples based on chromatography, involves a stationary phase, which can either be solid, liquid or a mixture of the two, and as is implied by the name is static, and the mobile phase, is gaseous. The gas mobile phase flow over or through the stationary phase, carrying with it the analytical sample (Figure 1.7). The choice of the stationary phase is entirely dependent on the compounds that are required for separation. In metabolomics experiments, GC separates metabolites based on molecular weight (18 – 300Da). The metabolite classes include sugars, amines, amides, organic and amino acids.
Figure 1.6 GC-ToF-MS

The image shows the GC machine with sampler is above the ToF-MS which is below and linked to a PC in a lab.

Figure 1.7 GC-MS and its function

Sample inlet, carrier gas, and output, is the chromatogram.

The major components of GC-MS include, the mobile phase which is the gas source, pumps gas through the column which is coated with the stationary phase, detector and a computer to capture the chromatogram. Samples are injected directly into the column and separated based on chemical affinity of sample components to the stationary phase.
A GC machine consists of an injection port, connected at one end to a metal column, which despite its name is actually coiled and is incubated inside an oven, and at the other end of the column is the detector (Figure 1.7 and 1.8). For a sample to undergo GC separation analysis, a small volume of liquid is initially injected into the injection port of the GC. The injected sample is then vaporised in a heated chamber and carried by a single chemically inert mobile gas usually helium, hydrogen or nitrogen. This mobile carrier gas forces the sample material down the column. The column itself is actually packed with material, with which the vaporized sample chemicals or molecules of the sample adhere to (Figure 1.7 and 1.8).

The forcing of these molecules directly through the column by the mobile phase gas is restricted because the sample molecules adhere or become adsorbed to the column material. The rate at which the molecules pass through the column is therefore dependent on the adsorption of molecules and their affinity for the stationary phase packed materials of the column. Different types of molecules move through the column at varying rates, the molecules of the sample are therefore separated at different times, as not all the material emerges at the end of the column simultaneously. This difference in emergence time is termed retention time. A detector at the end of the column monitors the emergence or elution of molecules and their relative amount. Eluting analytes are identified (qualitatively) by the order in which they emerge from the column and by their respective retention time. The detected output is depicted as peaks which correspond to each eluted molecule, and where the peak size is proportional to the amount of the molecule detected. This is termed the spectral output or chromatogram of the analysed sample. As is often the case different molecules may have the same retention time, and it may be difficult to
identify which molecule it may be. For the detection of peaks and the calculation of the peak areas is an automated process using computational software. The process of chromatographic deconvolution will be discussed later. But briefly this is the step needed to separate out overlapping chromatograms.

To counter any variation that may arise due to chromatographic separation conditions and the labile nature of the column, in that it degrades over sample analysis time, an internal standard is used. The internal standard should ideally be distinct and have near to or as similar to the physical properties of the test sample molecules. Known amounts of the internal standard are added prior to any sample extraction, and therefore undergo the same chemical reactions as in the sample processing stage. If there is any loss of the amount of standard at any time during the GC process, then this will be identical to any loss of the test sample analytes. The peak area associated with the fixed amount of internal standard.

The signal to noise ratio is an important component in the interpretation of chromatograms. The sensitivity of a detector is the minimum level of the sensitivity required to identify the eluted molecules from the column. Peaks are can become obscured when their peak heights becomes very similar in magnitude to the noise of the detector system. The detector noise is defined as the disturbance on the detector which is not related to an eluting solute, yet still generates peaks. The source of the noise can be due to a number of factors (the chromatographic system, the sensor or the associated electronics). To limit the impact if this disturbance or noise, the signal from the peak must be sufficiently greater than this noise to unambiguously identify the peak. The ratio of the signal size to the noise is termed the signal-to-noise ratio.
The choice of the numerical value for the signal-to-noise ratio is somewhat arbitrary and has been borrowed from the signal-to-noise ratio used in electronics i.e. 2. Thus, for the unambiguous identification of an eluted peak in chromatography the signal-to-noise ratio must be 2. That is, the peak height must be twice as big as compared to the noise.
GC-MS is used to analyse volatile and thermally stable, polar and non-polar metabolites. The metabolites in any sample contain both volatile and non-volatile species. The non-volatile metabolites need to be made volatile so that they can be eluted through the gas chromatography column. This is achieved by chemical derivatization (O’Hagan et al., 2005; O’Hagan et al., 2007 and Kenny et al., 2005). This two-stage sample chemical derivatization (oxime/silylation) is performed on the freeze dried sample, using $O$-methylhydroxylamine solution and heated at 40°C followed by addition of $N$-actyl-$N$-(trimethylsilyl) trifluoroacetaide (MSTFA) and then heated at a temperature of 40°C again. Low temperatures (of 40°C) are used so as not to interfere with the non-derivatized metabolites that are more stable at lower temperatures, as compared to higher temperatures (Dunn et al., 2005). Following derivatization, the sample is separated into vials for GC-MS analysis. The object of derivatisation is to reduce the boiling point of metabolites so that they can be effectively separated as they pass through the GC column. Derivatisation allows GC-MS to detect a large number of metabolite classes, including monosaccharides, disaccharides, amines, amino and organic acids.

High resolution chromatographic separations are achieved by vaporising the sample using high temperatures (up to 350°C), and passing it through a gas capillary chromatography columns (helium used as the carrier). The samples are analysed with a very small volume of liquid (1µl) injected on to the high resolution capillary columns, that are 30 - 60m columns, where the stationary phase is generally phenyl. The levels of detection are highly sensitive and are in the range of µM-nM.
This is followed by detection of the separated metabolite components, based on exploiting the electrical properties of the metabolites and detecting them based on their molecular weights. There are two main mass spectrometry detection methods, one of which a chemical ionisation method. The second of method is termed electron impact, an example of which is Time-of-Flight (ToF). ToF is a procedure in which there is further separation of the eluted sample (having undergone GC separation), based on mass and change of velocity. This TOF approach works by protonating (+) the metabolite fragments, and thus generating charged ions. The time taken for these ions to cross (migrate) through the instrument prior to being detected by an electrode, improves the ability to measure the mass to charge ratio (m/z) value of the ion (Dunn et al., 2005, Villas-Boas et al., 2005). The GC-ToF-MS analytical platform is therefore able to fully scan the metabolites from which a mass spectrum is generated. GC-ToF-MS was initially used in plant metabolomics, to profile plant metabolites (Fiehn et al., 2000).

**Liquid chromatography**

Most compounds which can dissolve in a liquid can be analysed using liquid chromatography (Meyer, 2004). Liquid chromatography separation techniques, are achieved by distributing a sample between a liquid (mobile phase) and a column packed with stationary phase substance, by a powerful pump. The end of the column, or outlet is connected to a detector which records the retention time (RT), other properties including the response for every detected analyte are also detected and recorded. LC can also be coupled to a MS. This coupling yields additional information, such as the concentration of metabolite peaks detected and the chemical structure of the metabolite peak. The data collected from the analysis of a sample
using an LC coupled to an MS, for each separated and detected analyte is retention
time, signal response or relative abundance, the molecular mass of the peak and the
ionic charge (see Figure 1.9).

In a biological sample, the separation and detection of constituent analytes, is
achieved on the basis of the affinity of the analyte with the mobile phase. That is
those analytes with lower affinity elute at a lower rate, that is they are usually
separated and detected at a slower rate. Analytes which have a higher affinity for
water (hydrophilic) elute at the beginning, whilst hydrophobic compounds elute at
the end. The retention time varies with respect to the types of compounds being
eluted. Further the chemical structure of analytes can impact on the retention time.

A consideration in LC-MS experiments is the time it takes to complete the analysis
of a sample. If the assay contains compounds which require longer retention times,
and this occurs when a sample contains very non-polar compounds, such as
hydrocarbons and lipids, the separation time required can be greatly lengthened
(Smith, 1988). For this reason a complex aqueous sample such as serum requires
extraction processes prior to analysis using LC-MS. In LC-MS the samples have to
be extracted in an organic solvent.

The mobile phase liquid used in LC-MS separation is usually two different solvents
referred to as solvent A and B. These solvents are used at different concentrations
both separately and as admixtures. The first, usually contains a high aqueous content
and an organic content e.g. methanol or acetonitrile. Methanol works better at higher
pressures, and does not impair sensitive separation. The high aqueous content liquid
is usually used to elute polar metabolites from the column, whilst a combination of
the aqueous and the organic solvent are used in tandem to elute the non-polar metabolites from the column. The organic solvent is introduced in increasing ratios, until the gradient is 100% organic solvent, in order to elute the non-polar compounds from the stationary phase. The organic solvent is also used to flush the column after each analysis.

1.8.3 UPLC LTQ Orbitrap-MS

UPLC or Ultra Performance Liquid Chromatography, uses a column which has been packed with extremely small particles (less than 2.0µm). This allows the column to withstand significant pressures in the region of 15 000 psi (see Figure 1.8 and 1.9). The combination of small stationary phase particles, and the ability to cope with significant pressures, enables complex biological samples, to be analysed at much higher sensitivity, improved resolution, and with time required for separation quickened (Wilson et al., 2006). The UPLC system has been used in a number of metabolomics studies involving biological fluids (Williams et al., 2006, Dunn et al., 2006, Guy et al., 2008, Zelena et al., 2009).

UPLC allows metabolites with a molecular weight range of 50 – 15, 000 Da, to be separated. UPLC is successful in efficiently retaining and separating non-polar metabolites. Metabolites which are of a large weight such as phospholipids and triglycerides, amino acids, are also successfully separated.

The preparation of samples using UPLC is less complex than GC-MS. UPLC, like LC does not require chemical derivatisation step. Lipids are present in samples at high concentrations and so can be removed as they interfere with the retention time.
Because ESI works in either positive or negative mode, samples have to be tested in both modes. This allows for a greater comprehensive number of ions to be generated. The ionisation mode, allows for greater analysis of the metabolome in a given sample, by exploiting the amenability of compounds to ionise either in the positive or negative mode. In order to profile by detection the largest number of metabolites this ESI seems a suitable approach. Compounds depending on their structures have an affinity for ionisation either in the positive or negative state.

**Hybrid LTQ/Orbitrap mass analyser**

The hybrid LTQ Orbitrap mass analyser is a hybrid MS, which is connected to an ESI source, ions from the ESI source are projected into a linear ion trap and via an Orbitrap are detected. The linear trap is a mass anlayser and the Orbitrap is a mass
detector. (Makarov et al., 2006; and Hu et al., 2005). Ions which are in a gaseous form are created by way of an ion source (in this context this could be the ToF ionisation technique or ESI). The ions are projected and then guided through pressure and electrode potential created by a series of guide and transport poles. The ions enter a third chamber which is termed the storage or linear ion trap, which contains a gas. Whilst in this storage chamber the ions speed is reduced as the ions try to pass through the gas. In doing so the ions are collected. The collected ions are released in small packets onwards to the Orbitrap. The ions are accelerated and are forced through on to the Orbitrap. The Orbitrap obtains optimal performance when only a small number of ions are required for detection or mass analysis. Information regarding the ion is then collected and amplified.
Figure 1.9 UPLC-LTQ/Orbitrap-MS system

On the left is the UPLC with solvents in glass jars on top of the machine. On the right is the LTQ/Orbitrap-MS.

Analysis of biological samples by UPLC coupled to an LTQ-Orbitrap mass spectrometry system, which is operated in either a positive or negative electrospray ionisation mode have been undertaken (Dunn et al., 2008; Kenny et al., 2008, Zelena et al., 2008, Wilson et al., 2007). Serum samples in these studies were analysed in separate positive and negative ion modes, where samples were reconstituted in 100µl or 200µl of water for electrospray ionisation mode – (ESI-) or electrospray ionisation mode + (ESI+) analysis respectively (Dunn et al., 2008; Kenny et al., 2008, Zelena et al., 2008, Wilson et al., 2007).

Ultra Performance Liquid Chromatography (UPLC) uses a column which has been packed with extremely small particles (less than 2.0 μm). This allows the column to
withstand significant pressures in the region of 15,000 psi. The combination of small stationary phase particles, and the ability to cope with significant pressures, means that complex biological samples, can be analysed at much higher sensitivity, improved resolution, and with time required for separation quickened (Wilson et al., 2006) (see Figure 1.10). The UPLC system has been used in a number of metabolomics studies involving biological fluid (Williams et al., 2006, Dunn et al., 2006, Guy et al., 2008, Zelena et al., 2009). Because UPLC-LTQ/Orbitrap offers the opportunity to measure metabolites with high levels of chromatographic separation and high levels of mass accuracy, this analytical platform is appropriate for metabolomic analyses.

**Other hyphenated separation technique coupled to a mass spectrometer**

Capillary electrophoresis mass spectrometry combines the use of a capillary electrophoresis separation technique, whereby ions which are in a liquid phase are separated on the basis of their charge and cross-sectional size, as they pass through a capillary. The capillary column is an open tubed column unlike packed columns, which are commonly used in GC and LC chromatography. The column or capillary is lined with an aqueous buffer solution. Samples as they move through the column are separated Sample constituents are separated on the basis of differences in the ionic charges and the interaction of the sample constituents with the aqueous buffer solution, relative to the size and shapes of the metabolites. In CE, positive and negative ions both pass along the column. CE allows the detection of hydrophilic and non-volatile compounds especially since such metabolites do not or are not amenable to and are detected by to separation using other chromatographic approaches. CE-MS has been used in a number of metabolomic studies, including
biomarkers for Parkinson’s disease (Soga et al., 2003, Bogdonov et al., 2007). CE-MS is comparative terms is similar to GC-MS in terms of resolution. However, there are issues pertaining to method reproducibility and like GC and LC the analysis time per sample is in the 30 minute range.

1.9 INFLUENCE OF ANALYTICAL VARIATION IN METABOLOMICS STUDIES

In any metabolomic study there is a need to ensure that samples collected for analysis are undertaken in a standardised manner, and the subsequent storage, and preparation before sample analysis is also undertaken in standardised manner, so as to reduce the inclusion and influence of external variables which could invalidate the study findings (Dunn et al., 2011).

GC-MS and LC-MS technologies have advantages and disadvantages. Whilst GC-MS based analyses are considered to be better suited to biological samples, to the type of separation, resulting in high resolution, and reproducibility. The limitation with GC-MS analysis is the complex process of sample preparation. Derivatization is an important step in the preparation of a sample, as this is needed to increase the volatility of non-volatile compounds, by adding because of their ability to produce high resolution and reproducibility.

In metabolomics experiments, metabolites have to suitably extracted from any biological sample in order to analyse the metabolites. Usually, such biological samples are obtained as saliva, urine, CSF, tissue or whole blood. The preparation of samples can be extensive depending on the metabolites of interest. Serum like
plasma contains proteins, electrolytes and moreover is home to a large number of metabolites, and is a complex aqueous solution with over 50% water content. In metabolomics experiments using serum samples, traces of water can interfere during sample analysis (Smith, 1988). This interference can be attributed to the metabolic changes that occur during the blood clotting processes, and can be reduced by collecting blood samples in a systemised process directly into a blood collection tube which contains serum clot activators. For repeatability and reproducibility purposes, clear procedures for the collection of blood samples, and subsequent handling procedures are important considerations (Tuck et al., 2009).

GC-MS and UPLC-MS are considered to be complimentary metabolomics techniques. These two analytical platforms for a diverse set of metabolites to be detected. In summary GC-MS allows metabolites such as carbohydrates, fatty acids, sugars, amino and organic acids to be detected. Whilst UPLC-MS allows the detection of high molecular weight metabolites to be detected. These include but may not be limited to, glycerolipids, phospholipids, fatty acids, bile acids and sterols. Whilst some metabolites maybe detected across both analytical platforms, the majority are not. This is because certain metabolites require specific sample preparation before they can be detected using chromatographic separation and MS analysis.

In metabolomics experiments, samples are analysed in both ESI+ and ESI- modes. This is because the ionisation modes allows those metabolites which would usually be detected in a single-ion mode to be detected (Dunn et al., 2011).
1.9.1 Controlling the metabolism in samples

When a biological sample is collected the metabolism in continues, because of enzymatic degradation. Therefore the metabolite numbers and concentrations remain in a dynamic state. Before the metabolome of a sample can be analysed the enzymatic activity and thus metabolism are stopped or quenched. Metabolism is quenched by freezing the sample as quickly as possible. Rapid freezing achieved through the use of liquid nitrogen, results in reduction of metabolite concentrations, and causes an irreversible binding of metabolites to bind to cell walls. The addition of acids to quench metabolic activity does not work with all metabolites. Therefore storing samples at -80°C, is effective at stopping reactions (Dunn et al., 2005). Once the metabolic activity is stopped, the sample metabolites undergo an extraction process.

1.9.2 Sample Extraction

Extraction of metabolites prior to analysis is necessary, if gas chromatography (GC) or liquid chromatography (LC) based separation is required. Extraction of metabolites can be based on their acidic, alkalinic, or ethanolic properties (Goodacre, 2007).

1.9.3 Deproteination

The sample is firstly spiked with an internal standard (Succininc d₄ acid). Following which the sample is deproteinated (removal of proteins) using acetonitrile, or methanol, and then centrifuged. The resultant supernatant is dried using a cooling trap. If the sample is to be separated chromatographically, it then undergoes a
further step termed derivatization. The volatility of some metabolites can be naturally low or non existent, in which case the metabolites have to undergo a derivatization process, whereby reagents are added to the sample to increase metabolite volatility (Villas-Boas et al., 2005).

1.10 METABOLOMICS EXPERIMENT WORK FLOW

There are a number of stages that are central to any metabolomics experiment (see Figure 1.10). These stages are the experimental design, sampling, sample preparation, sample analysis, data pre-processing and data processing (Dunn et al., 2005, Goodacre, 2004). These stages therefore allow for data to be collected, and for that data to be transformed to knowledge, and therefore allow hypotheses to be generated, for future testing.
Figure 1.10 An overview of a metabolomics experimental design

The design captures the sample collection (including metadata associated with the sample), analysis of the sample using an analytical platform, e.g. chromatographic separation, and mass detection (XC-MS), through to data analysis, through to the identification of detected biomarkers, and their role in known metabolic pathways.

The nature of metabolites is diverse both chemically and physically, and metabolites vary in size. The metabolome encompasses, low to high molecular weights (<1500Da), and the mass to charge ratio (<3000m/z) of ionized metabolites (Hollywood et al., 2006). The metabolites can also be volatile and non-volatile, and can exist as electrolytes (Lahner et al., 2003). In addition the concentrations of the metabolites vary over nine orders of magnitude from pica-molar to milli-molar (pM-mM) (Dunn et al., 2005). The information relating to these metabolites is derived from both experimental observations and publications. It is worth stating that
between the first HMDB publication in 2007 and 2009 the number of metabolites
reported to be identified has been increased from near 3000 to 7000 (Wishart et al,
2007 & 2009). The objective of mapping the human metabolome is still work in
progress. The combination of attempts by research groups to also reconstruct the
human metabolic network which used information from such databases as the
HMDB is a significant stride in better understanding and application of metabolites
(Duarte et al., 2007, and Ma et al., 2007 & Wishart et al, 2007 & 2009).

A number of resources are available online, which allow the synthesis of information
from the HMDB and the reconstruction of metabolic networks, which are open to the
scientific community. The Kyoto Encyclopaedia of Genes and Genomes (KEGG)
database (http://www.genome.jp/kegg), contains large amounts of data relating to
metabolic pathways, and the associated metabolites (Kanehisa et al; 2008). The
KEGG database allows the scientific community to work with complex metabolic
pathways and the enzymatic reactions (www.genome.jp/kegg/).
Figure 1.11 The sequential steps involved in processing raw metabolomics data.

Data derived following the separation and detection of metabolites, are required before any meaningful data can be analysed.

The data generated in metabolomics experiments undergoes a number of steps before any statistical analysis can be undertaken.

Despite the different analytical strategies that are employed in metabolomic experiments (Figure 1.11). The limitations of the techniques currently do not enable the capture of the entire metabolome present in a biological sample.

1.11 MACHINE LEARNING AND OPTIMIZATION OF GC-TOF-MS

Because of the large number of possible metabolites in a biological sample, it has been shown that combining machine learning to analyse serum samples using the GC-ToF-MS, can be used to optimize the parameter setting for the GC-ToF-MS (O'Hagan et al., 2005). Combining machine learning to GC-MS based separation,
can increase the sensitivity for separation and detection of metabolites (O'Hagan et al., 2007).

1.11.1 Machine learning

Machine learning is a part of artificial intelligence. It is the process by which a computer increases its performance and ability for a given task in an automated manner. This improvement or optimisation in performance is achieved through learning from previous attempts, in an iterative manner (Kell, 2006b).

The Robot Scientist (King et al., 2004), is an example of using machine learning in a heuristic manner to improve how a genomics experiment can be executed. The computer holds background biological information (regarding a specific biochemical process). This information is used to design and optimize an experiment, the experiment is executed, the results are evaluated from the experiment, and changes made to the conditions. The experiments are executed in an iterative manner, or a closed-loop with no human intervention, until an optimised final solution is arrived at (King et al., 2004).

![Figure 1.12 Closed-loop optimization](image)

**Figure 1.12 Closed-loop optimization**
Closed-loop optimisation using machine learning to generate optimized settings for GC-ToF-MS.
O’Hagan et al., 2005, optimised the use of GC-TOF-MS, to analyse in a non-biased manner, metabolite peaks from serum samples. This optimization was achieved through an entirely automated strategy, using a Robot chromatographer system, which ran the analysis of samples in a continuous loop, until the optimal parameter settings were determined. In an iterative manner, the instrument settings were adjusted, to aid peak detection, improve signal/noise ratio, and reduce run time. Genetic programming is central to this approach, as this is the way in which the selection of the optimal conditions for improvement is achieved. The optimized conditions resulted in reduced run time without compromising peak detection and signal/noise ratio, and a 3-fold increase in peaks identified (951 metabolite peaks) (O’Hagan et al., 2005).

1.12 METABOLOMIC PROFILES

1.12.1 Spectral analysis

Ion spectra is analysed using computer tools to focus in on or classify a subset of the spectra. This is achieved by initially processing (pre-processing) of the raw peak data, using software such as LECO™. This allows a data-matrix to be constructed, of metabolite peaks for each sample analysed. The initial pre-processing of raw data, using such software allows for a data matrix to be constructed of each peak in each sample. The data matrix is a composition of metabolite peaks from each analysed sample, constructed using information such as response ratios, and the peak area generated by the internal standard (Hollywood et al., 2006). This results in a metabolic profile, combining unique retention time and mass spectrum data.
1.12.2 Peak alignment and deconvolution

In order to identify the metabolites, alignment of chromatograms is a necessary step, due to machine drift. The phenomena of machine drift, is due to the analytical platform that is being used to analyse the same samples, producing variations in the retention times due to the instrumental parameters varying through the analysis process. This is problematical, especially when the analysis may take a week or longer. It is for this reason that an internal standard such as succinct d\textsuperscript{4} acid is spiked into the samples. Deconvolution software packages can be used to resolve metabolites whose chromatograms may not be completely resolved, and determine the s/n ratio (O'Hagan et al., 2005). Deconvolution is also employed to distinguish co-eluting compounds that have different mass spectra but the same chromatograms.

The mass spectra that is produced, is used to identify known compounds from mass spectra libraries. With large number of peaks being produced, there occurs an overlapping of metabolite peaks.

Having identified metabolites of a sample, the metadata of the sample donor (sex, age, disease status, medication etc.), is combined, to the metabolic data, to determine which metabolite(s) distinguish disease and control samples. Analysis of the combined data analysis can be a supervised or an unsupervised learning method (see Figure 1.14). The ultimate aim is to find patterns in the data, which shed light on the biology of the cell, and thus generate hypotheses.
Multivariate analysis
Machine Learning

unsupervised methods
The input information (metabolite/peak data), and are clustered into groups – revealing the overall structure of the data.

Principal components analysis (PCA)
- Reduces data dimensionality
- Investigate clustering
- Visualise the data
- PCA is followed by supervised methods

Hierarchical cluster analysis (HCA)
- Forms clusters of data set
- Represented as tree or dendrogram

supervised methods
The input information (metabolite/peak data), is paired to defined outputs e.g. disease or control (training data). A model is built to classify the data.

- Discriminant analysis – cluster analysis method
- Partial least-squares (PLS) – increases separation between groups – linear regression method
- PLS-DA – linear regression method
- Orthogonal projection to latent squares (O-PLS) pattern recognition technique which increases separation both between and within groups

Evolutionary computation
- Genetic algorithms/programming - based on Darwinian selection, to produce an optimized rule, code or tree linking input to output

significant biomarkers

Figure 1.13 Multivariate analysis methods for metabolomics data sets

The types of machine learning strategies employed, to identify biomarkers, involves unsupervised and supervised methods.

1.13 STATISTICAL ANALYSIS OF DATA

Once the peaks have been identified, and separated as metabolites and unique peaks, the next step is statistical analysis. This will be achieved univariate analysis to see if the peaks are correlating with time, by looking for peaks across runs that are consistent, to account for machine instability over time (machine drift).

Mathematical validation of biological data is not necessarily a biological validation. The biological data derived is based on analytical stability. The univariate analysis of the data (T-test, ANOVA), is usually followed by statistical analysis which is used to untangle the large number of variables (metabolite peaks) to manageable variables. The data can be further discriminated to extract significant differences.
due to biological variation. These variations can be used for predicting the differences, followed by biomarker validation.

There is a fundamental difference between the statistical approaches used in metabolomics experiments and statistics used in other biological research. In statistics there are two approaches that are used when dealing with data. Data falls into either a parametric or non-parametric category. Parametric data assumes that the data is derived from a normal distribution, whilst non-parametric data does not have a normal distribution (Field, 2005). Machine learning, assumes that data is not normally distributed, therefore the non-parametric data is used to find a hypothesis that best fits the data (i.e. build predictive models) (Breiman, 2001; Brown et al., 2005).

Obtaining knowledge from the large data produced from a metabolomics experiment requires the use of mathematical modelling techniques. As they are able to pick out any patterns or trends that otherwise would not be discerned.

1.13.1 Univariate analyses

Univariate analysis in metabolomics is important, because it has to overcome two fundamental problems, type I and II errors (Broadhurst and Kell, 2006). These problems are due to the fact that when comparing a large number of samples with hundreds of metabolites, a significance threshold of \( p < 0.05 \), is not a sufficient level to detect significance. There is a high probability that metabolites selected as being significant at \( p < 0.05 \) are not truly significant. Therefore to reduce the possibility of discovering false discoveries, a Bonferroni correction can be used, which is
considered too conservative, because it not only reduces the number of false positives, it also reduces the number of true discoveries (Broadhurst and Kell, 2006), or the alternative and less stringent False Discovery Rate (FDR) can be used (Benjamini and Hochberg 1995). The FDR therefore is the expected proportion of the null hypotheses amongst the discoveries. An FDR adjusted $p$-value (also termed the $q$-value) of 0.05 implies that 5% of significant tests will result in false positives.

### 1.13.3.1 False Discovery Rate

The classical multiple comparison procedures aim at controlling the probability of committing even a single type-I error within the tested family of hypotheses. The main problem with such classical procedures, which hinder their application in applied research, is that they tend to have substantially less power than uncorrected procedures.

The FDR is a new approach to multiple hypotheses testing. The FDR is the expected proportion of true null hypotheses rejected out of the total number of null hypotheses rejected. Multiple comparison procedures controlling the FDR are more powerful and are suited for use in large multiple comparison problems in which existing procedures lack power.

The FDR is very different from a $p$-value, and as such a much higher FDR can be tolerated than with a $p$-value. In the example above a set of 100 predictions of which 70 are correct might be very useful, especially if there are thousands of genes on the array most of which are not differentially expressed. In contrast $p$-value of .3 is
generally unacceptable in any circumstance. Meanwhile an FDR of as high as .5 or even higher might be quite meaningful.

Metabolites that are significant can be further analysed using the receiver-operator characteristic (ROC) curve. This is a plot of the degree of sensitivity (predicting a disease) versus specificity (predicting non-disease), and is used to identify cut-off points for metabolites (Hastie et al., 2001); and therefore to identify the most discriminatory metabolites, based on a ROC score.

1.13.2 Multivariate analyses

Unsupervised learning

In unsupervised learning, the aim is to look at the overall pattern of the data. This approach is therefore equivalent to clustering methods e.g. principle component analysis (PCA) (Brown et al., 2005). The limitations with unsupervised learning methods are that algorithms require a predetermined number of clusters (eventual outputs), prior to producing an output. The outputs can be control and patient data sets, though the partitioning between the clusters can be ambiguous with no clear demarcation (Brown et al., 2000).

PCA, is applied when there is multivariate data, it allows for patterns to be picked out and reduces the overall dimensionality of the variables. PCA allows for the data to be explored but does not allow any explanation to be concluded from the data (Trygg et al., 2007). PCA plots are of two types. The first a scores plot, reveals how
the data is spread, whilst the loadings plot reveals the variability of the data. Thus PCA is descriptive of the data variability.

PCA therefore by compressing or reducing the complexity of a data set can be used to identify hidden structures within that given data set. The hidden structures are amplified and can be visualised. The first PC usually accounts for the majority of the variability on a data set. All subsequent PC describe the remaining amount of variability.

**Supervised machine learning**

In supervised learning information on a number of paired input and output data is used. A number of statistical methods are used to model predictions, by reducing the multiple dimensions of the data into one or two dimensions (Broadhurst and Kell, 2006). The statistical strategies include, partial least square (PLS) which is a linear regression method; discriminant function analysis (DFA), and partial least square discriminant analysis (PLS-DA), which is also a linear regression method (Broadhurst and Kell, 2006). These techniques use a subset of the overall data (training data), to model the outcomes. The remaining data set is used to validate the predictive strength of the built model. The linear regression method of both partial least squares (PLS) and discriminant function analysis (DFA) algorithms have distinct advantages in their use, primarily because they are excellent modelling techniques, able to both identify and classify data (Jarvis and Goodacre, 2005), but are limited in providing information on the key contributing metabolites.
Discriminant function analysis and Canonical variate analysis

Canonical variate analysis (CVA) analysis is classed as a supervised multivariate method. In CVA, information about class membership is provided before the analysis is undertaken. The object of this analysis is to minimize the within class variation, and to maximise the between class variation or differences. There is a key issue with CV, whereby the number of variables considered to be crucial in determining class membership, have to be fewer than the number of samples or class members. The variables, which in this thesis would be detected metabolite spectra or peaks, are termed canonical variates, and their associated values are ascribed a weight, which determines how good the feature(s) are at determining accurate class membership. CVA is an appropriate statistical analysis when comparing two or more groups. Discriminant function analysis (DFA) can also be used when there are two groups to be separated using a set of discriminating variables. DFA classifies entities into groups using a classification criterion which maximises the correct classification and reveals differences amongst the two groups being considered in the analysis.
1.14 THE APPLICATION OF METABOLOMICS IN DISEASE

The application of metabolomics in scientific research of diseases can be used primarily to identify biomarkers of disease, and in doing so allow the accurate prediction of disease diagnosis, the ability to sub-classify diseases, to elucidate disease aetiology and mechanisms. Secondly, pharmacometabonomics, which is used to measure the effect of a drug on the metabolome of an individual, is an important area of research, which can help better understand the relative efficacy of treatments across patients of different sex, ages, and race (Kaddurah-Daouk et al., 2007). When both of these approaches can be synthesised, the knowledge of which could be derived through their metabolome, may lead to the potential of individualised drug therapy, whereby drugs could be tailored to patients (van der Greef et al., 2006).

1.14.1 Metabolomics application in disease

A number of analytical techniques have been used in metabolomics research. The most popular methods used in human diseases have been GC-MS, LC-MS, and NMR.

Analysis of the metabolome has been used in cardiovascular disease, in order to diagnose heart disease non-invasively using NMR (Brindle et al., 2002), Type 2 diabetes (Wang et al., 2005), and obesity (Hochberg, 2006). Denkert et al 2006 were able to distinguish ovarian cancer tissue and ovarian borderline tumour samples using GC-ToF-MS (Denkert et al., 2006). Holmes et al 2006 applied metabolomic
analysis profiling of cerebrospinal fluid (CSF) using NMR to identify change and search for biomarkers in schizophrenic patients. Their results indicated an abnormal biochemical profile (metabolic signature) in schizophrenic patients when compared to healthy controls. Holmes et al 2006 were able to show that the abnormal metabolic signature of drug naïve patients reverted to normal in half the participating patients (28 out of 54), when treated with antipsychotic medication (Holmes et al., 2006a). In another study, the plasma metabolome of schizophrenic twins was analysed. Differences in the metabolic profile, (lipoproteins) were identified in affected and unaffected twins (Tsang et al., 2006). Kaddurah-Daouk et al., 2007, attempted to measure the change in the metabolome before and after antipsychotic treatment in patients with schizophrenia, but no clear metabolic signature was determined (Kaddurah-Daouk et al., 2007).

Sabatine et al., 2005 were able to identify novel plasma biomarkers of myocardial ischemia in 18 patients with inducible ischemia (case), and 18 without (controls), following exercise. Samples were analysed using HPLC and mass spectrometry analysis. Nearly 480 ions were detected from the samples, of which 173 were known analytes. In this study, the researchers adopted a matched statistical analysis approach. Where the metabolites were normally distributed, they were analyzed using parametric tests, otherwise non-parametric tests were used. Metabolite levels were correlated with the extent of ischemic myocardium. Two ranked lists of metabolites, one for case and one for control were generated. Type I errors were limited by adjusting the probability value for the multiple comparisons. Receiver-operator characteristic (ROC) curve plot of the degree of sensitivity (predicting a disease) versus specificity (predicting non-disease), was used in this study to identify
cut-off points for metabolites; to identify the most discriminatory metabolites, based on a ROC score. A six-fold cross-validation using a training subset of the data (3 case and 3 control), followed by validation and testing of the outcomes in the remainder of the subsets, confirmed that the score used to discriminate case and control metabolites had excellent discriminatory ability. Amongst the metabolites found which were able to distinguish case and control samples was citric acid, as well as members of the citric acid pathway (Sabatine et al., 2005). This studied reveals the ability of univariate analysis to be sufficient in the identification of biomarkers.

Dunn et al., 2007 identified novel metabolite biomarkers of heart failure, using GC-TOF-MS. Serum samples from over 50 case to control matched patients revealed 272 metabolite peaks of which 38 were significantly different between the two sets of samples. Univariate analysis was employed, and type I error possibilities were reduced by modifying the p-value to $5 \times 10^{-5}$. Pseudouridine and 2-oxoglutarate were amongst the metabolites identified as robust individual biomarkers which could distinguish case and control samples. In this study univariate analysis was sufficient to identify from over 500 metabolite peaks, a number of robust biomarkers of heart failure (Dunn et al., 2007).

Kind et al., 2007, in a pilot study analysed from six patients, the urine metabolome suffering from kidney cancer, and six controls. Amongst the techniques used to analyse the metabolome was GC-ToF-MS (Kind et al., 2007). Supervised learning methods were used to classify the samples as case and control. PLS as well as PCA
were used to visualise the spread between control and healthy samples. The researchers also used PCA to validate the class separation.

Underwood et al 2006, analysed serum metabolites of Huntington disease patients as well as transgenic mice. Using GC-ToF-MS, 30 Huntington disease patients and 20 control subjects, were sampled, with detailed metadata recorded. Over 1300 metabolic peaks were recorded, of which approximately 600 could be assigned a molecular structure. In this study PCA was used to identify any major outliers, which were subsequently excluded from analysis. PLS-DA models were built using all but 17 of the 50 samples. Of the total of 50 samples, 17 samples (one third) were left out from any analysis as a hold out set, and used as a validation set. The models built were able to classify Huntington disease patients from controls, though this was not significant. The authors attributed the lack of significance to the heterogeneity of human metabolome. For in the transgenic mice model, there was significant discrimination between the transgenic and wild type mice. The key discriminatory metabolites between disease and healthy control, were suggestive of catabolic state preceding deterioration of symptoms in patients. The study also showed that within the Huntington disease patients, the biomarkers could distinguish between asymptomatic and symptomatic cases. In addition a the same metabolite (glycerol) was found between human Huntington disease patients and the transgenic mouse models of the disease (Underwood et al., 2006). A repeat of the experiment did not support the initial findings. Two experiments by Bogdanov et al., 2008, attempted to detect and identify metabolite biomarkers of Parkinson’s disease. Bogdanov and colleagues were also looking out for oxidative specific biomarkers e.g. 8-OHdG, a biomarker related to DNA damage in oxidative stress, and uric acid and glutathione.
Uric acid concentrations were lower in Parkinson’s disease patients, whilst 8-OHdG and glutathione levels were elevated. These findings were in line with previous studies, however, lacked sensitivity levels. In the CE-MS approach 1860 metabolites were detected, and using metabolites could differentiate disease and non-disease populations. In a follow-up experiment Johansen et al., 2009, using plasma metabolites were able to detect with high levels of specificity Parkinson’s disease from other Parkinson’s disease types, e.g. idiopathic form was distinguished from LRRK2 mutation form, using a panel of 12 metabolites. Michell and colleagues analysed 23 serum and urine samples from all female Parkinson’s disease patients age and sex matched with controls, using GC-MS to analyse the samples, did not find any biomarkers using leaving the researchers to conclude that any potential change in the 900 urine or 700 serum metabolite peaks in the metabolome associated with Parkinson’s disease is likely to be a subtle presence in the serum and urine samples (Michell et al., 2008).

Kaddurah-Daouk et al, 2010, have recently reported that there are in the CSF 33 metabolites following post mortem tissue analysis (n= 15), which can distinguish disease and non-disease samples.
1.15 SECTION SUMMARY - METABOLOMICS

In the past decade the rapid development of “omics” techniques have enabled scientists to target a wider area of any given biological sample when searching for biomarkers of disease. Whilst the human metabolome is chemically and physically heterogeneous, quantitatively at least, it is smaller than it’s “omics” counterparts, the genome, the transcriptome and the proteome. The key advantages of metabolomics are the potential to identify metabolites in an unbiased manner of a disease across a large sample set. Advances in the methods employed to systematically search the metabolome of any given biological sample have been used in a number of diseases, resulting in the identification of both known and unknown biomarkers (section 1.7.2).

There are generally three distinct steps in any metabolomics experiment: sample preparation, sample analysis and statistical analysis. For each of these steps there are a number of issues that need to be carefully considered to ensure that metabolomics experiment can be classed as both robust and reliable (sections 1.8-1.13).

During each of the stages, which can be broken down further to include sample collection, storage, preparation of a sample, followed by complex sample separation and analysis stage, there is ample room for the introduction of a number of extraneous variables. A significant degree of care is required when handling the generated metabolite data set(s) during the statistical analysis stage. This is why the literature reported above emphasise the need for careful consideration of the experimental design to minimise all known sources of analytical variation. A key issue with a number of publications which have identified potential disease
biomarkers has been the issue of over-fitting data, and the possibility of accepting metabolites as genuine biomarkers whilst rejecting actual biomarkers of a disease.

Metabolomics data can be a powerful tool, especially when a highly sensitive separation technique is coupled to sensitive mass analyser, thus allowing the parallel measurement of thousands of metabolites present within a biological sample. However, there are obvious limitations in the application of metabolomics and the detection of disease biomarkers. The primary limitation is that there is no single analytical platform that can help identify the entire human metabolome in any given biological sample. In order to overcome this limitation there is the possibility of using multiple analytical platforms to increase the detection coverage of a sample metabolome. There are also key limitations with regards to the ability of researchers to reproduce reliable data across different laboratories.

The second part of the literature review in this thesis has shown that metabolomics experimental techniques can be used to analyse potentially hundreds of biological samples in an automated manner, helping to sensitively detect thousands of metabolites in any given biological sample (Section 1.8). However, care has to be taken when measuring the metabolome so as to minimise the effect of biological, sample preparation and analytical variation. This is best overcome by using a standardised approach from sample collection through to data analysis. The identification of potential biomarkers of neurodegenerative disorders indicates the viability of using metabolomics to look at other complex diseases including neurodegenerative disorders (Section 1.14). As the reported literature has shown, the application of metabolomics in the discovery of biomarkers is a complex, multi-
staged process that can potentially reveal novel information of a disease such as Alzheimer’s disease (Section 1.14.1).

1.16 CONCLUSIONS FROM THE LITERATURE REVIEW

Summary of the literature review

There is currently an unmet need in the timely diagnosis of Alzheimer’s disease. Whilst the clinical profile of the disease and the associated symptoms are well established, there remains a significant need to identify a biomarker (s) which could significantly reduced the overall time taken to make a definite diagnosis of Alzheimer’s disease (Section 1.2 and 1.5).

Currently, the clinical diagnosis of the disease is achieved by using the NICDS-ADRDA criteria, which is the accepted gold standard (Section 1.2.1). However, the time it takes to diagnose a patient is usually over months rather than minutes or days. To date clinicians have attempted to reduce the affect of Alzheimer’s disease on cognitive decline using memory enhancers, but the drugs only work for a short duration relative to the course of the illness and are recommended for only patients with mild to moderate dementia (Section 1.2.3).

Research has indicated that there exists a potentially significant pre-symptomatic stage of Alzheimer’s disease (Section 1.1.2). It is therefore important to clinically identify Alzheimer’s disease in order to intervene with pharmacotherapy as early as possible and also to identify new drugs (Section 1.2.3 and 1.5). The current crop of Alzheimer’s disease biomarkers, whilst promising, have not resulted in a single or group of biomarkers that could be used to detect and predict a diagnosis of the
disease in a clinical setting, with significant focus of enquiry related to the search for such markers in the CSF (Section 1.5).

This literature review has shown that the three key areas of research in Alzheimer’s disease have yielded important data in understanding of the disease, the pathological basis, the clinical profiling and the search for biomarkers. This review has also shown that the need to identify robust biomarker of Alzheimer’s disease has been primarily focused on identifying known or proposed candidates, employing the traditional hypothesis testing scientific approach and usually using relatively small sample sets.

In recent years there have been advances in “omics” technologies and their application in identifying disease biomarkers (sections 1.5.6 and 1.7). These have resulted in the targeted and untargeted profiling of genes, proteins and metabolites to reveal potential known and unknown biomarkers. With the recent advent of technologies that can analyse large numbers of samples in a robust and repeatable manner the scope to interrogate potential biomarkers of Alzheimer’s disease has opened new avenues. The literature reveals that the coupling of high-throughput technologies combined to powerful data capturing computers has enabled researchers to interrogate both biological fluids and tissues in aiding discovery of known and unknown biomarkers of disease, whether at the genome, protein and metabolite level.

Metabolomics is one such area of research that has been used to identify disease biomarkers. The potential of employing metabolomics to identify biomarkers of a
variety of disease has been successfully employed (section 1.14). Similarly, metabolomics analytical platforms have been used in the search for neurodegenerative disease biomarkers (section 1.14). The literature cited shows that both known and unknown biomarkers have been identified using a number of chromatographic separation techniques coupled to mass analysers (section 1.14.1). The literature cited in this review also indicates that whilst there is a potential in the use of metabolomics technologies to identify disease biomarkers, a degree of caution is recommended when interpreting the vast quantities of data generated from any biological sample across all stages 1.14). These include, sample storage, preparation, machine drift associated with an analytical platform and statistical analysis (section 1.8-1.13).

The literature review has shown that employing a hypothesis testing methodology in biomarker research has possibly omitted other potential biomarkers from consideration (section 1.5). The application of metabolomics in scientific research of diseases can be used primarily to identify biomarkers of disease, and in doing so potentially allow the accurate prediction of disease diagnosis, the ability to sub-classify diseases, to elucidate disease aetiology and mechanisms (section 1.6).

1.17 APPLYING THE RESEARCH
There are many metabolites within the human body, which can be analysed using metabolomic techniques, to generate information on potential metabolite biomarkers in a non-biased manner and using a biological fluid which requires minimal intervention. Therefore, metabolomics has the potential to be employed to identify potential biomarkers which could be used reliably to diagnose Alzheimer’s disease and to monitor its progression.
This literature review has shown that there is sufficient scientific scope to employ a range of metabolomics technologies in a scientifically robust and repeatable manner to search for biomarkers of Alzheimer’s disease which could be used to for diagnostic purposes.

The experimental design when assessing the ability of metabolomics to detect Alzheimer’s disease biomarkers, would have to minimise the biological and analytical variation. To minimise the biological variation across disease and non-disease groups, the two groups would have to be compared and be matched as close as is feasible to age, sex and the absence/presence of significant cognitive deficits. This biological variation could be reduced if patients were followed up at regular intervals, and consistent metadata collected alongside the biofluid of interest. To minimise analytical variation, the collection of biofluids would have to take place in a standardised operating procedure. The subsequent sample handling, including the types of collection tubes, storage of samples, temperature, would have to be standardised. The preparation of samples prior to analysis depending on the type of metabolomic analysis technique likely to be undertaken, would also have to follow a standardised method. The potential of machine related “drift” would also have to be minimised where large number of samples are analysed. And finally robust statistical analysis techniques would be required to interrogate the metabolome data.
2 Study Rationale

The need to identify and validate a quick, non-invasive and above all safe diagnostic test for Alzheimer’s disease is a clinical need which is as yet unmet. Any biomarker for Alzheimer’s disease must be sufficiently reliable to diagnose and monitor disease progression. There are many metabolites within the human body, which can analysed using metabolomic techniques, and to generate information on potential metabolite biomarkers.

2.1 AIMS AND OBJECTIVES

The broad aim of this thesis was to examine the range of metabolomics technologies available and use repeatable and reproducible methods to generate metabolomics data to identify potential disease biomarkers for (i) diagnostic purposes and to measure (ii) disease progression, following the analysis of serum samples in a non-biased manner from Alzheimer’s disease patients (collected at two time-points) baseline and twelve months) and healthy controls (collected at baseline only).

2.2 SPECIFIC AIMS AND OBJECTIVES

(i) Alzheimer’s disease biomarker - serum samples from Alzheimer’s disease patients were compared to healthy controls (closely matched for age, sex and BMI), were analysed using metabolomic analysis.

(ii) Alzheimer’s disease progression - serum samples from Alzheimer’s disease patients collected at two points over a twelve month period, were analysed using metabolomic analysis.
(iii) Can metabolomics techniques be used to identify robust serum based biomarkers for the diagnosis of Alzheimer’s disease and for the measurement of disease progression.

In all the experiments, the same serum samples were used.

To achieve aims (i) – (iii), metabolomic analytical techniques, metabolic fingerprinting and metabolic targeting were employed, to detect any metabolites (whole, fragments or novel) which are related to disease diagnosis and disease progression.

(i) **Metabolic fingerprinting - FT-IR**

FT-IR was used to undertake metabolic fingerprinting of all serum samples, with the specific aim to analyse the metabolite groups a comprehensive manner, subject to the limitations of the techniques used.

(ii) **Metabolic targeting - GC-TOF-MS**

GC-TOF-MS was used to detect and measure metabolites within the serum samples.

(iii) **Metabolic targeting (UPLC-LTQ/Orbitrap)**

The UPLC separated serum samples were subjected to two different set of analyses. Eluted anlaytes from the UPLC, were ionised in two separate experimental runs. In the first experiment, the eluted ions were ionised in the positive mode and anlaysed. In the second experiment, serum samples following UPLC separation were ionised in the negative mode and analysed. The analysis of samples following two different
ionisation modes, was to allow the comprehensive capture of metabolites in the serum samples:

Positive ionisation mode (+)

Negative ionisation mode (-)

All the serum samples were analysed in the same running order across all analytical platforms.
2.2.1 Thesis aims and objective summary

Figure 2.1 Summary of the thesis design
3 Methods

3.1 STUDY PARTICIPANTS

The participants recruited in this study were collected as part of the Human Serum Metabolome (HUSERMET) project (www.husermet.org.uk) and were used in the experiments described in this thesis. Briefly, HUSERMET is investigating the serum metabolome of ‘healthy’ subjects and those from diseased populations (Ovarian cancer and Alzheimer’s disease).

3.1.1 Recruitment of patients with Alzheimer’s disease

Following approval by the local research ethics committee, old age psychiatrists in Manchester were informed about the study. These old age psychiatrists were asked to identify and refer potential patients with a clinical diagnosis of Alzheimer’s disease or mixed dementia. A total of 60 patients with either Alzheimer’s disease or mixed dementia were recruited from the old age psychiatry service units at South Manchester University Hospital NHS Trust, and North Manchester General Hospital.

Classification and diagnosis of Alzheimer’s disease

The patients were diagnosed as Alzheimer’s disease or mixed dementia according to the classification criteria set out in the Diagnostic and Statistical Manual, DSM-IV (APA, 1995).
All patients with clinical diagnosis of Alzheimer’s disease or mixed dementia seen by Old Age Psychiatry (OAP) services were eligible to take part in the study. Carers of all the patients who were thought to be suitable to approach by their consultant were sent a ‘Letter of Invitation’ and ‘Participant Information Sheet’ on behalf of their Consultant old age psychiatrist. Carers were contacted approximately one week later by phone to find out their interest and to arrange a face to face interview, with the patient in the presence of the carer to obtain written informed consent from the patient, and from the carer.

**Inclusion criteria**

For patients to be included in the study, they had to fulfil the following inclusion criteria.

- The patients had to satisfy the DSM-IV criterion for Alzheimer’s disease (APA, 1995);
- The patients had to satisfy the NINCDS/ADRDA criterion for ‘probable’ or ‘possible’ Alzheimer’s disease (McKhann et al., 1984).
- The Patients also had to attain a score of at least 1 or above on the Clinical Dementia Rating Scale (CDR) (Hughes et al., 1982).

If a patient fulfilled any of the below criteria they were excluded from the study from the outset.

**Exclusion criteria**

- The patients who fulfilled the NINCDS/ADRDA criterion for vascular dementia (Roman et al., 1993).
• The patients who were diagnosed with dementia with Lewy bodied (DLB) (McKeith et al., 1996).

The majority of the interviews of the patients were primarily carried out by a research nurse (SCG) and the author (MZ) also contributed in the interviewing of patients.

### 3.1.2 Recruitment of Controls

The identification and recruitment of controls was based on matching for age and sex and geographical locality. This was achieved by contacting the GP of each of the Alzheimer’s disease patients recruited into the study, and asking them to identify a maximum of six individuals registered with their practice, who were matched by sex, age (+/- three years), and not have any memory problems. Further by being registered at the same GP practice, the patients and controls were likely to be from the same geographical locality of the Alzheimer’s disease patient(s). The GP identified controls were sent information about the study. All the controls, who were interested in participation, were recruited into the study. The identification and recruitment of GP controls was the primary method of recruitment. Because not all of the GP surgeries were able to assist the study, a second approach of recruiting controls was also adopted. This involved recruiting carers, friends and family members of patients. Therefore any of these individuals who expressed an interest in the study were also recruited as controls, subject to eligibility. All the control participants were sent a ‘Letter of Invitation’ and ‘Control Information Sheet’. Controls were contacted approximately one week later by phone to find out their interest and to arrange a face to face interview and obtain written informed consent.
For controls to be included in the study, they had to fulfil the inclusion criteria.

**Inclusion criteria**

1. Not known to suffer from Alzheimer’s disease or dementia.
2. Same sex
3. Age (+/- 3 yrs where possible of the patient).

If the controls fulfilled any of the exclusion criteria they were excluded from the study at the outset.

**Exclusion criteria**

1. Any control subject who scored less than 24 out of 30 on the Mini Mental State Examination (Folstein, 1975), as this would have been indicative of significant cognitive impairment.
2. Scored 1 or more on the CDR scale (Hughes et al., 1982, Morris199?)

### 3.1.3 Study assessments

Following the assessment of dementia of the Alzheimer’s disease patients, the study assessments fell in to four categories: (1) medical and physical history, (2) cognitive assessments; (3) non-cognitive assessments (assessment of behavioural and psychological symptoms of dementia); and (4) Activities of daily living.
Physical illness assessment, and other physical measurements

Using the schema as outlined by Burvill et al., 1990, physical illness of all study participants was quantified. Patients and controls were asked to assess their own physical health and to measure the number of body systems affected by acute and chronic illness. The body systems were, central nervous system (CNS), “cardiovascular”; “endocrine”; “genitourinary”, “haematological”, “hearing/eyesight”, “musculoskeletal”, and “other” (Burvill et al., 1990). All current medications, including prescribed and over-the-counter medications were noted, as were all herbal supplements. Physical measurements including body mass index (BMI) composites height and weight were recorded. Blood pressure was also directly measured using standard techniques. A cuff was tied on the left arm, to measure the systolic and diastolic components of blood pressure.

Assessment of dementia and the classification of Alzheimer’s disease

For the patients only, Alzheimer’s disease was diagnosed using the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer’s Disease and Related Disorders Association (NINCDS/ADRDA) (McKhann et al., 1984). Patient psychiatric notes which contained subjective case histories from carers, neuroimaging report(s) (e.g. CT and MRI) were reviewed by two old age psychiatrists (BB or SB and NP). Following which, patients were then categorised as either ‘probable’ Alzheimer’s disease or ‘possible’ Alzheimer’s disease.
Cognitive function

In the patient population the primary outcome measure used to assess the cognitive function of all the patients was the Alzheimer’s disease Assessment Scale – Cognitive subscale (ADAS-Cog) (Rosen et al., 1984). The ADAS-Cog contains 11 sections, where each section evaluates a number of cognitive domains. These cognitive domains: attention, memory, orientation, language ability and praxis are usually susceptible to impairment in patients with Alzheimer’s disease. The ADAS-Cog is also the standard measure, used to assess change in cognitive function of patients undergoing drug trials. The default maximum score on the ADAS-Cog is 70. Performance on the ADAS-Cog is based on the number of errors made on the test. Therefore, the higher the number of errors, the greater the cognitive impairment.

In the control population the primary outcome measure used to assess the cognitive function of an individual was the Cambridge Cognitive Examination (CAMCOG), which is the cognitive section of the Cambridge Mental Disorders of the Elderly Examination Revised version (CAMDEX-R) (Roth et al., 1986 and Hupert et al., 1996). The CAMCOG contains eight major subscales which assess cognitive function. These subscales assess the performance of the following cognitive domains: orientation, language, memory, attention, praxis, calculation, abstract thinking, and perception. The maximum total score which can be attained is 105. The CAMCOG also includes all 19 items of the MMSE (see below) (Folstein, et al., 1975). The CAMCOG also contains items that can be used to calculate executive function of a participant. However not all the items used in the assessment of executive function are used to total the CAMCOG score. The CAMCOG scores can
be used to separate between dementia and non-dementia individuals, with a cut-point of 79/80 out of a maximum total of 106. This equates to high levels of sensitivity and specificity (92% and 96% respectively) (Roth et al., 1986). For this study the revised version of the CAMCOG was used (CAMCOG-R), for which the maximum total score is 105. The CAMCOG-R contains additional items to assess executive function. However the scores of these items are not used to contribute to the CAMCOG-R score. Similarly the coin recognition score was also omitted (Roth, Huppert, Mountjo and Tym, 1999).

The Mini Mental State Examination (MMSE) (Folstein et al., 1975), was the second outcome measure of cognitive function, used in the assessment of both patients and controls. The MMSE is probably the most widely administered cognitive assessment. The MMSE has a maximum score of 30 points, assessing (the same cognitive domains as the ADAS-Cog), attention, memory, orientation, language and praxis. Lower final scores are indicative of impaired cognitive function. However, in comparison to the other cognitive assessment scales in particular the ADAS-Cog, or the CAMCOG-R, the MMSE is less sensitive in detecting changes in cognitive function. Further whilst the MMSE can be limited by both a floor and a ceiling effect, the ADAS-Cog and CAMCOG-R can minimise these effects.

The Clinical Dementia Rating (CDR) scale is an interview based global severity scale that is used to rate both cognitive and functional changes (Hughes et al., 1982). The global CDR score is derived from scoring the daily performance of the patient in six categories, three of which are cognitive and three functional. Morris (1993) revised the CDR and the scoring of the CDR. Termed the CDR-Sum of Boxes (SB),
memory was the primary category, whilst the other five are considered secondary. The global CDR score was computed using an, which calculated the CDR stage. An alternative method is to sum the score of the category boxes. The CDR-SB is useful in distinguishing mild Alzheimer’s disease patients from normal controls, and for staging severity in Alzheimer’s disease.

**Non-cognitive functions**

To detect and assess behavioural and psychological symptoms of dementia, the 12 item neuropsychiatric inventory with caregiver distress scale (NPI-CD) was used (Cummings et al., 1994; Kaufer et al., 1998; Cummings, 1997). The NPI measured 10 behavioural symptoms that may occur in dementia patients over the past month, their respective frequency and severity, as informed by a caregiver. These behavioural symptoms were ("Delusions", "Hallucinations", "Agitation", "Dysphoria", "Anxiety", "Apathy", "Irritability", "Euphoria", "Disinhibition" and "Aberrant motor behaviour"). Later a caregiver distress scale was also added to the NPI to assess the impact of the individual behavioural disturbances on caregivers (Kaufer et al., 1998). Two additional behavioural symptoms ("Night-time behaviours" and "Appetite and eating disorders") were added to the original behavioural domains (Cummings et al., 1994). For each symptom present (for which the caregiver informed), a frequency and severity score was assigned. For frequency a score of between 1 to 4 was ascribed to each presented symptom, so 1 = symptoms occasionally present (less than once a week); 2 = symptoms often present (about once per week); 3 = symptoms frequently present (several times a week but less than every day); 4 = symptoms very frequently present (every day). Severity was classified as mild, moderate and severe, (scored 1 to 3 respectively). The NPI score
for each symptom was derived by multiplying the frequency and severity scores. A score of >3 was used to indicate the presence of symptoms (Aalten et al., 2008 Pt 1).

The NPI-CD was also used in the control group to identify the presence of any recent behavioural disturbances.

**Depression**

In order to detect, and track any changes in depression in both patients and controls, the Geriatric Depression Scale (GDS) was used (Yesavage et al., 1982). It has been shown that depressive symptoms are common in dementia patients as compared to the healthy elderly (Burns et al., 1990), and that in depressed patients, the level of cognitive dysfunction (especially the impairment of memory) is equivalent to dementia patients with no depression (Abas et al., 1990). The GDS is a self-report 30-item scale that is used to assess depression in the elderly. Each of the questions has a “yes” or “no” answer, with the scoring dependent on the answer given, that is for each question a score of 0 or 1 was given. “Normal” was defined as a score of 0-9, “Mild depressive” as 10-19, and “Severe depressive” as 20-30.

The Cornell Scale for Depression in Dementia (Alexopoulos et al., 1998), which was specifically designed for the assessment of depression in patients with cognitive impairment, was also used. It is an interview based assessment scale that has 19-items, which fall in to five broad categories of mood-related signs, behavioural disturbance, physical signs, diurnal variation and suicidal ideation. The CSDD measures symptoms of depression and rates the symptoms on a three-point score of
“absent”, “mild or intermittent”, and “severe”. A score of 8 or more is suggestive of depression.

Activities of Daily Living

The day-to-day activities of both patients and controls was assessed and recorded. The Bristol Activities of Daily Living (BADLS) is designed to assess activities of daily living in patients with dementia (Bucks et al., 1996). The scale consists of 20 daily living activities, and the corresponding performance for each activity in the past two weeks. The activities broadly cover the making and consumption of food and drink, dressing and personal hygiene, orientation to time and surroundings; ability to communicate including use of the telephone, to shopping and finance. For each of the 19 activities a box pertaining to the quality of the activity has to be ticked ranging from a-e, therefore scored as a = 0, b = 1, c =2, d = 3, e = 0.

To assess the daily functioning in the control population, the Functional Activities Questionnaire (FAQ) (Pfeffer et al., 1992) was used. The FAQ is a scale that assesses 10 common daily functioning activities in the non-demented elderly by focusing on more complex cognitive and social functioning. The 10 areas of daily activities that were assessed included, writing cheques and payment of bills; maintaining financial records; use of an accountant shopping independently; ability to make hot drinks; preparing a meal; current affairs; concentration; remembering important dates and orientation to environment.
3.1.4 Blood samples

Blood samples from the majority of the study participants were collected by the research study nurse (SCG). Samples were collected in the morning and in the homes of participants. No request was made to the participants to observe an overnight fast, prior to blood collection. No additional requests were made to the participants to either restrict their diets or conform to a pre-defined diet leading up to the morning of the blood samples being taken. Samples were collected from the forearm cephalic vein. Before any of the assessment scales were used, a volume of 30ml of venous, non-fasted blood samples was collected and stored immediately at 4°C prior to in a storage container appropriate for transport back to the laboratory (Mediporer), containing cooling packs, prior to the commencement of the questionnaires. All blood samples were collected into a 9mL Z serum clot activator Vacuette blood collection tube. Blood samples were allowed to clot for at least 1hr at 4°C. The samples were subsequently spun in a centrifuge at ~2500g for 15 minutes at 4°C and only the serum removed from the tube and aliquots of 500 and 250 µl prepared. The serum was then stored frozen and upright at -80°C, until required for metabolomic analyses.

3.1.5 Reduction of biological variation

The level of inter-individual variability of metabolome wa considered to be high in most metabolomics studies. Variations in the metabolome which are attributable to the lifestyle, age, sex and body mass index, were recorded. In order to minimise the inter-individual differences, 120 serum samples (60 controls + 60 Alzheimer’s disease baseline), were paired such that for each control sample the closest in age and sex were matched with the Alzheimer’s disease baseline sample. In order to
minimise the intra-individual differences for the disease progression analysis, the 60 Alzheimer’s disease baseline samples were paired with 60 Alzheimer’s disease 12 month follow-up samples to detect possible disease progression biomarkers.

Detailed information was captured on each study participant, in particular the Alzheimer’s disease population at baseline and follow-up to determine the clinical profile of each patient and to measure their decline using the neuropsychiatric instruments (section 3.1.3). Information on the memory enhancers used by the 60 Alzheimer’s disease patients (memantine, donepezil, galantamine and rivastigmine) was also recorded at baseline and follow-up.

The NINCDS/ADRDA (McKhann et al., 1984) criteria were used to categorise the Alzheimer’s disease patients as either “probable” or “possible” Alzheimer’s disease patients (section 3.1.3).

3.1.6 Power calculations and statistical analysis

At the start of this study there were no prior human metabolomic variability estimates that were available. Therefore no statistical power or precision calculations were performed. Overall sample size was based on feasibility of recruitment over time, and the need to minimise machine variation over time. In addition a review of the literature revealed that the proposed number of samples as detailed in this thesis were the largest numbers used for any neurodegenerative disease. Underwood and colleagues had reported that there is a “trade-off” when considering sample size and how much data can be captured, and that studies with a sample size of 20-40 in each group would be sufficient in identifying the key clinical
biochemical markers (Underwood et al., 2006). Further, by matching the disease and non-disease groups, any difference in metabolome would it was assumed be likely linked to the presence of disease in the serum samples. Similarly, the longitudinal study used patients acting as their own controls (baseline v follow-up), thereby reducing overall environmental and biological variability. Any change in the metabolite profile of the longitudinal sample group would therefore likely be associated with disease change.

Analysis of the metabolite profiles was undertaken using univariate analysis. To reduce the inclusion of false discoveries when undertaking the multiple comparisons, all data, and the False Discovery Rate (FDR) method of 5 or 10% was employed.

**Block design**

The HUSERMET study has been organised to look at large serum samples over time. The analysis of samples had been organised as a series of so-called “blocked” experiments. The use of block analysis is common to epidemiological studies (Altman, 1991), also in analytical terms; a block is termed a batch that is the continuous analysis of samples without the need to undertake instrument maintenance. For the purposes of the research detailed in this thesis, a block comprises of 60 subject serum samples (20 Alzheimer’s disease patient (baseline), and associated 20 Alzheimer’s disease patient (12 month follow-up); and 20 age, sex-matched control. In addition there were a total of 30 QC serum samples (10 lead-in QC serum samples and a QC every fourth sample analysed by the UPLC was a QC serum sample. An age-matched case-control experimental design was used for this study. This was based on optimisation work undertaken by the HUSERMET
consortium (Begley et al., 2009 and Zelena et al., 2009). A total of 180 serum samples were collected from participants for this thesis. Samples were collected from the 60 Alzheimer’s disease patients at two time points, baseline and at 12 months follow-up (120 serum samples); serum samples were collected from 60 controls at one time-point only (a 60 serum samples). Quality control (QC) pooled human serum was purchased from Sigma-Aldrich (Gillingham, UK). The QC samples used in this thesis originated from identical batches of serum, and were aliquots in equal volumes and frozen until the experimental stage.

The final data analysis involved comparing profiles within each block as well as between the block experiments run over the course of the research detailed in this thesis.

3.1.7 Ethics

The study was approved by the South Manchester Research ethics committee (Ref: 05/Q1403/151), the University of Manchester Senate Committee for ethics. NHS R&D approval was obtained from the Manchester Mental Health and Social care Trust and from the Pennine Care Trust (Appendix I).

3.1.8 Acknowledgment of activities undertaken

The majority of the controls were assessed by the author, with some Alzheimer’s disease patients also recruited by the author. The majority of the Alzheimer’s disease patients were assessed by a research nurse (SCG). The majority of the blood
samples were taken by the research nurse (SCG), with some samples taken by the author. The analysis of the metadata was undertaken entirely by the author.

3.2 METABOLOMIC ANALYSES

QC serum was prepared from human serum purchased from Sigma-Aldrich. The QC serum was used in each of the metabolomic analysis studies reported in this thesis. Briefly, the QC serum was a stock solution of serum with no information regarding origin of the number of donors from whom the serum was collected. The QC serum was considered to be identical and therefore equal aliquots of the serum.

3.2.1 Metabolic Fingerprinting of Serum samples using FT-IR

All blood serum samples were analysed using FT-IR machine equinox 55 (Brucker optics, Coventry, UK). The serum samples were aliquoted and run over a single 24 hour period on the FT-IR. The FT-IR was chosen as the preferred analytical platform as opposed to using an NMR for analysis, as it was the only analytical platform available to perform the metabolomic fingerprinting of the serum samples.

Preparation of FT-IR analysis

All the 96 well silicon plates in this study were soaked in 5% SDS (sodium dodecyl sulphate) (Sigma-Aldrich, Gillingham, UK) solution for at least 30 minutes and then rinsed three times in deionised water and dried using lint-free tissue paper. Following which the plates were rinsed three times with deionised water, dried with lint-free tissue paper and placed in an oven for 10 minutes.
Serum samples from patients and controls were thawed on wet ice including QC serum samples (pooled human serum, Sigma-Aldrich, Gillingham, UK). Aliquots containing of 1µl were spotted and spread evenly onto 96-well silicon plates. The samples were then dried in an oven at 50°C for 10 minutes and placed in a dessicater following which samples were analysed on the FT-IR instrument.

**FT-IR Analysis of Serum Blocks**

The 180 serum samples (60 controls + 60 Alzheimer’s disease baseline and 60 Alzheimer’s disease 12 month follow-up), were paired such that for each control sample the closest in age and sex were matched with the Alzheimer’s disease baseline and associated 12 month follow-up sample. Therefore there were a total of 60 paired samples (totalling 180 samples). The analysis order of the paired samples was randomised. And for each of the paired sample group (that is control and Alzheimer’s disease baseline + follow-up samples), the three samples were always randomised to three adjacent wells. The first well of the 96 well plate (A1) was left blank, as it was used for background measurement. The second well of each plate (A2) were spotted with Sigma QA serum. For each analysis of the paired samples a QC serum preceded and followed the paired samples. In total samples were spread across three plates, with three blank wells, three lead in QC serum; 120 preceding and followed QC serum, and 60 paired control and disease serum samples.

Three analytical replicates were analysed per well, this represented approximately five hours of instrument time per plate. With plates being prepared and stored in a dessicater until analysis was performed.
Operating parameters for FT-IR analysis of serum samples

The FT-IR instrument was operated under the control of the instrument software (OPUS). For the analysis of serum samples, measurement parameters were set using a pre-defined instrument method file ("serum.XPM"). The plate was loaded on to the FT-IR HTS (high-throughput screen) device and the stage calibrated in the motorised stage control window. A background spectrum was measured from well A1 (X position 94104 and Y position 18720). The position of each of the three replicate scans from each well was saved as an XY co-ordinates file ("adscoordinates.XY"). The experimental data files were converted to ASCII (a text file), and thus contained data pertaining to the samples analysed per plate using the FTIR Brucker converter programme.

FT-IR data files

The frequency spectra which showed high absorption of water vapour, CO$_2$ were removed from all the samples. The spectra for each of the three groups, Control, Alzheimer’s disease baseline and Alzheimer’s disease follow-up were combined into three group spectra, with each group containing 60 spectra from the corresponding groups as well as a fourth group for the QA samples. Any individual sample whose peak absorbance intensity were too high in comparison to the other grouping counterparts were removed as artefacts.
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**Table 3.1 The FT-IR measurement parameters for the analysis of serum**

The FT-IR was operated with four major setting parameters. These settings included the time taken to scan each well (64 seconds), the infra-red wavelength which was targeted on each well.
The measurement parameter settings fall into four categories: measurement settings, optics settings, acquisition settings and FT settings (see Table 3.1). Briefly, a mid-infrared region of 4000-600 cm\(^{-1}\) was used, and spectra were acquired at 4 cm\(^{-1}\). For sample and background scan a time of 64 interferograms were used to for a suitable signal-to-noise ratio for each of the spectrum. In order to capture the whole of the infra-red range frequencies (or the apodization function) of the combined interferograms, the Blackman-Harris 3-Term was used. Following which the data was Fourier-transformed.

All spectra were both corrected and normalised for baseline.

3.2.2 Acknowledgment of activities undertaken

The preparation of serum samples for FT-IR analysis was undertaken by the author.

The FT-IR analysis was run by Dr Catherine Winder.

The pre-processing and pre-treatment of the FT-IR data was undertaken by Dr Catherine Winder. The statistical analysis of the FT-IR data was undertaken by Dr David Broadhurst
3.2.3 Metabolomic profiling of Serum samples using GC-ToF-MS

The procedure for the analysis of blood serum samples by GC-MS is described in the sections below.

**Sample preparation**

Preparation of and analysis of blood serum samples for analysis by GC-ToF-MS, was as described previously (Dunn et al., 2008, Begley et al., 2008; Underwood et al., 2006, Broadhurst et al., 2007, Dunn et al., 2011). Briefly, 400µl blood serum samples and including stock QC serum (Sigma, UK) were spiked with 200µl of four sets of internal standard solution 1 (IS1), the nominal concentration of each component was 0.167mg/ml, MSG (malonic d$_2$, succinic d$_4$ acid, acid and glycine d$_5$; (Sigma-Aldrich, Gillingham, UK)), CFT (citric acid d$_4$, $^{13}$C$_6$ d-fructose, and l-tryptophan d$_6$ (Cambridge Isotope Labs Inc)) LA (l-lysine d$_4$, l-alanine d$_5$ (Cambridge Isotope Labs Inc)), SBO (stearic acid d$_{35}$, benzoic acid d$_5$, octanoic acid d$_{15}$ dissolved in methanol (Cambridge Isotope Labs Inc)); and vortex mixed (15 seconds). Internal standard solution 2 (IS2), was prepared fresh each day of sample analysis by combining 2ml of each IS1 stock (MSG, CFT, LA and SBO) and adding deionised water (Sigma, Cat no. 34877) to a final volume of 12ml. The nominal concentration of each component is 0.167mg/ml (Table 3.2).

Briefly, the application of internal standards is recommended for the use in GC-MS analyses to control for the chemical derivatisation step and errors associated with low sample volume injection. Therefore an internal standard was spiked into all the
serum samples and is measured for concentrations and is applied to correct analytical
variation throughout the sample analysis stage. A mix of metabolites was used as
the internal standard in order to compensate for the different metabolites likely to be
present in serum samples. This method was devised and applied for the analysis of
all serum samples as part of the HUSERMET project (Begley et al., 2009 and Dunn
et al., 2011)

**Deproteinating the sample**

Samples were deproteinated by adding 1200µl of methanol (Sigma-Aldrich,
Gillingham UK), vortex-mixed (15 seconds) followed by centrifugation (15, 800g,
15 min), and split into volumes of 370µl.
Transfer and freeze drying lyophilisation) of supernatant

The supernatant was transferred and freeze dried in a centrifugal vacuum evaporator (HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap; Thermo Life Sciences, Basingstoke, UK).

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<tbody>
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<td>MSG</td>
<td>MSG</td>
</tr>
<tr>
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<td>d4 Succinic acid</td>
<td></td>
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<tr>
<td>d5 Glycine</td>
<td>+</td>
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<td>CFT</td>
<td>CFT</td>
</tr>
<tr>
<td>d4 Citric acid</td>
<td>2ml</td>
</tr>
<tr>
<td>15C6  D-Fructose</td>
<td></td>
</tr>
<tr>
<td>d5 L-Tryptophan</td>
<td>+</td>
</tr>
<tr>
<td>LA</td>
<td>LA</td>
</tr>
<tr>
<td>d4 L-Lysine</td>
<td>2ml</td>
</tr>
<tr>
<td>d7 L-Alanine</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
</tr>
<tr>
<td>SBO (MeOH)</td>
<td>SBO</td>
</tr>
<tr>
<td>d35 Stearic acid</td>
<td>2ml</td>
</tr>
<tr>
<td>d5 Benzoic acid</td>
<td>+</td>
</tr>
<tr>
<td>d15 Octanoic acid</td>
<td>deionised H2O</td>
</tr>
</tbody>
</table>

Table 3.2  The composition of internal standard 1 (IS1) & internal standard (IS2)

Working internal standard solution “IS2” is prepared fresh each day by combining 2ml of each IS1 stock (MSG, CFT, LA and SBO) and adding deionised water (Sigma, Cat no. 34877) to a final volume of 12ml. The nominal concentration of each component is 0.167mg/ml.
Two-step derivatisation of serum extracts for GC-ToF-MS analysis

In order to increase the volatility of metabolites and to decrease the boiling point of the metabolites of the serum extracts, the dried serum underwent a two-step derivatisation process. Derivatisation allows metabolites to pass through the GC column during the separation analysis stage of the sample. For the first step oxime derivatives, ketone-functional groups were formed, by adding 50µl of 20mg/ml O-methoxylamine hydrochloride (Acros Organics, Loughborough, UK) dissolved in pyridine (Acros Organics, Loughborough, UK), to dried serum, vortex-mixed (15 seconds) and heated at 80ºC for 15 minutes. For the second step, the dried serum was treated with 50µl N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (Acros Organics, Loughborough, UK) silylating reagent to form trimethylsilyl derivatives of hydroxyl-, amino- and carboxylic acid functional groups, and heated at 80ºC for 15 minutes.

Addition of retention index

A 20µl volume of a retention index, consisting of 30mg docosane (Sigma, Cat no. D4509) and nonadecane (Aldrich, UK), 40µl each of decane (Aldrich, UK), dodecane (Aldrich, UK) and pentadecane (Sigma, UK) dissolved initially in 10ml hexane (Acros Organics, Loughborough, UK) followed by four parts of pyridine (Acros Organics, Loughborough, UK)) were vortex-mixed (15 seconds). The sample was then centrifuged (15,800g, 15 minutes), and the supernatant transferred for analysis using the Agilent 6890 gas chromatograph and 7683 autosampler (Agilent Technologies, Stockport, UK) coupled to a LECO Pegasus III time-of-flight
mass spectrometer (LECO Corp. St Joseph, USA), using optimised parameters (O’Hagan et al., 2005).

**Sample Analysis Run Order**

Serum samples were analysed in blocks. Each block of analyses consisted of 180 (Alzheimer’s disease baseline and 12 month follow-up + Control) prepared serum samples with QC serum and saline blanks. For analysis on the GC-ToF-MS, the samples were split into four blocks which were run in six daily batches. The order in which samples were analysed was paired Alzheimer’s disease and control samples (Table 3.3). The serum run order was the same as that used in the FT-IR analysis. The daily run batches were run on six consecutive days. The sample run order was five initial injections QC serum, followed by a saline blank. The remainder of the daily run batch comprised of 30 subject samples in the specified run order, interspersed with a QC serum injection after each third sample. A second saline blank was run between the 22nd and 23rd specimens in the daily run batch.

All chromatographic analyses followed by mass spectrometry analysis were analysed as single samples. The run-time for a single sample to be analysed using GC-ToF-MS was 21 min for the runtime. Therefore restricted by time and financial constraints, as well as the block design used in the metabolite profiling experiments, only single analyses were executed.
Table 3.3  Sample run-order for serum analysis.
Each block of 120 samples were run as four 24 hr batches (Alzheimer’s disease baseline +12 month follow-up and control) 180 sample block, six 24 hr batches) 30 samples per batch-5 QC serum lead-in, then 3 samples, QC serum, 3 samples, QC serum etc-2 saline blanks in each batch, injection 6 and 32.

GC-ToF-MS Settings and Parameters

The operating parameters for the analysis of the serum analysis was as optimized by O'Hagan et al., 2005. Each of the experiments were run on a GC-ToF-MS instrument (Agilent 6890N gas chromatograph and LECO Pegasus III TOF mass spectrometer) using the manufacturer's software (ChromaTof version 2.12). The loading of samples in to the GC-ToF-MS was automated using the autosampler.
(Gerstel MPS-2L) to load and inject the samples into the GC-TOF-MS. At the start of each batch sample analysis run, the chromatographic column would be washed through by injection of 5 solvent A prewashes, followed by 1 sample prewash, at an injection rate of 100ul/s followed by a 0.5s pre-injection delay and then followed by a 0.5s post-injection delay, followed by 5 solvent B post washes. The summarised set of instrument parameters chosen for optimization is given in Table 3.4. The gas chromatograph was operated in split mode using helium as carrier gas in constant flow mode (1ml/min), with an initial GC temperature of 70°C, with the temperature being increase (or ramped) at 20°C per minute to 300°C, with the final hold temperature time of 4 minutes. The sample volume injected into column was 1µL. The injection volume/split ratio was set at 4:1. A Varian VF-17MS GC column (Supelco, Gillingham, U.K.; 30m × 0.25mm × 0.25µm film thickness, was installed and conditioned according to the manufacturers; recommendations) was used. The transfer line and source temperatures used were 240 and 220°C, respectively. The mass range used was 45–600 Da with a detector voltage of 1700V. In the ChromaTOF software, the S/N threshold was set at 10, the baseline was offset at 1.0, the data points for averaging were set at 5, and the peak width was set at 3. The TOF mass spectrometer was able to collect mass spectra at up to 500 Hz; the manufacturers deconvolution software was used to discriminate overlapping peaks on the basis of their mass spectra.

The signal-to-noise ratio was calculated using the LECO software, as the peak height from the corrected baseline/noise for the ion of unique mass calculated by the LECO deconvolution software. The unique mass of ions varies between analyses, this means that the data are at actually estimates of the S/N ratio, as different unique ions
of different intensities result in differing S/N values. Most peaks, have good signal-to-noise, therefore by taking the average of the worst 20% of peaks, a threshold of 10 was used to maximize the sensitivity of the detector (10 is the lowest value of s/n permitted by the LECO software). The numbers of peaks that were considered to be noise (20%) were removed, and numbers of peaks that are retained are thus selected in a non-biased manner for analysis.

<table>
<thead>
<tr>
<th>Variable settings for the GC-ToF-MS</th>
<th>Setting for human serum analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample volume injected (µL)</td>
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</tr>
<tr>
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<tr>
<td>split ratio</td>
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<td>helium flow rate (mL·min⁻¹)</td>
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<tr>
<td>ramp speed (°C·min⁻¹)</td>
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</tr>
<tr>
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<tr>
<td>detector voltage (V)</td>
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</tr>
<tr>
<td>run time (min)</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 3.4 Optimal conditions for human serum analysis using the GC-ToF-MS

The preparation of and subsequent analysis of serum samples for GC-ToF-MS analysis was performed by Dr Warwick Dunn. The method described was the standard operating procedure for the analysis as adopted by the HUSERMET project.
Data pre-processing of GC-ToF-MS raw data

Raw data was processed using LECO ChromaTof software and the associated deconvolution algorithm. The resultant data matrix or metabolic profile (N samples x P metabolite peaks), was constructed based on the calculated response ratio of each metabolite peak relative to the internal standard mixture (peak area metabolite / peak area succinic-d$_4$ internal standard) for each sample. The data matrix numbered ~1400 unique peaks, with each individual metabolite peak being characterised by a unique retention time and mass spectrum.

A QA procedure was performed to remove metabolic features with low or poor repeatability. Data for all the metabolic features from the QC samples injections was used for this purpose. QC samples which are biologically identical and therefore it would be expected that identical results (within an expected error range) would be observed. Metabolic features that were detected in less than 50% of the QC samples were removed. Also removed were metabolic features which exceeded the 30% RSD across the QC samples. For GC-ToF-MS metabolic data, a higher threshold of 30% or more value is used as this represents the increased variability which can be introduced in the chemical derivatisation steps as well as the low sample volume injected during the analysis as compared to UPLC. Approximately 400 of these peaks from the 1400 detected were only derivatives of metabolites, and so were removed. The metabolite peaks were further reduced as only robust peaks were considered for further analysis. Any peaks which had more than 60% missing values, and could not be matched were removed. By using database matching approximately two thirds of these peaks can be assigned a molecular structure (Begley et al., 2009 and Dunn et al., 2011).
3.2.4 Acknowledgment of activities undertaken

The preparation of serum for GC-ToF-MS analysis was undertaken by Dr Paul Begley. The GC-ToF-MS analysis was also run by Dr Paul Begley. The pre-processing and pre-treatment of the GC-ToF-MS data was undertaken by Dr David Broadhurst. The statistical analysis of the GC-ToF-MS data was undertaken by the author.

3.2.5 Metabolomic profiling – UPLC-LTQ/Orbitrap-MS (+/- modes)

Metabolomic profiling of serum samples using the UPLC-LTQ/Orbitrap-MS (+/- mode)

Briefly, serum samples were analysed on the Acquity UPLC (Waters, Elstree, UK) coupled to a LTQ-Orbitrap mass spectrometry system operating in electrospray ionisation mode (Thermo Fisher Scientific, Bremen Germany). Identical serum samples were analysed in two different experiments, one in the positive mode and the other in the negative ion mode. Serum samples from study participants and QC serum samples were prepared in the same manner, and analysed in the same order as described for FT-IR and GC-ToF-MS.

Sample preparation

Analysis of samples by UPLC/LTQ-Orbitrap-MS was as previously described (Dunn et al., 2008; and Zelena et al., 2008). The required aliquots of frozen serum samples
collected from Alzheimer’s disease patients and controls (which had been stored at -80°C) were thawed on ice at 4°C. QC serum was also prepared in the same manner.

**Deproteinating samples**

The samples were deproteinated as described in section 3.2.

**Transfer and lyophilisation of supernatant**

The supernatant was transferred and heated at 45°C in a centrifugal vacuum evaporator (HETRO VR MAXI vacuum centrifuge attached to a Thermo Svart RVT 4104 refrigerated vapour trap (Thermo Life Sciences, Basingstoke, UK) and stored at 4°C until required for analysis.

**Sample analysis**

The lyophilised samples were reconstituted in 400µl HPLC grade water (a ratio of 1:1 of the original serum volume), vortex-mixed (15 seconds) followed by centrifugation (15 min at 15800 g). The resultant supernatant was transferred to analytical vials and stored in the automated sampler section of the UPLC at 4°C prior to UPLC/LTQ-Orbitrap-MS. All the reconstituted samples were analysed within 48 hours. The prepared samples were reconstituted in 200µl or 400µl of water for electrospray ionisation mode – (ESI-) or electrospray ionisation mode + (ESI+) analysis respectively (Dunn et al., 2011).
Run Order for serum sample analysis

Case and control samples were matched for age and sex. The run order and details of the serum samples being analysed were on a block by block basis. Each block consisted of: 60 subject serum samples (20 ‘Controls’, 20 ‘AD Baseline’, 20 ‘AD 12-month’), 30 QC samples (10 lead-in QCs and a QC for every fourth sample). All chromatographic analyses followed by mass spectrometry analysis were analysed as single samples. The run-time for a single sample to be analysed using UPLC-LTQ/Orbitrap (+/- modes) was between 21 and 24 min. Therefore restricted by time and financial constraints, as well as the block design used in the metabolite profiling experiments, only single analyses were executed.

UPLC separation

Serum sample volumes of 10µl were injected using an autosampler, an automated robotic process which is temperature controlled. Each 10µl volume of serum underwent chromatographic separation, following column elution (Acquity UPLC BEH 1.7 µm-C₁₈ column), with 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The column pressure at the beginning of the gradient was 100% A raised rapidly (ramped) to 100% B for 15 minutes, followed by 4 minutes at 100% B, and rapidly returned to 100% A followed by an equilibration period at 2 minutes. Column temperature was set at 50°C and sample temperature at 10°C, flow rate was 0.36 or 0.40 ml/min in positive and negative ion mode respectively.

The Orbitrap parameters for acquisition of mass spectra, mass range and resolution are detailed in (see Table 3.5). Mass spectra was acquired using mass range of 50-
1000Th, and mass resolution of 30, 000 and a scan time of 0.4 seconds. Mass accuracies were less than 3ppm over a wide dynamic range.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ESI +</th>
<th>ESI -</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary temperature (°C)</td>
<td>300</td>
<td>275</td>
</tr>
<tr>
<td>Capillary voltage (V)</td>
<td>7</td>
<td>-44</td>
</tr>
<tr>
<td>Ion trap full maximum ion time (ms)</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Resolving power (at m/z 400)</td>
<td>30000</td>
<td>30000</td>
</tr>
<tr>
<td>Mass range</td>
<td>50-1000</td>
<td>50-1000</td>
</tr>
<tr>
<td>MS run time (min)</td>
<td>22</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 3.5 LTQ/Orbitrap-MS key settings for positive and negative ionisation modes

The preparation of and subsequent analysis of serum samples for UPLC-LTQ/Orbitrap-MS analysis was performed by Dr Warwick Dunn. The method described was the standard operating procedure for the analysis as adopted by the HUSERMET project.

**Processing of UPLC-LTQ/Orbitrap-MS raw data**

The raw mass spectral data obtained was converted to the network Common Data Form (NetCDF) format. The FileConverter program available in the XCalibur software (ThermoFisher Scientific, Bremen, Germany) was used to transform the data into the NetCDF format.

Following sample analysis, the NetCDF format version of the raw spectral data was analysed using the freely available open-source XCMS software (Smith et al., 2006).
This data pre-processing is required to deconvolute the raw data into a usable raw data matrix. The raw analytical data was deconvoluted to the output for each sample with associated retention time, accurate mass and chromatographic peak are calculated in to peak responses (Dunn et al., 2011). Only peaks which were 60% peak reproducible were considered, i.e. if 50% of its QC measurements were outside its QC mean ± 20%, then they were discarded. All blocks were subsequently matched and stitched together. Matching was based on a mass tolerance of 0.02 m/z. Raw spectra, retention time and accurate mass of authentic chemical standards were used to compare metabolites of interest. The combination of UPLC-LTQ/Orbitrap-MS results in high chromatographic and mass resolution with high mass accuracy. This increases the number of metabolite peaks with assigned molecular structures.

### 3.2.6 Data pre-treatment

A two-step process was used for the pre-treatment of data originating from the analysis of QC samples. In the first step UPLC-MS feature responses for all QC samples with more than 50% of its data consisting of missing values were removed. This step was undertaken because metabolic features which were missing over 50% of the values revealed poor repeatability across the experiment and would not represent the actual distribution in normal samples. QC samples are biologically identical and therefore it would be expected that identical results (within an expected error range) should be observed. Secondly, for all the UPLC-MS feature vectors that pass through the first step of pre-treatment, the relative standard deviation (RSD) is calculated as a ratio of population standard deviation to population mean of intensity responses.
Feature vectors with RSDs higher than 20% in more than 50% of the samples were considered to show poor repeatability for biologically identical samples and were removed.

Features from QC samples that passed the pre-treatment process are expected to be highly reproducible. Analogous features were taken from data originating from normal samples of serum/plasma and the measured intensity response was normalised to the intensities of corresponding QC features. The process of normalisation involved the division of the median of feature intensity responses measured for QC samples with the intensity response of each feature for a normal sample. These pre-treated data were then submitted for further statistical analysis.

### 3.2.7 Acknowledgment of activities undertaken

The preparation of serum for UPLC-LTQ/Orbitrap-MS (+/- ionisation mode) analysis was undertaken by Dr Warwick Dunn. The UPLC-LTQ/Orbitrap-MS (+/- ionisation mode) analysis was run by Dr Warwick Dunn. The pre-processing and pre-treatment of the -LTQ/Orbitrap-MS (+/- ionisation mode) data was undertaken by Dr David Broadhurst and Dr Yun Xu.

### 3.3 STATISTICAL ANALYSIS

The statistical analysis of data was performed using scripts which were executed using the MATLAB application version 7.4 (MathWork, Massachusetts, USA). These scripts contain statements in the MATLAB programming language implementing the required statistical calculation and were provided by Dr. David
Further information about the statistical analyses used is described in each results chapter.

3.3.1 Clinical metadata

A matched case-control experimental design was used for the studies, and no assumptions were made regarding normal population difference. For the analysis of the clinical metadata univariate analyses were performed to identify significant differences between the groups. For comparisons of Alzheimer’s disease and control the Mann-Whitney test was employed, with $p < 0.05$ considered as significant. For the Alzheimer’s disease baseline and follow-up groups the Wilcoxon signed-rank test was employed, with $p < 0.05$ considered as significant.

3.3.2 Profiling studies

Univariate analyses were performed to identify significantly altered peaks between cases and controls; and that the peaks are from different populations (Mann-Whitney test and Wilcoxon signed-rank test was employed). Because a large number of parallel tests had to be performed, the probability of false discoveries (metabolite biomarkers that are significant) is increased (Broadhurst and Kell, 2006). In order to control for the multiple testing problems a FDR threshold of 0.05 was applied to all the analyses as the threshold controls for the number of false discoveries in those tests which result in a significant result. In quotidian statistical analysis a $p$ value of 0.05 is interpreted as 5% of all tests will be due to chance i.e. a false positive. The FDR adjusted $p$ value is referred to as the $q$ value of 0.05 is interpreted as 5% of significant tests will result in false positives. The $q$ value can be interpreted as the
estimation for all the tests which are considered significant, 5% of them will be false positives. Therefore each p-value obtained following univariate analysis was interpreted in light of the associated FDR q value statistic.

3.3.3 Statistical modelling

Unsupervised principal component analysis (PCA), was used to identify major outliers (removed from further analysis). Principal components canonical variate analysis (PC-CVA), a supervised modelling method, was used to build a predictive model able to optimally classify cases and controls. Models were built using two-thirds of the dataset, and validated using a hold-out or blind set (one third data set). Univariate analysis with a False Discovery Rate (FDR) \( \leq 0.10 \) was used for the p-values in the analysis of the metabolite data (Broadhurst & Kell). Metabolite peaks were analysed using univariate statistics to identify potential peaks which were significantly different between Alzheimer’s disease baseline v Control (Mann-Whitney test). A Wilcoxon signed-rank test was used to analyse between Alzheimer’s disease baseline v follow-up. A discriminant function analysis was performed on the top metabolite peaks to determine an overall classification percentage, and metrics for sensitivity and specificity of the key discriminating peaks (Figure 3.1).
Figure 3.1 Summary of data and statistical processes used in metabolomics experiments

The processing of metabolomic data sets is complex and time consuming. However, when the data sets are derived (post sample analysis) are converted to data sets representing peaks associated with the participant, univariate analysis can potentially identify significantly altered metabolite peaks between disease and non-disease groups.

All statistical analyses were undertaken using MATLAB Application 2009a (MathWork, Massachusetts, USA) and SPSS 16.0.

3.3.4 Acknowledgment of activities undertaken

The statistical analysis of the clinical metadata was undertaken by the author. The statistical analysis of the FT-IR data was undertaken by Dr David Broadhurst. The statistical analysis of the GC-ToF-MS and UPLC-LTQ/Orbitrap-MS (+/– ionisation modes) data was undertaken by the author.
4 CLINICAL METADATA FOR ALZHEIMER’S DISEASE PATIENTS AND CONTROLS

4.1 INTRODUCTION

Currently there are no known biological biomarkers that can be used in diagnosing Alzheimer’s disease and to differentiate it from normal ageing. Metabolites are a potentially useful source of detecting and identifying disease specific biomarkers. Metabolomics is the non-biased identification and quantification of all known and unknown metabolites in a biological system. The broad aim of this thesis was to examine the range of metabolomics technologies available and use repeatable and reproducible methods to generate metabolomics data to identify potential disease biomarkers for (i) diagnostic purposes and to measure (ii) disease progression, following the analysis of serum samples in a non-biased manner from Alzheimer’s disease patients (collected at two time-points) baseline and twelve months) and healthy controls (collected at baseline only).

In order to undertake metabolomic analysis of blood samples for the purpose of detecting and identifying potential Alzheimer’s disease associated metabolome changes, Alzheimer’s disease patients and controls with no known dementia were recruited, blood samples and associated metadata was collected.
4.2 MATERIALS AND METHODS

4.2.1 Recruitment and consent

The recruitment of Alzheimer’s disease patients and controls in this study was approved by the NHS local research ethics committee, and all participants were seen in their homes.

Alzheimer’s disease participants

Patients with a clinical diagnosis of Alzheimer’s disease, and seen by Old Age Psychiatric services in Manchester and Stockport during a 12 month recruitment period (August 2005 to August 2007) were recruited into the study. The inclusion criteria, for the Alzheimer’s disease group, was for patients to satisfy the DSM-IV criteria for Alzheimer’s disease (APA, 1995), NINCDS/ADRDA criteria for ‘probable’ or ‘possible’ Alzheimer’s disease (McKhann et al, 1984), and Clinical Dementia Rating (CDR) of 1 or above (Hughes et al, 1982). Written informed consent was obtained from the patients and their carers. The diagnosis was confirmed by old age psychiatrists (BB & NP).

Controls

Two methods were used in recruiting controls. Firstly, for each recruited Alzheimer’s disease patient, their general practitioner (GP) was asked to identify six individuals from their general practice list of the same sex and closest in age to the index patient. Secondly, carers of patients were asked to participate in the study. The inclusion criteria for the control group was, to be free of memory problems including known Alzheimer’s disease or dementia, a score of 0.5 or below on the
Clinical Dementia Rating (CDR) (Hughes et al, 1982), and a score of 24 or less on Mini-Mental State Examination (MMSE) (Folstein, 1975). Written informed consent was obtained from all participants who satisfied the criteria.

4.2.2 Metadata (metabolite and clinical data)

For this study we collected two sets of information for all study participants. The first set of data was to assess the cognitive symptoms, (MMSE, (Folstein, 1975); Alzheimer’s Disease Assessment Scale – cognitive subscale, ADAS-Cog, (Rosen et al, 1984) (Alzheimer’s disease only); Cambridge Cognitive Assessment (CAMCOG) from CAMDEX (Roth et al, 1986) (controls only); and the Clinical Dementia Rating, CDR, (Hughes et al, 1982)). A second set of information was data on all potential confounding sources of individual metabolite differences which could result in varying metabolic profiles. This included participant medical history (Burvill et al, 1990), diet, smoking, alcohol consumption, body mass index (BMI), and both prescribed and over-the-counter medication, including herbal supplements.

4.2.3 Serum collection

Serum samples were collected from Alzheimer’s disease patients (n=60) at baseline and at 12 months follow-up. These were age and sex matched with baseline control samples (n=60). All participants were assessed using cognitive and neuropsychiatric scales, and additional information collected included medical history, medication, diet, lifestyle and physical measurements.
Clinical and demographic characteristics were summarized using categorical variables, means +/- SD for normally distributed data and medians and interquartile ranges for non-normally distributed data. Statistical significance was set at $P < 0.5$. Independent t-tests and paired tests for normally distributed data and the equivalent tests for non-normally distributed data; Mann-Whitney and Wilcoxon signed-rank tests were used.

4.3 STATISTICAL ANALYSIS

Clinical and demographic characteristics were summarized using categorical variables, means +/- SD for normally distributed data and medians and interquartile ranges for non-normally distributed data. Statistical significance was set at $P < 0.5$. Independent t-tests and paired tests for normally distributed data and the equivalent tests for non-normally distributed data; Mann-Whitney and Wilcoxon signed-rank tests were used using SPSS 14.0.

4.4 RESULTS

In total 180 serum samples were prepared from 120 participants, (60 Alzheimer’s disease (baseline and 12 month follow-up) and 60 controls (baseline sample only). The 60 Alzheimer’s disease participants had a mean (SD) age of 79.4 (7.12) years and were 65% female. The control participants had a mean (SD) age of 77.92 (6.82) years and were 67% female. The 60 Alzheimer’s disease participants mean (SD) baseline MMSE score was 19.82 (6.28), and the 12 month follow-up MMSE score was 16.63 (7.73). The 60 control participants mean (SD) baseline MMSE score was 28 (1.69). The 60 Alzheimer’s disease participants had a mean (SD) age of 79.4
(7.12) years and were 65% female. The control participants had a mean (SD) age of 77.92 (6.82) years and were 67% female. The 60 Alzheimer’s disease participants mean (SD) baseline MMSE score was 19.82 (6.28), and the 12 month follow-up MMSE score was 16.63 (7.73). The 60 control participants mean (SD) baseline MMSE score was 28 (1.69).

BMI according to the WHO Global database on BMI of 25 or above is classed as overweight (http://apps.who.int/bmi/index.jsp?introPage=intro_3.html). The control and disease population baseline and follow-up were 27.18(4.48), 25.36 (4.64) and 25.6 (4.9). There were significant group differences between the control and disease and disease baseline and follow-up BMI. However, BMI did not correlate with MMSE or ADAS-Cog within the groups.

Patients with Alzheimer’s disease were recruited from specialist secondary care old age services in Manchester and Stockport, UK. Old age psychiatrists were asked to refer suitable patients to the researcher (MZ and SCG). Patients and their carers were assessed for suitability, and written informed consent was obtained. The Alzheimer’s disease sample of patients was therefore a convenience sample, rather than an epidemiological sample. Similarly controls were recruited primarily through the general practitioners of the Alzheimer’s disease patients.

There were no significant differences between the two populations on age or sex. The body mass indices were significantly altered and this is likely due to the cause and effect of Alzheimer’s disease. The BMI difference between the Alzheimer’s
disease baseline and Alzheimer’s disease follow-up sample was significantly reduced.
<table>
<thead>
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<th>+/-SD</th>
<th>AD FOLLOW-UP</th>
<th>+/-SD</th>
<th>P VALUE</th>
</tr>
</thead>
<tbody>
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<td>N</td>
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<td></td>
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<td>SEX (MALE)</td>
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Table 4.1 Demographic and clinical characteristics according to groups
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5 THE APPLICATION OF FOURIER TRANSFORM – INFRA RED TO DETECT A METABOLIC FINGERPRINT OF ALZHEIMER'S DISEASE

5.1 INTRODUCTION

Alzheimer’s disease is a neurodegenerative disorder with no known aetiology. In the developed world AD accounts for up to 60% of all dementia cases. The prevalence rate for dementia has been estimated at 25 million people worldwide. The diagnosis of Alzheimer’s disease develops over time, by clinical and neuropsychological tests, as well as with the use of neuroimaging. There remains a need to develop a non-invasive based quick and accurate diagnostic test, able to detect early Alzheimer’s disease changes in patients. The Fourier Transform infrared spectroscopy (FT-IR) induces and measures molecular vibrations of known chemical functional groups in biological samples. The resultant output is a characteristic metabolic fingerprint of the sample. Metabolic fingerprinting techniques have the potential to distinguish between disease and non-disease samples.

A metabolic fingerprinting analysis of control and Alzheimer’s disease samples could be performed using NMR and FT-IR. The choice to use FT-IR analysis rather than NMR was based on the availability the analytical platform to be used for the analysis of the of serum samples. The aim was to identify any metabolic fingerprint which could distinguish control and Alzheimer’s disease serum samples. Secondly, we wanted to identify any metabolomic fingerprint which could distinguish between disease samples based on changes in disease severity and progression over a one year period.
5.2 METHODS

Serum samples were collected from Alzheimer’s disease patients (n=60) at baseline and 12 month follow-up. Controls (n=60) were matched for sex and age with the Alzheimer’s disease baseline sample. A number of cognitive and neuropsychiatric scales were used, to stratify the control and Alzheimer’s disease groups. For FT-IR analysis, 1µl volume of control, Alzheimer’s disease, and stock QC serum samples were used. The transmission spectra were acquired over the mid-infrared range (4000-600cm⁻¹).

5.2.1 Preparation of FT-IR for analysis

All 96 well silicon plates were soaked in 5% SDS (sodium dodecyl sulphate: SIGMA) solution for at least 30 minutes and then rinsed three times in de-ionised water and dried using lint-free tissue paper. Plates were then rinsed three times with de-ionised water, dried with lint-free tissue paper and placed in an oven for 10 minutes.

Serum samples from patients and controls were thawed on wet ice including QA serum samples (pooled human serum: SIGMA). One µl aliquots were spotted and spread evenly onto 96-well silicon plates. The samples were dried in an oven at 50°C for 10 minutes and placed in a dessicater prior to being analysed on the FT-IR instrument.
5.2.2 FT-IR Analysis of Serum Blocks

The 180 serum samples (60 controls/ 60 Alzheimer’s disease at baseline/ 60 Alzheimer’s disease at 12-month follow-up) were paired to give the best matching in terms of age and sex between controls, Alzheimer’s disease at baseline and the associated 12-month follow-up (60 sets). The order of analysis of the paired samples was randomised. For each of the paired groups (control/ Alzheimer’s disease baseline/ follow-up) the three samples were always randomised to three adjacent wells. The first well of the 96 well plate (A1) was left blank, as it was used for background measurement. The second well of each plate (A2) were spotted with Sigma QA serum. For each analysis of paired samples a Sigma QA serum preceded and followed the paired samples. In total, samples were spread across three plates, each with three blank wells, followed by three wells each of which were spotted with QA sigma serum; QA sigma serum preceded and followed the 60 paired control and disease serum samples. Three analytical replicates were analysed per well.

5.2.3 Operating parameters for FT-IR Analysis of Serum Samples

The FT-IR instrument was operated under the control of the instrument software (OPUS). For analysis of serum samples, measurement parameters were set using a pre-defined instrument method file. The plate was loaded on to the FT-IR HTS (high-throughput screen) device and the stage calibrated in the motorised stage control window. A background spectrum was measured from well A1 (X position 94104 and Y position 18720). The position of each of the three replicate scans from each well was saved as an XY co-ordinates file (“adsoodordinates.XY”). The experimental data files were converted to ASCII.
All spectra were captured at the infrared region of the electromagnetic spectrum wavelength 4000-600cm-1. Spectra acquisition rate was 20s-1 with a resolution of 4cm-1. (The ASCII data was imported and analysed in Matlab (The Math works., Inc). CO₂ peaks at 2403-2272cm-1 and 683-656cm-1 were removed and spectra containing H₂O were also removed (4000-3036cm-1 and 2458-1706cm-1). All data were normalized by division of the intensity values for each wavenumber in the spectrum by its standard deviation prior to analysis. An absorbance range of between 0 and 1 was used. Three analytical replicates of the serum samples were analysed per well and the spectra was averaged, with a scan time of 64 seconds each (Table 3.1).

5.3 STATISTICAL ANALYSIS OF DATA

Unsupervised principal component analysis (PCA), was used to identify major outliers (removed from further analysis). Principal components canonical variate analysis (PC-CVA), a supervised modelling method, was used to build a predictive model able to optimally classify cases and controls. Models were built using two-thirds of the dataset, and validated using a hold-out or blind set (one third data set).

All statistical analyses were executed using MATLAB. The pre-processing and pre-treatment of data was undertaken by Dr Catherine Winder (FT-IR), and statistical analysis was performed by Dr David Broadhurst.
5.4 RESULTS

The frequency spectra showed high absorption of water vapour, CO₂, and so were removed from all samples. The spectra for each of the three groups, Control, Alzheimer’s disease baseline, and Alzheimer’s disease follow-up, were combined into three grouped spectra, with each group containing 60 spectra. Any individual sample whose peak absorbance intensity was too high in comparison to the other grouping counterparts was removed as an artefact.

Clinical and demographic characteristic data of the groups were as expected between Alzheimer’s disease and controls are presented in (Table 4.1). As expected there were significant differences between the MMSE scores. The decline in the Alzheimer’s disease group over a year is reflected in the ADAS-Cog scores and MMSE scores. However across the groups, the BMI was significantly different.

To determine any metabolic changes across the groups, spectra for each group were averaged (Figure 5.1). Variations of the spectra were also compared to identify any potential chemical difference between the groups. As this was a hypothesis-generating experiment, no single or specific band region was analyzed. Rather, the whole of the infrared region data was explored. None of the data across the groups showed significant differences.

The spectra obtained in the 4000-500 cm⁻¹ from the 180 serum samples were analysed using PCA plot of scores. The spectra from the groups did not reveal or indicate any degree of class separation in the PCA plot (Figure 5.2). The spectra from the 180 serum samples from the control, Alzheimer’s disease baseline and Alzheimer’s
disease follow-up groups was analysed using the PC-CVA analysis model of class discrimination. Using the first 40 PCA scores which explained the maximum variation between the groups a discriminant function analysis model was built and cross validated (Figure 5.3). The FT-IR spectra data was unsuccessful in discriminating between the three groups. The FT-IR spectra from the Alzheimer’s disease baseline and follow-up groups, derived from 120 serum samples was analysed using the first 15 PCA scores which explained the maximum variation between the two groups. A discriminant function analysis model was built using the first 15 PCA scores; the FT-IR PC-CVA analysis model of class discrimination did not reveal any separation of the serum FT-IR spectra. The discriminant function analysis models built were cross-validated using hold-out data sets. Principal components canonical variate analysis (PC-CVA), a supervised modelling method, was used to build a predictive model able to optimally classify cases and controls. The predictive modelling using two-thirds of the dataset to build the initial model and validated the model using a hold-out or blind set (one third data set) (Figure 5.3-5.4).

Differences which could be attributed to metabolic changes were investigated. The mean spectra obtained from the three groups, control, Alzheimer’s disease baseline and follow-up was compared. Any variation in the intensity and the presence or absence of any band regions within the obtained spectra would potentially reflect chemical differences between the groups. The spectra were obtained in the whole mid-infrared region (4000-500cm⁻¹), and processed with CO₂ vibrations removed. The spectra obtained for all three groups did not show any visual difference.
Figure 5.1 FT-IR absorbance spectra.

All spectra in the figures are displayed in terms of absorbance and were obtained in the whole mid-infrared region (4000-500 cm\(^{-1}\)). Processed with CO\(_2\) vibrations removed, baseline offset, spectra are inverted and offset. QC = Sigma serum sample, c = control, D1 = Alzheimer’s disease baseline and D2 = Alzheimer’s disease follow-up.
Figure 5.2 FT-IR Principal Component Analysis (PCA) plot of scores for class effect

PCA plot showing class effect for the four groups are represented QC = Sigma serum sample), c = control, D1 = Alzheimer’s disease baseline and D2 = Alzheimer’s disease follow-up. PC1 = the percentage of total variance in the 1st principal component (36.9%) and PC2 = the percentage of total variance in the 2nd principal component (22.8%). The PCA plot clearly shows that was no separation of the four groups of samples.
Figure 5.3 FT-IR PC-CVA analysis model of the class discrimination

The Principal Component-Canonical Variate Analysis (PC-CVA) was generated to reveal the class separation of the three sample groups: Control, Alzheimer’s disease baseline and Alzheimer’s disease follow-up, with QCs removed. The FT-IR spectra data was unsuccessful in discriminating between the all three groups.
Figure 5.4 FT-IR PC-CVA analysis model of the class discrimination between disease samples

DFA model built and cross validated using the first 15 PCA scores, D1 = Alzheimer’s disease baseline and D2 = Alzheimer’s disease baseline follow-up. There was no visual class separation between the two groups.
6 THE APPLICATION OF GC-ToF-MS TO DETECT A METABOLIC PROFILE OF ALZHEIMER’S DISEASE

6.1 INTRODUCTION

Alzheimer’s disease accounts for the majority of cases for dementia. With increasing aging populations the world ageing related disease such as Alzheimer’s disease present a significant health and socio-economic dilemmas (Ferri et al., 2005). Identifying biomarkers associated with Alzheimer’s disease and its progression could be used to detect and diagnose the disease at an earlier than currently. Potentially such biomarkers could be used in the development of effective drug therapies in the treatment and possible modification of the disease. To date research has not yielded the discovery of a biomarker which could be utilised for such purposes.

In recent years the use of a hypothesis-generating approach has been posited as an effective approach in the identification of disease biomarkers by measuring large numbers of molecules in biological samples to detect and identify biomarkers associated with a given disease (Kell, 2004; Kell and Oliver, 2004). The metabolome is considered to be closer to the phenotype of a disease, which is downstream from the genome (Fiehn et al., 2000). Metabolomics experiments have been used as to detect disease biomarkers. The use of the GC-MS system has been applied in a number of studies in the analysis of disease samples in order to identify disease specific metabolite profiles also referred to as metabolic profiling, including heart failure cancer, schizophrenia, depression Huntingdon’s disease.
The aim of the present experiment was to use GC-ToF-MS to analyse serum samples obtained from Alzheimer’s disease patients and controls, in order to detect metabolites associated with the disease and the progression of the disease.

6.2 MATERIALS AND METHODS

Detailed methodology is as detailed in section 3.2.3. Briefly, GC-ToF-MS was used to profile serum samples. This profiling of the serum sample metabolome was achieved by chromatographic separation in the gas phase, and using a sensitive detection method, which allowed metabolites to be identified based on two properties, mass spectra and the retention time of metabolites, and comparing these two properties against the pure standards which were analysed in the same runs at regular intervals. Therefore for each participant (total 180) a metabolic profile of the serum sample was constructed using GC-ToF-MS.

A total of 180 samples (matched by age and sex, and constrained by sample availability) and 60 QA samples, 8 blanks were analysed in four 24 hour blocks. The total number of metabolite peaks detected was 259. Of these peaks, 10 were significantly altered across the three groups (controls, Alzheimer’s disease baseline (0 month), and follow-up (12 month).

Following sample analysis, the raw output data from the GC-MS was deconvoluted, and the data from across the blocks was aligned. Quality control was ensured based on the reproducibility of Sigma QC samples across a single block. A metabolite peak was not considered for further data analysis if more than 40% of the sigma QC
measurement was outside the sigma QC mean +/- 20%. Blocks were matched and stitched together based on matching of mass tolerance of 0.02m/z.

Of the 180 samples subjected to GC-ToF-MS analysis, only 177 were analysed. This is because the final GC-ToF-MS set of serum samples, that is control matched to Alzheimer’s disease baseline and follow-up samples failed to be injected during the automated sample introduction in to the GC machine.

### 6.3 STATISTICAL ANALYSIS

Exploration of the metabolite peaks using PCA analysis did not reveal a separation in the three groups (Alzheimer’s disease baseline, follow-up and control.

The total number of metabolite peaks detected was 259. These peaks were selected for statistical analysis as they fell within the 30% RSD limit. Univariate analysis of the detected metabolite peaks were univariately analysed to identify potential peaks which were significantly different between Alzheimer’s disease baseline v Control (Mann-Whitney test). A Wilcoxon signed-rank test was used to analyse Alzheimer’s disease baseline v Alzheimer’s disease follow-up. A False Discovery Rate (FDR) ≤ 0.05 was used for the $p$-values in the analysis of the metabolite data (Broadhurst & Kell). This is a less conservative method than a Bonferonni correction for multiple tests, and is used in the analysis of large data sets.
Dr Paul Begley prepared the serum samples and operated the GC-ToF-MS and Dr David Broadhurst undertook the pre-treatment of the resultant data set including the selection of the peaks.

6.4 RESULTS

6.4.1 Clinical Metadata

Serum samples were collected and prepared from 60 controls were closely matched for age and sex with 60 Alzheimer’s disease patients collected at two time points baseline and 12 month follow-up, as described in section 4.2.3. Clinical metadata was also collected and has been described in clinical results (section 4). Briefly, a detailed history was taken from each participant including age, sex, and body mass index. Clinical and demographic characteristic data of the groups were as expected between Alzheimer’s disease and controls are presented in (Table 4.1). As expected there were significant differences between the MMSE scores. The decline in the Alzheimer’s disease group over a year is reflected in the ADAS-Cog scores and MMSE scores. However across the groups, the BMI was significantly different.

6.4.2 GC-ToF-MS Metabolic profiling

The total number of metabolite peaks detected was 259 in a minimum of 70% of the samples analysed (out of a total of 177). These peaks were selected for statistical analysis as they fell within the 30% RSD limit.

An initial unsupervised PCA plot of scores was performed to potentially identify any potential clusters which could be associated with the three groups, controls,
Alzheimer’s disease baseline and follow-up. No evidence of clustering of the metabolite peaks from the three groups was observed in the first two principal components. A number of metabolite peaks which were outliers in the PCA plot outliers (peaks 32, 185 and 187) were related to all three groups’ participants (Figure 6.1). Separate PCA scores plots for control and Alzheimer’s disease baseline; and Alzheimer’s disease baseline and follow-up also revealed some outlying peaks, but these were represented by each of the paired groups. Overall the distribution of the metabolite peaks detected in the groups was largely homogenous (Figure 6.2 and 6.3 respectively).
Serum samples (n=177), were analysed using the GC-ToF-MS which resulted in the detection of 258 peaks. The PCA plot shows that there was no observable separation of the three sample groups: Control, Alzheimer’s disease baseline (AD Baseline) and Alzheimer’s disease follow-up (AD Followup).
Serum samples (n=118), were analysed using the GC-ToF-MS which resulted in the detection of 258 peaks. The outlier peaks (Peaks 32, 185 and 187) have been labelled.

The PCA plot shows that there was no observable separation of the two sample groups: Control and Alzheimer’s disease baseline (AD Baseline)
Figure 6.3 GC-ToF-MS Principal Component Analysis (PCA) plot of scores for the Alzheimer’s patients

Serum samples (n=118), were analysed using the GC-ToF-MS which resulted in the detection of 258 peaks. The outlier peaks (Peaks 32, 185 and 187) have been labelled. The PCA plot shows that there was no observable separation of the two sample groups: Alzheimer’s disease baseline (AD Baseline) and Alzheimer’s disease follow-up (AD Followup).
6.4.3 Disease v control

A comparison of the 259 metabolite peak differences between control and Alzheimer’s disease baseline patients was performed using the Mann-Whitney test. There were no significant differences between the control and disease group metabolite peaks as determined by the combination of $p$- and $q$- values (false positive rate and false discovery rate). The top 20 peaks with the lowest $q$- values reveal that peak 87 was closer to significance than the remaining peaks ($p$ value 0.00035 and $q$ value 0.076) and was close to the FDR threshold of 0.05 (Table 6.1). The distribution of metabolite peak 87 across the three groups in the study was lower in the Alzheimer’s disease group as compared to the control group (Figure 6.4).
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Table 6.1 GC-ToF-MS metabolite peaks control v Alzheimer’s disease baseline

Control v Alzheimer’s disease baseline – from 258 peaks across 118 samples (59 Control v 59 Alzheimer’s disease baseline).

Following a Mann Whitney test comparing Control v Alzheimer's disease the top 20 peaks in order of p and FDR associated q values are reported.
6.4.4 Disease progression (baseline v follow-up)

A comparison of the 259 metabolite peak differences between Alzheimer’s disease baseline and follow-up was performed using the Wilcoxon signed-rank test. There were no significant differences between the control and disease group metabolite peaks as determined by the combination of $p$- and $q$-values (false positive rate and false discovery rate). Metabolite peak 128 which had the lowest $p$ value 0.009 and $q$ value 0.592 was not within the FDR threshold of 0.05(Table 6.2).
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Table 6.2 GC-ToF-MS metabolite peaks Alzheimer's disease baseline v follow-up

AD baseline v AD follow-up only – from 258 peaks across 118 samples (59 Alzheimer’s disease baseline v 59 Alzheimer’s disease follow-up).

Following a Wilcoxon sign-ranked test comparing Alzheimer's disease baseline v Alzheimer’s disease follow-up, the top 20 differences in order of $p$ and FDR associated $q$ values are reported.
Figure 6.4  Boxplot of GC-ToF-MS mode data peak no. 87

Group 1, 2 & 3 relate to healthy control, Alzheimer’s disease baseline & Alzheimer’s disease follow-up, respectively. Peak no. 87 was not significantly altered in both healthy control v Alzheimer’s disease baseline and Alzheimer’s disease follow-up. The outliers represent the subject number in the associated group. The red line indicates the median value of the peak intensity measurement for the specific metabolite peak.
7 THE APPLICATION OF UPLC-LTQ/ORBITRAP-MS (+/-) MODE DATA TO DETECT A METABOLIC PROFILE OF ALZHEIMER’S DISEASE

7.1 INTRODUCTION

An ideal biomarker for a disease as complex as Alzheimer’s disease should be obtained quickly with minimal invasive procedures and be relatively inexpensive to test. Further the biomarker of the disease must have a high specificity and sensitivity for the disease. The biomarker should ideally also offer the ability to detect the progress of the disease.

Blood based biomarkers offer a convenient biological fluid to access, and serves as a middle ground for the body’s physiological activity fitting between the CSF and urine. Recently blood based biomarkers associated with Alzheimer’s disease have been identified. Ray et al., tested 120 selected proteins extracted from plasma samples and observed that the concentration of 18 plasma proteins was sufficient to discriminate Alzheimer’s disease and control participants, with 90% accuracy (Ray et al., 2007). Another study utilised the hypothesis-generating approach adopting an unbiased methodology to identify 15 proteins in the plasma could discriminate between Alzheimer’s disease and controls (Hye et al., 2006).

The recent introduction of UPLC and the Orbitrap mass spectrometer have increased the speed, resolution and the degree of sensitivity in analysing biological fluids (Wilson et al., 2005 Dunn et al., 2008, Dunn et al., 2011). The net effect has been
that the volume of data which can be generated from any given biological fluid is far
greater than any other current analytical technique.

The experiment detailed in this chapter used the UPLC-LTQ/Orbitrap to analyse
serum samples obtained from 60 Alzheimer’s disease patients at two time-points,
baseline and a 12 month follow-up, closely matched with age and sex with 60
controls. The analysis of the samples was undertaken using two different ionisation
modes (+ and -). Therefore the experiment to analyse the serum samples was run on
two separate occasions and as such are two different experiments, exploiting the
ionisation properties of the separated metabolites prior to detection.

7.2 MATERIALS AND METHODS

The materials and methods for the UPLC-LTQ/Orbitrap-MS in the + and –
electrospray ionisation modes. Two identical sets of serum samples were prepared
in the order as detailed in METHODS, and was the same as reported for the FT-IR
and GC-ToF-MS data. A total of 180 samples were aliquot in to two 100 µl aliquots
to obtain duplicate samples for analysis by the UPLC-LTQ/Orbitrap-MS in the + and
– electrospray ionisation modes. All samples were produced in accordance to the
standard preparation protocol described in Section 2.3. Each identical set of serum
samples was analysed together with a standard metabolite mix, blank and stock
serum QC samples by the UPLC-LTQ/Orbitrap-MS system (Section 2.5.2). Stock
serum QC samples (Sigma) were analysed at the beginning of each analytical block.
Thereafter, a pooled QC sample was analysed after every fourth plasma sample.
7.2.1 DATA PRE-PROCESSING

All data files were transformed into netCDF format and deconvolved using XCMS software (Section 2.7).

7.2.2 DATA PRE-TREATMENT AND ANALYSIS

The resultant data produced from the analysis of the serum samples in the electrospray ionisation modes both + and -, was normalised to the peak areas of stock serum QC samples. Features showing low reproducibility were removed from each data set (Section 2.8). Both normalized data matrices originating from the two ionization modes were joined into a single matrix and the data normalized to TP 0. The normalization procedure enabled the analyst control for the metabolic differences among the control and disease samples and potentially to identify the changes in metabolome that are relative to the baseline. The pre-treatment of the raw data was undertaken by Dr David Broadhurst and Dr Yun Xu.

7.3 STATISTICAL ANALYSIS

Univariate analysis with a False Discovery Rate (FDR) ≤ 0.10 was used for the $p$-values in the analysis of the metabolite data (Broadhurst & Kell). Metabolite peaks were analysed using univariate statistics to identify potential peaks which were significantly different between Alzheimer’s disease baseline v Control (Mann-Whitney test). A Wilcoxon signed-rank test was used to analyse between Alzheimer’s disease baseline v follow-up. A discriminant function analysis was performed on the top metabolite peaks to determine an overall classification
percentage, and metrics for sensitivity and specificity of the key discriminating peaks.
7.4 RESULTS - UPLC-LTQ/ORBITRAP-MS (+ IONISATION MODE)

METABOLIC PROFILING

7.4.1 Clinical Metadata

In total 180 serum samples were prepared from 120 participants, (60 Alzheimer’s disease (baseline and 12 month follow-up) and 60 controls (baseline sample only). The 60 Alzheimer’s disease participants had a mean (SD) age of 79.4 (7.12) years and were 65% female. The control participants had a mean (SD) age of 77.92 (6.82) years and were 67% female. The 60 Alzheimer’s disease participants mean (SD) baseline MMSE score was 19.82 (6.28), and the 12 month follow-up MMSE score was 16.63 (7.73). The 60 control participants mean (SD) baseline MMSE score was 28 (1.69). The 60 Alzheimer’s disease participants had a mean (SD) age of 79.4 (7.12) years and were 65% female. The control participants had a mean (SD) age of 77.92 (6.82) years and were 67% female. The 60 Alzheimer’s disease participants mean (SD) baseline MMSE score was 19.82 (6.28), and the 12 month follow-up MMSE score was 16.63 (7.73). The 60 control participants mean (SD) baseline MMSE score was 28 (1.69).

7.4.2 UPLC-LTQ/Orbitrap-MS metabolic profiling

A total of 180 samples (matched by age and sex, and constrained by sample availability) and 60 QA samples, 8 blanks were analysed in four 24 hour blocks. The total number of metabolite peaks detected was 1954. Of these peaks, 12 were
significantly altered across the three groups (controls, Alzheimer’s disease baseline and follow-up.

The total number of metabolite peaks detected was 1954 in a minimum of 80% of the samples analysed. These peaks were selected for statistical analysis as they fell within the 20% RSD limit.

An initial unsupervised PCA plot of scores was performed to potentially identify any potential clusters which could be associated with the three groups, controls, Alzheimer’s disease baseline and follow-up. No evidence of clustering of the metabolite peaks from the three groups was observed in the first two principal components. A number of metabolite peaks which were outliers in the PCA plot outliers (peaks 1735) were related to all three groups’ participants (Figure 7.1). Separate PCA scores plots for control and Alzheimer’s disease baseline; and Alzheimer’s disease baseline and follow-up also revealed some outlying peaks, but these were represented by each of the paired groups. Overall the distribution of the metabolite peaks detected in the groups was largely homogenous (Figure 7.2 and 7.3 respectively).
Serum samples (n=180), were analysed using the UPLC-LTQ/Orbitrap-MS (+), which resulted in the detection of 1954 peaks. The PCA plot shows that there was no observable separation of the three sample groups: Control, Alzheimer’s disease baseline (AD Baseline) and Alzheimer’s disease follow-up (AD Followup).
Figure 7.2 UPLC-LTQ/Orbitrap-MS Principal Component Analysis (PCA) plot of scores for control and Alzheimer’s disease.

Serum samples (n=120), were analysed using the UPLC - LTQ/Orbitrap-MS (+), which resulted in the detection of 1954 peaks. The outliers (metabolite peaks 1735) have been labelled. There was no observable separation of the two sample groups.

The PCA plot shows that there was no observable separation of the two sample groups: Control and Alzheimer’s disease baseline (AD Baseline).
Figure 7.3 UPLC-LTQ/Orbitrap-MS Principal Component Analysis (PCA) plot of scores for the Alzheimer’s disease baseline and follow-up.

Serum samples (n=120), were analysed using the UPLC - LTQ/Orbitrap-MS (+), which resulted in the detection of 1954 peaks. The outliers (metabolite peaks 1735) have been labelled. The PCA plot shows that there was no observable separation of the two sample groups: Alzheimer’s disease baseline (AD Baseline) and Alzheimer’s disease follow-up (AD Followup).
7.4.3 Control v Alzheimer’s disease

A comparison of the 1954 metabolite peak differences between control and Alzheimer’s disease baseline patients was performed using the Mann-Whitney test. There were no significant differences between the control and disease group metabolite peaks as determined by the combination of \( p \)- and \( q \)-values (false positive rate and false discovery rate). The top 20 peaks were ranked according to the FDR associated \( q \) value. The lowest \( q \)-values revealed that peak 1632 was closer to significance than the remaining peaks (\( p \) value 4.27E-05 and \( q \) value 0.084) and was close to the FDR threshold of 0.05 (Table 7.1). The distribution of metabolite peak 1632 across the three groups in the study was higher in the Alzheimer’s disease group as compared to the control group (Figure 6.4). The next peak with the lowest \( q \) value was peak 626, (\( q = 0.40 \)). The box-plots depict a centre line which represents the median value, and the bottom and the top of the box represent the 25\(^{th}\) and 75\(^{th}\) percentiles respectively.

Although no direct comparisons were made between the control group and Alzheimer’s disease follow-up data, it was noted that the relative peak intensities of the top peaks were similar (Figure 7.4-7.8).
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Table 7.1 UPLC-LTQ/Orbitrap-MS (+) metabolite peaks control v Alzheimer’s disease baseline.

AD baseline v AD follow-up only from 1954 peaks across 120 samples (60 control v 60 Alzheimer’s disease). Following a Mann Whitney test comparing Control v Alzheimer’s disease the top 20 peaks in order of p and FDR associated q values are reported. No single peak fell within the 5% q value limit.
7.4.4 Alzheimer’s disease baseline v Alzheimer’s disease follow-up (disease progression)

A comparison of the 1954 metabolite peak differences Alzheimer’s disease baseline and follow-up patients using the Wilcoxon signed-rank test. A total of 12 metabolite peaks were significantly different between disease group, as determined by the combination of $p$- and $q$- values (false positive rate and false discovery rate). The top 20 peaks were ranked according to the FDR associated $q$ value. The lowest $q$-values revealed that peak 1636, 1632, 1669, 1594, 1248 were $q <0.0001$ (Table 7.2).

The distribution of metabolite peaks 1636, 1632, 1669, 1594 and 1248 across the three groups in the study was higher in the Alzheimer’s disease baseline group as compared to the follow-up group (Figure 7.4 – 7.8). The remaining key metabolite peaks in order of lowest $q$ value was 1498, 1671, 1079, 1324, 1387 and 657 (table 7.2). The remaining distribution of metabolite peaks for metabolite peaks 1498, 1671, 1079, 1324, 1387 and 657 was higher in the Alzheimer’s disease baseline group as compared to the remaining groups (Figures 7.9 – 7.15). The metabolite peaks did not correlate with any of the metadata, including BMI, MMSE, and ADAS-Cog.
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Table 7.2  UPLC-LTQ/Orbitrap-MS (+) metabolite peaks Alzheimer’s disease baseline v follow-up.

Wilcoxon signed-rank test, with FDR at 0.05, and associated \( q \) value reported.

Top 12 peaks fall within the 5% \( q \) value limit (1636, 1632, 1669, 1594, 1248, 1498, 1671, 1074, 1078, 1324, 1387, 657).
Figure 7.4 Boxplot of UPLC-LTQ/Orbitrap-MS (+) mode data peak no. 1636

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline vs Alzheimer’s disease follow-up, p value 3.88E-09 with an FDR adjusted q value of 0.000003 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outliers are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, \( p \) value 3.24E-09 with an FDR adjusted \( q \) value of 0.000003 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outliers are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.

Figure 7.5 Boxplot of UPLC-LTQ/Orbitrap-MS (+) mode data peak no. 1632
Figure 7.6 Boxplot of UPLC-LTQ/Orbitrap-MS (+) mode data peak no. 1669

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, $p$ value 3.10E-8 0.00497 with an FDR adjusted $q$ value of 0.00002 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outliers are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Figure 7.7 Boxplot of UPLC-LTQ/Orbitrap-MS (+) mode data peak no. 1594

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, p value 3.69E-07 with an FDR adjusted q value of 0.0001 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outliers are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Figure 7.8  Boxplot of UPLC-LTQ/Orbitrap-MS (+) mode data peak no. 1248

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, p value 3.50E-07 with an FDR adjusted q value of 0.0001 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outliers are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, $p$ value 1.64E-06 with an FDR adjusted $q$ value of 0.0005 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outliers are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Figure 7.10 Boxplot of UPLC-LTQ/Orbitrap-MS (+) mode data peak no. 1671

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, \( p \) value 2.25E-06 with an FDR adjusted \( q \) value of 0.0005 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outliers are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Figure 7.11 Boxplot of UPLC-LTQ/Orbitrap-MS (+) mode data peak no. 1074

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline vs Alzheimer’s disease follow-up, $p$ value 5.01E-06 with an FDR adjusted $q$ value of 0.01 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outliers are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Figure 7.12 Boxplot of UPLC-LTQ/Orbitrap-MS (+) mode data peak no. 1078

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, p value 7.95E-05 with an FDR adjusted q value of XXX was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outlier’s are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, $p$ value 7.75E-05 with an FDR adjusted $q$ value of 0.01 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outlier’s are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.

**Figure 7.13** Boxplot of UPLC-LTQ/Orbitrap-MS (+) mode data peak no. 1324
Figure 7.14 Boxplot of UPLC-LTQ/Orbitrap-MS (+) mode data peak no. 1387

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, $p$ value 0.00016 with an FDR adjusted $q$ value of 0.023 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outlier’s are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, $p$ value 0.0003 with an FDR adjusted $q$ value of 0.04 was significantly altered in Alzheimer’s disease baseline and Alzheimer’s disease follow-up. Outlier’s are the i.d. label for the individual participant in the group. Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
7.4.4.1 Processing Discriminant Function Analysis (DFA)

Using this peak information to use as a way in which to classify the disease vs non-disease and disease progression groups, DFA was used. In this analysis the peaks which are ranked in order of the $p$ value were used to classify the groups. The metabolite peaks which fell within the FDR threshold were subjected to discriminate function analysis, to identify the key discriminating metabolite peaks. The top five metabolite peaks were able to achieve the highest overall and sensitivity and specificity values. Overall the correctly classified 73.3% using the top 5 metabolite peaks (metabolite peaks 1636, 1632, 1669, 1594, 1248). The cross-validated model in SPSS also had an overall statistic of 73.3%. The sensitivity and specificity values for the 5 metabolite peaks to discriminate between the Alzheimer’s disease baseline and follow-up groups was 70% and 76.7% specificity.
The first 5 peaks in Table 7.2 which were below the FDR threshold 0.05 were used to build a model to discriminate Alzheimer’s disease baseline v follow-up. The overall actual v predicted classification in to the two groups was 73.3% in the cross-validated model. The sensitivity and specificity of the five peaks (1636, 1632, 1669, 1594, 1248) was at 70% and 76.7%.
7.5 RESULTS - UPLC-LTQ/ORBITRAP-MS (- IONISATION) MODE
METABOLIC PROFILING

7.5.1 Clinical metadata

This is as reported in section 7.4.1.

7.5.2 UPLC-LTQ/Orbitrap-MS metabolic profiling

A total of 180 samples (matched by age and sex, and constrained by sample availability) and 60 QA samples, 8 blanks were analysed in four 24-hour blocks. The total number of metabolite peaks detected was 1372. Of these peaks, 12 were significantly altered across the three groups (controls, Alzheimer’s disease baseline and follow-up).

The total number of metabolite peaks detected was 1372 in a minimum of 80% of the samples analysed. These peaks were selected for statistical analysis as they fell within the 20% RSD limit.

An initial unsupervised PCA plot of scores was performed to potentially identify any potential clusters which could be associated with the three groups, controls, Alzheimer’s disease baseline and follow-up. No evidence of clustering of the metabolite peaks from the three groups was observed in the first two principal components. A number of metabolite peaks which were outliers in the PCA plot
outliers (peaks 201, 410, 206) were related to all three groups participants however there was a large distance between these metabolites (Figure 7.17). Separate PCA scores plots for control and Alzheimer’s disease baseline; and Alzheimer’s disease baseline and follow-up also revealed some outlying peaks, but these were represented by each of the paired groups. Overall the distribution of the metabolite peaks detected in the groups was largely homogenous, however, there was a split in the groups whereby the metabolite peaks were occupying space in the plots that was different in direction to the majority of the peaks an was pronounced in the Alzheimer’s disease baseline v follow-up group (Figure 7.18 7.19 respectively).
Figure 7.17  UPLC-LTQ/Orbitrap-MS (-) Principal Component Analysis (PCA) plot of scores for the Control and Alzheimer’s patients (baseline and follow-up) groups.

Serum samples (n=180), analysed using the UPLC - LTQ/Orbitrap-MS (-) mode, data for 1372 peaks is represented. Top panel represents the entire PCA with outliers; the bottom panel is zoomed in to the overlying groups. There is no observable separation of the three sample groups.
Figure 7.18 UPLC-LTQ/Orbitrap-MS (-) Principal Component Analysis (PCA) plot of scores for Control and Alzheimer’s disease (baseline).

Serum samples (n=120), analysed using the UPLC - LTQ/Orbitrap-MS (-) mode which resulted in the detection of 1372 peaks. The outliers (metabolite peaks 177, 409, 583, 1365, 1364, 409, 366 and 95) have been labelled. The PCA plot shows that there was no observable separation of the three sample groups: Control, Alzheimer’s disease baseline (AD Baseline)
Figure 7.19 UPLC-LTQ/Orbitrap-MS (-) Principal Component Analysis (PCA) plot of scores for Control and Alzheimer’s disease (baseline).

Serum samples (n=120), analysed using the UPLC - LTQ/Orbitrap-MS (-) mode which resulted in the detection of 1372 peaks. The outliers (metabolite peaks 177, 422, 413, 366, 583, 364 and 219), have been labelled. The PCA plot shows that there was no observable separation of the three sample groups: Alzheimer’s disease baseline (AD Baseline) and Alzheimer’s disease follow-up (AD Followup)
7.5.3 Control v Alzheimer’s disease

A comparison of the 1372 metabolite peak differences between control and Alzheimer’s disease baseline patients was performed using the Mann-Whitney test. There were no significant differences between the control and disease group metabolite peaks as determined by the combination of $p$- and $q$- values (false positive rate and false discovery rate). The top 20 peaks were ranked according to the FDR associated $q$ value. The lowest $q$- values revealed that peak 413 was closer to significance than the remaining peaks ($p$ value 0.00072 and $q$ value 0.081) and was close to the FDR threshold of 0.05 (Table 7.3). The distribution of metabolite peak 413 across the three groups in the study was higher in the Alzheimer’s disease baseline group as compared to the control group (Figure 7.20). The next peak with the lowest $q$ value was peak 831, ($q = 1.1$). The box-plots depict a centre line which represents the median value, and the bottom and the top of the box represent the 25th and 75th percentiles respectively.
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<th>p-value Mann-Whitney</th>
<th>q value</th>
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Table 7.3  UPLC-LTQ/Orbitrap-MS (-) metabolite peaks Control v Alzheimer’s disease baseline.

Mann-Whitney test with FDR at 0.05, and associated q value reported.

No single peak fell within the 5% q value limit.
7.5.4 Alzheimer’s disease baseline v Alzheimer’s disease follow-up (disease progression)

A comparison of the 1372 metabolite peak differences Alzheimer’s disease baseline and follow-up patients using the Wilcoxon signed-rank test. A total of 3 metabolite peaks were significantly different between disease group, as determined by the combination of $p$- and $q$- values (false positive rate and false discovery rate). The top 20 peaks were ranked according to the FDR associated $q$ value. The lowest $q$-values revealed that peak 302, 173, 413 were $q <0.005$ (Table 7.4).

The distribution of metabolite peaks 302, 173, and 413 across the three groups in the study was higher in the Alzheimer’s disease baseline group as compared to the follow-up group (Figure 7.20 – 7.22). The metabolite peaks did not correlate with any of the metadata, including BMI, MMSE, and ADAS-Cog.
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Table 7.4 UPLC-LTQ/Orbitrap-MS (-) metabolite peaks Alzheimer’s disease baseline v follow-up.

Wilcoxon signed-rank test, with FDR at 0.05, and associated $q$ value reported.

The top 3 peaks fall within the 5% $q$ value limit (302, 173 and 413).
Figure 7.20 Boxplot of UPLC-LTQ/Orbitrap-MS (-) mode data peak no. 302.

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, $p$ value 7.62E-06 with an FDR adjusted $q$ value of 0.005 was considered significant. Outlier’s are the i.d. label for the individual participant in the group.

Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Figure 7.21 Boxplot of UPLC-LTQ/Orbitrap-MS (-) mode data peak no. 173.

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, $p$ value $1.63E-05$ with an FDR adjusted $q$ value of $0.007$ was considered significant. Outliers are the i.d. label for the individual participant in the group.

Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
Figure 7.22 Boxplot of UPLC-LTQ/Orbitrap-MS (-) mode data peak no. 413.

Following Wilcoxon signed-rank test of Alzheimer’s disease baseline v Alzheimer’s disease follow-up, \( p \) value 7.61E-06 with an FDR adjusted \( q \) value of 0.005 was considered significant. Outliers are the i.d. label for the individual participant in the group.

Group 1 = healthy control, Group 2 = Alzheimer’s disease baseline and group 3 = Alzheimer’s disease follow-up. The red line indicates the median score of the peak intensity measurement for the specific peak.
7.5.4.1 UPLC-LTQ/Orbitrap-MS (- ionisation mode)

The total number of metabolite peaks detected was 1372. Statistical analysis of the healthy Control and Alzheimer’s disease baseline samples did not reveal metabolite peaks which had been significantly altered. Statistical analysis of the Alzheimer’s disease baseline and follow-up serum samples revealed three metabolite peaks which were significantly altered. Some of these peaks were also altered in the healthy Control and Alzheimer’s disease baseline samples, but did not achieve statistical significance.

Analysis of the peak data for longitudinal disease change produced numerous peaks that fell within the significant $p$ value and $q$ value threshold. These peaks did not correlate with any of the metadata collected, e.g. medication, or MMSE or CDR. These peaks which had significant $p$ values when subjected to classification statistics did not yield high percentage in sensitivity and specificity values. Despite this, metabolomic profiling was able to identify significant alteration of metabolites between baseline and follow-up is promising. This is because the variability in serum samples owing to biological variation is greatly reduced as these samples are from the same 60 individuals over a 12 month period. The only significant alteration in the metadata was BMI and blood pressure. The peaks detected could not be correlated with the metadata. Before links can be made to any changes in metadata with the metadata the properties of the peaks will need to be explored. A repeat of the analysis of a fresh set of serum samples would help answer the two key questions. Is serum an appropriate biological fluid to be used in metabolomic profiling experiments? If similar features can be identified, using a similar
experimental set-up as used in the experiments for this thesis, then the next step would be to identify these metabolite features, and to identify any putative link with known or unknown Alzheimer’s disease aetiology.
8 DISCUSSION

8.1 CLINICAL METADATA

The clinical data obtained from healthy controls and the Alzheimer’s disease patients revealed that the disease and control sample were closely matched for age and sex. However, a key physical difference between the two populations was related to BMI, which was significantly different with the disease group having a lower BMI as compared to controls.

Body mass in old age has been shown to be associated with Alzheimer disease pathology in persons with and without dementia (Buchman et al., 2006). BMI is an indicator of the energy metabolism in humans and the literature states that systemic metabolic changes are present early in the Alzheimer’s disease process (Burns et al., 2010). The earliest stages of Alzheimer’s disease have been shown to be associated lower body mass with and without Alzheimer’s disease (Johnson, Wilkins and Morris, 2006). Specific factors contributing to weight loss are unknown and may potentially play a role in weight loss before the development of Alzheimer’s disease (Vidoni et al., 2011). Hence, weight loss may be a preclinical indicator of Alzheimer disease especially as individuals who go on to develop Alzheimer’s disease lose a significant amount of weight 4 to 6 years before the diagnosis of the disease, with mild weight loss, doubling in the year before the clinical detection of the mildest behavioural symptoms and psychological symptoms (Johnson, Wilkins and Morris, 2006).
Biomarker levels associated with Alzheimer’s disease pathophysiology has been associated with lower BMI in a sample Alzheimer’s disease participants (Vidoni et al 2011). This is inline with autopsy evidence links Alzheimer’s disease neuropathological changes with low and declining BMI, even in individuals with normal cognition, suggesting that Alzheimer’s disease related brain changes may influence BMI, amyloid and tau (Vidoni et al., 2011 and Buchman et al., 2006).

There were as expected differences in the MMSE scores between the control and Alzheimer’s disease group, with the average MMSE score lower in the Alzheimer’s baseline group. The decline in MMSE score over a one year period in the Alzheimer’s disease baseline and follow-up group was in line with the reported literature which show that the annual decline in MMSE is approximately between 2.5 to 2.8points (Corey-Bloom and Fleisher, 2005). The ADAS-cog change over a one year period in the disease groups was also in line with the reported literature that there is an increase in the score by 7 – 9 points (Thal et al., 2000). Whilst it is accepted that the measurement of Alzheimer’s disease progression is difficult due to the variability between and within subjects (Corey-Bloom and Fleisher, 2005), the measurement of the rate of cognitive decline is appropriate in short term studies and as such clinical trials use the ADAS-Cog and the MMSE.

The majority of Alzheimer’s disease patients were receiving memory enhancers and in addition, a number of psychiatric medication were also prescribed. Over half the control group reported having blood pressure problems, and this was supported by the number of medications required for the treatment of blood pressure (usually hypertension). Hypertension has been associated with cognitive decline and
Alzheimer disease in some studies (Luchsinger and Mayeux, 2004, Luchsinger, 2008). However, the use of antihypertensive treatments has been associated with lower reported rates of Alzheimer’s disease (Petrovitch et al., 2000). Potentially this could be an important issue in the data reported and a key limiting factor.

The control and the Alzheimer’s disease population would be thought to be an adequate cross-section of the population (based on the convenience recruitment sampling method employed). As a pragmatic healthy control population in the community, there is the possibility that the control population could have an underlying Alzheimer’s disease pathology, which was not manifested in the clinical metadata.

8.2 METABOLIC FINGERPRINTING – FT-IR

In the first experiment a metabolic fingerprinting technique (FT-IR), was applied to provide serum fingerprints for Alzheimer’s disease patients and non-Alzheimer’s disease participants. The study was not focused on a particular or specific band region, and as such specific metabolite groups were not targeted or analysed. Rather the whole of the infrared region data was explored. Variation of the spectra was compared as this was likely indicative of any chemical difference between the groups. The FT-R analysis of serum samples did not reveal a disease specific fingerprint. The analysis of the spectra did not reveal any serum changes with respect to a disease such as Alzheimer’s disease or any class separation. Multivariate analysis of the sample spectra, showed no vibrational differences between the Control and Alzheimer’s disease groups. It was not possible to
discriminate between controls and Alzheimer’s disease baseline and follow-up spectra. The FT-IR spectra data could not be used successfully in classifying the longitudinal changes in Alzheimer’s disease when comparing the baseline and follow-up samples.

The aim of the research in this thesis was to adopt a hypothesis-generating approach in searching for metabolite features which could be used to discriminate disease and non-disease features, and discriminate features associated with disease progression. From the outset, the experiment was not focused on a particular or specific band region, and as such specific metabolite groups were not targeted or analysed. Rather the whole of the infrared region data was explored.

The use of FT-IR as a metabolic fingerprinting technique may not be suitable when using serum samples from Alzheimer’s disease patients and healthy controls. This can be attributed to the possibility that FT-IR is not sufficiently sensitive to discriminate between disease and non-disease samples, or longitudinal changes (over a twelve month period) within a set of disease samples. The results suggest that that the magnitude of analytical variation using FT-IR may potentially be of a similar magnitude to the biological variation of the serum samples. In comparison to serum other biological fluids such as CSF, may be amenable to metabolomic fingerprinting techniques, as there are potentially fewer sources of variations in the CSF (Wishart et al., 2008). FT-IR has been successfully used to discriminate disease and non-disease samples including diabetes (Liu et al., 2002) and more recently the FT-IR analysis of saliva samples from disease and healthy patients, was applied to identify diabetes patients using differentiating spectral features including lipid proteins and glucose
Bovine spongiform encephalopathy using brain tissue has also been successfully used to discriminate brain tissue samples (Lasch et al., 2003). Peuchant et al., 2008, recently analysed plasma samples from 25 Alzheimer’s disease patients and 75 healthy controls using the FT-IR. Spectra were captured in the mid-infra red range (4000-500 cm$^{-1}$). The spectra captured in the infra-red region 1480-1428 cm$^{-1}$ allowed for optimal separation between the disease and control groups above 95%, specifically a decrease in the intensity of the peaks in the disease group. The region represented specifically fatty acids linked to oxidative stress. The researchers concluded that the FT-IR spectra revealed a decrease in the intensity associated with vibrational changes of CH$_2$ groups and specifically fatty acids at peak 1455 cm$^{-1}$. The researchers used a large number of healthy controls to Alzheimer’s disease patients (~ 3:1), and the use of overnight fasted-plasma are the two key differences between the FT-IR data reported in this thesis. The impact of a fasted sample and the quality of plasma data may well significantly alter the potential spectra output from samples. The spectra obtained for each of the plasma samples from disease and control groups clustered into two groups. The research team also used the spectra from the entire 25 Alzheimer’s disease samples to build a multivariate model to differentiate disease and non-disease samples are too few to adequately build a model which can differentiate disease and control samples. The validation test of the classification model built by the research team involved the use of only three Alzheimer’s disease and six control samples. The problem with using too few input variables when constructing a classification model is that the learning algorithm memorises the characteristics of the variable rather than using specific features to build a model, as too few inputs have been used.
The data analysed in this thesis had sufficient sample numbers across the groups to build a viable classification model with sufficient variation to avoid the possibility of memorisation by the machine learning algorithm as used in the CVA models. Had such a technique such as FT-IR resulted in discriminating disease versus non-disease samples and in detecting disease progression, the use of such a technique would have major clinical implications. The use of a hypothesis-generating approach for metabolic fingerprinting with the FT-IR may not be appropriate for a disease such as Alzheimer’s disease. Rather a technique which is far more sensitive than FT-IR or using FT-IR to specifically target spectra within a certain band area, e.g. fatty acids which may be linked with known mechanisms of Alzheimer’s disease pathophysiology may be worth investigating.

The use of serum samples for FT-IR analysis offers a relatively cheap and quick method in the diagnosis of a disease state. However, the number of publications which have adopted a hypothesis-generating approach in researching a neurodegenerative disease is few. The analysis of disease tissue may be more amenable to the discovery of disease based features, but a biologically rich biological fluid such as serum may potentially overpower the analytical capabilities of the FT-IR.

That samples do not require significant sample preparation before-hand and thus reducing the increase in extraneous variables within samples, the use of FT-IR in the interrogation of different biological fluid and specific tissues ante-mortem is a possibility which merits further investigation.
8.3 METABOLIC PROFILING EXPERIMENTS

8.3.1 GC-ToF-MS

The metabolome consists of metabolites which number a wide variety of different compound classes. Metabolic profiling techniques can provide detailed information on the metabolome of a biological fluid. In comparative terms metabolic profiling is able to provide far more detailed and information rich data in comparison to basic metabolic fingerprinting techniques. The aim of the research in this thesis was to adopt a hypothesis-generating approach in searching for metabolite peaks which could be used to discriminate disease and non-disease features, and discriminate features associated with disease progression.

A total of 257 GC-ToF-MS derived metabolite peaks were detected during the analysis. Statistical analysis of the data using an FDR of at 5% of the healthy control and Alzheimer’s disease baseline samples did not reveal metabolite peaks which had been significantly altered. Statistical analysis of the Alzheimer’s disease baseline and follow-up serum samples also did not reveal metabolite peaks which had been significantly Metabolite peak number 87, was the only peak across both control versus disease and the disease progression samples which had the lowest $q$ value (0.076) of all the peaks. This metabolite peak would be of interest in any follow-up experiment.

GC-ToF-MS has been used successfully in the analysis of metabolic disorders and in brain associated disorders. Huntington’s disease metabolite peaks associated with a change in the Huntington’s disease samples have been identified using the GC-ToF-MS, revealing a pro-catabolic phenotype in early Huntington’s disease is linked.
directly to the onset of symptoms (Underwood et al., 2006). Other publications detailing the analysis of samples from Parkinson’s disease patients have shown that metabolic profiling using the GC-ToF-MS produced only subtle differences in the metabolome of healthy and disease urine samples only (Michell et al., 2008), concluding that GC-ToF-MS cannot discriminate disease features.

The use of GC-ToF-MS as a metabolic profiling technique has a higher affinity for tissue based sample analysis, in particular in the use of cancer tissues (Denkert et al., 2006; Denkert et al., 2008) and potentially metabolic specific disorders (Wang et al., 2005; Yi et al., 2008). Wuolikainen et al., 2011 recently analysed CSF samples from controls and patients with amyotrophic lateral sclerosis using GC-ToF-MS detecting a total of 120 metabolite peaks. Patients with a genetic predisposition (superoxide dismutase-1 gene mutation) to the disorder had a specific metabolite signature with 8 metabolites thought to be important as compared to patients with the sporadic version of the disease. Interestingly the authors were not able to detect a metabolite signature for the drug naive patients who had CSF samples taken either prior to the commencement of treatment or after patients on the drug treatment riluzole in this study. Interestingly, the researchers could not identify a riluzole specific metabolite signature. Further none of the 20 metabolite peaks were significantly altered.

The use of GC-ToF-MS in experiments for this research, were highly controlled at the sample analysis stage. Care was taken to control the extent of machine variability which is a common limitation for chromatographic techniques. The serum samples were collected with associated metadata. The collection of this
metadata, whilst rich in information did not correlate with any of the serum metabolite features with the lowest $p$ values.

The likelihood that GC-ToF-MS can be used to produce Alzheimer’s disease specific features was explored. That no significant features were identified from the analysis of serum samples should not preclude GC-ToF-MS as a metabolomics technique in the detection of disease biomarkers. However, its use in the analysis of Alzheimer’s disease serum samples highlights the possibility that it lacks potentially sensitivity in detecting serum based diseased features for Alzheimer’s disease and disease progression.

The reproducibility of data with GC-ToF-MS separation is higher than what is achieved with LC, and that the separation of compounds in a sample is higher in the gas phase than the liquid phase (Mishur and Rea, 2011). There is no evidence in the literature where the GC-ToF-MS has been used to analyse serum samples from Alzheimer’s disease samples and healthy controls.

The method employed to analyse the serum samples in this thesis used standardised procedures as reported by the HUSERMET consortium (www.husermet.com) (Dunn et al., 2011). GC-MS provides separation and detection of metabolites in a molecular weight range of 18 Da (e.g. ammonium) to 350 Da (cholesterol), including a range of relatively polar metabolite classes including amino acids, organic acids, amines and amides and sugars among others. The methods described in this thesis enabled the reliable detection of 100–200 metabolic features in a serum or plasma sample (Begley et al., 2009), where one metabolite is detected as multiple metabolic
features, a direct consequence of the formation of multiple derivatization products. The number of peaks therefore relates to a smaller number of actual metabolites.

The findings of this thesis suggest that there may be limited utility in the use of GC-ToF-MS as a metabolic profiling technique in the identification of potential metabolite peaks as biomarkers of Alzheimer’s disease.

8.3.2 UPLC-LTQ/Orbitrap-MS (+/- ionisation modes)

UPLC chromatographic techniques coupled with a technologically advanced MS (the Orbitrap), offers the most sensitive technique available to researchers involved in the pursuit of metabolic profiling. To date the UPLC coupled to the Orbitrap has not been used in the profiling of metabolic profiling experiments. The experimental data reported in this thesis are to the best of my knowledge represent the first use of UPLC Orbitrap (+/- ionisation) in the analysis of disease samples. UPLC-MS has been used in metabolic profiling, but the use of the Orbitrap is reported here for the first time.

The exploration of metabolic profiles in positive and negative mode was used as a method in a hypothesis-generating approach to analyse serum samples.

UPLC-LTQ/Orbitrap-MS (+/- ionisation mode)

The total number of metabolite peaks detected with UPLC-LTQ/Orbitrap-MS (+/-) mode was 1954 and 1372 peaks respectively. The PCA plots did not indicate any obvious groupings in the data set, and so there was no obvious class discrimination.
associated with disease and non-disease or disease progression. Statistical analysis of the data using an FDR at 5% of the control and Alzheimer’s disease baseline samples did not reveal metabolite peaks which had been significantly altered. However the $q$ value of a single peak fell within the 10% cumulative error range. The ability to replicate this finding in a repeat experiment is required.

Analysis of the positive ionisation mode derived metabolite peak data for longitudinal disease change produced numerous peaks that fell within the significant $p$ value threshold and $q$ value. These peaks were present in higher concentration in the serum at baseline than follow-up. These peaks did not correlate with any of the metadata collected, age sex BMI, MMSE, ADAS-Cog, and CDR. The peaks which had significant $p$ values when subjected to classification statistics did not yield high percentage in sensitivity and specificity values. That a number of metabolite peaks were significantly altered may be attributable to the variability in serum samples owing to biological variation. The only significant alteration in the metadata was BMI and blood pressure. The peaks detected could not be correlated with the metadata.

Analysis of the negative ionisation mode derived metabolite peak data for longitudinal disease produced three peaks that fell within the significant $p$ value threshold and $q$ value. Similar to the positive ionisation mode data, the peaks were present in higher concentration in the serum at baseline than follow-up.

Classification statistics were performed only on the positive ionisation derived metabolite peak data were used to interrogate the sensitivity and specificity of the
top twelve metabolite peaks, in distinguishing disease versus non-disease serum samples, as well as distinguishing baseline and follow-up samples. Only the top five metabolite peaks yielded the highest level of discrimination using discriminant function analysis. These statistics were used primarily as an assessment of the strength of these features in a basic classification model. Similar to the results reported by Underwood et al., 2006 and Michell et al., 2008, an over-fitting of data, is considered a fundamental limitation.

It was observed from the data that the first five peaks which were responsible for the maximum discrimination between Alzheimer’s disease baseline and 12 month follow-up samples, revealed increased levels of peak intensity for peaks 1636, 1632, 1669, 1594, 1248 and 1498 relative to control and follow-up.

Possible reasons for the increase in baseline levels of the top five discriminatory peaks for the UPLC-LTQ/Orbitrap (+) ionisation mode data may be attributable to either a systemic contamination during the sample preparation stage involving the Alzheimer's disease baseline samples, hence the increase in the metabolite peak intensities. However, if this was the case then the PCA plot would reveal a distinct clustering of the metabolite peaks for this group, and also a significant difference of the metabolite peaks detected (disease versus non-disease). Another possibility which may explain in some part the increased peak intensities of the Alzheimer’s disease baseline metabolite peaks is that these increases maybe linked to possible changes at the pathological level. Savva and colleagues have shown that with age the pathological features of Alzheimer’s disease are less prominent, whereby the neuropathology of older patients converged with an increase in the level of plaques.
and tangle pathology in non-demented patients (Savva et al., 2004). In addition the researchers observed evidence of a decrease in the prevalence of Alzheimer’s disease related pathology in the hippocampus and neocortex decreased between the ages of 70 and 90, whilst in non-demented elderly there was an increase in the neuropathology, indicating a convergence over a 20 year period. Holland et al., 2012 examined the extent of MRI measured changes in the brain of Alzheimer’s disease patients and non-disease patients, revealing a similar convergence over time of not just MRI measured change but also within the levels of CSF based biomarkers including Aβ and tau which also converged in a similar manner (Holland et al., 2012).

Whilst it is clear that a change in pathology over a one year period may not result in a significant level of change in the brain, these changes nonetheless may be cause a subtle perturbation of the metabolome which can be detected in a longitudinal sample analysis using a sensitive analytical platform such as UPLC-LTQ/Orbitrap.

A repeat of the analysis of a fresh set of serum samples would help answer the two key questions. Is serum an appropriate biological fluid to be used in metabolic profiling experiments? If similar features can be identified, using a similar experimental set-up as used in the experiments for this thesis, then the next step would be to identify these metabolite features, and to identify any putative link with known or unknown Alzheimer’s disease aetiology.

Greenberg et al 2009., also analysed plasma samples from Alzheimer’s disease patients, healthy controls and MCI patients using UPLC-MS analysis using ESI in
the negative mode. A number of metabolites were identified which were altered across the population groups, the concentrations of three bile acids (glycocholate, glycodeloxycholate and glychenodeoxycholate) was noticed to be increased in the in the Alzheimer’s patients and mild cognitive impairment patients as compared to the healthy controls. Greenberg and colleagues were unable to discriminate the groups using these key metabolites and the discriminant analysis of the data revealed a model with low predictive power. The number of participants in the study was 16 patients with Alzheimer’s disease, 12 patients with MCI and 10 healthy controls. The research groups did not use FDR nor was any other conservative \( p \) value correction not reported. The researchers concluded that the reason for the low predictive ability of the model constructed using the key metabolite peaks, was possibly due to the high levels of inter- and intra-subject variability and the small number of samples employed in the analysis (Greenberg et al 2009).

Li et al., 2010, recently also analysed plasma samples from 20 age and sex matched Alzheimer’s disease patients and healthy controls using the UPLC-MS operated in the positive mode. The BMI of the two groups was not significantly different and the Alzheimer’s disease patients were not receiving any drug treatment. The researchers were able to separate the disease and non-disease populations on the basis of PCA scores plot, with tryptophan, dihydroshingosine, phytosphingosine, hexadecasphinganine and lysophosphatidylcholines as potential biomarkers which discriminate between disease and the non-disease populations (Li et al., 2010). Further the data reported by Li et al., used drug free Alzheimer’s disease population, and this it seems was a powerful difference between the two experiments and may be reason to explain the potential metabolite peaks which could be used to discriminate
between the two populations. There is a potential that the serum samples used in this thesis were from patients who were receiving memory enhancers at the time of sampling.

A repeat of the analysis of a fresh set of serum samples would help answer the two key questions. Is serum an appropriate biological fluid to be used in metabolomic profiling experiments? If similar features can be identified, using a similar experimental set-up as used in the experiments for this thesis, then the next step would be to identify these metabolite features, and to identify any putative link with known or unknown Alzheimer’s disease aetiology.

8.3.3 How these findings fit in with recent findings

The most promising data reported in this thesis arises from the analysis of disease progression serum samples using the UPLC-LTQ/Orbitrap operated in the positive and negative ionisation modes. In line with the data reported in this thesis it is interesting to know that the Greenberg et al., and Li et al., using two different ionisation modes were able to detect peaks which were indicative of differences across healthy controls and Alzheimer’s disease patients. Whilst Li et al., 2009, were able to use PCA to differentiate healthy and disease groups, Greenberg et al were unable to show a significant level of separation. The data in this thesis does not support the findings of the Li et al., and Greenberg et al., studies, when comparing disease and healthy controls. The only significant alteration in metabolites was found within the patient group using data from UPLC (+) mode data.
As explained previously, it seems likely that the reason why no significant changes could be determined between the patient and healthy control groups using the UPLC (+) and (-) mode data could be attributed in part to the inter- and intra-subject variability. However, the results from the comparison of the Alzheimer’s disease baseline and follow-up, using the UPLC-LTQ/Orbitrap (+) mode derived data revealed a number of significantly altered metabolites. This shows that there are differences that can potentially be attributing to disease change over a 12 month period.

Secondly, this thesis shows that using different ionisation modes does affect the detection of metabolites. Using different ionisation modes permit different metabolites to be detected using MS and to exploit the nature of the metabolite based on how readily it can be ionised in to a cation or an anion. The use of different metabolomics technologies to interrogate the metabolome of a sample is both complementary and necessary.

The use of GC-ToF-MS as a potential tool for the identification of Alzheimer’s disease biomarkers may be limited. Much like the use of FT-IR as a fingerprinting technique, the data support the idea that the analytical variation versus biological variation is likely to be of a similar magnitude, and such techniques potentially lack the ability to discriminate disease and non-disease samples as well as disease progression. Whether this limit in the sensitivity of these techniques can be overcome by analysing larger sample numbers, and potentially using more sensitive analysis technology remains to be seen.
Clinical phenotypes

As described by Jorm, there is no pure Alzheimer’s disease, rather it is a heterogeneous disorder that is subject to the temporal and spatial staging of the disease pathology (Jorm 1984).

The literature describes subtypes of Alzheimer’s disease which are thought to be linked to the symptoms manifested at any given time during the course of the disease (section 1.4), the disease population for the purposes of this thesis was categorised using the current clinical gold standard diagnosis (McKhann et al., 1984), and categorised as $2/3$ with “probable” and the remaining as “possible” Alzheimer’s disease. It is possible that the total number of “probable” patients may have been too few to help reasonably detect a disease specific metabolite profile. A larger Alzheimer’s disease population with an excess of 80% patients classified as “probable” would be required to prove whether the metabolite profiling technologies used in this thesis in particular the UPLC-LTQ/Orbitrap (+/- ionisation modes), can or cannot detect a disease specific metabolite profile. Further categorisation of the Alzheimer’s disease population could be achieved by using the CDR scores to determine the global severity scale to rate both the cognitive and functional changes (Hughes et al., 1982).

The clinical changes over a one year period are recorded as a decline in a number of assessment tools used to assess the cognitive domains, including memory. The clinical metadata reported in this thesis conform to the reported general trend of significant decline in the measurement of the cognitive domains using e.g. the MMSE and the negatively scored ADAS-Cog. However, the pathological changes
over a year may not be as pronounced as the deterioration in the clinical symptoms of the disease, and may be reflected as potential subtle changes in the metabolome. In the absence of more large clinical pathological research of population-based patients, it will be difficult to understand the rate of neuropathological change over a year (Brumback-Peltz et al., 2011 and Holland et al., 2012).

One other key confounder when considering an Alzheimer’s disease population is a recent study which following the autopsy of 1100 dementia patients, found that mixed Alzheimer’s disease and vascular dementia were the most common pathological finding (Jellinger et al., 2010). Furthermore, according to Savva and colleagues the presence of vascular and other pathologies combine to lower the impact of Alzheimer’s disease specific pathology and therefore may result in potential confounders in the search of a disease specific biomarker and also that the burden of Alzheimer’s disease pathology and overall seems to decrease with age (Savva et al., 2009). Taken together it seems that the progression of Alzheimer’s disease biologically and clinically may not be causally manifested over a short period of time (e.g. one year), and may not follow a linear disease course.

**Biological variation**

In metabolomics studies, it is important to minimise the number and impact of variation, both biological and analytical. As part of the experimental design for the metabolomics data reported in this thesis, keen attention was paid to the analytical and biological variation. Whilst analytical variations were easily controlled following standardised such as sample storage, preparation, sample analysis and data analysis, biological variation was difficult to control. Inter-individual differences are
considered to be large and can mask the detection of subtle disease biomarker(s) (Scalbert et al., 2009, Dunn et al., 2011, Mamas et al., 2011). These inter-individual variability differences are largely attributable to genome, diet, age, and gender. For the data reported in this thesis, the samples used for analysis were closely matched for age and sex. For most metabolomics research studies investigating disease biomarkers, it is difficult to match the disease and non-disease samples by all measurable parameters (Lenz et al., 2004; Maher et al., 2007; and Scalbert et al., 2009). Further, in order to detect any meaningful change between disease and non-disease samples, the detected metabolites were averaged between individuals, and there is the possibility that meaningful metabolites were potentially discarded. However, the analysis of intra-individual samples is a robust method whereby each Alzheimer’s disease patient acted as his/her own control, thereby allowing the control of biological variation.

The influence of diet on the metabolome as highlighted above is one area of variation which could not be controlled in the experimental design. As part of the experimental design for this thesis, there was potential scope to further minimise the influence of the dietary metabolome by requesting all participants to undertake an overnight fast followed by blood collection on the next day. Indeed it has been proposed that in metabolomic research studies, participants should be asked to commit to a standardised diet to minimise the variation of the metabolome as measured in a biofluid, and lifestyle restriction are needed to establish disease specific perturbations of the metabolome (Lenz et al., 2004 and Walsh et al., 2006). Whilst a request for compliance with an overnight fast or a specific diet may have been a suitable proposition for most other disease studies, this was a particular issue
for Alzheimer’s disease patients. The primary reason was ethical concerns related to asking patients and their carers to ensure the patient for example did not eat prior to the blood collection visit. However this issue would need to be considered alongside the possibility that short and long term dietary habits have been shown to be an important source of biological variation. There is evidence that that diet and changes to diet can be detected using metabolite profiling, including the discovery of novel biomarkers of dietary intake (Altamaier et al., 2011, and Primrose et al., 2011). There is also evidence that the collection of fasting morning samples may introduce further variation in collected serum samples, as changes have been reported between samples collected with a 2 hour separation (Maher et al. 2007).

Where diets are controlled, there are questions which relate specifically to how long the diet should be controlled of community dwelling patients and healthy participants (Lenz et al., 2004). Highly controlled studies set within a clinical research facility could potentially be undertaken in clinical research facilities where in particular the vulnerabilities of the patients could be adequately addressed by qualified staff, whilst monitoring a strict control of the diet of patients and healthy participants.

**Alzheimer’s disease medication**

Almost all of the Alzheimer’s disease patients recruited at baseline and follow-up had been prescribed memory enhancer drugs, details of which were recorded. The impact of memory enhancers on the metabolome may therefore have served as a potential differentiator of the disease and non-disease metabolite profile. The majority of the patients used for the studies in this thesis had blood samples taken by
09:30 hours on each occasion. However, there was no specific disease versus non-disease metabolite profile signature. Some possible explanations for this anomaly are thought to relate to the possibility of the primary metabolites of the drugs were excreted by the time the blood samples were collected, or that the metabolites that were present in the serum were outside the range of detection by the instruments used in this thesis. In the main drugs bind to plasma protein and tissue protein (Alavijeh et al., 2005), in the research presented, serum samples were de-proteinated. This may explain why potential drug specific metabolite profile may not have been detected. One further possibility which cannot be ignored is that the patients were not taking their medication. One plausible explanation for the absence of a memory enhancer metabolite profile is that the primary metabolites become bound to the albumin (the albumin was removed prior to serum sample analysis).

**Power calculation**

This thesis underlies the limitations of measuring biological variability versus analytical techniques. The data presented in this thesis revealed that 60 samples per group is likely to be underpowered to detect any metabolomic changes in serum samples when comparing disease and non-disease samples, but may be sufficient to detect changes in the same population over a 12 month period, using the FDR method. The data reported in the thesis revealed that where biological differences are likely to be reduced (i.e. the longitudinal Alzheimer’s disease samples versus disease/non-disease samples), the use of the FDR method is a valid method to detect potential metabolite peaks which may be described as biomarkers of disease progression. At the outset of the research the use of 60 samples for each group was also limited by the need to control the technical variation and biological variation,
which had to be minimised to conclude with some degree of certainty whether, metabolomic profiling can be used to detect serum based metabolome biomarkers or not. Also at the experimental design stage, a sample size of 20-40 in each group was thought to be sufficient in identifying the key clinical biochemical markers (Underwood et al., 2006).

In most “omics” studies, and specifically in metabolomics studies which follow the hypothesis-generating methodology, no assumptions are made regarding the effect a disease on the metabolome. Generally, it is difficult to estimate the effect size primarily because it is unknown. Moreover, the primary aim of metabolite profiling experiments is to detect and identify different metabolite peaks which are common to the sample sets under investigation. Despite no assumption regarding the effect of Alzheimer’s disease on the metabolome being made, for the purpose of this thesis, it was however possible to obtain an estimate of an effect size based on the literature related to metabolomics and biomarker discovery in neurodegenerative disorders (section 1.14.1). The use of the number of disease and non-disease samples was a pragmatic choice and limited by time, cost and the need to minimise analytical variation.

The Alzheimer’s population recruited for this study was a convenience sample and not an epidemiological sample. The overall sample size was based on feasibility of recruitment over time, and the need to minimise machine variation over time. In addition a review of the literature revealed that the proposed number for samples as detailed in this thesis were the largest numbers used for any neurodegenerative disease (section 1.14.1).
Recently, it has been suggested that metabolomics studies should fall into two or three categories: discovery, validation and cohort validation (Dunn et al., 2011 and Mamas et al., 2011). Briefly, a small study would involve between at least 10 samples per group, with sources of biological variation including age and sex strictly controlled and thereby leaving disease and non-disease as the major biological variable. Any potential biomarker discovery could then be replicated in a new large-scale study involving 1000 plus samples per group. Potentially, these types of large-scale clinical studies would be able to validate earlier findings and be applicable to the general population (Broadhurst and Kell, 2006 and Dunn et al., 2011)

**Statistical analysis**

In metabolomic experiments the primary objective is an inductive data-driven approach which results in the attempted capture of complete or near complete biological data from any given sample. This is necessary especially when no assumption is made regarding which metabolites whole or in part known or unknown will be present in any given sample. This capturing step is a pre-requisite for the generation of hypotheses and their subsequent testing (Kell and Oliver, 2004). There are a large number of statistical multivariate data reduction and analysis techniques available which are used to interrogate metabolomics data sets (Kell, 2002; Brown et al., 2005; Broadhurst and Kell, 2006). Principal Component Analysis (PCA) as an unsupervised technique which assumes no *a priori* knowledge of class structure of the data set, reduces the number of data dimensions of the multivariate data set and projects the data into usually two reduced dimension spaces or principal components. The observed metabolite peaks from healthy controls and
Alzheimer’s disease baseline; Alzheimer’s disease baseline and Alzheimer’s disease follow-up, were observed in the PCA space to be in close proximity. Therefore one could assume that the groups overall were metabolically similar (Dunn et al., 2008). Univariate statistical analysis was performed in order to assess the characteristics of the metabolite peaks. Because of the large number of metabolite peaks detected in the metabolomic profiling experiments and the need to perform an equally large number of multiple comparison tests, type I errors (that is falsely rejecting the null hypothesis that the detected metabolite peaks came from the populations with the same mean), had to be controlled for. The FDR was used precisely because it lacks the conservative nature of the Bonferroni correction.

No adjustments for co-variates was undertaken in the data reported in this thesis. The primary justification for not adjusting for the co-variates was that the study was a pilot study and a hypothesis-generating methodology was applied. Therefore no regression analyses were performed in each study sample to examine the relation of each metabolite(s) to: BMI, blood pressure and drugs.

In comparison to any serum biomarkers, the combining of the three CSF based biomarkers (Aβ 1-42), total tau and phosphor tau 181 have been established and validated as diagnostic biomarker of Alzheimer’s disease results in >95% sensitivity and >85% specificity Marksteiner et al., 2007; Blennow et al., 2010 and Humpel, 2011).

However obtaining CSF is a complicated invasive procedure in comparison to obtaining blood, saliva or urine. The relative ease, with which blood can be obtained
from patients, is offset by the complex composition of this bio fluid and its analysis. The analysis of the components of blood, e.g. genes, proteins and metabolites are in turn subject to technological limitations. Further biological fluids such as blood are likely to contain and be affected by concurrent clinical ailments which could potentially mask the discovery of an Alzheimer’s disease specific biomarker(s). In light of the ongoing and continued advance in the detection and separation technologies which can be used in the detection of metabolites, metabolomics is still in its infancy and has potential as a method of detecting disease biomarkers for Alzheimer’s disease.

Metabolic profiles were constructed for each participant. Across the three profiling methods used, an important step would be to chemically identify each of the metabolite peaks. The ability to comprehensively characterise the biochemical signature of the serum samples would be an important forward step. This can be achieved by two methods. The first is to use online databases to compare the mass spectra of the peaks, and secondly, by exploiting two specific properties which are recorded when using the sensitive mass detection method in such the profiling methods, by comparing the mass spectrum and retention time, and comparing these properties with those of pure metabolites i.e. the pure standards which were used in the experiments. This approach would be analogous to the approach used in identifying genes based on gene sequence properties.
8.4 LIMITATIONS

Although this research was carefully prepared, I am still aware of its limitations and shortcomings.

First of all, the research was conducted on a relatively small number of participants, with only one time point blood sample for controls to ascertain whether the controls were in fact not potential converters to Alzheimer’s disease in the short term, and would have reduced the influence of biological variation. Second, the time to detect pathological change in the Alzheimer’s disease population over a period greater than the 12 month samples used in this thesis was needed. Third, since the research presented in this thesis was to investigate the possibility of using metabolomics applications to detect Alzheimer’s disease associated metabolite peaks to identify disease specific biomarkers, it seems the number of 60 samples per group used was insufficient to allow metabolite profiling experiments to detect such peaks in disease and non-disease samples, due to the magnitude of biological variation across the sample groups and within. Fourth, the metabolite peaks detected in the disease progression samples may not be related to a specific metabolite signature linked to the pathology of Alzheimer’s disease, and therefore weaken the potential impact of the findings. Finally, more exclusive inclusion criteria restricted only to “probable” Alzheimer’s disease patients should have been used, to increase the likelihood of detecting disease specific biomarkers.
Alternative Strategies

This thesis underlies the limitations of measuring biological variability versus analytical techniques in an Alzheimer’s disease, which has highly complex neuropathology and wide ranging neuropsychiatric symptoms. The data presented in this thesis reveal that a sample set of n=60 are likely to be underpowered to detect any metabolomic changes in serum samples when comparing disease and non-disease samples. However, the limitations of the technologies especially GC and UPLC to analyse a maximum of 60 samples per block is a limiting factor that can only be overcome with the ability of these machines to improve the balance of machine drift and the challenge of robust and reproducible findings. Until this can be achieved the complementary use of UPLC (+ and -) mode data to analyse serum samples collected at regular time-intervals from the same patient population and at risk control population may be the most effective way forward in maximising the identification of metabolite peaks associated with the onset and progression of Alzheimer’s disease.

Validation of potential biomarkers, would be a logical follow-on after a repeat of experimentation on a new set of serum samples from a separate group of Alzheimer’s disease patients and healthy controls.

One additional avenue worth exploring and maximising the metabolome data would be to couple imaging techniques in individuals with mild cognitive impairment, to predict the likelihood of conversion to Alzheimer’s disease. This is a pertinent approach as recent studies have indicated that so-called neuroimaging candidate biomarkers include, volume changes in the hippocampus and entorhinal cortex, the
nuclei of the basal forebrain, and cortical thickness. A combination of neuroimaging and neurochemical biomarkers could provide an optimum course of investigation.

8.5 STRENGTHS

The data reported in this thesis are the first to be undertaken and reported to the best of the author’s knowledge. For the first time a metabolomics analysis of serum samples using a different metabolomics analyses and techniques has been reported.

The experimental methods reported in this thesis were based on developed and optimised standard operating procedures and have been used to analyse approximately 7000 serum samples as part of the HUSERMET project. These procedures do provide some level of confidence when considering issues of experimental repeatability and reproducibility.

The subjects recruited as part of the research reported in this thesis were real world disease patients and healthy controls (that is with no known memory problems), and were selected and recruited based on strong clinical criteria directly from memory clinics in the Greater Manchester region. Further, there was no specific screening of the Alzheimer’s disease patients when compared to clinical trials.

The selection of patient and control samples was automated using a computer program.

It is important to note that there have to date been no detailed studies which have assessed the stability of the metabolites in serum at -80°C. The recommendations of
the HUSERMET Consortium regarding the stability of frozen serum samples is that multiple freeze/thaw processes were not used during the sample preparation stages, as the serum samples were divided into multiple aliquots at the pre-freezing stage. Only a single aliquot for each participant was used for each type of analytical analysis of the samples during the experimental stage (Dunn et al., 2011).

The mechanism of EI ionization had minimal instrument-to-instrument variability and provides highly reproducible and characteristic fragmentation patterns, enabling the resulting mass spectra to be used for determination of chemical structure (Dunn et al., 2011).
9 CONCLUSION

This thesis shows that metabolic profiling techniques of serum samples have a practical application in the discovery biomarkers of Alzheimer’s disease. The use of the UPLC-LTQ/Orbitrap where ions have been generated in both the positive and negative ESI mode is reported for the first time. This is the first study to have analysed disease progression serum samples. The detected metabolite peaks which were significantly altered within an Alzheimer’s disease population over a 12 month period are reported for the first time, and may be associated with the progression of Alzheimer’s disease in the serum over a one year period (within the same population). Metabolic profiling techniques involving UPLC-LTQ/Orbitrap (where ions have been generated in both the positive and negative ESI mode) are sufficiently sensitive to overcome the biological variation inherent in the serum samples when comparing the same disease population over one year period.

The metabolic fingerprinting technique involving FT-IR lacks the technical sensitivity to overcome the biological variation inherent in the entire serum samples. Metabolic profiling techniques involving GC-ToF-MS may lack the technical sensitivity to overcome the biological variation inherent in the serum samples. Metabolic profiling techniques involving UPLC-LTQ/Orbitrap are less likely to detect metabolite peak differences between two different populations owing to the limited technical sensitivity which is required to overcome the biological variation inherent in the serum samples.
The use of different metabolomics technologies to interrogate the metabolome of a biological fluid such as serum is practicable, complementary and important in the discovery of disease related changes in the metabolome. Further studies are warranted to validate the findings presented in this thesis that the UPLC-LTQ/Orbitrap can be used to detect metabolite peaks in a prospective longitudinal study.

10 FUTURE DIRECTIONS AND INVESTIGATIONS

Future research in this field should investigate samples from people with and without Alzheimer’s disease; in addition the findings of the profiling experiments as detailed in this thesis should also be repeated. First, the individual metabolite peaks which have been shown to be altered significantly in the data presented in this thesis should be identified and new experiments undertaken to see if these findings can be replicated, using multiple blood samples collected over a number of years. Close monitoring of diet and selection of participants would be required in order to minimise potential environmental influence of biological variation of the samples. If such peaks are replicated, then large-scale of prospective studies involving multiple groups of patients with Alzheimer’s disease and controls should be undertaken in order to validate these findings. The set of validated metabolite peaks would then need to be identified using online databases and linked according to their function to known metabolic networks. These metabolic peaks would need to be investigated following a literature review to identify links to any known neurodegenerative processes. Studies should also be undertaken involving healthy controls and patients, who are assessed and blood samples obtained over a number of years to detect meaningful metabolomic changes, with each study participant acting as
his/her own control. This would minimise the potential influence of inter-individual variation, which could potentially otherwise mask the detection of meaningful metabolome change linked to the onset of Alzheimer’s disease and the progression over a period of five years. Future studies should also aim to recruit patients who have not been administered memory enhancers, and over a period of 12 months obtain blood samples in order to identify a drug response signature by comparing and categorising patients as drug responders and non-responders to treatment, mapping such changes would enable the identification as possible future targets for pharmacotherapy or personalised medication in the treatment of Alzheimer’s disease.
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28 September 2005

Dear Professor Burns

Full title of study: Metabolomics in health and Alzheimer's disease, version 1.0

REC reference number: 05/Q1403/151

Thank you for your letter of 01 September 2005, responding to the Committee’s request for further information on the above research and submitting revised documentation.

The further information was considered at the meeting of the Sub-Committee of the REC held on 21 September 2005. A list of the members who were present at the meeting is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Application</td>
<td>4.1</td>
<td>03 June 2005</td>
</tr>
<tr>
<td>Investigator CV</td>
<td>1</td>
<td>03 June 2005</td>
</tr>
<tr>
<td>Protocol</td>
<td>1.0</td>
<td>01 June 2005</td>
</tr>
<tr>
<td>Covering Letter</td>
<td></td>
<td>03 June 2005</td>
</tr>
<tr>
<td>Letter from Sponsor</td>
<td>1</td>
<td>18 October 2004</td>
</tr>
</tbody>
</table>

An advisory committee to Greater Manchester Strategic Health Authority.
Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q1403/151 Please quote this number on all correspondence

With the Committee’s best wishes for the success of this project

Yours sincerely

Dr Philip G Haji-Michael
Chair

Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments
Standard approval conditions SL-AC2
Site approval form

Copy to: Manchester Mental Health and Social Care Trust
Research and Development Manager
Chorlton House, 70 Manchester Road
Chorlton cum Hardy
M21 9UN

An advisory committee to Greater Manchester Strategic Health Authority
South Manchester Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 21 September 2005

Committee Members:

<table>
<thead>
<tr>
<th>Name</th>
<th>Profession</th>
<th>Present?</th>
<th>Notes</th>
</tr>
</thead>
</table>

Chair: Dr Philip Haji-Michael, Consultant Anaesthetist, Christie Hospital NHS Trust

Vice-Chair: Dr Ann Wakefield, University Representative, Coupland Building

Ms Bridget Simpson, Research Nurse, SMUHT

Also in attendance:

<table>
<thead>
<tr>
<th>Name</th>
<th>Position (or reason for attending)</th>
</tr>
</thead>
</table>

Ms Cynthia Carter, REC Co-ordinator

An advisory committee to Greater Manchester Strategic Health Authority
South Manchester Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

<table>
<thead>
<tr>
<th>REC reference number:</th>
<th>05/Q1403/151</th>
<th>Issue number:</th>
<th>1</th>
<th>Date of issue:</th>
<th>28 September 2005</th>
</tr>
</thead>
</table>

Chief Investigator: Professor Alistair Burns

Full title of study: Metabolomics in health and Alzheimer's disease, version 1.0

This study was given a favourable ethical opinion by South Manchester Research Ethics Committee on 21 September 2005. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

<table>
<thead>
<tr>
<th>Principal Investigator</th>
<th>Post</th>
<th>Research site</th>
<th>Site assessor</th>
<th>Date of favourable opinion for this site</th>
<th>Notes (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Professor Alistair Burns</td>
<td>Professor of Old Age Psychiatry</td>
<td>Memory Clinic, Laureate House, Wythenshawe Hospital</td>
<td>South Manchester Research Ethics Committee</td>
<td>28/09/2005</td>
<td></td>
</tr>
<tr>
<td>Dr Nilin Purandare</td>
<td>Senior Lecturer / Hon. Consultant in Old Age Psychiatrist</td>
<td>Park House, Manchester Mental Health &amp; Social Care Trust, North Manchester General Hospital</td>
<td>North Manchester Local Research Ethics Committee</td>
<td>28/09/2005</td>
<td></td>
</tr>
</tbody>
</table>

Approved by the Chair on behalf of the REC:

\[\text{Signature of REC Co-ordinator}\]

\[\text{Cynthia Carter} \quad \text{Name}\]

\((1)\) The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.