METABOLOMICS ANALYSIS OF BLOOD AND URINE TO IDENTIFY ALCOHOL-DEPENDENCE BIOMARKERS

by

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بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

۞ يَسۡـَٔلُونَكَ عَنِ ٱلۡخَمۡرِ وَٱلۡمَيۡسِرِۖ قُلۡ فِيهِمَآ إِثۡمٞ كَبِيرٞ وَمَنَٰفِعُ لِلنَّاسِ وَإِثۡمُهُمَآ أَكۡبَرُ مِن نَّفۡعِهِمَاۗ وَيَسۡـَٔلُونَكَ مَاذَا يُنفِقُونَ قُلِ ٱلۡعَفۡوَۗ كَذَٰلِكَ يُبَيِّنُ ٱللَّهُ لَكُمُ ٱلۡأٓيَٰتِ لَعَلَّكُمۡ تَتَفَكَّرُونَ ٢١٩

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(They ask you about wine and gambling. Say, "In them is great sin and [yet, some] benefit for people. But their sin is greater than their benefit." And they ask you what they should spend. Say, "The excess [beyond needs]." Thus Allah makes clear to you the verses [of revelation] that you might give thought).

Quran, Surah 2 (Al-Baqara) : 219

DEDICATION

I would like to dedicate this work to my parents and my siblings Arwa, Moaaz, Anas, Baraa and Somaya, for their unconditional encouragement and support throughout my Master.

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LIST OF SYMBOLS AND ABBREVIATIONS

AD	Alcohol-Dependence
ADH	Alcohol dehydrogenase
ALDH	Acetaldehyde dehydrogenase
ALT	Alanine Aminotransferase
ANNs	Artificial Neural Networks
AST	Aspartate Aminotransferase
ASM	Acid Sphingomyelinase
APTT	Activated Partial Thromboplastin Time
AUC	Area under the Curve
AUDIT	Alcohol Use Disorders Identification Test
AUDIT-C	Alcohol Use Disorders Identification Test-Consumption
AUROC	Area under the receiver operating characteristic
B-BIOREFCODE	Bruker Biofluid Reference Compound Database
BML	Birmingham Metabolite Library
BMRB	Biological Magnetic Resonance Data Bank
CDT	Carbohydrate-Deficient Transferrin
COPD	Chronic Obstructive Pulmonary Disease
CPMG	Carr Purcell Meiboom Gill
CVD	Cardiovascular Diseases
CYP2E1	Cytochrome P450
df	Degrees of Freedom
DFA	Discriminant Function Analysis
D_2O	Deuterium Oxide
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders-IV

ETG	Ethyl Glucuronide
ETS	Ethyl Sulfate
FDA	Food and Drug Administration
g	Gravity
g	Gram
GGT	Gamma Glutamyltransferase
HIV	Human Immunodeficiency Virus
HMDB	Human Metabolome Database
HPP	Hospital Pulau Pinang
ICC	Intra-Class Correlation Coefficient
Jres	J-resolved
KMO	Kaiser-Meyer-Olkin
LC	Liquid Chromatography
LD	Light Drinkers
MAST	Michigan Alcoholism Screening Test
MCV	Mean Corpuscular Volume
MHD	Moderate to High Drinkers
ml	milliliter
mM	millimolar
MREC	Medical Research and Ethics Committee
MS	Mass Spectroscopy
NAD	Nicotinamide Adenine Dinucleotide
Na_2HPO_4	Di-Sodium Hydrogen Phosphate
NaH_2PO_4	Sodium Dihydrogen Phosphate
NaN_3	Sodium Azide

NIH	National Institute of Health
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
OPLS-DA	Orthogonal Partial Least Square-Discriminated Analysis
PC	Principal Component
PCA	Principal Component Analysis
PEth	Phosphatidylethanol
PLS	Partial Least Square
ppm	Parts Per Million
PT	Prothrombin Time
SD	Standard Deviation
SE	Standard Error
SIMCA	Soft Independent Modeling Of Class Analogies
TSP	Sodium 3-(Trimethylsilyl)-Propionate-2,2,3,3-d_4
UV	Unit Variance
VIP	Variable Influence on Projection
WHO	World Health Organization
µL	microliter

LIST OF PUBLICATIONS AND CONFERENCES

Journal Paper /Abstract

Hamza Mohamed Amin Mostafa, Arwa Mohamed Amin Mostafa, Nor Hayati Arif, Chin-Hoe Teh, Vikneswaran a/l Murugaiyah&Baharudin Ibrahim (2014).Metabolomic analysis of blood andurine to identify alcohol-dependencebiomarkers. The Medical Journal of Penang Hospital, Supplement 2014. P.9.

Hamza Mostafa, Arwa M. Amin, Nor Hayati Arif, Chin-Hoe Teh, Vikneswaran a/l Murugaiyah, Baharudin Ibrahim (2015). Nuclear magnetic resonance spectroscopy based metabolomics to identify novel biomarkers of alcohol dependence. Asia-Pacific Psychiatry journal.Submitted.

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ANALISIS METABOLOMIK DARAH DAN URIN UNTUK MENGENAL PASTI PENANDA BIOLOGI KEBERGANTUNGAN ALKOHOL

ABSTRAK

Penyalahgunaan alkohol boleh membinasakan kesihatan masyarakat dan mengakibatkan masalah sosial yang teruk.Kebergantungan alkohol merupakan fasa penyalahgunaan alkohol sebagai akibat pengambilan alkohol dalam kuantiti yang berlebihan dan sentiasa terdorong untuk mengambil alkohol secara berterusan.Kaedah sedia ada untuk mengenalpasti kebergantungan alkohol adalah melalui soal selidik dan beberapa penanda biologi.Malangnya, kedua-dua kaedah ini mempunyai sensitiviti dan spesifisiti yang rendah.Metabolomik adalah lapangan saintifik yang novel yang menyediakan kaedah novel untuk mengenalpasti kebergantunagn alkohol dengan menggunakan teknik sensitif and spesifik seperti resonans magnet nukleus (NMR).Tujuan kajian ini adalah mengenalpasti pencapjarian metabolit ("metabotype") yang dapat membezakan antara kebergantunagn alkohol, peminum tetapi tidak bergantung pada alkohol dan individu bebas alkohol dengan meggunakan kaedah metabolomik.Kajian ini turut dikaji dalam kebolehulangan. Sampel darah dan urin dikumpul daripada 30 individu yang bergantungan kepada alkohol (purata umur: 45.7), 54 peminum sosial (purata umur: 39.5) dan 60 kawalan (purata umur: 37.1). Plasma dipisahkan melalui pengemparan, kemudian sampel plasma dan urin dicampur bersama larutan tampan fosfat yang disediakan dengan melarutkan di-natrium hidrogen fosfat (Na_2HPO_4), natrium dihidrogen fosfat(NaH_2PO_4), natrium 3-(trimetilsilil)- propionat -2,2,3,3-d_4 (TSP), natrium azida (NaN_3) and deuterium oksida (D_2O) seterusnya analisis melalui spektroskopi NMR dijalankan. Data dianalisis menggunakan analisis multivarian termasuk analisis komponen utama dan analisis perbezaan ortogon separa kuasa dua

terkecil (OPLS-DA) diikuiti regresilogistik univariate dan multivariat untuk membangunkan model bagi pengenalpastian penanda biologi AD.Manakala, analisis kebolehulangan menggunakan pekali kolerasi antara-kelas (ICC).

Kajian dalam plasma, model OPLS-DA mendedahkkan 39 pembolehubah yang mempengaruhi unjuran(VIPs) secara signifikan yang dapat membezakan antara kumpulan AD dengan peminum sosial dan kawalan. Kesensitifan, kespesifikan dan kejituan model ini masing-masing adalah 64.29%, 98.17% dan 91.24%. Manakala dalam urin, model OPLS-DA menunjukkan 59 VIPs yang membezakan secara signifikan antara AD daripada peminum sosial dan kawalan. Kesensitifan, kespesifikan dan kejituan model ini masing-masing adalah 86.21%**,** 97.25% and94.93%. Analisis dalam plasma menggunakan regresi logistik univariat mendapati 9 puncak yang berkaitan secara signifikan dengan AD dengan nilai $p \leq 0.1$. Seterusnya analisis dalam regresi logistik multivariat membuktikan 4 puncak secara signikan berkait dengan AD dengan luas permukaan koefisi lingkungan penerima (AUROC), 0.961 . Kesensitifan, kespesifikan dan kejituan model ini masing-masing adalah 78.6%, 98.2% dan 94.2%. Manakala analisa dalam urin menggunakan regresi logistik univariate, 30 puncak dikenalpasti mempunyai kaitan signifikan dengan AD dengan nilai $p \leq 0.1$. Analisis menggunakan PCA, mendapati 5 PCs mempunyai kaitan yang signikan dengan AD. Kemudian melalui analisis regresi logistik multivariate, mendedahkan 2 PCs yang mempunyai 18 puncak adalah berkait secara signifikan dengan AD dengan AUROC, 0.909. . Kesensitifan, kespesifikan dan kejituan model ini masing-masing adalah 65.5%**,** 99.1%and92%.

Melalui kajian ini, aplikasi teknik metabolomik menggunakan plasma dan urin dapat membezakan antara individu kebergantungan alkohol , peminum sosial dan kawalan. Metabolomik berdasarkan resonans magnetik nukleus dapat mengenalpasti penanda biologi yang novel dalam plasma dan urin bagi diagnosis AD untuk kegunaan masa hadapan.

METABOLOMICS ANALYSIS OF BLOOD AND URINE TO IDENTIFY ALCOHOL-DEPENDENCE BIOMARKERS

ABSTRACT

Alcohol misuse is a ravaging public health and social problem. Alcohol-dependence (AD) is a phase of alcohol misuse in which the drinker consumes excessive amount of alcohol and have a continuous urge to consume alcohol. The current methods of alcohol dependence diagnosis are questionnaires and some biomarkers. However, both methods lack specificity and sensitivity. Metabolomics is a novel scientific field which may provide a novel method for the diagnosis of alcohol-dependence by using a sensitive and specific technique such as nuclear magnetic resonance (NMR). Our aim was to identify the metabolic fingerprint (metabotype) that is able to discriminate between AD, non-AD alcohol drinkers and controls using metabolomics approach. The reproducibility of the outcomes was also investigated. Blood and urine samples were collected from 30 alcohol-dependent (mean age: 45.7), 54 social drinkers (mean age: 39.5) and 60 controls (mean age: 37.1). Plasma was separated by centrifugation, then both plasma and urine samples were mixed with phosphate buffer which was prepared by dissolving di-sodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 (TSP), sodium azide (NaN_3) and deuterium oxide (D_2O)and then analyzed using NMR spectroscopy. Data analysis was done using multivariate analysis including principal component analysis (PCA) and orthogonal partial least squarediscriminateanalysis (OPLS-DA) followed by univariate and multivariate logistic regression to develop a model to identify the AD biomarkers. The reproducibility was done using intraclass correlation coefficient (ICC).

For plasma, the OPLS-DA model revealed 39 bins with VIP (variable influence on projection) value more than 1 significantly discriminated AD from social drinkers and controls. The sensitivity, specificity and accuracy of the model were 64.29%, 98.17% and 91.24% respectively. For urine, the OPLS-DA model revealed 59 bins with VIP value more than 1 significantly discriminated AD from social drinkers and controls. The sensitivity, specificity and accuracy of the model were 86.21%**,** 97.25% and94.93% respectively. In the univariate logistic regression analysis of plasma, 9 peaks were significantly associated with AD with the p value $\leq$ 0.1. In the multivariate logistic regression analysis, 4 peaks were significantly associated with AD with area under the receiver operating coefficient (AUROC) 0.961. The sensitivity, specificity and accuracy of the model were 78.6%, 98.2% and 94.2% respectively. In the univariate logistic regression analysis of urine,30 peaks were significantly associated with AD with the p value $\leq$ 0.1. The PCA revealed 5 PCs were significantly associated with AD. In the multivariate logistic regression analysis, 2 PCs which consisted of 18 peaks were significantly associated with AD with AUROC 0.909. The sensitivity, specificity and accuracy of the model were 65.5%**,**99.1%and92% respectively.

In this study, we have shown that the applied plasma and urine metabolomics technique was able to differentiate between the alcohol-dependent and the social drinkers and the controls. Nuclear magnetic resonance based metabolomics was also able to identify novel biomarkers in plasma and urine which can be useful to diagnose AD in the future.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 Background

1.1 The global burden of alcohol drinking

Alcohol is one of the most historical substances that has been misused by man until today. Alcohol drinking is a ubiquitous public health problem. Its detrimental effect does not only ravage the consumers but also their families and societies. The World Health Organization (WHO) reported 2.5 million deathsevery year due to the hazardous consumption of alcohol(IAS, 2013; WHO, 2011). The United States reported 24,518 death cases due to alcohol consumption in 2009, of which 15,183 of them died due to alcoholic liver cirrhosis(NCHS, 2009). Many studies have shown that the harmful effects of alcohol consumption is the highest compared to other illicit drugs(van Amsterdam & van den Brink, 2013). Besides its harmful effect on vital organs such as liver and kidney(Lieber, 2003), alcohol also has negative effects on cognitive perception, mood and behavior. Alcohol intoxication usually leads to drastic and destructive behavior. This will create social problems to the community. It also increases the incidence of accidents, injuries and crimes(Couture, Brown, Tremblay et al., 2010; Vaaramo, Puljula, Tetri et al., 2012).

Alcohol consumption is estimated to be the third risk factor for disease burden(Lieber, 2003).It was reported that there are some caseswhere alcohol consumption causedimmune system impairment(Karavitis & Kovacs, 2011). Therefore, chronic alcohol consumers usually have high susceptibility to acquire fatal infections(Cheng & Currie, 2005; Romeo, Warnberg, & Marcos, 2010).

Moreover, it has a negative impact on the vascular system by causing hemorrhagic stroke, arrhythmia and high blood pressure(McKee & Britton, 1998; J. Rehm, Room, Graham et al., 2003).

1.2 Alcohol consumption in Malaysia

In Malaysia, according to the WHO report in 2005, alcohol consumption has increased since 2001 with beer as the highest consumed type of alcohol (WHO, 2005).

A research that was conducted to investigate the effects of alcohol drinking and drug abuse on fatal road accidents in urban areas of Kuala Lumpur, between year 2006 and year 2009, indicated that 23.3% of fatal drivers were positive to alcohol, 11% positive to drug abuse and 2.3% were positive to both(Norlen Mohamed, Batcha, Nurul Kharmila Abdullah et al., 2012). A recently published study, that aims to find the etiologies of liver cirrhosis in Malaysia, revealed that alcohol is the main cause of liver cirrhosis among Indian Malaysians, and the fourth cause of liver cirrhosis among Chinese Malaysians(Qua & Goh, 2011). Such scrutiny sheds light on the increasing harmful effects of chronic alcohol consumption in Malaysia. In fact, this requires a thorough investigation of possible solutions that can early prevent the devastating consequences of chronic consumption of alcohol.

1.3 Organ damage induced by alcohol consumption

A growing body of literature has asserted the harmful effects of alcohol consumption to the vital organs. Although some studies advocated cardio protective

role of moderate alcohol consumption(Boto-Ordonez, Urpi-Sarda, Queipo-Ortuno et al., 2013; Wallerath, Poleo, Li et al., 2003), other studies contradicted this by indicating that the protective role is overvalued(Fillmore, Stockwell, Chikritzhs et al., 2007; Plunk, Syed-Mohammed, Cavazos-Rehg et al., 2013; van Amsterdam & van den Brink, 2013). Such contentious literature robustly defeat any suggested beneficial effect of alcohol in the prevention of cardiovascular diseases.

In addition to cardiovascular diseases (CVDs), previous studies have found that the average volume of alcohol consumption is positively correlated with liver cirrhosis, depression, gastric ulcer and osteoporosis(Aguilera-Barreiro Mde, Rivera-Marquez, Trujillo-Arriaga et al., 2013; Fini, Salamanna, Veronesi et al., 2012; McKee & Britton, 1998; J. Rehm et al., 2003; van Amsterdam & van den Brink, 2013). It was concluded that alcohol consumption causes malabsorption, oesophageal reflux and diarrhea(Bujanda, 2000). Besides,alcohol drinkingalso reduces fertility in both males and females (van Amsterdam & van den Brink, 2013). The literature has asserted that different patterns of alcohol consumption is a risk factor of several types of cancer such as liver cancer, breast cancer, colon cancer, renal cell cancer and oropharyngeal cancer(Baan, Straif, Grosse et al., 2007; Bagnardi, Rota, Botteri et al., 2013; Bujanda, 2000; Radoi, Paget-Bailly, Cyr et al., 2013; J. Rehm et al., 2003). A recent meta analysis that aims to investigate the association between light alcohol drinking and different types of cancer has concluded that light drinking is associated with oropharyngeal cancer, breast cancer and oesophageal cancer(Bagnardi et al., 2013). In other words, chronic alcohol consumption can lead to injury and then to subsequent failure of vital organs. This requires some effort to be made to encourage the avoidance of alcohol consumption. Moreover, the early identification of people

who might be under the harmful effects of chronic alcohol consumption will help to save their vital organs.

1.4 Alcohol metabolism in human

The alcohol is metabolized in the human body to acetaldehyde using three enzymes. These enzymes are alcohol dehydrogenase (ADH), cytochrome P450 (CYP2E1) and catalase, which work by oxidizing ethanol to acetaldehyde. After that, the acetaldehyde will be metabolized to acetic acid by acetaldehyde dehydrogenase (ALDH). These two steps of metabolizing ethanol to acetic acid will increase the ratio of NADH/NAD^+ ratio (Zakhari, 2006).

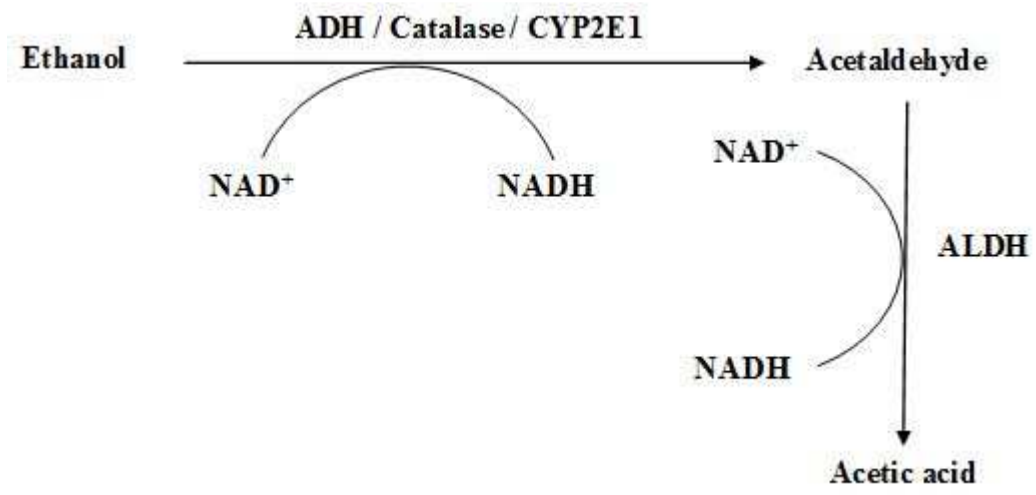

Figure 1.1: Ethanol metabolism in human body

1.5 Alcohol-dependence (AD)

The National Health Committee, Wellington-New Zealand, in their guidelines for recognizing, assessing and treating alcohol and cannabis abuse in

primary care, has adapted a definition for alcohol dependence (AD) based on the American Psychiatric Association's classification system (DSM-IV), as the following(BPAC, 2010; "Guidelines for Recognising, Assessing and Treating Alcohol and Cannabis Abuse in Primary Care," 1999):

"*A maladaptive pattern of alcohol use leading to clinically significant impairment or distress*".

This distress has many symptoms, such as a desire to drink more amounts of alcohol to attain intoxication effect, spending a lot of time either consuming alcohol or recovering from it, refraining fromsocial life activities, searching for events or places that will include drinking alcohol, making ineffective efforts to cut down alcohol consumption and having physical disorders after reducing the amount of consumed alcohol such as tremors, anxiety, hallucination, nausea and vomiting(BPAC, 2010; "Guidelines for Recognising, Assessing and Treating Alcohol and Cannabis Abuse in Primary Care," 1999).

Unfortunately, the data shows that when the habit of drinking alcohol starts earlier in age, the susceptibility to develop AD is higher(Grant & Dawson, 1997). It could be inferred that when individuals become known as alcohol dependent, they would already have gone through a long period of exposure to the detrimental effects of alcohol consumption and hence will suffer failure of some of their vital organs. As an early diagnosis of diseases will lead to an early start of treatment, early detection of AD is crucial for preventing organs failure. For instance, the early detection of AD might prevent the progression of liver injury to anirreversible phase.

AD can be treated by ALDH inhibitor such as disulfiram (Carroll, Nich, Ball et al., 1998), or by opioid antagonist such as naltrexone (Volpicelli, Alterman,

Hayashida et al., 1992), or by topiramate(Johnson, Ait-Daoud, Bowden et al.) in a standardized medication compliance management of AD.

1.5.1 Current diagnosticmethods of alcohol-dependence (AD)

Currently, AD questionnaires are the main clinical methods to diagnose the disease in clinical practice. Some biomarkers can also be used to aid the diagnosis. As biomarkers are usually produced by the body in response to disease or organ injury(Kumar & Sarin), a group of biomarkers were deduced from studies which investigated the harmful effects of alcohol consumption on body organs(Adias, Egerton, & Erhabor, 2013; Freeman & Vrana, 2010; Litten, Bradley, & Moss, 2010). Therefore, the biomarkers of organ injury, such as liver injury, are used as biomarkers of AD as well(Adias et al., 2013).However, their specificity to AD is reduced because they are biomarkers of organ injury regardless of the cause of injury(Adias et al., 2013; Freeman & Vrana, 2010). These biomarkers may lead to a confusionof diagnosis of AD with other diseases, such as non-alcoholic liver cirrhosis(Litten et al., 2010).

1.5.1.1 AD questionnaires

Several questionnaires have been developed to indicate alcohol dependence diagnosis. The commonly used questionnaires are the alcohol use disorders identification test (AUDIT), the short version of AUDIT (AUDIT-C) and Michigan alcoholism screening test (MAST)(BPAC, 2010). These questionnaires consist of a set of questions that can differentiate between harmful and dependent alcohol consumption, and predict the leading risk factors of some diseases associated with alcohol consumption.

The main drawback of AD diagnostic questionnaires is their subjective nature of measurement. They may not provide an actual estimation of an individuals' alcohol consumption due to the denial and under reporting of the regular consumed amount of alcohol. Moreover, there are limitations in the validations of these questionnaires due to the lack of standard questionnaires or definite diagnostic tool to be used as validation reference(Kroke, Klipstein-Grobusch, Hoffmann et al., 2001).

1.5.1.2 Biomarkers

Biomarkers are biological indicators of a specific medical condition or disease which can be tested or measured by using lab tools. The National Institute of Health (NIH)Biomarkers Definitions Working Group had defined a biomarker as " *a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention*" (NIH Biomarkers Definitions Working Group, 2001)(Freeman & Vrana, 2010). For example, the increase in serum creatinine level and consequently the decrease in creatinine clearance are used as biomarkers to diagnose and monitor renal diseases(Duncan, Heathcote, Djurdjev et al., 2001; Myers, Miller, Coresh et al., 2006).

The second method to diagnose AD is by testing AD biomarkers in biofluids. In fact, studies that were conducted to investigate the mechanisms of alcohol induced organ damage have found several mechanisms/pathophysiology of injuries involving certain biomarkers. For example, the rise of liver enzymes such as Alanine Aminotransferase (ALT), Aspartate aminotransferase (AST) and Gamma Glutamyltransferase (GGT) in AD individuals was obviously an indicator of alcohol induced liver injury(Adias et al., 2013). These differences in the levels of AST and

GGT in AD individuals compared to their abstainers had been found to be significant in several studies(Adias et al., 2013; Quaye, Nyame, Dodoo et al., 1992).The altered level of inflammatory mediators such as (cytokines) due to alcohol consumption had been correlated with the harmful effects of alcohol on bone, lung, liver and other tissues(Birkedal-Hansen, 1993; Fini et al., 2012; N., Freeman, & Vrana, 2011). Furthermore, as coagulating factors are synthesized in the liver, a study has concluded that prothrombin time (PT) and activated partial thromboplastin time (APTT) are significantly elevated in AD individuals (Adias et al., 2013).

Therefore, today it is a common practice to use blood and urine biomarkers of organ damage to diagnose AD. There are many biomarkers that have been investigated in research to diagnose AD such as Gamma glutamyl transferase (GGT), Mean Corpuscular Volume (MCV), Carbohydrate-Deficient Transferrin (CDT), Phosphatidylethanol (PEth), Ethyl Glucuronide (EtG) and Ethyl Sulfate (EtS)(Freeman & Vrana, 2010; George G. Harrigan, Maguire, & Boros, 2008; John C. Lindon, Nicholson, & Holmes, 2007 ; Litten et al., 2010; Rinck, Frieling, Freitag et al., 2007).

Gamma glutamyl transferase (GGT) is available in cell membranes of the tissues of some organs such as liver, kidney, spleen, pancreas and heart(Litten et al., 2010). Chronic alcohol consumption results in inflammation and necrosis of these cells, consequently this might increase the leakage of GGT from the destroyed cells (especially hepato-cells) thereby leading to an elevation in serum GGT(Litten et al., 2010; Tavakoli, Hull, & Michael Okasinski, 2011).GGT has a long window of assessment, it remains elevated for two to three weeks after alcohol cessation, and it takes two weeks to elevate after the relapsing of heavy drinking(BPAC, 2010).It has 20% sensitivity and 65.2% specificity (Larkman, 2013).

The Mean Corpuscular Volume (MCV)is a measurement of the size of red blood cells. It is an indicator of the chronic use of alcohol rather than the acute intake(Tavakoli et al., 2011), as chronic use increases the size of red blood cells(BPAC, 2010).The level of MCV can remain high for several months after cessation (Litten et al., 2010). Compared to GGT,the sensitivity of MCV in women is greater than that in men(Conigrave, Davies, Haber et al., 2003).

Carbohydrate-Deficient Transferrin (CDT) is the widely used AD biomarker in clinical practice. Transferrin is glycoprotein synthesized and released by the liver, and transports iron throughout the body. Regular high alcohol intake leads to a decrease in the number of carbohydrate residues (sialic acid) attached to transferrin, thereby increasing carbohydrate deficient sites(BPAC, 2010). Serum CDT elevates with regular and heavy drinking(60-80 g per day) and usually returns to normal after two to three weeks of cessation(Stibler, 1991). Because of the improvement in CDT measurement methods, the sensitivity and specificity of its testing have increased. Therefore, the Food and Drug Administration (FDA) affirmed CDT as a biomarker of heavy alcohol consumption(Litten et al., 2010).

Phosphatidylethanol (PEth) is a phospholipid formed by phospholipase D enzyme in the presence of ethanol. It remains elevated in the blood for one to two weeks after cessation of moderate to heavy alcohol intake(Stewart, Reuben, Brzezinski et al., 2009). The advantage of PEth is that it is more sensitive than other biomarkers as it is not affected by the liver state(Stewart et al., 2009). Although other biomarkers can be increased by non-alcohol liver diseases, PEth can be used to monitor alcohol consumption with hepatic disease(Litten et al., 2010).

Ethyl Glucuronide (EtG) is a direct metabolite of ethanol, as ethanol is conjugated in hepatic endoplasmic reticulum with glucuronic acid through glucuronosyltransferase enzyme into EtG. It can be measured in blood, urine and hair. Urine EtG is usually used rather than serum EtG due to the short half-life of serum EtG which is between 14-20 hours. Whereas, urine EtG can be detected up to several days. Currently, there are developed methods to measure EtG in hair and this will help to detect EtGfor several months after alcohol cessation(Litten et al., 2010).

Ethyl Sulfate (EtS) is a metabolite of alcohol. It is yielded from the sulfate conjugation of alcohol by sulfo-transferase enzyme. It was discovered that there is a positive correlation between EtG and EtS in urine samples(Litten et al., 2010).

1.5.1.2.1 Limitations of thecurrentAD biomarkers

Although current AD biomarkers are being used to assist in making AD diagnosis, there are some limitations that hinder their uses. The drawbacks are mainly due to their low specificity and sensitivity which indeed lead to false diagnosis.

The AST and ALT are not specific to AD, they are usually used to screen liver damage regardless of the cause(Adias et al., 2013; Litten et al., 2010).The GGT is also not specific to AD. It can be elevated in non-alcoholic liver diseases**,** smoking, obesity, diabetes mellitus, age, nutritional factors and metabolic disorders(Adias et al., 2013; Litten et al., 2010). Moreover, GGT can be elevated by some medications such as barbiturates, anticonvulsants and anticoagulants(Litten et al., 2010). In fact, the low sensitivity and specificity will hinder the use of GGT, AST and ALT in the discrimination between alcoholic and non-alcoholic liver diseases.

The MCV is elevated in both folate and vitamin B12 deficiencies, hypothyroidism, non-alcoholic liver diseases, hemolysis, and bleeding disorders. It can also be elevated in patients on treatment with medications that can induce bone marrow disorders and toxicity(Litten et al., 2010).

Though PEth has the advantage of being a specific biomarker of alcohol consumption and not affected by liver condition, it is limited by its lower differentiation ability between moderate and heavy drinkers(Stewart et al., 2009). Similarly, the EtG has its limitations as well. For example, if urine sample contains yeast, this will convert urine glucose to alcohol and then to EtG.This may lead to false positive results,especially of diabetic patients who have high levels of glucose in urine(Litten et al., 2010).Moreover, washing the hair may remove EtG from hair shaft(Tavakoli et al., 2011).The EtG's window of assessment isbetween one to two days after alcohol cessation which is too short a time to detect AD.

The CDT sensitivity is similar to GGT, but with higher specificity. A recent study to evaluate and to compare the range of AD biomarkers of a group of heavy drinkers in Russia concluded that CDT might be the best biomarkers with 67% sensitivity and 71% specificity to detect a daily average alcohol consumption of 40g and above(McDonald, Borinskya, Kiryanov et al., 2013). However, the use of CDT as AD biomarker is hindered by the possibilities of false positives due to rare genetic transferrin variants, chronic end-stage liver disease, smoking, body weight, female gender, primary biliary cirrhosis and hepatocarcinoma(Litten et al., 2010).

In an approach to increase sensitivity and specificity of AD biomarkers, some combinations had been examined. The best suggested combination is CDT, GGT and MCV(Larkman, 2013; Litten et al., 2010). This combination might help in males and

females, heavy drinkers with or without liver diseases(Litten et al., 2010).Another combination is CDT and MCV which has been shown to improve sensitivity and to perform better than either one of the biomarkers alone. However, the risk of false diagnosis cannot be excluded(Tavakoli et al., 2011).

Nevertheless, there were some reports which stated that the CDT, GGT and MCV are not reliable enough biomarkers of AD,the current practice uses them to aid questionnaires(Larkman, 2013).Therefore, a combination of biomarkers was suggested to limit but not rule out the risk of false diagnosis(Larkman, 2013). In general, the questionnaires and current AD biomarkers are not the perfect methods to diagnose AD accurately. Therefore, it is reasonable to argue that the finding is a more objective measurement of novel AD biomarkers that would be of an advantage. The diagnosis of AD patients will help to reduce AD patients' organ failure and social problems. It has been suggested that the use of any of the systems biology disciplines, particularly metabolomics, could eventually prove valuable in this area(Kumar & Sarin).

1.6 Systems Biology

System biology is a biological approach that aims to investigate and to explore the complexity of molecular perturbation by the comprehensive integration of different bio-databases of molecular, genetic and metabolic networks, and the individual interaction between the components of each network(Breitling, 2010; Kuster, Merkus, van der Velden et al., 2011; G. Louridas & Lourida, 2012; G. E. Louridas, Kanonidis, & Lourida, 2010). This can give an in-depth understanding of the medical condition and hence improvesdrug discovery and personalization of

therapy(G. Louridas & Lourida, 2012). In fact, the power of system biology is based on the concept that although the clinical phenotype of a molecular disturbance is not obvious, the consequent compensatory adaptation due to this disturbance will be reflected in the transcriptome, proteome or metabolome(Kuster et al., 2011).

1.6.1 Disciplines of Systems Biology

Systems biology combines a group of (-omics) disciplines, particularly the main majoromics such as genomics, transcriptomics, proteomics and metabolomics.While genomics focuses on the genome sequencing, transcriptomicsstudies the transcription and expression of genes sets using gene expression microarrays or RNA sequencing (Cappola & Margulies, 2011; Piran, Liu, Morales et al., 2012). These expressions will yield proteins which can be measured by gel electrophoresis or mass spectrometry (MS). The biochemical modifications to proteins can reflect a state of specific medical conditions. Lastly, the metabolic process in cells and organs ends with the production of metabolites which constitute the metabolome or metabolic profile.Metabolomics aim to discover and measure the changes in metabolome. Each one of the systems biology disciplines has been found to give fingerprints of prognostic value in diagnosing diseases.However, metabolomics is the ultimate screen that reflects other omics in addition to environmental factors. The use of systems biology disciplines to diagnose complicated medical conditions such as cancer, cardiovascular diseases and liver disease, has the advantage of being less invasive and fast(Cappola & Margulies, 2011; Piran et al., 2012).Figure1.1 shows how the systems biology disciplines help in the understanding of the pathophysiology of diseases and the pathways behind specific phenotypic manifestation of the disease. The figure shows that metabolomics

is the ultimate internal picture of systems biology which can reflect the preceding disciplines.

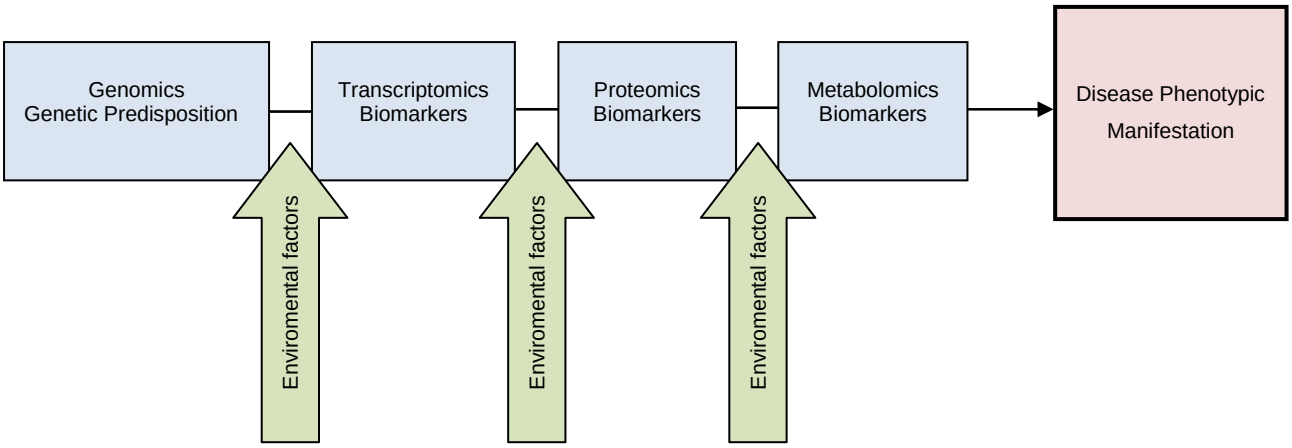

Figure 1.1: The role of systems' biology disciplines (omics) in disease diagnosis, where metabolomics represents the ultimate biomarker for detection of the disease before the phenotypic manifestations (adapted from(Piran et al., 2012)).

1.7 Metabolomics

Metabolomics (ormetabonomics in some literature) is one of the systems' biology disciplines which comes in a chaincircle after genomics, transcriptomics and proteomics. It deals with the identification and quantification of small molecular metabolites in the metabolic profile (metabolome) of the living organism(Corona, Rizzolio, Giordano et al., 2012; G. Louridas & Lourida, 2012). The metabolome of each organism is highly affected either byinternal or external environmental factors. Therefore, the variation in metabolomemight be associated witha particular phenotype, specific nutrition, drugsand diseases. This variation can be considered as the metabolic finger print or surrogate biomarkers of medical condition or disease. Metabolomicsalso helps in the ability to understand the pathways that lead to changes in the levels of body metabolites due to disease, drug and/or environmental

effect(George G. Harrigan et al., 2008).The reason for the use of metabolomics in the exploration of medical conditionsin recent medical approach is because it doesnot only reflect the variation in genetics, transcriptomics and proteomics which might be associated with the condition, but also the environmental factors associated with the condition (Gutiu, Andries, Mircioiu et al., 2010). Moreover, it can give a predictionof body response to these internal and external factors. This can help in drug discovery and personalization of therapy.Recent studies have used metabolomics analysis to identify novel biomarkers of diseases such as asthma, COPD, cancer, diabetes mellitus, cystic fibrosis and metabolic disorders (Hocquette, 2005; Hunt & John, 2007; Montuschi, Paris, & Melck, 2009; Robroeks, Van Berkel, Dallinga et al., 2010).

The metabolomics analysis involves the identification and measurement of small molecularcompounds inbiological samplessuch as blood, urine, cerebrospinal fluids CSF, breath, seminal fluids and tissues. These small molecular compounds are metabolites. Thetechniqueinvolveschemometric studies of the spectroscopic data of instruments such as nuclear magnetic resonance (NMR)spectroscopy and mass spectrometer (MS)(John C Lindon, Nicholson, & Holmes, 2011).

There are two terms that are commonly used to describe the study of body metabolites which are metabolomics and metabonomics. Metabonomics is defined as the study of metabolic response of living organism to biological or genetic variation (Nicholson & Lindon, 2008), while metabolomics is defined as the exploration of all metabolites inside the living organism(Fiehn, 2001; R. Goodacre, Vaidyanathan, Dunn et al., 2004). The distinction between these two terms; metabolomics and metabonomicsremains controversial and both terms are being used interchangeably in the literature to refer to the same discipline.In this context we are going to use the

term **metabolomics**, however, we might also refer to some literature which used the term metabonomics instead for our literature review.

1.8 Application of metabolomics in disease diagnosis

As the pathophysiological changes in the body are usually reflected on the metabolome, it can be inferred that different medical conditions can have their distinct metabolic finger print (metabotype)on the metabolome. The metabotype is a group of certain metabolites that, eitherby their presence or absence, increased or decreased concentration,are distinctive for a specific clinical status or disease(Semmar, 2012).Researchers have investigated the use of metabolomics techniques to explore different medical conditions, in particular the conditions which have no definitediagnosis or their clear-cut diagnosis which needfurther invasive procedures.

1.8.1 Application of metabolomics techniques to identify novel-biomarkers of diseases

The aim of metabolomics studies is to find a metabotypewhich has diagnostic or classifying value among patients andto exploreand measure the metaboliteswhich will further assistin the diagnosis or classification(John C. Lindon et al., 2007).

Many metabolomics studies have been conducted on biological fluid samples to identify biomarkers of diseases such as liver disease, diabetes, asthma, cancer and critical illnesses(Serkova, Standiford, & Stringer, 2011; Wang, Zhang, Han et al., 2012). These studies have identified metabotypes which are able to

discriminatepatients from their controls. Table 1.1presents examples of metabolomics studies to diagnose diseases.

Some of the metabolomics studies aim to investigate metabolic changes consequent to the exposure to environmental factors or toxin, such as smoking and other harmful substances. In an approach to explore the effects of smoking and smoking cessation, researchers have used metabolomics techniques to quantify 140 metabolites in fasting serum of three groups, namely current smokers, non-smokers and quitters (who have quitted during the follow up period of the study) in a longitudinal analysis (Xu, Holzapfel, Dong et al., 2013). It was discovered that 21 smoking related metabolites were significantly different from those of current smokers and non-smokers. Interestingly, the study discovered that 19 out of the 21 metabolites were reversible in quitters (Xu et al., 2013). In another study aimed to study the systemic toxic effect of welding fumes on humans, Wei and colleagues used liquid and gas chromatography-MS to investigate the plasma metabolome of boilermakers pre-welding and post-welding fumes exposure in a two stage-study (Wei, Wang, Chang et al., 2013). The first stage (stage one) of the study was conducted in 2011 on 11 boilermakers. The second stage (stage two) was conducted in 2012 on 8 boilermakers, and five of them participated in stage one in addition to three new recruited boilermakers. The results showed that the high exposure to high metal welding fumes causes a decrease in the level of unsaturated fatty acids (Wei et al., 2013).

The aforementioned metabolomics studies are part of continually growing body of evidences on the preeminent role that metabolomics can play not only in diseases diagnosis, but also in the pathogenic understanding of diseases and variable

medical conditions. This will help to monitor, guide and evaluate the current therapy, and help to find new drug target for future drugs.

Hitherto, to our knowledge, there were no metabolomics studies that had been conducted in humans to identify biomarkers of chronic use of alcohol or AD. Therefore, we propose the use of metabolomics in this research to find novel biomarkers that can discriminate AD patients from their matched controls and their matched social drinkers.

Table 1.1: Examples of metabolomics studies to diagnose diseases

Study	Disease	Analytical Method	Specimen	Findings
Ibrahim et al.,2011 (Ibrahim, Basanta, Cadden et al., 2011)	Asthma	GC-MS	Breath	Development of a discriminatory model which classifies asthma patients with accuracy of 86%
Basanta et al.,2012 (Basanta, Ibrahim, Dockry et al., 2012)	COPD	GC-MS	Breath	Development of a discriminatory model which discriminate COPD patients from their healthy controls with 85% sensitivity and 50% specificity
Schicho et al.,2012 (Schicho, Shaykhutdinov, Ngo et al., 2012)	IBD	^{1}HNMR	Serum, Plasma and Urine	Characterization of 44 serum, 37 plasma and 71 urine metabolites to differentiate between diseased and non-diseased individuals
Motsinger-Reif et al., 2013 (Motsinger-Reif, Zhu, Kling et al., 2013)	Al-zheimer with Dementia	LC-ECA GC-TOF MS	CSF	Identified biomarkers which discriminate Al-zheimer patients from their healthy controls
Lewitt et al., 2013 (Lewitt, Li, Lu et al., 2013)	Parkinson's disease (PD)	UPLC and GC- MS	CSF	Identification of 19 compounds which were able to discriminate PD patients from similar age healthy controls with a false discovery level of 20%

GC-TOF MS: gas chromatography-time of flight mass spectroscopy, CSF: cerebrospinal fluid, UPLC: ultra performance liquid chromatography, IBD: Inflammatory bowel disease, COPD: Chronic obstructive pulmonary disease, LC-ECA: liquid chromatography-electrochemical coulometric array

1.8.2 Using metabolomics to investigate alcohol consumption

There are metabolomics studies that investigated the effect of alcohol consumption by analysing biological samples of animals on alcohol-containing diets. Further, researchers started to explore this in humans.

1.8.3 Using metabolomics to investigate alcohol consumption in animal models

There are some studies that used the metabolomics approach to study the pathogenesis of alcohol consumption in animal models and to find novel biomarkers of alcohol consumption. Most of these studies were oriented by expected consequences of alcohol's detrimental effect such as liver injury or alcoholic liver disease (Bradford, O'Connell, Han et al., 2008; Fernando, Kondraganti, Bhopale et al., 2010; Loftus, Barnes, Ashton et al., 2011).

Bradford and colleagues used ^{1}H-NMR and MS metabolomics techniques to evaluate the metabolic profile of urine and liver extract samples of mice with alcohol induced liver injury (Bradford et al., 2008). The mice were divided into two groups, one was fed with isocaloric (control group) and the other group was fed with alcohol containing liquid diet (alcohol group) of which the steatohepatitis was confirmed by 5-fold increase of serum ALT, 6-fold increase in liver injury score and the increase of lipid peroxidation in the liver. The ^{1}H-NMR principal component analysis of both urine and liver extract showed obvious discrimination between the two groups. For instance, the lactate was high in both liver and urine of those mice in the alcohol group. N-oleoylethanolamine metabolite was found to be elevated as well. Both lactate and N-oleoylethanolamine indicated hypoxic injury of the liver. Tyrosine was found to be elevated which might reflect an alteration in metabolism due to alcohol.

Moreover, there was a decrease in the excretion of taurine (glutathione metabolite) in the urine of those mice in the alcohol group. Additionally, it was found that there was an increase in prostacycline inhibitor 7,10,13,16-docosatetraenoic acid which is vital in the regulation of platelets formation (Bradford et al., 2008).

In another study, by using LC-MS metabolomics technique, Loftus and colleagues explored the metabolomics changes in non-polar metabolites of rodents' livers due to alcohol consumption (Loftus et al., 2011). The study was conducted on rats and mice fed with intragastric alcohol feeding model. The analysis of liver derived samples revealed a significant increase of fatty acyls, fatty acid ethyl esters, in addition to octadecatrienoic acid and eicosapentaenoic acid metabolites (Loftus et al., 2011). Similarly, but by targeting the lipidomic profile, Fernando and colleagues extracted lipids from the plasma and liver of rats fed with alcohol diets and their controls (Fernando et al., 2010).It was concluded from both ^{1}H-NMR and ^{31}P-NMR data analysis that a significant alteration in lipid metabolism was induced by alcohol consumption.

To the best of our literature review we have found that most of the metabolomics studies which assessed alcohol consumption in animal models, focused on specific organ injury, particularly liver injury. None of these studies aimed to find the biomarkers of chronic use of alcohol or AD.

1.8.4 Using metabolomics to investigate alcohol consumption in humans

To date, there are few metabolomics studies that have been conducted to study the effects of alcohol consumption on human metabolome. Jaremek and colleagues investigated the effects of alcohol consumption on human serum metabolome(Jaremek, Yu, Mangino et al., 2013). Researchers compared the serum

metabolome of two groups, light (LD) and moderate to heavy drinkers (MHD). The results showed that 40 identified metabolites in males and 18 in females differed significantly in concentration between these two groups. Out of these metabolites, 10 in males and 5 in females were specific metabolites to discriminate LD from MHD. The investigators concluded that alcohol consumption mostly affect metabolic profile classes of diacylphosphatidylcholines, lysophosphatidylcholines, ether lipids and sphingolipids. These results indicated that the stimulatory effect of alcohol consumption on acid sphingomyelinase (ASM) activity causes the accumulation of ceramide and a decrease of sphingomyelins(Jaremek et al., 2013). However, the study did not explore the metabolic variation associated with chronic use of alcohol or AD.Additionally, it did not investigate urine metabolomics which is non invasive compared to blood.

In another study, the researchers used ^{1}H-NMR metabolomics technique to find serum metabolic finger print that discriminate alcoholic liver cirrhosis from hepatitis B virus liver cirrhosis (Qi, Tu, Ouyang et al., 2012). The investigators found that five metabolites were able to distinguish between the two different types of cirrhosis. Similar to the studies mentioned earlier, the study did not explore urine metabolic profile and did not look for metabolic finger print of chronic use of alcohol.

1.9 Application of metabolomics to find novel biomarkers of ADin blood and urine samples

As metabolomics studies aim to find a specific metabolic fingerprint (metabotype) that is associated with a particular medical condition or

disease,samplinga suitable biological sample is required.Blood and urine samples are usually considered stable and less invasive biological sampleswhere urine is leastinvasive and easy to get in abundance as it is the waste product (Decramer, de Peredo, Breuil et al., 2008; Down, 2010; Griffiths, 2008; Vaidyanathan, Harrigan, & Goodacre, 2005; Want, Wilson, Gika et al., 2010). Therefore, researchers preferplasma, serum and urine in metabolomics studies.

1.10 Using ^{1}H-NMR spectroscopicinstrument in metabolomics

The proton nuclear magnetic resonance ^{1}H-NMR is one of the main analytical tools that isbeing widely used in metabolomics, in addition to mass spectroscopy (MS) and fourier transform infrared spectroscopy (FT-IR) (Basanta et al., 2012; Lloyd, William Allwood, Winder et al., 2011; Schicho et al., 2012). It is being used increasingly in metabolomics recently because it has the advantage of quickly revealing the metabolic profile of the biological sample depending on the magnetic properties of the widely spread hydrogen atoms in the chemical structure of the metabolites(Dunn, Bailey, & Johnson, 2005). The concept that^{1}H-NMR analysisprocesses rely on every compound or metabolite contains hydrogen atoms in different chemical structure forms that will give specific and different peaks in the NMR spectra when the compound enters the magnetic field. These peaks are characteristic for each compound(Dunn et al., 2005).This, with the help of a chemometricsoftware,will help to speculate the chemical structure of the unknown metabolites in the biological samples**.**Although the sensitivity of ^{1}H-NMR is low when compared to MS (Silva Elipe, 2003), the^{1}H-NMR analysis is less complex as the sample does not need a prior derivatization, extraction and separation as required

by MS (Clayton, Lindon, Cloarec et al., 2006). In addition, ^{1}H-NMR acts as an independent instrument compared to MS which requires a separate instrument like GC or LC(Dunn et al., 2005). The ^{1}H-NMR is able to analyze hundreds of samples per day and the sample can be reused for further analysis (Nicholson & Lindon, 2008; Shulaev, 2006). Therefore, the^{1}H-NMR is considered cheap and non destructivewhen compared to MS. Figure 1.2 illustrates a Google scholar based literature search for publications containing (metabolomics & NMR) and (metabonomics & NMR). The search showed an increase in the number of publications from a total of 67 articles in year 2000 to 5870 articles in 2014. This reflects the great interest in using NMR in metabolomics studies due to the simplicity of sample preparation and the rapidyield of results.

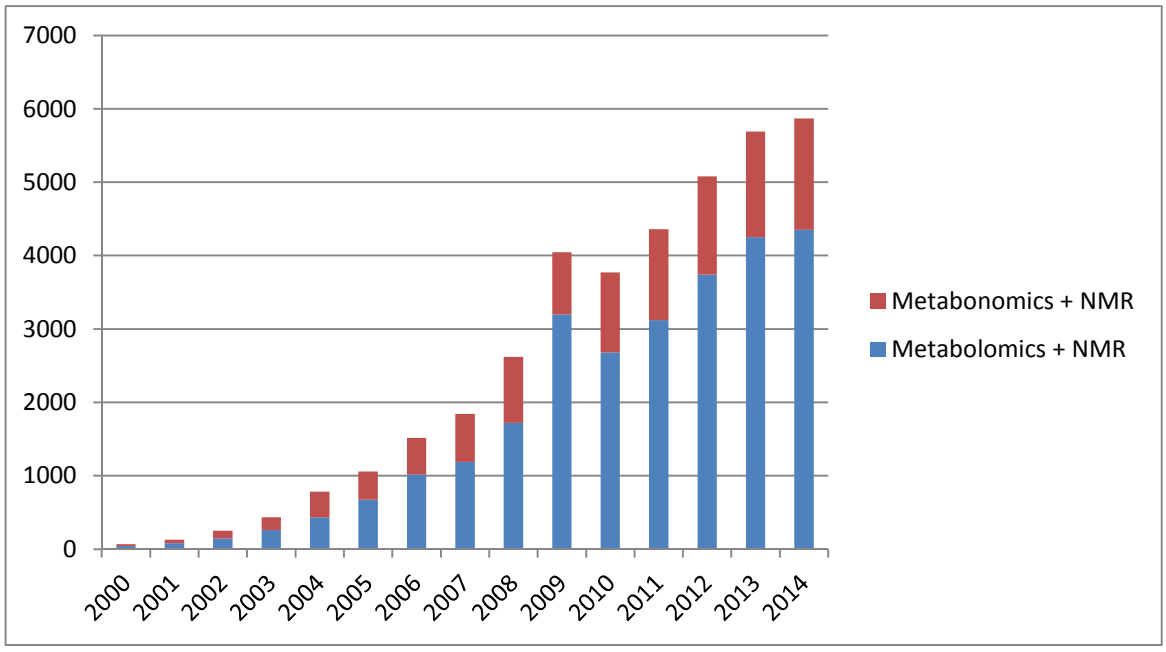

Figure1.2: Literature search at Google scholar for publications of (metabolomics + NMR) and (Metabonomics + NMR)