



Review Article

Nuclear magnetic resonance spectroscopy based metabolomics to identify novel biomarkers of alcohol-dependence

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Abstract

Alcohol misuse is a ravaging public health and social problem. Its harm can affect the drinkers and the whole society. Alcohol-dependence is a phase of alcohol misuse in which the drinker consumes excessive amounts of alcohol and has a continuous urge to consume alcohol. Current methods of alcohol dependence diagnoses are questionnaires and some biomarkers. However, both methods lack specificity and sensitivity. Metabolomics is a scientific field which deals with the identification and the quantification of the metabolites present in the metabolome using spectroscopic techniques such as nuclear magnetic resonance (NMR). Metabolomics helps to indicate the perturbation in the levels of metabolites in cells and tissues due to diseases or ingestion of any substances. NMR is one of the most widely used spectroscopic techniques in metabolomics because of its reproducibility and speed. Some recent metabolomics studies were conducted on alcohol consumption and alcohol misuse in animals and humans. However, few focused on identifying alcohol dependence novel biomarkers. A sensitive and specific technique such as NMR based metabolomics applied to find novel biomarkers in plasma and urine can be useful to diagnose alcohol-dependence.

Keywords: alcohol-dependence, diagnosis, metabolomics, metabotype, nuclear magnetic resonance

1. Introduction

Alcohol drinking is 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 death cases every year due to the hazardous consumption of alcohol (Institute of Alcohol Studies [IAS], 2013; World Health Organization [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 (National Center for Health Statistics [NCHS], 2009). Studies have shown that the harmful

effects of alcohol consumption are the highest compared to that of other illicit drugs (van Amsterdam & van den Brink, 2013). Alcohol intoxication usually leads to drastic and destructive behaviour such as accidents, injuries and crimes which may create social problems (Couture *et al.*, 2010; Vaaramo, Puljula, Tetri, Juvela, & Hillbom, 2012). A growing body of literature had asserted the harmful effect of alcohol consumption on vital organs. Although some studies advocated cardio protective role of moderate alcohol consumption, (Boto-Ordóñez *et al.*, 2013; Wallerath, Poleo, Li, & Förstermann, 2003), other studies contradicted this by indicating that the protective role is overvalued (Fillmore, Stockwell, Chikritzhs, Bostrom, & Kerr, 2007; Plunk, Syed-Mohammed, Cavazos-Rehg, Bierut, & Grucza, 2013; van Amsterdam & van den Brink, 2013). The average volume of alcohol consumption is associated with liver cirrhosis,

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depression, gastric ulcer, malabsorption, oesophageal reflux, diarrhea and osteoporosis (Aguilera-Barreiro Mde, Rivera-Marquez, Trujillo-Arriaga, Ruiz-Acosta, & Rodriguez-Garcia, 2013; Bujanda, 2000; Fini *et al.*, 2012; Rehm *et al.*, 2003). Besides, it causes immune system impairment (Karavitis & Kovacs, 2011). Therefore, chronic alcohol drinkers usually are highly susceptible to acquiring fatal infections (Romeo, Warnberg, & Marcos, 2010). Different patterns of alcohol consumption are risk factors for liver cancer, breast cancer, colon cancer, renal cell cancer and oropharyngeal cancer (Baan *et al.*, 2007; Bagnardi *et al.*, 2013). A meta-analysis that aimed 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 drinking can lead to vital organs injury and then to subsequent failure. This requires efforts to encourage the avoidance of chronic alcohol drinking. The early identification of people who might be chronic consumers of alcohol will help save their health and the quality of their lives.

2. Alcohol-dependence (AD)

The National Health Committee, Wellington-New Zealand, in the 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: "A *maladaptive pattern of alcohol use leading to clinically significant impairment or distress*" (National Health Committee, 1999). This distress has many symptoms, such as a desire to drink more amounts of alcohol to attain the intoxication effect, spending more time in consuming or recovering from alcohol, refraining from social 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 (Best Practice Advocacy Centre of New Zealand, 2010).

The data showed that when the habit of drinking alcohol starts earlier in age, the susceptibility of developing AD is higher (Grant & Dawson, 1997). This implies that when individuals become alcohol dependent, they have already spent long period of exposure to the detrimental effects of alcohol consumption and hence will start to suffer failure of vital organs. Because early diagnosis of disease can lead to early start of treatment, the early detection of AD is crucial for preventing organs failure. For instance, the early detection of AD might prevent the progression of liver injury due to alcohol consumption to the irreversible phase of liver failure in AD individuals.

3. Current Diagnostic Methods of Alcohol-dependence

Currently, AD questionnaires are the main clinical methods of diagnosis 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, 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; Kumar, 2010). Therefore, the biomarkers of organ injury, such as liver injury, commonly used as biomarkers of AD. However, their specificity to AD is reduced by being biomarkers of organ injury regardless of the cause of the injury. Such biomarkers may lead to confusion of AD diagnosis with other diseases, such as non-alcoholic liver cirrhosis (Litten, Bradley, & Moss, 2010).

3.1 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) (Best Practice Advocacy Centre of New Zealand, 2010). These questionnaires consist of 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 questionnaires is the subjective nature of its measurement. They may not provide an actual estimation of an individuals' alcohol consumption due to the denial and under reporting of regularly consumed alcohol amounts. Moreover, there are limitations in the validations of these questionnaires due to the lack of standard questionnaires or definite diagnostic tools to be used as validation reference (Kroke *et al.*, 2001).

3.2 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 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*" (National Institute of Health [NIH], 2001).

Studies that were conducted to investigate the mechanisms of alcohol induced organ damage have found several mechanisms/pathophysiology of injuries involving certain biomarkers. Therefore, today, it is common practice to use

blood and urine biomarkers of organ damage to diagnose AD. For example, the raise of liver enzymes such as Alanine Aminotransferase (ALT), Aspartate aminotransferase (AST) and Gamma glutamyl transferase (GGT) in AD individuals is indicator of alcohol induced liver injury (Adias *et al.*, 2013). The differences in the levels of AST and GGT in AD individuals compared to abstainers were found to be significant in several studies (Adias *et al.*, 2013; Quaye, Nyame, Dodoo, Gyan, & Adjei, 1992). The GGT is available in cell membranes of the tissues of some organs such as the liver, kidney, spleen, pancreas and heart (Litten *et al.*, 2010). Chronic alcohol consumption results in an inflammation and necrosis of those cells. Consequently, this might increase the leakage of GGT from the destroyed cells (especially hepatic cells) thereby leading to an elevation in serum GGT (Litten *et al.*, 2010; Tavakoli, Hull, & Michael, 2011). The 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 (Best Practice Advocacy Centre of New Zealand, 2010). It has 20% sensitivity and 65.2% specificity (Larkman, 2013).

Other AD biomarkers include the mean corpuscular volume (MCV), carbohydrate-deficient transferrin (CDT), phosphatidylethanol (PEth), ethyl Glucuronide (EtG) and ethyl sulfate (EtS) (Beckonert *et al.*, 2007; Freeman & Vrana, 2010; Harrigan, Maguire, & Boros, 2008; Litten *et al.*, 2010; Rinck *et al.*, 2007). The MCV which is measurement of red blood cells size can be used as 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 (Best Practice Advocacy Centre of New Zealand, 2010). The level of MCV can remain high for several months after cessation (Litten *et al.*, 2010). The CDT is frequently used AD biomarker. Transferrin is glycoprotein that is synthesized and released by the liver which transports iron throughout the body. Regular high alcohol intake leads to decrease in the number of carbohydrate residues (sialic acid) attached to transferrin, thereby increasing carbohydrate deficient sites (Best Practice Advocacy Centre of New Zealand, 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 has increased. Therefore, the food and drug administration (FDA) has affirmed CDT as a biomarker of heavy alcohol consumption (Litten *et al.*, 2010). The 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 *et al.*, 2009). The PEth is more sensitive than other AD biomarkers as it is not affected by the liver state. The EtG is direct metabolite of ethanol conjugation in hepatic endoplasmic reticulum with glucuronic acid through glucuronosyltransferase enzyme. It can be measured in blood, urine and hair. Urine EtG can be detected up to one to two days after alcohol consumption which is longer than the life

span of EtG in serum. Similarly, EtS is metabolite of ethanol conjugation with sulfur by sulfa-transferase enzyme. Both EtG and EtS levels are positively correlated in urine samples (Litten *et al.*, 2010). Of further interest, the altered level of the inflammatory mediators (cytokines) due to consumption which is correlated with the harmful effects of alcohol on bone, lung, liver and other tissues were suggested as AD biomarkers (Achur, Freeman, & Vrana, 2010; Birkedal-Hansen, 1993; Fini *et al.*, 2012). Furthermore, as coagulating factors are being synthesized in the liver, a study has concluded that prothrombin time (PT) and activated partial thromboplastin time (APTT) are significantly elevated in AD individuals and therefore, they can be used to aid AD diagnoses (Adias *et al.*, 2013).

3.2.1 Limitations of current AD biomarkers

Although most of current AD biomarkers are commonly used to aid in the diagnosis of AD, their accurate productivity is limited. This is mainly due to their low specificity and sensitivity, which may lead to false diagnosis. The AST and ALT are not specific to AD; they are usually used to screen liver damage regardless of the etiology (Adias *et al.*, 2013; Litten *et al.*, 2010). Similarly, GGT can be elevated in non-alcoholic liver disease, smoking, obesity, diabetes mellitus, age, nutritional factors, metabolic disorders and by some medications such as barbiturates, anticonvulsants and anticoagulants. The MCV might be elevated in folate and vitamin B12 deficiencies, hypothyroidism, non-alcoholic liver disease, haemolysis, bleeding disorders and in patients whom are on medications that can induce bone marrow disorders (Litten *et al.*, 2010). The EtG may indicate false positive results if urine contains yeast as urine glucose will be converted to alcohol and then to EtG. This may be seen particularly in diabetic patients who have high levels of glucose in urine. Moreover, the EtG's window of assessment is between one to two days after alcohol cessation, which is too short time to detect AD. The PEth has lower discrimination between moderate and heavy drinkers (Stewart *et al.*, 2009). The CDT sensitivity is like 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 biomarker with 67% sensitivity and 71% specificity to detect a daily average alcohol consumption of 40g and above (McDonald *et al.*, 2013). However, the use of CDT as an 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 the sensitivity and specificity of AD biomarkers, some combinations had been examined. However, combination of biomarkers could limit, but not rule out the risk of false diagnosis (Larkman, 2013). The best suggested combination is CDT, GGT and MCV. 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). In general, the questionnaires and current AD biomarkers are not the accurate methods to diagnose AD accurately. Therefore, it is reasonable to argue that finding more objective measurement of novel AD biomarkers would be of an advantage.

4. Systems Biology

Systems biology is a biological approach that aims to investigate and to explore the complexity of molecular perturbation by the comprehensive integration of different bi-databases of molecular, genetic and metabolic networks, and the individual interaction between the components of each network (Breitling, 2010; Kuster, Merkus, van der Velden, Verhoeven, & Duncker, 2011; Louridas & Lourida, 2012; Louridas, Kanonidis, & Lourida, 2010). This can give an in-depth understanding of the medical condition and hence improves the disease diagnosis and personalized medicine (Louridas & Lourida, 2012). In fact, the power of systems 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).

4.1 Disciplines of systems biology

Systems biology combines group of (-omics) disciplines, particularly the main major omics such as genomics, transcriptomics, proteomics and metabolomics. While genomics focuses on the genome sequencing, transcriptomics studies the transcription and expression of gene sets using gene expression microarrays or RNA sequencing (Cappola & Margulies, 2011; Piran, Liu, Morales, & Hershberger, 2012). These expressions will yield proteins, which can be measured by mass spectroscopy (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 perform 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. Figure 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 disease phenotype, which can reflect the perturbation in other omics fields which precede metabolomics.

5. Metabolomics in Disease Diagnosis

Metabolomics (or metabonomics in some of the literatures) deals with the identification and quantification of small molecular compounds (metabolites) in the metabolic profile (metabolome) of the living organism (Corona, Rizzolio, Giordano, & Toffoli, 2012; Louridas & Lourida, 2012). The metabolome of each organism is highly affected either by internal or external environmental factors. The variations in the metabolome usually are associated with disease phenotype, specific nutrition exposure and drug response or toxicity. The pattern of this variation can be considered as the metabolic fingerprint or surrogate biomarkers of the clinical condition or disease under investigation. Different medical conditions can have their distinct metabolic finger print (metabotype). The metabotype is a group of certain metabolites that, either by their presence or absence, increased or decreased concentration, are distinctive for a specific clinical status or disease (Semmar, 2012). This metabotype gives better understanding of the pathways that lead to changes in the metabolites due to disease, drug and/or environmental effects (Harrigan *et al.*, 2008). A metabotype does not only reflect the variation in genetics, transcriptomics and proteomics, which might be associated with the condition, but also the environmental factors interfering with them (Gutiu *et al.*, 2010). In the diagnosis of some diseases, it is difficult to have definite diagnosis without further invasive procedures such as cancer and cardiovascular diseases (Corona *et al.*, 2012; Shah, Kraus, & Newgard, 2012). Therefore, identifying a metabotype associated with the disease is a non-invasive tool for disease diagnosis. Similarly, metabolomics can be used to identify a metabotype which can predict body response to drugs and xenobiotics. This will help to monitor, guide and evaluate the current therapy, and help to find new drug target. The metabolomics analysis involves analyzing biological samples such as blood, urine, cerebrospinal fluids (CSF), breath, seminal fluids and tissues using instruments such as nuclear magnetic resonance (NMR) spectroscopy and MS (Clayton *et al.*, 2006). The blood and urine samples are usually considered stable and less invasive biological samples

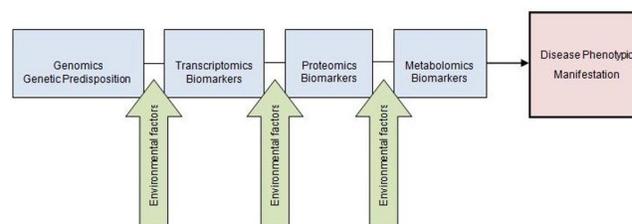


Figure 1. The role of systems' biology disciplines (omics) in disease diagnosis, where metabolomics represents the ultimate biomarkers level for detection of the disease before the phenotypic manifestations (adapted and modified from (Piran *et al.*, 2012)).

where urine is less invasive and easy to get in abundance (Decramer *et al.*, 2008; Down, 2010; Griffiths, 2008; Vaidyanathan, Harrigan, & Goodacre, 2005; Want *et al.*, 2010). Therefore, the researchers prefer plasma, serum and urine in metabolomics studies.

Metabolomics studies have been able to identify metabolotypes of diseases such as liver disease, diabetes, asthma, COPD, cancer and metabolic disorders using different biological fluid samples of patients and healthy controls (Hocquette, 2005; Hunt & John, 2007). The discriminating metabolotypes usually have high specificity and sensitivity. Table 1 presents examples of metabolomics studies to diagnose diseases. These metabolomics studies are part of a continually growing body reflecting the preeminent role of metabolomics in diseases diagnosis and the pathogenic understanding of diseases and variable medical conditions.

5.1 Metabolomics in the prediction of substance exposure

Some of the metabolomics studies aimed to investigate metabolic changes consequent to the exposure to environmental factors or toxins, such as smoking and other harmful substances. In an approach to explore the effects of smoking and smoking cessation, researchers had 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 *et al.*, 2013). They had found that 21 smoking related metabolites were significantly different from current smokers and non-smokers. Interestingly, the study discovered that 19 out of the 21 metabolites were reversible in quitters. In study which aimed to test the systemic toxicity of welding fumes, Wei and

colleagues used liquid and gas chromatography-MS to investigate the plasma metabolome of boilermakers pre-welding and post-welding fumes exposure in two stage-study (Wei *et al.*, 2013). The first stage was conducted in 2011 on 11 boilermakers. The second stage was conducted in 2012 on 8 boilermakers, where five of them participated in first stage in addition to three new recruited boilermakers. The results showed that the high exposure to high metal welding fumes causes decrease in the level of unsaturated fatty acids.

5.2 Using ¹H-NMR spectroscopy in metabolomics

The proton nuclear magnetic resonance ¹H-NMR is one of the main analytical tools that is being widely used in metabolomics, in addition to MS and fourier transform infrared spectroscopy (FT-IR) (Basanta *et al.*, 2012; Lloyd *et al.*, 2011; Schicho *et al.*, 2012). It is being widely used in metabolomics 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 atom in the chemical structure of the metabolites (Dunn, Bailey, & Johnson, 2005). The concept that ¹H-NMR analysis relies on is that every compound or metabolite contains hydrogen atoms in different chemical structure forms will give specific and different peaks in the NMR spectra when the compound enters the magnetic field. These peaks are characteristic for each compound. Although ¹H-NMR has lower sensitivity when compared to MS (Silva Elipe, 2003), the ¹H-NMR analysis is less complex as the sample does not need prior derivatization, extraction and separation as required by MS (Clayton *et al.*, 2006). In addition, ¹H-NMR act as an independent instrument compared to MS which requires separation instrument like GC or LC (Dunn *et al.*, 2005).

Table 1. Examples of metabolomics studies to diagnose diseases.

Study	Disease	Analytical Method	Specimen	Findings
(Ibrahim <i>et al.</i> , 2011)	Asthma	GC-MS	Breath	Development of a discriminatory model which classifies asthma patients with accuracy of 86%
(Basanta <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 2013)	Alzheimer's with Dementia	LC-ECA GC-TOFMS	CSF	Identified biomarkers which discriminate Alzheimer's patients from their healthy controls
(Lewitt <i>et al.</i> , 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.

In NMR metabolomics, plasma and urine samples will be mixed with buffer before analyzing them in the NMR machine. Chemometrics software will be used to process the obtained NMR spectrum to elucidate and identify the chemical structure of the metabolites in the samples. Metabolites web-based and software databases can be used to assist the identification. This will help to assign the metabolite of interest. The $^1\text{H-NMR}$ can analyze many samples per day and the samples can be stored and reused for further analysis (Nicholson & Lindon, 2008; Shulaev, 2006). Therefore, the $^1\text{H-NMR}$ is considered cheap and non-destructive when compared to MS. Figure 2 illustrates a Google scholar based literature search for publications containing (metabolomics and NMR) and (metabonomics and 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.

5.3 Using metabolomics to investigate alcohol consumption

There are metabolomics studies that investigated the effects of alcohol consumption by analyzing the biological samples of animals on alcohol containing diets. Furthermore, researchers explored this in humans as well.

5.3.1 Using metabolomics to investigate alcohol consumption in animal models

Some metabolomics studies used the metabolomics approach to study the pathogenesis of alcohol consumption in animal models. Most of these studies were oriented by expected consequences of alcohol's detrimental effect on liver (Bradford *et al.*, 2008; Fernando *et al.*, 2010; Loftus *et al.*, 2011). Bradford and colleagues used $^1\text{H-NMR}$ 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 iso-caloric (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 $^1\text{H-NMR}$ principal component analysis of both urine and liver extracts 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 the alcohol group. Additionally, there was an increase in prostacycline inhibitor 7,10,13,16-docosatetraenoic acid which is vital in the regulation of platelets formation. Similarly, Loftus and colleagues used LC-MS metabolomics analysis to explore 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. In another study, Fernando and colleagues extracted lipids from the plasma and liver of rats fed with alcohol diets and their controls (Fernando *et al.*, 2010). From both $^1\text{H-NMR}$ and $^{31}\text{P-NMR}$ analysis, they concluded 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.

5.4.2 Using metabolomics to investigate alcohol consumption in humans

There are few metabolomics studies that studied the effect of alcohol consumption on human metabolome. Jaremek and colleagues investigated the effects of alcohol consumption on human serum metabolome (Jaremek *et al.*, 2013). Researchers compared the serum metabolome of two

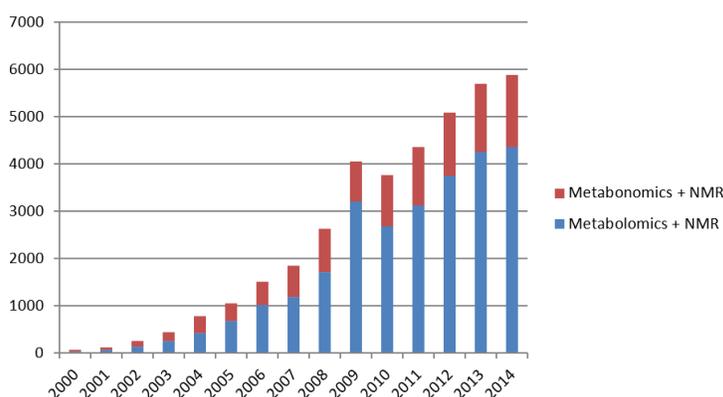


Figure 2. Literature search at Google scholar for publications of (metabolomics + NMR) and (Metabonomics + NMR).

groups, light drinkers (LD) and moderate to heavy drinkers (MHD). The results showed that the concentration 40 identified metabolites in males and 18 in females differed significantly between the 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 sphingo-myelinase (ASM) activity causes the accumulation of ceramide and decrease of sphingomyelins. 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 ¹H-NMR metabolomics technique to find serum metabolic fingerprint that discriminate alcoholic liver cirrhosis from hepatitis B virus liver cirrhosis (Qi *et al.*, 2012). The investigators found that five metabolites could distinguish between the two different types of cirrhosis. However, the study did not explore urine metabolic profile and did not look for metabolic fingerprint of chronic use of alcohol. Recently, we evaluated using ¹H-NMR metabolomics to identify novel biomarkers of AD in plasma and urine (Mostafa *et al.*, 2016; Mostafa *et al.*, 2017). We analyzed plasma and urine samples of three groups from East Asians population; AD patients, social drinkers and naïve control. The analysis showed that ¹H-NMR is able to discriminate between AD subjects and both naïve and

social drinkers subjects with high accuracies. In plasma, the accuracy of the identified biomarkers was 94.2%. In urine, the accuracy of the identified biomarkers was 92%. These findings showed that ¹H-NMR metabolomics can be a pre-eminent approach to accurately diagnose AD. It is necessary to validate these findings in other populations. Table 2 shows a summary of metabolomics studies conducted on alcohol consumption and AD.

6. Conclusions

AD is a disease which burdens patients and society, therefore, the early diagnosis of AD is imperative to start the optimum treatment and to prevent the detrimental effects of chronic consumption of alcohol. Indeed, this detection might encourage AD individuals to seek treatment for their illness. Besides, it is substantial for the society to detect individuals suffering from AD to address them with awareness programs on the harmful effects of chronic alcohol consumption. This will not only have a positive impact on AD individuals themselves but also on their families and the society. As the optimum treatment of diseases requires precise diagnosis, finding accurate method to diagnose AD is crucial for the treatment. The benefits of the rapid, non-selective and non-destructive analytical tool such as ¹H-NMR has distinctly increased its use in metabolomics recently. Applying this technique in AD diagnosis can provide fast and reliable precise diagnosis.

Table 2. Examples of metabolomics studies to study alcohol consumption.

Study	Class	Analytical Method	Specimen	Main Findings
(Bradford <i>et al.</i> , 2008)	Mice	¹ H-NMR and MS	Liver extract and urine	The analysis showed significant discrimination between the control and alcohol groups.
(Fernando <i>et al.</i> , 2010)	Rats	¹ H-NMR and ³¹ P-NMR	Plasma and liver samples	A significant alteration in lipid metabolism was induced by alcohol consumption.
(Loftus <i>et al.</i> , 2011)	Rats and mice	LC-MS	Liver samples	The study revealed a significant increase of fatty acyls, fatty acid ethyl esters, in addition to octadecatrienoic acid and eicosapentaenoic acid metabolites.
(Qi <i>et al.</i> , 2012)	Human	¹ H-NMR	Serum	Five metabolites could distinguish between alcoholic liver cirrhosis and hepatitis B virus liver cirrhosis.
(Jaremek <i>et al.</i> , 2013)	Human	LC-MS	Serum	Ten metabolites in males and 5 in females were specific metabolites to discriminate light drinkers from moderate to high drinkers.
(Mostafa <i>et al.</i> , 2016)	Human	¹ H-NMR	Urine	Six biomarkers discriminated between alcohol dependence from social drinkers and alcohol-naïve.
(Mostafa <i>et al.</i> , 2017)	Human	¹ H-NMR	Plasma	Two biomarkers could differentiate between individuals with AD from social drinkers and alcohol-naïve.

References

- Achur, R. N., Freeman, W. M., & Vrana, K. E. (2010). Circulating cytokines as biomarkers of alcohol abuse and alcoholism. *Journal of Neuroimmune Pharmacology*, 5(1), 83-91.
- Adias, T. C., Egerton, E., & Erhabor, O. (2013). Evaluation of coagulation parameters and liver enzymes among alcohol drinkers in Port Harcourt, Nigeria. *International Journal of General Medicine*, 6, 489-494. doi: 10.2147/ijgm.s43472
- Aguilera-Barreiro Mde, L., Rivera-Marquez, J. A., Trujillo-Arriaga, H. M., Ruiz-Acosta, J. M., & Rodriguez-Garcia, M. E. (2013). Impact of risk factors for osteoporosis on bone mineral density in perimenopausal women of the City of Queretaro, Mexico. *Archivos Latinoamericanos de Nutrición*, 63(1), 21-28.
- Baan, R., Straif, K., Grosse, Y., Secretan, B., El Ghissassi, F., Bouvard, V.,...Cogliano, V. (2007). Carcinogenicity of alcoholic beverages. *Lancet Oncology*, 8(4), 292-293.
- Bagnardi, V., Rota, M., Botteri, E., Tramacere, I., Islami, F., Fedirko, V.,... La Vecchia, C. (2013). Light alcohol drinking and cancer: a meta-analysis. *Annals of Oncology*, 24(2), 301-308. doi:10.1093/annonc/mds337
- Basanta, M., Ibrahim, B., Dockry, R., Douce, D., Morris, M., Singh, D.,...Fowler, S. J. (2012). Exhaled volatile organic compounds for phenotyping chronic obstructive pulmonary disease: a cross-sectional study. *Respiratory Research*, 13, 72. doi:10.1186/1465-9921-13-72
- Beckonert, O., Keun, H. C., Ebbels, T. M., Bundy, J., Holmes, E., Lindon, J. C., & Nicholson, J. K. (2007). Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature Protocols*, 2(11), 2692-2703. doi:10.1038/nprot.2007.376
- Best Practice Advocacy Centre of New Zealand. (2010). Best Tests: Investigation of hazardous drinking. *Best Tests*.
- Birkedal-Hansen, H. (1993). Role of cytokines and inflammatory mediators in tissue destruction. *Journal of Periodontal Research*, 28(6 Pt 2), 500-510.
- Boto-Ordóñez, M., Urpi-Sarda, M., Queipo-Ortuno, M. I., Corella, D., Tinahones, F. J., Estruch, R., & Andres-Lacueva, C. (2013). Microbial metabolomic fingerprinting in urine after regular dealcoholized red wine consumption in humans. *Journal of Agricultural and Food Chemistry*. doi:10.1021/jf402394c
- Bradford, B. U., O'Connell, T. M., Han, J., Kosyk, O., Shymonyak, S., Ross, P. K.,...Rusyn, I. (2008). Metabolomic profiling of a modified alcohol liquid diet model for liver injury in the mouse uncovers new markers of disease. *Toxicology and Applied Pharmacology*, 232(2), 236-243. doi:10.1016/j.taap.2008.06.022
- Breitling, R. (2010). What is systems biology? *Frontiers in Physiology*, 1. doi:10.3389/fphys.2010.00009
- Bujanda, L. (2000). The effects of alcohol consumption upon the gastrointestinal tract. *American Journal of Gastroenterology*, 95(12), 3374-3382. doi:10.1111/j.1572-0241.2000.03347.x
- Cappola, T. P., & Margulies, K. B. (2011). Functional genomics applied to cardiovascular medicine. *Circulation*, 124(1), 87-94. doi:10.1161/circulationaha.111.027300
- Clayton, T. A., Lindon, J. C., Cloarec, O., Antti, H., Charuel, C., Hanton, G.,...Nicholson, J. K. (2006). Pharmacometabonomic phenotyping and personalized drug treatment. *Nature*, 440(7087), 1073-1077. doi:10.1038/nature04648
- Corona, G., Rizzolio, F., Giordano, A., & Toffoli, G. (2012). Pharmacometabonomics: an emerging "omics" tool for the personalization of anticancer treatments and identification of new valuable therapeutic targets. *Journal of Cellular Physiology*, 227(7), 2827-2831. doi: 10.1002/jcp.24003
- Couture, S., Brown, T. G., Tremblay, J., Ng Ying Kin, N. M., Ouimet, M. C., & Nadeau, L. (2010). Are biomarkers of chronic alcohol misuse useful in the assessment of DWI recidivism status? *Accident Analysis and Prevention*, 42(1), 307-312. doi:10.1016/j.aap.2009.08.016
- Decramer, S., de Peredo, A. G., Breuil, B., Mischak, H., Monsarrat, B., Bascands, J.-L., & Schanstra, J. P. (2008). Urine in clinical proteomics. *Molecular and Cellular Proteomics*, 7(10), 1850-1862. doi:10.1074/mcp.R800001-MCP200
- Down, S. (2010). Biofluid sampling techniques: Critical influences on metabolomic studies by GC/MS. Retrieved from <http://www.spectroscopynow.com/details/ezine/sepspec24396ezine/Biofluid-sampling-techniques-Critical-influences-on-metabolomic-studies-by-GCMS.html?tzcheck=1>
- Dunn, W. B., Bailey, N. J., & Johnson, H. E. (2005). Measuring the metabolome: current analytical technologies. *Analyst*, 130(5), 606-625. doi:10.1039/b418288j
- Fernando, H., Kondraganti, S., Bhopale, K. K., Volk, D. E., Neerathilingam, M., Kaphalia, B. S.,...Shakeel Ansari, G. A. (2010). (1)H and (31)PNMR lipidome of ethanol-induced fatty liver. *Alcoholism Clinical and Experimental Research*, 34(11), 1937-1947. doi:10.1111/j.1530-0277.2010.01283.x
- Fillmore, K. M., Stockwell, T., Chikritzhs, T., Bostrom, A., & Kerr, W. (2007). Moderate alcohol use and reduced mortality risk: systematic error in prospective studies and new hypotheses. *Annals of Epidemiology*, 17(5 Suppl.), S16-23. doi:10.1016/j.annepidem.2007.01.005
- Fini, M., Salamanna, F., Veronesi, F., Torricelli, P., Nicolini, A., Benedicenti, S.,...Giavaresi, G. (2012). Role of obesity, alcohol and smoking on bone health. *Frontiers in Bioscience (Elite Ed.)*, 4, 2686-2706.
- Freeman, W. M., & Vrana, K. E. (2010). Future prospects for biomarkers of alcohol consumption and alcohol-induced disorders. *Alcoholism Clinical and Experimental Research*, 34(6), 946-954. doi:10.1111/j.1530-0277.2010.01169.x

- George G. Harrigan, Maguire, G., & Boros, L. (2008). Metabolomics in Alcohol Research and Drug Development. *Alcohol Research and Health*, 31(1).
- Grant, B. F., & Dawson, D. A. (1997). Age at onset of alcohol use and its association with DSM-IV alcohol abuse and dependence: results from the National Longitudinal Alcohol Epidemiologic Survey. *Journal of Substance Abuse Treatment*, 9, 103-110.
- Griffiths, W. J. (2008). *Metabolomics, metabonomics and metabolite profiling*. Cambridge, England: RSC Publishing.
- National Health Committee. (1999) *Guidelines for Recognising, Assessing and Treating Alcohol and Cannabis Abuse in Primary Care*. Wellington, New Zealand: National Health Committee.
- Gutiu, I. A., Andries, A., Mircioiu, C., Radulescu, F., Georgescu, A. M., & Cioaca, D. (2010). Pharmacometabonomics, pharmacogenomics and personalized medicine. *Romanian Journal of Internal Medicine*, 48(2), 187-191.
- Hocquette, J. F. (2005). Where are we in genomics. *Journal of Physiology and Pharmacology*, 56(Suppl. 3), 37-70.
- Hunt, & John. (2007). If it smell like a duck, it might be an asthma subphenotype. *American Journal of Respiratory and Critical Care Medicine*, 175(10), 975-976. doi:10.1164/rccm.200703-302ED
- Institute of Alcohol Studies. (2013). Health impacts of alcohol factsheet. Retrieved from: <http://www.ias.org.uk/uploads/pdf/Health%20Impacts%20docs/Health%20impacts%20of%20alcohol%20factsheet%20July%202013.pdf>
- Jaremek, M., Yu, Z., Mangino, M., Mittelstrass, K., Prehn, C., Singmann, P.,... Wang-Sattler, R. (2013). Alcohol-induced metabolomic differences in humans. *Translational Psychiatry*, 3, e276. doi:10.1038/tp.2013.55
- Karavitis, J., & Kovacs, E. J. (2011). Macrophage phagocytosis: effects of environmental pollutants, alcohol, cigarette smoke, and other external factors. *Journal of Leukocyte Biology*, 90(6), 1065-1078. doi:10.1189/jlb.0311114
- Kroke, A., Klipstein-Grobusch, K., Hoffmann, K., Terbeck, I., Boeing, H., & Helander, A. (2001). Comparison of self-reported alcohol intake with the urinary excretion of 5-hydroxytryptophol:5-hydroxyindole-3-acetic acid, a biomarker of recent alcohol intake. *British Journal of Nutrition*, 85(5), 621-627.
- Kumar, P. (2010, November 23). How excess alcohol affects human metabolism: A metabolomic perspective. *Biotech Articles*. Retrieved from: <http://www.biotecharticles.com/Healthcare-Article/How-Excess-Alcohol-Affects-Human-Metabolism-A-Metabolomic-Perspective-489.html>
- Kuster, D. W., Merkus, D., van der Velden, J., Verhoeven, A. J., & Duncker, D. J. (2011). 'Integrative Physiology 2.0': integration of systems biology into physiology and its application to cardiovascular homeostasis. *Journal of Physiology*, 589(Pt. 5), 1037-1045. doi:10.1113/jphysiol.2010.201533
- Larkman, N. (2013). Towards evidence-based emergency medicine: best BETs from the Manchester Royal Infirmary. BET 1: Can biological markers predict alcohol withdrawal syndrome? *Emergency Medicine Journal*, 30(6), 512-513. doi:10.1136/emered-2013-202697.1
- Litten, R. Z., Bradley, A. M., & Moss, H. B. (2010). Alcohol biomarkers in applied settings: recent advances and future research opportunities. *Alcoholism Clinical and Experimental Research*, 34(6), 955-967. doi:10.1111/j.1530-0277.2010.01170.x
- Lloyd, A. J., William Allwood, J., Winder, C. L., Dunn, W. B., Heald, J. K., Cristescu, S. M.,... Mur, L. A. J. (2011). Metabolomic approaches reveal that cell wall modifications play a major role in ethylene-mediated resistance against *Botrytis cinerea*. *The Plant Journal*, 67(5), 852-868. doi:10.1111/j.1365-313X.2011.04639.x
- Loftus, N., Barnes, A., Ashton, S., Michopoulos, F., Theodoridis, G., Wilson, I.,... Kaplowitz, N. (2011). Metabonomic investigation of liver profiles of nonpolar metabolites obtained from alcohol-dosed rats and mice using high mass accuracy MSn analysis. *Journal of Proteome Research*, 10(2), 705-713. doi: 10.1021/pr100885w
- Louridas, G., & Lourida, K. (2012). The new biology: a bridge to clinical cardiology. *Hippokratia*, 16(2), 106-112.
- Louridas, G. E., Kanonidis, I. E., & Lourida, K. G. (2010). Systems biology in heart diseases. *Hippokratia*, 14(1), 10-16.
- McDonald, H., Borinskya, S., Kiryanov, N., Gil, A., Helander, A., & Leon, D. A. (2013). Comparative performance of biomarkers of alcohol consumption in a population sample of working-aged men in Russia: the Izhevsk family study. *Addiction*, 108(9), 1579-1589. doi: 10.1111/add.12251
- Mostafa, H., Amin, A. M., Teh, C.-H., Murugaiyah, V., Arif, N. H., & Ibrahim, B. (2016). Metabolic phenotyping of urine for discriminating alcohol-dependent from social drinkers and alcohol-naive subjects. *Drug and Alcohol Dependence*, 169, 80-84. doi:10.1016/j.drugalcdep.2016.10.016
- Mostafa, H., Amin, A. M., Teh, C.-H., Murugaiyah, V. a. I., Arif, N. H., & Ibrahim, B. (2017). Plasma metabolic biomarkers for discriminating individuals with alcohol use disorders from social drinkers and alcohol-naive subjects. *Journal of Substance Abuse Treatment*, 77, 1-5. doi:10.1016/j.jsat.2017.02.015
- National Center for Health Statistics. (2009). *Alcohol use in US*. Washington, DC: Author.
- National Institute of Health. (2001). Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clinical Pharmacology and Therapeutics*, 69(3), 89-95. doi:10.1067/mcp.2001.113989
- Nicholson, J. K., & Lindon, J. C. (2008). Systems biology: Metabonomics. *Nature*, 455(7216), 1054-1056. doi: 10.1038/4551054a

- Piran, S., Liu, P., Morales, A., & Hershberger, R. E. (2012). Where genome meets phenome: rationale for integrating genetic and protein biomarkers in the diagnosis and management of dilated cardiomyopathy and heart failure. *Journal of the American College of Cardiology*, 60(4), 283-289. doi:10.1016/j.jacc.2012.05.005
- Plunk, A. D., Syed-Mohammed, H., Cavazos-Rehg, P., Bierut, L. J., & Grucza, R. A. (2013). Alcohol Consumption, Heavy Drinking, and Mortality: Rethinking the J-Shaped Curve. *Alcoholism Clinical and Experimental Research*. doi:10.1111/acer.12250
- Qi, S., Tu, Z., Ouyang, X., Wang, L., Peng, W., Cai, A., & Dai, Y. (2012). Comparison of the metabolic profiling of hepatitis B virus-infected cirrhosis and alcoholic cirrhosis patients by using (1) H NMR-based metabolomics. *Hepatology Research*, 42(7), 677-685. doi:10.1111/j.1872-034X.2011.00964.x
- Quaye, I. K., Nyame, P. K., Dodoo, D., Gyan, B., & Adjei, A. A. (1992). Biochemical and haematological markers of alcohol intake in Ghanaians. *West African Journal of Medicine*, 11(3), 199-202.
- Rehm, J., Room, R., Graham, K., Monteiro, M., Gmel, G., & Sempos, C. T. (2003). The relationship of average volume of alcohol consumption and patterns of drinking to burden of disease: an overview. *Addiction*, 98(9), 1209-1228.
- Rinck, D., Frieling, H., Freitag, A., Hillemacher, T., Bayerlein, K., Kornhuber, J., & Bleich, S. (2007). Combinations of carbohydrate-deficient transferrin, mean corpuscular erythrocyte volume, gamma-glutamyltransferase, homocysteine and folate increase the significance of biological markers in alcohol dependent patients. *Drug Alcohol Depend*, 89(1), 60-65. doi:10.1016/j.drugalcdep.2006.11.022
- Romeo, J., Warnberg, J., & Marcos, A. (2010). Drinking pattern and socio-cultural aspects on immune response: an overview. *Proceedings of the Nutrition Society*, 69(3), 341-346. doi:10.1017/s0029665110001904
- Schicho, R., Shaykhtudinov, R., Ngo, J., Nazyrova, A., Schneider, C., Panaccione, R., ...Storr, M. (2012). Quantitative Metabolomic Profiling of Serum, Plasma, and Urine by 1H NMR Spectroscopy Discriminates between Patients with Inflammatory Bowel Disease and Healthy Individuals. *Journal of Proteome Research*, 11(6), 3344-3357. doi:10.1021/pr300139q
- Semmar, N. (2012). Metabotype concept: Flexibility, usefulness and meaning in different biological populations. In U. Roessner (Ed.), *Metabolomics* (pp. 131-166).
- Rijeka, Croatia: InTech. Retrieved from: <http://www.intechopen.com/books/metabolomics/metabotype-concept-flexibility-usefulness-and-meaning-in-different-biological-populations>
- Shah, S. H., Kraus, W. E., & Newgard, C. B. (2012). Metabolomic profiling for the identification of novel biomarkers and mechanisms related to common cardiovascular diseases form and function. *Circulation*, 126(9), 1110-1120.
- Shulaev, V. (2006). Metabolomics technology and bioinformatics. *Briefings in Bioinformatics*, 7(2), 128-139. doi:10.1093/bib/bbl012
- Silva Elipse, M. V. (2003). Advantages and disadvantages of nuclear magnetic resonance spectroscopy as a hyphenated technique. *Analytica Chimica Acta*, 497(1-2), 1-25. doi:10.1016/j.aca.2003.08.048
- Stewart, S. H., Reuben, A., Brzezinski, W. A., Koch, D. G., Basile, J., Randall, P. K., & Miller, P. M. (2009). Preliminary evaluation of phosphatidylethanol and alcohol consumption in patients with liver disease and hypertension. *Alcohol and Alcoholism*, 44(5), 464-467. doi:10.1093/alcal/agp039
- Stibler, H. (1991). Carbohydrate-deficient transferrin in serum: a new marker of potentially harmful alcohol consumption reviewed. *Clinical Chemistry*, 37(12), 2029-2037.
- Tavakoli, H. R., Hull, M., & Michael Okasinski, L. (2011). Review of current clinical biomarkers for the detection of alcohol dependence. *Innovations in Clinical Neuroscience*, 8(3), 26-33.
- Vaaramo, K., Puljula, J., Tetri, S., Juvela, S., & Hillbom, M. (2012). Mortality of harmful drinkers increased after reduction of alcohol prices in northern Finland: a 10-year follow-up of head trauma subjects. *Neuroepidemiology*, 39(3-4), 156-162. doi:10.1159/000341241
- Vaidyanathan, S., Harrigan, G. G., & Goodacre, R. (2005). *Metabolome analyses: Strategies for systems biology*. Berlin, Germany: Springer.
- van Amsterdam, J., & van den Brink, W. (2013). The high harm score of alcohol. Time for drug policy to be revisited? *Journal of Psychopharmacology*, 27(3), 248-255. doi:10.1177/0269881112472559
- Wallerath, T., Poleo, D., Li, H., & Förstermann, U. (2003). Red wine increases the expression of human endothelial nitric oxide synthase: a mechanism that may contribute to its beneficial cardiovascular effects. *Journal of the American College of Cardiology*, 41(3), 471-478.
- Want, E. J., Wilson, I. D., Gika, H., Theodoridis, G., Plumb, R. S., Shockcor, J., ...Nicholson, J. K. (2010). Global metabolic profiling procedures for urine using UPLC-MS. *Nature Protocols*, 5(6), 1005-1018. doi:10.1038/nprot.2010.50
- Wei, Y., Wang, Z., Chang, C. Y., Fan, T., Su, L., Chen, F., & Christiani, D. C. (2013). Global metabolomic profiling reveals an association of metal fume exposure and plasma unsaturated fatty acids. *PLoS One*, 8(10), e77413. doi:10.1371/journal.pone.0077413
- World Health Organization. (2011). Alcohol Fact Sheet. Retrieved from: <http://www.who.int/mediacentre/factsheets/fs349/en/>
- Xu, T., Holzapfel, C., Dong, X., Bader, E., Yu, Z., Prehn, C., ...Wang-Sattler, R. (2013). Effects of smoking and smoking cessation on human serum metabolite profile: results from the KORA cohort study. *BMC Medicine*, 11(1), 1-14. doi:10.1186/1741-7015-11-60