Metabolomics of diet-related diseases using mass spectrometry

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ABSTRACT

Increased caloric intake associated with decreased physical activity and the presence of thrifty genes that are theoretically adapted to enhance the energy storage efficiency, cause metabolic changes that result in diet-related diseases or disorders. Such phenotypes are prevalent in populations of developed countries and their incidence is continuing to rise. Therefore, early diagnosis of diet-related diseases is an exciting field of research. The application of 'omics' technology, particularly metabolomics, has revealed the metabolic changes associated to diet-related diseases and also consequences of diet intervention in a global un-targeted way. The on-going development of dietary ideal models could elucidate the sequence of events, starting with the interaction between dietary habits and genetic adaptations that cause the metabolic changes induced as well as auxiliary symptoms and associated diseases. In this review, a range of mass spectrometry techniques applied to metabolomics of diet-related diseases is discussed, including the combination of metabolomics with other studies to reveal systems properties of the diseases. Since it is difficult to set up a clinical study based on the probability of finding exploratory biomarkers to be applied in wide-population screening, many metabolomics studies have revealed biomarkers of the complications of the disease, which could have power as prognostic biomarkers.

Keywords: diabetes; cardiovascular disease; cancer; metabolic fingerprinting; nutrigenomics; systems biology

1. Introduction

1.1. Analysis of the Metabolome: Metabolomics

The metabolome is the complete set of small molecules (typically, less than 1500 Da) arising from protein activity (anabolism and catabolism) in living systems. There are four major approaches used in metabolomics studies: (i) targeted analysis, (ii) metabolite profiling, (iii) metabolic fingerprinting and (iv) metabolic flux analysis. Targeted analysis is the classical analytical approach to measure the concentration of a limited number of known metabolites precisely. Metabolite profiling, which can be considered an extension of target analysis is the simultaneous measurement of a set of related metabolites that are (bio)chemically related. Metabolic fingerprinting does not attempt to identify or precisely quantify all the metabolites in the sample; it considers the fingerprint as a unique pattern characterising a snapshot of the metabolism in a particular cell line or tissue and is most useful in biomarker discovery and diagnostics [1-3]. Metabolic flux analysis involves tracing certain elements through metabolic pathways that results in a detailed quantification of fluxes, usually through central carbon metabolism. The latter is an important approach in metabolomics, but has not been included in this review since its application in diet-related diseases is only pioneering.

Due to the large variability in physico-chemical properties of analytes, together with the enormous differences in concentrations, there is no single analytical technique that can fulfil all the requirements to provide adequate signal for all. Metabolome analysis is generally conducted through two detection techniques: nuclear magnetic resonance (NMR) or mass spectrometry (MS). Metabolomics with NMR has been recently reviewed elsewhere [4-6], and was the topic for a special issue . Although sensitivity is poorer in NMR than MS, the elucidation capabilities are unquestionable; the NMR profile could contain qualitative and quantitative information on hundreds of different small molecules present in the sample. Although metabolomics was first developed with NMR, in recent years MS has been the most commonly employed technique; from 2007 to date there have been more than 70% of papers from this topic published. With regard to MS, two approaches can be considered, with different subtypes: the MS can be

hyphenated to a separation technique (gas chromatography-GC, liquid chromatography-LC, capillary electrophoresis-CE, supercritical fluid chromatography-SFC) or not. MS benefits from detection permitted at high sensitivity and also structural elucidation based on spectral libraries and tandem mass spectrometry, even in complex biological samples.

GC-MS is very well suited for the analysis of low-molecular weight (typically, less than 400 amu). GC is excellent for characterisation of energy metabolism as it is highly appropriate for volatile derivatives of metabolites; amino acids, monosaccharides, fatty acids, disaccharides and cholesterol are easily identified in the chromatogram [7, 8]. Such metabolites are mostly hydrophilic, and therefore freely soluble in typical bio-fluids such as serum/plasma or urine. CE-MS can be applied to the analysis of similar compounds to a certain extent, benefiting from reduced sample treatment compared to GC-MS [9, 10] but has different strengths and drawbacks. The most employed technique for metabolic fingerprinting is LC-MS, which involves the minimum requirement for sample treatment and alteration or hydrolysis of the metabolites during the analysis among the hyphenated techniques. [11-13]. The most common separation mode for LC-MS based metabolomic studies involves reversed-phase chromatographic separation of analytes. This is especially suited to metabolites with medium-low polarity, although analysis is limited since polar metabolites including sugars or amino acids that CE-MS and GC-MS typically analyse are not detected. Such metabolites can be analysed by LC-MS, however it requires the application of hydrophilic liquid chromatography (HILIC) [14-16]. The most high-throughput approach for MS in metabolomics, with respect to data acquisition, utilises direct injection with no prior separation of analytes in the sample. For this reason, it is often the preferred technique for clinical trials where sample sizes are large but the intricacy of information required from each is not high. A considerably less high-throughput form of direct MS that is pioneering in metabolomics is mass spectrometry imaging (MSI). This technique involves the analysis of a sample surface from which a chemical map can be created based on collecting spectra-per-pixels.

1.2. **Metabolomics in clinical research**

In the field of clinical research there are recent reviews which highlight both the potential and relevance of metabolomics for biomarker discovery and the design of new therapeutics [17-21]. Epidemiological and clinical studies have concluded that many diseases with high rates of morbidity and mortality worldwide are associated with diet-related incidences and include cardiovascular disease (heart disease and stroke), diabetes and cancers. The goals of metabolomics for diet-related disease research (Figure 1) are to improve the search for biomarkers that would permit determination of the cause of the disease as well as to elucidate the biochemical mechanisms involved in the development of the disease status. Ultimately, it may be possible using this approach to improve early diagnosis and accurate prognosis, as well as aid adequate monitoring including detecting early markers of target organ damage.

The term diet-related disease can refer to a wide variety of diseases and disorders affecting different organs and systems. Nevertheless, not all diseases related to diet are considered diet-related diseases: the lack of nutrients (proteins, vitamins, and minerals) may give rise to specific complications known as deficiency diseases. In addition, the management of inborn errors of metabolism is mainly based on specific diets without food constituents that are safe for general population but harmful for those affected. For example, the onset of phenylketonuria (PKU) can be prevented through a diet deficient in phenylalanine [22]. Diabetes mellitus (DM), cardiovascular Disease (CVD), ischemic heart disease (IHD), and cerebrovascular disease (CBVD) are classified as the main diet-related diseases in addition to a range of cancers [23].

Research on human nutrition metabolomics has proven valuable [24-28], highlighting the potential for the interactions between nutrients and metabolism leading to metabolic alterations [29, 30]. It is accepted that in modern life, increased caloric intake associated with decreased physical activity and the presence of thrifty genes, theoretically adapted to enhance the energy storage efficiency, will result in metabolic changes that could result in diet-related diseases or disorders. Regardless of the disease, the risk is modified by diet, nutrient uptake, genetic profile and environmental factors (alcohol consumption, smoke, sedentary lifestyle). In some cases one disease or disorder can lead to others. For example, metabolic syndrome, impaired glucose tolerance, diabetes, obesity and dyslipidaemia increase the risk of cardiovascular events [31]. Conditions

such obesity, hyperlipidaemia, and insulin resistance have also been described to increment the risk of heart diseases [32].

. DM is classified as type 1 when there is insulin deficiency and type 2 when genetic and metabolic defects in insulin action or secretion cause hyperglycemia. Although the management of all types of diabetes must include careful control of diet, nutrition disorders are associated only with type 2 diabetes; therefore most of the data herein mentioned are related to this. The worldwide prevalence of DM is increasing; the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 [33]. For this reason, the disease has been extensively researched and the application of metabolomics has been reviewed [34-38], including its association with CVD [39, 40].

CVD covers an extended group of diseases including hypertension, heart failure and rheumatic heart disease. IHD and CBVD are also closely related. In general, CVDs have been studied through metabolomics and this has been previously reviewed [41-44]. IHDs (e.g. myocardial angina, heart attack) are characterised by inadequate blood supply to a portion of the myocardium. The main cause of myocardial ischemia is atherosclerotic disease of the coronary arteries that are responsible for irrigating the myocardium. Its incidence is increasing not only in developed countries but also in developing countries where it has been estimated to rise by 120 % in women and 127 % between 1990 and 2020 [32].

Cancers of the mouth, throat, oesophagus, lung, breast, endometrium, stomach, colon, and rectum can be modified by food and nutrition (including alcohol), and by physical activity and therefore can be considered diet-related diseases. While consumption of processed food increases the risk of some cancers, epidemiological studies have suggested that the consumption of diets rich in whole cereals reduce the risk of cancer [45]. Therefore diet can modify the risk of cancer both positively and negatively. The particular effect of carbohydrate modification on serum metabolic profile has been studied with metabolomics [46]. Results suggest that the dietary carbohydrate modification alters the serum metabolic profile, especially in lyso-phosphatidylcholine (lysoPC) species, and may, thus, contribute to pro-inflammatory processes which in turn promote adverse changes in insulin and glucose metabolism.

Obesity, hypertension, atherosclerosis and metabolic syndrome are the main disorders associated with diet related diseases. Atherosclerosis that is one of the main disorders associated with CVDs is characterised by the accumulation of lipids in large arteries and it is estimated that over 25 million people in the US have clinical manifestation of ASVD [47]. The process of atherosclerosis involves lipid disturbances, platelet activation, endothelial dysfunction, chronic inflammation, oxidative stress and altered matrix metabolism and as a result the vessel wall thickens affecting blood flow. It is known that its development involves lipid and inflammatory components and that metabolomics can contribute to an increased understanding of its mechanisms [48, 49].

Metabolic Syndrome is associated to and increases the risk of CVD and DM. It is clinically recognised by hypertriglyceridemia, low levels of high-density lipoprotein (HDL), hyperglycaemia, hypertension and central obesity. According to the third national health and nutrition examination survey (NHANES III), approximately 47 million adults suffer from metabolic syndrome in the US [50]. The pathogenesis of metabolic syndrome is still unknown; however the principal cause is insulin resistance due to an inability of peripheral tissues to increase input and utilisation of glucose, which is especially problematic in the liver, skeletal muscle and adipose tissue. Metabolic syndrome is treatable and changes in lifestyle can reduce the risk. The metabolic mechanisms of this syndrome have been reviewed [24, 51, 52].

The purpose of this review is to discuss the contributions from metabolomics in the study of diet-related diseases with particular focus on MS based analytical techniques. Examples will be given to highlight the effectiveness of MS coupled with prior separation of metabolites (LC-MS, GC-MS and CE-MS) as well as direct analysis techniques in MS, where the advantages and disadvantages of each will become apparent. In addition to the analytical platforms, the value of systems biology to study complex diseases such as diet-related diseases will be discussed through reviewing the combination of metabolomics with other 'omic' techniques.

2. **Analytical platforms for metabolomics of diet-related diseases**

The choice of platform depends largely on the application with respect to the samples for analysis as well as the desired information required from the metabolomics experiment. Some of the main considerations for selecting an appropriate platform are summarised in **¡Error! No se encuentra el origen de la referencia.**

Table 1: Choosing the correct MS tool for metabolomics. Techniques are assigned stars (* = lower, ** = medium, *** = higher) based on their appropriateness to satisfy each parameter. Techniques are classified into 5 categories: gas chromatography – mass spectrometry (GC-MS), liquid chromatography – mass spectrometry (LC-MS), capillary electrophoresis – mass spectrometry (CE-MS) and direct mass spectrometry (DMS) and imaging mass spectrometry (MSI)

		LC-MS	GC-MS	CE-MS	DMS	MSI
Metabolite chemistry	High LogP	**	*	*	***	**
	Low LogP	**	***	***	*	**
	Negative charge	***	***	**	**	**
	Positive charge	**	**	***	*	*
	m/z < 80	**	***	*	**	**
	m/z > 80	***	***	**	***	***
Sample type	Tissue	**	**	**	**	***
	Bio-fluids	***	***	***	**	*
	Cell culture	**	**	**	**	**
Metabolomics approach	Targeted	**	***	*	*	*
	Non-targeted	***	**	**	**	**
Analytical specifications	Sample preparation	***	*	***	***	**
	Throughput	**	**	**	***	*
	MS Mass accuracy/resolution	**	*	*	***	**
	Inter-day reproducibility	**	***	*	**	*
	Nº metabolite features	***	**	**	**	*
Data	Databases	**	***	**	*	*
	Data analysis	**	**	**	***	**
	Information	**	**	**	*	***

Choices can be made based on a number of parameters: for example the chemical class of the metabolites to be analysed, the type of sample, whether or not the experiment will follow a targeted approach etc. Additionally, a technique can be chosen based on the ease of data handling with respect to the availability of resources for compound identification (databases) or the ease of data analysis but also the level of information each offers with respect either to the level of metabolite coverage offered or to how well data can be biologically interpreted. The table provides a guide to which techniques are stronger or weaker for different aspects of a metabolomics experiment. Figure 2 shows the features of each MS platform for studying diet-related diseases that can be distinguished both by the classes of compounds they are best for analysing and by the key feature that sets them apart from the other techniques.

2.1. **Gas chromatography - mass spectrometry**

GC-MS is limited to volatile, thermally stable, and energetically stable compounds and those that can be made volatile by derivatisation. For that reason, the main drawbacks

are i) limited metabolite coverage; ii) laborious sample pre-treatment and iii) derivatisation introduces variability and produces artefacts; however, the main strengths include i) involvement of a standardised ionisation source (electronic impact) at 70 eV that leads to reproducible mass spectra and highly transferable electron ionisation MS spectral libraries that allow compound identification through mass spectral library matching; ii) highly reproducible retention indices can also be incorporated to spectral libraries and improve identification of isomers; iii) metabolites covered by GC-MS are related to central carbon metabolism and other fundamental metabolic pathways.

Sample treatment strategies are well established [53] and are based on the trimethylsilyl(TMS)-methoxime(MeOx) derivatives after protein precipitation, where tissues are previously extracted. In that sense a method was validated for colon tissue profiling [54]. Each colon tissue sample was ultra-sonicated with 1 mL of a mixture of chloroform/methanol/water in the ratio of 20:50:20 (v/v/v), followed by centrifugation and collection of supernatant that is later derivatised in the same way.

Urine poses a problem in GC-MS fingerprinting, because urea concentration in urine is so high that it influences the appearances of other compounds with lower concentration and is easy to contaminate the column and MS detector. The role of urinary metabolic profiling in systems biology research has been reviewed recently including urine collection and storage, GC-MS and data pre-processing methods (including depletion of urea *via* treatment with urease, protein precipitation with methanol, and trimethylsilyl derivatisation), chemometric data analysis and urinary marker metabolite identification. The authors conclude that GC-MS information is complementary to NMR and LC-MS [55]. A metabolomics method to investigate the urinary metabolic differences between hepatocellular carcinoma [56] (HCC, $n = 20$) male patients and normal male subjects ($n = 20$) has utilised GC-Q-MS. The urinary endogenous metabolome was assayed using chemical derivatisation followed by GC-MS. After GC-MS analysis, 103 metabolites were detected, of which 66 were annotated as known compounds. By a two sample *t-test* statistics with $p < 0.05$, 18 metabolites were shown to be significantly different between the HCC and control groups.

Recently a time-effective microwave-assisted oximation and silylation approach for metabolomic study of plasma samples has been described [57]. The results showed that microwave irradiation decreased the sample preparation time from approximately 180 min to 5 min without loss of information for the metabolites in plasma samples. This

may prove to be an attractive alternative for high-throughput sample preparation in plasma metabolomics for studies with a large number of samples. After a study showing that sarcosine could be a potential marker for the diagnosis and prognosis of prostate cancer (PCa), a metabolomics approach utilising isotope dilution (ID) GC-MS evaluated sarcosine using [methyl-D3]-sarcosine as an internal standard. Microwave-assisted derivatisation (MAD) together with GC-MS was utilised to obtain the urinary metabolomic information [58]. Due to the derivatisation step, specific data validation, correction/filtering and normalisation procedures are necessary to ensure comparability between profiles and to avoid assigning biological significance to experimental biases, which are due mainly to incomplete derivatisation and the formation of multiple derivatives for some compounds [59, 60].

Regarding MS analysers, GC is often coupled to either TOF-MS or single quadrupole MS. The latter have the advantages of high sensitivity and good dynamic range but suffer from slower scan rates and lower mass accuracy relative to TOF-MS detectors. However, the availability, reliability, effectiveness, and affordable cost of GC-quadrupole-MS analysers have made them a popular and robust metabolomics platform. Another alternative that shares similar characteristics to single quadrupole MS in terms of mass accuracy is the utlilisation of ion trap MS, although this analyser adds the option of isolating an specific ion and doing MS/MS

Plasma samples from children with normal weight have been compared to those who are overweight or obese through metabolic profiling by GC-Q-MS [61]. Multivariate analysis revealed that the metabolic patterns of the three groups were different. Furthermore, several metabolites, including isoleucine, glyceric acid, serine, 2,3,4 trihydroxybutyric acid and phenylalanine were screened as potential biomarkers of childhood obesity. In other study, a diagnostic model for gastric cancer has been constructed using GC-Q-MS and principal component analysis (PCA) [62]. Results showed that 18 metabolites were detected differently between the malignant tissues and the adjacent non-malignant tissues of gastric mucosa. Five metabolites were also detected differently between the non-invasive tumours and the invasive tumours.

GC-Iontrap-MS has been employed to evaluate the major metabolic changes in low molecular weight plasma metabolites of patients with acute coronary syndrome (n = 9) and with stable atherosclerosis (n = 10) *vs*. healthy subjects without significant

differences in age and sex (n = 10) [63]. The three groups were successfully classified and citric acid, 4-hydroxyproline, aspartic acid, as well as fructose were observed to decrease while lactate, urea, glucose, and valine were increased in acute coronary syndrome patients *vs*. healthy people. This analytical platform was also employed in the fingerprinting of patients with stable carotid atherosclerosis *vs*. healthy subjects. In this study, 19 metabolites (isocitrate, glutarate, fructose, citrate, aspartate, lactate, tyrosine, glucose, 3-hydroxybutirate, serine and alanine, among others) were found altered in the plasma of patients with stable carotid atherosclerosis *vs.* healthy subjects [64]. Metabolomics has aided in the study of IHD by allowing identification of biomarkers useful for diagnosis. Vallejo *et al*. [63] used GC-MS to evaluate the metabolic changes associated with acute coronary syndrome and atherosclerosis, whereby a group of 29 subjects: 9 with acute coronary syndrome without ST segment elevation (NSTEACS), 10 with stable atherosclerotic disease of the carotid and 10 controls. Clear differences were observed in the profile of cases and controls, with a significant decrease in the levels of citric acid in NSTEACS patients. Hydroxyproline was also found to be decreased in NSTEACS patients and this fact could be related to the atheromatous plaque instability and increased risk of coronary heart disease.

The GC-MS metabolic profiles of plasma samples from mice maintained on 12- or 15-month long low (10 kcal%) or high (60 kcal%) fat diets were also obtained through GC-Iontrap-MS [65]. The profiles of 48 out of the 77 detected metabolites were used in multivariate statistical analysis. Data mining suggested a decrease in the activity of the energy metabolism with age. In addition, the metabolic profiles indicated the presence of subpopulations with different physiology within the high and low-fat diet mice, which correlated well with the difference in body weight among the animals and current knowledge about hyperglycemic conditions.

GC-TOF-MS technology offers high mass resolution, high mass accuracy, and fast scan speeds. The relatively faster scan rates associated with TOF-MS are extremely useful for the accurate deconvolution of overlapping high resolution or ultrafast GC peaks such as those encountered during complex metabolic mixture analyses. A recent application of GC-TOF-MS in metabolomics included large-scale metabolite profiling of human serum [66]. Apart from genetic alterations, development and progression of colorectal cancer has been linked to influences from nutritional intake,

hyperalimentation, and cellular metabolic changes that may be the basis for new diagnostic and therapeutic approaches. A set of paired samples of normal colon tissue and colorectal cancer tissue was analysed with GC-TOF-MS, resulting in robust detection of a total of 206 metabolites. Subsequent supervised analysis found 82 metabolites to be significantly different at $p < 0.01$. Intermediates of the tricarboxylic acid (TCA) cycle and lipids were found down-regulated in cancer, whereas urea cycle metabolites, purines, pyrimidines and amino acids were generally elevated compared to normal colon mucosa [67].

The serum metabolome of a primate model of *in utero* high-fat exposure has also been characterised by GC-TOF-MS. Data suggest that high-fat diet exposure as well as a maternal obese phenotype results in metabolome variations with 2-hydroxybutyrate, ascorbic acid, a-tocopherol, cholesterol and 3-hydroxybutyrate as significant metabolite markers [68]. Serum samples from 52 patients with systolic heart failure and 57 controls were analysed by GC-TOF-MS [69] and the raw data reduced to 272 statistically robust metabolite peaks. A significant difference between case and control was observed for 38 peaks ($p < 5 \cdot 10^{-5}$). Two such metabolites were pseudouridine, a modified nucleotide present in tRNA and rRNA and marker of cell turnover, as well as the TCA intermediate 2-oxoglutarate.

A unique innovation in GC was the development of GC × GC, which offers dramatically increased separation efficiencies and peak capacities. In GC × GC, two capillary columns of different stationary-phase are selectivity coupled in series through a flow modulator. Effluents from the first column (usually a long non-polar column) are captured and transferred by the modulator onto the second column. The second column is normally a short polar or semi-polar column that quickly separates the effluent within seconds before the next effluent enters the column. The sharp and narrow peaks generated in fast GC or GC × GC require the use of fast scanning analyzers such as TOF-MS.

Comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GC×GC-TOF-MS) coupled with pattern recognition methods was applied to analyse plasma from diabetic patients and healthy controls. Five potential biomarkers including glucose, 2-hydroxyisobutyric acid, linoleic acid, palmitic acid and phosphate were

identified using this method [70]. In another example, glutamic acid, N-acetyl-glycine, 3-hydroxy-2-methyl-butanoic acid and nonanedioic acid were shortlisted from GC×GC-TOF-MS analysis as markers of recurrent breast cancer [71]. Despite this massive separation effort, the markers identified herein could be obtained with simpler procedures such as GC-Q-MS.

A complementary approach to metabolite profiling by GC-MS is taken by employing stable-isotope labelling for flux analysis. Flux analysis emphasises the turnover of molecules through a number of enzymes. In most cases, stable-isotope glucose is used and is differentially labelled at one or more atom positions within the molecule. Flux through glycolysis or adjacent pathways (pentose-phosphate pathway, TCA cycle and gluconeogenesis) is then estimated by analysing relative enrichments in isotopes of metabolites comprised in these pathways in a dynamic way [72]. Flux analysis by GC-MS has also been shown to be useful to characterise the impact of pancreatic tumours on metabolic fluxes in different organs by use of a rat model [73].

2.2. **Capillary electrophoresis - mass spectrometry**

Charged and polar, water soluble metabolites are best suited to analysis by CE-MS. CE is considered a highly efficient, flexible separation technique that is both comprehensive and quantitative. One of its main assets for fingerprinting, where samples require minimum manipulation, is the capability to analyse complex matrices such as urine directly. This is possible because once compounds of interest have been measured, the capillary is rinsed and apparently no irreversible adsorptions take place, except when uncoated capillaries are used that can be modified with proteins and other compounds from the sample. Unfortunately, CE-MS has inherent limitations too. These are mainly low sensitivity and poor reproducibility. CE can be interfaced with various MS analysers, however TOF-MS is the most commonly used CE-MS analyser due to its fast acquisition rates which are necessary to statistically sample the narrow CE peaks while ionisation in CE-MS typically employs ESI.

One of the earlier developments of CE-MS based methods for metabolic fingerprinting to study diet-related diseases came from Soga's group in 2003 [74]. This involved the

development of a non-targeted method for biomarker discovery in several types of cancer-specific profiles in human saliva [75].

CE-TOF-MS has been used to explore new serum biomarkers with high sensitivity and specificity for diabetic nephropathy (DN) diagnosis, through comprehensive analysis of serum metabolites with 78 diabetic patients [76]. Of the 289 profiled metabolites, 19 metabolites were identified that could distinguish between DN patients with macro-albuminuria and diabetic patients without albuminuria. These identified metabolites included creatinine, aspartic acid, γ-butyrobetaine, citrulline, symmetric dimethylarginine (SDMA), kynurenine, azelaic acid, and galactaric acid.

In an attempt to improve sensitivity, new interphases are being tested avoiding the dilution originated by the sheath liquid. Sheath-less CE-MS, using a porous tip sprayer, has been proposed for metabolic profiling of human urine [77]. However, these systems are still under development and do not work in a routine base.

Another study involving CE-MS is related to metabolomics of CBVD. So far there aren't tools that allow an early diagnosis of CBVD and its patho-physiology is not completely understood. Hattori *et al.* (2010) [78] described a metabolomics study of cerebral artery occlusion in a mouse model, through the application of imaging mass spectrometry by matrix-assisted laser desorption ionisation (MALDI-MS) and CE-MS with ESI. Utilising both complementary techniques made possible the analysis of a major fraction of metabolites, including ATP, ADP, and AMP, all of them polar or ionic, by CE-MS and also allowed discrimination along the spatial distribution of the molecules by MALDI-MS. The authors distinguished metabolically two spatial areas in the brain after CBVD; the penumbra zone and the core area. This study made a valuable contribution in terms of development of new strategies to treat patients with CVBD.

2.3. **Liquid chromatography - mass spectrometry**

The advantages of LC-MS in metabolomics are numerous, in terms of sample treatment, robustness and reproducibility, together with the amount and quality of the information that can be achieved. From a theoretical point of view, the versatility of the systems can be highlighted too, because different columns and mobile phases can be employed in

order to obtain the maximum coverage of the metabolites contained in one single sample.

The objective of sample treatments for LC-MS based metabolomics is to obtain a solution containing as many metabolites as possible, free from large molecules (mainly proteins, but complex carbohydrates and nucleic acids must be also removed) dissolved in a solvent compatible with the mobile phase. As reviewed by Álvarez-Sánchez *et al.* in 2010 [79], this involves sampling and metabolite quenching, followed by metabolite extraction. The procedures for sample preparation are different according to the type of sample that is going to be analysed: tissues (organs) or bio-fluids (urine, plasma, serum, cerebrospinal fluid, etc.), free cells (culture) or tissue homogenates. For the specific field of diet-related diseases, it is worthy to mention that another bio-fluid - saliva opens new possibilities for metabolomics studies.

Urine and plasma/serum have been extensively used for research with metabolomic tools, and more clinical applications of such approaches have been already proposed [20, 80]. Regarding urine, all the steps of sample pre-treatment (sampling time and collection, quenching, preservative addition, volume correction, pH adjustment, deproteinisation by centrifugation, normal filtration, ultrafiltration, ultrasound-assisted filtration and freeze-thawing) and sample-preparation steps (dilution, enzymatic hydrolysis, sample clean-up and preconcentration – by solid-phase extraction, liquid-liquid extraction, evaporation, and lyophilisation) have been reviewed elsewhere, for all types of metabolomics analysis based on MS [81]. Sample treatment for blood-derived samples has also been recently reviewed [82], and according to the authors, the most common procedure for LC-MS analysis is based on protein precipitation with cold organic solvent such as methanol or acetonitrile of three-times their volume.

Diet as an origin for disease is not easy to study in humans, and therefore animal models are often used. Insulin resistance was studied by Li *et al.* [83] in wild-type mice and mice lacking a gene involved in lipid metabolism, with diets differing in the fat content. This involved non-targeted fingerprinting with UHPLC-MS (linear trap quadrupole) and GC-MS on plasma, liver and adipose tissue. From this, metabolites not previously associated with insulin resistance were revealed, which could be important in understanding the pathophysiology of diabetes. In another study from Lin *et al*. [84],

rats were used to study these biochemical aspects but with another approach: a fructose-high diet known to promote insulin resistance. The effects of the oral glucose tolerance test were studied by RP-HPLC-qTOF in plasma, liver, skeletal muscle and brain, and a biochemical network could be inferred. These results were confirmed in humans, by UPLC-qTOF analysis during oral glucose tolerance testing of 16 normal individuals where free fatty acids, acylcarnitines, bile acids, and lysophosphatidylcholines were the most discriminating biomarkers of the glucose bolus [85].The development of the atheroma plaque mediated by the diet has been studied by Jové *et al*. [86] in hamsters after consumption of an atherogenic diet through obtaining a global fingerprint, and a lipid fingerprint (lipidomics) by UHPLC-qTOF.

With regard to the effect of diet on cancer, animal models have been applied to clinical studies, such as that published by Tan *et al.* [87]. In this study, hepatocellular carcinoma was induced in rat by diethylnitrosamine, a compound commonly found in food, and sera were fingerprinted by RP-UHPLC-qTOF. Three compounds appeared as strong classifiers of the groups, and a targeted analysis of these metabolites was applied to samples from 150 patients with different liver diseases and 262 patients with liver cancer. The association of these metabolites showed good sensitivity and specificity and were proposed as diagnostic biomarkers of the disease.

The effect of diet (origin) has been studied not only in the disease, but also in the associated disorders, in order to gain a deeper insight into the metabolic alterations (mechanisms). Overweight/obese men showed differences in classical parameters (triglycerides, cholesterol, etc.) that were studied together with changes in the metabolite fingerprinting performed by RP-UPLC-qTOF [88]. Three lysoPCs were identified as potential plasma markers and confirmed eight known metabolites for overweight/obesity men. Results confirmed abnormal metabolism of branched-chain amino acids and aromatic amino acids as well as fatty acid synthesis and oxidation in overweight/obese men. The same research group has investigated obesity in an animal model, whereby liver and serum metabolites of obese and lean mice fed on high fat or normal diets have been analysed using UPLC-qTOF and GC-MS [89]. It is worth noting that the results from the first study [88] were different to those highlighted in another study by Wang *et al*. [90], which involved RP-UPLC-qTOF fingerprinting of urine of a special subset of obese people (under 26 year-old). In the latter study, significant

metabolites were associated with changes in some metabolic intermediates and more notably variations in signalling compounds (including hormone metabolites).

Most of the applications of metabolomics to study diet-related diseases have been addressed to study whether the pathology can be distinguished from the control-healthy situation, and papers have been published where the main goal was to achieve separation of samples by means of multivariate analysis techniques, with no identification of the metabolites responsible for the classification [91]. More recently, it is considered obligatory to propose biomarkers responsible for such differences that can be proposed as diagnostic biomarkers. Moreover, diagnostic markers can be considered also as possible predictive biomarkers for certain diseases, and propose ways to improve the therapeutics. Jiang *et al.* [92] found in a study with sera from 67 patients with cerebral infarction analysed by RP-UPLC/TOF that some molecules from 1-carbon metabolism such as S-adenosyl homocysteine and folic acid were differentially expressed, and that the prognostic for the evolution of the disease was associated to their levels. This opened the possibility of an intervention based on B-vitamins to manage such acute disease.

In the case of DM, pathology is typically irreversible by the time the disease is diagnosed. A reliable test for predicting the risk could allow earlier implementation of intervention measures. Type 2 DM has been one of the most studied diseases by metabolomics, and in order to obtain the most comprehensive list of changes, the metabolic footprint of the variations in 482 metabolites in 40 patients and 60 matched controls have been studied by DMS, UHPLC-MS/MS, GC-MS and NMR [93]. In addition, the risk of developing DM was studied in the Framingham Offspring Study with baseline samples from 189 patients that afterwards had developed Type 2 DM, and 189 matched controls with similar characteristics that had not [94]. This study was performed with HILIC/QqQ, and revealed a panel of amino acids that could be used to predict the risk for developing diabetes. Wang *et al*. used high-throughput metabolomics to uncover significant associations between the concentrations of five branched-chain (leucine, isoleucine and valine) and aromatic (phenylalanine and tyrosine) amino acids in blood and predisposition to diabetes.

One of the main issues to be taken into account about the quest of biomarkers by means of LC-MS is what is known in science as the Maslow´s hammer, popularly phrased as "if all you have is a hammer, everything looks like a nail" [95]: If RP-LC-MS is applied to biological samples, differences will be found in the metabolites that can be analysed with such a technique. If the sample for analysis is serum; probably a large number of lysophospholipids will be significant for the model. In this sense, changes in lysoPCs have been associated to different types of cancer such as pancreatic cancer [96], hepatocellular carcinoma [97], renal cell carcinoma [98], oesophageal squamous cell carcinoma [99], *etc*.

HILIC chromatography has been applied to the study of paraffin-stored tumours, seeking to correlate general changes in metabolites in the different phenotypes of cancer, including five soft tissue sarcomas and five paired normal samples [94]. Researchers used LC-MS/MS with selected reaction monitoring (SRM) to study a total of 249 endogenous water soluble metabolites. Significant changes were detected in an average of 106 metabolites, most of which were related to changes in glucose metabolism, including glycolysis, glutamate metabolism, and the TCA cycle. Although the pre-treatment of samples with formalin leads to degradation, the findings are correlated to the published literature on metabolic alterations present in cancer [65]. HILIC is very well suited for the analysis of urine [97] but it is more commonly used as a complementary, orthogonal technique to RPLC, usually as separated analyses, although the possibility of applying it as a second dimension in metabolomics studies has also been published [100]. HILIC has been widely applied in combination with RPLC to cancer [87, 97-99, 101].

For finding diagnostic biomarkers of pre-diabetes, other orthogonal stationary phases such as pentafluorophenyl [102], or phenyl-hexyl and amino phases [103], coupled to triple quadrupole MS detectors have been employed in combination with reverse phase. Sabatine *et al.* [103] monitored 477 parent/daughter ion pairs through six SRM experiments for each sample. A metabolic ischemia risk score was created based on differences in some metabolites before and after exercise stress tested in 18 patients and 18 controls. The score yielded a highly statistically significant relation to the probability of ischemia. This analytical approach was used by Shaham *et al*. to study insulin sensitivity [104].

An interesting example from the cancer research field used RPLC-MS/MS and GC-MS to perform non targeted profiling on >1,100 individual metabolites in prostate tumour explants, blood, and urine from biopsy-positive cancer patients and biopsy-negative control subjects [70]. Statistically meaningful increments were found in a small subset of metabolites in tumour explants, particularly in metastatic tumours relative to benign prostate. Six metabolites were found to increase with progression from benign prostate to localised cancer to metastatic cancer, including sarcosine, a glycine metabolite. Importantly, the authors then developed a targeted stable isotope-dilution method for quantitative measurement of sarcosine and found it to be elevated by 10- to 20-fold in metastatic tumours compared with benign prostrate. They also showed that manipulation of enzymes of sarcosine metabolism influenced prostate cancer invasion. This combination, developed as "non-targeted profiling" has been applied to different diet-disease conditions, such as insulin resistance [83], cardiomyopathy [105], obesity [106], kidney cancer [107], and has been implemented to find new biomarkers even in tumours that have been stored in paraffin for a long time [108].

In the LC-MS based metabolomics of silent myocardial ischemia (SMI), a form of coronary heart disease, Lin *et al.* [109] studied a group of 39 human adults and 25 controls by UPLC-qTOF. This identified plasma concentration differences of four kinds of phospholipids closely related with the occurrence of SMI, among which 1-linoleoyl glycerophosphocholine (C18:2) was statistically decreased in SMI population. Furthermore, the plasma phospholipid changes were previous to enzymatic alteration in SMI, which might be a useful complementary reference to facilitate SMI diagnosis.

Metabolomics with MS has been used to assess whether metabolites discriminate coronary artery disease (CAD) and predict risk of cardiovascular events [110]. To evaluate discriminative capabilities of metabolites for CAD, two groups were profiled: 174 CAD cases and 174 sex/race-matched controls ("initial"), and 140 CAD cases and 140 controls ("replication"). To evaluate the capability of metabolites to predict cardiovascular events, cases were combined ("event" group); of these, 74 experienced death/myocardial infarction during follow-up. A third independent group was profiled ("event-replication" group; 63 cases with cardiovascular events, 66 controls). Two PCA-derived factors were associated with CAD: 1 comprising branched-chain amino

acid metabolites and one comprising urea cycle metabolites. A factor composed of dicarboxylacylcarnitines predicted death/myocardial infarction and was associated with cardiovascular events in the event-replication group. In a related study, LC-MS/MS on 2023 patients at risk of CAD revealed 45 acylcarnitines, 15 amino acids and other lipid-related metabolites can be used to predict further events [111].

Finally, Tulipani *et al*. [112] studied by RP-HPLC-qTOF the urine of 42 volunteers with metabolic syndrome before and after an intervention in the diet, adding 30 g of nuts daily. They found markers of the intake of the nuts (microbial and phase II metabolites of polyphenols) together with other markers of energy balance metabolism (serotonin metabolites).

2.4. **Direct mass spectrometry**

Although direct mass spectrometry can be less informative, advancements in instrumentation have improved the resolution, accuracy and its relevance for tandem mass spectrometry [113]. For example, Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) can have a mass resolution exceeding 1,000,000 and mass accuracy sub 1ppm [113]. An alternative advanced instrument for direct mass spectrometry is the Orbitrap mass spectrometer that is more commonly used in LC-MS. This is also associated with high mass resolution (over 100,000) and mass accuracy in the range 2-5ppm [114]. As a consequence of these specifications, instruments are also costly both for the initial outlay as well as for each analysis and therefore routine use is often not an option. This can make these instruments inaccessible to many researchers, especially in a clinical setting. Nevertheless, examples in the literature represent them as alternative methods to the more commonly used techniques in different applications.

The use of FTICR-MS has been reported in the discovery of biomarkers that pose the risk of lifestyle and diet on the susceptibility of colorectal cancer [115]. Ultra-long-chain fatty acids: *m/z* 446, *m/z* 448 and *m/z* 450 were observed to be significantly reduced in the serum of colorectal cancer patients relative to healthy controls, as determined by FTICR-MS followed by structural characterisation of biomarkers using tandem LC-MS/MS and NMR [115]. This provided new, specific evidence for lipid profiles in colorectal cancer since it was previously suggested that fibre rich diets

prevent colon cancer while high dietary fat promotes it [116]. It is thought that the initiation or promotion of neoplastic growth could be prevented through diet control. In this way chemoprevention could be possible through revealing dietary agents or strategies and that different diets can be associated with increasing or decreasing the risk of cancer.

FTICR-MS has also been reported in a direct mass spectrometry approach to assess the hepatic effects of polychlorinated biphenyls in mice. These pollutants were previously associated with abnormal liver enzymes and suspected to contribute to a range of diet-related diseases including obesity, metabolic syndrome and non-alcoholic fatty liver disease [117]. Mice were fed control or high fat diets with or without exposure to polychlorinated biphenyl 153 and metabolite extracts were obtained from the livers after 12 weeks of exposure. The analysis involved a linear ion trap FTICR mass spectrometer coupled with direct infusion nano-electrospray ionisation. The results indicated 18 metabolites that that were decreased and 6 metabolites that were increased with the high fat diet combined with polychlorinated biphenyl 153 exposure compared to the control diets (with and without polychlorinated biphenyl 153). Moreover, when comparing high fat diets, the addition of polychlorinated biphenyl 153 caused a rise in erythronic acid while decreasing the levels of glutathione and creatine [117]. This and the fact that no significant effect of polychlorinated biphenyl 153 was observed for control diets indicated that the metabolic effect of polychlorinated biphenyls on the liver are highly dependent on the level of fat in the diet [117].

Metabolic syndrome can be associated with obesity and insulin resistance which are the major risk factors for many diet-related diseases including hypertension. In a study to determine whether the effect of hypertension on the lipidome is independent to the effects of obesity and insulin resistance, the plasma lipidomes of 19 men with hypertension compared to 51 controls were assessed using direct mass spectrometry involving an LTQ Orbitrap hybrid mass spectrometer [118]. From this, obesity was observed to increase lipid load in blood plasma in all subjects, involving a specific and dramatic increase in tri- and diacylglycerols while insulin resistance had little effect on the lipidome. In response to hypertension, ether lipids were decreased, specifically ether phosphatidylcholines and ether phosphatidylethanolamines, as well as free cholesterol [118]. Since these alterations in subjects with hypertension were independent of those

induced by obesity or insulin resistance, it was suggested that preventative dietary strategies may alleviate the severity of hypertension.

In another application of the LTQ Orbitrap in metabolomics of diet-related diseases, the modified phospholipid products of low density lipoprotein oxidation associated with atherosclerosis and cardiovascular disease has been assessed. Lipoprotein-associated phospholipase A2 acts on phospholipid oxidation products to generate pro-inflammatory lysophospholipids and oxidised non-esterified fatty acids. Furthermore, free lipoprotein-associated phospholipase A2 can act as a predictor of cardiovascular disease and inhibitors of this compound could be used in therapy of atherosclerosis [119]. A metabolomics approach to study the effects of *in vitro* oxidation in the presence and absence of one of these inhibitors on the phosphatidylcholine composition of human low density lipoprotein has been performed. From direct mass spectrometry analysis using an LTQ Orbitrap, three main classes of phosphatidylcholine were revealed including truncated forms represented by peaks in the range *m/z* 594-666, non-truncated oxidised forms in the range *m/z* 746-830 and the major products of lipoprotein-associated phospholipase A2: saturated and mono-unsaturated lyso-phosphatidylcholine [119]. From this it was concluded that phosphatidylcholines can be markers of atherosclerotic disease progression as well as markers of the response of lipoprotein-associated phospholipase A2 inhibitor therapy.

Cholesterol esters are linked to a range of diet-related diseases. Direct infusion electrospray ionisation mass spectrometry was involved in the targeted and quantitative analysis of cholesterol esters in plasma to determine their correlation with diet [120]. When assessed in the plasma lipid extracts of mice fed a western diet, it was revealed that cholesterol esters containing mono-unsaturated fatty acids were elevated compared to normal chow diets [120].

An alternative approach in direct mass spectrometry is imaging mass spectrometry. This is not usually high-throughput and therefore not always applicable in metabolomics, but offers the distinct advantage that metabolites or lipids can be spatially localised within a sample. In this way, imaging mass spectrometry is a novel method for the analysis of biological samples *in situ*, combining mass spectrometry with microscopic imaging. The chemical organisation of a sample is likely to be correlated with its physical

features and therefore spatially localising and co-localising groups of chemicals elucidates properties of structure and potentially reveals information about their function in a biological system. Imaging mass spectrometry is currently of paramount importance and receiving significant attention in the mass spectrometric community [121]. Since these techniques are based on surface analysis, samples are most commonly tissue sections with an approximate thickness in the μm range and more recently imaging mass spectrometry of single cells has been reported [122, 123]. Two techniques utilised in metabolomics based studies of biological samples are time of flight-secondary ion mass spectrometry (ToF-SIMS) and desorption electrospray ionisation mass spectrometry (DESI-MS) due to their capability of analysing low weight compounds in the metabolite and lipid range.

Current ToF-SIMS instruments available for imaging benefit from remarkable spatial resolution (sub 1 μm) without compromising mass resolution [124-126]. The way in which samples should be prepared to optimise results has been explored [127, 128]. As a technique, it is ideal for analysing at low mass, often but not restricted to masses <1000 Da, making it an ideal choice for metabolite studies. ToF-SIMS has had reported success in imaging both cells [129] and tissues [130] and although there have been few examples to date of studies focussing specifically on metabolites rather than lipids [131, 132], its potential for metabolite identification has been explored [133]. It has already been applied to lipid analysis but could also be a contender for metabolomics with respect to diet-related diseases in the future.

Alcoholic/non-alcoholic fatty liver disease caused by steatosis, the abnormal retention and accumulation of lipids in liver cells, is strongly influenced by diet and lifestyle. The distribution of lipids has been assessed *in situ* utilising ToF-SIMS, revealing the location of accumulated tri- and diacylglycerols as well as monoacylglycerols in steatosis regions of fatty livers compared to control livers [134]. Imaging of vitamin E confirmed previous knowledge that this group of metabolites (tocopherols and tocotrienols) is reduced with steatosis but further revealed a periportal predominance in control liver [134].

Diabetes can often be associated with atherosclerosis and characterising the metabolome (or lipidome) of atheroma plaques could help determine its contribution to

inflammation. ToF-SIMS imaging has revealed that a significant increase in non-esterfied fatty acids occurs in diabetic atheroma plaques compared to non-diabetic subjects and that they are co-localised with areas of inflammation within the plaques [135]. Specifically, linoleic acid, palmitic acid and oleic acid were found to significantly differ in the plaques as a consequence of diabetes, however using this technique, other lipids including cholesterol and triglycerides that are known to be associated with atherosclerotic pathogenesis were not significantly different as a consequence of diabetes [135]. This may suggest that non-esterified fatty acids are specifically associated to inflammation within the plaques that occurs with diabetes.

Desorption electrospray ionisation (DESI) is an alternative imaging mass spectrometry technique that can be employed for *in situ* analysis of biological samples. DESI was the first reported mass spectrometry technique to utilise ionisation under ambient conditions [136]. Other techniques involve post ionisation in ambient conditions, including laser desorption methods such as laser ablation electrospray ionisation (LAESI) [137] and thermal desorption methods such as direct analysis in real time (DART) [138], however these techniques are not associated with imaging. DESI can therefore be considered one of the most advantageous techniques for sample preservation since metabolites are analysed *in situ* and without requiring vacuum. Methods in imaging mass spectrometry for biological analysis have been reviewed previously, where the advantages and limitations of different techniques are discussed [139, 140].

One key example of the application of DESI to study diet-related diseases has been in the imaging analysis of colorectal adenocarcinoma tissue. In this study, a DESI ion source was employed in addition to an Orbitrap mass spectrometer for data acquisition in negative ion mode in the range *m/z* 600-1000 with a spatial resolution of 100 µm, mass resolution of 30,000 and mass accuracy of 4 ppm [141]. The aim of the study was to reveal differences in lipid profiles caused by cancer and to generally compare its application to current histological methods in imaging to assess its usefulness in tissue analysis of disease states. It was concluded that the technique can be applied for single biomarker discovery in individual cases, however this approach may not be reproducible enough for global analysis [141]. When combining the technique with multivariate analysis however, it was elucidated that global tissue identification can be possible and furthermore, the specificity exceeds the limitations of traditional microscopic techniques. The lipid profiles of colorectal carcinoma tissue could be

clearly distinguished from smooth muscle tissue and healthy intestine mucosa also imaged.

As presented, direct mass spectrometry offers some advantage over mass spectrometry coupled with a pre-separation technique (such as the high resolution and accuracy of instrumentation that can be employed and more over the capability for imaging that is not possible using pre-separation techniques). However, a major limitation is that they can often be susceptible to ion suppression or enhancement that means the matrix effect in complex metabolomics samples can affect the quality of the experiment. In addition, the matrix effect can complicate data interpretation and unique metabolite ions can often not be distinguished from adduct and product ions from other metabolites [113]. For this reason, its application is most useful when the complexity of samples is not a limitation or the necessity for high-throughput analysis (in the case of non-imaging direct mass spectrometry) or imaging (in the case of imaging mass spectrometry) outweighs these issues.

3. **Systems biology in the study of diet-related diseases**

Systems biology draws upon knowledge and techniques from various disciplines across the physical, information and life sciences with an aim to fill gaps in biological knowledge. It is an approach commonly used to study the biochemical interactions of genes, proteins and/or metabolites as emergent properties of a biological system, rather than as isolated biological features. Many systems biology experiments are driven by a hypothesis or biological question that has not been resolved using traditional techniques. The experiment is carefully designed and samples collected for analysis using often high-throughput technologies capable of generating reproducible, qualitative or (semi-) quantitative data that can be analysed using intelligent computational methods including multivariate analysis and modelling. Data and data models are usually reported in an accepted systems biology language that if made publically available in databases can be compatible with other data across the world. Subsequently a collection of genomic, transcriptomic, proteomic and metabolomics data is generated for a range of biological systems and used to reveal structural and functional properties that can lead to further hypotheses. Recent advancements in technology especially in the physical and information sciences have enhanced the quality of experimental data and

its analysis. Furthermore, the increased use of standardised languages and databases for data sharing enables combining of data on one biological system.

Incorporating high-throughput data from genomics, transcriptomics, proteomics and/or metabolomics can be useful for global analysis of diseases. Moreover, it can highlight the effect of external factors such as diet on each level of function and how this changes interaction between hierarchical levels. For example, in a study combining metabolomics data with gene expression data it has been suggested that dietary energy restriction in pre-menopausal women can reduce the risk of breast cancer in post-menopause [142]. The study aimed to reveal the effect and effectiveness of dietary energy restriction (approximately 60 % lower than in a normal diet) on reducing mammary tumourigenesis. From the combined microarray RNA and GC-MS metabolomics data collected, it was found that the most down-regulated metabolic processes in the breast after dietary energy restriction were related to fat synthesis and glycolysis. Furthermore, biomarkers of breast cancer risk including leptin, cholesterol and triglycerides were reduced with dietary energy restriction. [142]. The lipidome has been described previously as a composite biomarker for breast cancer. For example, a combined elevation in mono-unsaturates and lowering of the ratio between *n*-6 and *n*-3 fatty acids has been associated with reducing the risk of breast cancer [143]. In this way, the lipidome could be considered a modifiable feature to control breast cancer risk.
In another study combining metabolomics with genomics data, metabolite biomarkers have been proposed to identify pre-diabetes. Three metabolite biomarkers were identified by for impaired glucose tolerance: acetylcarnitine C2, lyso-PC (18:2) and glycine [144]. The underlying mechanisms for their control was investigated by studying their associations with type II diabetes related genes through analysing protein-metabolite interaction networks, revealing seven genes related to these metabolites. The gene expression analysis elucidated the transcriptional activity of four enzymes related to these metabolites [144]. This relationship between metabolites and enzymes determined experimentally suggested the association of these metabolites with the genes identified through network analysis.

In a related study, serum metabolites found to be strongly associated with free fat mass index in humans included amino acids, acylcarnitines, phosphatidylcholines (PCs), sphingomyelins and hexose [145]. This was observed in a study of free fat mass and

muscle mass changes in the human body related to diet and lifestyle. Assuming additive genetic effects, linear models were used to analyse the relationships between the serum metabolite concentration/ratios and single nucleotide polymorphisms known to be associated with diet and lifestyle. Since the observed changes were related to obesity, it was advocated that accumulation of fat from diet and lifestyle may have a detrimental effect on skeletal muscle metabolism including a limited induction of fatty acid oxidation [145].

In an analysis of transcriptome and metabolome profiles to assess the alterations in fatty liver induced by high-fat diets in rats, it was observed that 130 genes are regulated by high fat diets and this causes a marked downstream increase in serum cholesterol, triglycerides, glucose and insulin [146]. Metabolomics results were obtained from rat serum analysed by GC-MS, gene expression analysis was performed on rat livers using DNA microarrays and transcriptomics results were obtained using real-time reverse transcriptase polymerase chain reaction, where samples were collected 16 weeks after continuous diet exposure (high fat or control chow diet). Specific gene expressions were observed through the transcriptomics approach revealing that lipid metabolism regulators such as sterol regulatory element binding factor 1 and stearoyl-coenzyme A desaturase 1 were up-regulated, whereas the expression of peroxisome proliferator-activated receptor, carnitine palmitoyltransferase 1, and 3-hydroxy-3 methylglutaryl-co-enzyme A reductase were repressed in high fat livers. From a global analysis of all metabolomics and transcriptomics data together, the integrated systems analysis led to conclusions that long-term exposure to high fat diets can result in multi-dimensional alterations in fatty acid metabolism and lipogenesis as well as inflammatory and stress response related metabolic pathways [146].

In an alternative combination of 'omics' data, novel markers of body fat mass changes associated with obesity have been revealed from the combined analysis of metabolomics and proteomics data [147]. This involved studying the difference between lean and obese individuals where 39 unique proteins were identified and 12 metabolites were significantly related obesity. Integrating proteome and metabolome data in a bioinformatics evaluation, group separation was improved [147], highlighting the appropriateness of the systems biology approach employed.

Computational modelling is a widely used and highly applicable technique to understand disease mechanisms through a systems biology approach. Models can integrate metabolomics data or be used to predict metabolomics data, most commonly through studying (genome scale) metabolic networks. A mathematical model of ordinary differential equations has been developed for pancreatic β-cell glucose transport using metabolic data describing the alterations that occur as a result of type II diabetes [148]. Sensitivity analysis was performed to assess the effect of model perturbations on different pathway components. Findings indicated that a physiological and metabolic threshold exists whereby glucose entry, and not glucose kinase activity, is the rate limiting step in glucose-6-phosphate production [148]. It was shown that β -cell glucose transport is below this threshold in diabetes but well above it in healthy subjects. Furthermore, preservation of a glycan linkage by a corresponding glycosyltransferase may be the most effective way to maintain glucose transporter expression in β -cell glucose and to retain normal glucose transport [148]. This feature determined computationally was consistent with experimentally determined gene expression of glucose transporters in the literature.

The use of models for data interpretation and combination of data from different hierarchical levels is a key feature of systems biology. It may eventually be possible to build a parameterised computational model of any biological system that has the potential to illustrate function possible. By elucidating a greater understanding of diseases through testable and quantifiable models, it is hoped that new targets for therapy will be revealed. Building a good model for a disease may be key to recognising dynamic features, which may be useful to identify parts of the system that can be perturbed to disrupt disease progression, while also moving towards improved diagnostics [149]. The approach to model a silicon cell is a key theme in systems biology research that, 'aims to describe the intracellular network of interest precisely, by numerically integrating the precise rate equations that characterise the ways macromolecules interact with each other' [150].

4. **Conclusion**

There are a range of analytical techniques in mass spectrometry that have shown useful in the characterisation of a wide variety of diet-related diseases, and some clues have

been provided for their selection, although for a good coverage combination of several techniques is usually necessary. However, further validation of the results using larger and independent new datasets is one of the pending aspects of most metabolomics studies. Currently, analytical capabilities are impressive; however, data treatment and interpretation are the real bottleneck of this type of studies.

Systems biology is a useful approach, particularly for combining metabolomics with data obtained through other 'omics' experiments to reveal more information about pathway regulation and mechanisms of diseases. Furthermore, the bioinformatics element of systems biology allows a metabolomics researcher to use metabolomics data to generate computational models that can be used as predictive tools for further omic analysis. One final feature of systems biology that has been particularly useful in the metabolomics of diet-related diseases is the availability of databases that are accessible to researchers employing a wide range of techniques. This is particularly advanced for GC-MS which is beneficial since this is often used for studies based on energy metabolism which is a key feature of diet-related diseases.

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FIGURE CAPTIONS

Figure 1: The goals of metabolomics for diet-related disease research. Metabolic fingerprinting is commonly employed for biomarker discovery that can be useful for diagnosis, prognosis, monitoring and staging of the disease as well as to understand its cause and mechanism. The transition from health to disease can be predisposed by diet and the reverse can be positively influenced by diet. The main diet-related diseases are shown along with the associated symptoms.

Figure 2: Best features of different MS platforms to study diet-related diseases. Choosing the correct instrument for the application can be based on the classes of compounds best analysed by each respective technique (shown below each technique) or by the analytical aspect that sets each instrument apart from the rest (shown above each technique).

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