NMR-Based Pharmacometabonomics: A New Paradigm for Personalised or Precision Medicine

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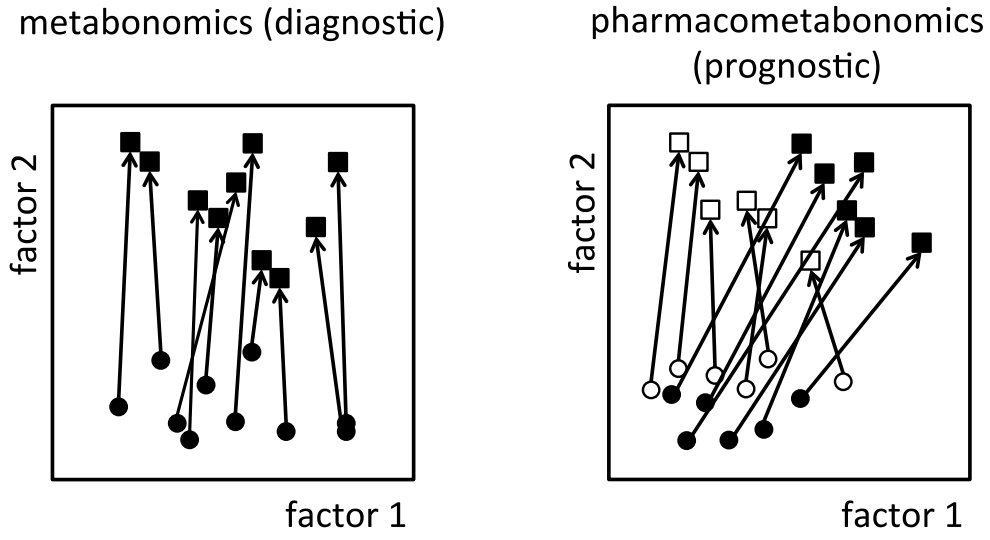
Abstract

Metabolic profiling by NMR spectroscopy or hyphenated mass spectrometry, known as metabonomics or metabolomics, is an important tool for systems-based approaches in biology and medicine. The experiments are typically done in a diagnostic fashion where changes in metabolite profiles are interpreted as a consequence of an intervention or event; be that a change in diet, the administration of a drug, physical exertion or the onset of a disease. By contrast, pharmacometabonomics takes a prognostic approach to metabolic profiling, in order to predict the effects of drug dosing before it occurs. Differences in pre-dose metabolite profiles between groups of subjects are used to predict post-dose differences in response to drug administration. Thus the paradigm is inverted and pharmacometabonomics is the metabolic equivalent of pharmacogenomics. Although the field is still in its infancy, it is expected that pharmacometabonomics, alongside pharmacogenomics, will assist with the delivery of personalised or precision medicine to patients, which is a critical goal of 21st century healthcare.

Highlights

- metabonomics or metabolomics involves metabolic profiling by NMR or MS methods
- metabonomics is used in a diagnostic mode to study the effects of an intervention
- pharmacometabonomics is a prognostic method to predict the effects of drugs
- pharmacometabonomics is a special case of predictive metabonomics
- pharmacometabonomics will help to deliver personalised medicine in the future

Graphical Abstract



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1. Introduction

1.1 Personalised or Precision Medicine

It is an unfortunate fact that many drugs currently prescribed are either ineffective or unsafe for significant numbers of patients.[1] In an ideal world, each patient would be prescribed a treatment that had the best chance of achieving efficacy and the lowest risk of toxicity. A key goal of 21st century healthcare is to deliver the promise of personalised medicine, or precision medicine as it is sometimes known; that is to use genomic, molecular and clinical information to select treatments or medicines that are more likely to be both effective and safe for that patient.[2]

The cost of drug-related morbidity and mortality due to lack of drug safety was estimated to be greater than $177 billion in 2000 in the United States alone, which is a staggering figure. Earlier, Pomeranz et al estimated that in US hospitals in 1994 over 2 million patients had serious adverse drug reactions (ADRs), with an estimated 100,000 patients having fatal ADRs.[3] This represents a massive personal and financial burden.

Since earliest days, physicians have practised personalised medicine; that is the use of information to select treatments that are most likely to be efficacious and least likely to be harmful for the particular patient. Thus treatments would be selected based on factors such as the patient's individual personal history, their family history, gender, age and body mass index. However, in the past 50 years or so, genetic information has become increasingly available, enabling clinicians to base treatment decisions upon a patient's individual genetic make-up as well. This approach to personalised medicine is known as pharmacogenomics: the study of how genes modulate drug responses among individuals.[4]

Pharmacogenomics is now having some significant clinical successes, for example, selecting breast cancer patients for treatment with trastuzumab on the basis of a test for the over-expression of the *erbB2* oncogene in the tumour, or deselecting treatment with the EGFR-specific antibodies cetuximab and panitumumab, for colorectal cancer patients that have activating mutations in their *KRAS* gene.[4] In addition, pharmacogenomics can inform on drug pharmacokinetics and hence dosing schedules for drugs like warfarin, which has a very narrow therapeutic index and whose dose is critically dependent upon the patient's *VKORC1* and cytochrome P450 genotypes. *VKORC1* codes for a subunit of the vitamin K oxide reductase complex, which is the target for warfarin, whereas the P450s are 'drug metabolising' enzymes that are responsible for metabolising warfarin, with the *CYP2C9* genotype being particularly important. The pharmacogenetics approach has been demonstrated to improve the accuracy of warfarin dose setting and reduce the risk of hospitalisation from incorrect dosing.[4]

However, pharmacogenomics faces challenges due to a number of factors: (1) in a complex, multi-factorial disease, involving many genes, there may not be a simple correlation between a mutation in one gene and the subsequent disease phenotype or its response to a particular treatment, and in any event there are many steps and much uncertainty between having a gene mutation and having an altered phenotype; (2) many diseases and many human phenotypes have an environmental component as well as a genetic component and human genetics is blind to these factors, especially the influence of the bacteria in the human gut microbiome and finally; (3) the issue of phenoconversion, where genotypic extensive metabolisers may be converted into phenotypic poor metabolisers by drug administration and thereby confound a pharmacogenomics prediction.[5] Thus, whilst the promise of pharmacogenomics to help deliver personalised medicine is clear, it has recently been shown to have delivered less than was expected in randomised clinical trials in the areas of cardiovascular disease,[6] diabetes[7] and depression.[8]

Given the importance of improving the selection of medicines for patients, so as to maximise efficacy and to minimise adverse effects, there is thus clearly a need for new technologies to work in tandem with pharmacogenomics to improve the current personalised or precision medicine paradigm. The rest of this article will introduce pharmacometabonomics as one such methodology that can help achieve that.

1.2 Metabolic profiling: metabonomics and metabolomics

Metabolic profiling has a long history, going back at least several hundred years, [9] but has come to the fore in the past few decades with the advent of powerful NMR and mass spectrometers capable of providing exquisite details of the metabolic compositions of biological fluids and tissues.[10] Metabolic profiling has proven enormously valuable in many areas of biology and medicine, including disease diagnosis, drug discovery and development, and the

understanding of human biochemistry and physiology from a systems biology perspective.[9-11]

The term metabonomics was coined in 1996 by Everett and Nicholson to provide a framework for a set of metabolic profiling experiments done in parallel with proteomics and transcriptomics studies, in a collaboration between Birkbeck College and Pfizer Global R & D on the discovery of early drug safety biomarkers. Metabonomics has the following interventional definition: "the quantitative measurement of the multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification."[12] The alternative term metabolomics has an observational definition: "a comprehensive analysis in which all the metabolites of a biological system are identified and quantified"[13] that is more difficult to achieve, as the identification of all the metabolites in a biological system can only ever be an aspiration. In this review, we shall use the original term metabonomics throughout.

To date, most applications of metabolic profiling have been diagnostic in nature; that is the effects of an intervention are interpreted and understood through the changes observed in the metabolic profile of, for example, a patient, before and after the intervention. In the case of the intervention being drug dosing to a patient, a typical metabonomics experiment would interpret the effects of the drug by an assessment of the impact of the drug administration on the endogenous metabolic profiles of the patient post-dose compared with pre-dose.

In order to be useful for personalised medicine and the selection of optimal treatments for patients, a new prognostic approach to metabolic profiling is required where pre-dose metabolic profiles can be interpreted in order to predict drug effects in advance of dosing. Pharmacometabonomics delivers this new prognostic approach, as will be demonstrated (see Section 3 below).

2. Metabonomics: Metabolic Profiling in Practice

2.1 Key elements of metabolic profiling experiments

Regardless of the objective, the analytical technology, or the intervention used, a metabolic profiling experiment has a number of important stages, including: 1) definition of study aims and experimental design, 2) ethical approval where necessary, 3) sample collection and storage, 4) sample preparation, 5) data acquisition, 6) data quality control, 7) spectroscopic data pre-processing 8) statistical data pre-processing, 9) statistical analysis to determine the biomarkers that are responsible for any differences in metabolite profiles due to the intervention used, 10) identification of biomarker metabolites, 11) biological and biochemical interpretation of the role of biomarker metabolites including pathway analysis, and 12) reporting of results and deposition of the data.

Each of these steps has been well investigated for either NMR-[14-20] or mass spectrometry (MS)-based[21-24] approaches to metabolic profiling. The step that presents the most difficulties to investigators using either NMR-based or MS-based approaches is metabolite identification (see Section 2.4 below).

2.2 NMR and MS technologies for metabonomics

NMR spectroscopy, and MS hyphenated with a separation technology such as ultra performance liquid chromatography, gas chromatography or capillary electrophoresis, are the two main analytical methodologies used for both the detection and the identification of metabolites in biological samples. The methodologies are both very powerful and can often be used synergistically.[25] Table 1 gives a comparison of the relative strengths and weaknesses of the two analytical technologies.

Table 1. A comparison of the analytical capabilities of NMR spectroscopy and mass spectrometry for metabolic profiling

NMR spectroscopy	mass spectrometry
• powerful structure elucidation capability for small molecules in solution giving information on molecular structure, conformations and dynamics	• powerful structure analysis capability to generate metabolite mass and some fragments and molecular formulae at high resolution
• relatively insensitive, but sensitivity improved recently with digital spectrometers, cryoprobes and low volume probes	• highly sensitive
• instrumentation expensive but per sample cost relatively low	• instrumentation relatively inexpensive but isotopically labelled reference standards for quantitation can be expensive
• absolute quantitative measurements and no reference standard required with ERETIC technology[26]	• not absolutely quantitative in absence of specific reference standards, but has relative quantification capability
• highly stable as no contact between sample and spectrometer • little effect of history on data	• relatively unstable • column and spectrometer performance can be affected by history
• minimal sample preparation and direct analysis of biological samples	• generally requires a chromatographic separation step prior to MS analysis • gas chromatographic (GC) analysis requires metabolite derivatisation in order to effect volatilisation
• simple spectra corresponding to	• soft ionisation mass spectra

each metabolite	complicated by possibilities of multiple adduct formation with different metal ions and solvent adducts observed separately for each metabolite • GC-MS analyses may be complicated by multiple derivatisation species

2.3 Metabonomics Data Analysis

The following steps would be typical of a data analysis workflow for an NMR-based metabonomics experiment:

1. NMR spectra quality control, checking resolution, lineshape, sensitivity and water suppression quality
2. spectroscopic data pre-processing, which for NMR data would include zero-filling, apodisation, Fourier transformation, phasing, baseline correction, chemical shift referencing and removal of regions of no interest (usually as follows: > ca 10 ppm, < 0.4 ppm, the residual water region, any regions due to administered drugs or their metabolites, and for urine spectra the urea region)
3. statistical data pre-processing, including peak alignment, scaling and normalisation, in order to optimally prepare the NMR data for high quality statistical analysis
4. multivariate statistical analysis of the data to determine the biomarkers that are responsible for any differences in metabolite profiles due to the intervention used

Whereas a decade ago it was common to use a bucketing approach and divide the NMR spectrum into multiple (hundreds) regions each of e.g. 0.04 ppm spectral width for multivariate analysis, it is now more usual to adopt a full data point analysis of the spectral data, resulting in multivariate analysis in a metabolic space with tens of thousands of dimensions: one dimension for each data point. This poses no particular technical issues with modern computers and statistical analysis software. However, this approach does open the possibility of false discoveries due to multiple hypothesis testing, and this must be taken into account in the data analysis protocol.

Two philosophically distinct approaches to multivariate analysis are available: 1) unsupervised methods such as principal components analysis (PCA)[27] are typically used for the initial analysis of all studies, in order to discover subject outliers and to determine any structuring of the data, such as differential clustering of pre-dose and post-dose subjects, in a completely unbiased fashion; and 2) supervised methods such as projection to latent structures (PLS, partial least squares)[28] or maximum margin criteria (MMC)[29] where the algorithm is fed the information about which treatment grouping e.g. high fat diet group versus control diet group, each subject's spectrum belongs to. The algorithm then uses this information in order to find metabolic variables that maximally

correlate with and discriminate between the different treatment groups. Considerable care has to be taken with the use of supervised multivariate methods in a high dimensional space, as it is possible to overfit the data and arrive at false conclusions, especially with low subject numbers. It is therefore good practice not only to apply a false discovery rate (FDR, typically set to 10%) filter to the multivariate analysis to reduce the probability of false outcomes, but also to test the outcome of the multivariate analysis in an independent data set i.e. use external validation.[30-33] With an FDR approach, the critical p value for statistical significance is often reduced to well below the typical value of 0.05 or 5% in order to protect against false discovery by multiple comparisons.

The outcome from a successful multivariate analysis of the spectroscopic data is a set of spectroscopic features that are associated with different sub-groups of subjects and that discriminate between those subgroups e.g. treated and untreated patients.

2.4 Metabolite Identification

This stage of metabolic profiling is the assignment of metabolite identities to the discriminating spectroscopic features identified by the multivariate data analysis (see Section 2.3 above). This is one of the most important steps in metabolic profiling, as correct identification of metabolites that are discriminating between groups of subjects in an experiment, e.g. drug-dosed vs placebo-dosed subjects, is critical to the correct biological interpretation of the results. If metabolites are mis-identified, the ensuing analysis will be worthless. Unfortunately, in the current metabolic profiling literature, it is often impossible to determine the confidence with which metabolites have been identified.

The metabolite identification problem is of two types: first, the identification of truly novel metabolites, either previously unknown, or known metabolites for which no relevant spectroscopic data is available; and second, the identification of known metabolites for which the relevant spectroscopic data are available. The structure elucidation of novel metabolites requires the high standards associated with the identification of novel natural products[34] or novel drug degradation products[35] and would normally involve either: (i) the isolation and purification of the metabolite from the biological matrix and its full molecular structure characterisation by the standard array of methods including UV, IR, MS and NMR, or X-ray crystallography, or (ii) the synthesis of an authentic reference standard of the metabolite for comparison of its spectral properties with those of the same metabolite in the biological matrix. This novel *structure elucidation* work can be clearly differentiated from the second type of identification problem, that of *structure confirmation* of known metabolites, for which spectroscopic data is available in databases such as the Human Metabolite Database (HMDB)[36], the BioMagResBank (BMRB)[37] and COLMAR.[38] The key question for this structure confirmation work is what confidence the investigator has in the identification of the known metabolites.

The Metabolomics Standards Initiative's Chemical Analysis Working Group first proposed a four-level classification scheme for the identification of known

metabolites in 2007.[39] However, these recommendations have not been widely adopted and are currently being updated.[40] In the meantime, new proposals have emerged for both MS-[41, 42] and NMR-based[38, 43-45] known metabolite identification. It is expected that the Metabolite Identification Task Group of the Metabolomics Society (http://metabolomicssociety.org/board/scientific-task-groups/metabolite-identification-task-group) will publish improved and unifying recommendations for this important area shortly.

3. The Discovery of Pharmacometabonomics

Twenty to thirty years ago, it was typical to observe large differences in the outcomes of drug dosing experiments in animal species such as rats and mice and this was often ascribed at the time to 'biological variability', although the latter was never defined, nor understood. These differences in outcome could be striking and so large that it would appear that some animals had been dosed whereas others had not, often leading to concerns about the quality of the experiments. These concerns were discussed at a project review meeting on 18 October 2000, in Amboise France, between Pfizer Global R & D and Imperial College collaborators working on a drug safety prediction project. During the course of this meeting, the radical notion was proposed that the supposedly equivalent rats under study were in fact not identical, and that metabolic profiling of the animals *prior* to dosing might identify sub-group differences that were correlated with different post-dose responses, such as radically different drug excretion, metabolism and safety outcomes. This was the birth of the notion of pharmacometabonomics: the use of pre-dose metabolite profiles to explain post-dose differences in response to drug treatment. A series of experiments were designed to test this hypothesis that led to a study dosing paracetamol/acetaminophen to rats. This study demonstrated that pre-dose metabolite profiles could be used to predict, to some extent at least, both the metabolism and the toxicity of paracetamol.[46]

This first pharmacometabonomics experiment dosed paracetamol at a toxic threshold dose of 600 mg/kg to 65 Sprague-Dawley rats, with 10 further controls receiving an equal volume of dose vehicle. 600 MHz ^{1}H NMR spectroscopy was performed on both pre-dose and post-dose urine samples, with comparisons made between pre-dose metabolite profiles and post dose drug metabolism and toxicity outcomes.

A projection to latent structures (PLS) supervised model, based on pre-dose urine metabolite profiles, was built and validated that could predict the post-dose ratio of the concentrations of the glucuronide metabolite of paracetamol to that of the parent paracetamol drug (G/P, see Figure 1). This model was validated by permutation of the G/P data relative to the pre-dose data, and by a leave-one-out strategy (Figure 2).[46]

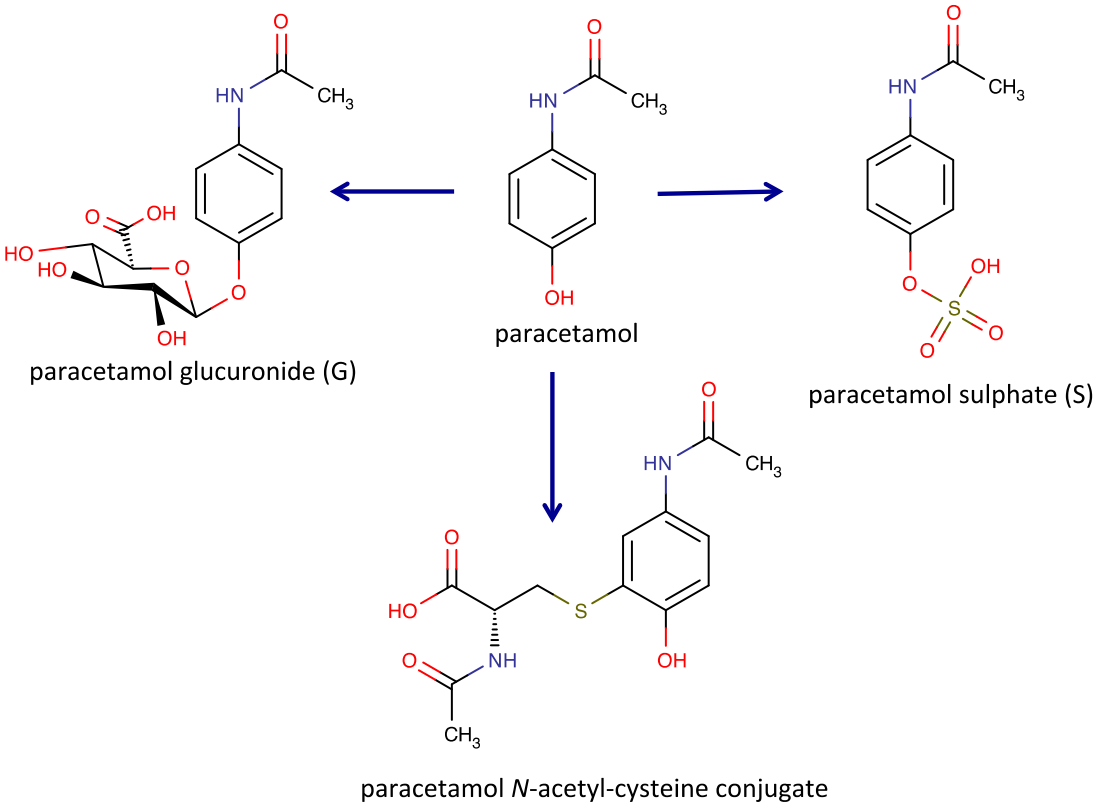

Figure 1: the molecular structures of paracetamol and its major metabolites

The most significant feature in the pre-dose urine metabolite profile that correlated positively (r = 0.48) with the post-dose G/P ratio was the integral of the region from 5.06 to 5.14 ppm, the region where the glucuronide of paracetamol itself resonates in post-dose urine spectra. It was hypothesised that the signals in this region pre-dose were from endogenous ether glucuronides and that it was reasonable that the propensity to form these ether glucuronide metabolites pre-dose was linked to the extent of paracetamol glucuronide formation post-dose.[46]

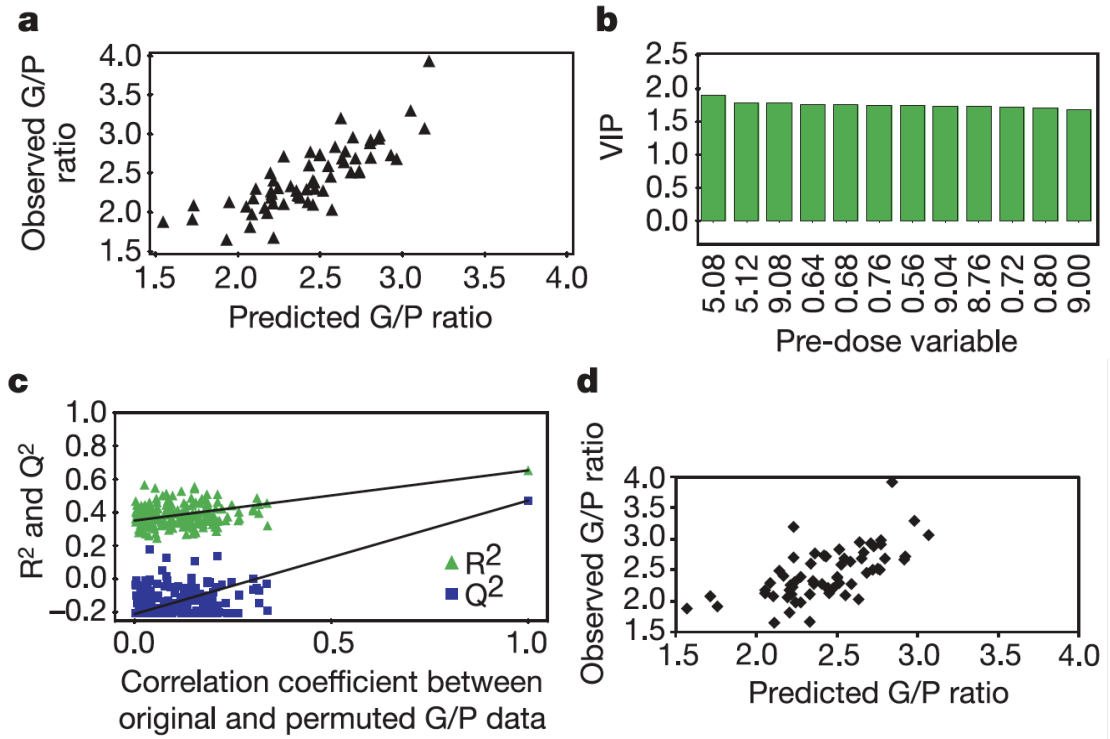

Figure 2. a) observed paracetamol glucuronide to paracetamol ratio (G/P) plotted against predicted G/P ratio from a 2-component projection to latent structures (PLS) model in which all the predictions relate to model-building data. b) the 12 regions of the pre-dose 600 MHz ^{1}H NMR spectra that are most important in deriving the PLS G/P model and have the highest variable influence on projection, VIP (each region is identified by the centre-point of its 0.04 ppm wide 'bucket'). c) internal validation of the PLS model by showing the decreases in R^2 (measure of how well the model fits the data, and is the proportion of the sum of squares explained by the model) and in Q^2 (the predictive ability of the model: the cross-validated R^2), as the G/P data are randomly permuted relative to the pre-dose data and d) results of a seven-round cross-validation is which every point represents test data that was not used in building the models. Figure reproduced with permission from Nature Publishing Group.[46]

The degree of liver toxicity observed was measured using a mean histology score (MHS) calculated histopathologically across all five liver lobes, and this was also shown to be statistically significantly associated with pre-dose metabolic profile features (Figure 3).

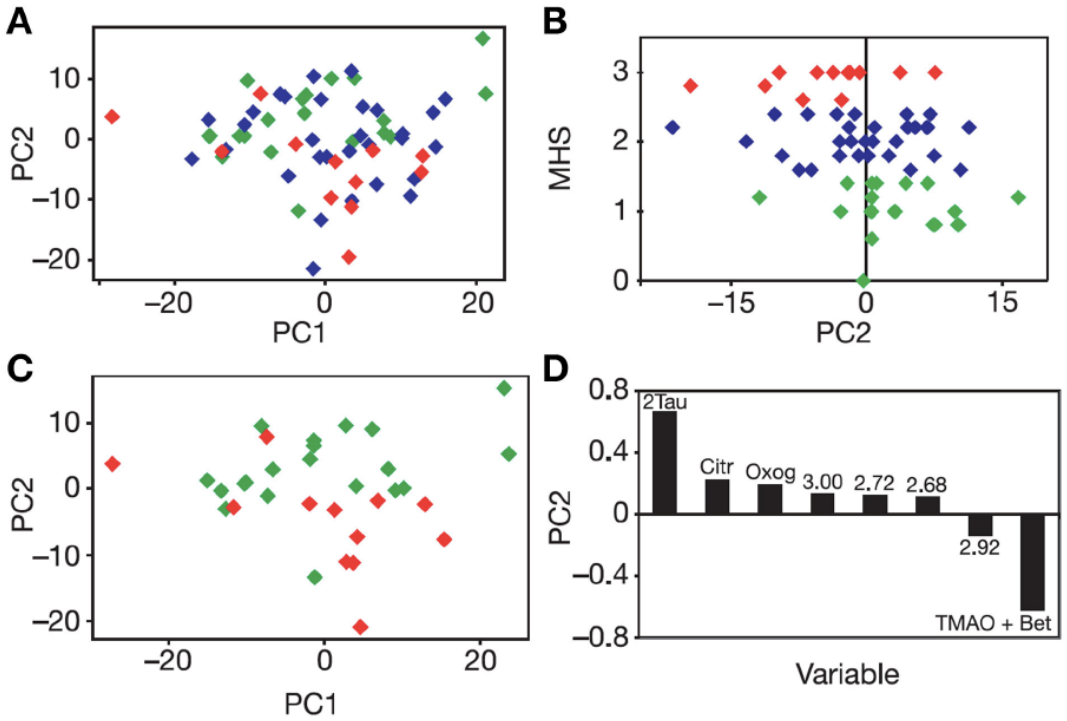

Figure 3. A) Unsupervised principal components analysis of the pre-dose urine spectral features in 65 rats dosed with paracetamol and with each animal represented by a diamond colour-coded by post-dose histopathology class: green, Class 1, minimal or no hepatocellular necrosis): blue, Class 2, mild necrosis and red, Class 3, moderate necrosis. A partial separation between Class 1 and Class 3 is observed across PC2. B) A plot of mean histopathology score (MHS) against PC2 showing a weak but significant correlation. C) Unsupervised PCA of the pre-dose urine spectral features with the same colour coding as in a) but for Classes 1 and 3 only. D) A PCA loadings plot showing the pre-dose spectral bins (denoted by bin centre-point in ppm) and metabolites (where identified), responsible for the separation across PC2 and the direction of that feature's influence. Tau, taurine; Citr, citrate; Oxog, 2-ketoglutarate; TMAO + Bet, trimethylamine-*N*-oxide (TMAO) and betaine. Figure reproduced with permission from Nature Publishing Group.[46]

An unsupervised principal components analysis (PCA) comparing the pre-dose metabolic profiles of rats with Class 1 (minimal or no hepatic necrosis) versus Class 3 (moderate necrosis) showed partial but statistically significant separation across principal component 2 (PC2, Mann Whitney *U*-test, p = 0.002). The main pre-dose metabolites responsible for the discrimination between minimal and moderate hepatic necrosis were shown to be taurine (high pre-dose levels associated with minimal necrosis) and trimethylamine *N*-oxide (TMAO) where high pre-dose TMAO levels were associated with moderate necrosis. The association of high pre-dose levels of taurine with minimal necrosis is consistent with literature findings of the protective effects of taurine, if administered prior to, or soon after, a toxic dose of paracetamol. Lower levels of taurine may be due to lower levels of the paracetamol-sulphating agent phosphoadenosine phosphosulfate (PAPS, a co-factor required by sulphotransferase enzymes such as SULT1A1), since most of the rats with a higher degree of post-dose necrosis (MHS > 2.5) excreted a relatively low amount of paracetamol sulphate in their urine. The association of high levels of TMAO with moderate necrosis may indicate an involvement of gut bacteria in determining the degree of toxicity, since TMAO is formed by oxidation of bacterially derived trimethylamine by mammalian flavin monoxygenase 3 (FMO3).[47]

This paper[46] was a landmark in terms of demonstrating the ability of a metabolic profile to operate in a prognostic as opposed to diagnostic mode, but it was a pre-clinical study in rats.

The team responsible for the pre-clinical paracetamol study then turned to gain funding and ethical approval for a pharmacometabonomics experiment involving paracetamol dosing in human subjects. The aim of this follow-on study was to determine if pre-dose metabolite profiles could be used to predict post-dose drug metabolism in humans given a normal oral dose of paracetamol. The drug paracetamol was chosen again, as the degree of metabolism to the major sulphate or glucuronide metabolites was known to be variable in humans. The Pfizer Global R & D Phase I Unit in Kent UK recruited a total of 99 fit and healthy, male volunteers. Each volunteer was given a normal 2 x 500 mg dose of paracetamol together with water (250 ml) and their pre-dose, 0-3 hour and 3-6 hour post-dose urines collected. 600 MHz flow-mode ^{1}H NMR spectroscopy was used to characterise the metabolite profiles in these urines. Illustrative pre-dose and post-dose NMR spectra from the urines of two volunteers are shown in Figure 4.

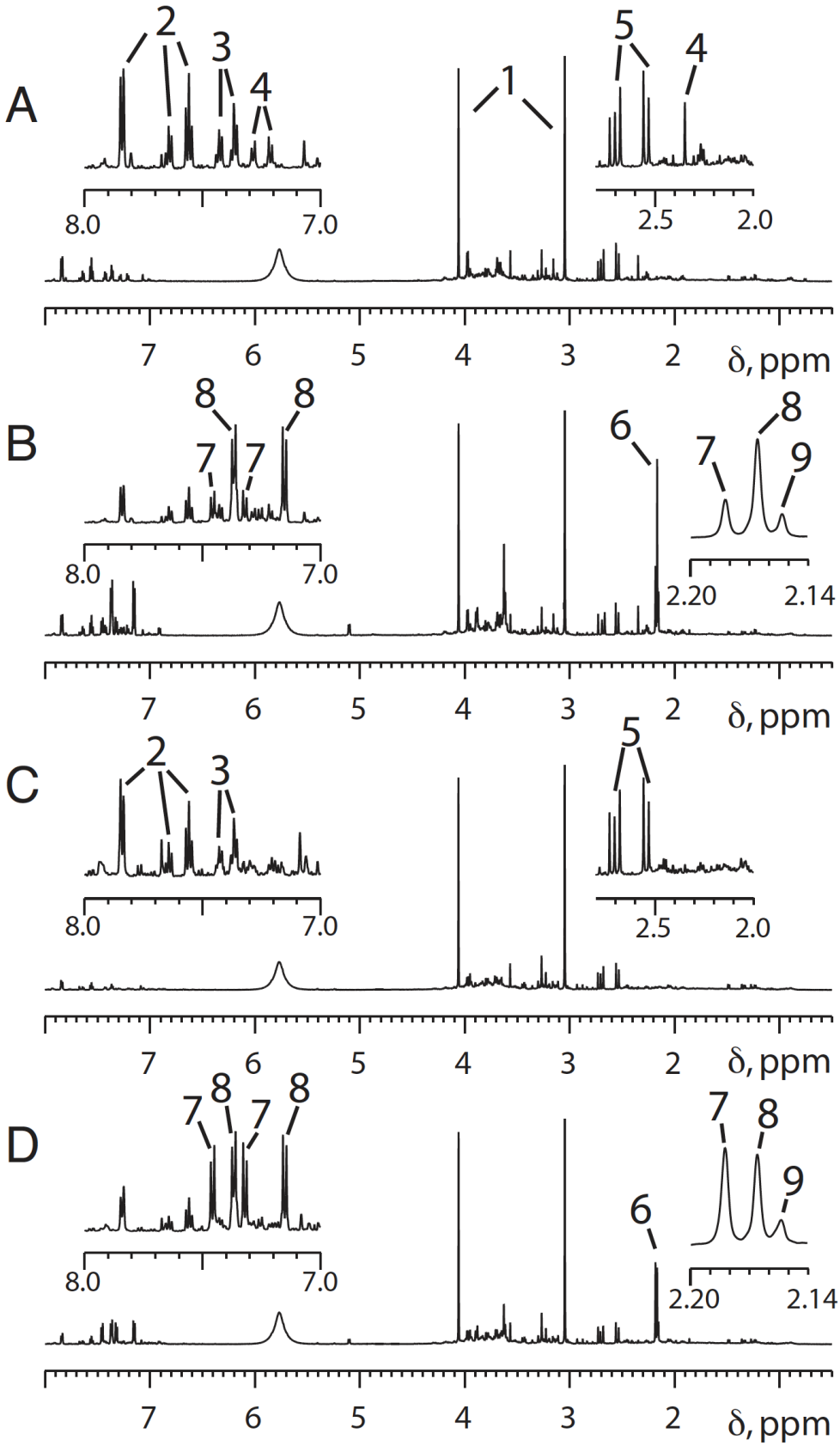

Figure 4. 600 MHz ^{1}H NMR spectra of the urine from volunteers on a paracetamol trial. A: the spectrum of the pre-dose urine from volunteer 1. B. the spectrum of the 0 – 3 hour post-dose urine from volunteer 1. C and D: the corresponding pre-dose and post-dose urine spectra of volunteer 2 respectively. Key to NMR signal numbers: 1, creatinine; 2, hippurate; 3,

phenylacetylglutamine; 4, unknown metabolite 4 (see text); 5, citrate; 6, cluster of signals from *N*-acetyl groups from paracetamol-related compounds that resolves into 7, 8 and 9 on expansion; 7, paracetamol sulfate; 8, paracetamol glucuronide; 9, other paracetamol-related compounds. Reproduced with permission from PNAS.[48]

The pre-dose urine of volunteer 1 (Figure 4A) shows signals from a number of endogenous metabolites, including the methyl (ca 2.35 ppm) and aromatic signals (ca 7.2 - 7.3 ppm) from an unknown metabolite, 4. In the 0-3 hour post-dose urine (Figure 4B), this volunteer excreted a relatively low amount of paracetamol sulphate metabolite (S, peak 7) relative to the paracetamol glucuronide metabolite (G, peak 8). By contrast, the pre-dose urine NMR spectrum of volunteer 2 (Figure 4C) shows no significant levels of endogenous metabolite 4 and a higher ratio of S/G (peak 7 / peak 8) in the 0-3 hour post-dose urine spectra (Figure 4D). Close analysis of all of the data showed that this pattern was repeated across the sample set: higher-pre-dose levels of metabolite 4 normalised to creatinine were associated with lower post-dose S/G ratios (Figure 5).

A Mann-Whitney *U*-test in conjunction with a Bonferroni correction of 100 (p value for statistical significance becomes $0.05/100 = 5.0 \times 10^{-4}$), to correct for multiple hypothesis testing, showed that pre-dose levels of metabolite 4 normalised to creatinine of > 0.06 were statistically significantly associated with 0-3 hour post-dose S/G ratios of < 0.8 ($p = 1.0 \times 10^{-4}$, Figure 5A). On the other hand, if the creatinine-normalised, pre-dose levels of 4 were < 0.06 then the post-dose S/G ratio was variable and not predictable. In addition, if the pre-dose levels of metabolite 4 normalised to creatinine were > 0.06, then the 3 - 6 hour post-dose ratios of S/G were always < 0.6, a smaller value than that at 0 - 3 hours, with $p = 1.2 \times 10^{-4}$, and also statistically significant (Figure 5B).[48] Conversely, if the post-dose ratios of S/G were high, then the pre-dose levels of metabolite 4 normalised to creatinine were always low (Figure 5 A and 5B).

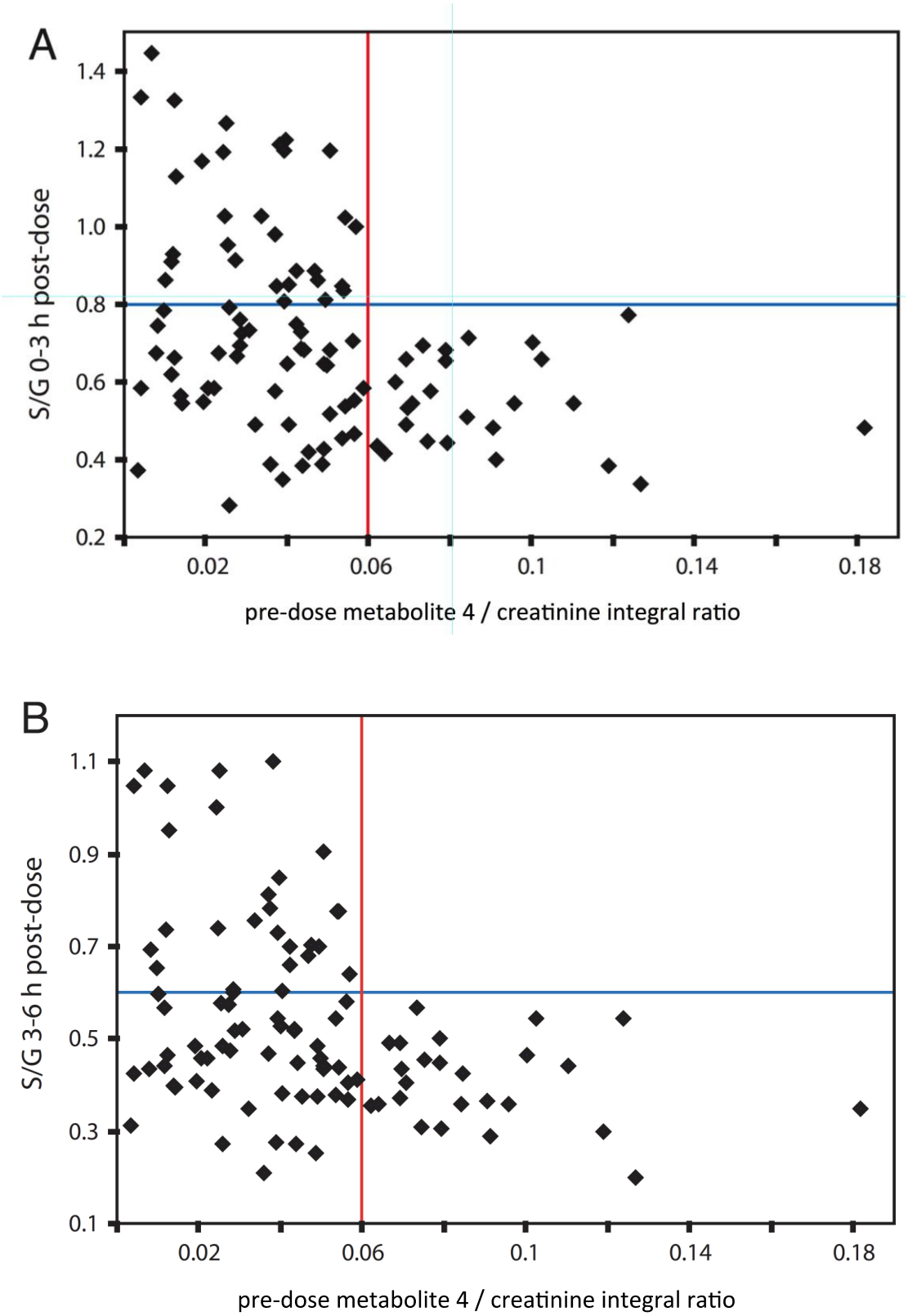

Figure 5. A. Plot of 0 – 3 hour post-dose urinary paracetamol sulphate to paracetamol glucuronide metabolite ratios (S/G) plotted against the pre-dose urinary ratios of metabolite 4 normalised to creatinine. Critical cut-offs in the S/G and metabolite 4/creatinine ratios are shown with horizontal blue and vertical red lines (see text). B. Corresponding plot for the 3-6 hour post-dose S/G ratios plotted against pre-dose ratios of metabolite 4 normalised to creatinine. Reproduced with permission from PNAS.[48]

Given the discovery of unknown metabolite 4 as a biomarker that could at least partially predict low S/G excreting volunteers from high S/G excreting volunteers, it became important to identify this metabolite. Metabolite 4 was characterised by a singlet methyl resonance at 2.348 ppm that was linked by statistical correlation spectroscopy (STOCSY)[49] to a pair of second order, aromatic pseudo-doublets at ca 7.210 and 7.285 ppm. It thus appeared that metabolite 4 contained a 4-substituted benzene ring with a methyl group at position 1. The metabolite was shown *not* to be 4-cresol itself by spiking an authentic reference standard of the latter into a representative pre-dose urine. Metabolite 4 was finally identified as 4-cresol sulphate, (1) by incubation of a representative pre-dose urine sample with a sulphatase enzyme that resulted in the reduction in the signals for 4, and the production of signals for 4-cresol, and (2) by unambiguous synthesis of metabolite 4, by reaction of 4-cresol with chlorosulphonic acid.[48]

Given the importance of these results, the entire NMR experiment was repeated in 2007 (4 years after the original analysis) in 5 mm NMR tubes, instead of the previous flow mode, using stored frozen urine samples, and with no significant changes in results. A year later, in 2008, the original S/G ratio analysis was repeated using UPLC-MS instead of NMR and a correlation coefficient of 0.99 was found between the two studies, with no outliers. Confidence having been gained in the data, the results were published in 2009.[48]

The identification of metabolite 4 as 4-cresol sulphate came as a surprise, as this key pre-dose biomarker that can partially predict human post-dose paracetamol sulphate to glucuronide metabolite ratios does not originate as a human metabolite. 4-cresol sulphate is produced in humans by the sulphation of 4-cresol, which is itself largely produced in the gut microbiome, particularly by *Clostridia* species. Thus, the human metabolism of paracetamol, one of the most widely taken drugs worldwide, is partly under the control of human gut bacteria; a result that was striking at the time as the degree of microbiome involvement in human biology and human drug metabolism was not well understood.

The rationale for the involvement of metabolite 4, that is 4-cresol sulphate, in paracetamol metabolism is as follows. In humans, as opposed to rodents, 4-cresol is eliminated from the body almost exclusively by sulphation, with no significant glucuronidation of the compound. However, the sulphation pathway in humans is considered not have a high capacity[50] and will be limited (1) by availability of the sulphate donor 3-phosphoadenosine 5-phosphosulfate (PAPS), and (2) by competition for the active site of the sulphotransferase enzyme involved.

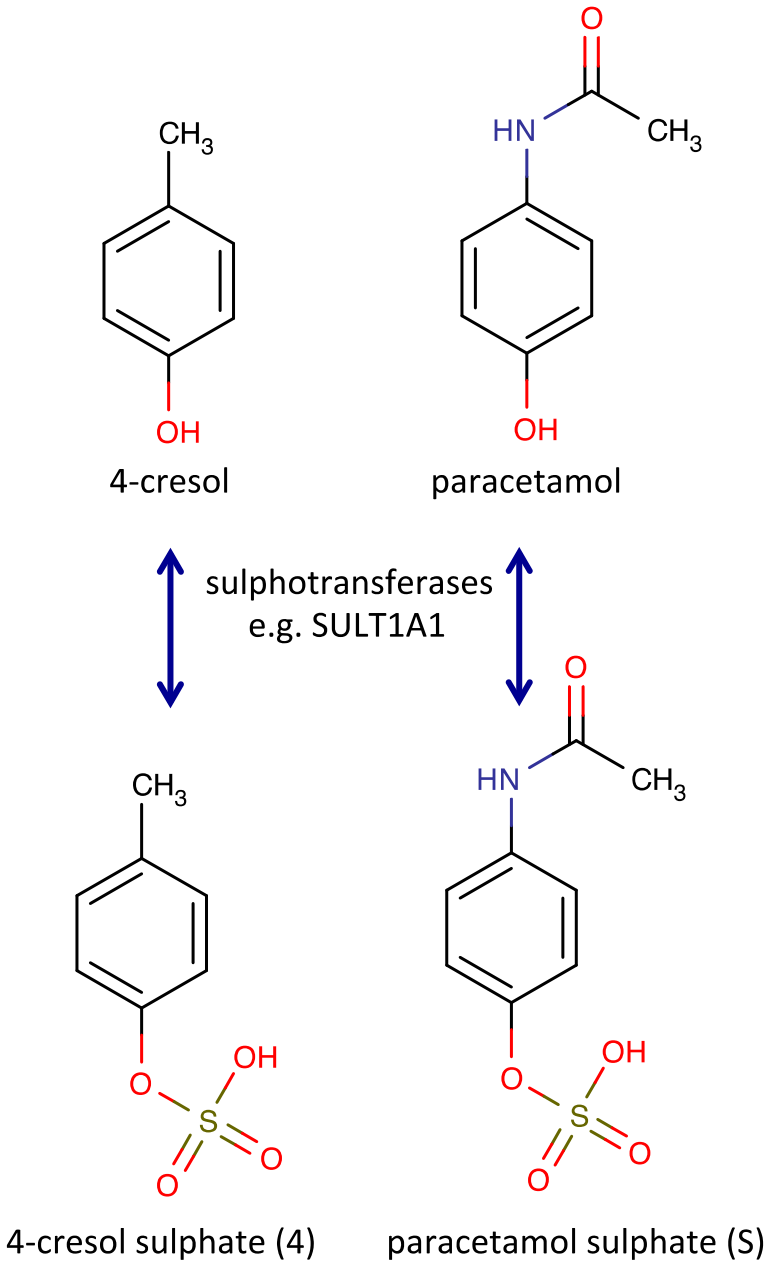

Figure 6. A comparison of the molecular structures of 4-cresol and paracetamol and their corresponding sulphates, the latter produced by the action of human sulphotransferases such as SULT1A1.

Figure 6 shows the striking molecular structural similarity between 4-cresol and paracetamol; they will compete for sulphation, especially by the enzyme sulphotransferase 1A1 (SULT1A1). Thus, in a human with a gut microbiome excreting significant quantities of 4-cresol, there is potentially a drain on the limited sulphation resources that the body uses (together with glucuronidation) to eliminate this toxic compound. If that person then takes a dose of paracetamol, the metabolism of the drug will tend to use glucuronidation more, due to depletion of sulphation capacity. On the other hand if a person has a gut microbiome that does not excrete significant levels of 4-cresol, then there is no excess demand on that person's sulphation capacity from that source and the

ratio of paracetamol sulphate to paracetamol glucuronide metabolites excreted will be variable and not predictable by this method.[48] What is clear, however, is that a person excreting a high ratio of S/G paracetamol metabolites post-dose will have had a low level of 4-cresol sulphate normalised to creatinine in their pre-dose urine.

Given the number of drugs that are metabolised, at least in part, by sulphation, the results found for the influence of 4-cresol excretion on paracetamol sulphation could have relevance for other such drugs. In addition, sulphation is an important endogenous metabolic process, which could therefore be influenced by gut microbiome excretion of 4-cresol.[48]

Since these first demonstrations of the ability of pharmacometabonomics to predict the outcomes of drug administration in animals and humans, a host of other studies have been published (Table 2). The rest of this article will review these later studies, with a focus on those using NMR spectroscopy as the metabolite detection, quantification and identification technology.

Table 2. A compilation of human and pre-clinical pharmacometabonomics studies and the detection technologies employed, published up to March 2017[a,b,c]

class of experiment	human studies	pre-clinical studies
prediction of pharmacokinetics (PK)	prediction of tacrolimus PK in healthy volunteers [LC-MS] [51]	prediction of pharmacokinetics of triptolide in rats [GC-MS] [52]
	prediction of atorvastatin pharmacokinetics in healthy volunteers [GC-MS] [53]	
	prediction of methotrexate clearance in patients with lymphoid malignancies [GC-MS] [54]	
	prediction of midazolam clearance in female volunteers [GC-MS] [55]	
	pharmacometabonomic prediction of busulfan clearance in hematopoietic cell transplant recipients [LC-MS] [56]	
prediction of drug metabolism	prediction of metabolism of paracetamol / acetaminophen in human volunteers [NMR] [48] **** first demonstration of pharmacometabonomics in humans**	prediction of paracetamol/acetaminophen metabolism in rats [NMR] [46] **** first demonstration of pharmacometabonomics**
	prediction of CYP3A4 induction in volunteer twins [NMR] [57]	
	prediction of CYP3A activity in healthy volunteers [GC-MS] [58]	
prediction of drug efficacy	prediction of simvastatin efficacy in patients on the Cholesterol and Pharmacogenomics study (TLC plus GC and GC-MS respectively] [59, 60]	
	prediction of citalopram/ escitalopram response in patients with major	

	depressive disorder (MDD) [GC-MS] [61] **** first demonstration of pharmacometabonomics-informed pharmacogenomics approach to personalised medicine** See also [62] and [63] the latter using LC-electrochemical coulometric array detection (LC-ECA)	
	prediction of sertraline and placebo responses in patients with MDD [LC-ECA and GC-MS] [64], [65] and [66]	
	prediction of efficacy of anti-psychotics in schizophrenia patients [LC-ECA] [67]	
	prediction of response to aspirin in healthy volunteers [LC-MS and GC-MS] [68-70]	
	prediction of efficacy with anti-TNF therapies in rheumatoid arthritis [NMR] [71]	
	prediction of thiopurine-*S*-methyltransferase phenotype in Estonian volunteers [HPLC] [72]	
	prediction of efficacy of L-carnitine therapy for patients with sepsic shock [NMR] [73]	
	prediction of acamprosate treatment outcomes in alcohol-dependent patients [UPLC-MS] [74]	
	prediction of blood pressure lowering in hypertensive patients treated with atenolol and hydrochlorothiazide [GC-MS] [75]	
	prediction of response in lung cancer patients [NMR and GC-MS] [76]	
	prediction of patient response to trastuzumab-paclitaxel neoadjuvant therapy in HER-2 positive breast cancer [LC-MS] [77]	
	prediction of blood pressure lowering in Caucasian hypertensive patients on the PEAR study with atenolol [LC-MS] [78]	
prediction of adverse events	prediction of weight gain in breast cancer patients undergoing chemotherapy [NMR] [79] **** first demonstration of pharmacometabonomics in patients**	prediction of toxicity from paracetamol/acetaminophen dosing in rats [NMR] [46] **** first demonstration of pharmacometabonomics**
	prediction of liver injury markers in patients treated with ximelagatran [NMR, GC-MS and LC-MS] [80]	prediction of onset of diabetes in rats administered with streptozotocin [GC-MS] [81]
	prediction of toxicity of paracetamol / acetaminophen (not strictly pharmacometabonomics, but 'early-onset pharmacometabonomics')	prediction of nephrotoxicity of cisplatin in rats [NMR] [83]

	[NMR] [82]	
	prediction of toxicity in patients with inoperable colorectal cancer treated with capecitabine [NMR] [84]	prediction of toxicity of isoniazid in rats [NMR] [85]
		prediction of variability in response to galactosamine treatment in rats [NMR] [86]
		prediction of toxicity from lipopolysaccharide treatment in rats [LC-MS and GC-MS] [87]
		prediction of nephrotoxicity of cisplatin in rats [GC-MS and LC-MS] [88]

Footnotes:
a) significant papers are highlighted with a double asterisk with explanatory text in bold blue font
b) abbreviations: GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; ECA, electrochemical coulometric array detection
c) the reader should beware that many papers with pharmacometabonomics or pharmacometabolomics in their titles are in fact just diagnostic metabonomics papers with no predictive or prognostic elements: these papers have been ignored in this compilation

3.1 NMR-Based pre-clinical pharmacometabonomics studies

Kwon et al[83] used an NMR-based pharmacometabonomics approach to predict the nephrotoxicity (kidney toxicity) of the anti-cancer drug cisplatin in rats. Nephrotoxicity is a significant issue for cisplatin and can be dose-limiting for some patients. Significant inter-animal variations in toxicity to cisplatin were observed. When challenged at a toxic dose of 10 mg / kg, 10 rats developed toxicity (T), but 5 did not (NT), and the differences in haematology markers were very large (p-values ca 10^{-6}), with the NT rats showing few differences from the control rats. Allantoin, succinate and creatinine were at higher levels in the urines of the non-toxic (NT) group and 2-ketoglutarate was at higher levels in the urines of the toxic group (T). With a leave-one-out approach their OPLS-DA (orthogonal projection to latent structure - discriminant analysis) model could predict the toxicity class of unknown samples with a modest 66% accuracy. The metabolic differences between the T and NT groups were interpreted as indicating that in the T group insufficient NADH was being produced to detoxify reactive oxygen species generated by cisplatin, the main mechanism of its toxicity.

Cunningham et al[85] also used an NMR-based approach to predict the central nervous system (CNS) toxicity of the anti-tubercular drug isoniazid in rats when dosed at 200 and 400 mg/kg. Isoniazid has a complex metabolic fate, including conjugating with pyruvate and pyridoxal to form pyruvate isonicotinylhydrazone and isoniazidyl-pyridoxal metabolites respectively. The neurotoxicity of isoniazid is associated with the depletion of cellular pyridoxal, which then leads to depletion of gamma-amino-butyric acid (GABA) levels in the brain and consequent seizures. Again, differences were seen between rats that exhibited toxic CNS responses at the high dose (responders, R) and those that did not (non-responders, NR). An OPLS-DA model showed that pre-dose urine levels of an unidentified phenolic ether-glucuronide metabolite were higher in the high dose

CNS responders than in the non-responders. Even in the low-dose rats, there were higher pre-dose urinary levels of this glucuronide in those animals that went on to excrete low levels of the drug metabolite acetylisoniazid post-dose: the latter drug metabolite also lower in the high dose CNS responders. It was hoped that this pre-clinical study, the first to identify multiple isoniazid urinary metabolites, would have the ability to translate its finding to the human clinical setting.[85]

Coen et al[86] used an NMR-based approach to investigate variability in the response of rats to the toxicity of galactosamine, a drug whose variable responses in rats triggered the design of the original pharmacometabonomics experiment.[46] Male Sprague-Dawley rats were dosed intraperitoneally with 415 mg/kg galactosamine and classified as responders or non-responders based on clinical pathology. After an 11-day drug washout period, the animals were given a second dose of the drug and their responses monitored again. Some rats that were non-responders to the first dose were responders to the second dose: these rats were termed induced responders. An OPLS-DA model based on the pre-dose urine metabolite profiles was able to distinguish the profiles of non-responders to dose 1 from induced responders to dose 2, with significantly discriminating metabolites including hexanoic acid and the *N*-acetyl resonances of some α1 acid glycoproteins.[86] A similar discrimination, was observed from an OPLS-DA model of fecal extract profiles, with decreased levels of γ - aminobutyrate (GABA), α-ketoisovalerate, and lactate in predose fecal profiles of induced responders.

3.2 NMR-based human pharmacometabonomics studies

3.2.1 Prediction of drug metabolism studies.

Ahmadi, Kemsley et al[57] used an NMR approach to predict variability in the activity of the 'drug-metabolising' enzyme cytochrome P450 3A4 (CYP3A4) in 301 female, volunteer twins who were administered St John's Wort (a potent CYP3A4 inducer) for 14 days. The activity of CYP3A4 was then assessed by measuring the ratio of 3-hydroxyquinine (3-OH Q) to quinine (Q) after administration of the CYP3A4 substrate quinine sulphate (300 mg) to the volunteers at day 14. The best model for the prediction of the 3-OH Q / Q ratio explained around 38% of the variation in the cohort ratios and included 7 pre-dose urine NMR bins plus BMI, volunteer alcohol and smoking status and, strikingly, the batch code of the UPLC-MS data used to measure the 3-OH Q/Q ratio. None of the metabolites identified as most significantly contributing the predictive 3-OH Q / Q ratio model were linked in a direct fashion to CYP3A4 induction, but the majority were known to have urine levels substantially influenced by diet.

3.2.2 Prediction of drug efficacy studies.

Kapoor et al[71] used 500 MHz ^{1}H NMR spectroscopy of pre-dose urines from 16 rheumatoid arthritis patients to show that the profiles of several pre-dose metabolites, including histamine, glutamine, xanthurenic acid, and ethanolamine, could be used to discriminate between those patients who either would, or would not have a good response to anti-TNF therapy according to European

League Against Rheumatism criteria, with a sensitivity of 88.9% and a specificity of 85.7%. A correlation between baseline metabolite profiles and the change in the disease activity score in 28 joints from baseline to 12 months had a p value of 0.04. The positive association of degree of response with the strongly discriminating, pre-dose metabolite histamine was interpreted as potentially indicating the degree of pre-dose inflammation or the degree of breakdown of histidine. Larger, additional studies were recommended.

Puskarich et al[73] used 500 MHz ^{1}H NMR of pre-dose serum samples to show that lower baseline blood serum levels of 3-hydroxybutyrate ($p < 0.001$), acetoacetate ($p < 0.001$) and 3-hydroxyisovalerate ($p < 0.001$) were significantly associated with survival for septic shock patients on L-carnitine therapy (4 g bolus followed by 8 g infusion over 12 hours). The 31 patients were randomised between placebo (n = 15) and drug treatment (n = 16). Patient survival analysis indicated a marked trend towards greater survival in L-carnitine-dosed, low ketone patients (3-hydroxybutyrate < 153 uM) compared to L-carnitine-dosed, high ketone patients (baseline 3-hydroxybutyrate > 153 uM, $P = 0.007$), or compared to placebo treated patients with either high or low baseline ketone levels. These precision healthcare results were seen as important clinically as the differentiation of responders from non-responders was not evident from traditional clinical biomarkers such as lactate levels and the Sequential Organ Failure Assessment (SOFA) score. Additional method validation was recommended.

Hao et al[76] have used both 600 MHz ^{1}H NMR and GC-MS to study serial blood serum samples from 25 lung cancer patients undergoing chemotherapy with or without radiation. The metabolites detected by ^{1}H NMR were associated with cancer type and stage. However, metabolites such as hydroxylamine, tridecan-1-ol, and octadecan-1-ol, detected by GC-MS, were indicative of patient survival ($P < 0.05$) and other GC-MS-detected metabolites including tagatose, hydroxylamine, glucopyranose, and threonine were indicative of disease progression ($P < 0.05$). It was concluded that metabolic profiling has the potential to provide prognostic clinical outcome information.

3.2.3. Prediction of drug safety studies.

In the first validated study with human patients, as opposed to volunteers, Keun et al[79] used 600 MHz ^{1}H NMR to show that pre-treatment serum levels of alanine and lactate together with % body fat were predictive of weight gain in women undergoing chemotherapy treatment for breast cancer. This is important as weight gain is associated with a risk of cancer recurrence.

Andersson et al[80] used a wide array of analytical methodologies, including 600 MHz ^{1}H NMR, GC-MS, LC-MS and proteomics, to show that the blood plasma levels of a wide variety of metabolites and proteins, including kininogen 1 (KNG1), formate, L-cystine, creatinine, lecithin-cholesterol acyltransferase (LCAT), glutamic acid, pyruvic acid, alanine, 2-ketoglutaric acid, apolipoprotein (APO) A2, APOA4, and APOE, were all statistically significant in predicting liver injury (as determined by alanine aminotransferase (ALT) levels) in patients treated with the anticoagulant agent ximelagatran. The systems biology

approach used in this study was successful in developing new hypotheses for the unknown mechanism of toxicity. Unfortunately, the development of this compound was ultimately terminated due to its liver toxicity. The ability to determine which patients could benefit from a drug without toxicity could be crucial in such a situation in the future, and potentially avoid the loss of a drug which is toxic to just a subset of patients as opposed to all patients.

Backshall et al[84] used 600 MHz ^{1}H NMR to show that high pre-treatment serum levels of low-density lipoprotein–derived lipids, including polyunsaturated fatty acids and choline phospholipids, were predictive of greater toxicity, in 54 patients with locally advanced or metastatic colorectal cancer being treated with capecitabine. Although the authors recommended validation by repetition in a larger cohort, this is an important result, as capecitabine toxicity is not only a significant issue for patients' quality of life, it also limits the drug dose range available, and may thereby diminish the likelihood of patient cure.

4. Conclusions and the future for pharmacometabonomics

The development of personalised medicine (or precision or stratified medicine as it is sometimes known) is an important goal for 21st Century healthcare, in order to avoid unnecessary drug adverse events in patients and to maximise efficacy in patients by prescribing the most appropriate drugs for the particular patient. The current paradigm for personalised medicine is built upon the use of pharmacogenomics, i.e. the use of patient genomic information to predict the influence of patient gene mutations on drug metabolism, pharmacokinetics, efficacy and safety. However, this paradigm is limited by the fact that the effects of drugs on the patient (efficacy, safety) and the effects of the patient on the drugs (metabolism, transport, pharmacokinetics) involve a variety of environmental factors, especially the status of the patients' microbiomes, in addition to their genetic profiles. In these circumstances it is unsurprising that pharmacogenomics has had less influence on personalised medicine in clinical practice than was first hoped for. By contrast, pharmacometabonomics is able to sample both genetic and environmental factors that influence the efficacy, safety, metabolism, transport and pharmacokinetics of drugs. The metabolic profiles of biological fluids such as urine and plasma contain human metabolites, bacterial metabolites (from the microbiome) and human/bacterial co-metabolites. It is therefore to be hoped that a combination of pharmacogenomic and pharmacometabonomic technologies will be able to provide more sensitive and specific predictions of drug efficacy and safety that pharmacogenomics alone is currently able to do.

In the decade or so since the first publication on pharmacometabonomics, an array of publications have demonstrated the ability of both NMR-based and mass spectrometry-based pharmacometabonomics studies to predict drug metabolism, pharmacokinetics, efficacy and safety: see Table 3. It is striking that pharmacometabonomics approaches are now being used to predict drug efficacy in situations that are literally life-and-death, such as the report by Puskarich et al[73] involving septic shock patients on L-carnitine therapy. Many of the studies

reported are early stage and exploratory, rather than large-scale clinical trials, and many of the studies will require validation in larger cohorts of subjects. However, the promise of using human metabolic information in combination with human genomic information in order to optimise the choice of medical treatments for patients in the future is compelling.

The delivery of the promise of metabolic profiling in pharmacometabonomics studies will be considerably assisted by the recent development of large number of Biobanks: repositories of human biofluids and tissues that can be accessed for large-scale phenotyping and crucially, in addition, the advent of Phenome Centres such as those in London and Birmingham in the UK and in Singapore, that can deliver large-scale metabolic profiling using NMR and MS technologies on hundreds or thousands of patients' samples with high efficiency, high speed and high quality.[89]

It is also worth considering that pharmacometabonomics itself is just one example of a wider array of prognostic metabolic profiling experiments that can be generally termed predictive metabonomics.[90] In pharmacometabonomics, a pre-dose metabolite profile is used to predict the effects of drug treatment, be that efficacy, safety, or indeed metabolism or pharmacokinetics.[91] In predictive metabonomics, a pre-event metabolite profile is used to predict a broader array of interventions such as change in diet, exercise, or just the passage of time. It was envisaged from the point of discovery of pharmacometabonomics that the methodology would have this broader utility.[46] If the intervention is just passively the passage of time, the methodology can be used to predict disease onset in subsets of a population, and this has been demonstrated in several important cases, including the prediction of the onset of type-2 diabetes, [92-94] as well as in other diseases.[90] The power of metabolic profiling to provide prognosis of outcome in a wide variety of situations is one of the most exciting current aspects of this new science.

In summary, personalised medicine will benefit enormously from the combined implementation of metabolic profiling by pharmacometabonomics, together with genomic profiling by pharmacogenomics. It is expected that the synergistic combination of these technologies[61] will lead to much improved drug choices for patients in the future, which is a critical goal of current and future healthcare.

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6. Terminology[95]

BIOFLUID: A biological fluid, typically from an animal or human, such as urine, blood plasma, cerebrospinal fluid, saliva, sweat, or tears.

METABOLITE: A small molecule product of enzyme-mediated biotransformation in an organism.

METABONATE: A small molecule product of non-enzymatic, chemical transformation in an organism. This term is also used to describe molecular artefacts produced chemically during sample extraction, isolation, purification or analysis procedures.[96]

METABONOMICS: "The study of the metabolic response of organisms to disease, environmental change or genetic modification."[12, 97] Metabonomics has an interventional definition describing a diagnostic, systems biology approach to understanding global metabolic regulation of organisms and their commensal, parasitic and symbiotic partners.

METABOLOMICS: The comprehensive and quantitative measurement of all the metabolites in a biological system.[98] Metabolomics, by contrast to metabonomics, has an observational definition which is also aspirational, as it will not, in the foreseeable future, be possible to detect, let alone quantitate, *all* the metabolites in a biological system.

METABOLOME: The full set of metabolites within, or that can be secreted from, a biological system such as a cell type or tissue.[99]

METABONOME: The sum of the cellular metabolomes in a multi-cellular organism and their interaction components plus the products of chemical transformations and extra-genomically generated metabolites.[100]

METABOTYPE: The metabolic phenotype of an organism.

MICROBIOME: The collection of microorganisms present both in and on an organism, in a variety of environmental niches

PHARMACOGENOMICS: The prediction of the outcome of a drug or xenobiotic intervention in an individual based on an analysis of that individual's genetic profile.

PHARMACOMETABONOMICS: The prediction of the outcome of a drug or xenobiotic intervention in an individual based on a mathematical model of pre-intervention metabolite signatures.[46]

PHARMACOPROTEOMICS: Sometimes defined as the use of proteomic technologies in drug discovery and development [101], but logically, this would be better defined as: 'The prediction of the outcome of a drug or xenobiotic intervention in an individual based on an analysis of that individual's proteome.'

PHARMACOMETABOLOMICS: This later prognostic term is used synonymously with pharmacometabonomics, but is sometimes erroneously used to describe the investigation of the effects of a drug on an organism: this is just diagnostic metabonomics.

PREDICTIVE METABONOMICS: The prediction of the outcome of an intervention in an individual based on a mathematical model of pre-intervention metabolite profiles. The intervention could be a change in diet, exercise, the passage of time, surgical treatment etc. Pharmacometabonomics is one case of predictive metabonomics, which covers the prognosis of any intervention.

TRANSCRIPTOMICS: the study of the transcriptome: the complete set of ribonucleic acid transcripts produced by an individual genome under a given set of conditions.

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8. Glossary

Term	Meaning
ADR	adverse drug reaction
BMI	body mass index
CYP2C9	the gene encoding the 9th member of the cytochrome P450 enzyme in family 2, subfamily C
ERETIC	Electronic REference To access In vivo Concentration
FDR	false discovery rate
GC-MS	gas chromatography - mass spectrometry
HER-2	human epidermal growth factor receptor 2
LC-ECA	liquid chromatography - electrochemical coulometric array detection
LC-MS	liquid chromatography - mass spectrometry
MHS	mean histopathology score
MMC	maximum margin criteria
NADH	reduced form of nicotinamide adenine dinucleotide (NAD)
OPLS-DA	orthogonal projection to latent structure - discriminant analysis
PC1	principal component 1 etc.
PCA	principal components analysis
PLS	partial least squares / projection to latent structures
STOCSY	statistical correlation spectroscopy
SULT1A1	member 1 of the sulphotransferase enzyme family 1A
TMAO	trimethylamine *N*-oxide
UPLC-MS	ultra performance liquid chromatography - mass spectrometry
VIP	variable influence on projection