Why Metabonomics?

Ivan K S Yap

Metabonomics can be used to quantitatively measure dynamic biochemical responses of living organisms to physiological or pathological stimuli. A range of analytical tools such as high-resolution nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) combined with multivariate statistical analysis can be employed to create comprehensive metabolic signatures of biological samples including urine, plasma, faecal water and tissue extracts. These metabolic signatures can reflect the physiological or pathological condition of the organism and indicate imbalances in the homeostatic regulation of tissues and extracellular fluids. This technology has been employed in a diverse range of application areas including investigation of disease mechanisms, diagnosis/prognosis of pathologies, nutritional interventions and drug toxicity. Metabolic profiling is becoming increasingly important in identifying biomarkers of disease progression and drug intervention, and can provide additional information to support or aid the interpretation of genomic and proteomic data. With the new generation of post-genomic technologies, the paradigm in many biological fields has shifted to either top down systems biology approaches, aiming to achieve a general understanding of the global and integrated response of an organism or to bottom up modelling of specific pathways and networks using a priori knowledge based on mining large bodies of literature. Whilst metabolic profiling lends itself to either approach, using it in an exploratory and hypothesis generating capacity clearly allows new mechanisms to be uncovered.

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Introduction

The advent of molecular profiling tools in the post-genomic era facilitated extraction of extensive and comprehensive description of the molecular sequelae involved in mammalian systems in response to a particular stressor. Increasingly, integrated systems biology study of disease and physiological state involving gene (transcriptomics), protein (proteomics) and metabolite (metabonomics) profiling are being used to better understand the underlying mechanistic interactions between host genotype and the environment. Metabonomics was introduced into the scientific community in the late 90's[1] and has since proven invaluable in uncovering and decoding metabolic signatures of health, disease and biological challenges. The most widely used definition for metabonomics is "… the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification"[1]. A statement, coined by its pioneer Professor Jeremy Nicholson of Imperial College London (UK), that captures the essence of the approach, which is to analyse spectroscopic data through multivariate statistical analyses for an objective differential diagnosis based solely on the biochemical compositional differences[2]. The emphasis of metabonomics, originally, was on the global metabolic responses of the organism, which is not to be confused with the term 'metabolomics' that essentially studies the local cellular metabolome. In this review, the term 'metabonomics' will be used to represent both approaches. 'Omics' approaches yield complementary information, however, coupled with advanced statistical pattern recognition analyses, metabonomics offers an unbiased untargeted approach to identifying and understanding metabolic pathways that are perturbed in a disease state as compared to transcriptomics and proteomics. In addition, the metabolome is a downstream product of both the transcriptome and proteome, therefore it has the potential to be a better indicator of pathway activity than the proteins and genes involved in that particular pathway. A range of analytical tools such as high-resolution nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) combined

Department of Life Sciences, School of Pharmacy and Health Sciences, International Medical University, Bukit Jalil, Kuala Lumpur, MALAYSIA

Address for Correspondence:

Dr Ivan K S Yap, Department of Life Sciences, School of Pharmacy and Health Sciences, International Medical University, 126, Jalan Jalil Perkasa 19, Bukit Jalil, 57000 Kuala Lumpur, MALAYSIA

with multivariate statistical analysis can be employed to create comprehensive metabolic signatures of biological samples including, but not exclusively, urine, plasma, faecal water and tissue extracts. These metabolic signatures can reflect the physiological or pathological condition of the organism and indicate imbalances in the homeostatic regulation of tissues and extracellular fluids. This technology has been employed in a diverse range of application areas including investigation of disease mechanisms[3], diagnosis/prognosis of pathologies[4], nutritional interventions[5] and drug toxicity[6]. Metabonomics is becoming increasingly important in identifying biomarkers of disease progression and medical intervention[7], and can provide additional information to support or aid the interpretation of genomic and proteomic data[8].

Understanding our virtual 'organ'

The mammalian gut contains hundreds of species of commensal and symbiotic microbes that mainly reside in the large intestine. This collective of microbes, regarded as the virtual 'organ', contributes to ~60% of the total faecal mass produced[9, 10]. The gut microbiota contributes to myriads of mammalian processes, including defence against pathogens at the gut level, immunity, intestinal microvilli development, non-digestible dietary fibre fermentation and protein putrefaction, which results in recovery of metabolic energy for the host[11-13]. In man, dietary preferences, lifestyle and genetics influence microbial population and ecological development, and these interactions determine the health of both individuals and populations (Figure 1)[12,14]. Thus, the gut microbiome–mammalian 'Superorganism'[15] represents a level of biological evolutionary development where true symbiosis is characterised by extensive 'transgenomic' modulation of metabolism and functions between the two entities. The gut microbiota has also been implicated in the etiology of many gut disorders including irritable bowel syndrome and colon cancer[16,17]. Moreover, the microbiome has recently been reported to vary significantly between obese individuals and normal individuals, and it is known to provide refined control mechanisms on energy recovery through catabolism of otherwise poorly digestible nutrients, such as resistant starch and other polysaccharides[18-21]. Perhaps, one of the greatest challenges in modern biology is to interrogate and classify these critical transgenomic interactions and to understand their role in diverse human disease processes. A metabonomics approach offers a robust strategy for capturing and characterising contribution of gut microbiome in human physiological functions and disease state in a topdown systems biology approach. Depending on the analytical instrument used, metabonomics can be utilised in a targeted or untargeted approach to identify key metabolites that occur in the gut that are derived from microbial processes. Previous research revealed the influence of gut microbiota on urinary metabolite composition in animal and human population studies using metabolic profiling methods based on ^{1}H nuclear magnetic resonance (NMR) spectroscopy[22-26]. In particular, diet, medication and other environmental factor induced effects on aromatic components such as hippurate, 4-cresyl sulphate, phenylacetylglycine (or phenylacetyleglutamine in human) and 3-hydroxyphenylpropionic acid (HPPA), which are known to be generated or co-metabolized by gut microbiota, are well-documented in conjunction with a variety of other metabolic pathways such as those involved with bile acid catabolism, choline metabolism and utilization of short chain fatty acids (SCFAs)[27-29].

Nicholls and colleagues demonstrated the potential of metabonomics strategy as a metabolic tracking tool for monitoring dynamic metabolic flux in mammalian system[30]. In the study, gnotobiotic (germ-free) rats were 'conventionalised' following removal of these animals from the aseptic barrier unit housing them (Figure 2). A metabonomics strategy was employed to monitor the biochemical changes and influence of the microbiome on host metabolism as the microbiome establishes their population in the gastrointestinal tract. The changes in biochemical flux through time can be non-invasively tracked using metabonomics urine analysis, which can

then be visualised *via* pattern recognition analysis in the form of metabolic trajectory (Figure 2). Sequential metabolic changes observed in the study, reflects the dynamics of host gut microbiota colonizing the gastrointestinal track[30]. A further study using gnotobiotic animals showed that the gut microbiome is involved in modulation of bile acid metabolites, which suggested the involvement of gut microbiota in regulation of lipid absorption as well as regulation of nuclear receptor family of ligand-modulated transcription factors such as the farnesoid X receptor and pregnane X receptor[27,31].

Another example of microbial effects on mammalian metabolism studied using pseudo-integrated metabonomics and metagenomics approaches was provided by Yap *et al.* in an antibiotic model[25]. The antibiotic, vancomycin, was used in the study to deliberately reduce host gut microbiota population in order to further understand microbial-mammalian co-metabolism. Time course changes in host metabolic phenotype were monitored using NMR spectroscopy-based metabonomics approach in tandem with 16S rRNA gene polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) analysis to track changes in gut microbial community following antibiotic treatment. The study showed that vancomycin treatment profoundly affects microbial co-metabolites (Figure 3) including short chain fatty acids, phenylacetylglycine and hippurate, which correlated with changes in several microbial species mainly within the Firmicutes. This study not only highlighted the potential of metabonomics approach in unraveling microbial-mammalian biocomplexity, but also the danger of reckless uncontrolled use of antibiotics in human populations that may irreversibly altered the stable core symbiotic microbiome in addition to increased risk of antibiotic resistance[12,25,32,33].

Parasitology in 'Omics' dimension

One of the major challenges in characterising infectious diseases is the fact that parasitic infection induces metabolic changes in multiple physiological compartments within the host that affects both local and systemic homeostasis. Contact with multiple host tissues is very likely with protozoan infection (e.g., *Trypanosoma* spp. and *Plasmodium* spp.) since they can be distributed by host systemic circulation during the blood stages in their life cycle[34]. In the case of parasitic worms, contact with multiple host organs is minimal since the worms are often restricted to a single organ once they have reached their place of maturation as in the case of *Fasciola* spp. (bile duct), or *Echinostoma* spp (small intestine)[35]. However, irrespective of parasite topographical location, parasitic infections are known to induce pathology in remote organs such as neuroshistosomiasis, where parasite eggs migrated to the central nervous system and induced local tissue inflammation[36]. In addition, the composition and function of host gut microbiota play an important role in many physiological and pathological processes[37,38]. Several studies have shown that parasitic infections exert effects on the host gut microbiota composition and activity[39,40].

Metabonomics approaches were first introduced into parasitology in an initial study on *Schistosoma mansoni*-infected mice[41]. Metabonomics strategy was utilised in the study to obtain urinary metabolic profiles of mice infected with *S. mansoni* cercariae as compared to controls. The study revealed that *S. mansoni* infection induced metabolic perturbations in tricarboxylic acid cycle intermediates (citrate, succinate), amino acid metabolism (taurine, 2-oxoglutarate, tryptophan) and gut microbial-related co-metabolites (hippurate, trimethylamine, phenylacetylglycine), indicating that *S. mansoni* infection not only affects host endogenous metabolism but host gut microbiome as well. Since then, the ^{1}H NMR spectroscopy-based metabolic profiling strategy has been used to investigate four other rodent host-parasite models including *Trichinella spiralis*-mouse[37], *S. japonicum-hamster*[42], *Echinostoma caproni*-mouse[43] and *Trypanosoma brucei brucei*-mouse[40] with the aim of characterising the metabolic responses of hosts to

these parasites at the molecular level and establishing the sensitivity and specificity of potential diagnostic biomarkers in the biofluids.

Recent development in the area of infectious disease incorporated both metabolite and immune profiles, as demonstrated by Saric *et al.*[3], in an attempt to give a holistic systems biology view of the complex multilevel interactions of host physiology in response to infection. Saric and colleagues investigated host immune responses to single- and multicellular parasites (*Plasmodium berghei*-mouse, *Trypanosoma brucei brucei*-mouse, *Schistosoma mansoni*-mouse and *Fasciola hepatica*-rat) infection. Metabonomics strategy was used to obtain urinary and plasma metabolic profiles, together with a multiplex panel of cytokine profiles generated from a multi cytokine assay platform. The data were then analysed independently and integrated to understand host metabolic responses that are directly and indirectly associated with immune responses. The study showed that host metabolic and immune responses were differentiated according to parasite species whereby the protozoan (*P. berghei* and *T. b. brucei*)-induced responses were quite similar but those induced by helminth (*S. mansoni*) infection were clearly differentiated from the protozoan. For example, both *P. berghei* and *T. b. brucei* infection triggered a change in plasma levels of acetyl glycoproteins that correlated with interferon-g, however, in the case of *S. mansoni* infection, changes in the levels of lactate, choline and D-3-hydroxybutyrate were correlated with changes in levels of cytokines interleukin (IL)-4 and IL-5. From the study, one could infer that differential host immune responses to parasitic infection are not only apparent in their immune responses but are also imprinted in the metabolite signature, which indicated a link between immunoregulatory and metabolic pathways[3].

The ability to obtain metabolic information from multiple compartments and analyse the data simultaneously allows metabonomics to play an important role in bridging the gap in understanding parasitic infection in a multi compartmentalised 'superorganism'. In addition, such a fingerprinting strategy proved to be a reliable, robust and easily acquired method of diagnosis and could ultimately be used to monitor host-parasite response to therapeutic intervention.

Metabolome-wide association studies

Until recently, scientists could only envisage the use of 'omics' technology to understand gene-environment interactions in diseases at the population level. The introduction of high-throughput molecular screening and spectroscopic approaches has made this a reality. Utilisation of high-throughput genomic screening to understand genetic variation in relation to disease state has revolutionised the world of molecular biology and led to the creation of genome-wide association (GWA) study. The GWA study approach on large human population genotype and statistically linking genotypic variation to epidemiological data such as cardiovascular disease (CVD) incidence and diabetes has proved successful for the detection of common variants associated with a range of diseases[44]. However, capturing and understanding human biocomplexity requires a level of knowledge beyond just genetic programming but one that encompasses the study of population proteome and metabolome, which together will give a holistic view of individual and population gene-environment interactions and its relation to diseases and risk factors[45]. The concept of metabolome-wide association (MWA) study is analogous to that of GWA, that is, to capture environmental and genomic influences that relate population phenotypic variations to disease risk factors using advanced molecular spectroscopic approaches[46,47].

The first proof-of-concept MWA study was demonstrated by Holmes *et al*, who successfully identified discriminatory markers and their associations with known disease risk of CVD, blood pressure (BP), at the population level[48]. The study involved high resolution NMR spectroscopic analyses of urine samples from 4630 individuals from 17 different population subgroups in China, Japan, United Kingdom and United States of America, and extensive multivariate statistical analyses

to model population metabolic phenotypes in relation to geographical variations and BP. The study was part of the National Institutes of Health (USA)-funded INTERMAP (International Collaborative Study of Macronutrients, Micronutrients and Blood Pressure) epidemiological study[49], that aimed to understand the effect of micro- and macronutrient intake on human BP. Results from the study showed extensive metabotype variation both between and, more interestingly, within countries, and successfully identified 10s of discriminatory biomarkers that significantly differentiate the populations. The study reported that these biomarkers were derived from four main sources of phenotypic variation namely dietary, gut microbiomic, xenometabolomic (population drug use) and genetic. Four of the biomarkers: alanine (linked to diet), formate (folate-related, one-carbon metabolism and starch breakdown by gut microbes), *N*-methylnicotinamide (diet) and hippurate (diet and microbiome activity) were further examined as these have strong environmental connections. Quantitative values of these biomarkers, derived from NMR spectroscopy, were investigated for their relationship to BP using multiple linear regression. Formate was found to have the strongest inverse relationship with BP. Formate is involved in chlorine (Cl) exchange in the kidney through the Cl-formate exchange (CFEX) transporter, which itself is related to a complex series of solute carrier 26 (SLC26) anion exchangers that handle renal ion balance, including Na^+ and Cl^-[50]. This MWA-generated putative biomarker (formate) is linked by a series of complex physiological factors to factors (including salt handling) that are linked to BP regulation[48].

Recent work by Yap *et al.* applied and extended the concept of MWA study on epidemiological urinary samples collected from subjects in north and south China where rates of heart disease and stroke vary markedly[51]. ^{1}H NMR spectroscopy-based MWA approach was used to identify urinary metabolites that discriminated between southern and northern Chinese population samples as well as population biomarkers that might relate to the difference in CVD risk. The study involved analyses of NMR spectra from two 24-h urine specimens per person for 523 northern and 244 southern Chinese participants in the INTERMAP study. Discriminating metabolites were identified and assessed for statistical significance with a conservative family wise error rate (< 0.01) to minimize false positive discoveries. The study revealed a wide range of discriminatory metabolites (Figure 4) that differentiated the two populations that were derived from dietary (alanine, prolinebetaine), gut microbial (hippurate, 4-cresyl sulfate, phenylacetylglutamine, 2-hydroxyisobutyrate) and endogenous metabolism (succinate). These findings indicated the importance of environmental influences that may help explain north-south China differences in CVD risk[51].

Results from these pioneering MWA studies are beginning to reveal its untapped potential in uncovering new aspects of disease pathophysiology. The MWA approach is complementary to GWA studies. Future fully aligned and integrated MWA-GWA studies may offer scientists the potential to identify genetic basis of diseases with complementary sets of putative biomarkers that form parts of established metabolic or physiological pathways, which may translate into novel therapeutic targets[47].

What is in the future?

This article is by no means an exhaustive review on all the application areas of metabonomics, but the author's mere opinion on the areas of scientific research where metabonomics can contribute greatly particularly in Malaysia. One of the biggest challenges in modern biology lies in elucidating and understanding human biocomplexity, which involves interactions between the single-genome host and the highly dynamic gut microbiome and their genomes. Improvement in instrument sensitivity and evolution in metabonomic modelling techniques have enabled broader application in areas unfathomable a decade ago. An example is the

application of metabonomics in surgical metabonomics and personalised health care currently pioneered by Imperial College London (UK) stirred by the founder himself, Professor Jeremy Nicholson and his team of experts to bring translational systems biology research from laboratory to clinical setting, where metabonomics will be used as a tool to provide real-time diagnostics and information to clinicians on patient metabolic status that may influence surgical outcomes[52]. The author wishes to end with a paragraph from Professor Jeremy Nicholson, "Although it might not be possible to predict the unpredictable, we might be able to predict the likelihood of unpredictability. Our ability to do this in the future will depend on the construction of new modelling paradigms that are not limited by the horizons of metabolic knowledge of the last century[53]." An exciting future awaits those who dare to grab the challenge and go beyond what scientists have thus far achieved.

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Figure 1. Complexity of mammalian biology: A summary of some key factors that limit or augment individual genetic complement and affect the probability of particular disease outcomes later in life. More details in Nicholson and Wilson, Nat Rev Drug Discovery, 2003[53].

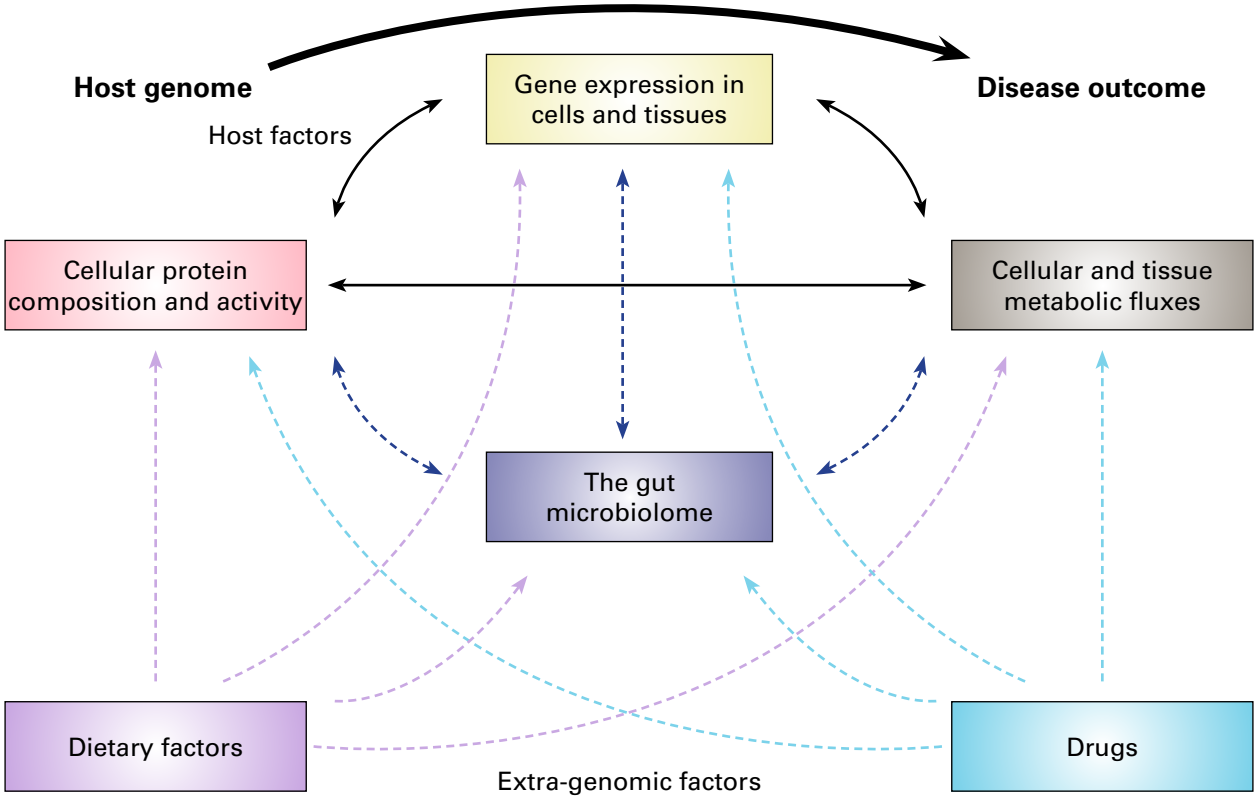

Figure 2. Urinary metabolites and gut microbiota conventionalisation in rats. Pattern recognition analysis (principal components (PC) analysis) and partial NMR spectroscopic data from gnotobiotic (germ-free) sequential rat urine samples showing corresponding diagnostic regions of the NMR spectra indicating dominant microbiotal metabolites for time point T1 & T5. It takes at least 3 weeks for complete conventionalization – T5 closely resembles the profile of a normal control adult Sprague–Dawley rat[30].

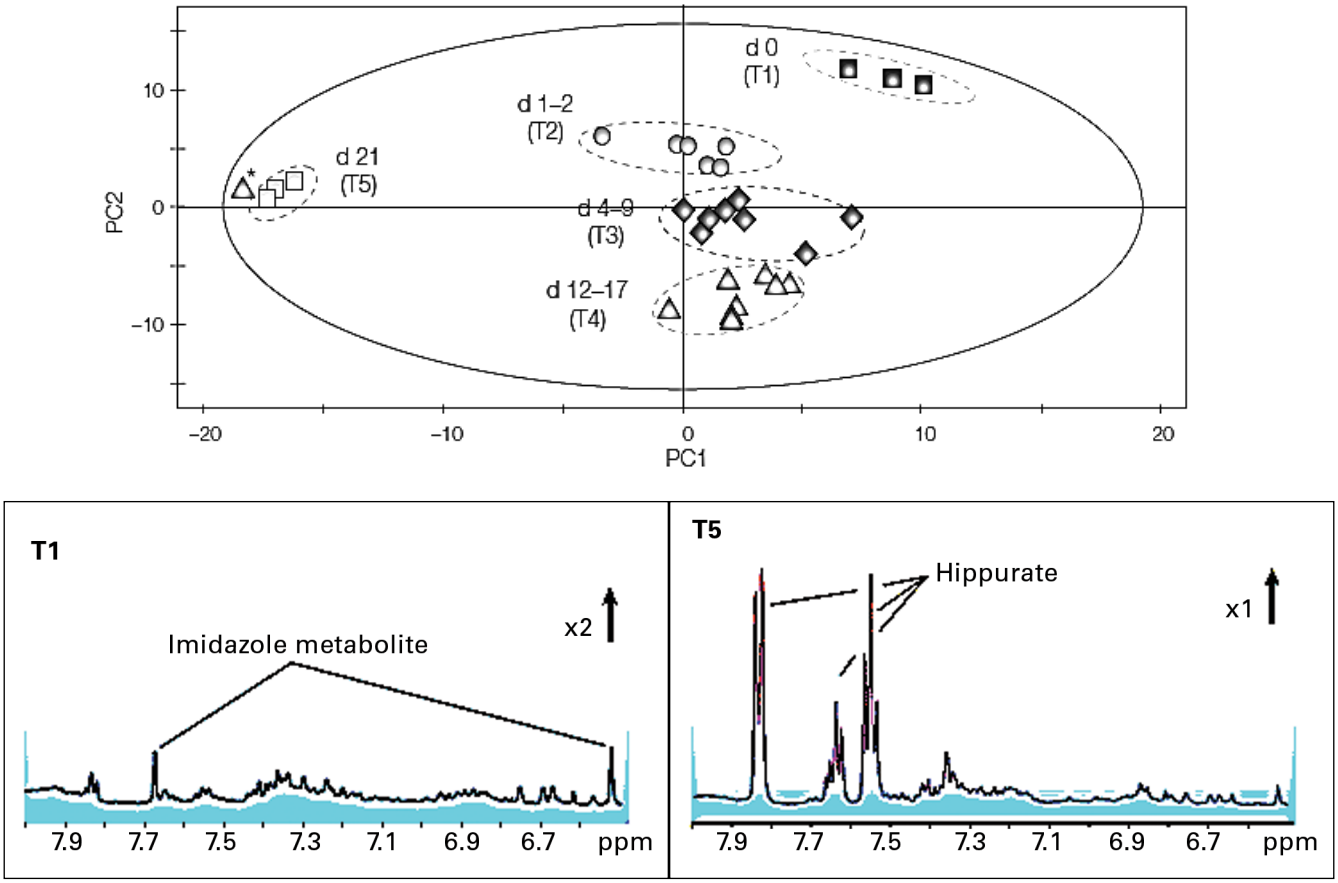

Figure 3. Integrals of selected peaks from hippurate, creatine and phenylacetylglycine from total area-normalised urinary NMR spectra at 8 time points in a vancomycin treatment mice model, demonstrating the variation in the concentrations of metabolites with the progression of time after antibiotic treatment. Error bars represent the standard error[25].

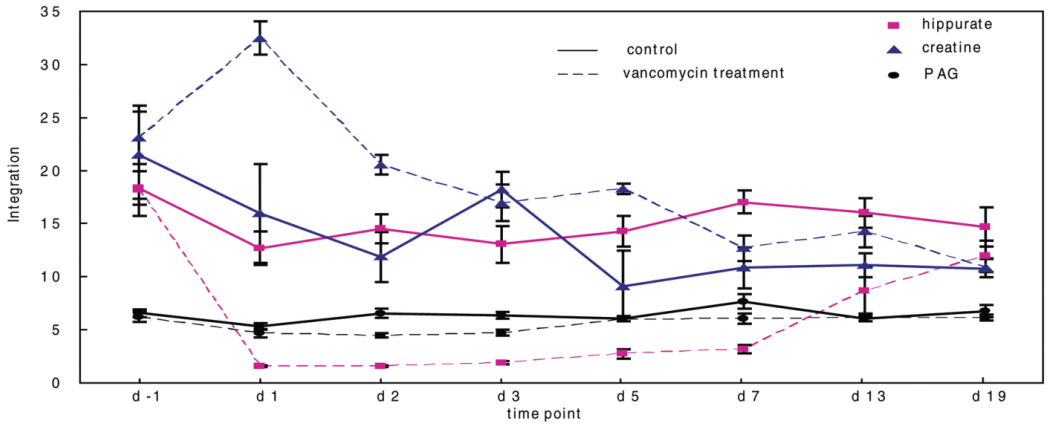

Figure 4. (A) Cross-validated orthogonal-partial least squares-discriminant analysis scores plot derived from urinary NMR spectra of northern and southern Chinese population samples, based on the first urine collection. (B) Covariance plot showing color-coded significance of urinary metabolite differences between northern and southern Chinese populations, based on the first urine collection. Mean north-south differences in peak intensity for 7100 spectral variables were assessed for statistical significance using family wise error rate < 0.01, corresponding to $P < 4 \times 10^{-6}$ for group mean north-south differences by Student's *t* test, for the two urine collections considered separately[51].

1, Pentanoic/heptanoic acid; 2, Branched-chain amino acids; 4, Lactate; 5, 2-hydroxyisobutyrate; 6, Alanine; 8, *N*-acetyls of glycoprotein fragments (including uromodulin); 9, *N*-acetyl neuraminic acid; 11, 4-cresyl sulphate; 12, Succinate; 16, Methylguanidine; 18, Dimethylglycine; 19, Creatine; 21, Prolinebetaine; 23, *Scyllo*-inositol; 10, Phenylacetylglutamine; 26, Hippurate; 28, *Trans*-aconitate.