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Overview

An Introduction to Metabolomics and its Potential Application in Veterinary Science

Oliver AH Jones[1,*] and Victoria L Cheung[2]

Metabolomics has been found to be applicable to a wide range of fields, including the study of gene function, toxicology, plant sciences, environmental analysis, clinical diagnostics, nutrition, and the discrimination of organism genotypes. This approach combines high-throughput sample analysis with computer-assisted multivariate pattern-recognition techniques. It is increasingly being deployed in toxico- and pharmacokinetic studies in the pharmaceutical industry, especially during the safety assessment of candidate drugs in human medicine. However, despite the potential of this technique to reduce both costs and the numbers of animals used for research, examples of the application of metabolomics in veterinary research are, thus far, rare. Here we give an introduction to metabolomics and discuss its potential in the field of veterinary science.

Abbreviations: GC-MS, gas chromatography–mass spectrometry; LC-MS, liquid chromatography–mass spectrometry; NMR, nuclear magnetic resonance; PC, principal component; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis

Metabolomics can be defined as the analysis of thousands of the small molecules (metabolites)—such as sugars, organic acids, amino acids, and nucleotides—that are the products of cellular metabolism. The full metabolite complement (metabolome) of a cell, tissue, or organism can be used to get an idea of the underlying biochemistry. As in related techniques, such as genomics and proteomics, the metabolome is context-dependent and will change in response to external factors, such as disease or exposure to a toxin. The advantages of studying metabolites include cost-effectiveness and rapid measurements. These features enable large numbers of samples to be processed quickly, thereby providing a high-throughput analytical tool. In addition, unlike many genes and proteins, metabolites are conserved across species, and so the detection methods and equipment used in one organism can easily be applied to another without the need for recalibration. This versatility means that metabolomics-based approaches can be applied to studies in a wide variety of disciplines, including (but not limited to) drug toxicity and gene function,[38] nutrition,[61] microbiology,[6] cancer research,[11] pharmacology,[37] plant sciences,[49] and environmental studies.[59] A summary of the workflow for standard metabolomics based studies is shown in Figure 1.

The concept of metabolic analysis is not new, but metabolomics as a distinct field dates from around the mid-1990s.[47] One aspect of the technique which distinguishes it from previous metabolically based studies is the attempt to measure all metabolites simultaneously, often termed the 'global approach.' When coupled with pattern recognition techniques, metabolomics makes a powerful investigative tool, with great potential for studying the biochemical effects of disease, as well as a screening method for potential pharmacological agents in a wide range of species. Indeed, metabolomics has proven to be highly sensitive for this type of analysis, since metabolic perturbations often present much earlier than either tissue accumulation of toxins or induced histopathologic changes.[22]

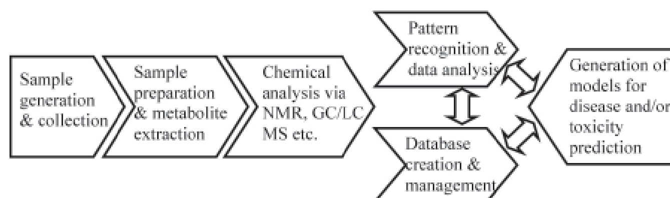

Figure 1. Diagram of the workflow for a standard metabolomics-based study (after reference 18).

Somewhat confusingly, several terms have been coined to describe the process of combining global analytical tools and pattern recognition analysis to define the metabolic status of a tissue or organism. Although 'metabolomics' is probably the most common, the terms 'metabonomics,' 'metabolic fingerprinting,' 'metabolic footprinting,' and 'metabolic profiling' are all in common usage. They often are used interchangeably, despite subtle differences in their definitions, thus making terminology complicated (and slightly perplexing) even for those working in the field. Throughout this paper, the term 'metabolomics' will be used and taken to mean the combination of analytical tools with pattern recognition processes used to define a metabolic phenotype (metabotype) by means of a global approach.[39] This definition places the technique alongside those such as genomics and proteomics, which represent the complete genetic profile and the complete protein expression in a cell, tissue, organ or individual organism respectively. This paper will focus on the different analytical methods available

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[1]The Hopkins Building, Department of Biochemistry, University of Cambridge, Cambridge, UK; [2]Veterinary Medicine Research and Development, Pfizer Animal Health, Sandwich, Kent, UK.
[*]Corresponding author. Email: oahj2@mole.bio.cam.ac.uk

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to study metabolites and how the metabolomic approach could be used in veterinary science, especially its potential for drug discovery and development.

Analytic methodology. A major challenge in metabolomics is to address the extremely diverse and complex nature of the subject matter.[12] Metabolites may range in concentration to the order of approximately 10^9, have mass ranges of the order of approximately 1500 amu, and have polarity ranges of approximately 10^{20}.[1] Unsurprisingly therefore, no single analytical approach can provide universal coverage of the metabolome, and multiple analytical techniques and sample preparation strategies are necessary for complete analysis. A brief outline of some of the more prevalent technologies is given below.

NMR. The primary analytical technique for metabolomic studies has for many years been nuclear magnetic resonance (NMR) spectroscopy. NMR works by the application of strong magnetic fields and radio-frequency pulses to the nuclei of atoms, exploiting a quantum mechanical property of nuclei known as spin. The most commonly used nuclei are those with a spin number of ½, including the proton (^{1}H) and ^{31}P, ^{13}C, and ^{19}F. Absorption of radio-frequency energy allows these nuclei to be promoted from a low- to high-energy state; the radio-frequency radiation emitted as they relax back to the low-energy state can then be detected. A detailed explanation of the principles and practices of the technique is given in Keeler.[30] NMR has a number of advantages in that it requires minimal sample preparation (although urine samples must usually be buffered) and is a highly reproducible, robust, nondestructive technique that allows simultaneous measurement of many kinds of small-molecule metabolites. Recent improvements in NMR probes also enable analysis of very small sample volumes, for example, mouse cerebral spinal fluid, which may be present in quantities as low as approximately 1 to 3 µl.[20]

Other NMR-based approaches allow the quantification of metabolite concentrations in intact tissue, either in vivo or ex vivo. For example, magnetic resonance spectroscopy (MRS) allows noninvasive assessment of metabolite concentrations directly in vivo within a specific localized region; this technique has previously led to the generation of proposed mechanisms of disease pathology.[23] MRS can be complicated by inherently low spectral resolution and the presence of broad resonances from rapidly relaxing molecules present in vivo (especially lipids). These effects can be reduced dramatically by spinning the sample at the so-called 'magic angle' (54.7°). Indeed, using high-resolution magic-angle–spinning NMR, it is possible to produce spectra from intact tissue ex vivo that are comparable to those from tissue extracts and to investigate metabolic compartmentalization.[3]

Although capable of detecting all of the high concentration metabolites in a solution, NMR-based approaches have 1 major disadvantage: they lack the sensitivity offered by mass spectrometry (MS)-based techniques. Strategies are currently being developed to improve the sensitivity of NMR spectrometers, including the use of stronger magnetic fields and cryoprobes (which can increase the signal-to-noise ratio 3- to 4-fold by reducing thermal noise) and through the introduction of combined techniques, such as liquid chromatography (LC)–NMR. The LC-NMR approach involves using LC to separate high- and low-concentration metabolites before NMR analysis. Sensitivity is thus improved by reducing the likelihood of coresonant peaks as well as by allowing the dynamic range of the spectrometer to be tuned for each LC peak. The spectra of low-concentration metabolites can therefore be collected within an appropriate dynamic range, preventing low-concentration signals from becoming lost in baseline noise. One of the newest products in the field of combined NMR techniques is that of capillary LC-NMR. Here the high spectral resolution of NMR is maintained while its sensitivity is improved by increasing the sample concentration through reducing the volume of solvent or buffer used in the NMR sample.[24] Nevertheless, it is unlikely that NMR will offer better sensitivity than MS in the near future.

Gas chromatography–mass spectrometry. Gas chromatography–mass spectrometry (GC-MS) is a combined analytical system. Compounds are first separated by GC, with eluting compounds then being detected via MS, traditionally by using electron-impact ionization. GC-MS is increasingly being used for metabolomic investigations and has proven to be a very useful technique. For instance, low-molecular–weight metabolites in a gaseous phase (for example, those in breath) can be sampled and analyzed directly.[10]

The main advantages of GC-MS are a) increased sensitivity and b) the fact that compound identification is greatly facilitated by the availability of extensive, easily searchable databases of molecular fragmentation patterns.[56] It has also proven to be a very robust and reproducible metabolomic technique. Some modern instruments also allow 'two-dimensional' chromatography.[51] Here, a short polar column is used in combination with the main analytical column, increasing the resolution of the device. The use of time of flight- and ion-trap mass spectrometers has also increased sensitivity compared with that of quadrupole-based instruments.[19] For these reasons GC-MS is often seen as the 'gold standard' in metabolomics.

A disadvantage of GC-MS is that it is only useful for volatile, thermally stable compounds or those that can be rendered as such by chemical derivatization,[35,56] which markedly increases the processing and analysis time per sample. In addition, the similarity of molecular fragmentation patterns from structural isomers (such as those of sugar diastereomers) can make compound identification difficult. In such cases, accurate assignment must be resolved by using retention time indices of standards (such as those available at the Max Planck Institute of Molecular Plant Physiology online database [http://www.mpimp-golm.mpg.de/mms-library/]) in conjunction with spectral analysis.

Liquid chromatography–mass spectrometry. LC-MS is another combined system, similar in principle to GC-MS but using a liquid mobile phase rather than gas. This technique is still at an early stage in terms of use for metabolomic experiments but shows great promise in drug development studies.[44] Like GC-MS, LC-MS is more sensitive than NMR and has the added advantage that, because the need for sample volatility is eliminated, high analysis temperatures and derivatization reactions are unnecessary (although derivatization can help to improve chromatographic resolution).[13] This attribute makes LC-MS a potentially universal technique. Recent innovations such as ultra-performance liquid chromatography have also shortened chromatographic run times considerably.

The disadvantages of LC-MS include the fact that several different ionization techniques are in use and often these differ from those used for GC-MS. Typically electrospray (rather than electron impact) ionization is used, but several other ionization techniques such as atmospheric pressure chemical ionization and atmospheric pressure photo ionization, are also in common use. As a result, spectral libraries are not so readily available as with

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electron impact ionization and fragmentation patterns between instruments and laboratories can therefore vary significantly. Additional problems include ion suppression (matrix effects) and poor reproducibility of results. These issues make LC-MS–based metabolomics particularly demanding, although there is considerable potential for targeted analysis, especially for large or very polar compounds, such as many hormones and lipids, which are not easily analyzed by GC-MS.

Alternative methods. Although most metabolic studies use one or more of the above techniques, specific platforms are not a prerequisite for metabolomic analysis. In fact, any technique capable of generating comprehensive metabolite measurements can potentially be used.[45] Other analytical methods that have been used include fourier transform infrared spectroscopy,[27] fourier transform ion cyclotron resonance mass spectrometry, sometimes known as fourier transform mass spectrometry,[4] raman spectroscopy,[14] high-performance liquid chromatography, either on its own[42] or in conjunction with coulometric array detectors,[34] thin-layer chromatography,[36] capillary electrophoresis–mass spectrometry,[52] and direct-injection mass spectrometry.[29]

Pattern recognition and data analysis. The analysis of a large number of biologic samples by any technique will usually produce an equally large number of extremely complex datasets, often with considerable overlap of analytes. This type of data consists of the measurements of a range of metabolites (variables) for a number of individuals (observations). The identification and quantification of changes in metabolite concentration across such large and complex datasets often proves very difficult.

Standard univariate techniques (such as the Student *t* test) do not consider multiple colinearities within the dataset, where the variation in 1 variable is related to the variation of 1 or more covariables. In addition, it is usually impractical to assess the metabolic effect of stressors by univariate methods due to the volume of data produced and the richness of spectral information. Although metabolomic data may involve hundreds, if not thousands, of variables, hundreds of independent events are certainly not occurring in the biologic system under test.[40] Important information is therefore more likely to be found within the patterns of correlation between variables as opposed to within individual signals. By measuring the changes that occur (or do not occur) across many compounds and metabolic pathways, a much richer picture of the overall effects of a disease- or drug-related perturbation on the metabolic network is obtained than if the concentrations of only 1 or 2 directly affected compounds are measured. In such metabolomic analyses, the use of multivariate statistics coupled with sophisticated pattern-recognition techniques have proven to be of value, since they consider all of the variables in a dataset simultaneously. A basic tenet of these techniques is to calculate a smaller number of latent variables. Latent variables are linear combinations of correlated variables. They account for the same amount of variation present in the larger dataset while reducing dimensionality and minimizing loss of information. Popular software used for this type of analysis includes SIMCA-P, (Umetrics, Umeå, Sweden), Pirouette (InfoMetrix, Bothell, WA), Matlab (The MathWorks, Natick, MA), and R (freeware, http://www.r-project.org/).

The primary multivariate statistical techniques used in metabolomics can be described as either unsupervised or supervised. Unsupervised techniques, such as principle component analysis (PCA), form the basis for multivariate data analysis. They model the intrinsic variation within the dataset and are defined as unsupervised techniques because they do not take class membership in account. The starting point for these analyses is a data matrix with *N* rows (observations) and *K* columns (variables). PCA transforms the variables in the dataset into a smaller number of new latent variables called principal component (PCs). These PCs are combinations of the initial variables but highlight the variance within the dataset and remove redundancies. The components are orthogonal (uncorrelated) to each other and are calculated in order of decreasing contribution to the total variance of the original dataset. Most of the information in the dataset is described by the first few PCs, thus the reduction in the dimensionality of the data. Observations are assigned scores according to their projection in principal component space. When displayed graphically on a scores plot, samples with similar scores, and therefore similarly correlated metabolic changes, will cluster together—away from groups with different scores[15] (see Figure 2 for an example of a scores plot).

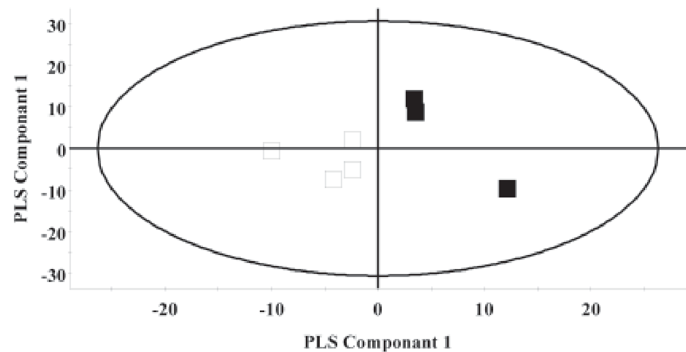

Figure 2. An example of a partial least squares–discrimination analysis (PLS-DA) scores plot based on NMR analysis of liver tissue from rats exposed to 1.572 ± 0.132 (µg/g BW/d) of cadmium(II) chloride for 94 d (open squares) and controls (solid squares) in an ecotoxicology study (unpublished data). The ellipse was calculated by using the Hotelling T^2 test, a multivariate generalization of the Student *t* test that checks the multivariate normality of the dataset. The area within the ellipse corresponds to the 95% confidence region of the model.

To understand what each PC represents in relation to the original measurements, the loading or coefficient lists for the original PCA model (which can also be displayed graphically) must be examined. They describe the magnitude and direction of the contribution each variable makes to a PC; a large value for a metabolite indicates that changes in concentration of that metabolite contribute strongly to the variation along that PC, whereas a small value means that a metabolite has little or nothing to do with variation along that PC.

Supervised techniques, such as partial least squares–discrimination analysis (PLS-DA), use prior knowledge of class membership (for example, control and diseased animals) or regression trends to maximize separation between groups or correlate data matrices by searching for changes in variables which are correlated with class membership. Since supervised techniques specifically identify variation associated with group membership, they can be used to examine class separation, which would otherwise be spread across 3 or more PCs. However, care must be taken to ensure data is not overfitted. Supervised techniques therefore require some form of crossvalidation to ensure the statistical validity of results. This verification usually is achieved by dividing the dataset into a training set (samples used to build the model) and a

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test set (samples not used to build the model). The model is built using just the training set and used to predict either class membership (for example, presence of absence of a drug) or the trend variable (for example, species type) for the test set. By repeating this process a number of times using different subsets of the dataset for the test and training sets, an indication of the robustness of the models built using the dataset can be obtained.[15]

With GC- and LC-MS data, multivariate techniques are applied to a collection of integrated values, with each value corresponding to a chromatographic peak representing a metabolite. In contrast, because each metabolite can give rise to multiple peaks in an NMR spectrum (and due to the considerable signal overlap of peaks typically observed), some form of data reduction prior to analysis frequently is applied. A common method for data reduction in metabolomic studies is to split the spectra into integral regions, known as buckets or bins, and then to use these integral buckets for statistical analysis. Buckets may be of any size but are usually set to a default width of 0.04 ppm.[57] Alternative techniques such as intelligent bucketing can also be used to set bucket sizes for groups of spectra.[26] All signals in each bucket are summed, and each variable then corresponds to the total signal intensity observed within each individual bucket. At this stage, normalization of the data (for example, to the total signal intensity or to a specific metabolite or standard) is used to account for any differences in the mass of tissue or biofluid present between samples. This approach has several advantages, not least of which is the ability to analyze spectra whose integration may present difficulties due to resonance overlap and line-fitting difficulties and to potentially reveal the presence of compounds otherwise masked through resonance overlap.[8]

Much information may still be lost even in a rigorous statistical analysis. For this reason, multivariate statistics used in conjunction with targeted univariate methods are most likely to yield the best and most complete results. In addition, computer-based, supervised schemes have recently been developed for application in metabolomic studies. These include techniques such as neural networks and genetic algorithms, which use machine-learning–based methods for the classification of samples.[17,31]

The potential of metabolomics in veterinary science. Metabolomics enables noninvasive systems assessment of untoward effects induced by candidate compounds, thereby characterizing a broad spectrum of biologic responses on an individual-animal basis in a relatively rapid-throughput fashion. This characteristic makes it an ideal addition to early preclinical safety assessment.[46] It is a rapidly developing tool in the human health drug discovery field and could potentially impact all stages of veterinary drug discovery and development.

Veterinary drug discovery. Endogenous metabolites are the end products of gene expression and are the main effectors of cellular signaling in response to changes in a biochemical pathway, whether due to disease or a toxic xenobiotic. This theoretically means that biochemical pathways can be traced back to identify a target (or targets) of a specific disease. This in turn could potentially lead to the identification of more druggable endpoints for future studies.

Metabolomics can also be used to identify specific biomarkers, or perhaps more usefully, biomarker profiles of disease.[21] This application may be especially useful in the development of veterinary medicines, because the drug candidate goes into the target animal at a much earlier stage than in equivalent human studies (earlier drug testing in target animals and culling of animals are the main differences between human health and veterinary medicine). Research animals must be killed if their organs are to be evaluated to assess drug efficacy or residue levels. This is where a noninvasive technique such as metabolomics has the greatest potential. Ethically, reducing the need to euthanize animals during the course of drug discovery is an important factor contributing to the implementation of 'the 3 Rs' (replacement, reduction, and refinement) in line with current European Union policy on animal testing.[25]

Toxicology and pharmacokinetics. The financial cost of developing any new therapeutic agent is considerable. An effective early screen of toxicity is therefore extremely desirable because the earlier that a toxic molecule or molecular class can be removed from the developmental pipeline, the lower the economic cost.[38] Metabolomics offers the potential for identifying and assessing toxic effects during the early stages of drug development, thus reducing costs. For this reason it is used widely by pharmaceutical companies involved in the development of human (but rarely veterinary) medicines.

A metabolomics-based approach is attractive to toxicologists because it measures the responses of the whole system, unlike classic assays which often focus on selected organs. Additionally, samples for metabolomic analysis can easily be incorporated into many types of traditional toxicology studies and the results integrated with routinely measured end points.[32] Furthermore, because it aims to identify metabolic changes through the measurement of all metabolites, rather than just those thought to be of interest, metabolomics (in common with other 'omics'-based approaches) can be used to generate hypotheses. This attribute makes metabolomics useful for studying systems in which the mechanism of toxicity is unknown. For interested readers, Robertson[45] has reviewed the use of metabolomics in toxicology in more detail.

Noninvasive analysis of body fluids may be carried out to enable monitoring of both efficacy and toxicity of a given compound or group of compounds. Plasma and urine are the main body fluids used. Of these, urine is less invasive to collect and, if using metabolism cages, samples can be taken while the animal is left unattended, thus removing the need for off-hour sample collections. In fact, providing they are not contaminated (for example, with bacteria), most biological fluids are suitable for metabolomic investigations, including whole blood, milk, and saliva.

Using metabolomics it is possible to track how the metabolism in a cell, tissue, or body fluid is perturbed after administration of a test compound, as well as how it returns to normal after treatment has ceased. A useful example of such a study is given by Robosky and colleagues,[47] who looked at male rats (*Rattus norvegicus,* Wistar strain) exposed to carbon tetrachloride, a well-known hepatotoxin. The results of NMR-based metabolomics were compared with traditional clinical chemistry analysis, and both sets of results were found to correlate remarkably well. The work is one of many which demonstrate that metabolic measurements provide a reliable endpoint for assessing toxicity and that they can be potentially included in standard screening programs.

Studying metabolite levels may also be useful in pharmacokinetic studies since, in addition to looking for toxic effects or biomarkers of toxicity, it is also theoretically feasible to use the same analytical techniques to follow a particular substance and its breakdown products over a period of time to ascertain how they accumulate within, and are eliminated from, tissues and organs after dosing.

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Clinical applications. Examples of veterinary-based studies in the metabolomics literature are, so far, quite rare. The only directly related work is a general overview of functional genomics and systems biology and their relevance to veterinary science[63] and a study by Whitfield and colleagues who used metabolomics to distinguish canine congenital portosystemic vascular anomalies from acquired hepatopathies.[62] In that study, plasma samples were collected from 25 dogs (comprising 9 with congenital portosystemic vascular anomalies, 6 with acquired hepatopathy, and 10 with nonhepatic disorders) and analyzed by use of LC-MS. Multivariate analysis of the results produced clear segregation among all 3 study groups. Disturbances were identified in the plasma, bile acid, and phospholipid profiles of dogs with portovascular anomalies.[62] The study demonstrates the potential of metabolomics as a diagnostic tool for naturally occurring hepatic disease in dogs and, potentially, other animals.

It may also be possible to develop biomarkers or metabolic profiles to detect the effects of illegally administered drugs and/or growth promoters (doping) even after the substance itself is no longer present.[63]

Related work has involved the analysis of cadmium toxicity in rodents,[22] the study of liver tumors in wild flatfish,[55] the assessment of sublethal stress in aquatic organisms,[58] as well as the extensive use of rodent models in the study of human disease.[19,28] For instance, a study by Pears and colleagues used metabolomics to demonstrate that there was a neurotransmitter cycling deficit in cerebral tissue from a mouse model of batten disease (a progressive neurologic disorder primarily affecting children).[41] In additon, a combined ^{1}H NMR spectroscopy and mass spectrometry-based approach was used in a metabolomic study of the PPAR-α null mutant mouse as a model for the metabolic syndrome in humans.[1]

Potential drawbacks. There are limits in using metabolites as markers for toxicity and disease. Not least is that their concentrations are often heavily influenced by a variety of other factors such as age,[50] diurnal and estrus cycles,[2,43] gender,[43,53] parasite load,[60] as well as the species and strain background of the animal under study.[16,43] Strain is of particular importance in veterinary medicine because individual strains are often inbred, and variability among specific strains of animals may be substantial. Diet is another major factor affecting metabolic phenotypes,[54] and even environmental stressors such as temperature can play an important role.[48]

The ability of metabolomic studies to reveal meaningful differences between samples is useful only if the observed differences originate from sample characteristics of interest, rather than from unknown or uncontrollable effects.[9] The influence of external factors potentially can be significant, and therefore including adequate control groups is crucial to the study design. For example, Brindle and colleagues previously reported that ^{1}H NMR analysis of human blood sera could rapidly and noninvasively predict angiographically defined advanced coronary heart disease with >90% accuracy and specificity.[5] However, a later study demonstrated that once potential confounding factors (such as gender and drug treatment) were taken into account, accurate predictions between patients with heart disease and those without were only 61.3% for men treated with statin drugs and 80.3% for men who were not treated.[33] Compared with a random correct prediction rate of 50%, the detection rate of heart disease by use of metabolomics was thus demonstrated to be poor when compared with angiography.

Metabolomics also suffers from the phenomenon of 'the usual suspects.' This refers to the fact that the levels of a very similar group of metabolites have been found to be altered in a wide range of unrelated studies.[45] It is theorized that this effect may be due to the fact that many toxicants, and indeed diseases, often affect central hubs of metabolism, such as energy generation.[40] Perturbation at such hubs may then quickly be transferred to other biochemical pathways. However, because the metabolites in the hubs affect many different pathways, it is they that are often found to be most important in the resultant analysis.

Although this effect is not considered to be an artifact of the analytical or data-processing methods used, it often masks the less obvious effects of a biologic perturbation. For example, Connor and colleagues demonstrated that many of the usual suspects often cited as biomarkers of liver and kidney toxicity could in fact be explained by diet or food restriction (which are themselves frequent indicators of toxicity), with resultant weight loss.[7] Therefore they may in fact be markers of general stress, rather than of a specific effect. To avoid this problem, many pharmaceutical companies now use weight- (in addition to sex-, strain-, and age-) matched control animals in drug development studies. These controls are 'pair-fed' with treated animals so that they achieve a similar weight change. Although this practice increases the numbers of animals used in the study (with associated costs and ethical considerations), it enables the changes in metabolic profiles of each group to be compared and enables the effects of weight loss to be separated out from any underlying toxicity.

It is also important to bear in mind that, since metabolomics is a static technique, it cannot generate data on dynamic processes, such as the flux rates of specific metabolic pathways. This problem is common to many -omic sciences but can potentially be addressed by the use of isotope labeling studies.

In conclusion, metabolomics is a rapidly developing technique that already has made great impacts in a wide range of fields, including many of interest to the veterinary scientist. This approach has potential drawbacks which are important to consider (such as its sensitivity to external influences). However, if studies are carefully designed and monitored and the data interpreted with care, metabolomics shows considerable potential in veterinary pharmacology, toxicology, and clinical diagnostics and therapeutics. Metabolomics also shows great promise in significantly reducing the number of animals used for drug discovery and development and related studies.

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