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Commentary

Metabolomics and its potential in drug development

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ABSTRACT

Metabolomics is the global and unbiased survey of the complement of small molecules (say, <1 kDa) in a biofluid, tissue, organ or organism and measures the end-products of the cellular metabolism of both endogenous and exogenous substrates. Many drug candidates fail during Phase II and III clinical trials at an enormous cost to the pharmaceutical industry in terms of both time lost and of financial resources. The constantly evolving model of drug development now dictates that biomarkers should be employed in preclinical development for the early detection of likely-to-fail candidates. Biomarkers may also be useful in the preselection of patients and through the subclassification of diseases in clinical drug development. Here we show with examples how metabolomics can assist in the preclinical development phases of discovery, pharmacology, toxicology, and ADME. Although not yet established as a clinical trial patient prescreening procedure, metabolomics shows considerable promise in this regard. We can be certain that metabolomics will join genomics and transcriptomics in lubricating the wheels of clinical drug development in the near future.

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1. Metabolomics

1.1. What is metabolomics?

There is no universal and agreed wording regarding a definition of metabolomics. A host of scientific articles and reviews each defines the word differently. This is perhaps not too surprising for a field that is less than 15 years old. For the purposes of this commentary, metabolomics is defined as the global and unbiased survey of the complement of small molecules (<1 kDa) in a biofluid, tissue, organ or organism. This is essentially a definition that has been used before [1,2] and distinguishes the use of the word metabolomics from that of metabonomics [3] and small molecule or metabolite profiling [4,5]. However, all these terms are essentially describe the same process and distinction between them is largely semantic. The word "metabolomics" is finding growing favor, almost certainly because of its snug fit into the other principal omics fields of genomics, transcriptomics, and proteomics. But, as has been pointed out, metabonomics has a more precise etymology [6].

What matters is whether or not the search for metabolites is targeted. The screening of plasmas from diseased patients and controls for their pattern of amino acids, for example, is not metabolomics, because the screen is restricted and not global. Here, metabolite profiling would be a more correct description, although it must be said that many authors would describe such work as metabolomics. An easy to remember maxim for metabolomics comes from the movie *Forrest Gump*, when Forrest says, "Mama always said life was like a box of chocolates. You never know what you're gonna get." Anyone familiar with the execution of mass spectrometry (MS)-based metabolomic studies will understand this. Nuclear magnetic resonance (NMR)-based studies usually produce findings within the confines of about a dozen relatively high concentration "usual suspects", including 2-oxoglutarate, acetate, citrate, lactate, succinate, and glucose, particularly in studies of urine [6]. It would be appropriate if the term "metabolomics" was restricted to untargeted metabolite surveys that are unrestricted by the usual suspects.

1.2. What is required to perform a metabolomic study?

Metabolomics is a toolbox for examining differences in metabolite concentrations between different sets of biological

Abbreviations: MS, mass spectrometry; UPLC, ultraperformance liquid chromatography; UPLC–ESI-QTOFMS, UPLC–electrospray ionization-quadrupole time-of-flight MS; LCMS, liquid chromatography–mass spectrometry; GCMS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; ESI+, electrospray ionization in positive ion mode; ESI−, electrospray ionization in negative ion mode; MDA, multivariate data analysis; PCA, principal components analysis; PC, principal component; PLS-DA, projection to latent structures-discriminant analysis; OPLS-DA, orthogonal PLS-DA; NME, new molecular entity; POC, proof of concept; ADME, absorption, distribution, metabolism, and excretion; IVA, influenza A virus; HSV-1, Herpes simplex virus type-1; HCMV, human cytomegalovirus; PPARα, peroxisome proliferator-activated receptor alpha; PXR, pregnane X receptor; PCN, pregnenolone 16α-carbonitrile; CCl_4, carbon tetrachloride; ANIT, α-naphthylisothiocyanate; SOM, site of metabolism.

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samples. To accomplish this, an investigator must first have access to a high-resolution, high-throughput analytical platform capable of resolving and quantitating hundreds, if not thousands, of small biological molecules. In addition, the researcher would need access to appropriate biological materials, for example, urines from gene knockout and wild-type mice [7], urines from animals dosed with a drug and their sham-dosed controls [1], urines from animals subjected to a stress, such as ionizing radiation exposure, and their sham-exposed controls [8,9], plasmas from cancer patients and controls [2] or urines from human volunteers administered a drug, including pre-dose control samples [10,11]. In all of these aforementioned studies, biological samples were analyzed using ultraperformance liquid chromatography–electrospray ionization-quadrupole time-of-flight mass spectrometry (UPLC–ESI-QTOFMS). Other metabolomics investigators have utilized alternative MS platforms, such as electrostatic axially harmonic orbital trapping (orbitrap-MS) [12], Fourier transform ion cyclotron resonance MS [13], and tandem quadrupole MS [14], each of which is perceived as having its own advantages. In addition to liquid chromatography–mass spectrometry (LCMS) platforms, one of the commonest instruments used in metabolomics has been the gas chromatograph–mass spectrometer (GCMS) [15–17], including the introduction of gas chromatography–time-of-flight mass spectrometry with fast scanning into plant metabolomics by Fiehn and colleagues a decade ago [18].

The early literature was dominated by work using either GCMS (calling itself "metabolomics") or NMR (calling itself "metabonomics"), although the aims of these two branches were in effect the same. The analysis using NMR spectroscopy of biological fluids such as rat urine was pioneered by Nicholson. Administering model toxins to rats, Nicholson's group used NMR to report the appearance of urinary biomarkers that mapped to specific organ toxicities. Here, metabonomics was born [3].

How samples are prepared for metabolomic analysis may be of critical importance to the outcome of a metabolomic experiment. First, it should be considered whether plasma or serum should be acquired in a metabolomic experiment. For 1H NMR studies, which report a relatively small number of analytes (see above), serum and plasma give similar results [19]. However, clear advantages of plasma over serum have been reported using both GC–TOFMS- and UPLC–ESI-QTOFMS-based metabolite profiling in small cell lung cancer patients [20]. It has also been reported that peak shape can be improved by the addition of trace amounts of the metal chelating agent EDTA to a variety of biological samples [21].

How data are acquired using a UPLC–ESI-QTOFMS is of critical importance to the outcome of a metabolomic experiment. It was recently reported that the number of data points collected per peak and sufficient dilution of samples, in the case of urines, significantly increased the peak signal-to-noise and reduced the number of missing values that can weaken subsequent statistical analyses [22]. In the case of UPLC–ESI-QTOFMS, the output of the MS instrument typically comprises 1000–5000 mass/charge ratio (m/z) and retention time (RT) pairs that essentially constitute at least one thousand single-ion chromatograms, each with m/z values to four decimal places, with ion abundances in each case. These ions with unique m/z and RT values are referred to as "features". The number of features can be doubled if samples are injected in both positive (ESI+) and negative (ESI−) electrospray ionization modes. If the experiment calls for 100 samples to be injected, for example, the total data output may comprise a data matrix comprising millions of data. Many stages of data manipulation and analysis are needed to tease out the metabolomic differences between sample sets. The next requirement for the metabolomics investigator involves data handling, storage, and analysis.

For all analytical platforms, it is essential that the raw data be preprocessed with peak detection, alignment, and area extraction algorithms. There are several software packages that can do this for chromatographic data, including XCMS [23], centWave [24], MZmine [25], and MarkerLynx [2,9,26]. Further information on both open-source and commercial programs is available [27,28]. Recently, it has been recognized that variance stabilizing transformation and normalization are critical preprocessing steps that greatly enhance recovery of metabolite data [28]. Multivariate data analysis (MDA) can then be applied to extract metabolite information. In the NMR metabonomics field, it was recognized early on that computer-based MDA was required to extract biomarkers from metabonomic data sets and principal components analysis (PCA) became a tool of first choice [29,30]. The same situation obtained in the GCMS metabolomics field [15]. PCA is a mathematical procedure that reduces the dimensionality of a dataset, in a way like light shone onto a 3D object produces a 2D shadow on a screen. This lower-dimensional view of the data is then used to generate the principal components (PC) of the variance within the dataset. The first principal component (PC1) represents the largest component of the variance, PC2, the second largest, *etc.* PCA is particularly helpful in permitting insight into the internal structure of the dataset and also for identifying outliers. PCA has been employed in medical research for over four decades, for example in the evaluation of drug efficacy [31].

Fig. 1A shows a typical so-called PCA scores plot in an experiment where mice were administered a drug and 0–24 h urine was collected. A control group of sham dosed animals was used where only the vehicle was administered, as in several published studies [1,32]. The differential concentration of chemical entities in the dosed and sham groups gives rise to differential patterns of ions derived from the UPLC–ESI-QTOFMS analysis. The sham urines cluster together and separate from the cluster of drug treated urines. The only exception is for a single outlier from the drug treated group, which falls outside the Hotelling T2 ellipse that represents a 95% confidence interval (Fig. 1A). Note that the separation of drug treated and control urines occurs in the first principal component of the variance, indicating that this is the major difference between the sample groups. However, within each cluster it can be seen that there is considerable variation, mostly in PC2. Natural variation in urine composition plus individual variation in metabolite formation and excretion contribute to the intraphenotype variation in PC1 and PC2. These PCA were conducted using the software package SIMCA-P+ (Umetrics Inc.) and are so-called unsupervised analyses, meaning that no class information is used in the analysis. When the class of sample is considered, in this case, drug treated and sham, the test is known as supervised. There are many supervised multivariate data analyses, but one commonly used test is projection to latent structures-discriminant analysis (PLS-DA), also called partial least squares-discriminant analysis [33]. Fig. 1B shows a so-called loadings plot from a PLS-DA analysis, where the weights (or loadings) of the first two principal components are plotted against each other. This then shows the ions that are responsible for the group separation, such as that seen in Fig. 1A. The shaded area has been designated as the "metabolic space" [32] where ions most likely derived from the administered drug and its metabolites can be found. M1 and M2 are the two most abundant drug metabolites in these mouse urines but other metabolites are also visible within this metabolic space. In this particular example [34], a total of 11 drug related compounds were identified in mouse urine. The mouse urinary metabolome is represented by the cloud of ions that is centered on the 0.0,0.0 coordinate. The chemical and enzymic behavior of metabolites and their mass spectra, together with the use of authentic standards, are all employed in the identification of individual metabolites. In all cases where UPLC–ESI-QTOFMS

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