Archives of Biochemistry and Biophysics 589 (2016) 177–186

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Review article

Metabolome analyses in exposome studies: Profiling methods for a vast chemical space

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ARTICLE INFO

Article history:
Received 29 July 2015
Received in revised form
30 September 2015
Accepted 9 October 2015
Available online 19 October 2015

Keywords:
Metabolic profiling
Metabonomics
Metabolomics
Exposome
Environmental epidemiology

ABSTRACT

Metabolic profiling (metabonomics/metabolomics) is now used routinely as a tool to provide information-rich datasets for biomarker discovery, prompting and augmenting detailed mechanistic studies. The experimental design and focus of any individual study will be reflected in the types of biomarkers that can be detected; toxicological studies will likely focus on markers of response to insult, whereas clinical case-control studies may yield diagnostic markers of disease. Population studies can make use of omics analyses, including metabonomics, to provide mechanistically-relevant markers that link environmental exposures to chronic disease endpoints. In this article, examples of how metabolic profiling has played a key role in molecular epidemiological analyses of chronic disease are presented, and how these reflect different aspects of the causal pathway. A commentary on the nature of metabolome analysis as a complex mixture problem as opposed to a coded, sequence or template problem is provided, alongside an overview of current and future analytical platforms that are being applied to meet this analytical challenge. Epidemiological studies are an important nexus for integrating various measures of the human exposome, and the ubiquity, diversity and functions of small molecule metabolites, represent an important way to link individual exposures, genetics and phenotype.

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http://dx.doi.org/10.1016/j.abb.2015.10.007
0003-9861/

1. Introduction

The completion of the of the human genome project promised to provide bioscientists, clinicians and epidemiologists with a new way to identify underlying genetic causes of chronic disease, estimate disease risk, conduct population/patient stratification, and identify new drug targets for therapy [1]. There are numerous examples of how candidate gene approaches, and genome-wide association studies (GWAS) have proved useful in understanding disease, and the post-genomic era has been characterized by incalculable innovation and progress [2,3]. However, it is now well recognised that the proportion of chronic disease explained by genetic variation is relatively small when compared with the influence of the environment [4–7]. Precise definitions of '*environment*' vary, but for the purposes of this commentary, it is taken to mean all non-genetic factors, which corresponds well with the concept of a human '*exposome*' as a complement to the human genome, suggested by Wild in 2005 [8–12]. The initial exposome definition that it "*… encompasses life-course environmental exposures (including lifestyle factors), from the prenatal period onwards*.", has been subsequently expanded and reinterpreted, but all definitions retain much of the same scope and scale [13–15]. Conceptually, the exposome is relatively simple, and has highlighted the need to devote effort to understanding environmental exposures in relation to health in addition to genomics. This approach represents a dramatic shift away from a candidate, chemical-oriented approach, to embrace the totality of exposures across different scenarios and timeframes (Fig. 1). While traditional methods for estimating exposure (e.g. personal exposure monitoring, geographic information systems) remain important in the overall exposure story, characterizing the exposome is predominantly led by the application of omics platforms that provide rich individual-level biological profiles. The blend of these techniques is clearly important to those designing studies of the exposome, and many factors play a part in determining which are used (e.g. life-course studies vs adult exposures vs mother-child studies), which has been highlighted in recent articles [16,17]. Metabolic profiling (metabonomics/metabolomics) [18,19] is central to these analyses, and several reviews provide further rationale and examples of how this is being implemented [20–23].

2. Throwing down the gauntlet: characterizing chemical and biological space

The full complement of small molecules in a given tissue, biofluid or compartment is known as the metabolome. Biological systems efficiently create complex metabolomes through the combinatorial action of multiple enzyme systems with varying substrate affinities and reaction rates. For example, a simple xenobiotic may (although not always) be metabolized to a dizzying array of phase 1 and phase 2 metabolites, with these distributed unequally at cellular, tissue and system level, and each having a unique set of interactions and responses. The human body also exists in close companionship with a wide variety of other organisms, each with their own genomes and metabolomes; the term '*superorganism*' has appropriately been used to describe the ensemble [24]. Human gut microbiota exhibit both spatial and temporal dynamics, and are intimately involved in co-metabolism across a wide range of substrates, meaning that the already complex picture of the human metabolome presented above is incomplete. The gut-host metabolic interface can give rise to diverse compound metabolism, with both potentially beneficial and adverse consequences [25]. Furthermore, the microbiotic composition is modulated by the gut environment - including its metabolic profile – meaning it is not a simple exposure, but inherently plastic and variable. There is currently much interest in understanding the role of microbiota in human health, as a potential dietary and/or therapeutic modifiable factor. Researchers have attempted to partition subsets of chemicals relevant to their own biological interest area, resulting in a variety of metabolomes being defined, echoing efforts in genomics to sequence species/individual genomes. These include the human blood and urine metabolomes [26,27], and those relating to nutrition [28], herbs [29], and pharmaceuticals, supplements, cosmetics, toxins, and substances of abuse [30–32].

2.1. Chemical space

The chemical space in which these processes occur is vast. Estimates of how vast vary considerably, but many estimates from the pharmaceutical industry put the number of chemicals that might have drug-like (i.e. potentially biologically active) properties at around 10^{60}, and dwarfs the current list of human-synthesized compounds (listed in the Beilstein database) [33–36]. The authors of these papers comment that ennumeration of all compounds is nether helpful nor possible – the search space is so large – but focused libraries of compounds help cover the chemical space efficiently. In a similar way, while any of these compounds has the potential to exist – they could be synthesised given sufficient effort - most are irrelevant in terms of a real exposure that has a meaningful effect on the metabolome, and consequently the health status of an individual. The use of analytical methods that are sufficient broad so as to help cover the potential exposure/metabolome space are likely to be most useful. Interestingly, not only is the chemical space vast, but so to is the range of concentrations experienced, both inside and outside of the body. Rappaport et al. (2014) compiled the available, published literature values for 1500 chemicals observed in human serum or plasma samples. These compounds included dietary components, drugs, and environmental pollutants – and revealed these span 11 orders of magnitude. Interestingly, these authors demonstrated that environmental pollutants were present at much lower levels (typically 10–1000-fold) than those of other sources, which were comparable in magnitude [37]. If we are to attempt to dissect exposures from various sources, and the responses that are elicited by their ensemble action across the vast physicochemical and concentration ranges, then appropriate, high-capacity methods for interrogating biofluids are needed.

2.2. Codes

As described above, the human metabolome is a complex, responsive, dynamic part of the exposome, with comparable complexity to that of the human genome. However, the

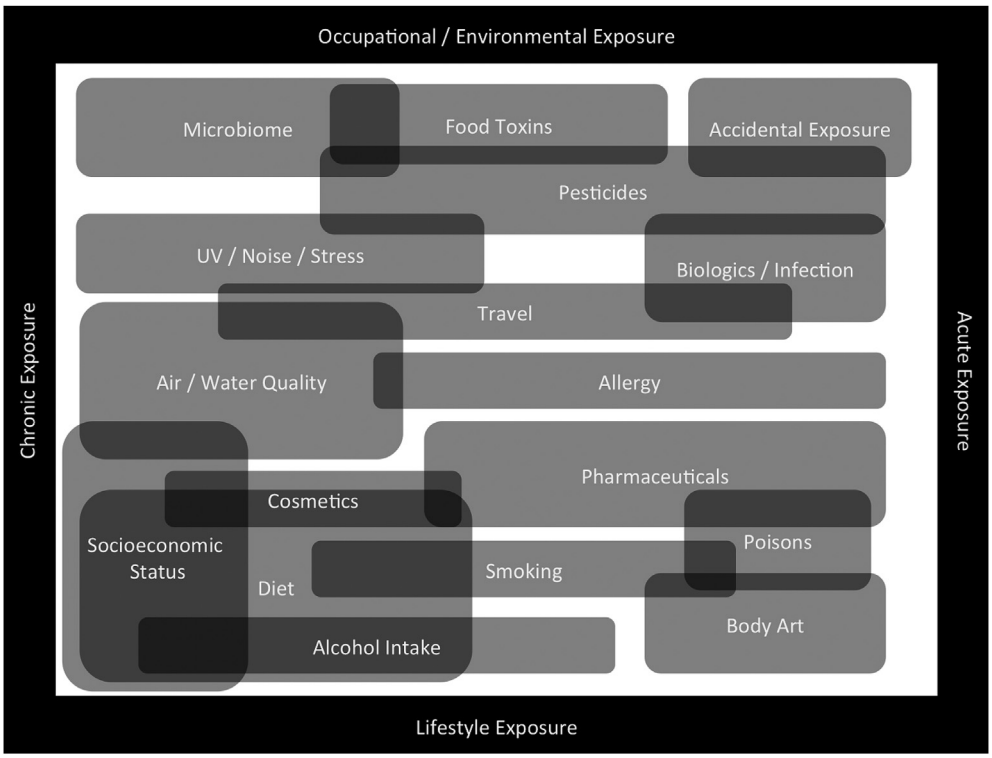

Fig. 1. Cartoon illustrating the chemical and non-chemical environmental influences on the human metabolome. They incorporate both acute and chronic exposures that may originate from lifestyle/behaviors and interaction with the wider environment, including occupation. Such diversity generates a considerable challenge when attempting to delineate relations between exposures – many of which may occur simultaneously – and biological responses encoded in the metabolic phenotype of individuals.

metabolome presents a different type of analytical challenge to other omics approaches that are based on profiling *encoded* molecules. Here, we define encoded molecules as those formed from the linkage of a limited number of subunits (for example, DNA incorporates the nitrogenous bases cytosine, guanine, thymine, and adenine that are used as the molecular code). For identification and to a large extent, function, of these molecules, the order is what matters, and the combination of the limited set of subunits gives rise to the observed (and required) complexity. Sequencing technologies that rely on incremental detection of these limited subunits (e.g. by pyrosequencing [38], or nanopore analysis [39]) need only be able to distinguish between the limited number of possibilities for a particular part of the code. As is evident from our ability to sequencing an entire human genome in a short timeframe, such analyses have been well optimized, made possible by the relatively simple analytical step that is required, coupled with the ability to repeat the step at a very high rate (Fig. 1).

Differentiation of metabolites in the context of complex biofluid profiling cannot be conducted in such a way, as the analytical step is not simply to choose between one of a limited number of options. Two similar metabolites (for example, two phospholipids differing only by the degree of fatty acid chain saturation) may be separable/differentiated using a highly specific assay, but the assay will have limited applicability elsewhere in the metabolome. A further issue is that sequencing can be applied to *any* coded molecule of a particular type (DNA, RNA, proteins), as the critical analytical step (e.g. measurement of a particular base) is independent of its molecular context. By contrast, not all metabolites are amenable to the same analytical platforms/techniques, with each giving rise to a different/unique response. Thus, we are not attempting an analogous *sequencing* of the metabolome, but a very complex chemical *mixture* analysis. An alternative to molecular sequencing is to use knowledge of the various codes to generate templates that have highly specific affinity for target coded molecules, such as is used in transcriptomic microarrays. The specificity comes from the requirement for a large number of possible interactions to be present only for the target (or those with very similar sequence). For small molecules, template recognition approaches are possible, using techniques such as molecular imprinted polymers (MIPs) whereby analytes have high affinity for specific target, but these have not yet been readily deployed in a metabolic profiling context. Some panels of metabolites (e.g. lipids) can now be detected using multiplex ELISA-based kits, suggesting that the template/affinity approach may have value for targeted applications in certain metabolite classes. The differences between these approaches are summarised in Fig. 2.

3. Rising to the challenge: global metabolome profiling methods and population studies

The inherent complexity of the metabolome means we must look for methods that are capable of differentiation, identification, and measurement of metabolites across a wide chemical space. Detailed accounts of the main analytical platforms have been extensively reviewed and appraised [40]. In brief, global profiling methods are used to provide an agnostic view of sample similarity, identify outliers, and uncover metabolites that differentiate sample classes; targeted methods may be used to focus analysis on particular subsets of metabolites. Typically, these platforms rely on a combination of chromatographic separation, mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy as these techniques can be used to detect a wide range of small molecules in an untargeted manner. Molecular epidemiological analyses present their own unique set of challenges for the application of metabolic

profiling, which are well met by the characteristics of existing analytical platforms.

NMR spectroscopy is used extensively in metabolic profiling, typically by application of 1D ^{1}H experiments for many reasons. These include the non-destructive nature of the technique, wide coverage of chemical classes in key biofluids, highly reproducibility and robustness, and direct comparability of spectra obtained on different instruments. NMR spectroscopy does not require physical instrumental contact with the sample, and no carry over is possible (unless using flow cell apparatus, which is rare in such applications). In the context of large-scale profiling exposome studies, NMR may also present an additional benefit in that it can be used as a screening tool for inappropriate/outlier samples (e.g. gross contamination, protocol non-compliance, etc.), that can be removed/quarantined before subsequent analysis. This may improve the representative nature of the overall sample set, and reduce adverse effects on other analytical platforms.

In the case of mass spectrometry, it is commonplace for the analyte detection step to be preceded by physical chromatographic separation of sample components, either by gas chromatography

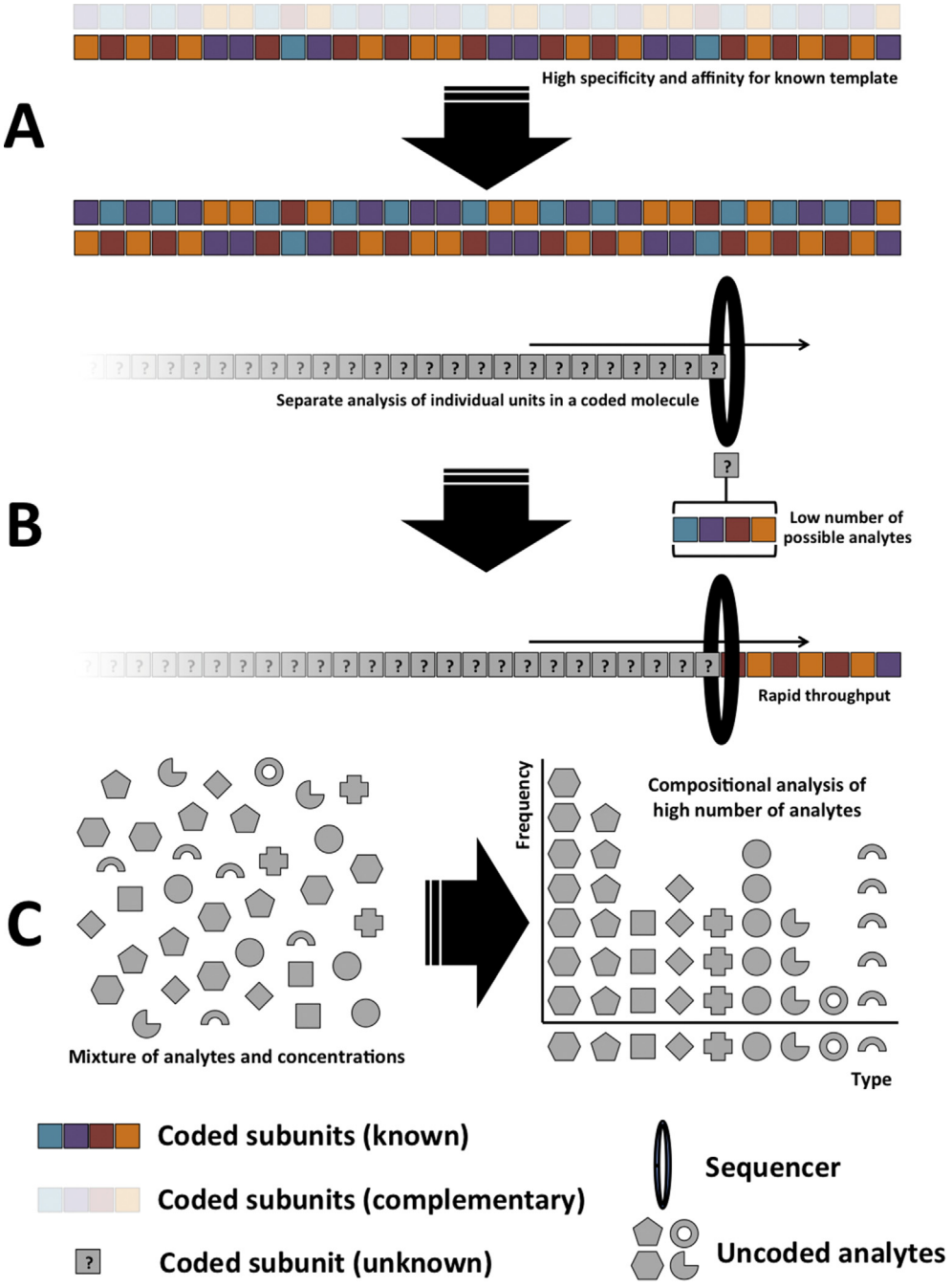

Fig. 2. The analytical challenge of metabolome profiling is different to that presented by coded molecules such as DNA or RNA. The analysis of encoded macromolecules either relies on the complementarity of the analyte with a (known) template of high specificity and affinity (e.g. in a microarray) (A), or the identification of subunits drawn from a small number of possibilities (e.g. ribonucleotides) in the sequence the analytical challenge being to conduct a relatively simple analysis with very high throughput and fidelity (B). Conversely, metabolite measurement requires analytical methods that can differentiate multiple analytes drawn from a very large and chemically diverse pool, based on selected physico-chemical properties (C).

(GC), liquid chromatography (LC) or capillary electrophoresis (CE). This provides a much greater resolution of metabolites, and affords the opportunity to separate isobaric species, that would be inseparable by MS alone. This increase in resolution comes with the price of longer analytical runtimes and an additional (chromatographic retention) dimension in the resulting data. However, recent advances in system/column technology such as ultraperformance liquid chromatography UPLC and supercritical fluid chromatography (SFC), have resulted in systems greatly improving sample throughput while maintaining resolution. The main benefits for exposome studies are the high sensitivity and resolved coverage, and both reversed-phased UPLC and SFC exhibit particular utility in the separation of lipids, which represent a large subset of the blood metabolome [41–43].

NMR- and MS analyses provide detailed spectral information about detected metabolites, and therefore can be used for structural elucidation, beneficial in the assignment of unknowns and biomarker identification. Protocols for global/untargeted/agnostic and panel/targeted metabolic profiling analysis of biofluids and tissues by NMR spectroscopy, FT-MS, UPLC-MS, GC-MS and related platforms are widely available in the literature, and have been reviewed on numerous occasions [44–49].

3.1. Requirements

Platforms for population and individual level profiling require similar properties: high throughput, high information density and coverage, robustness, reproducibility, adequate coverage, and low cost; samples sets in population studies are typically (and understandably) large, and therefore the high-throughput of NMR- and MS-based metabolic profiling techniques are well suited to the task. Analysis times are dependent on individual assay, but typically permit up to ten samples per hour. Analysis cost is also relatively low, as consumables are typically restricted to solvents, selected standards, and chromatographic column chemistries are now typically very robust to heavy usage [48,50]. While it is almost irrelevant to compare the per-sample costs of platforms that provide completely different genotypic/phenotypic information, experience indicates that in terms of total financial outlay, inclusion of metabolic profiling in multi-omic large-scale studies is likely to represent only a fraction of the analytics budget.

Unlike other omics platforms (commonly used in parallel), NMR and MS analyses will provide a metabolite profile for a biospecimen irrespective of its composition, and as such does not have an inherent measure of '*quality*' (c.f. RNA integrity number; RIN). This is a double-edged sword, as it does mean virtually all samples will yield a useable profile, but the need to establish if that profile is truly representative is greatly increased. This is of significance in the context of molecular epidemiological studies, as archived samples may have been collected many years before analysis, using a variety of different protocols, and may lack information relating to pre-analytical factors that could potentially affect or bias metabolic profiles (e.g. collection time, centre, clotting time, etc.). A number of studies have focused on understanding the influence of these pre-analytical factors on the metabolome, including bias associated with collection protocol parameters [51], storage/transport conditions [52], and the identification of metabolites that are differentially affected (i.e. those that are most reliable and stable) by pre-analytical factors [53]. These and other aspects of the collection-to-analysis workflows have recently been the subject of a detailed review [54]. With respect to the specific requirements of large-scale metabolome profiling in exposome studies - that may require the use of both freshly collected and archived biospecimens - efforts to establish suitability/preanalytical criteria for biobank samples have been made. As part of a multi-platform evaluation, Hebels et al. (2013) compared metabolome profiles obtained from freshly-collected blood plasma samples with varying anticoagulant preservatives (EDTA, heparin, citrate), bench time (0–24 h), storage temperature (−80 C or liq. N_2) [55]. The factors found to be most influential on the global metabolite profiles could be related to chemical interactions with the anticoagulant (potentially directly influencing the analytics), and periods in which the plasma had the opportunity to undergo post-draw metabolic changes (bench time).

3.2. Overcoming limitations

A combination of preanalytical factors, in addition to inter- and intra-instrumental variability means that one major limitation of global metabolic profiling in large-scale studies is data comparability. Within individual (campaigns of) untargeted metabolomics studies, data comparability can be assessed on a feature-wise basis using standard quality control (QC) methods (typically characterizing the coefficient of variation of individual spectral features across repeated analysis of a pooled sample), which also serve to track instrument performance, identify outliers and permit batch-wise normalization [56,57]. Such measures are critical to enable combined analysis of large sample sets. For example, after implementing appropriate quality control measures and establishing in the HUSERMET study, Dunn et al. (2014) have generated comparable metabolic profiles for 1200 individuals [48,50,57].

As described in numerous previous commentaries, the trade-off for using global profiling methods that allow for a hypothesis-free approach is that subsequent identification, annotation and validation of putative markers is currently a potential bottleneck. This is particularly acute in the context of molecular epidemiological analysis, where collaborators are used to having annotated datasets (e.g. measures of single nucleotide polymorphisms or gene methylation). Targeted methods that provide a validated panel of metabolite concentrations can help bridge this gap for metabolic profiling, at the expense of excluding unexpected associations (e.g. AbsoluteIDQ p180 Kit). Many studies have combined both approaches to provide both readily amenable readouts for core metabolites, while retaining the ability to conduct agnostic profiling.

Irrespective of the platforms used to characterize the metabolome, one of the main limitations to their application and subsequent interpretation of metabolic profiles is the sparsity of data that details normal variation in population across the measureable/detected metabolites; among other things, this limits *a priori* power calculations. Despite the severe lack of metabolome variability information, a recent report by Yousri et al. (2014) showed that there is long-term conservation of individual metabolic phenotype (over seven years) and apparent heritability of signatures [58]. In light of this, the view of this author is that appropriate study designs that utilize i) participants as their own control, and ii) multiple repeat samples may be well placed to identify and integrate systematic patterns of change that align with exposures profiles. A recent exemplar has been the 'Oxford Street' study of diesel exhaust exposure and respiratory health, that is due to be expanded to include metabonomics analysis in the EU FP7 EXPOsOMICS study [59]. Once combined with longer-term data describing key developmental periods, and early markers of disease onset, it will start to enable the deconvolution of metabolic changes occurring over varying timescales in response to environmental exposures.

Another limitation includes the difficulty in annotation of metabolic profiles, largely a result from the requirement to perform additional spectroscopic analyses/sample cleanup, separation, and authentic standards synthesis/addition to conform the identity of unknowns sufficiently well. An issue with this is that the annotation stage occurs after an unannotated feature of interest has been identified during the study data analysis, and therefore may result

in delays between identifying metabolites of interest and biological interpretation or analysis that requires annotation to be in place (e.g. over-representation analysis or joint pathway analysis).

4. Emerging and future analytical methods

While NMR, LCMS, GCMS and CEMS comprise the main analytical platforms for large-scale analysis, there are several other auxiliary approaches that may address the need to increase the coverage of the metabolome by improving resolution, sensitivity, or throughput. One important aspect to note is that expansion of the coverage puts additional demands on the bioinformatics and potential number of putative metabolites requiring annotation.

4.1. Ion-mobility

Ion-mobility (IM) spectrometry is a well-established analytical platform for separations performed on a millisecond timescale, intermediate between that of chromatography (seconds) and time-of-flight mass spectrometry (microseconds) [60–62]. Ions are moved through a drift tube by an electric field from a source and into a detector. Several different ion mobility approaches for achieving separation exist but all rely on a drift gas (e.g. helium) to be present in the drift tube to impede the motion of the ions [63]. IM provides several potential benefits for metabolic profiling, particularly those involving samples obtained from human populations in exposome studies [64]. Firstly, the resolution of the analysis is greatly increased through the ability to conduct an orthogonal separation within the typical timescale of a chromatographic peak elution that is amenable to multiple MS scans. Secondly, the observed mobility drift time (that relates to the ion collision cross section) can provide an additional identifier to aid structural elucidation, and in certain circumstances, separate isobaric species [65,66]. Thirdly, common sample contaminants/additives that co-elute with metabolites of interest are detected separately; and can be edited from the profile data in post-acquisition data processing stages.

While IM can greatly increase the resolution of the metabolic profiles available, the additional complexity may result in additional headaches for the analyst. For example, ion mobility may reveal protomeric ions (i.e. ions of the same parent molecule, protonated at different sites) formed in the instrument source, that have differing mobilities (and potentially different fragmentation spectra) as a consequence of the site of protonation altering the conformation/analyte-gas collision cross section. Another limitation of IM is the dramatic increase in the computational overhead and data storage requirements that are concomitant with the increased dimensionality of the spectral data. For large-scale studies, this may prove prohibitive for individuals without access to dedicated computational hardware, at least in the short-medium term.

IM can also be used as a stand-alone separation step, permitting very rapid acquisitions, albeit at lower resolution than when coupled to other chromatographic systems. This has been developed for metabolic profiling applications [67,68] and has potential utility for deployment in a variety of exposome study scenarios using less complex instrumentation (e.g. high-frequency longitudinal studies). Other direct methods are also being developed, and may find utility in the analysis of very large datasets due to their relative simplicity, and speed. For example, Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) analysis of direct infusion of biofluid preparations have been used extensively by some metabolomics research groups, utilizing the very high resolution and mass accuracy on this type of mass analyzer. Methods and applications have been reviewed by Brown et al. (2005) [69]. More recently, interfaces for MS that are capable of direct sampling without the need for complex matrices, and can be used under ambient conditions. These include desorption electrospray ionization (DESI) [70,71] and rapid evaporative ionization mass spectrometry (REIMS) methods [72,73].which have been demonstrated to provide very rapid, interpretable MS data on biofluids and tissues (particularly in clinical applications), and will undoubtedly influence future directions for high-throughput metabolome analysis in the context of exposome studies.

4.2. Chirality

Metabolite stereochemistry is one largely underexplored dimension of the chemical space in metabolic profiling. Chiral analytical methods are readily available, but typically have very high specificity for particular analytes. We have previously explored the use of chiral co-solvation agents as a potential route to efficient enantiodifferentiation in routine NMR biofluid spectra [74]. For chromatographic separations of enantiomeric species, several different strategies are potentially viable, including use of a chiral stationary phase, enantiopure solvents, addition of ion-pairing agents, and sample derivatization to produce diastereomeric species that can be separated on a chiral column. Some evidence that IM-MS might also be feasible for providing chiral separations has been provided by Dwivedi et al. who have performed drift-cell enantiodifferentiation using chiral drift gas modifiers [75]. Replication and expansion of these findings for metabolome profiling platforms potentially provide a route to efficient capture of the chiral metabolome, notwithstanding the need to explore and optimize such separations across a wide variety of metabolite classes. Other similar approaches, based on derivatization of stereoisomers to form diastereomers have been suggested [76–78]. In addition to the limitations that targeted methods such as these place on metabolome coverage, derivatization methods also require more time and money, and methods may suffer from additional pre-analytical issues.

The analytical challenge of chiral metabonomics results form a combination of the inherent complexity of metabolic profiles, and the specificity of chiral interactions – simultaneous enantiodifferentiation of multiple species is not only difficult to achieve, but results in an even more complex profile. Nevertheless, harnessing chiral methods to explore this facet of metabolic variation is of interest as biological systems are intrinsically chiral environments, and the absolute configuration of small molecules can fundamentally alter their biological function (e.g. thalidomide, serine). As is the case with the metabolome as a whole, understanding sources of variation is a key step in being able to interpret observed differences in enantioselective. Visser et al. previously compared multiple LCMS based approaches for amino acid analysis, and reported the concentrations and enantiomeric excess in urine, blood plasma and cerebrospinal fluid [79]. Recently, Lorenzo et al. demonstrated the use of an analysis in which eight amino acid enantiomeric pairs were separated and detected using a chiral GCMS method [80]. The method was applied to urine samples obtained form a small gestational diabetes cohort, and indicated an association of phenylalanine enantiomeric ratio with presence of the condition.

5. Incorporating metabolome analyses into studies of environment and health

5.1. Metabolomes vs exposure

Biomarkers of exposures have particular utility in molecular epidemiology studies, as they can help alleviate some of the problems related to the misclassification of study participants'

exposure or co-exposure (e.g. through inaccurate recall, inaccurate behavior modeling, or protocol non-compliance) that reduce study power [81]. The human metabolome has the potential to report directly on a very large number of environmental exposures of chemical origin; many environmental, occupational, and lifestyle exposures fit the common definition of '*small molecule metabolite*' (typically < 1500 Da), and are potentially detectable using the common metabolic profiling analytical platforms discussed above.

While environmental chemicals retain importance from an epidemiological and public health perspective, the diet provides arguably the greatest quantitative influence on the human metabolome, in terms of both diversity of exposures and range of concentrations; dietary components from animals and plants each have their own metabolomes, are consumed in different quantities, combinations, and proportions, and over different periods, with components modified by storage, preparation, and cooking. Therefore, compositional analysis of foods, provides a natural route to characterizing key exposures, and can be conducted using either traditional analytical methods, or untargeted metabolomics. In particular, the plant biology community has an expansive literature relating to metabolomics, with established profiling protocols [82]. Community resources for food composition (e.g. FooDB; www.fooddb.ca) have been established, and can be used to help annotate metabolome signatures, and identify biomarkers of food intake [28]. The current interest in developing dietary exposure markers, and the role of diet as a major, modifiable chronic disease risk factor [7] suggest it is an opportune time to bring together those working on non-human metabolome characterization to help capture the broad range of nutritional exposures in much more detail.

As highlighted by the analysis of Rappaport et al. [37], major dietary inputs are typically observed at concentrations many times higher than environmental pollutant chemicals that are traditionally the focus of environmental toxicology. In this respect, current untargeted metabonomics methods are limited in their role for global environmental exposure assessment as they do not deliver sufficient sensitivity and coverage, although some efforts have been made to provide extensive panels of pesticide exposure markers using typical profiling instrumentation and (semi)targeted methods [83].

While the wide coverage of untargeted/targeted NMR- and MS-based profiling analyses are by far the most prevalent methods for characterising biofluid composition, the field can also benefit from measurements made by a variety of means, particular if many are made in a highly parallel manner. A good example of this is the panel of biomarkers routinely collected from individuals enrolled on the National Health and Nutrition Examination Survey (NHANES) – an ongoing research programme run by the National Center for Health Statistics in the USA. In addition to periodic physical examinations and detailed questionaire data collection for around 5000 participants each year, the study also archives and analyses urine and/or blood measurements for a range of environmental exposures and nutritional indicators. Patel et al. have developed a model for interpreting the wide range of laboratory measurements made in this study to realise the concept an environment-wide association study (EWAS). As the first of its kind, this study has been extensively discussed elsewhere as a major step towards tools that can integrate environment and health within an exposome context [84,85]. However, one point that is worth highlighting is that one of powerful aspects of these studies is that they do not rely on cutting-edge instrumental analysis of archived samples, but the relative consistency of repeated measures made over time on a vary large scale. In order to capture aspects of the life-course in the human exposome, accessing such datasets may prove useful in the extreme.

5.2. Responses to exposure

While many individual exposures may be currently beyond the reach of routine metabolome analyses, the responses they provoke are manifest as biochemical perturbations that may be more easily captured. Identifying responses at the cellular, organ and organism level are central to the concept of defining Adverse Outcome Pathways (AOPs), an emerging framework in chemical risk assessment. As the name suggests, AOPs are aimed at integrating a variety of knowledge relating to the causal chain of key events that link exposures at '*Molecular Initiating Events*' (MIEs) to '*Adverse Outcomes*' (AOs). Definition of AOPs may help interpret the effect of simultaneous exposure to multiple agents; those with the same MIE will share common toxicological pathways. Metabolic phenotypes are ideal for helping characterize events in AOPs, contribute to their definition, and provide linkage between population-based exposome studies and mechanistic toxicology used in risk assessment [86]. For example, dioxins are a class of persistent environmental contaminants that are byproducts of a range of industrial processes, and represent an almost ubiquitous human chemical exposure, with the main route being in foodstuffs, particularly those of animal origin. The observed toxicity of these dioxins and dioxin-like compounds differs according to their specific chemical structure [87], with the most potent being 2,3,7,8-tetrachlorodibenzodioxin (TCDD), which is used as a comparator for other compounds (toxicity equivalence factor; TEF of 1) [88] and has been used as an exemplar AOP based around the aryl hydrocarbon receptor [89]. Exposure to TCDD can give rise to a wide range of adverse effects across multiple tissue types, and is classed as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC), although limitations of the epidemiological and other evidence, and potential influence of publication bias on the overall assessment have been highlighted [90]. Hosnijeh et al. (2013) employed an LCMS based metabonomics approach to identify systemic metabolic changes related to TCDD exposure in serum samples obtained from a cohort of occupationally-exposed workers (n = 81) in chlorphenoxy herbicide factories and control worker (n = 63) factory locations in the Netherlands [91–93]. Jeanneret et al. (2014) also investigated long-term responses to dioxin, in a Czech cohort who were exposed at high levels of TCDD in 1960 as a consequence of working in a herbicide production facility [94]. The authors incorporated the existing biological knowledge relating to TCDD perturbation of steroid hormones, and focused on the urinary androgen (conjugate) profile that could be generated using a broad analysis of this chemical class. The authors also accessed urine samples collected from an extreme case of TCDD exposure (poisoning of V. Yuchchenko in 2004; [95]) and compared their profiles with freshly collected healthy control individuals. Studies such as these, that capture real human exposures and responses, may be useful in the development of AOPs, as they can help anchor other existing mechanistic knowledge (e.g. *in vitro* or *in vivo* toxicological data) relating to adverse human health endpoints.

Responses to non-chemical exposures are also highly relevant to the exposome concept, as these may elicit specific responses, and also contribute to the overall context in which other exposures and responses occur. Variation related to system–level cycles and timeframes such as diurnal variation [96], circadian rhythm [97], aging [98], and many others [99] influence metabolic status of the individual. For example, in recent work, Davies et al. (2014) investigated the influence of sleep deprivation on the human plasma metabolome using an LC-MS profiling platform [100]. The authors showed that the daily rhythms exhibited by a large number of metabolites were influenced by the disruption of normal sleep patterns. Interestingly, the patterns of some metabolites, (e.g. some

branched-chain amino acids) remained unaffected by sleep or meal patterns. Variation of this kind is likely to be a consequence of several highly correlated factors such as day length, food intake, temperature, activity, etc., and this study highlights the challenge faced by those attempting epidemiological analyses in free-living populations, with potential impact on sampling times/logistics, and the overall ability to attribute observed metabolic changes to specific environmental sources.

5.3. Metabolomes vs disease risk factors and health endpoints

Recently, several studies have applied similar approaches to metabolic phenotypes in metabolome-wide association studies (MWAS), and provided several examples of linking metabolic traits to health [101,102]. For example, NMR-based metabolic profiles of urine were obtained the INTERMAP study, and showed several metabolites to be associated with BMI [103].

The responses to environmental agents are, in part, conditional on health status at the time exposure, and therefore the metabolome can provide useful context in the form of a detailed phenotype; exposures may not only give rise to specific metabolic phenotypes, but be modulated by them. For example, dysregulation and inflammation associated with metabolic syndrome will likely alter an individual's subsequent response to exposures (of all types), and therefore modulate their risk of disease. This complexity is encompassed by the exposome concept; while the totality of exposures represents the scope, it is important to recognize that prior exposures alter the context of subsequent exposures. Maitre et al. (2014) conducted a ^{1}H NMR-based metabolome urinalysis of pregnant mothers from the Rhea birth cohort [104]. Using a case-control design comparing adverse pregnancy endpoints (preterm birth (PB), fetal growth restriction (FGR), small for gestational age (SGA)) and matched controls ($n = 438$), the authors explored associations to urinary metabolites in first trimester collections. Previous analysis had shown PB to be associated with maternal metabolic syndrome in this cohort and in this work, the predictive signatures derived differed between spontaneous and medically indicated/induced PB. Additionally, a number of metabolites showed an inverse relationship with both FGR and blood insulin, suggesting a potential complex relationship between metabolic syndrome and adverse birth outcomes.

5.4. Metabolic profiling in current exposome studies

One conceptual framework that has been proposed as a way of engaging with the human exposome and to help develop appropriate experimental designs has been the '*Meet-in-the-Middle*' (MITM) concept [105,106]. This augments traditional epidemiological approaches of determining exposure-disease associations, by using case-control studies, nested in prospectively collected cohorts to delineate intermediate markers of response. On the one hand, these markers can be related to exposures (modeled or measured in biospecimens), and on the other, are predictive of the subsequent health endpoint (case-control status). It has been proposed that analysis of these biospecimens using multiple omics platforms will reveal signatures that report on the causal events linking exposures and disease. This approach has most recently been applied in the EU FP7 EnviroGenomarkers study (http://www.envirogenomarkers.net) which utilized prospectively collected biospecimens from the European Prospective Investigation into Cancer and Nutrition (EPIC) and Northern Sweden Health and Disease Study (NHSDS) biobanks, and integrated multiple omics analyses (transcriptomics, epigenomics, metabonomics) with biomarkers of high-priority environmental exposures. Two ongoing large-scale exposome studies have incorporated the MITM approach into their analytical plan (EU FP7 EXPOsOMICS and HELIX projects) [107,108]. Limitations of the MITM approach are largely due to the complexity of gene-environment (GxE) interactions [109], and the multifactorial influences on the metabolome/transcriptome/proteome/epigenome that confound the relationship(s) of interest; samples collected may not capture similar stages in the exposure-disease pathway, or the pathway may be exhibit nonlinear and multicomponent behavior.

5.5. Metabolic profiles in understanding gene-environment interactions

Partitioning the variation that is attributable to G and E respectively on a metabolite-wise basis is important as it provides a context for differences observed between individuals and sub-populations in molecular epidemiological studies. Cohort studies of twins provide a valuable opportunity to examine the effect of environmental variables on the metabolome, by permitting the genomics component to be kept effectively constant. Nicholson et al. (2011) used the TwinsUK (http://www.twinsUK.ac.uk) cohort of 154 twins to estimate spot urine and blood plasma metabolite variation that was attributable to the biologically-factors familiality (28–58%) and individual environment (9–22%) which were shown to be relatively stable, and two less stable factors relating to short-term variations occurring at the timescale of the collection visit of the study [19]. Longitudinal sampling in this study allowed this biologically-related variation of each metabolite measured to be deconvoluted from the non-biological variation, such as technical variation and random noise. Importantly, the argument is not to focus on either the genome or the exposome, but to integrate data relating to both to understand their interaction. Where genomic and metabolic datasets exist in parallel, it has been possible to start linking genotype to phenotype; understanding the influences of genome and epigenome on metabolic phenotypes has been conducted in several studies [110–117] and reviewed by Kastenmüller et al. (2015) [118].

6. Future perspectives

The use of metabolic profiling in human environmental health studies has become ever more relevant as the concept of the exposome has developed, and the centrality of metabolome in mediating gene-environment interactions recognised. Application of metabolic profiling to large-scale studies is associated with a number of unique challenges that are well met by current methods including throughput, cost, and suitability for archived biospecimens. Areas currently requiring substantial further development are data comparability, coverage, and feature annotation. This raises the question of what we can realistically expect metabolic profiling platforms to provide in terms of metabolome/exposome characterization. Should we be aiming for sensitivity/depth or coverage/breadth? Alongside national and international initiatives to provide harmonized analytical protocols, the application of complementary analytical techniques may actually yield both depth *and* breadth. A more detailed view on the continuum of metabolism that connects genes, environment, health, will ultimately provide new insight into the determinants of chronic disease.

Acknowledgments

TJA wishes to thank colleagues at Imperial College London, particularly Prof Paolo Vineis, Dr Hector Keun, Dr Muireann Coen, and Dr Elizabeth Want for thought-provoking discussion relating to the exposome and the role that metabolic profiling can play, prior to the writing of this article. TJA acknowledges the EU FP7

EXPOsOMICS (Grant Agreement No. 308610) and HELIX (Grant Agreement No. 308333) projects for support in this area of research.

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