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Omic techniques in systems biology approaches to traditional Chinese medicine research: Present and future☆,☆☆

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ABSTRACT

Omic techniques have become key tools in the development of systems biology. As the holistic approaches underlying the practice of traditional Chinese medicine (TCM) and new tendencies in Western medicine towards personalised medicine require in-depth knowledge of mechanisms of action and active compounds, the use of omic techniques is crucial for understanding and interpretation of TCM development, especially in view of its expansion in Western countries. In this short review, omic applications in TCM research are reviewed which has allowed some speculation regarding future perspectives for these approaches in TCM modernisation and standardisation. Guidelines for good practice for the application of omics in TCM research are also proposed.

1. Introduction

Identification of molecular mechanisms and targets is a critical step in the validation of a biological effect. When using phytocomplexes[2] as in Chinese herbal medicine (CHM) research, this is often hampered by the complexity of the molecular mixtures, with many different molecules participating to the overall effect, either positively or negatively.

With the advent of information-rich techniques such as genomics, proteomics and transcriptomics as well as various profiling approaches, including metabolomics (a non-targeted analytical approach, usually concentrating on molecules of low molecular weight) and metabonomics (similar studies but involving studying the effects of perturbation of a system [see precise definitions in Section 3.3]), it has recently become possible to examine simultaneous molecular effects of mixtures of chemical

☆ The literature contains many abbreviated forms to describe the techniques often collectively known as the omic techniques. We prefer the use of the simple brief term omics; without complicating punctuation.

☆☆ For clarity throughout this article, Latinised pharmacopoeial names are shown in bold italics to differentiate them from Latin binomial plant names which are italicised only. The Pinyin versions of Chinese and Japanese formula names are shown in bold.

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[2] The term "phytocomplexes" is taken here to mean the use of a whole plant or part, or its extract (thus containing many components) or indeed mixtures of such plant parts or extracts, as in common in TCM.

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agents (Ulrich-Merzenich et al., 2007) and, with the help of bioinformatics, to look at such effects with a global view on the biological system affected. Factorial analytical models can decode the large quantity of raw information derived from these omic techniques, sometimes allowing correlation of the multiple components of phytocomplexes with their biological effects (Wang et al., 2010a,b).

At the same time high-throughput, information-rich assays can be used to fingerprint herbs and botanical extracts (Heubl, 2010; Kim et al., 2011; Liang et al., 2010; Sucher and Carles, 2008; Van der Kooy et al., 2009). A number of studies have thus begun to address the other key issue of quality control and sample variability, which is pivotal for reproducibility and standardisation of biological effects in the study of herbal preparations. Many papers, particularly those reporting clinical data, do not adequately address the quality (or even the identity) of the test material, so significantly reducing the validity of the results. Although some international journals are beginning to require authors to give details of their test materials, not all yet do so and one of the recommendations of the present group relates to such a proposal (see Section 6.2).

Such applications of information-rich approaches make the use of omic techniques particularly appropriate for addressing many of the problems encountered in traditional Chinese medicine (TCM) research that have hampered its acceptance in the Western biomedical mainstream and its integration with more orthodox Western medical practice.

Today, a holistic systems biology approach provides a new perspective in pharmacological sciences which goes beyond target specificity and single molecule pharmacology, and embraces the entire equilibrium of a biological system undergoing simultaneous perturbations on primary and secondary multiple molecular targets (Auffray et al., 2009). This approach is even more relevant when using multichemical mixtures, as in CHM and other TCM formulae which comprise mixtures of mixtures (Cooper, 2009; Ulrich-Merzenich et al., 2009; Wang et al., 2009).

The advent of such assays with advanced bioinformatics has thus brought an opportunity to change the way the different experimental levels are considered, long viewed almost as different fields. The operation of the different omic techniques are applied in an identical fashion whether used *in vitro*, *in vivo* or in clinical research. With the new systems biology approach the difference is only seen in terms of its application to increasing system complexity: molecular, cellular, multicellular, organ and whole organism levels. Thus, when reviewing omic techniques in experimental models, it is important to include *in vitro*, *in vivo* and clinical research applications. Omic techniques are of equally useful application in all such contexts, and researchers from different levels should be able to readily share and transfer information within the research community and to the clinic.

These approaches obviously have great potential, as shown by recent applications of omic techniques in TCM research, which are the focus of the present review. It is to be emphasised that the term 'holistic' is often used to describe the application of omic technologies (being non-reductive in that they do not focus on single active principles), the information derived from them is obviously at the level of physical matter (albeit by considering many compounds and their effects at once). This is attractive in the context of research on Chinese herbal medicine which is described as having a holistic, multidimensional approach to treating patients but the use of the term must be carefully differentiated according to context. The authors nonetheless contend that the use of non-reductive technologies is highly appropriate to begin to understand the complexities of the use of herbal materials at the molecular level.

2. Development of the use of omic techniques in TCM research

Since their origin at about the beginning of this century, omic techniques have found application in virtually all biomedical fields and have rapidly found complementarity with many classical experimental models. Systems biology and, on the clinical research side, personalised medicine, have given rise to an emerging holistic approach in research, which zooms out the focus from the static detail of single molecular targets to try to embrace the biological system in its dynamic complexity. Omic techniques, allowing a simultaneous vision of classes of molecules in a given system, are the main experimental drivers of such an approach. Accordingly, information-rich assays can be applied to the fine description of a complex formulation, a multi-target biological context as well as to the observation of their interactions and the subsequent perturbation of the biological equilibrium. Despite the apparent potential for application of omic analytical techniques and bioinformatics in studies involving herbal medicine, high cost and the range of multidisciplinary expertise required for their application have initially constrained the speed of their acceptance and dissemination to a pace slower than might have been expected.

From a PubMed search restricted to subjects of pharmaco-toxicological interest, using as keywords (contained in the titles or abstracts) the commonly used omic techniques together with herbal studies[3]; it has been found that in the last ten years or so; some 3300 papers have been published on omic techniques applied in the study of medicinal plants. More than one third of them have appeared in the last four years. However; as there are only about 130 titles having a clear mention of the area of Chinese medicine in their titles or abstracts; omics applied to TCM research still represents only a small fraction of the total of omic work published; even though this proportion is increasing.

Interestingly, when looking into the individual omic techniques and their applications, it is clear that a linear path cannot be traced in the evolution of omics techniques in CHM. The application of newer generation omic strategies, such as metabonomics, is rapidly catching up those from the first generation of omics that were based mostly upon genomic and transcriptomic techniques. This is illustrated in Fig. 1.

During the early years of their application in CHM research, omic techniques were used almost exclusively as tools for botanical identification purposes, using genomic techniques like DNA sequencing and fingerprinting, or DNA microarrays (Cai et al., 1999; Mihalov et al., 2000; Zhang et al., 2003). Until 2007, about 50% of original papers on omics and TCM related to botanical issues, mostly addressing authentication and quality control of Chinese herbal medicinal products (Zhao et al., 2006). During this period, in addition to DNA-based tools, other omic analytical techniques had been introduced to the field of herbal identification, for example, metabolomic profiling has been used for herbal authentication and fingerprinting (Murch et al., 2004). This methodology has recently gained momentum (Frédérich et al., 2011; Kang et al., 2008; Xie et al., 2008; Xiang et al., 2011), together with the improvement of DNA fingerprinting performed with novel techniques, like DNA barcoding (Ma et al., 2010a; Zuo et al., 2011).

Since 2008, in parallel with the increase in pharmacological studies, a steady trend has been established for papers on omics applied to botanical studies of TCM, and now represents one quarter of the total number of articles published.

Pharmaco-toxicological studies of CHM using omic techniques addressing biological effects and pathway perturbation began in

[3] The complete string for the Boolean search on PubMed performed on 1 October 2011 is shown in the supporting information.

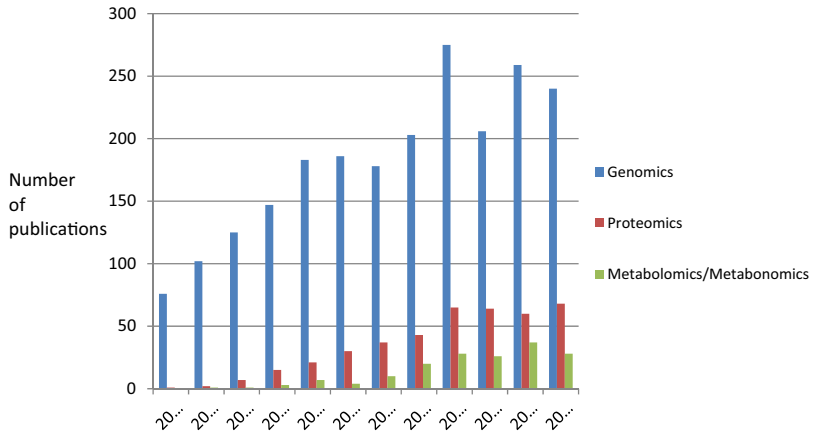

Fig. 1. Histogram showing the total number of pharmaco-toxicological publications in the broad field of the application of omics to medicinal herbs (not restricted to Chinese medicine) 2000–2011 (up to September only for 2011) from a bibliographic search carried out in October 2011.

the early 2000s (Lee et al., 2002), following mainstream scientific evolution of their application to pharmacological studies (Zanders, 2000). Since then there has been a limited, but significant, production of experimental work in the field using both reductionist as well as holistic approaches. In the last two years about twenty papers per year were published, very few considering that they represented slightly more than 5% of all papers dealing with omics and herbal preparations in this period. Nevertheless, this small number represented a jump from a production of only few articles per year before 2008, thus setting a positive trend.

From the beginning, most activity-oriented papers have used omic techniques with a holistic approach, with experimental models appropriate for the examination of multi-chemical perturbation of multiple targets. Indeed this represents the real context in which CHM should be characterised pharmacologically (Kang, 2008), thus exploiting the full potential of the systems biology approach (Auffray et al., 2009; Naylor and Chen, 2010). Taken together, systems biology and omic techniques represent a unique opportunity to understand the complex biological perturbations as the explanation of and the basis for the application of TCM for therapeutic intervention and disease prevention. TCM offers a large number of examples in which several active ingredients in one prescription are aimed at numerous targets and work together to provide therapeutic benefits, a concept that has been given the definition of "magic shrapnel", or "herbal shotgun" as opposed to the "magic bullet", or "silver bullet", concept (Chen et al., 2006; Williamson, 2001), which for many years has driven the quest for pharmacologically active molecules. Phytopharmaceutical research has indeed focused for long on the search for the (mostly single) "active principle", but single components can behave in completely different ways from a phytocomplex. A change of paradigm towards the application of complex mixtures in medicine may thus be possible with the application of omic technologies, paving the way to new fields of phytogenomics, -proteomics and -metabolomics (Ulrich-Merzenich et al., 2007).

The present Boolean search indicates that, as with botanical characterisation studies, experimental pharmacological models in CHM have followed the mainstream evolution of omics techniques (Fig. 2). Thus, initially DNA microarrays for transcriptomic analysis were the tools most frequently used, then the use of proteomic strategies became more frequent in the mid-2000s, while wider application of metabonomic approaches emerged later, and are now looked at with increasing interest in the TCM research community.

Thanks to their unique ability to simultaneously monitor the expression of thousand of genes, cDNA and/or oligonucleotide microarrays have been used *in vitro* and *in vivo* to identify pathways affected by phytocomplexes from CHM, both from single medicinal plants and from Chinese formulae. Such a transcriptomic approach has been used to study the biological effect of CHM in a wide range of conditions, from cancer to fibrosis, and gastrointestinal, neurological, metabolical and immune disorders (Cheng et al., 2008; Efferth et al., 2011; Guo et al., 2010; Kang et al., 2005; Meng et al., 2010; Pan-Hammarström et al., 2006; Sakai et al., 2007; Sun et al., 2008; Watanabe-Fukuda et al., 2009; Wang et al., 2011a,b,c; Yin et al., 2004; Zhuang et al., 2004). An interesting recent development in the use of transcriptomics indicated that this method can be used as a platform for translational research in CHM, by comparing the transcriptomic patterns with genetic databases of drug-altered genes (Lamb, 2007), drug metabolism-related genes and toxicity-associated genes (Hayes et al., 2005). Indeed these combinations might allow improved therapeutic prediction, drug development and safety evaluation (Cheng et al., 2010; Wen et al., 2011).

Proteomic techniques have been applied to CHM treatment in, for example, cardiovascular disease, epilepsy, cancer and psychiatric conditions (Fan et al., 2010; Hung et al., 2010; Lo et al., 2010; Pennington et al., 2009; Tian et al., 2010). In almost all cases proteomic analysis was performed in *in vitro* experimental models using two-dimensional gel electrophoresis coupled with mass spectrometric peptide analysis, but the affected pathways were not always thoroughly characterised.

In contrast to proteomics, metabonomic studies have been performed in *in vivo* models or involving human samples, in most cases using urine. Metabolic profiles are most frequently obtained using ultra-performance liquid chromatography coupled with mass spectrometry (UPLC/MS) and followed by multivariate analysis. The use of metabonomics is only relatively recent in the TCM research field, and only a few articles have been published so far, but there seems to be a rapidly increasing consensus among researchers that metabonomics might actually develop into the most feasible omic technique for CHM studies, not only for its ability to generate a complete perspective of the functional consequences of ectogenic stimuli, but also for the noninvasive, simple and less expensive characteristics of the methods (Wang et al., 2010a,b). Indeed metabonomics is based on the analysis of entire patterns of low molecular weight compounds, rather than focusing on individual metabolites whether of high or low molecular weight, thus allowing a vision on complete organism pathways following exposure to a perturbation (Lu et al., 2011; Su et al., 2011). This strategy coincides well with the integrative and systemic features of TCM, and has been chosen by several to study Chinese medicine syndrome patterns (Lu et al., 2011; Mamtimin et al., 2011; Wang et al.,

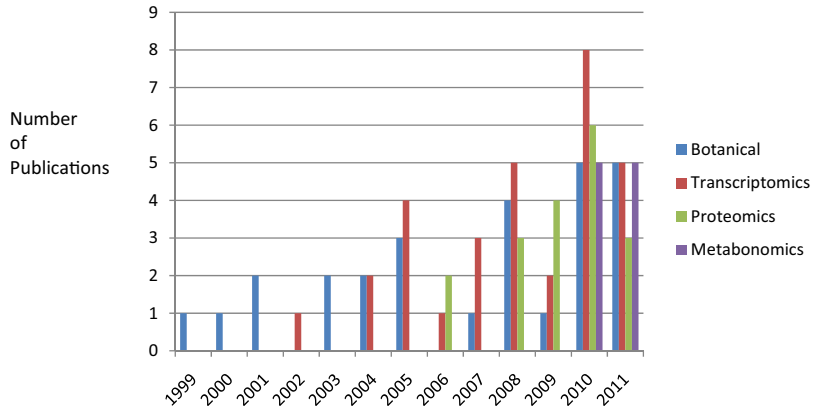

Fig. 2. Histogram showing the proportional increase in 'omic-related publications in the years 1999–2011 (up to September only for 2011) from a bibliographic search carried out in October 2011. The first main application of all techniques was in plant definition but in the last few years transcriptomic, proteomic and metabonomics publications have increased.

2010a,b) involving cancer, psychiatric conditions, cardiovascular disease, and gastric and renal diseases and dysfunctions (Liang et al., 2010; Ma et al., 2010b; Mamtimin et al., 2011; Su et al., 2011). Notably, most authors extol the advantages of using metabonomic approaches.

The examples given in this paper cannot be exhaustive and are simply meant to be representative of the literature (emphasizing the sectors in which work has been done and to indicate the likely direction of development in the application of the techniques mentioned).

3. Omic techniques most frequently used in TCM research

3.1. Genomics

Quality control and sample variability of TCM materials and products are among the first issues successfully addressed by omics techniques in the TCM research field. A number of DNA polymorphism-based assays have been developed for the identification of herbal products, where DNA is amplified by polymerase chain reaction (PCR) and the cDNA can be sequenced, a procedure utilised in TCM research to identify different species of *Fritillaria* as early as 1999 (Cai et al., 1999). DNA chips with DNA sequences unique to a given species of medicinal plant were soon developed for plant material identification and applied to CHM samples (Carles et al., 2001). This proved to be a more powerful tool that can also be used for analyzing mixed herbal preparations, which for instance, allowed the determination of *Dendrobium nobile* in a Chinese medicine formulation containing nine different herbal components (Zhang et al., 2003). Thus DNA chip technology can provide a rapid, high-throughput and information-rich tool for genotyping, quality assurance and species authentication (Chavan et al., 2006; Tsoi et al., 2003).

A further development of DNA chip technology is represented by the "DNA barcoding" method, which can be used for recognition and identification of plant samples through comparison of sequences of a standard short DNA fragment, "DNA barcode", from an unknown specimen to a library of reference sequences from known species. An optimal DNA barcode region is a small fragment present in all species of a major taxonomic group, having invariable nucleotide sequence in all members of the same species, but with sufficient variation to discriminate species (Hollingsworth et al., 2011). This method has been used for establishing an accurate and effective identification system for *Panax* species. Using 95 ginseng samples, representing all the species in the genus, considerable differences were found in the performance of the potential barcoding regions and an efficient method for identifying all the species and clusters in the genus was established (Zuo et al., 2011). This is a more powerful methodology compared to the previous techniques used for identifying ginseng samples, such as DNA fingerprinting by rapid amplification of polymorphic DNA (Mihalov et al., 2000), microchip electrophoresis coupled with the PCR-short tandem repeats (STR) technique, and fluorescence detection (Qin et al., 2005).

New innovative automated assays and specific tools for DNA analysis are continuously emerging, including mini sequencing, nanoscale DNA sequencing or microsphere-based suspension arrays which have a high multiplexing capacity and great potential for genotyping and future taxon identification. These will undoubtedly contribute to the next generation of genomic technologies (Heubl, 2010).

DNA-based techniques thus represent an effective platform for CHM identification. Nevertheless, they have certain limitations, mostly related to the quality and quantity of the DNA from dried or processed materials, as well as high concentrations of secondary plant metabolites which may influence DNA extraction or PCR reaction. In addition, different parts of the same plant and herbs of the same species grown under different conditions have the same DNA sequences but their quality, clinical utility and indications might be very different.

Microarray analysis of gene expression is a mature gene chip technology that allows rapid and detailed analysis of thousands of transcripts. When used in this way, the microarray and other relevant methods are referred to as transcriptomic technologies. Given their ability to identify simultaneously the variations in expression of multiple genes, this represents one of the most powerful tools for elucidating the molecular mechanisms and networks underlying the complex pharmacological function of CHM preparations and, properly coupled with bioinformatics and statistical tools, it can be applied to pharmacodynamic, pharmacokinetic and toxicological characterisation of herbal drugs. A positive correlation between the herbal drug induced transcriptional pattern and a transcriptomic database profile of a known therapeutic agent, by comparison, can suggest target specificity, mechanism of action and downstream effects. Likewise it is possible to gain insight into toxicological effects by comparing gene expression profiles with those known to be relevant to toxicity, or for drug metabolism (Chavan et al., 2006). In addition, several molecular mechanisms and targeted pathways have been elucidated in CHM using transcriptomics. Some examples of *in vitro* and *in vivo* transcriptomic studies of CHM are given below.

cDNA microarrays have been used to investigate the mechanism of action of *Tripterygium hypoglaucum*-induced apoptosis in HL-60 cells. Sixteen genes were identified to be differentially expressed upon treatment, including NF-κB-related genes, c-myc binding protein, caspase-3 and caspase-8. This allowed insight into the mechanisms of its pro-apoptotic activity (Zhuang et al., 2004). Cell growth arrest and apoptosis were also the subject of a study investigating the *in vitro* effect of an extract of ***Coptidis rhizoma*** (the rhizome of *Coptis chinensis*) on human breast cancer cells. In this case the use of DNA microarray revealed that IFN-β mRNA was dramatically upregulated, suggesting that the antiproliferative effect might be mediated by interferon-β (Kang et al., 2005). The induction of cytokines was also investigated in both peripheral blood mononuclear cells and spleen cells upon treatment with **VI-28**, a TCM formula comprising principally ***Radix Ginseng***, ***Cornu Cervi Pantotrichum*** and ***Fructus Cnidii***, to investigate its effect on immune function. The cytokine gene expression profile showed several cytokine and cytokine-related genes to be differentially regulated, suggesting an effect on both innate and acquired immune functions (Pan-Hammarström et al., 2006).

The effect of **San Huang Xie Xin Tang**, a TCM formula traditionally comprising a mixture of roots of *Scutellaria baicalensis* and *Coptis chinensis* with the root and rhizome of rhubarb species (*Rheum* sp.), on a human hepatoma cell line (HepG2 cells), was investigated *in vitro* using DNA microarrays. Gene set enrichment analysis indicated that the cytotoxic effect of the phytocomplex was in some way associated with changes in the p53 signalling pathway. Using a network analysis to visualise the possible relationship between p53 and other regulated genes, it was established that most of them were connected to p53, thus indicating a central role for p53 in the apoptosis-inducing network. Moreover, using hierarchical cluster analysis, it was shown that one of the plants in the formula, ***Rhizoma Coptis***, showed a similar pattern, thus allowing the identification of the likely principal component of the formula for this effect (Cheng et al., 2008).

DNA microarrays used *in vivo* were usually associated with the characterisation of the pharmacological effect of TCM. In an experimental mouse model of Parkinson's disease, microarray analysis was performed using Genechip® Mouse Genome Array, following treatment with **Tokito**, a TCM-derived Japanese herbal remedy comprising ten medicinal plants (***Angelicae Radix***, ***Pinelliae Tuber***, ***Cinnamoni Cortex***, ***Ginseng Radix***, ***Magnoliae Cortex***, ***Paeoniae Radix***, ***Astragali Radix***, ***Zanthoxyli Fructus***, ***Zingiberis Siccatum Rhizoma*** and ***Glycyrrhizae Radix***), that had been previously shown to alleviate symptoms in some patients with Parkinson's disease. The analysis allowed the identification of a down-regulated gene, the serum and glucocorticoid regulated kinase (SGK), believed to be involved in the pathogenesis of Parkinson's disease, thus suggesting a molecular mechanism for the effect of **Tokito** (Sakai et al., 2007).

Orengedokuto, another TCM-derived Japanese herbal medicine (usually comprising ***Coptidis Rhizoma***, ***Scutellariae Radix***, ***Phellodendri Cortex*** and ***Gardeniae Fructus***), used for various indications including gastric ulcers and gastritis, protects mice from indomethacin-induced enteropathy. This effect was studied *in vivo* using a transcriptomic approach to guide the search for the molecular mechanism(s). Gene expression data indicated that the adenosine system, especially adenosine deaminase, might be involved in the effect–this result was then validated by biological experiments (Watanabe-Fukuda et al., 2009). As reviewed in this special issue by Liu and Cheng (2012), PHY906 is a standardised TCM formula under clinical evaluation as an adjuvant to relieve the side effects associated with chemotherapy. It comprises a formulation of four Chinese herbs: *Glycyrrhiza uralensis*, *Paeonia lactiflora*, *Scutelleria baicalensis* and *Ziziphus jujube*. Using a murine tumour model, gene expression was studied in groups of animals treated by an antitumour compound (CPT-11), PHY906 or their combination. Gene expression microarray analysis was performed using RNA from tumour tissues or control tissues. The results indicated that the networks activated by CPT-11 and PHY906 were different, though both acted on pathways relevant to the antitumour effect. Importantly, when used in combination, the gene expression pattern changed and the antitumour effect of CPT-11 was enhanced, with prevalent induction of pro-apoptotic and pro-inflammatory pathways that may favour tumour elimination (Wang et al., 2011a,b,c).

The full transcriptomic potential though can be more appreciated when using genetic association databases. By comparing the gene expression signatures of formulae with those of disease states or known drugs, it is possible to predict unknown biological effects or either identify or exclude unwanted interactions with drugs or toxicological effects (Youns et al., 2010). One such example was carried out by Cheng et al. (2010).

Transcriptomics was actually used as a platform of translational medicine, and DNA microarray technology was used to analyse the biological events induced in mice by 15 different herbal formulae to predict their therapeutic potential, as well as their safety. The assays were performed using Mouse Whole Genome OneArray™; groups of up-regulated genes were detected and 352 pathways were analysed using several on-line databases. Then a connection analysis of formulae-altered genes and diseases-altered genes was performed using the genetic association database (Becker et al., 2004). The expression signatures of formulae-regulated genes were connected with those of drug-regulated genes using the Connectivity Map (Lamb, 2007). Analysis of expression levels of genes associated with drug metabolism and toxicity was also performed. Using this strategy it was possible to establish significant similarities with expression signatures and a surprisingly high number of findings was achieved, allowing the prediction of both therapeutic potential and safety of the formulae analysed (Cheng et al., 2010). A systematic approach using DNA microarrays, bioinformatics and the Connectivity Map was also used *in vitro* to examine TCM-induced changes in gene expression.

The herbal formula **Si Wu Tang**, comprising ***Radix Angelicae Sinensis***, ***Rhizoma Chuanxiong***, ***Radix Paeoniae Alba*** and ***Radix Rehmanniae Preparata***, traditionally used in oestrogen-related disorders, was added to MCF-7 human breast cancer cells. Significant gene expression changes were observed and the **Si WuTang**-induced gene expression profile was compared with those of 1309 compounds in the Connectivity Map database. Consistent with the formula's claimed use for women's health, the Connectivity Map of estradiol-treated MCF-7 cells showed an excellent match with **Si Wu Tang** treatment, suggesting a phytoestrogenic effect (Wen et al., 2011).

Transcriptomic information can be adapted into microRNomics, a technical approach devoted to expression of miRNAs – the expression of approximately one third of the transcripts is controlled by miRNAs, small non-coding RNAs that regulate gene expression and emerge as critical mediators of diseases (for review, see Bartel, 2009). miRNA studies are likely to be important in TCM research although existing data are scarce and most of those that have been reported concern cases where TCM compounds were used as chemotherapy co-adjuvants. For example, it was reported that a TCM product, **Aidi injection** (prepared from cantharidin, together with species of the genera *Panax*, *Astragalus* and *Acanthopanax*) changed breast-cancer miRNA profiling which might suggest that miRNA alterations could be one of the mechanisms of action in TCM (Zhang et al., 2011).

3.2. Proteomics

Proteomics emerges as an area that promises to transform biology and medicine, since changes in mRNA expression often correlate poorly with changes in protein expression, and protein function depends on many post-translational modification and varies with protein localisation. Thus proteomic research aims at understanding the expression and functions of proteins on a global level and requires to be integrated with other information such as profiles of genes, mRNAs and metabolites, in order to fully understand how biological systems work. The integrative proteomic approach thus exactly maps to the holistic concept and practice of TCM. Proteomic technologies have been used to study pharmacological effects and mechanisms of action of relevant CHM, to identify target molecules for CHM or even to identify new bioactive components.

Currently, two approaches based on mass-spectrometry are the most frequently used for global quantitative protein profiling: (1) two-dimensional electrophoresis (2DE) followed by staining, selection and identification by mass-spectrometry; (2) isotope tags to label proteins, separation by multidimensional liquid chromatography and mass spectrometry analysis (for review, see Kandal et al., 2009; Weston and Hood, 2004). Both basic proteomic approaches can be supplemented with useful information provided by molecular imaging. In fact, combination of proteomic approaches and *in vivo* imaging has been successfully used for the study of the metabolism of flavonoids from ***Herba Epimedii*** in zebra fish (Li et al., 2011).

One of the most interesting applications of proteomics in CHM is the ability of these techniques to identify different species as in the case of *Panax* (*P. ginseng vs. P. quinquefolium*) (Lum et al., 2002). This will be a very valuable tool for quality control, toxicity studies and standardisation of TCM preparations and decoctions, key points in the use of CHM in Western countries.

Complementary to genomic and transcriptomic approaches, proteomic assays have been successfully used for describing mechanisms of action of many different TCM preparations, such as **Si Wu Tang** decoction effects in general health (Guo et al., 2004), *Ganoderma* properties in nerve injury (Zhang et al., 2006), *Salvia miltiorrhiza* effects on atherosclerotic lesions (Hung et al., 2010) as well as several other effects of preparations in cancer, which have been investigated at the proteomic level (Hung et al., 2010). In addition, the use of proteomic data has also shed light into the effect of the TCM formula **Shuanglong** on differentiation and use of pluripotential cells in myocardial ischemia repair (Fan et al., 2010).

Although interpretation of proteomics as well as other omic techniques is difficult due to their inherent complexity, compounded in view of the variety of the components in TCM decoctions, proteomics is nonetheless becoming a useful tool for elucidating multitarget effects of complex herbal preparations, as well as for the discovery of single bioactive compounds, development of active fractions, characterisation of safe herbal prescriptions and eventually personalised molecular diagnosis for TCM treatments (Cho, 2007).

3.3. Metabolomics and metabonomics

"Metabonomics broadly aims to measure the global, dynamic metabolic response of living systems to biological stimuli or genetic manipulation. The focus is on understanding systemic change through time in complex multi cellular systems. Metabolomics seeks an analytical description of complex biological samples, and aims to characterise and quantify all the small molecules in such a sample. In practice, the terms are often used interchangeably, and the analytical and modelling procedures are the same" (Nicholson and Lindon, 2008).

Metabonomics in pharmaceutical research and development has been reviewed (Lindon et al., 2006). The term **metabolomics** was actually coined for comprehensive, nonbiased, high-throughput analyses of complex metabolite mixtures typical of plant extracts (Hall et al., 2002), and the use of this term is mostly restricted to this field. Chromatographic fingerprinting of CHM has proved to be a comprehensive strategy for assessing quality of herbal medicines (Liang et al., 2010). Metabolomic profiling using Fourier transform ion cyclotron mass spectrometry (FTMS) has been used for screening extracts from different germplasm lines of *Scutellaria baicalensis* to profile, identify and quantitatively compare metabolites, with the aim of identifying the best phytochemical profiles and to compare the effects of different growth conditions (Murch et al., 2004). NMR spectroscopy-based metabolomics has been used to differentiate *Panax ginseng* roots of different origins. The methodology was efficient not only at distinguishing ginseng roots from different origin, but could also discriminate material of the same origin, but of different age (Kang et al., 2008). In a different study five *Panax* herbs were profiled using ultra-performance liquid chromatography–quadrupole TOF MS (UPLC–QTOFMS) and multivariate statistical analysis (Xie et al., 2008). UPLC has the ability to generate many discrete peaks in a short time, thus facilitating the simultaneous analysis of complex samples with diverse chemical characteristics, while the high precision of the mass information generated with TOF MS analysis allows its application even in untargeted metabolite profiling. The method proved reliable for the rapid analysis of a group of metabolites present in the herbal extracts (Xie et al., 2008). Another methodology used in metabolomic profiling of CHM is hyphenated gas chromatography–mass spectrometry. This methodology, coupled with multivariate statistical analysis, has been used to discriminate and assess the quality of samples of three *Curcuma* species from different ecotypes (genetically distinct geographic varieties, populations or races within species adapted to specific environmental conditions). Samples were analysed, and using principal component analysis, partial least squares and discrimination analysis, characteristic components with an important influence on the separation of the different groups were identified. The methodology thus not only discriminated efficiently among samples, but the identification of characteristic components provided new valid chemical markers that have been proposed to be used for discrimination and quality control of *Curcuma* samples (Xiang et al., 2011).

The use of **metabonomics** has been welcomed by some as the ultimate phenotyping. Using a systemic approach, it applies a "top down" strategy to study the functions and perturbations of a biological system, from the end products of the metabolic network. This endows metabonomics with a holistic view, making it closer to TCM thinking and particularly fit to study pharmacological and toxicological effects of CHM (Lao et al., 2009; Wang et al., 2005, 2011a,b,c; Zhang et al., 2010). Used with a holistic approach, metabonomics has been also exploited to study and characterise Chinese medicine syndromes in several experimental models, recently reviewed (Wang et al., 2011a,b,c; Zhang et al., 2010). Metabonomics involves global low molecular weight metabolite profiling in complex biological fluids or tissues to study downstream homoeostatic perturbations in biochemical pathways. One of the major benefits of metabonomics is that urine or plasma samples can be used for analysis, thus allowing large scale research. Also metabolic profiling can, in the same experimental context, give information of pathophysiological, pharmacodynamic, pharmacokinetic and diagnostic value. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), are the most used analytical techniques for metabonomic applications, following the separation of metabolic components using gas chromatography or, more often, UPLC. Combination of UPLC/MS is often used to obtain the largest possible biochemical profile. All metabolomic

and metabonomic studies result in complex multivariate datasets that need computer programmes for visualisation and chemometric as well as bioinformatic methods for interpretation (Zhang et al., 2010). The therapeutic activity of a classical TCM formula, **Liu Wei Di Huang Wan** (LW), comprising a mixture of ***Radix Rehmanniae Preparata***, ***Fructus Corni Officinalis***, ***Cortex Moutan Radicis*** (***Paeonia Suffruticosa***), ***Rhizoma Dioscoreae Oppositae***, ***Sclerotium Poriae Cocos*** and ***Rhizoma Alismatis Orientalis***, was investigated in a rat model of shen yin deficiency, using a metabonomic approach. The experimentally induced condition of shen yin deficiency was characterised by changes induced in the animals' urine metabolic profile, which were restored to their baseline values following treatment with LW (Wang et al., 2010a,b).

A further study assessed the efficacy of another TCM formula, **Shuanglong** (a so-called 'blooming tea', usually comprising the flowers of *Gomphrena globosa* [red globe amaranth] and jasmine [often *Jasminum sambac*] in a base of tea [*Camellia sinensis*] leaves) in myocardial infarction in rats. Urinary samples from treated and control rats were collected and analysed with UPLC/ESI-SYNAPT^{TM}-HDMS^{TM}. Potential urine biomarkers correlated well with serum biochemistry and histopathology of myocardial infarction, and also with the beneficial effect of the formula. The analysis of urinary metabolic pathways also suggested that the **Shuanglong** effect is linked with the regulation of myocardial energy metabolism (Liang et al., 2011).

The therapeutic effect of ***Rhizoma Drynariae*** on an experimental rat model of shen yang deficiency syndrome was investigated by comparing urine profiles from diseased and treated rats. The metabonomic patterns indicated that energy metabolism, amino acid metabolism and gut microflora pathways were implicated in the effect (Lu et al., 2011). Metabonomics has also been used to profile the metabolic fingerprints of urine obtained from rats with stress-induced chronic depression with and without treatment with **Chaihu Shu Gan San** (CSGS), a TCM formula, comprising *Bupleurum chinense*, *Citrus reticulata*, *Paeonia lactiflora*, *Citrus aurantium*, *Cyperus rotundus*, *Ligusticum chuanxiong* and *Glycyrrhiza uralensis*, widely used for the treatment of depression. Metabolites with significant changes were characterised as potential biomarkers involved in the pathogenesis of depression. Most of them were regulated by CSGS treatment suggesting that the effect of CSGS on depression might involve energy metabolism, tryptophan metabolism, bone loss and liver detoxification (Su et al., 2011).

A recent development has been the proposal of so-called 'Fangjiomics' as an approach involving in the design, production and pharmacological evaluation of herbal recipes (Wang et al., 2011a,b,c), acting through multiple targets, modes of action and biological pathways. There are around 50 ***Fangji*** preparations. In a similar to classical TCM use, every ingredient in a ***Fangji*** (formula) is classified into 1 of 4 categories: in order of importance, ***Jun*** (sovereign), ***Chen*** (minister), ***Zuo*** (assistant) or ***Shi*** (envoy). Genomics, proteomics and metabonomics are key approach of Fangjiomics, since it is characterised by integration of information from many levels of study. Systems-based therapeutics could be the next big challenge in the generation of new combination drugs, and their optimal use can be investigated in experimental models with omics.

4. Good practice in the application of omics to TCM research – report from GP-TCM discussion group on functional genomic techniques

GP-TCM (*Good practice in traditional Chinese medicine research in the post-genomic era*) is an EU-China consortium on TCM research. It was formed under the EU's Seventh Framework Programme with the objective to revise the state of the art of TCM research and propose guidelines for good practice in TCM research (Uzuner et al., 2010).

Regarding the application of omics in TCM research, a questionnaire was circulated in spring 2011 among a group of GP-TCM researchers "*D4.14 – Discussion group on use of functional genomic techniques for in vitro CHM research*" (the original document can be consulted on http://www.gp-tcm.org/about/deliverables). The questionnaire addressed issues related to the use of omic techniques in TCM research and most researchers agreed on several pros and cons. The most relevant conclusions were:

- Quality control of the test material at all stages of preparation and production and its standardisation still remain the main barrier to meaningful research, but omic techniques can and increasingly do contribute significantly to this important aspect.
- Thanks to its wider view of biological systems and closer view of simultaneous multiple effects exerted by phytocomplexes, systems biology can contribute to increased participation of CHM in the scientific mainstream.
- Omic technologies are excellent at providing information on whole collections of molecules. These can then either be studied further using a reductionist approach to establish the properties and function of each or to try to understand and validate the relationship between them and their functions.

For these reasons, one single methodology is not considered sufficient to investigate mechanisms of action of CHM. A well-consolidated pipeline should be used comprising *in silico* evaluation, *in vitro* and *in vivo* validation through a combination of classical biochemical signalling work, conventional molecular biology, omics technology and bioinformatics.

5. Conclusions and future directions

Omic methodologies were developed in response to the need for information-rich, coherent molecular biological strategies and have been applied to different phases of CHM research starting from standardisation and quality control of herbal formulae, characterisation of target-mediated and downstream effects, the identification of molecular mechanisms to prediction of side effects and interactions with other drugs. Thanks to their potential to unveil the complex pharmacological networks induced by complex herbal preparations, omic techniques can thus be considered a powerful tool to address many open questions in CHM research.

Despite the many advantages of omic techniques, caution still surrounds their application to CHM, and to pharmacognosy in general, mainly because of the current limited experience. Nevertheless, there is a common and general recognition that a systems biology approach is the best answer to study phytocomplexes. The results obtained so far with the applications of omic technologies have been promising but much more work needs to be undertaken to examine their full potential in CHM and wider TCM research. Indeed, it could be argued that the study of TCM is a perfect field for the demonstration of the benefits of systems biology using all the emerging technologies because of the recognition that a disease is usually not a single target issue, but a complex perturbation involving multiple pathways, a recognition of the fact that TCM follow just this principle (Wang et al., 2005). While all the omic techniques are slowly but firmly pushing their way further in CHM research, the youngest of them, metabonomics, seems to be rapidly gaining ground with respect to the others. This is probably due to the simplicity of the experimental design and to the better affordability of the method, which allows direct and detailed analysis of large numbers of biological samples which, like urine, can be obtained very easily. What is really attractive about metabonomics though is the

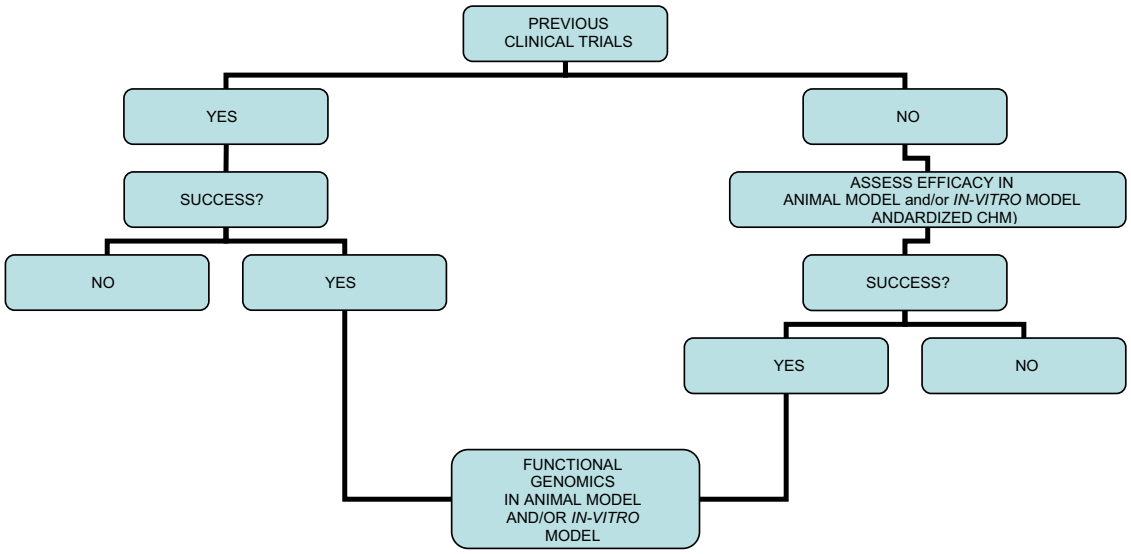

Fig. 3. The possible place of an omic study in TCM research.

possibility of examining complete metabolic pathways and their intermingled interactions, in just one snapshot, taking a whole picture of the downstream outcomes of any biological perturbation. This would appear as the ultimate systems biology phenotyping and is particularly fit for studying TCM, with its holistic view of biological effects. Accordingly, not only metabonomics is used to study the action of Chinese formulae, but is being increasingly used to successfully characterise TCM syndromes. Characterizing both diagnostic Chinese medicine profiles and CHM pharmacological activities with Western science-developed metabonomics, might actually lead to a faster and more efficient way to bridge modern Western medicine with Chinese medicine. Considering the rapid evolution of personalised medicine as a new model of systems biology approach to TCM (Naylor and Chen, 2010; Van der Greef et al., 2006), the time might have come to open new avenues towards an integrative medical approach exploiting the knowledge of both oriental and Western medicine backgrounds.

6. Suggestions for further guidelines

The consortium has identified at least two main issues which need to be addressed in the field of omics in CHM studies using experimental models of disease:

6.1: There is an intrinsic problem of replicating the patterns of human disease in animal or cellular models. Both types of model are rarely a close representation of the clinical scenario and are not widely accepted as authentic, or standard – indeed some animal or disease models are not even clearly characterised or validated. Therefore prior validation and standardisation of animal models where omics could be applied to the study of TCM is an absolute requirement, as has been the case in Western medicine research.

6.2: Omics methods are extremely powerful but since they approach the basic components of the systems studied, they are also liable to variability. A particular source of variability however that has not been generally considered in most publications on CHM in experimental models of disease is the generalised use of *non-standardised* research materials (in terms of the constituent herbs and herbal preparations), which itself promotes variation. This lack of rigour significantly reduces the scientific value and impact of these studies. This implies that before using difficult and expensive omic technologies, it is necessary to have a robust control of plant mixture preparation (batch to batch variability), as well as the experimental model: cell cultures and animal system (organism variability as well as technical procedures). In addition, pharmacokinetic (absorption, distribution, metabolism and excretion) profiling of a given CHM will identify *in vitro* and *in vivo* bioavailable drug-like components and reveal determining factors for availability, dynamics and individual variations of 'real' active components to target sites of action.

Without addressing these two basic problems, omics research in TCM may fall foul of discipline, and descend to anarchy. Given that omic technologies may help elucidate the mechanism of action of a given CHM treatment, we suggest that these studies have to be applied on those CHM treatments the efficacies of which have been previously demonstrated. Since most relevant pieces of evidence on efficacy come from clinical trials, it is thus advisable to start applying omic technologies to animal models of diseases for which efficacy has been proven in clinical trials. This would render a number of TCMs relatively easy to deal with.

In this context, we propose the scheme in Fig. 3 for starting successfully to apply omics in TCM research. Additionally, we propose the following workflow for applying omics in experimental *in vitro* and *in vivo* models:

(1) To use a TCM proven to be efficacious in an appropriate experimental model by gold-standard measurement methods.
(2) To assess variability in TCM composition and select a uniform, appropriately defined batch.
(3) To assess variability in the organism population (cell cultures or animals) by carrying out pre-testing omic studies, including metabolic profiles.
(4) To assess the known levels of analytical variation in relation to the changes observed (whether at the transcript, protein or metabolism levels).
(5) To define precisely the experimental groups as well as their size in terms of number of cell types or animals to make precise statistical analysis. It will also be desirable to choose a homogeneous experimental population as far as possible.
(6) To check that the effects observed in cell cultures or animal after TCM use, especially if chronic administration is necessary, are due to the treatment and not to some other variable (*e.g.*, cell passage, proliferation, aging or body weight changes). As much data must be collected as possible in case any of the above differences need to be explained.

(7) To perform the appropriate omic technique and analysis accordingly to the available guidelines on omics standardisation in the literature, such as MIAME (minimum information about a microarray experiment) for transcriptomics (Brazma et al., 2001), MIAPE (minimum information about a proteomics experiment) for proteomics (Taylor et al., 2007), and MSI (*the metabolic standards initiative*; website: http://msi-workgroups.sourceforge.net) for metabolomics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2012.01.055.

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