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Challenges and developments in tandem mass spectrometry based clinical metabolomics

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ABSTRACT

'Clinical metabolomics' aims at evaluating and predicting health and disease risk in an individual by investigating metabolic signatures in body fluids or tissues, which are influenced by genetics, epigenetics, environmental exposures, diet, and behaviour.

Powerful analytical techniques like liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) offers a rapid, effective and economical way to analyze metabolic alterations of pre-defined target metabolites in biological samples. Novel hyphenated technical approaches like the combination of tandem mass spectrometry combined with linear ion trap (QTrap mass spectrometry) combines both identification and quantification of known and unknown metabolic targets.

We describe new concepts and developments of mass spectrometry based multi-target metabolome profiling in the field of clinical diagnostics and research. Particularly, the experiences from newborn screening provided important insights about the diagnostic potential of metabolite profiling arrays and directs to the clinical aim of predictive, preventive and personalized medicine by metabolomics.

1. Introduction

The term "metabolome" describes the total quantity of small molecular weight components (metabolites) presented in biological systems. "Metabolomics" is commonly defined as the study of all metabolites (<1500 Da) expressed in a cell, tissue or organism. The concept of metabolomics is complementary to the other 'omics' sciences (genomics and proteomics) (Goodacre et al., 2004). In the field of clinical drug safety assessment, environmental, plant and microbial science the metabolome approach is already established to gain a more global picture of the molecular networks (Hall, 2006; Mashego et al., 2007; Dunn, 2008).

'Clinical metabolomics' aims at evaluating and predicting health and disease risk in an individual by investigating metabolic signatures in body fluids or tissues. These signatures are influenced by the individual genome, possible epigenetic effects by environmental exposures, diet behaviour and life style. To this end, metabolome concepts add significant new information to the individual phenotype beyond genome and proteome analysis. From the analytical point, the sample complexity is less interfering for analytical identification and quantification of metabolites. Metabolome analyses with liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) are rapid, reproducible and much economically and therefore applicable in clinical routine and large-scale clinical studies. Technical advances particularly in mass spectrometry increased tremendously the use of the metabolome approach (Dunn et al., 2005). Powerful analytical techniques like LC–MS/MS offer a rapid, effective and economical multi-targeted metabolite profiling. Novel hyphenated techniques, like tandem mass spectrometry coupled with a linear ion trap (QTrap mass spectrometry) combine both identification and quantification of known and unknown targets. In the following we summarize established and new concepts of multi-target metabolome profiling in the field of clinical metabolomics by LC–MS/MS.

2. Mass spectrometric concepts in clinical metabolomics

Metabolites are a class of heterogeneous low molecular-weight components, which are characterized by a wide variation in physical and chemical properties (e.g. polarity, volatility, and solubility). Therefore, different analytical approaches are required according to the different metabolite properties (Dunn et al., 2005; Dettmer et al., 2007). Investigations of metabolites can be performed in two ways: (a) 'non-targeted' analyses, in which a large number of metabolites after minimal sample pre-treatment is analyzed non-biasedly under high throughput conditions or (b) 'targeted'

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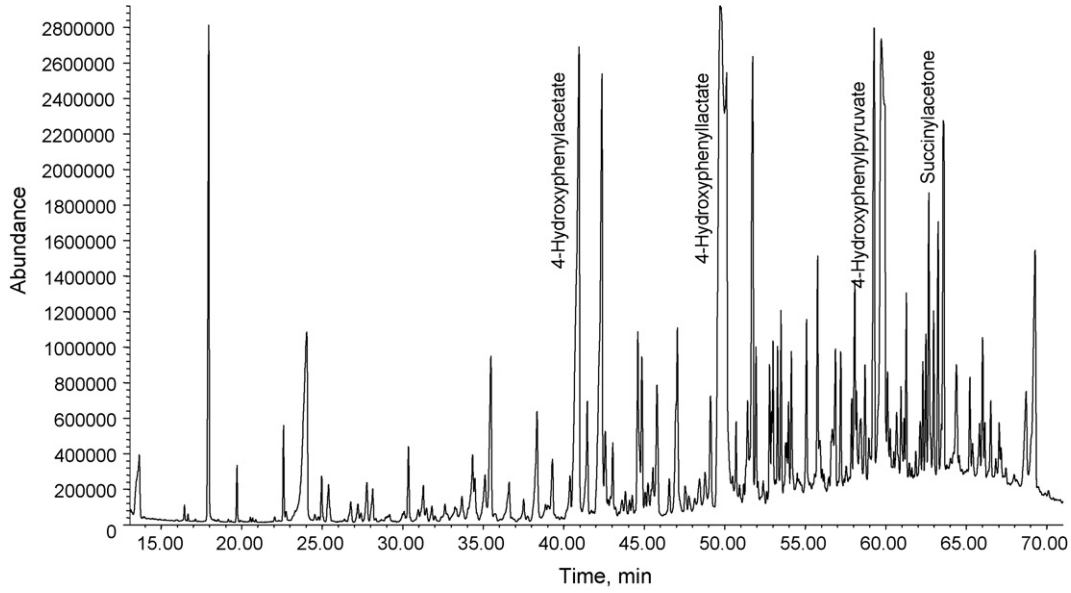

Fig. 1. GC–MS chromatogram of an organic acid analysis in urine from a patient affected with hepatorenal tyrosinosis, an inborn error of metabolism in the tyrosine-metabolism. Only the excretion of the hepatotoxic succinylacetone is evidentiary for the diagnosis of this disease.

analyses including identification and quantification of pre-known metabolites or metabolite classes. The aim of the first approach is the detection of new biomarkers related to the 'healthy vs. diseased' study concept as known from clinical proteomics and genome wide studies (Williams et al., 2005; Wilson et al., 2005; Pattin and Moore, 2008). Time-of-flight mass spectrometry (TOF), ^{1}H NMR spectroscopy and direct injection ion trap mass spectrometry are widely used in this field (Dunn, 2008).

For 'targeted' metabolomics, mass spectrometry with and without chromatography is mainly applied. Gas chromatography combined with electron impact mass spectrometry is the gold standard for the identification and quantification of volatile and thermally stable components. The quantitative analysis of organic acids in biological samples by mass spectrometry was already described in the 1970s (Jellum, 1977; Hoffmann et al., 1989). This approach is still used for diagnostics of a variety of metabolic disorders in urine in the clinical laboratory. The majority of metabolites analyzed by GC–MS requires laborious sample pre-treatment (hydrolyzation, derivatization) and is therefore limited for clinical diagnostic use and large-scale studies. Fig. 1 shows as an example an organic acid urine profile of a patient with hepatorenal tyrosinosis (Weigel et al., 2007). The chromatograms are complex, containing more than 100 metabolite peaks in a run time greater 60 min. Only the detection of the metabolite succinylacetone is indicative for the diagnosis of hepatorenal tyrosinosis, whereas 4-hydroxyphenylacetate, 4-hydroxyphenyllactate and 4-hydroxyphenylpyruvat may be elevated non-specifically in bacterial contaminations and liver immaturity, too.

The quadrupole tandem mass spectrometry platforms, established in the 1990s, are today increasingly used in the field of targeted metabolomics. The application of LC–MS/MS enables the simultaneous quantification of chemically different classes of metabolites without time-consuming and laborious sample pre-treatment (Chace and Kalas, 2005; Dunn et al., 2005). Electrospray (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI) are available for ionization of polar to non-polar components (Marquet, 2002; Baumann et al., 2005). In Fig. 2 the different ionization behaviour of the similar molecular structured steroids testosterone and estradiol by ESI, APCI and APPI is shown. The source-dependent generation of differing charged molecular ion species indicates that ionization conditions dramatically influence the sensitivity of the mass spectrometric detection.

Performing specific MS/MS experiments (precursor scan PS, neutral loss scan NL, and multiple reaction monitoring MRM) selective and sensitive target analysis can be achieved. The remaining limitation of restricted number of MRM transitions per run might be overcome by new software solutions, which enable the use of up to 1000 MRMs by 'scheduled MRM' in one method (Martin et al., 2008).

Novel hybrid linear ion trap-triple quadrupole mass spectrometers (QTrap) combine the selective scans of the triple quadrupole with the high speed and high sensitivity of the ion trap allowing metabolite quantification and characterization in one single scan (King and Fernandez-Metzler, 2006). Additionally, the system enables MS^{3} experiments and time delayed fragmentation

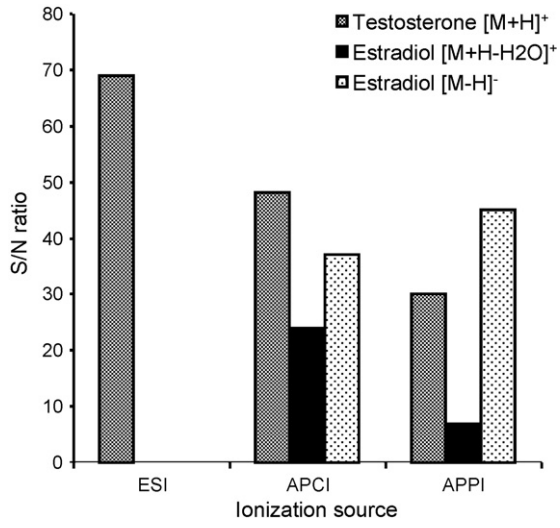

Fig. 2. Signal/noise ratios of the different ion species for testosterone and estradiol (c = 10 ng/mL) dependent from the ionization source and charge; ESI: electrospray ionization, APCI: atmospheric pressure chemical ionization, APPI: atmospheric pressure photo ionization.

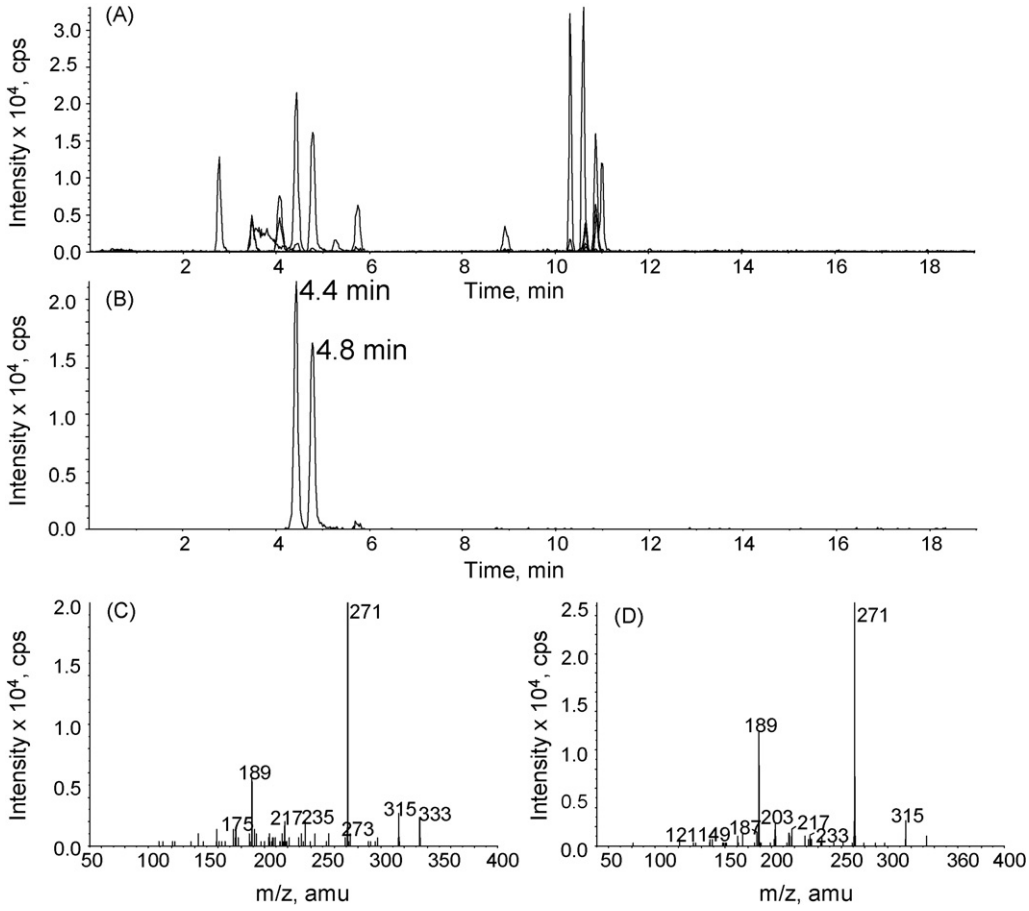

Fig. 3. (A) Total ion current of an eicosanoid standard mixture containing prostaglandin E2 (PGE) and prostaglandin D2 (PGD) (c = 1 ng/mL); (B) MRM transition 351/271 for the isobaric prostaglandins E2 and D2 (C) corresponding fragment spectra for PGE at the retention time 4.4 min and (D) corresponding fragment spectra for PGD at the retention time 4.8 min.

scans for structural elucidation of molecules. In principle the third quadrupole of a tandem mass spectrometer can be used for quantification (quadrupole function) or structural identification (ion trap function). In Fig. 3 the principle of QTrap mass spectrometry is exemplified by an eicosanoid profile analysis. Fig. 3A presents a total ion current (TIC) of a standard mixture of 15 eicosanoids. The analytical method is described by Deems et al. (2007) in detail. Specific MRM transitions were used for quantification of each analyte (Fig. 3B, MRM 351/271). The ion trap function allows a simultaneous structural elucidation via enhanced product ion experiment (EPI). The EPI experiment generates for all mass transitions, exceeding a pre-defined intensity threshold, a simultaneous fragment spectrum without significant loss in signal intensity compared to normal MS/MS analysis. The isobaric prostaglandins PGE 2 and PGD 2 at 4.4 and 4.8 min can be identified by their characteristic fragment pattern using the linear ion trap function (Fig. 3C and D). This can be simply performed by comparison with a spectra library.

In the future, the LC–MS/MS approach may replace GC–MS in the field of high-throughput targeted metabolomics due to the easier sample pre-treatment and the shorter analysis times. On the other hand, the combination of GC with electron impact (EI) mass spectrometry offers advantages like high chromatographic resolution and the availability of spectral libraries, which facilitate the identification of diagnostic markers. Additionally, novel analytical approaches like two-dimensional GC coupled two time-of-flight mass spectrometry enable the simultaneous detection and identification of about 1200 components (Pasikanti et al., 2008). Therefore, both LC–MS/MS and GC–MS should be used complementary to get the best coverage of metabolites inside the biological sample.

3. Metabolite analysis in laboratory diagnostics

Single metabolite tests (e.g. glucose, creatinine, bilirubin, lactate and ammonia) are performed in the daily clinical routine. However, this single test approach limits the diagnostics of certain metabolic and major organ-related diseases.

In contrast, the multiplex metabolomics approach is aimed at the simultaneous analysis of a large number of biological relevant small molecules of various pathways to unveil disease-related metabolic changes. In this context, low molecular weight species like amino acids, fatty acids, carbohydrates, vitamins and lipids in body fluids and tissue are from clinical interest.

The first routine application of tandem mass spectrometry driven targeted metabolite profiling was introduced by Millington and Chace in the 1990s (Rashed et al., 1995; Chace and Kalas, 2005). This innovative technology boosted the worldwide newborn screening programs and enhanced the panel of screening diseases (Tarini, 2007). In the screening panel about 30 metabolic parameters of amino acid and fatty acid metabolism were applied for the detection of inherited metabolic diseases. This was the first multi-parametric metabolic approach which proved as valuable and effective preventive diagnostic strategy (Schulze et al., 2003; Arn, 2007). Today, metabolome approaches for the newborn screening, including pre-analytics, instrumental analysis, data processing

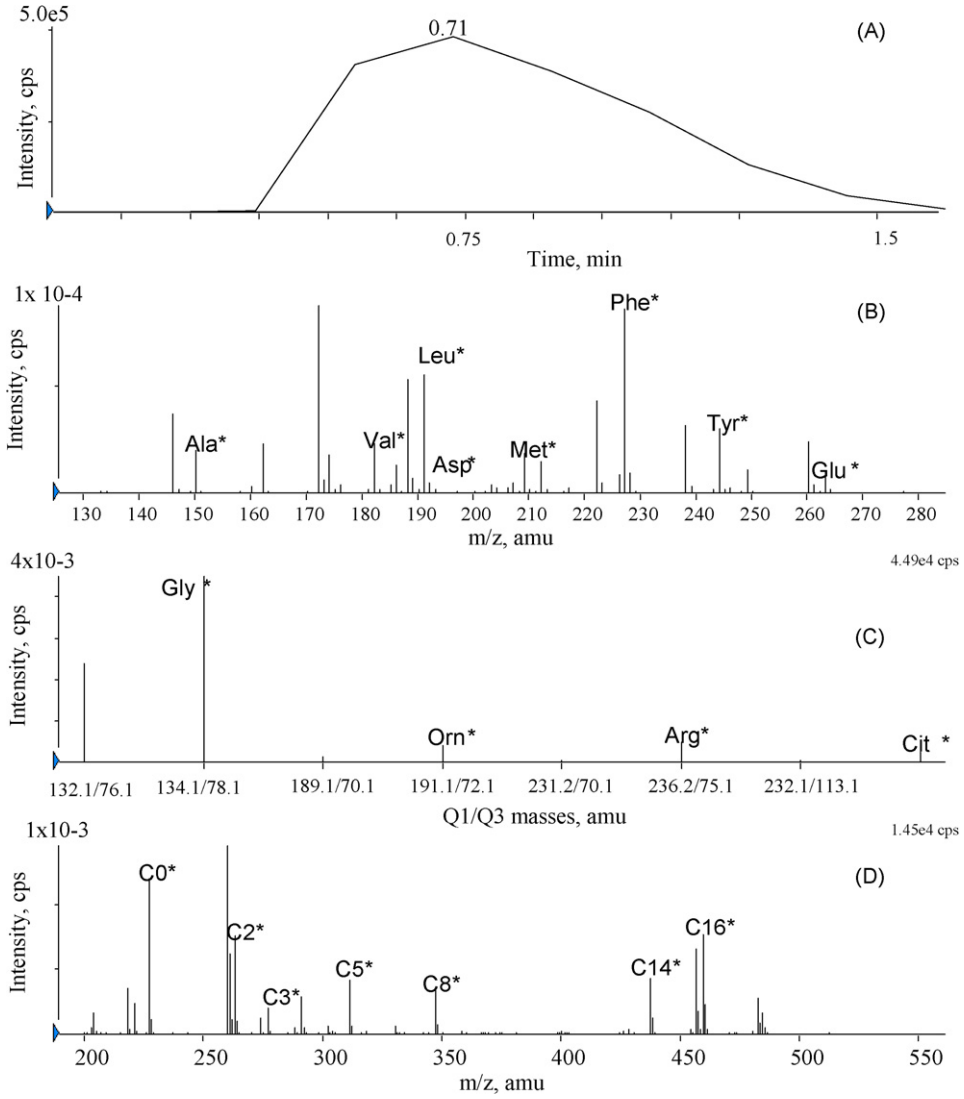

Fig. 4. (A) Total ion current of a tandem-MS analysis for the determination of amino acids and acylcarnitines following the direct injection of a derivatized extract of a dried blood newborn sample; (B) neutral loss experiment ($m/z = 102$); (C) selective MRM-transitions for the determination of amino acids and (D) precursor ion experiment ($m/z = 85^+$) for the determination of acylcarnitines.

Table 1
Newborn screening result sheet for selected acylcarnitines: C0 – free carnitine, C5 – isovaleryl carnitine, C5/C3 Ratio – isovaleryl carnitine/propionylcarnitine, C6 – hexanoylcarnitine, C8 – octanoylcarnitine, C8/C10 – octanoylcarnitine/decanoylcarnitine ratio, C8/C12 – octanoylcarnitine/dodecanoylcarnitine ratio, C8/C10 – octanoylcarnitine/hexadecanoylcarnitine ratio.

Sample	Diagnosis	Parameter with upper cut offs in μmol/L							
		C0 50	C5 0.6	C5/C3 0.22	C6 0.24	C8 0.3	C8/C10 4.6	C8/C12 2.9	C8/C16 0.16
S 1	NAD[a]	35.3	0.18	0.07	0.04	0.05	0.25	0.14	0.01
S 2	NAD	15.7	0.19	0.08	0.06	0.11	0.86	1.36	0.05
S 3	IVA[b]	20.7	*5.30*	*2.29*	0.05	0.08	0.31	0.30	0.02
S 4	NAD	14.8	0.05	0.05	0.07	0.13	0.53	0.45	0.04
S 5	NAD	29.5	0.14	0.09	0.08	0.14	0.65	1.05	0.04
S 6	NAD	19.8	0.08	0.01	0.04	0.04	0.34	0.16	0.01
S 7	MCADD[c]	10.1	0.10	0.04	*1.35*	*7.42*	*11.24*	*41.36*	*5.14*
S 8	NAD	24.9	0.10	0.04	0.03	0.05	0.36	0.27	0.01
S 9	NAD	20.1	0.03	0.04	0.03	0.00	0.00	0.00	0.00
S 10	NAD	33.1	0.08	0.04	0.05	0.08	0.83	0.31	0.02

[a] NAD – no abnormality detected.
[b] IVA – isovaleric aciduria.
[c] MCADD – medium-chain acyl coenzyme A dehydrogenase deficiency.

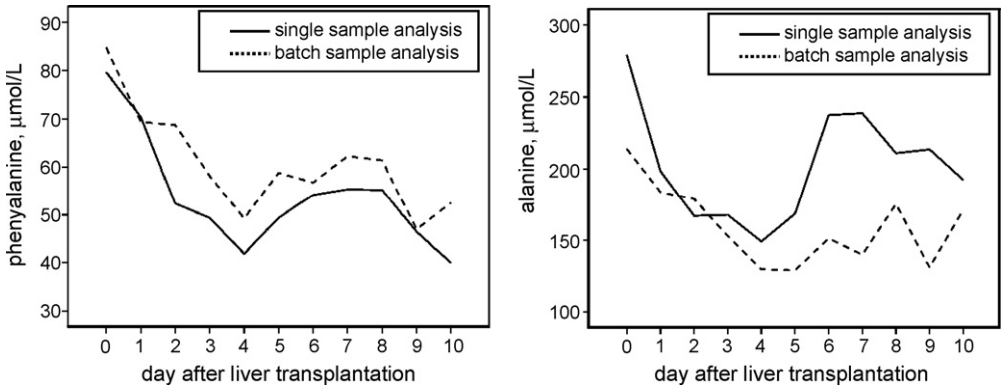

Fig. 5. Variability of the tandem mass spectrometric amino acid concentrations over a period of 2 month. Dried blood samples of 8 patients before and 10 days after liver transplantation were collected over a period of 2 month. Aliquots of set A were separately prepared and separately measured directly after each blood taking over a period of 2 month, aliquots of set B were stored till the end of the study at −80 °C and prepared and measured in one single batch; (A) comparison of the time dependent phenylalanine concentration (B) comparison of the time dependent alanine concentration.

and data interpretation are world-wide established (Kayton, 2007; McCabe and McCabe, 2008; Wilcken and Wiley, 2008). Fig. 4A shows the characteristic MS/MS analysis following the direct injection of a derivatized extract of a dried blood spot from a newborn. Within a run time of 1.5 min more than 30 amino acids and acylcarnitines are analyzed using a neutral loss experiment ($m/z = 102$) for amino acids (Fig. 4B), selective MRM-transitions (Fig. 4C), and a precursor ion experiment ($m/z = 85^+$) for acylcarnitines (Fig. 4D). The corresponding Table 1 demonstrates an extract of the computational generated excel result sheet, which is generated automatically using a commercially available database. Analyte concentrations are calculated automatically using spiked isotope labeled internal standards. Acylcarnitines results of unaffected newborns, one newborn screening result with isovaleric aciduria (IVA) and medium chain dehydrogenase deficiency (MCADD) are presented. Diagnostic sensitivity and specificity of >99.9% were reported, which underlies the diagnostic potential of the newborn screening approach (Ceglarek et al., 2002; Fingerhut, 2008). The technology can be applied to a much wider range of compounds and is therefore extendible to others diseases (Wilcken, 2007).

4. Pre-analytical and analytical aspects of clinical metabolomics

Based on the experiences from newborn screening it is well known that pre-analytical and analytical aspects significantly influence the consistency of metabolome data sets. Several questions have to be answered, particularly regarding reproducibility and accuracy of metabolome data generation. The following example presents the time-dependent variability of the tandem mass spectrometric amino acid results (Fig. 5). Dried blood samples of 8 patients before and 10 days after liver transplantation were collected over a period of 2 month. Aliquots of set A were separately

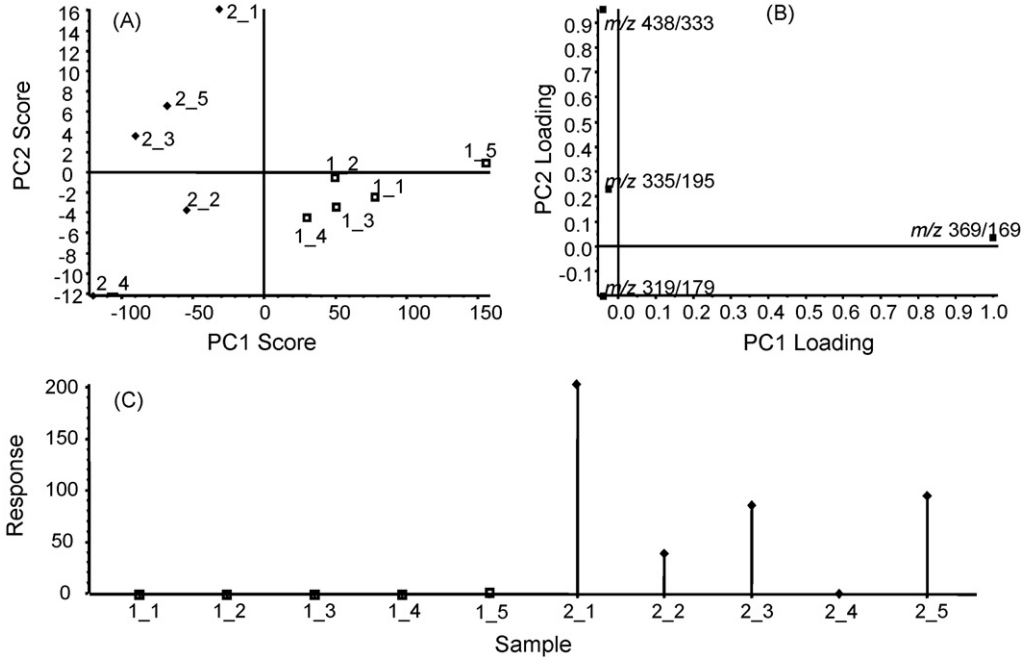

Fig. 6. Data interpretation of an eicosanoid stimulation experiments: human macrophages were stimulated with two different inflammatory activators (lipopolysaccharide, LPS and zymosan). The eicosanoid profile was measured by LC–MS/MS using 54 MRM transitions. Computational assisted data interpretations were performed using the commercial software tool MarkerView (SCIEX, Toronto). (A) loading plot for data comparison of the two subsets (B) results of the principle component analysis (C) plotting of the discriminating signal MRM 438/333 (Leukotrien E2) in each of the four replicates.

prepared and separately measured directly after each blood taking over a period of 2 month, aliquots of set B were stored till the end of the study at −80 °C and prepared and measured in one single batch. For phenylalanine no time-dependent differences could be observed between both data sets, but for alanine significant different distributions were obtained. The presented data variance of normal-range alanine concentrations is without relevance for the newborn screening evaluation. However, for metabolome analysis this may have an important impact for the data consistency and should therefore be minimized. As previously shown in the field of clinical proteomics well-defined protocols for precise metabolome analysis and the pre-analytical procedure of blood sampling, sample storage and sample processing are necessary to minimize the data variability (Baumann et al., 2005; Fiedler et al., 2007).

5. Data analysis and biostatistics

Data analysis is a predominant bottleneck of all 'omics' sciences. In the field of metabolomics a large number of quantitative data can be generated per single run. For example measuring 200 newborn screening samples/day yield 14800 quantitative data (74 parameters/sample), which have to be immediately processed and clinically validated. Hence, computational tools to handle and interpret the large amounts of data are demanded for data interpretation (Goodacre et al., 2004). Simple statistical approaches frequently provide an appropriate starting point for further bioinformatic analysis, since more sophisticated bioinformatic tools require special expertise and therefore are limited to direct diagnostic use.

In Fig. 6 an example for the analysis of eicosanoids is presented. Human macrophages were incubated with two inflammatory agents. The concomitant modification of the eicosanoid synthesis and oxidation was measured by LC–MS/MS according to the method described by Deems et al. (2007). 54 time-resolved MRM transitions were monitored for each experiment. As result about 1000 mass signals had to be assessed. Computational assisted data interpretations were performed using the commercial software tool MarkerView (SCIEX, Toronto). In Fig. 6A the loading plot and the results of the principle component analysis (PCA) (Fig. 6B) is presented. PCA is a multivariate analysis calculating the factors, which explains the variance of complex data sets. The results of PCA are usually visualized by loading plots or component scores (Steinfath et al., 2007). Visual evaluation was performed by automatic plotting the discriminating signals in the different subsets (Fig. 6C). As shown for the example the best discriminating component between the two subsets of the experiment was the lipoxygenase pathway product leukotriene E4. This example demonstrates that already commercial software tools are sufficient for rapid analysis of the large number of metabolome data.

6. Conclusion

The concept of clinical metabolomics aims at investigating disease-related metabolic signatures with the focus on a direct diagnostic application. The experiences from newborn screening provide important insights about the diagnostic potential of metabolome analyses. Novel hyphenated mass spectrometry based analytical techniques enable a rapid, effective and economical identification and quantification of metabolites in different biological specimens. Before starting metabolome experiments standardized protocols for sample pre-treatment and analytical performance are required to minimize data variability. Moreover, the huge amount of metabolome data demands novel bioinformatic strategies. The promising concepts of clinical metabolomics direct to the future of predictive, preventive and personalized medicine.

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