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New Insights Into Cardiovascular and Lipid Metabolomics

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ABSTRACT

Metabolomics is the study of metabolite profiles in biological samples, particularly urine, saliva, blood plasma and fat biopsies. The metabolome is now considered by some to be the most predictive phenotype: consequently, the comprehensive and quantitative study of metabolites is a desirable tool for diagnosing disease, identifying new therapeutic targets and enabling appropriate treatments. A wealth of information about metabolites has been accumulated with global profiling tools and several candidate technologies for metabolomic studies are now available. Many high-throughput metabolomics methodologies are currently under development and have yet to be applied in clinical practice on a routine basis. In the cardiovascular field, few recent metabolomic studies have been reported so far. This minireview provides an updated overview of alternative technical approaches for metabolomics studies and reviews initial applications of metabolomics that relate to both cardiovascular disease and lipid metabolism research. J. Cell. Biochem. 105: 648–654, 2008.

KEY WORDS: METABOLOMICS; CARDIOVASCULAR DISEASES; LIPIDS

The aim of metabolomics is the characterization of a phenotype through analysis of its metabolites which provide a sum of enzymatic activities in a living sistem. It is difficult to associate a pathological state with values for a single metabolite, therefore scientists are interested in all, rather than some, of the metabolites in a given system [Weckwerth, 2003]. The most useful biological samples for metabolomics studies are urine [Kind et al., 2007; Salek et al., 2007], saliva [Walsh et al., 2006], and blood plasma sample [Boernsen et al., 2005]. The contemporary qualitative and quantitative evaluation over time of a large number of metabolites, such as those detectable by Nuclear Magnetic Resonance (NMR) [Pan and Raftery, 2007] or Mass Spectrometry (MS) [Kusano et al., 2007; Vaidyanathan and Goodacre, 2007; Want et al., 2007] in biological fluids, can supply, with acceptable probability, the description of the biochemical state of an organism, providing information on the interrelations between the various metabolic processes that define this state [van der et al., 2007]. Therefore while genomics and proteomics reveal possible functions of the system, metabolomics represents its actual state.

TECHNICAL APPROACHES

Metabolomics has a higher level of variability than proteomics, in fact protein turnover can be measured in minutes to days or more while metabolite turnover is in the range of seconds. Therefore, any event perturbing a given metabolic state can have a rapid effect on turnover of a given metabolite and delete crucial information from the collected samples. Any degradation of metabolites after sample collection should be avoided, to ensure appropriate quantitation of each analyte and reproducibility among samples. Metabolomics studies generally use cell, tissue extracts or biofluids; samples from biofluids like urine and plasma are obtained essentially noninvasively and hence can be used more easily. However, fat biopsies can also be analyzed for selected features that may play roles in the clinical evaluation of cardiovascular risk, such as the elevation of growth factors and cytokines. Sometimes, there is uncertainty on information that might come from the analysis of cellular components. Yet, several techniques have a low degree of invasivity, like microdialysis of interstitial tissue fluid, adipose tissue vein cannulation and needle biopsy [Summers, 2006]. These techniques provide different results, in fact microdialysis is used to study low molecular weight hydrophylic molecules, whereas the cannulation system gives information about all substances released by tissues; finally needle biopsy provides small amount of cells from tissues, although in the case of subcutaneous adipose tissue samples up to 300 mg can be obtained [Summers, 2006]. If possible, a power analysis should be performed to ensure that a sufficient number of samples are acquired and to reduce the influence of biological

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variability in obtaining statistically validated data [Smit et al., 2007].

The metabolome consists of a vast number of components that belong to a wide variety of compound classes, such as amino acids, peptides, lipids, organic acids, nucleotides, etc. The exact number of unique metabolites in humans is unknown, but is estimated to be about 20,000; this is a lower estimate than for genes (24,000) and proteins (100,000). In addition, their wide concentration range spreading over nine orders of magnitude frequently requires application of several instrument platforms.

The two most important platforms employed presently in metabolomics are: NMR and MS. NMR multinuclear spectroscopy facilitates the analysis of hundreds of metabolites in a single sample, with or without pretreatment. It is widely used in chemistry as it provides detailed information on molecular structure, both for pure compounds and in complex mixtures. It does not require the separation of analytes for their detection, and, being a non-destructive technique, permits the recovery of the sample for further analyses [Betz et al., 2006]. Multivariate analysis of data can estimate, in an integrated way, specific variations that describe the effect of a perturbation in a given system, showing interconnections and interdependences of multiple analytes [Kemsley et al., 2007]. Importantly, it is also possible to determine the isotopic distribution of intermediate analytes in metabolic pathways using precursor labeled with stable isotopes (^{13}C or ^{2}H) [Birkemeyer et al., 2005]. The acquisition time of a NMR spectra typically takes only a few minutes; moreover, this technique allows also automatic sample preparation and injection. Furthermore, it is the only technique by which in vivo experiments can be performed, although it is restricted to animal models. However, NMR has poor sensitivity compared to mass spectrometry-based techniques, and spectra can be very difficult to interpret for complex mixtures because of overlapping among metabolite signals (Fig. 1). In addition, NMR analysis has poor baseline reproducibility among samples due to difference in salt concentration and pH so that baseline artifacts are sometimes larger than the intensity of the peaks. Therefore, many spectra of each sample must be acquired to provide statistical significance at each peak [Aranibar et al., 2006]. Conversely, MS possesses high sensitivity and selectivity, and can be used as a stand alone technique by direct injection of a sample into the ionization source of the mass spectrometer. Its performance, however, is greatly enhanced by coupling with upstream separation methodologies like gas chromatography (GC) [Styczynski et al., 2007] or liquid chromatography (LC) [Kristal et al., 2007] (Fig. 2). GC, coupled with mass spectrometry (GC-MS), is one of the most powerful methods. It has a very high resolution, but requires chemical derivatization for non-volatile molecules. Substances generally can be identified through their fragmentation pattern by comparison with a database of fragments generated from standards (Fig. 3). On the other hand, this technique has the disadvantage that some large and polar metabolites cannot be analyzed. High performance liquid chromatography (HPLC) has a lower chromatographic resolution compared with GC but is more amenable to co-analysis of varying types of compounds. Recently, pumps with higher operating

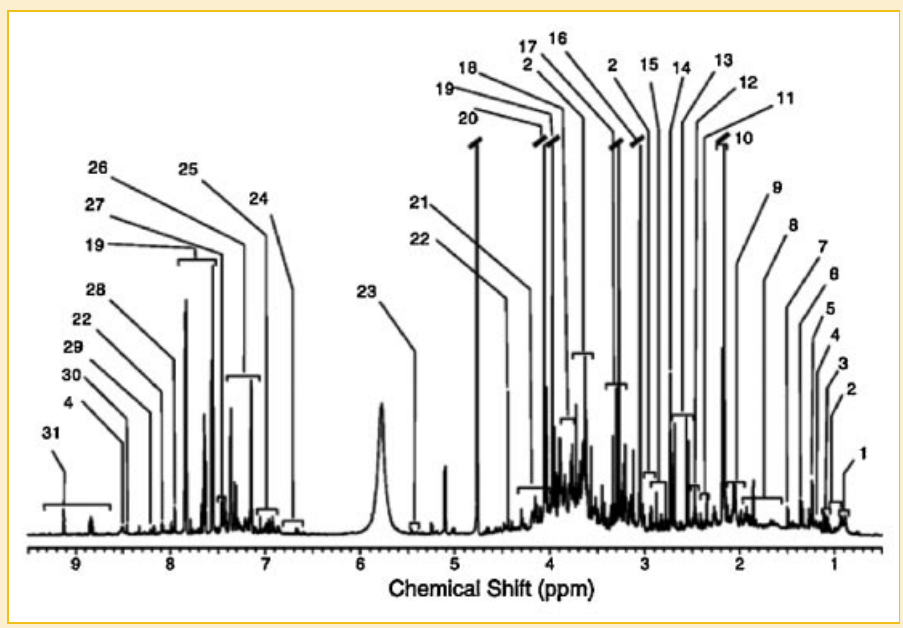

Fig. 1. Analysis of human urine from a healthy control volunteer. 700-MHz resolution 1H-NMR spectrum of an aqueous urine sample with the relevant resonance assignments shown. Each resonance corresponds to a chemical moiety within a particular metabolite with the intensity concentration of that metabolite. (1) α-hydroxybutyrate/valerate, (2) amino acids, (3) valerate, (4) unassigned, (5) β-hydroxybutyrate, (6) lactate, (7) alanine, (8) amino acids/ornithine, (9) *N*-acetyl groups/aspartate/glutamate, (10) methionine, (11) oxalacetate/pyruvate, (12) β-hydroxybutyrate/glutamine/glutamate, (13) citrate, (14) DMA, (15) TMA/DMG, (16) creatine/creatinine, (17) taurine, (18) PAG, (19) hippurate, (20) creatine/creatinine, (21) uridine bases, (22) NMN acid, (23) allantoin, (24) unassigned pyrimidine, (25) 3-hydroxypropionic acid/tyrosine, (26) metahydroxyphenyl-propionic acid (mHPPA) sulfate/indoxyl sulfate, (27) PAG, (28) *N*-methyl-2-pyridone-5-carboxamide (2PY), (29) NMN amide, (30) formate, (31) NMN amide/NMN acid.

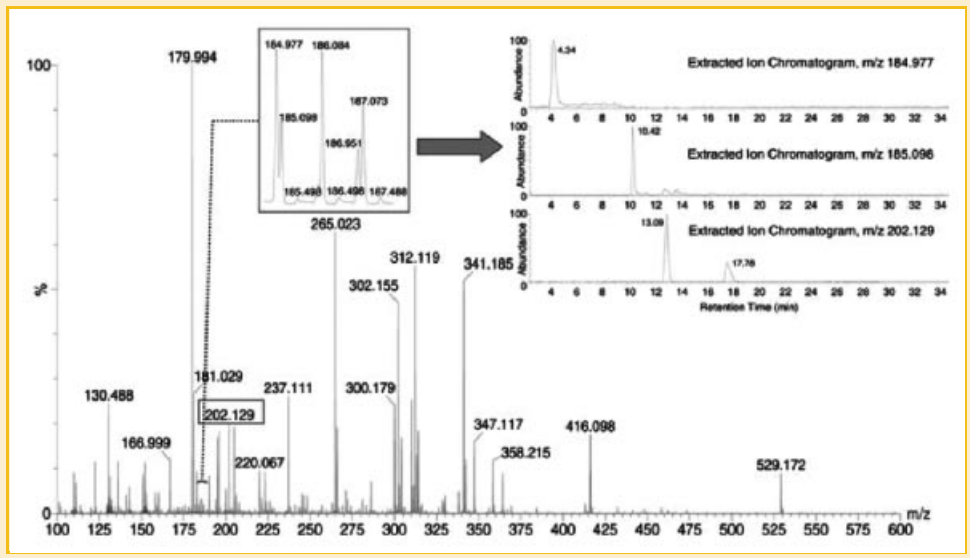

Fig. 2. Combined MS spectra from 2.5 to 35 min obtained after online-SPE extraction of 10 μl human urine, reversed phase HPLC separation, and TOF-MS detection (LCT, Micromass, UK) with ESI in positive mode. Expanded section of the spectrum illustrates the need for high-mass resolution to distinguish isobaric peaks, which originate from different metabolites as shown by the extracted-ion chromatogram. The third extracted-ion chromatogram shows that m/z 202.129 originates from two different metabolites that can be chromatographically separated. Direct infusion would not readily distinguish these two metabolites. Reprinted with permission of John Wiley & Sons, Inc. from Dettmer K, Aronov PA, Hammock BD. 2007. Mass spectrometry-based metabolomics. Mass Spectrom Rev 26(1):51–78. Copyright © 2006 John Wiley & Sons, Inc.

pressure have been developed which can be joined to columns packed with 2 μm particles thus increasing the resolution power. This technology called ultra-performance LC (UPLC) has been successfully employed in metabolomic applications [Shen et al., 2005; Kind et al., 2007]. Indeed, since a large fraction of metabolites are ionic, they can be separated easily by capillary electrophoresis (CE) which separates analytes according to their mass-to-charge ratios. The advantage of CE is its high efficiency and speed in separation, low cost per analysis, and small sample requirement (few nanoliters) [Monton and Soga, 2007]. Its main drawback is its poor sensitivity when fitted with a photometric detector, but this can be overcome by using MS as a detector [Soga, 2007].

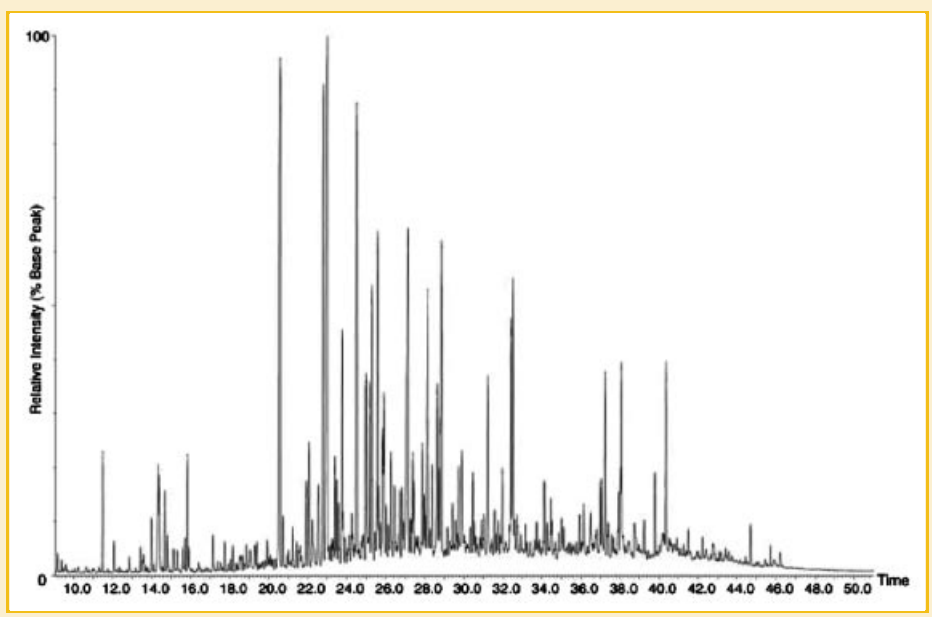

Fig. 3. Total ion chromatogram of a human urine (1 ml) extract after silylation with MSTFA and GC-MS analysis. Peak deconvolution using AMDIS resulted in 1,582 components. Reprinted with permission of John Wiley & Sons, Inc. from Dettmer K, Aronov PA, Hammock BD. 2007. Mass spectrometry-based metabolomics. Mass Spectrom Rev 26(1):51–78. Copyright © 2006 John Wiley & Sons, Inc.

Imaging mass spectrometry (IMS) is an emerging technology that uses the MS platform [Cornett et al., 2007]. This technique combines the multichannel (m/z) measurement capability of mass spectrometers with a surface sampling process based on matrix assisted laser desorption ionization (MALDI) or bombardment by energetic particles such as Cs^{+} Au^{3+}. Using this technique, the molecular mass of hundreds of molecules and their spatial distribution can be detected in a few cells.

MALDI-IMS employs the same process of matrix-assisted laser desorption/ionization to promote a gas-phase ionization of molecules in a limited sample area. A thin section (5–20 μm thick) of a tissue is coated with MALDI matrix on a plate. MS or MS/MS spectra are acquired at discrete positions on the sample surface and associated to their position along the *x*,*y* axis. The software then reconstitutes the bidimensional image by associating the intensity of any given signal, or combinations of signals with the original coordinates.

Although this technique has found several applications in proteomics, it has been employed recently for small molecules [Reyzer and Caprioli, 2007]. Using MALDI-IMS with the aid of fragmentation of parent ions (MS/MS), phospholipids were unambiguously identified and their localization in tissues assessed [Jackson et al., 2007; McLean et al., 2007]. This technique also has been applied to drug metabolism, providing an exclusive opportunity to monitor drug localization in a particular cellular compartment and to follow each metabolite which differs in mass from the parent drug in the same experiment [Drexler et al., 2007]. This approach is particularly useful when analysis is restricted to a class of compounds that are related to a specific pathway or to intersecting pathways, rather than to a complete metabolite profiling.

DATA HANDLING

Data processing in metabolomics requires several steps [Katajamaa and Oresic, 2007]: first of all, raw proprietary data produced by different instruments must be converted into a common raw data format. Then raw data must be filtered to separate the relevant signals from noise or baseline; subsequently, each peak must be normalized and statistically validated; and finally, every peak associated with a metabolite. The last step is quite difficult to perform and depends upon the analytical platform chosen. Indeed, if NMR is used, each metabolite can produce several peaks arising from different chemical shifts thus producing peaks that overlap among metabolites. This reduces the efficiency of metabolite identification and quantitation. If mass spectrometry is used, the attribution of each peak to a single metabolite is more simple because modern instruments like MALDI-TOF can resolve mass differences as little as 0.05 Da, in addition, if two isobaric metabolites fall in the same range they can be fragmented, and each fragment mass can be analyzed. In mass spectrometry metabolite quantitation is achieved by comparison with an isotope-labelled internal standard [Sun et al., 2007]. Moreover, mass spectrometry selectivity can be increased further if it is coupled with a HPLC system via an electrospray module. Given that different analytical platforms are used in metabolomics the standardization of results is a prerequiste for data exchange among laboratories [Fiehn et al., 2006].

Metabolomics researchers also need databases that contain a Minimum Information About Metabolomics Experiment (MIAMET) together with the data values (Metabolomics standards initiative, http://msi-workgroups.sourceforge.net). This database can be searched using NMR and MS spectra, raw or structural formula; others are currently under development, for example, the Human Metabolome Database (HMDB, http//www.hmdb.ca) [Wishart et al., 2007], and a metabolomics extension to the BioMagResBank (BMRB, http://www.bmrb.wisc.edu), [Markley et al., 2007; Ulrich et al., 2008], providing additional information about physiological concentrations, known disease associations, biofluid or tissue and cellular locations, and chemical and physical properties.

APPLICATION OF METABOLOMICS IN CARDIOVASCULAR DISEASES AND LIPID METABOLISM

At present metabolomics represents a field of unrealized potentiality. Scientists have long recognized that the search for novel biomarkers is fundamental to understand how molecular pathways are interconnected in cells and organisms, but stringent relationships between a given set of markers and a particular pathological state are largely unestablished. As key metabolite markers are identified, these will contribute to our understanding of the physiological or pathological processes and serve as starting points for drug discovery and therapeutics evaluation. Metabolomic investigations are likely to be of great interest in pharmaceutical evaluations and toxicology, contributing to our understanding of the mechanisms of action of drugs or toxic substances [Dix et al., 2007; Materi and Wishart, 2007]. Metabolic profiling of urine or blood plasma samples can be used to detect changes produced by xenobiotics or related to specific syndromes [Constantinou et al., 2007b; de Boer et al., 2008].

Metabolomic studies of adipose tissues have been demonstrated to be useful in discovery of new biomarkers related to metabolic and cardiovascular diseases (CVD) [Watson, 2006; Glisic et al., 2008; Kola et al., 2008; Tsai et al., 2008]. In fact, adipose tissue, once only considered as an energy storage tissue, has now been shown to play an important role in the homeostasis of non-esterified fatty acids, and its endocrine role has been evaluated [Summers, 2006]. Several hormones and factors produced in this tissue have been associated with obesity, insulin resistance and CVD. In particular the accumulation of visceral adipose tissue, as opposed to subcutaneous fat, increases the risk of developing metabolic diseases and CVD [Bays et al., 2008].

Metabolomics has been used for profiling of low-molecular-weight metabolites particularly relevant to myocardial ischemia. Sabatine et al. [2005] used mass spectrometry-based technology to identify differences in plasma metabolites among 18 subjects with ischemia induced by exercise stress testing compared with nonischemic individuals who also exercised. Highly statistically significant changes in circulating levels of metabolites belonging to the citric acid pathway were found in myocardial ischemic group

[Zhao et al., 2008]. Furthermore γ-aminobutyric acid, citrulline and argininosuccinate were found to decrease. All three of these metabolites are in some way related to the citric acid cycle showing that in acute ischemia, maintenance of citric acid cycle intermediates is of a great importance to preserve the ATP synthesis.

NMR based metabolomics proved to be a useful tool in the investigation of myocardial ischemia-reperfusion, ischemic preconditioning and antioxidant intervention in rabbits [Constantinou et al., 2007a]. The ^{1}H NMR signal intensity ratios of lactate/glucose and lactate + alanine/acetate related to glycolysis pathways correlate with the different ischemia-reperfusion conditions.

Further evidence for the effect of melatonin and indole derivative C6458 were shown and the results were in agreement with previous findings based on measurement of biomarkers [Andreadou et al., 2004].

Although a wide range of risk factors for coronary heart disease have been identified from population studies, these measures, singly or in combination, are insufficiently powerful to provide a reliable, noninvasive diagnosis of the presence of coronary heart disease [Brindle et al., 2002; Miller et al., 2007].

Moreover, from very recent population studies, differential risk of CVD and disparity in response to drug treatments were found related to the genetic variability of such patients [Shiffman et al., 2008; Iakoubova et al., 2008a,b]. In these studies a genetic polymorphism of the kinesin-like protein KIF6 was associated with coronary heart disease. Indeed, patients carrying the KIF6 719Arg allele were found to have a higher risk of coronary events, but they received greater benefit from statin therapy compared to patients non carrying this mutation. These investigations lead to the conclusion that the metabolomic evaluation coupled with the well assessed classical risk factor evaluation can increase the prediction of coronary events improving drug treatment. Moreover, a careful and detailed lipidomic analysis may promote further beneficial effects from large clinical trials.

Metabolomics is also used for mapping the metabolic pathways of drugs in order to investigate their homeostasis and the species of catabolites produced. With this aim, ascorbic acid homeostasis and degradation in diabetics rats using 6-deoxy-6-fluoro ascorbic acid was investigated by NMR spectroscopy. The results demonstrated that diabetes led to a remarkable increase in urinary loss of ascorbic acid and a relative decrease in most other urinary ascorbate catabolites. Furthermore, oxidation of ascorbate took place preferentially in liver, spleen, kidney, and plasma [Nishikawa et al., 2003].

In the last years several papers have been published on ''lipidomics,'' which can be considered a sub-class of metabolomics, but has been proposed as a stand-alone ''omics'' [German et al., 2007]. Compared to metabolomics, lipidomics has several advantages: the number of lipid metabolites is undoubtedly much lower than the whole number of metabolites in a cell. Lipid analyses use a single platform: mass spectrometry as stand alone technique [Han and Gross, 2005a] or coupled to HPLC [Han and Gross, 2005b]. Lipids are involved in critical cellular functions, for example in membranes; they are also a source of second messengers like diacylglycerols, phosphatidic acids, eicosanoids, lysolipids, and ceramides. These lipids are generated by the actions of a variety of intracellular enzymes whose activity can be modified in pathological states [Griffin and Nicholls, 2006].

Diseases like atherosclerosis, diabetes, obesity and stroke have been linked to alterations in lipid metabolism. In diabetic rat myocardium severe lipid alterations occur, in fact a 46% increase in phosphatidylinositol together with a fivefold increase in tripalmitin and a 60% decrease in triacylglycerol molecular species containing polyunsaturated acyl acids has been observed. These alterations cannot be suppressed by routine insulin administration alone [Han et al., 2000].

Moreover, a severe loss of cardiolipin was found at the very earliest stages of diabetes accompanied by substantial remodeling of the remaining cardiolipin molecular species. These alterations occur within days after the induction of the diabetic state and precede the triacylglycerol accumulation manifest in diabetic myocardium [Han et al., 2005, 2007]. Diabetes is also linked to enhanced cellular oxidative stress of lipid and protein components [Liguori et al., 2001; Balestrieri et al., 2008].

A relation between altered lipid metabolism and Alzheimer's disease was also established. Han et al. [2001] found that the ethanolamine glycerophospholipids have a different profiles in brain gray matter compared to white matter. In addition, in patients with the earliest clinically recognizable stage of Alzheimer's disease a remarkable reduction of plasmenylethanolamine in gray matter and white matter was observed. The myelin sheath component sulfatides also dramatically decreased both in gray and white matter whereas a consistent (>3-fold) increase of ceramide content in white matter took place [Han et al., 2002]. Finally, recent developments in myocardial nuclear lipidomics showed that different profiles of nuclear choline and ethanolamine glycerophospholipids subjected to pathophysiological stresses may provide important information on the role of the myocardial nuclear lipidome on long-term cardiac cell function [Albert et al., 2007].

CONCLUSIONS

It is reasonable to expect that within the next few years, the metabolomic approach, together with functional genetics and proteomics, will have substantial impact on development of diagnostics, therapeutics and biotechnology drugs. We propose that metabolomics and lipidomics will help physicians and basic scientists extend laboratory investigation and clinical diagnosis in CVD. Moreover, these applications may increase effectiveness of detecting toxicity in drug development at both the preclinical and clinical phases, and support screening and stratification of patients leading to discovery of new biomarkers or sets of diagnostic biomarkers. In addition to reducing times and costs for research and experimentation with new drugs, metabolomics may predict new indications for drugs already in production.

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