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Clinical Implementation of Metabolomics

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1. Introduction

1.1 Overview

Metabolomics, which is also referred to as metabonomics, metabolic profiling or metabolic fingerprinting, is the comprehensive quantitative measurement of endogenous metabolites within a biological system (Fiehn, 2002; Kaddurah-Daouk et al, 2008; Spratlin et al, 2009). Detection of metabolites is in general carried out in cell extracts, tissue specimens, or various biological fluids including serum, plasma, urine and cerebrospinal fluid (CSF) by liquid chromatography mass spectrometry (LC-MS), gas chromatography–mass spectrometry (GC-MS), capillary electrophoresis–mass spectrometry (CE-MS) or nuclear magnetic resonance spectroscopy (NMR). Metabolomics captures the status of diverse biochemical pathways in a particular situation and can define the metabolic status of an organism (Aranibar et al, 2011; DeFeo et al, 2011; Lu et al, 2008; Roux et al, 2011; Soga, 2007; Yuan et al, 2007). In clinical settings, biomarkers generated from metabolomics have become one of the most essential diagnostic criteria that can be objectively measured and evaluated as indicators of normal or pathological states, as well as a tool to assess responses to therapeutic interventions (Hunter, 2009; Spratlin et al, 2009; van der Greef et al, 2006; Zeisel, 2007). As we describe in this chapter, novel metabolomic markers, for instance, for cancer therapy, glucose intolerance, hepatic steatosis, nephrotic and psychiatric disorders, and their incorporation into clinical decision-making may considerably change future health care.

In order for metabolomics to be successful in clinical settings, it must surpass conventional methods in reliability and predictive capability, and/or should be more informative about disease pathogenesis. Utilizing a systems biology approach in

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biomarker investigation may allow for a deeper understanding of disease associated metabolism. (Jenkins et al, 2004; Kell, 2006). A systems biology approach does not focus on identifying a single target or mechanism of an observed phenotype. Instead it seeks to identify the biological networks or pathways that connect the differing elements of a system (Wheelock et al, 2009). When a shift in equilibrium is observed in a disease, such as altered metabolic fluxes or enzymatic activities, it can be elucidated that those components of the network that are associated with the observed shift are characteristic and potentially descriptive of the disease, and that they accordingly represent potential targets for intervention. Thus, the systems approach in combination with metabolomics, may lead to the discovery of panels of metabolites that more accurately capture the disease status and help acquire information valuable for individualized clinical care (Quinones & Kaddurah-Daouk, 2009). Clinical metabolomics is expected to be a promising technology for personalized medicine and nutrition. A metabolic marker designed to predict individual response including efficacy and side effects during therapeutic intervention for each patient will enable administration of optimal treatments and improve clinical outcome.

1.2 Comprehensive vs. focused metabolomics

The spectrum of biochemicals in a clinical specimen, range from organic acids, amino acids, lipids, nucleic acids and their metabolic intermediates to complex secondary metabolites with signaling functions. Today, however, clinicians in human health care utilize only a very small part of the information contained in the metabolome. Although NMR or MS technology enables a comprehensive (i.e. global) measurement of various small molecules, in many cases, it is simply too difficult to quantify each molecule and understand underlying mechanisms from a global dataset by a single measurement (Steuer, 2006). This has led a number of researchers to look at a focused set of (i.e. local) metabolites such as amino acids or lipids (German et al, 2007; Kimura et al, 2009), where data from multiple measurements such as transcriptomics, proteomics and metabolomics can be effectively integrated to allow more insight into the underlying metabolic alternations, by projecting multiple datasets onto biochemical pathways and analyzing their interactions under a particular physiological state (Caesar et al, 2010; Momin et al, 2011; Noguchi et al, 2008; Zhang et al, 2011).

Recently, there have been reports of trials in integrating different types of 'omics' datasets for the systemic understanding of metabolic phenotypes at multiple levels. Various software packages are available in integrating nonuniform 'omics' datasets (Grimplet et al, 2009; Gruning et al, 2010; Taylor & Singhal, 2009). The link between information and modeling can be achieved by two major types of complementary approaches, a data-oriented exploratory approach, in which data generates information about the structure and relationships between the observed variables in a given system, and a model-based bottom-up approach, in which cybernetic and systems–theoretical knowledge are used to create models that describe mechanisms and dynamics of a system. Formerly, a model-based approach had been used for studying in-vitro cellular or organ systems; however, because of the complexity in modeling whole body systems, recently this approach has been replaced by a data-oriented approach, particularly when dealing with in-vivo 'omics' data in various models including animal models and clinical studies (Dunn et al,

2011). The critical step is the construction of models from the raw dataset of transcriptomics, proteomics, and metabolomics. This may be achieved by using different mathematical techniques ranging from simple Pearson correlations to the use of ordinary differential equations (Wheelock et al, 2009). Through this modeling, fundamental concepts in the understanding of biological systems like robustness, modularity, emergence, etc. are incorporated.

Most studies currently remain focused on local level networks within a set of related genes or protein expressions (Bapat et al, 2010; Kirouac et al, 2010). Yet a combination of different levels of networks can be connected to overview the whole system. A change in the gene regulatory network may have a corresponding effect in the protein–protein interaction network, the metabolic network, etc., which collectively may manifest changes in the pathological phenotype. To understand the whole system, it is critical to integrate knowledge from different datasets. Although some progress has been made in amino acid metabolism, the integration of different types of datasets is still difficult due to differences in dynamic range, scales, or analytical errors, particularly in metabolomic analysis (Ishii et al, 2007; Momin et al, 2011; Noguchi et al, 2008). Therefore, focused-metabolomics, with well managed measurements in terms of accuracy and reproducibility, for lipid, amino acid and glucose metabolism appears to be a realistic approach to illustrate how the phenotype is altered when the metabolic network itself is modified through the alteration of endogenous or environmental factors.

1.3 Generation of multiple metabolite markers

When generating biomarkers from metabolomic analysis, marker identification, verification, and also statistical and experimental evaluations, using bioinformatic techniques of identified candidate markers are required. Recently, various data mining methodologies have been reported for identifying and prioritizing reliable metabolomic markers with high diagnostic capability (Caruana, 2006; Duda, 2001; Gu et al, 2011; Kim et al, 2010; Maeda et al, 2010; Montoliu et al, 2009). In cohort studies, the definite diagnoses of the patients are normally known beforehand. In such trials, "supervised" statistical methods which consider patient classification tend to be more efficient in information utilization and suitable for obtaining targeted metabolite markers.. In contrast, when phenotypes in patients are undetermined, "unsupervised" analysis such as cluster analysis are useful tools for biomarker identification and classification of specimen groups. Moreover, improvement in discriminatory power has been reported when multivariate mathematical models are constructed combining multiple metabolite markers. These approaches include discriminant analysis methods such as linear discriminant analysis, logistic regression analysis, decision trees, the k-nearest neighbor classifier (k-NN), an instance-based learning algorithm, support vector machines or artificial neural networks (Duda, 2001). The Receiver Operating Characteristics (ROC), or the area under the ROC curve (AUC) of multivariate markers is used to represent its discriminatory performance as a trade off between selectivity and sensitivity(Hanley & McNeil, 1982). Obtained metabolomic markers are also required to be experimentally validated using larger datasets from multiple clinical trials and also statistically validated using cross validation, leave-one-out cross validation, and bootstrapping.

2. Practical Issues in the clinical implementation of metabolomics

2.1 Sample stability issues

Enormous information can be obtained by analyzing large numbers of metabolites, and it is utilized for various fields such as health and nutrition. However, the chemical and enzymatic stabilities of most metabolites are unknown. Therefore, inappropriate handling of samples can lead to inaccurate measurements. In this section, blood sampling issues for amino acids analysis as a typical case of sample handling are described. There are mainly four steps in the blood sampling process for amino acids analysis; 1) blood collection, 2) centrifugation, 3) sample storage, and 4) deproteinization. In this section, the crucial points for each step are outlined to highlight the importance of sampling processes in metabolomic studies.

2.1.1 Blood collection

The concentrations of amino acids are known to show circadian rhythms and some of them vary 30% within a day (Forslund et al, 2000). Therefore, it is desirable to collect the blood at a fixed time point. Moreover, since the amino acid concentrations increase after a protein containing meal, blood collecting between 7am and 10am in a fasting state is desirable.

The concentrations of some amino acids are known to be quite different between blood cells and plasma. The differences of essential amino acids are small, but the concentrations of nonessential amino acids can be greater by severalfold in blood cells (Filho et al, 1997). There are also many metabolic enzymes such as arginase in blood cells which will act on the plasma free amino acids (PFAAs). Therefore it is important to verify that haemolysis dose not occur in blood samples. If the blood sample shows heavy haemolysis, it is desirable to take another sample.

If blood samples are left at room temperature after collection until centrifugation, many amino acids are metabolized due to metabolic enzymes from blood cells. In particular, there are many enzymes for metabolizing nonessential amino acids. For instance, glutamine and asparagine are well known to be metabolized to glutamate and aspartate. The concentration change of glutamate at different temperatures is shown in Figure 1. This suggests that it is desirable to cool blood samples after collecting. In another study, we also found that it is essential to cool down the blood samples to 0°C immediately after collecting and that Ice-water is better than the refrigerator or ice because of the faster cooling rate.

However it is not always easy to prepare ice-water in the medical institutions at the time of blood collection. For this reason, we have developed a portable blood tube cooler (CubeCooler™, Figure 2). This cooler is composed of high thermal conductive container (aluminum) and insulator (polyethylene form), which enables the quick cooling of blood samples as well as ice-water and maintains the temperature for 12h (Figure 3). There are many coolers which is commercially available. As far as we have examined, these coolers, however, could not achieve a cooling rate as close to that of ice-water and could not cool blood samples for a long time without differences in temperature arising between tubes inserted in different holes. Thus, the cooler we have developed may be a useful tool not only for amino acid analysis but also for sample management in other metabolomic studies.
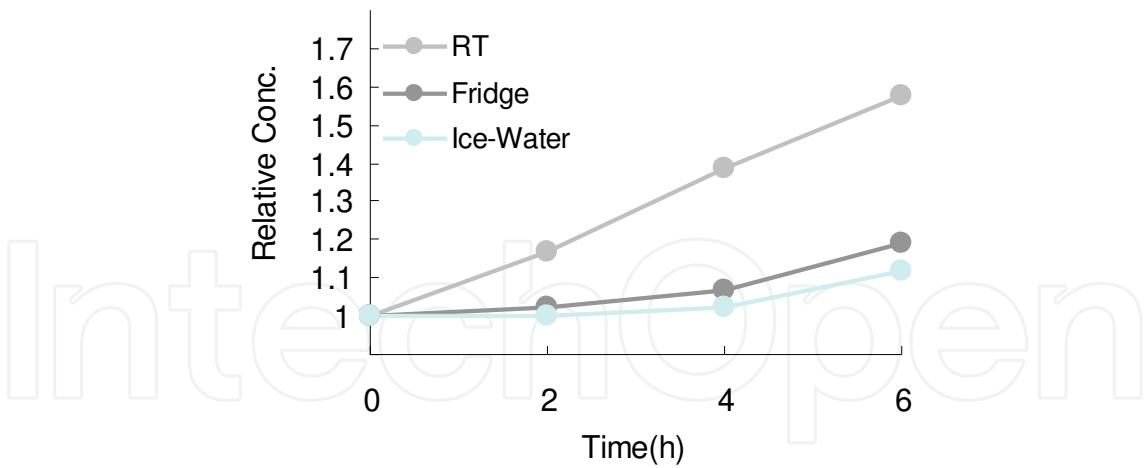
Fig. 1. Effect of cooling on concentration of glutamate in whole blood

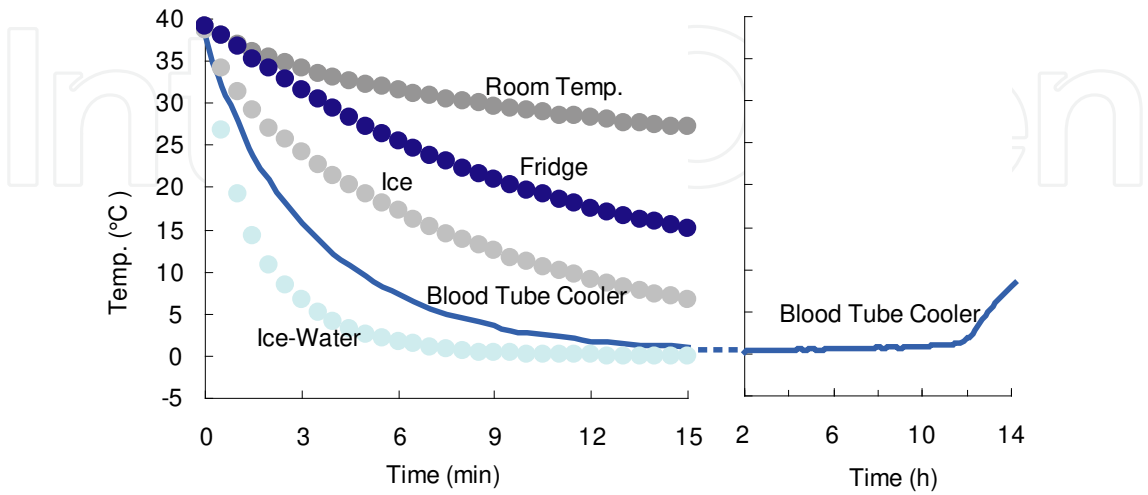

Fig. 2. View of the blood tube cooler (CubeCooler™)

Fig. 3. Cooling rate when the blood tubes are set in various conditions and cooling duration of the blood tube cooler

2.1.2 Centrifugation

It is desirable to store blood samples in ice-water after collection and to separate the plasma from the blood cells within a few hours. As mentioned above, since blood cells contains many amino acids and enzymes, it is important not to contaminate the plasma with platelets. If contamination occurs, the concentrations of some amino acids, such as glutamate, aspartic acid and taurine can be high.

2.1.3 Sample storage

It is necessary to store the plasma in a freezer in case of long term storage. When stored at -20°C, some amino acids, especially glutamate, aspartate and cysteine can gradually decrease. Therefore -80°C freezer should be used for long term storage of plasma samples. When transporting the samples, the samples should be carried in a box filled with dry-ice.

2.1.4 Deproteinization

Since plasma contains proteins such as albumin, deproteinization is necessary before amino acid analysis. When analyzed with amino acid analyzer, plasma is generally mixed with trichloro-acetic acid or sulfo-salicylic acid and the precipitate is centrifuged. Since these reagents are strong acids, it is necessary to rapidly analyze amino acids or store in -80°C freezer so that some amino acids like glutamine are not decomposed due to acid hydrolysis. When analyzing with LC-MS or LC-MS/MS, organic solvents such as methanol and acetonitrile is useful for deproteinization. In this case, the organic solvent may influence the derivatization reaction and separation of amino acids. Since recovery rates for amino acids depend on the procedure of deproteinization, it is desirable to unify the procedure. When analyzing with LC-MS or LC-MS/MS, recovery rates can be calculated by adding stable-isotope-labeled amino acids as internal standards before deproteinization.

2.2 Analytical issues

Nuclear magnetic resonance (Bollard et al, 2001), mass spectrometry (Piraud et al, 2003), gas chromatography mass spectrometry (Thysell et al, 2010), liquid chromatography mass spectrometry (LC-MS) (Lin et al, 2011a), and capillary electrophoresis mass spectrometry (Sugimoto et al, 2010) have been used as primary tools employed for metabolomics.

A clinical metabolomics approach with LC-MS can be broadly classified into comprehensive and targeted analysis. Comprehensive analysis aims to identify and quantify all detectable metabolites in a single run. This analysis offers the advantage of giving much information. In the past, the retention and separation of polar metabolites had been difficult in LC-MS analysis. This was a weakness of LC-MS analysis, and LC-MS was limited to the analysis of hydrophobic metabolites such as lipids. However, the development of column technology enabled the retention and separation of hydrophilic metabolites(Alpert, 1990; Yoshida et al, 2007). This technology has been applied for the research of drug metabolites (Plumb et al, 2003), galactosamine toxicity (Spagou et al, 2011), and renal cell carcinoma diagnosis, staging, and biomarker discovery (Lin et al, 2011a).

In targeted analysis, a selected number of predefined metabolites are quantified. This analysis is sometimes used for quantification of metabolites, which is extracted from comprehensive

analysis. Derivatization methods, based on specific reactions to targeted functional groups are major tools in targeted analysis. This method allows for sensitive and selective quantification of endogenous metabolites with amino and carboxyl groups (Tsukamoto et al, 2006; Yang et al, 2006). An advantage of this method is to be able to select a suitable sample preparation for each endogenous metabolite with the same functional group, because of the similar physical and chemical properties. This method is also very important for accurate quantification, because sample stability is different for each endogenous metabolite.

The analysis of amino acids with an amino group has a long history. In 1958, a key application for physiological amino acid analysis was supplanted by ion exchange column chromatography separations on an automated apparatus designed and built by postdoctoral fellow Darrel H. Spackman at the request of his mentor William H. Stein, and Stanford Moore at Rockerfeller University (Moore et al, 1958). This automated system reduced the analytical time from a few weeks to a full day and provided easy to use operation. The present system is used for the study of inborn errors of amino acid metabolism in clinical laboratories (Qu et al, 2001).

Recently, pre-column derivatization reagents for amino acid analyses have been developed, mainly to achieve greater sensitivity and selectivity, and much attention is paid to the design of derivatization reagents for LC-MS (Yang et al, 2006) and LC-MS/MS (Shimbo et al, 2009a; Shimbo et al, 2009b). These reagents have three notable characteristics (Figure 4). First, the reagent must have sufficient hydrophobicity to enable the retention of amino acids. Secondly, is should have a desirable structure which will increases ionization efficiency. Thirdly, it should be designed to provide characteristic and selective cleavage at the bonding site between the reagent moiety and the amino acid in the collision cell of the triple-stage quadrupole mass spectrometer. Using precursor ion scanning, endogenous metabolites with amino groups are can be extracted on ion chromatograms, even in crude biological samples.

3-aminopyridyl-N-hydroxysuccinimidyl carbamate (APDS) reagent is known to provide rapid analysis and separation of amino acids of the same charge to mass ratio on a column (Shimbo et al, 2009b) (Figure 5). This reagent is applied to the modelling of a diagnostic index, "AminoIndex technology", from differences in PFAA profiles between non-cachectic colorectal/breast/lung cancer patients and healthy individuals. (Maeda et al, 2010; Okamoto et al, 2009).
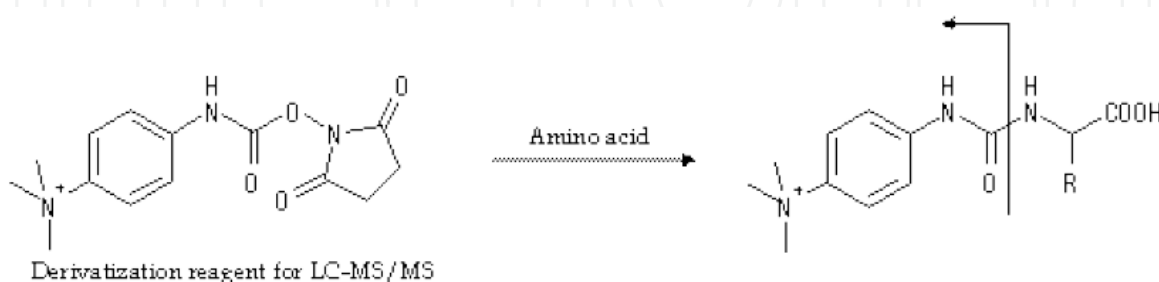

Fig. 4. Typical reaction of amino acids with a derivatizaiton reagent for LC-MS/MS. This reagent has three notable characteristics; 1) sufficient hydrophobicity (benzene ring) 2) increases ionization efficiency (quaternary amine) 3) characteristic and selective cleavage (the reagent moiety and the amino acid).
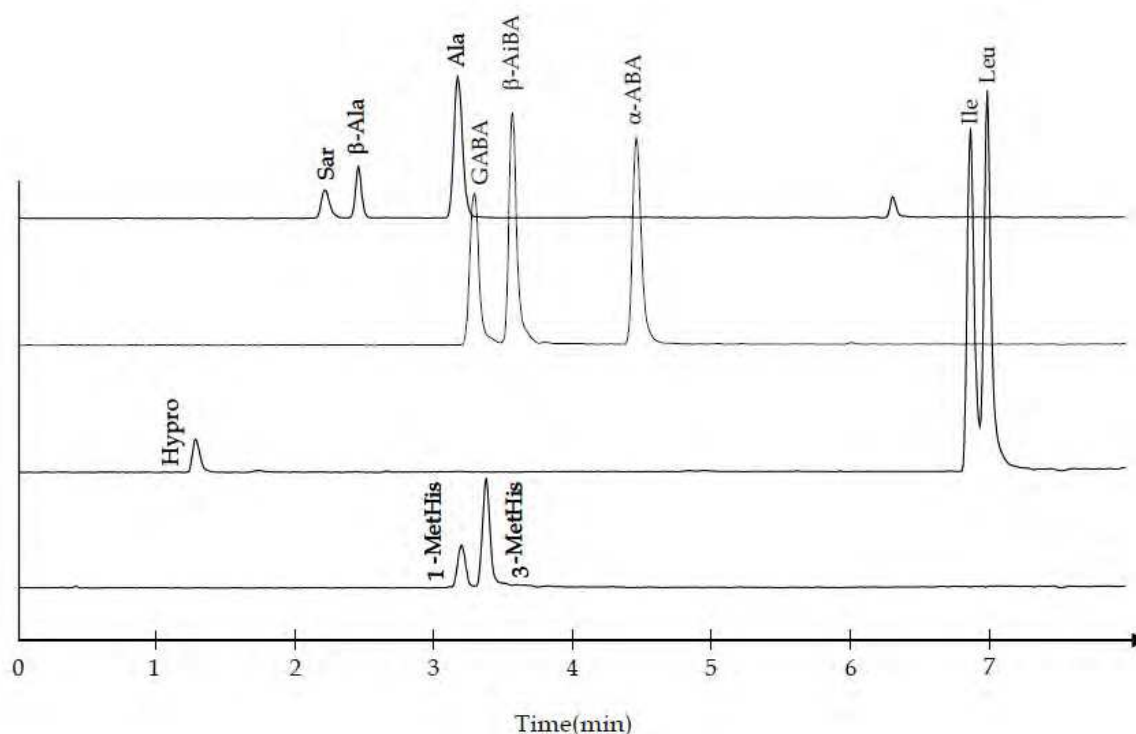
Fig. 5. Typical chromatograms of amino acids which were the same charge to mass ratio on a column.

2.3 Statistical issues

Recently, several applications of metabolome analysis based on computer-aided detection and diagnosis (CAD) has been demonstrated (Duda, 2001; Gu et al, 2011; Kell, 2002; Kim et al, 2010; Montoliu et al, 2009; Righi et al, 2009; Serkova et al, 2011; Taylor et al, 2010). The importance of objective indices for diagnosis based on empirical and statistical knowledge are increasing due to the trend called "Evidence Based Medicine (EBM)". Although CAD follows this trend, the required level of statistical analysis is also increasing and becoming more complex. The requirement of clinical investigation includes not only statistical significance but also feasible and in-depth clinical protocols in which necessary and sufficient conditions need to be satisfied. In this section, multivariate statistical aspects of metabolome analysis focused on the establishment of medical evidence and investigation of biomarkers will be introduced and discussed.

Reproducibility is the most important point of a diagnostics index. It is more complicated to guarantee the statistical reproducibility by multivariate analysis than univariate analysis. Adequate experimental design prior to data collection is therefore crucial for the quality control of the analysis (Hulley, 2006). In general, knowledge obtained from statistical analysis is only capable within the realm in which the data was analyzed and therefore cannot extrapolate beyond the realm. Generally, larger sample size is required in case of multivariate analysis because freedom of variable space is higher than univariate analysis. For example, multivariate analysis of variance (MANOVA) and data simulation are used to determine the appropriate sample size. Additionally, it is sometimes necessary for a data set to be normalized or scaled for unbiased analysis.

The most important point of analysis is algorithm selection. It is well-known as the "no free-lunch theorem", that it is impossible to determine the most suitable algorithm a priori, and that the pros and cons of each algorithm are not always specific, but dependent on each situation. Therefore, preliminary analysis to determine the most felicitous algorithm is necessary in each case. Univariate analysis can be performed to figure the behavior of each metabolite and to select the variable, i.e. dimensionality reduction of variable space, prior to multivariate analysis. It should be noted that the metabolome data are often so connected that there is a potential pitfall of statistical analysis, so-called multicollinearity, where the excess reduction of dimension sometimes can lead to the loss of latent network structure of metabolites. Multivariate analytical methods are applicable for simplification or dimensionality reduction of data to easily figure out visualized images of the "metabolite space" which has huge body of dimensions (metabolites).

Algorithms for multivariate analyses are categorized into two different groups, i.e., unsupervised methods and supervised methods. Unsupervised methods do not require objective variables such as subject status, other observed data, etc., while supervised methods require them for the data set to be analyzed. The examples of multivariate algorithms are listed in Table 1. Unsupervised learning methods are especially useful for investigating the latent structure and decreasing the redundancy of data and therefore they are sometime performed in combination. The advantages of unsupervised methods are that they minimize the loss of information (Maeda et al, 2010). However, whether the results of unsupervised methods can provide the appropriate interpretation or not depends on the setting of parameters or the problem to be analyzed.

Models	Unsupervised learning	Supervised learning Continuous	Discrete
Linear model	Factor analysis	Multiple linear regression (MLR)	Linear discriminant analysis (LDA)
	Principal component analysis (PCA)	Canonical correlation analysis	Canonical discriminant analysis
	Independent component analysis (ICA)	Partial least square regression (PLS)	Partial least square discriminant analysis (PLS-DA)
Nonlinear model	Hierarchical cluster analysis (HCA)	Logistic regression analysis	Naïve Bayes classifier
	K-means cluster analysis	Conditional logistic regression analysis	Support vector machine (SVM)
	Mixture of Gaussians	Generalized linear model (GLM)	Decision trees

Table 1. Algorithm examples for multivariate analysis

On the contrary, supervised methods (Caruana, 2006) themselves contain the objective variables. Therefore the goal of analysis is to find a model (or classifier) in which the error between the model's response and the target traits is minimized to fit the target traits. Target traits can be discrete (e.g., disease vs. healthy, grade of disease) or continuous (e.g., measurement value). Supervised methods are also applicable to discover and predict which metabolites are responsible for the target traits (Maeda et al, 2010; Okamoto et al, 2009; Zhang et al, 2006). However, the generality of the model obtained from those methods can not be always guaranteed because of the potential overfitting or bias of data. Therefore,

validation of the obtained model is necessary to establish the usefulness for practical use. Validation methods are categorized into two classes. The first is cross- validation in which single or multiple samples are iteratively left out from the training data set, and the remaining samples are used to evaluate the predictive performance of the model. The other is usage of external validation data set which must not be used for construction of models. Ideally, the latter case in which blinded data set is used is the most appropriate validation. However, it is sometimes difficult to perform the validation test itself.

Various metrics are used as criterion of the performance of diagnosis. In the case of the model in which the object variable contains only two classes (e.g., controls and patients), receiver-operator characteristic (ROC) curve analysis is the most appropriate criteria for evaluating the model because this analysis is independent of both sample size of each group and threshold. As threshold metrics, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy are used. Among them, both sensitivity and specificity is independent of sample size and ratio of each group while the others are dependent. Therefore, to determine threshold in terms of PPV, NPV, and accuracy, it is necessary to take into account the "real" distribution of subjects.

3. Examples of clinical implementation of focused metabolomics

3.1 „AminoIndex technology": Example for early cancer diagnosis

Several investigators have reported changes in plasma free amino acid (PFAA) profiles in cancer patients (Cascino et al, 1995; Lai et al, 2005; Lee et al, 2003; Maeda et al, 2010; Naini et al, 1988; Norton et al, 1985; Okamoto et al, 2009; Proenza et al, 2003; Vissers et al, 2005; Zhang & Pang, 1992). Despite evidence of a relationship between PFAA profiles and some types of cancer, few studies have explored the use of PFAA profiles for diagnosis because although PFAA profiles differ significantly between patients, the differences in individual amino acids do not always provide sufficient discrimination abilities by themselves (Cascino et al, 1995; Lai et al, 2005; Naini et al, 1988; Norton et al, 1985; Proenza et al, 2003; Vissers et al, 2005). To address this issue, we have studied using diagnostic indices based on PFAA concentrations that compress multidimensional information from PFAA profiles into a single dimension to maximize the differences between patients and controls.

In previous studies, the alterations in PFAA profiles in cancer patients sometimes seem inconsistent, and some discrepancies existed between our study and those reported (Cascino et al, 1995; Lai et al, 2005; Naini et al, 1988; Norton et al, 1985; Proenza et al, 2003; Vissers et al, 2005). This discrepancy may be due not only to the statistical aspect of data, for example, sample size, the biased distribution of cancer stages, etc., but also to some other factors such as amino acid measurement methods. In contrast to previous studies, we performed analyses using samples in which PFAAs were measured in a unified protocol to guarantee the robustness of analysis in terms of the quality of data (Shimbo et al, 2009a; Shimbo et al, 2009b; Shimbo et al, 2009c).

As a pilot study, we investigated the possibility for early detection of colorectal cancer (CRC) and breast cancer (BC) patients (Okamoto et al, 2009). PFAA profiles were compared between cancer patients (who had CRC or BC) and control subjects. The plasma concentrations of several amino acids in the CRC patients were significantly different from

those observed in the controls. The alteration of the PFAA profile in BC differed from that in CRC, with fewer changes observed. Multiple logistic regression analyses with selected variables using each data set resulted in AUC of ROC of0.860 for CRC and 0.906 for BC, respectively when using training data sets. To confirm the performance of the obtained classifier, ROC curves were also generated from the split test data. These reproduced similar diagnostic performances, with AUC of 0.910 for CRC, and 0.865 for BC, respectively.

We then investigated the possibility for early detection of non-small-cell lung cancer (NSCLC) using a larger size of samples (Maeda et al, 2010). 141 NSCLC patients and 423 age-matched, gender-matched healthy controls without apparent cancers were used as the study data set. As a result, fifteen amino acids (Ser, Gly, Ala, Cit, Val, Met, Ile, Leu, Tyr, Phe, His, Trp, Orn, Lys, and Arg) were identified whose profile in plasma were associated with NSCLC. Multiple logistic regression analyses by conditional likelihood methods were performed with variable selection and LOOCV cross-validation using the study data set. The resulting conditional logistic regression model included six amino acids: Ala, Val, Ile, His, Trp, and Orn. The AUC of ROC for the discriminant score was 0.817 in the study data set. It should be noted that conditional logistic (c-logistic) regression analysis can correct the effects of age, gender, and smoking statuses which are potential confounding factors in the discrimination. To verify the robustness of the resulting model, a ROC curve was also generated using the split test data set, which had not been used to construct the model. An AUC of ROC for the discriminant score was 0.812 in the test data set, again demonstrating that the obtained model performed well (Figure 6).
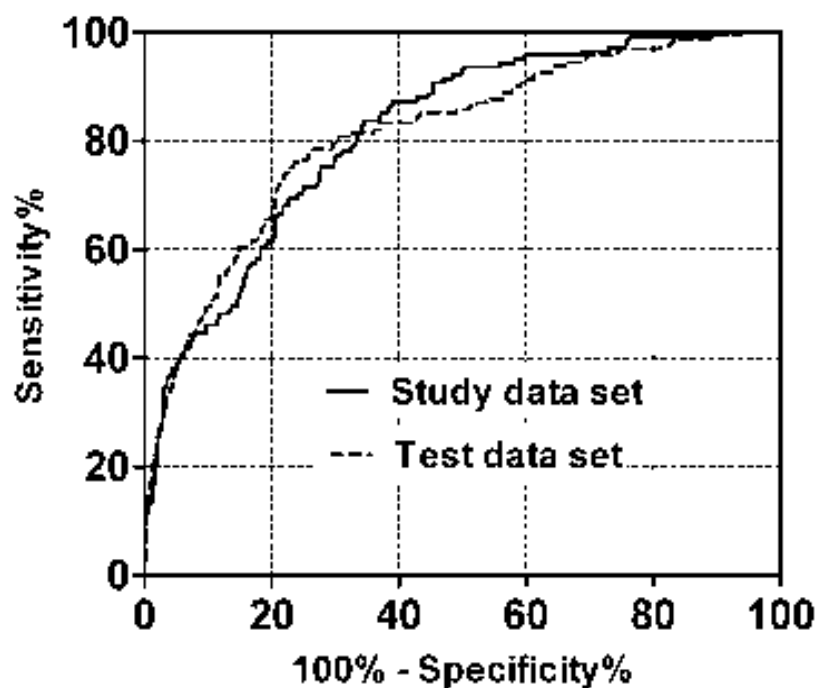

Fig. 6. ROC curves for discriminant scores for the discrimination of NSCLC(Maeda et al, 2010).

It was indicated that the model could discriminate lung cancer patients regardless of cancer stage or histological type. Furthermore, the distribution of the discriminant scores for small-cell lung cancer (SCLC) patients was similar to that for NSCLC patients (Figure 7).
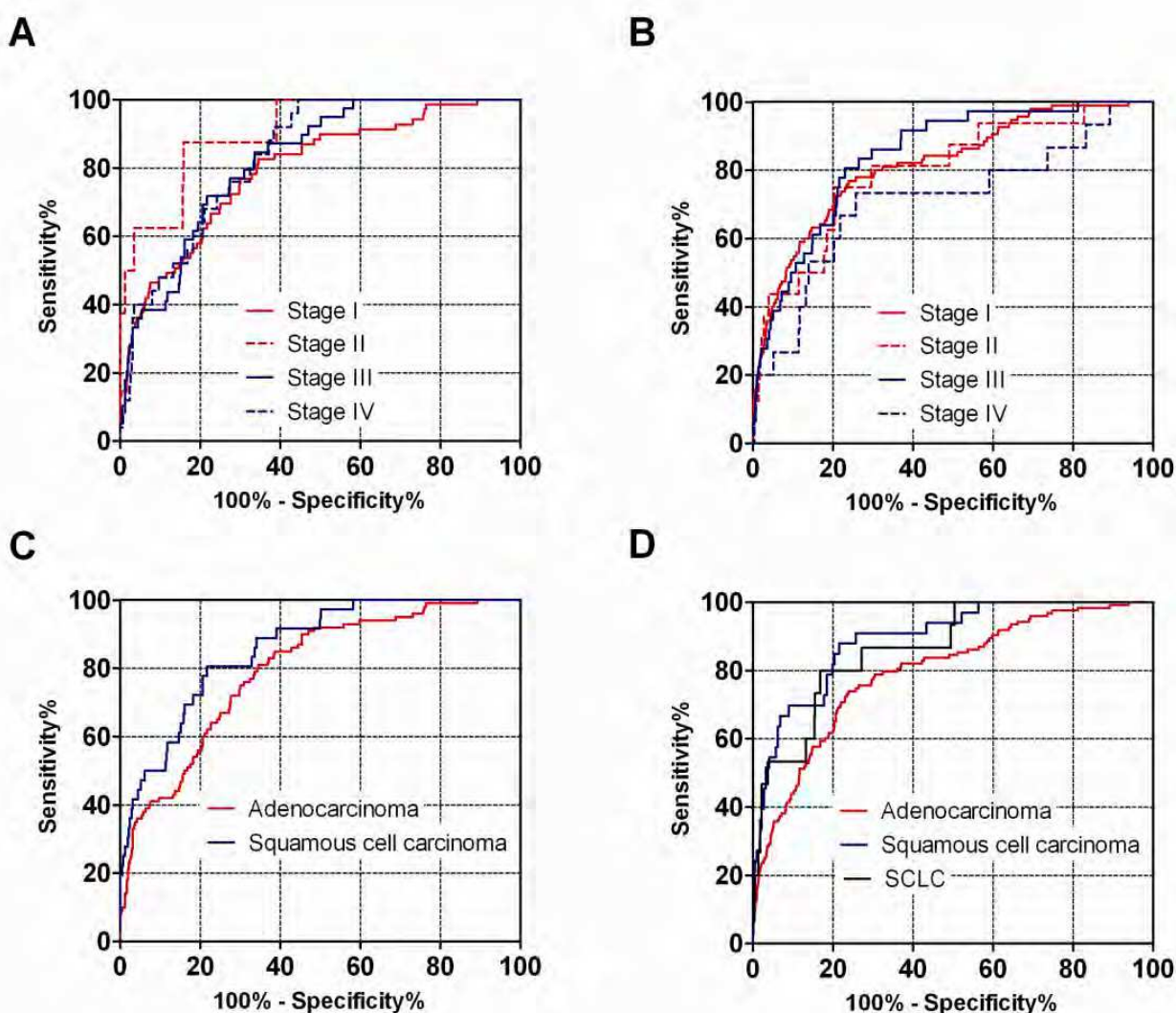
Fig. 7. ROC curves for discriminant scores subgrouped by NSCLC stage and histological type (Maeda et al, 2010). A. ROC curves for cancer stage of study data set. B. ROC curves for cancer stage of test data set. C. ROC curves for histological type of study data set D. ROC curves for histological type of test data set (including SCLC patients).

These studies demonstrated the potential use of PFAA profiling as a focused metabolomics approach for the early detection of patients with various types of cancer. Combining novel analytical techniques and statistical analyses, previously unknown aspects of amino acid metabolism in humans have been revealed. The analysis using considerably larger sample size provided sufficient statistical power to test the robustness of PFAA profiling for cancer diagnosis. We also demonstrated the possibility of detecting cancers, both specifically and broadly, using multivariate analysis to compress the PFAA profile data, even for patients with early stage cancer. Following the further accumulation of data (not shown), AminoIndex® Cancer Screening (AICS) has been commercially released from Ajinomoto Co., Inc., in Japan in April 2011. AICS enables multiple cancer diagnoses simultaneously of gastric, lung, colorectal, prostate and breast cancer.

3.2 „AminoIndex technology": Example for diagnosis of liver fibrosis

In the clinical pathway of patients with chronic hepatitis C infection, the progression of liver fibrosis leads to cirrhosis and eventually increases the risk of hepatocellular carcinoma (Poynard et al, 2003). The efficacy of current therapy depends on the fibrosis grade, and therefore the detection of fibrosis stage is desirable for determining the clinical settings, i.e., whether treatment is necessary, and what treatment is appropriate (Aspinall & Pockros, 2004; Fried, 2002; Shiffman, 2004). Although fibrosis grading based on biopsy has been considered as a gold standard, there is a high demand for less invasive but effective alternative methods.

In searching for surrogate markers other than biopsy, several methods ranging from the serologic marker-based test (Fibrotest)(Imbert-Bismut et al, 2001) to the ultrasonic-based transient elastography (Fibroscan)(Castera et al, 2005), and others(Lin et al, 2011b) have been suggested. On the other hand, since the liver is an important organ for the metabolism of amino acids, glucose synthesis, fatty acid synthesis, urea synthesis and protein synthesis(Cynober, 2004), it is reasonable to expect any metabolic derangement due to liver failure like liver fibrosis may induce the variation of amino acid metabolism and eventually the variation of PFAA concentration.

In this section we describe the PFAA profiling which was first applied to the diagnosis of liver fibrosis using clinical data(Zhang et al, 2006). The aim of this study was to develop a diagnostics index for the diagnosis of liver fibrosis as a less invasive and effective method using PFAA profiles. The liver specimens were analyzed histologically and graded with the METAVIR scoring system(Metavir., 1994), where F0 means no fibrosis, F1 portal fibrosis without septa, F2 fibrosis with rare septa, F3 portal fibrosis with numerous septa, and F4 cirrhosis. The distribution and variation of the 23 PFAAs of all patients over fibrosis stages is represented in a radar chart, Figure 8.

In the progression of fibrosis from F01 to F4, the decrease of BCAA and inversely the increase of aromatic amino acids, Phe and Tyr, can be observed typically in the profiles of the radar chart. In the non-parametric multi-stage comparison test (Kruskal-Wallis test) , for each amino acid among different fibrosis stages, significant changes in concentration of Phe, Val, Ile, Tyr, Gln, Leu, Met ($p < 0.01$) and ABA (alpha-amino butyric acid, $p < 0.05$) were observed. Dataset including fibrosis stage and PFAA concentrations were analyzed to obtain the diagnostics index for liver fibrosis (AI_fibrosis) in fractional form, (Phe)/(Val) + (Thr+Met+Orn)/(Pro+Gly), which was optimized as a surrogate marker for the liver stages obtained through biopsies. The distribution of molar ratios in two fractional forms over fibrosis stages are shown in Figure 9.

The observation of two molar ratios in the classifier revealed that the former ratio mainly contributed to the F4 discrimination, whereas the latter mainly contributed to discrimination of advanced fibrosis (F3 and F4). For the discriminative power assessment of the surrogate AI_fibrosis as a whole, the area under the curve of receiver operator characteristic curve (ROC AUC) was used. The classifier exhibited high discriminative power for advanced fibrosis (fibrosis stages F3 and F4) from the earlier stages F0-2 and also for cirrhosis (F4) from all other stages, with ROC AUC (95% CI) 0.92 (0.84-1.00) and 0.99 (0.96-1.00), respectively.
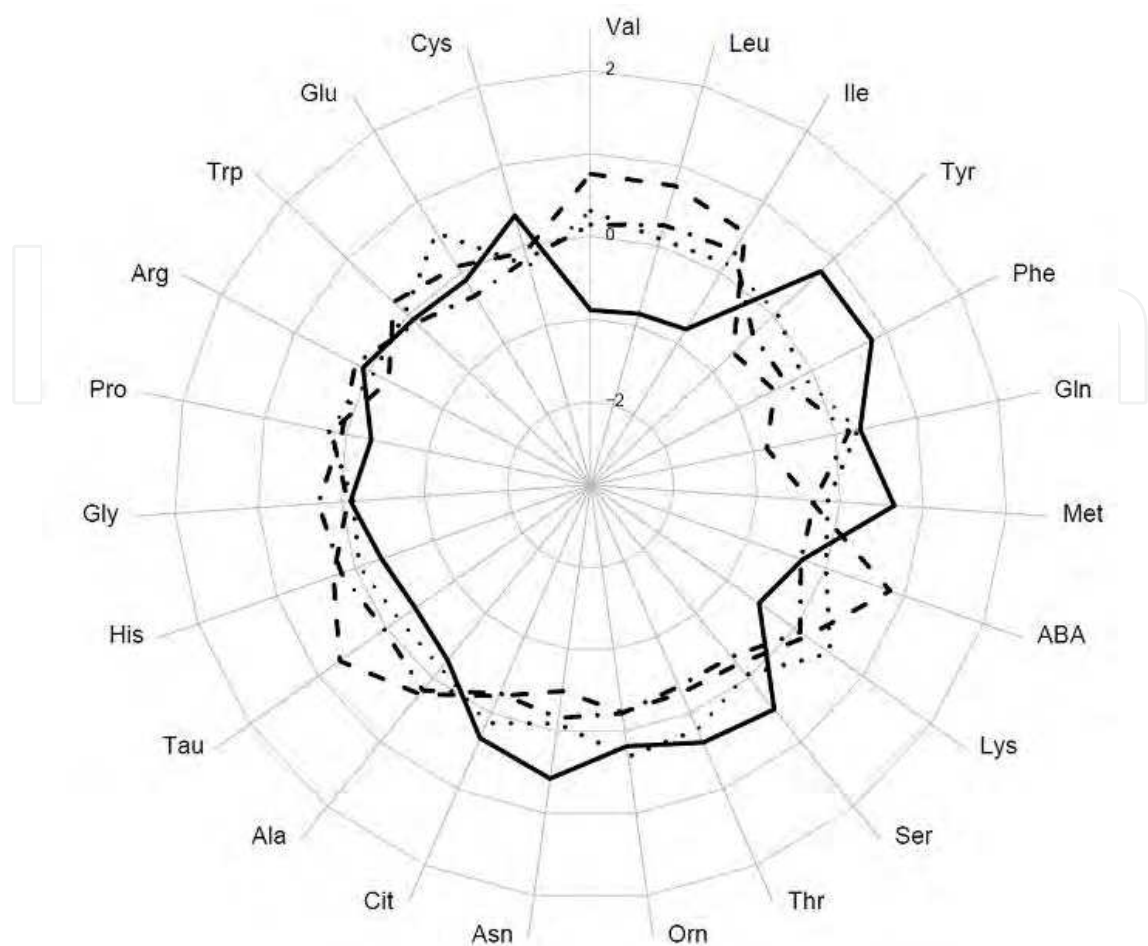
Fig. 8. Radar chart of mean values of PFAAs over fibrosis stages. F01: dashed, F2: dot-dash, F3: dotted, F4: solid. Mean values are scaled in z-score.
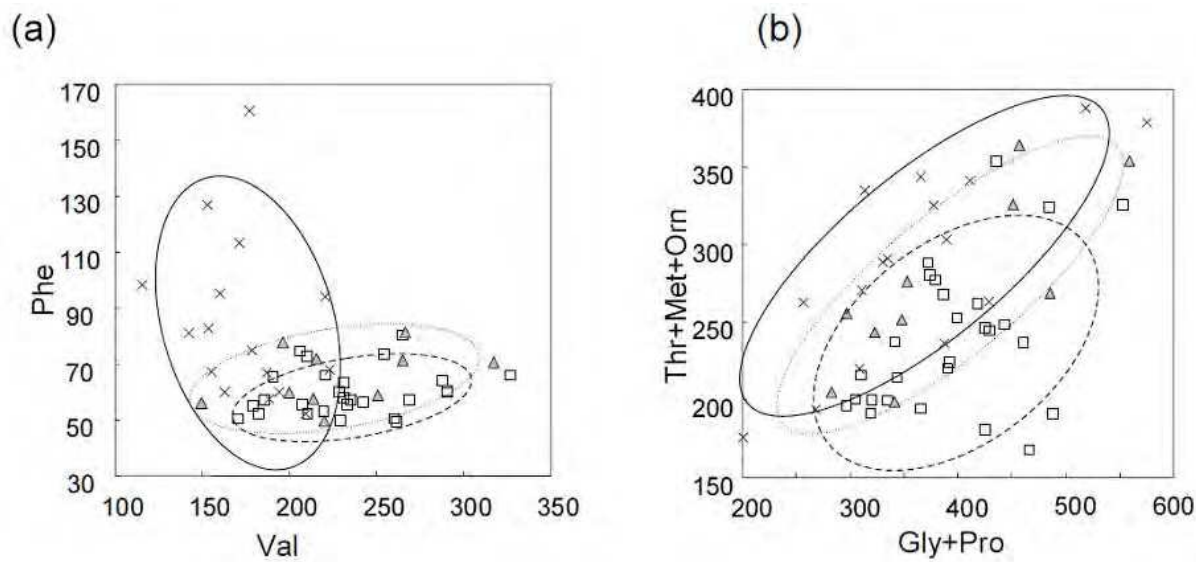

Fig. 9. Molar ratio variation over fibrosis stages. The change in distribution among F0-F2,F3 and F4 stages indicated a stage-dependent trend. Circles are 80% regions of each stage, F0-F2: dashed and square, F3: dotted and triangle, and F4: solid and christcross.

The Fischer's ratio (Val+Leu+Ile)/(Phe+Tyr) was originally created for diagnosis of hepatic encephalopathy (Fischer et al, 1975; Fischer et al, 1976) and has been reported to show good performance in assessing chronic hepatitis (Kano et al, 1991). Therefore a comparison study between the Fischer's ratio and the classifier was undertaken, where the index was generated to have a positive correlation with the degree of fibrosis, showing an inverse pattern to Fischer's ratio. The AI_fibrosis indicated ROC AUC values larger than Fischer's ratio: the ROC AUC values of Fischer's ratio being 0.87 (0.77-0.96) for advanced fibrosis and 0.91 (0.83-0.99) for cirrhosis, respectively. There is a close relationship between the AI_fibrosis and the Fischer's ratio as partially supported by the fact that the ratio Phe/Val correlated well with the inverse of Fischer's ratio (r = 0.95) because the BCAAs exhibited good mutual correlation, as did Tyr and Phe. In summary, these results suggest that the AI_fibrosis based on amino acid concentration can be applied to evaluate liver fibrosis as an effective and less invasive method as a surrogate marker for liver biopsy, although future extended validation study is still necessary.

3.3 Lipidomics: A review on the use of lipid metabolomics for clinical use

Lipidomics, a type of focused metabolomics, is the comprehensive measurement of a variety of lipid classes: free fatty acids (FFA), triglycerides (TAGs), cholesterol esters (CEs), lysophosphatidylcholines (LPCs), phosphatidylcholines (PCs), lysophosphatidyl ethanolamines (LPEs), diacylglycerols (DAGs), and sphingomyelins (SMs) and ceramides, generally using LC-MS/MS (Bou Khalil et al, 2010; Bucci, 2011; Dennis, 2009). Several studies have reported the potency of lipidomic analyses for biomarker discoveries in humans in diabetes, non-alcoholic fatty liver disease (NAFLD) (Puri et al, 2009), Alzheimer's disease (Han et al, 2011; Valdes-Gonzalez et al, 2011) and cancers (Hilvo et al, 2011; Min et al, 2011). For instance, Rhee et al reported the LC-MS–based lipid profiling of 189 individuals who developed type 2 diabetes and 189 matched disease-free individuals, with over 12 years of follow up in the Framingham Heart Study (Rhee et al, 2011). They found that lipids of lower carbon number and double bond content were associated with an increased risk of diabetes, whereas lipids of higher carbon number and double bond content were associated with a decreased risk. In addition, Barr et al demonstrated differential serum lipidomics in both NAFLD patients and in a mouse model of NAFLD by ultra performance liquid chromatography-mass spectrometry (UPLC-MS) (Barr et al, 2010). Multivariate statistical analysis of the UPLC-MS datasets revealed metabolic similarities between NAFLD mice and human NAFLD patients in relative serum metabolite levels compared to normal subjects. Lipidomic analysis is also applicable to other biological fluids such as cerebrospinal fluid (CSF), in addition to plasma and serum (Fonteh et al, 2006). For instance, phospholipid profiling in the CSF by nano-HPLC-MS has been reported in Alzheimer's disease (AD) patients, and a statistically significant increase of SMs were observed in CSF from probable AD patients compared to normal subjects (Han et al, 2011).

4. Elucidation of mechanisms underlying metabolomic diagnosis

4.1 Introduction

Many living systems have homeostatic mechanisms to continuously maintain their biological activity. Yet, when a dynamic multi-parametric metabolic response to patho-

physiological stimuli is evoked in many disease-associated cells and tissues, it leads to the formation of disease-specific enzymatic metabolite profiles quite different from that of the healthy hosts, and the blood components are significantly influenced as a result.

Blood amino-acid contents are included in such components (referred to as a blood amino-acid profile). It is well known that in the process of feeding, exercising, sleeping, and other activities, the blood amino-acid profile temporarily fluctuates, but within a few hours returns to the normal level through intrinsic homeostatic mechanisms. By contrast, disease-mediated disturbances in the local amino-acid metabolisms may result in formation of a disease-specific change in the blood amino-acid profile. Based on these findings and discussions, we have introduced the AminoIndex® Cancer Screening (AICS) system as a tool for providing new biomarkers to enable the early detection of various cancers.

4.2 Tumor-specific blood amino-acid profile

In order to explain the effects on PFAA profiles by the various tumors, we propose the following simple idea consisting of "three components". As shown in Figure 10, these three components are as follows: a) Metabolic changes in the tumor-bearing organs; b) Metabolic changes in response to the inflammatory reactions; and c) Metabolic changes in various remote healthy organs. In patients with tumors, these three metabolic changes may be evoked simultaneously and their overall effects may be reflected in the tumor-specific blood amino-acid profile. Yet, it is highly unlikely that they contribute evenly to such formation of the tumor-specific blood amino-acid profile during the entire course of the tumor development. It seems more reasonable that these three components contribute individually and differently to the formation of the tumor-specific blood amino-acid profile in the early, the mid and the late (cachexia) stages.
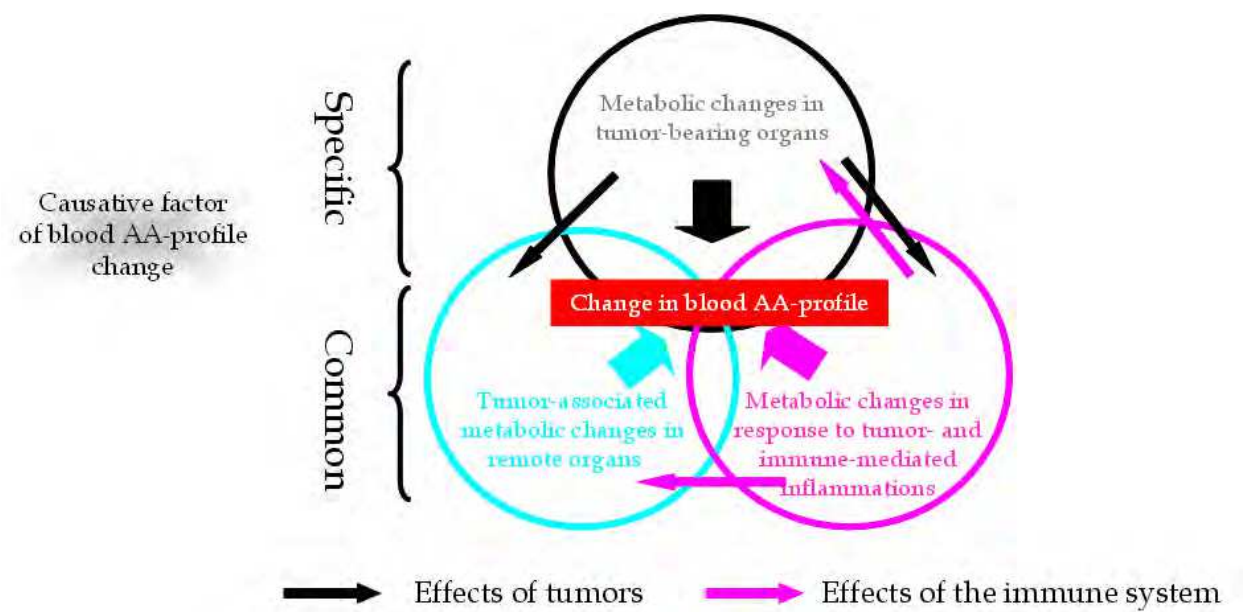

Fig. 10. Scheme for mechanisms underlying tumor-specific metabolic changes

In tumor-bearing hosts, "metabolic changes in tumor-bearing organs", "metabolic changes in response to inflammations" and "metabolic changes in remote organs" are all evoked simultaneously, leading to formation of the tumor-specific amino-acid profile.

4.3 Metabolic changes in the tumor-bearing organs

It has been shown that cancer cells, which can proliferate extraordinarily faster than healthy normal cells, obtain the biological energy required to proliferate by switching to aerobic glycolysis from oxidative phosphorylation (Matthew et al, 2009)(known as "Warburg effect"): several intermediates obtained from the glycolysis pathway might be more easily utilized in nucleotide synthesis and glucose might be used as a carbon source in fatty acid generation (Dang, 2010). At the same time, glutaminolysis was found to be stimulated profoundly, meaning that cancer cell energy generation is highly dependent on glutamine content (Wise & Thompson, 2010). A change in the amino-acid metabolism has been documented even in several noncancerous areas of the tumor-bearing organs. Douvlis has proposed the possibility that many normal tissues show their own specific pattern of the amino-acid absorption but such normal-cell functions may be impaired by amino acids abnormally excreted from neighboring tumor cells (Douvlis, 1999).

4.4 Metabolic changes in response to inflammation

Tumor-associated persistent inflammatory responses are regarded as one of the causative factors for changes in amino-acid metabolism. In addition, inflammation-mediated proliferation of immune competent cells and synthesis of various inflammatory proteins including cytokines and chemokines are also involved (Fox et al, 2005). In almost all of the solid tumors, a variety of inflammatory responses are shown to be evoked in the tumor-surrounding tissues (Mantovani et al, 2008). The tumor-associated inflammation is characterized by a mixture of the anti-tumor inflammatory response, which directs tumor-cell killing, and the tumor-induced inflammation, which stimulates tumor-cell proliferation and promotes neovascularization (Schetter et al, 2010). Therefore, such tumor-associated metabolic changes can be substantially different from those observed in other inflammatory processes seen in pneumonia and colitis. In addition, these tumor-associated inflammations can induce immunocompetent-cell proliferation and antibody production both in tumorous tissues, regional lymph nodes, and the bone marrows, leading to further changes in metabolism (Youn & Gabrilovich, 2010).

4.5 Metabolic changes in various remote normal organs

With an increase in amino-acid demands closely associated with elevated synthesis of nucleotides and proteins in tumor cells, the amounts of amino acids recruited from other tissues and organs are increased by means of enhancing whole body protein turnover, elevating hepatic nonessential amino-acid biosynthesis, and stimulating proteolysis in skeletal muscles accompanied by reduced protein synthesis (Rossi Fanelli et al, 1995). It is well known that in liver and skeletal muscles, tumor-induced negative nitrogen balance can promote intracellular production and extracellular release of glutamine (Medina et al, 1992). In addition, it was reported that in a chronic inflammatory process, the amino-acid metabolism could be influenced even in many remote organs: For instance, absorption of blood cysteine and methionine were both elevated immediately after glutathione synthesis was increased in the liver (Mercier et al, 2002).

5. Future expectations

Although the applications of "AminoIndex technology" are still limited, the foundations for their use for diagnostic purposes are in progress as described above. Studies with clinical data indicate that even with individual variability, the "AminoIndex technology" can be used to separate certain disease and physiological states. We believe that the amino acids are a convenient metabolomic subset to use as a model for the development of metabolomics based diagnostics, and that in the near future, other metabolites could be added to the current analytical platform as practical issues such as stability are solved. At the same time, the universality of the findings must be examined and it should be studied whether the data set we have obtained for the Japanese population is applicable to other populations. We believe that there is a great potential to use metabolome based markers in preliminary diagnostic screening for multiple diseases in which a single measurement of a metabolomic subset can lead to multiple diagnoses. One further advantage of the focused metabolomics multiple metabolite marker approach is that since the biomarkers are generated from a combination of already measured markers, new markers can be generated against any measured target parameter. This means that if a focused metabolomic subset data is obtained at the beginning of a treatment or an experiment, the generation of predictive markers can be attempted with the outcome of the treatment or experiment as the target parameter. We believe this would be of great use in tailor-made medicine and nutrition, as it may be possible to discriminate populations for which certain pharmaceutical or nutritional interventions would be useful or not.

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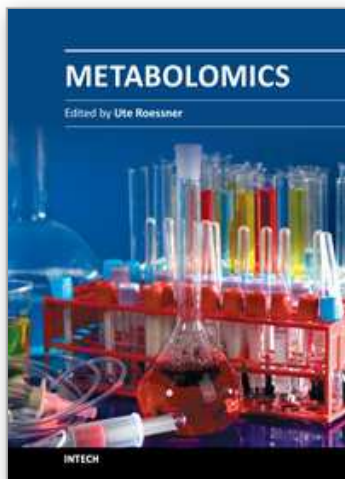
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Metabolomics is a rapidly emerging field in life sciences, which aims to identify and quantify metabolites in a biological system. Analytical chemistry is combined with sophisticated informatics and statistics tools to determine and understand metabolic changes upon genetic or environmental perturbations. Together with other 'omics analyses, such as genomics and proteomics, metabolomics plays an important role in functional genomics and systems biology studies in any biological science. This book will provide the reader with summaries of the state-of-the-art of technologies and methodologies, especially in the data analysis and interpretation approaches, as well as give insights into exciting applications of metabolomics in human health studies, safety assessments, and plant and microbial research.

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