Supplement Article

Use of plasma-free amino acids as biomarkers for detecting and predicting disease risk

Kenji Nagao and Takeshi Kimura

This paper reviews developments regarding the use of plasma-free amino acid (PFAA) profiles as biomarkers for detecting and predicting disease risk. This work was initiated and first published in 2006 and was subsequently developed by Ajinomoto Co., Inc. After commercialization in 2011, PFAA-based tests were adopted in over 1500 clinics and hospitals in Japan, and numerous clinician-led studies have been performed to validate these tests. Evidence is accumulating that PFAA profiles can be used for diabetes prediction and evaluation of frailty; in particular, decreased plasma essential amino acids could contribute to the pathophysiology of severe frailty. Integration of PFAA evaluation as a biomarker and effective essential amino acid supplementation, which improves physical and mental functions in the elderly, could facilitate the development of precision nutrition, including personalized solutions. This present review provides the background for the technology as well as more recent clinical findings, and offers future possibilities regarding the implementation of precision nutrition.

INTRODUCTION

Amino acids play important roles in many metabolic pathways, and the quantification of free amino acids in biological fluids and tissues has historically provided nutritional information used in the diagnosis of certain diseases, especially metabolic deficiencies. Specific abnormalities in amino acid concentrations are reported in the context of various diseases, including liver failure,[1] renal failure,[2] cancer,[3] diabetes,[4] fatty liver,[5] muscle dysfunction,[6,7] Alzheimer's disease,[8] and protein malnutrition.[9,10] The ratio of branched-chain amino acids (BCAAs) to aromatic amino acids, known as Fischer's ratio, is an established diagnostic marker used to monitor the progression of liver fibrosis and the effectiveness of drug treatments.[11–13] Additional methods have been recently developed to generate indices composed of multiple plasma-free amino acid (PFAA) concentration data that correlate with specific target physiological parameters or that discriminate between 2 physiological states, thus expanding the potential use and commercialization of such amino acid-based indices.[14,15] This review focused on these new developments in the use of PFAAs as biomarkers for disease risk prediction and their potential application for monitoring nutritional and disease risk in the elderly, outlining methodological issues that needed to be overcome in order that research could be translated into a commercial service available in the clinical setting.

METHODOLOGICAL ISSUES

To ensure the reproducibility, accuracy, and high throughput of PFAAs for application in a commercial clinical setting, it was initially necessary to overcome a number of methodological issues. First, to overcome the meal-dependent variability in amino acid concentrations,[16] blood collections were performed in the

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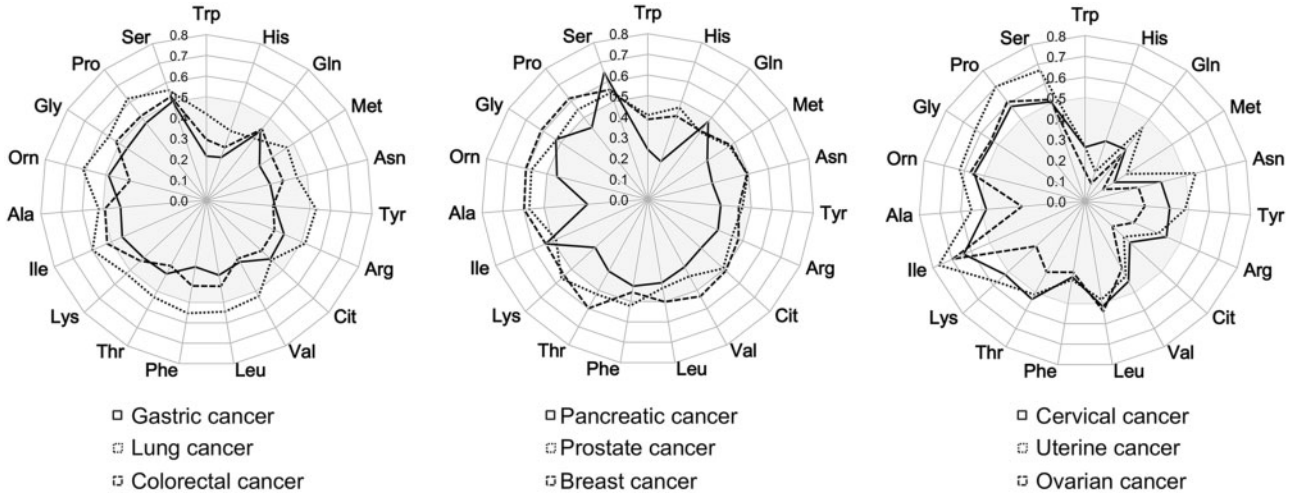

Figure 1 **Plasma amino acid profiles of various cancer patients**. For each amino acid, an area under the receiver operating characteristic (ROC) curve that distinguishes each cancer patient from a healthy control is shown. For healthy control, the value is set at 0.5, and when the area under the ROC curve for cancer patients is less than 0.5, the amino acid concentration is lower. When it is greater than 0.5, the amino acid concentration is higher than the control value. Modified from Miyagi et al (2011)[3], Fukutake et al (2015)[32], and Ihata et al (2014).[33]
Abreviations: Ala, alanine; Arg, arginine; Asn, asparagine; Cit, citrulline; Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine

morning after an overnight fast. If blood samples are left at room temperature after collection, many amino acids are metabolized through the enzymatic activities of blood cells.[17] For this reason, a portable blood tube cooler (CubeCooler), to enable the rapid cooling of blood samples, was developed.[18] To increase throughput for handling a large number of samples for commercial use, a novel derivatization reagent, 3-aminopyridyl-N-hydroxysuccinimidyl carbamate, was developed for liquid chromatography–mass spectrometric analysis, allowing for both rapid separation of amino acids and their highly sensitive detection.[18–22] A dedicated liquid chromatography–mass spectrometry system with an automatic derivatization device that uses this reagent is available from the Shimadzu Corporation.[18] Reference intervals, authorized by the Japan Society of Clinical Chemistry, were established for 21 PFAAs using 7685 reference individuals from the Japanese population.[23]

EARLY CANCER DETECTION

Many investigators have reported changes in PFAA profiles in cancer patients.[24–31] By themselves, differences in individual amino acids do not always provide sufficient discrimination capability, and therefore diagnostic indices that compress the multidimensional information from PFAA profiles into a single dimension were studied to maximize the differences between patients and controls. Comparison and analysis of the ratios of PFAAs between cancer patients and healthy individuals revealed that the PFAA concentration ratios were altered in 7 types of cancers.[3,32,33] The changes in PFAA profiles in various cancer patients are shown in Figure 1.[3,32,33] Some amino acid changes were common in any cancer type, while other changes were characteristic only of certain cancer types. Moreover, such changes in the PFAA profile were observed in the early stages of cancer.

These findings led to the development and clinical implementation of the AminoIndex Cancer Screening (AICS) system as an early screening test that evaluates the risk of having specific cancers.[34–37] The AICS system was commercially released from Ajinomoto Co., Inc., in April 2011, and is available in more than 1500 clinics and hospitals in Japan. The AICS formula for each cancer type is a combination of 6 amino acid concentrations as variables depending on the cancer type. The discrimination capability was improved by combining amino acids common to various cancer types with amino acids that vary characteristically between cancer types. Table 1 summarizes the area under the receiver operator characteristic curve, an indicator of discrimination capability. It shows a value of 0.68 or more for each cancer type, which suggests good discrimination capability for clinical use.

CHARACTERISTICS OF AMINOINDEX CANCER SCREENING

In AICS, the risk of having cancer is indicated by a numerical value (AICS value) calculated from the PFAA

Table 1 **Area under the ROC curve for each cancer type**

Cancer type	ROC-AUC
Gastric	0.85
Lung	0.83
Colorectal	0.77
Pancreatic	0.86
Prostate	0.78
Breast	0.68
Uterine/ovarian	0.87

Abbreviation: ROC-AUC, receiver operating characteristic–area under the curve.

data that ranges from 0.0 to 10.0. A specificity of 80% for each cancer type was set to an AICS value of 5.0, and a specificity of 95% was set to a value of 8.0. The higher the risk of cancer, the higher the AICS value. In addition, as a guideline for judging the risk indicated by the AICS value, the AICS value was classified into 3 ranks, with values of 0.0–4.9 indicating "Rank A," values of 5.0–7.9 indicating "Rank B," and values of 8.0–10.0 indicating "Rank C." The AICS test shows the risk of having cancer at the time of blood collection and is not a test to determine whether or not one has cancer. Someone with a "Rank C" result has a 4.0- to 11.6-fold higher risk of having cancer than the general population and should undergo a detailed examination.

The discriminative capability of AICS does not depend on the tissue type or the site of cancer.[38,39] For example, the AICS (stomach) test has a sensitivity of 65% for poorly differentiated adenocarcinoma, which is difficult to detect by means of a pepsinogen test, and a sensitivity of 52% for signet-ring cell carcinoma. The AICS (uterus/ovary) test has a sensitivity of 72% for cervical adenocarcinoma, which can be missed by cervical cytology. The AICS (pancreas) test also has a sensitivity of 62% for cancer that occurs in the pancreatic tail, which may be missed by ultrasonography.

A multicenter clinical validation study in a cohort comprising 10245 individuals who underwent AICS was recently reported.[40] The sensitivities of "Rank C" for cancer diagnosis within 1 year after AICS examination were 83.3% (10/12) for gastric cancer, 50.0% (2/4) for lung cancer, 46.2% (6/13) for colorectal cancer, 50.0% (8/16) for prostate cancer, 43.8% (7/16) for breast cancer, and 50.0% (1/2) for uterine/ovarian cancer. The total cancer detection rate determined via the AICS system was 0.33% (34/10245), compared with the general detailed examination (Ningen-Dock) cancer detection rate of 0.26%. The performance of the AICS system compares favorably with that of other systems,[41] and the AICS (lung) test is reported to be more sensitive than routine chest X-ray for the detection of tumors with a diameter of 1.0 cm or less.[42]

Recently, pre- and postoperative AICS values were examined for AICS (colorectal),[43] AICS (lung),[44] and AICS (uterine/ovarian).[45] The pre- and postoperative AICS (colorectal) values were examined in 62 colorectal cancer patients who had undergone curative resection.[43] The postoperative AICS (colorectal) value was lower than the preoperative value in 57 of the 62 patients and the rank was also lower in 49 patients with no recurrence during the study period. In a different study, pre- and postoperative AICS (lung) values were compared in 72 lung cancer patients who underwent curative resection.[44] Of the 44 preoperative Rank B–C patients, 23 experienced postoperative reductions in the AICS rank (Figure 2[44]). Further, the absence of a postoperative reduction in the AICS (lung) rank was associated with cancer recurrence, suggesting that AICS (lung) rank is a sensitive marker of postoperative recurrence.

POSSIBLE MECHANISMS OF PLASMA-FREE AMINO ACID PROFILE CHANGES IN CANCER PATIENTS

The mechanism of PFAA changes can be examined from a number of viewpoints, such as amino acid metabolism of the cancer itself, metabolism of distant normal tissues, and metabolic effects caused by the immune system. In cancer tissues, the recruitment pathway from glutamine to the citric acid cycle is enhanced and Gln transporter expression is increased.[46] In addition, metabolomic analysis of surgical specimens of colorectal cancer patients shows increased concentrations of valine and methionine in local cancers.[47,48] A decrease in glutamine is commonly observed in cancer patients (Figure 1), and a decrease in valine and methionine is observed in patients with colorectal cancer, so certain PFAAs may be decreased owing to increased amino acid uptake by the cancer cells. Studies of rats with subcutaneous cancer cell transplantation demonstrated that gluconeogenesis from alanine is suppressed in the liver.[49] High mobility group box protein 1 derived from colon cancer induces skeletal muscle autophagy and PFAA changes in mouse models of colon cancer[50] and is reportedly increased in early cancer patients.[51] Furthermore, a metabolite of tryptophan, kynurenine, is reported to contribute to a suppressed immune environment, and the tryptophan : kynurenine ratio in the blood has been found to decrease in patients with early lung cancer.[52] These data seem to suggest that cancer cells can change the metabolism in distant tissues to create an environment conducive to their growth. Further research is needed, however, to unveil the mechanism of PFAA changes in the context of cancer.

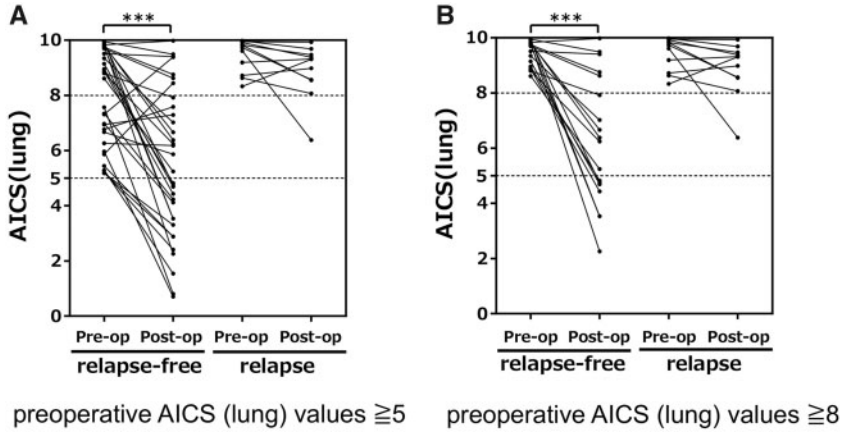

Figure 2 **Postoperative changes in AICS (lung) values**. Paired pre- and postoperative AICS (lung) values are shown for patients with a preoperative AICS (lung) rank of (A) B–C, and (B) C. The dotted line shows a cutoff AICS (lung) value of 5.0 corresponding to a specificity of 80.0%, and a cutoff value of 8.0 corresponding to a specificity of 95.0%. ***$P < 0.001$ (Wilcoxon signed-rank). Modified from Anayama et al (2018).[44] *Abbreviations:* AICS, AminoIndex Cancer Screening; pre-op, preoperative; post-op, postoperative

DIABETES PREDICTION

Diabetes risk assessment is an area of interest in metabolomics research, and several changes in metabolites, including elevations of BCAAs and aromatic amino acids, have been found to be associated with visceral obesity[53] and insulin resistance.[54–57] A longitudinal analysis confirmed that PFAA analysis can predict the near future development of lifestyle-related diseases.[58,59] PFAA levels can be used to predict the development of diabetes, metabolic syndrome, dyslipidemia, or hypertension within a 4-year period, even after adjusting for commonly accepted risk factors, such as age, sex, body mass index, fasting plasma glucose level, insulin resistance, waist circumference, blood pressure, and lipid variables.[59] Similarly, the Framingham Offspring Study over a 12-year period revealed that BCAA and aromatic amino acid concentrations are significantly related to the future development of type 2 diabetes.[58] A combination of 3 amino acids (isoleucine, phenylalanine, and tyrosine) predicted future type 2 diabetes, whose results were replicated in an independent, prospective cohort.[58] Numerous other studies have observed the predictive capability of PFAA analysis in evaluating the risk of developing lifestyle-related diseases and associated cardiovascular diesases.[60–66] Interestingly, these PFAA patterns, including elevations of BCAAs, are related to the overall dietary pattern rather than the dietary intake of BCAAs.[67] These obesity-related elevations in BCAAs may be the result of changes in amino acid metabolism, especially metabolic changes in adipose tissue.[68,69]

Analysis of general health checkup data from 8070 participants revealed that participants who developed diabetes within 4 years already had PFAA profiles similar to those in patients with diabetes, and based on these PFAA changes, an index known as the AminoIndex LifeStyle diseases (AILS) test was developed.[4] AILS values indicate the risk of developing diabetes, from 0.0 to a maximum value of 10.0. An AILS value of 5.0 was set with a specificity of 40% for the onset of diabetes within 4 years, and a value of 8.0 with a specificity of 80%. In addition, the rank based on AILS is set to "Rank A" when the diabetes risk value is less than 5.0, "Rank B" when it is 5.0–8.0, and "Rank C" when it is 8.0 or more. The higher the AILS value, the higher the risk of developing diabetes within 4 years, with Rank C representing a relative risk of 16.7 compared with Rank A (Figure 3[4]).

AILS (diabetes risk)	Proportion of diabetes onset within 4 years	Odds ratio to rank A	
		Odds ratio	Confidence interval
Rank A	0.4% (12/3008)	1.0	
Rank B	3.1% (96/3091)	8.0	4.4 – 14.6
Rank C	6.7% (107/1604)	17.8	9.8 – 32.5

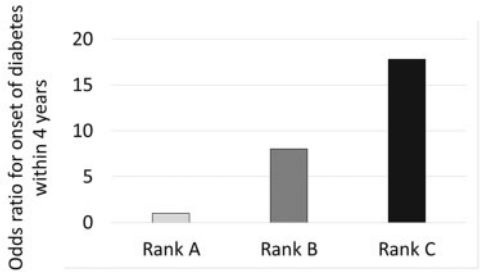

Figure 3 **Risk of developing diabetes within 4 years by AILS**. The odds ratio of diabetes onset risk was 8.0 (relative risk is 7.8) in Rank B, and 17.8 (relative risk is 16.7) in Rank C, compared with Rank A; both ranks were significantly higher than Rank A. Adapted from Yamakado et al (2018)[4]. *Abbreviation:* AILS, AminoIndex LifeStyle Diseases

One study examining whether the AILS value normalizes owing to interventions such as nutrition and

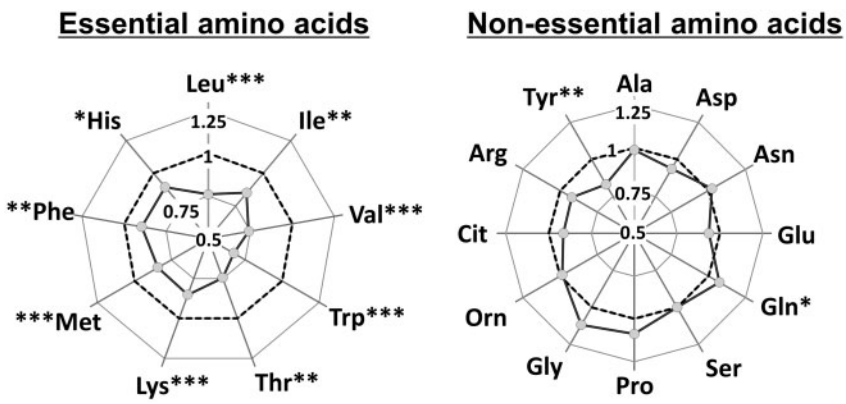

Figure 4 **Comparison of PFAA concentrations of severely frail elderly persons (bold line) and nonfrail elderly persons (dotted line).** The concentration of each amino acid is shown as a ratio compared to nonfrail elderly persons. Compared to the nonfrail elderly persons (n=31; average age 80.3 ± 9.0 y), the severely frail elderly persons (n=28; average age 85.9 ± 9.6 y) had significantly lower essential amino acid plasma concentrations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Welch's t-test). Adapted from Adachi et al (2018)[79]. *Abreviations:* Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartate; Cit, citrulline; Gln, glutamine; Glu, glutamate; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; PFAA, plasma-free amino acid; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine

exercise guidance for 3 months demonstrated that AILS values decreased significantly in those who successfully reduced their weight and waist circumference,[70] suggesting that early-risk assessment using AILS could aid early interventions in at-risk populations.

AGE-RELATED FRAILTY AND LOW PLASMA-FREE AMINO ACID CONCENTRATIONS

Age-related conditions are associated with abnormal PFAA profiles,[6,9,71] suggesting that abnormal PFAA metabolism, especially decreased concentrations of essential and semi-essential amino acids in the plasma, could contribute to the pathophysiology of severe frailty. At the same time, there is evidence that amino acid supplementation, particularly essential amino acids, has beneficial effects on both physical and mental functions in the vulnerable elderly.[72–78] One study recruited 59 elderly patients, aged ≥65 years, and the PFAA profile and clinical characteristics were compared between nonfrail and severely frail groups.[79] The severely frail group had lower body mass index, serum albumin, serum pre-albumin, hemoglobin, and blood pressure values, and higher C-reactive protein values, and 79% of the severely frail patients exhibited cognitive impairment. These severely frail patients had significantly lower concentrations of essential amino acids in their plasma (Figure 4[79]).

Regarding muscle function, essential amino acids, especially leucine (Leu), which is one of the BCAAs, stimulate protein synthesis via the mammalian target of rapamycin complex.[80,81] The beneficial effect of Leu-enriched amino acid ingestion is clinically well demonstrated.[82–85] In a study recruiting community-dwelling older Japanese women (n = 232; 79.4 ± 7.0 y), PFAA concentrations at each stage of sarcopenia (normal, pre-sarcopenia, dynapenia, and sarcopenia) were examined.[86] Significant differences were observed for concentrations of Leu, BCAAs, and essential amino acids among the 4 stages, and the dynapenia and sarcopenia groups showed significantly lower concentrations of Leu. A positive relationship between plasma Leu, BCAA, and essential amino acid concentrations and muscle function was also observed. In a cross-sectional analysis of the Maastricht Sarcopenia study in the Netherlands,[7] lower blood concentrations of essential amino acids, BCAAs, and Leu were associated with lower skeletal muscle index and strength, and longer time to complete the chair stand, whereas no association was found for total amino acids. Furthermore, essential amino acids act as precursors for important neurotransmitters, such as catecholamines, histamine, and serotonin. Thus, low PFAA concentrations could affect health conditions through several mechanisms. A substantial amount of evidence indicates that essential amino acid supplementation improves physical and mental functions through direct and indirect effects.[72–78,82–85] Therefore, stratifying elderly persons using PFAA profiles to supplement the diet with essential amino acids may be an effective way to implement precision nutrition for the elderly.

CONCLUSION

PFAAs can be used as biomarkers for cancer risk detection, prediction of diabetes risk, and potentially for identifying frail persons. The finding that amino acid profiles are similar in early and late cancer patients as

well as in prediabetic and diabetic patients is significant as this offers the possibility of early detection and early intervention or prevention. Whether this observation holds true for other diseases is unknown, but current work on cardiovascular disease and Alzheimer's disease may shed light on this issue. Amino acids can be regarded as a metabolomics subset, and other non–amino acid metabolite data could be integrated into the analysis providing such data can be measured accurately and reproducibly. Eventually, various "omics" data may be combined to increase the power of the analysis. Integration of these technologies and their utilization as a new means of health examination could facilitate the development of personalized solutions, leading to disease prevention.

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