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Towards a Personalized Approach in Pancreatic Cancer Diagnostics Through Plasma Amino Acid Analysis

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Abstract. *Background/Aim: Body fluid biomarkers may provide means for early pancreatic cancer diagnosis, patient stratification, application of personalized approaches and, finally, improved outcomes. Amino acids are the most frequently distinguished metabolite class in the metabolomics of pancreatic cancer patients. They have been identified as pre-diagnostic and diagnostic markers and associated with pancreatic cancer risk factors. Materials and Methods: Deep phenotyping and quantitative amino acid analysis were performed in patients scheduled for pancreatic surgery due to pancreatic tumors (n=75). Results: Significant differences in plasma amino acid concentrations were observed between diagnostic categories (malignant vs. benign lesions and histological cancer types) and pancreatic ductal adenocarcinoma stages. Characteristic patterns of plasma amino acid concentration dynamics according to cancer stage were identified. Conclusion: Standardization of metabolomics methods and deep phenotyping may provide means for improved patient stratification and effective personalized approaches in pancreatic cancer prevention, early diagnosis and treatment.*

The incidence of pancreatic cancer is growing. The highest mortality has been registered in Eastern Europe (1). Absence of validated biomarkers and sensitive methods preclude early diagnosis; even in patients with a known increased risk (*e.g.*, due to familial cancer), timely diagnosis may be difficult (2). Due to the highly heterogenous nature of the disease and pancreatic cancer cell plasticity, the usual oncological treatments are ineffective in the majority of patients, while proper stratification measures for personalized approaches are missing (1). The only curative option is surgical treatment, however, surgery is applicable to a minority of patients (up to 20%) and results in significant morbidity (3). Moreover, due to limited abilities to differentiate between malignant and benign lesions and due to the frequently imprecise staging prior to surgery, a substantial number of patients with benign lesions (up to 25%) (1) and those with disseminated unresectable cancer (4) are subjected to potentially avoidable, highly morbid surgeries. In the era of personalized medicine, proper stratification of patients that takes into consideration all factors with an impact on outcomes (*i.e.*, risk factors, cancer biology, general clinical state) will lead to tailored treatments for every patient, will help avoid ineffective and inadequate options and improve outcomes. Due to the high heterogeneity that involves both cancer biology and clinical parameters, the identification of one single biomarker does not seem feasible; rather, sets of biomarkers should be used.

Search for pancreatic cancer biomarkers using both tissue and systemic "omics" strategies was very intensive in recent years. Cancer tissue "omics" revealed the major genetic drivers (5) and the main genomic and metabolic cancer phenotypes (6), studies on proteomic (7) and epigenetic (8) biomarkers are also making good progress. "Omics" of body fluids resulted in the identification of many potential metabolomic (9, 10), proteomic (11), non-coding RNA, exosome and circulating DNA biomarkers (12, 13). However, the huge amount of acquired knowledge still has not been translated to clinical practice.

Due to a signal amplification in the direction genome – proteome – metabolome, metabolomic biomarkers may be more sensitive, besides, metabolome comprises a snapshot of the whole network of metabolic processes at a given

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Table I. *Inclusion and exclusion criteria*.

Inclusion criteria	Exclusion criteria
• Patients 21-85 years age, scheduled for pancreatic surgery	• Pancreatic cancer stage ≥3 according to TNM classification of Malignant Tumours UICC (8th.ed.) • The Eastern Cooperative Oncology Group (ECOG) grade ≥3.

time, including both cancer and systemic factors (14). In patients with pancreatic cancer, amino acids are the most frequently distinguished metabolite biomarker class in both targeted and untargeted studies of body fluid metabolomics (10). Amino acid concentration changes were associated with pancreatic cancer risk factors – obesity and insulin resistance (15), identified as pre-diagnostic (16) and disease markers (10).

The aim of this study was to perform quantitative plasma amino acid analysis in patients referred for pancreatic surgery due to pancreatic tumours and to evaluate amino acid concentration changes in regards to histological cancer types, cancer staging and grading, and clinical parameters.

Materials and Methods

Patients and data. The study was approved by the relevant institutional review boards (Vilnius Regional Biomedical Research Committee permission 2016-01-12 No. 158200-16-810-341, the State Data Protection Inspectorate permission 2016-03-21 No. 2R-1807 (2.6-1). All consecutive patients scheduled for pancreatic surgery at Vilnius University Hospital Santaros Klinikos between January 2016 and November 2018 were recruited into the study according to the inclusion/ exclusion criteria (Table I). The age of patients ranged from 21 to 85 years old. The patients were scheduled for surgeries after regular multidisciplinary team meeting discussions. All study participants provided informed consent to participate. The final diagnoses of pancreatic adenocarcinoma (PDAC; n=50), other pancreatic cancers (OPC; n=6) and benign pancreatic lesions due to chronic pancreatitis (CP; n=7) were obtained after histological examination of surgical tissues. Other pancreatic cancers (OPC) included periampullary carcinoma (n=11), neuroendocrine pancreatic cancer (n=3), pseudopapillary solid tumour (n=1), mucinous pancreatic adenocarcinoma (n=1) and acinar cell pancreatic cancer (n=1).

For each patient, clinical and laboratory testing information was collected: demographics, medical history including risk factors (diabetes, obesity), clinical and nutritional evaluation, performance status data, results of laboratory testing and radiologic evaluation, and histological examination of specimens removed during surgery. For further analyses, deidentified data were used. The MIDAS archive was used for data capture and storage. The system automatically generated backups and data protection systems.

Amino acid analysis. Fasting blood samples (5 ml) were collected just before surgery from antecubital veins into tubes containing lithium heparin according to a standard procedure. Samples were prepared for quantitative amino acid analysis according to routine procedure: plasma separated from the whole blood by centrifugation at 3,000 rpm at 4˚C for 15 min, deproteinized with 5% sulfosalicylic acid and centrifuged at 3,000 rpm at 4˚C for 15 min. Separated supernatant (400 μl) was filtered through 0.2 μm pore size cellulose acetate filters and stored at –80˚C until analysis. After thawing, quantitative amino acid analysis was performed using Biochrom30+ amino acid analyzer (ion-exchange chromatography, ninhydrin post-column derivatization, single-point calibration). Norleucine was used as an internal standard and the method was certified by participation in the external quality assurance scheme (ERNDIM; https://erndim.org). Concentrations of 39 amino acids were measured and analysed: phosphoserine (Phser), taurine (Tau), phosphoethanolamine (Pea), aspartate (Asp), threonine (Thr), serine (Ser), asparagine (Asn), glutamate (Glu), glutamine (Gln), sarcosine (Sarc), alpha-aminoadipic acid (Ama), glycine (Gly), alanine (Ala), citruline (Cit), alpha-aminobutyric acid (Aaba), valine (Val), cystine (Cys), methionine (Met), cysteine (Cyst), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), beta-alanine (Bala), phenylalanine (Phe), beta-aminoisobutyricacid (Baiba), gamma-aminobutyric acid (Gaba), ethanolamine (Etha), hydroxylysine (Hlys), ornithine (Orn), lysine (Lys), 1-methylhistidine (1-Mhis), histidine (His), tryptophan (Trp), 3-methylhistidine (3MHis), anserine (Ans), carnosine (Car), arginine (Arg), hydroxyproline (Hpro), proline (Pro).

Statistical analysis. All data were analyzed with SPSS version 23.0 (IBM SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to check for normal distribution. Parametric data were analyzed by Student's *t*-test and expressed as means and standard deviations. Non-parametric data were analyzed with the use of Mann-Whitney *U*-test and reported as medians and ranges. Categorical variables were analyzed by Fisher's exact test. p-Values < 0.05 were considered as statistically significant.

Two models were comprised to discriminate amino acid concentration differences between study groups: Model 1 – PDAC *vs*. OPC and CP (merged group); Model 2: PDAC *vs*. OPC (without CP).

Results

Characteristics of patients. Table II summarizes the characteristics of study participants. There were no differences between study groups regarding age, BMI and gender. Significantly more patients with advanced cancer stages (IIb – III) were identified in PDAC group. Besides, significantly more patients with PDAC had cachexia or risk of malnutrition (92% of patients with PDAC *vs*. 72.2% of patients with OPC).

Table II. *Characteristics of study participants*.

	PDAC			Other pancreatic cancers			Chronic pancreatitis			Total		
	Mean	N	Percentage	Mean	N	Percentage	Mean	N	Percentage	N	Mean	Percentage
Number		50	66.7%		18	24%		7	9.3%	75		100.0%
Age	65.5			63.5			58				64.47	
BMI (kg/height m^2)	26.55			27.59			22.5	7		75	26.6	
Jaundice		10	20%		3	16.7%				13		17.3%
Stent		25	50%		9	50%		2	14.3%	36		48.0%
Diabetes mellitus		11	22%		4	22.2%		1	14.3%	16		21.3%
Gender (Male)		28	56%		10	55.6%		4	57.1%	42		56%
Stage	IA	4	8.0%	I	11	61,1%						
	IB	4	8.0%									
	IIA	5	10.0%	II	3	16.6%						
	IIB	21	42.0%									
	III	16	32.0%	III	4	22.2%						
Nutritional status												
Normal		4	8%		5	27.8%				9		13.2%
Cachexia		31	62%		9	50%				40		58.8%
Risk of malnutrition		15	30%		4	22.2%				19		28%
Total		50	100%		18	100%				68		100%

Cachexia: Defined as unintentional weight loss >5% over the last 6 months; or body mass index (BMI) <20 kg/m^2 with unintentional weight loss >2%; or sarcopenia (lumbar skeletal muscle index (LSMI) (n.r.: male<55 cm^2/m^2; female<39 cm^2/m^2) with unintentional weight loss >2%. Risk of malnutrition: mandatory to fulfil criteria for being "at risk" of malnutrition by NRS 2002. Weight loss (unintentional) >10% indefinite of time, or >5% over the last 3 months, combined with either BMI <20 kg/m^2, if <70 years of age, or <22 kg/m^2, if ≥70 years of age, or FFMI <15 and 17 kg/m^2 in women and men, respectively.

Amino acid analysis. Significant differences in amino acid concentrations were identified between study groups. In Model 1, concentrations of Orn, Thr, Phe, Gly, Arg, His, Gln, 3-Mhis, and Citr differed in PDAC *vs*. OPC and CP. In Model 2, differences in the concentrations of Orn, Thr, Phe, Lys, Val, Arg, His, Asn, Gln, 3-Mhis, and Citr were identified in PDAC *vs*. OPC (Table III).

In patients with PDAC, a significant inverse correlation between plasma His concentrations and PDAC stage was observed (Table IV). Importantly, U-shaped curves from stage I to stage IV were observed for Tyr, Pro, Gly, Arg, Ser and Thr (Figure 1).

Discussion

Radiologically identified pancreatic tumors encompass a group of various pathologies, including different histological types of pancreatic cancer, pre-cancerous lesions and benign pancreatic masses. While the main aim for a surgeon is to identify candidates for surgery, there is a need to differentiate between malignant and benign lesions and between early and advanced stages of cancer. In practice, this task presents significant challenges. The only serum biomarker that is currently used in clinical practice, CA 19-9, has a poor sensitivity of 50% to 80% and a specificity of about 90% in patients with clinical symptoms (17). The diagnostic rate of pancreatic biopsies is only about 71%, with sensitivity of 85% and negative predictive value of only about 64%, therefore, negative biopsies do not aid in clinical decision making (4). Hence, there is an urgent need for novel biomarkers that could aid in the differential diagnosis of pancreatic tumors and proper decision-making in every clinical situation.

In patients with pancreatic cancer, amino acids were the most frequently distinguished metabolite biomarker class in both targeted and untargeted studies of body fluid metabolomics (10). In this study, characteristic plasma amino acid biomarker profiles were identified for discrimination between pancreatic cancer histological types (PDAC *vs*. other pancreatic cancers) and stages (in patients with PDAC). The deadliest of all pancreatic cancer histological types, PDAC, could be discriminated from other pancreatic tumors, encompassing variable group of pathologies.

Many studies to date revealed potential plasma amino acid biomarkers for pancreatic cancer diagnosis. A limited number of studies investigated patient groups with various pathologies, that may present with pancreatic tumor (*e.g*., benign pancreatic lesions or various rare pancreatic cancer subtypes). Most frequently, amino acid concentrations in healthy controls *vs*. pancreatic cancer patients were analyzed. Some major trends could be identified from these studies (Table V). The most concordant findings include decreased

Table III. *Inter-group differences of plasma amino acid concentrations for Model 1 and Model 2.*

	Model 1							Model 2					
Amino acid	PDAC (N=50)	OPC and CP (N=24)	Mann-Whitney U	Wilcoxon W	Z	Asymp. Sig. (2-tailed)		PDAC (N=50)	OPC (N=17)	Mann-Whitney U	Wilcoxon W	Z	Asymp. Sig. (2-tailed)
Orn	64.34	73.80	389.00	1715.00	–2.307	0.021	Orn	64.34	75.33	294.00	1569.00	–1.888	0.059
Thr	99.29	113.51	378.00	1704.00	–2.435	0.015	Thr	99.29	118.92	223.00	1498.00	–2.911	0.004
Tyr	62.57	64.88	509.00	1835.00	–0.905	0.365	Tyr	62.57	67.36	321.00	1596.00	–1.499	0.134
Phe	54.56	59.78	415.00	1741.00	–2.003	0.045	Phe	54.56	59.87	275.00	1550.00	–2.161	0.031
Trp	48.44	50.16	572.00	1898.00	–0.169	0.866	Trp	48.44	49.76	390.00	1665.00	–0.504	0.614
Ile	68.85	70.13	564.00	840.00	–0.263	0.793	Ile	68.85	76.90	348.00	1623.00	–1.109	0.267
Leu	123.14	132.98	552.00	1878.00	–0.403	0.687	Leu	123.14	143.25	322.00	1597.00	–1.484	0.138
Lys	158.75	180.19	433.00	1759.00	–1.793	0.073	Lys	158.75	186.81	244.00	1519.00	–2.608	0.009
Gly	187.71	217.02	366.00	1692.00	–2.575	0.010	Gly	187.71	208.88	304.00	1579.00	–1.743	0.081
Ala	299.52	336.04	489.00	1815.00	–1.139	0.255	Ala	299.52	340.16	315.00	1590.00	–1.585	0.113
Val	200.75	225.29	495.00	1821.00	–1.069	0.285	Val	200.75	243.71	261.00	1536.00	–2.363	0.018
Arg	61.98	74.15	356.00	1682.00	–2.692	0.007	Arg	61.98	74.26	224.00	1499.00	–2.896	0.004
His	65.94	72.95	414.00	1740.00	–2.015	0.044	His	65.94	72.79	284.00	1559.00	–2.032	0.042
Pro	138.51	150.32	453.00	1779.00	–1.559	0.119	Pro	138.51	150.67	321.00	1596.00	–1.499	0.134
Ser	100.57	110.72	427.00	1753.00	–1.863	0.062	Ser	100.57	112.42	311.00	1586.00	–1.643	0.100
Asn	48.34	55.36	444.00	1770.00	–1.664	0.096	Asn	48.34	56.86	280.00	1555.00	–2.089	0.037
Glu	198.11	138.40	439.00	715.00	–1.723	0.085	Glu	198.11	147.51	314.00	467.00	–1.599	0.110
Gln	362.32	450.94	353.00	1628.00	–2.636	0.008	Gln	362.32	446.32	248.00	1473.00	–2.471	0.013
Asp	8.62	8.23	565.00	841.00	–0.251	0.802	Asp	8.62	8.17	401.00	554.00	–0.346	0.729
3-Mhis	4.21	6.07	324.00	1650.00	–3.066	0.002	3-Mhis	4.21	5.97	228.50	1503.50	–2.831	0.005
Citr	24.26	30.41	357.00	1683.00	–2.680	0.007	Citr	24.26	29.36	253.00	1528.00	–2.478	0.013
Met	19.70	19.87	578.00	1904.00	–0.099	0.921	Met	19.70	20.52	366.00	1641.00	–0.850	0.395

plasma concentrations of His, Val, Gln, Ala, Pro, Asn, Tyr, Lys in patients with pancreatic cancer *vs*. healthy controls and decreased concentrations of Gln, Ala, His, Pro, Asn, Thr, Lys in patients with pancreatic cancer *vs*. chronic pancreatitis. This study revealed the same trends of characteristic amino acid concentration profiles in PDAC *vs*. other pancreatic tumours and PDAC *vs*. other pancreatic tumours and chronic pancreatitis groups. A small number of studies preclude comparisons between rare pancreatic tumour lesion groups.

Unfortunately, discordant study results are also common (*e.g*., Arg, Glu, Ile, Leu in table 5). The reasons for these discrepancies may be variable, including differences in methods and study design, biological heterogeneity of pancreatic cancer and variable clinical parameters of patients. A range of methods is applied in amino acid studies, including both untargeted semi-quantitative (*e.g*., H1-NMR or GC/MS-based) and targeted quantitative (*e.g*., LC/MS-based) approaches. Limited sensitivity of H1-NMR may present difficulties in identifying subtle metabolite concentration changes, especially a decrease of concentrations (18). Indeed, in all discordant pairs of findings in table 2, at least one of the results was obtained using H1-NMR.

Dynamic changes of amino acid concentrations in relation to disease progression were described for branched-chain amino acids (BCAA). In a study by Mayers *et al*. (16), elevation of BCAA concentrations was identified in plasma samples, collected 2 to 5 years prior to pancreatic cancer diagnosis, but not later. Plasma concentrations of BCAA are

Table IV. *Significant inverse correlation between plasma His concentrations and PDAC stage.*

Pearson correlation			
		Stage	His
Stage	Pearson Correlation	1	–0.391**
	Sig. (2-tailed)		0.005
	N	51	51
His	Pearson Correlation	–0.391**	1
	Sig. (2-tailed)	0.005	
	N	51	51
Spearman's rank correlation coefficient			
		Stage	His
Stage	Correlation Coefficient	1.000	–0.390**
	Sig. (2-tailed)		0.005
	N	51	51
His	Correlation Coefficient	–0.390**	1.000
	Sig. (2-tailed)	0.005	
	N	51	51

**Correlation is significant at the 0.01 level (2-tailed).

Table V. *Concordance of blood-based AA analyses in (1) patients with pancreatic cancer vs. healthy controls and in (2) patients with pancreatic cancer vs. chronic pancreatitis.*

Authors	Fukutake *et al.*, 2015 (24)	Kobayashi *et al.*, 2013 (25)	Hirata *et al.*, 2017 (26)	OuYang *et al.*, 2011 (27)	Zhang *et al.*, 2012 (28)	Xie *et al.*, 2014 (29)	Bathe *et al.*, 2011 (4)	Mayerle *et al.*, 2018 (30)	Zhang *et al.*, 2012 (28)		
Methods	HPLC/ ESI-MS, quantitative	GC-MS, semi-quantitative	GC-MS/ MS, quantitative	H1 NMR, semi-quantitative	H1 NMR, semi-quantitative	LC-MS and GC-MS	H1 NMR, semi-quantitative	GC-MS, semi-quantitative	H1 NMR, semi-quantitative		
Amino acid	PC vs. HC						PC vs. benign or CP			Concordance: PC vs. HC	Concordance: PC vs. CP
Ala	↓				↓			↓	↓	++	++
Arg	↓						↑	↓			D
His	↓	↓	↓		↓			↓	↓	++++	++
Pro	↓					↓	↓	↓		++	++
Asn	↓	↓					↓	↓		++	++
Glu					↓	↑	↑		↓	D	D
Gln		↓			↓	↓	↓	↓	↓	+++	+++
Tyr	↓	↓							↓	++	
Thr	↓						↓	↓			++
Lys	↓				↓		↓		↓	++	++
Ile				↑	↓				↓	D	
Leu	↓			↑	↓				↓	D	
Val	↓	↓			↓	↓			↓	++++	
Met	↓	↓								++	

*Only 4 amino acids were investigated. HPLC/ESI-MS: High-performance liquid chromatography (HPLC) – electrospray ionization (ESI) - mass spectrometry (MS); GC/MS: gas chromatography – mass spectrometry; GC-MS/MS: gas chromatography – tandem mass spectrometry; LC-MS: liquid chromatography – mass spectrometry; H1 NMR: 1H NMR spectroscopy.

directly associated with muscle mass in healthy subjects (19), and could be a sign of cancer-associated muscle wasting in patients with early pancreatic cancer in a study by Mayers *et al*. Besides, plasma BCAA levels increase in prediabetes and diabetes, presumably due to insulin resistance in skeletal muscle (20). Patients with cancer cachexia usually have diminished BCAA concentrations (21). Indeed, in our study, Val concentrations were significantly lower in PDAC patients (cachexia or risk of malnutrition identified in 92% of patients) as compared to other pancreatic cancer groups (cachexia or risk of malnutrition identified in 72.2% of patients). Moreover, plasma concentrations of 3-MHis, a specific biomarker of muscle, showed the same direction of differences between these groups.

In our study, significant differences of plasma His concentrations between the different PDAC stages were observed, with diminishing levels from stage I towards stage IV. Of major importance, concentrations of other amino acids, including Thr, Lys, Arg, Gly, Ser, and Asn, obtained U-shaped curve across cancer stages. This observation may also explain some discordances in results of different studies: when study groups include variable proportions of different cancer stages, results may not be comparable. The same applies to comparisons of patients with early *vs*. advanced cancer stages.

The major limitation of this study was the small number of patients with other pancreatic cancers. Due to the rarity of these disorders, it is very difficult to collect representative patient cohorts, as was also the case in other studies (22). In many previous studies of other cancers, cancer type-specific amino acid changes were identified (23). Various histological cancer types may also give differing plasma amino acid profiles. Recently, cancer tissue genomics and metabolomics provided measures to identify several PDAC subtypes with unique properties (6) that may also have a variable impact on plasma amino acid profiles through local or systemic effects.

In conclusion, although metabolomic studies in pancreatic cancer provided us with some promising results, further efforts are required to translate these achievements into clinical practice. Standardization of metabolomics methods and deep phenotyping of patients may provide means for improved patient stratification, more precise diagnostics and, eventually, more effective personalized approaches in pancreatic cancer prevention, early diagnosis and treatment.

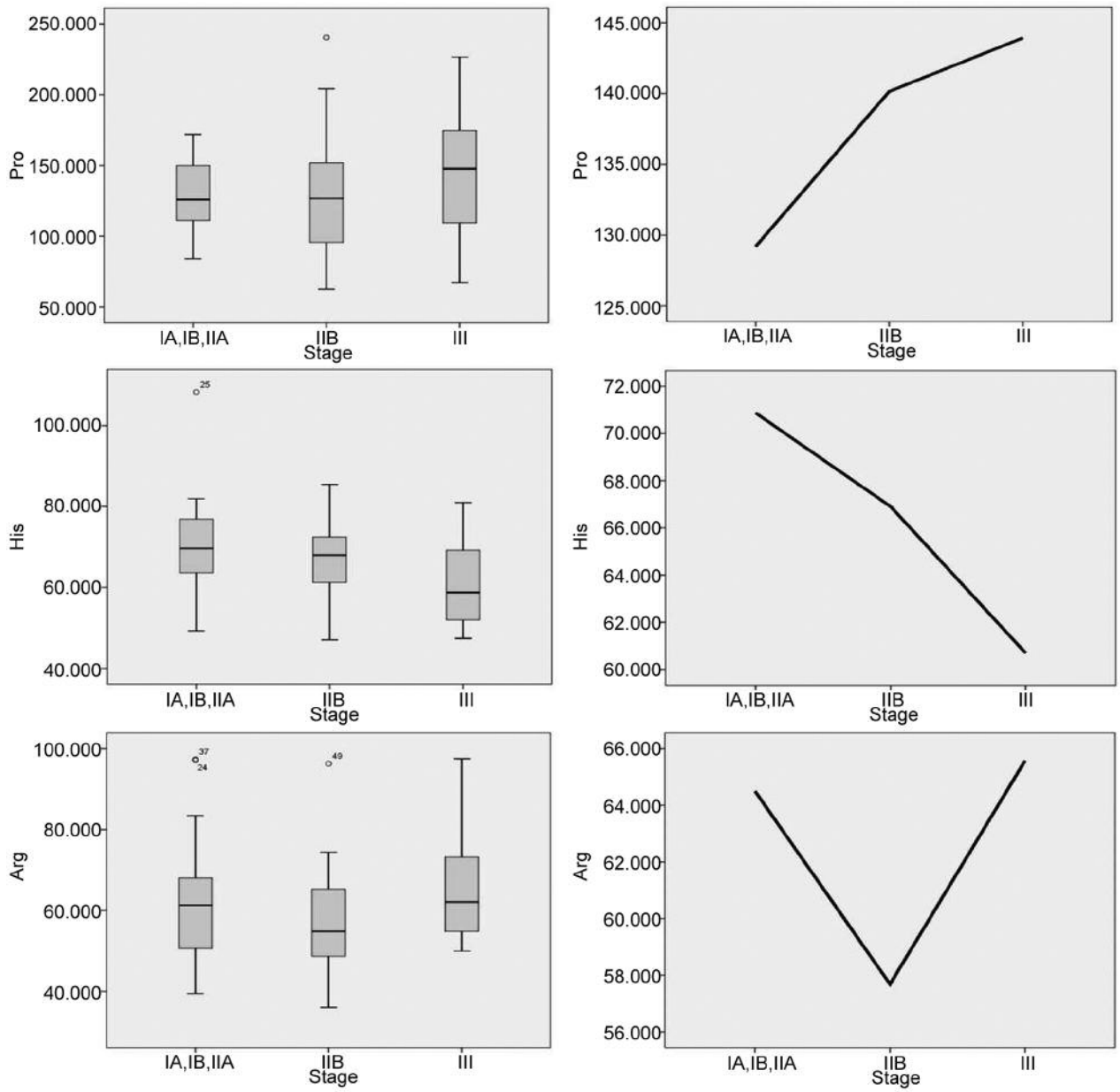

Figure 1. *Patterns of dynamics of plasma amino acid concentrations according to PDAC stage. The mean plasma concentrations of Pro are increasing from stage I to stage III; the mean plasma concentrations of His are decreasing from stage I to stage III; the mean plasma concentrations acquire U-shaped form from stage I to stage III.*

Conflicts of Interest

Authors declare that they have no conflicts of interest regarding this study.

Authors' Contributions

Jaroslav Tumas designed the study, collected samples and drafted the manuscript. Inga Baskirova and Tomas Petrenas performed amino acid analyzes and analyzed results. Kestutis Strupas and Audrius Sileikis supervised the overall study, advised on study design and implementation and revised the manuscript.

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