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Plasma tryptophan pathway metabolites quantified by liquid chromatography-tandem mass spectrometry as biomarkers in neuroendocrine tumor patients

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Abstract

A good and accessible biomarker is of great clinical value in neuroendocrine tumor (NET) patients, especially considering its frequently indolent nature and long-term follow-up. Plasma chromogranin A (CgA) and 5-hydroxyindoleacetic acid (5-HIAA) are currently used as biomarkers in NET, but their sensitivity and specificity are restricted. 5-HIAA is the main metabolite of serotonin, an important neurotransmitter of the tryptophan pathway. The aim of this study is to estabish a sensitive and accurate method for the quantification of tryptophan pathway metabolites in plasma. We further aimed to evaluate its utility as a clinical tool in NET disease. We obtained plasma samples from NET patients and healthy controls recruited from the University Hospital of North Norway, Tromsø. Samples were analyzed by liquid chromatography-tandem mass spectrometry (LC–MS/MS), and eight metabolites of the tryptophan pathway were quantified. We included 130 NET patients (72/130 small intestinal [SI] NET, 35/130 pancreatic NET, 23/130 other origin) and 20 healthy controls. In the SI-NET group, 26/72 patients presented with symptoms of carcinoid syndrome (CS). We found that combining tryptophan metabolites into a serotonin/kynurenine pathway ratio improved diagnostic sensitivity (92.3%) and specificity (100%) in detecting CS patients from healthy controls compared with plasma 5-HIAA alone (sensitivity 84.6%/specificity 100%). Further, a clinical marker based on the combination of plasma serotonin, 5-HIAA, and 5OH-tryptophan, increased diagnostic capacity identifying NET patients with metastasized disease from healthy controls compared with singular plasma 5-HIAA, serotonin, or CgA. In addition, this marker was positive in 61% of curatively operated SI-NET patients compared with only 10% of healthy controls ($p < .001$). Our results indicate that simultaneous quantification of several tryptophan metabolites in plasma, using LC–MS/MS, may represent a clinically useful diagnostic tool in NET disease.

KEYWORDS
biomarker, carcinoid syndrome, liquid chromatography-tandem mass spectrometry, neuroendocrine tumor, tryptophan metabolites

1 | INTRODUCTION

Neuroendocrine tumors (NET) are a relatively rare disease originating from neuroendocrine cells with the gastroenteropancreatic (GEP) system and lungs as their main localizations. The incidence of NET is increasing worldwide, in Norway the incidence of NET was 4.01/100,000 per year in 2006–2010.[1] NETs are often indolent tumors with few clinical symptoms, making early diagnosis challenging, and ~40% of NET patients have metastasized disease at diagnosis.[1] Consequently, a good and accessible tumor marker for diagnosis is of great value. In addition, a good clinical tool in NET follow-up is needed, as the majority of patients have an indolent disease course requiring several years of follow-up.

Current biomarkers used in the diagnosis and follow-up of NET disease are plasma chromogranin A (CgA) and 5-hydroxyindoleacetic acid (5-HIAA) in urine or blood. Although CgA has demonstrated a correlation with tumor burden, there is conflicting evidence of its use as a marker of response to therapy. CgA alone in a diagnostic setting, is not recommended due to low specificity.[2] In addition, several GEP-NETs show normal levels of CgA, indicating impaired sensitivity.[2–5] 5-HIAA is the main metabolite from serotonin (5-hydroxytryptamine), primarily secreted from small intestinal NETs (SI-NETs). Urinary 5-HIAA has demonstrated high specificity, but low sensitivity in NET disease, possibly due to diverse expression.[6,7] Serum and plasma 5-HIAA have shown results comparable to urine analyses.[8–10]

Serotonin is derived from the dietary essential amino acid tryptophan.[11] Tryptophan is metabolized via three main pathways. The majority (about 90%) is metabolized through the kynurenine pathway and <5% via the serotonin pathway, Figure 1. In total, 95% of serotonin is synthesized and released in the gastrointestinal tract.

Carcinoid syndrome (CS) is a constellation of symptoms present in about 19%–35% of SI-NET patients, and is a result of increased secretion of bioactive substances from the tumor, with serotonin as a pivotal contributor.[12–14] Main symptoms of CS are flushing and diarrhea, and its presence represents a negative prognostic factor reducing median overall survival to 4.7 versus 7.1 years without CS.[14] In NETs and CS, the tryptophan metabolism is altered, and up to about 70% of dietary tryptophan may be utilized in the serotonin pathway.[12,13]

In this study, we quantified tryptophan metabolites in plasma from NET patients and healthy controls using liquid chromatography-tandem mass spectrometry (LC–MS/MS). Our aim is to establish a sensitive and accurate method for quantification of tryptophan metabolites in plasma, and further evaluate its utility as a clinical tool in NET patients.

2 | METHODS

2.1 | Materials

All solvents, acids, and salts used for sample preparation and analytical work were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and were of LC–MS quality or better. Ultrapure water was obtained from Milli-Q equipment (SINTEF Industry, Trondheim, Norway). All analytical standards were bought from Sigma-Aldrich and isotope labeled internal standards were bought from Merch (Darmstadt, Germany) or TRC (Toronto Research Chemicals, Toronto, Canada). Analyses were performed at SINTEF Trondheim, Norway.

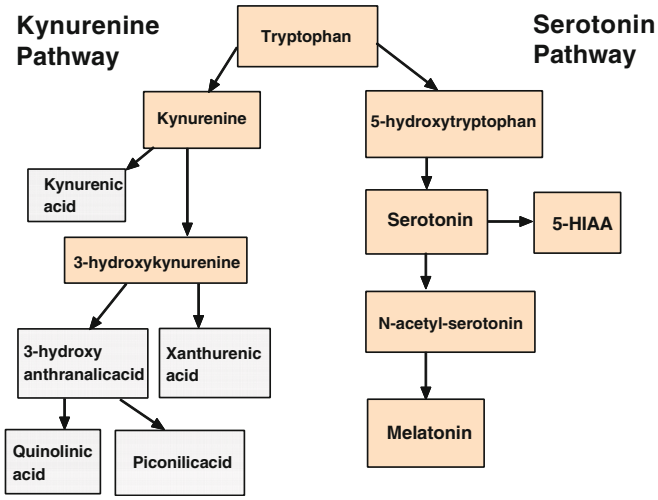


FIGURE 1 The kynurenine and serotonin pathway of tryptophan metabolism. Metabolites in orange are quantified in our study.

2.2 | Study population

We obtained plasma samples from NET patients ($n = 130$) and healthy controls ($n = 20$) from November 2016 to March 2022. NET patients were recruited from the Gastrointestinal Department at the University Hospital of North Norway, Tromsø. Patients were included if they were 18 years or above and had a histologically confirmed NET diagnosis based on the WHO Classification of Digestive System Tumours 2019[15] into well-differentiated NETs Grade 1 (G1, Ki-67 index <3%), Grade 2 (G2, Ki-67 index 3%–20%), or Grade 3 (G3, Ki67 index >20%). All cases were reviewed by gastroenterologists and dedicated pathologist. Patients with CS were included and were defined based on typical clinical symptoms such as cutaneous flushing and/or diarrhea. Patients with neuroendocrine carcinoma (poorly differentiated tumors with Ki-67 >20%) were excluded from the study. Plasma samples were drawn at time point of scheduled clinical controls, and they were nonfasting and with no prior diet restrictions. Healthy controls were recruited from hospital staff. Nine of these controls gave fasting samples in addition to sampling 2–3 h after a breakfast meal.

All study participants were informed and gave written consent. The study was approved by the Regional Committee for Medical and Health Research Ethics, Northern Norway (ref: no. 2016/1664) and conducted according to the Helsinki Declaration.

2.3 | Sampling and sample preparation

Blood samples were drawn from the antecubital vein into 9 mmol/L EDTA tubes. Good blood flow was emphasized and the first tube was discharged. Samples were aliquoted into four small tubes and, without delay,

centrifuged at 12,000*g* for 5 min at 22°C. Two thirds of the supernatant was aliquoted to new clean tubes and stored at −70°C until analysis.

Sample stability was evaluated using four quality control (QC) plasma samples. QC samples were aliquoted into five tubes, stored at room temperature, and analyzed at Days 0, 1, 3, 5, or 7. Samples were considered stable if the mean concentration of QC samples were between 85% and 115% of the mean concentration at Day 0.

Samples were analyzed at the Department of Biotechnology and Nanomedicine, SINTEF Industry, Trondheim, Norway. After thawing of samples, 100 µL of plasma was added 400 µL ice-cold methanol and vortexed vigorously. After 5 min of incubation, the samples were centrifuged at 14,000*g* for 10 min at 4°C. 100 µL sample was removed for analysis of tryptophan and metabolites.

2.4 | Liquid chromatography-tandem mass spectrometry

Quantification of tryptophan metabolites was conducted by an Agilent 1290 Infinity II LC-system (Agilent, Santa Clara, USA) coupled to an Agilent 6495 triple quadrupole utilizing two different methods. The triple quadrupoles were operated in multiple-reaction monitoring mode with unit resolution of both quadrupoles. For all analytes and internal standards, two transitions were used, one transition for quantification and one transition for qualification.

Quantification of tryptophan and metabolites were conducted by diluting the samples 1:10 and 1:100 prior to isotope labeled internal standards were added to the samples. Calibration curves of all analytes were made in the range from 0.5 to 5000 ng/mL. Satisfactory chromatography was achieved by utilizing an Ascentic Express 2.1 × 150 mm fluorophenyl columna and 25 mM ammonium format in water and methanol as solvents.

2.5 | Assay validation

Eight metabolites of the tryptophan pathway were quantified in plasma: tryptophan, 5-OH-tryptophan, *N*-acetyl-5-hydroxytryptamine, 5-hydroxytryptamine (serotonin), 5-HIAA, melatonin, kynurenine, and 3-hydroxy-kynurenine. Analysis on melatonin and *N*-acetyl-5-hydroxytryptamine were below lower limit of quantification (LLOQ) in all patients and controls (<2.15 and <2.29 nmol/L, respectively) and where thus not used further. Residue stability is shown in Figure S1. Plasma serotonin, 5-HIAA, and 5OH-tryptophan were stable for 3 days at room temperature. Hydroxykynurenine was stable only Day 1 at room temperature and plasma tryptophan and kynurenine demonstrated stable values within Day 7.

2.6 | CgA sampling

In total, 123/130 of patients had plasma analyzed for CgA as part of their clinical follow-up. CgA samples were drawn the same day, or within 2 weeks, of the tryptophan metabolite sampling, and consecutively analyzed by enzyme-linked immunosorbent assay at the Oslo University Hospital, Rikshospitalet, Oslo, Norway. In addition, CgA analysis was performed in healthy controls.

2.7 | Statistical methods

We used IBM SPSS version 29.0 (Armonk, New York, USA) and GraphPad Prism 9.1.2 for Windows (La Jolla, CA, USA) for statistical analyses including descriptive statistics, Receiver operating characteristic (ROC) analysis, Chi-Square test for categorical variables, independent samples *t*-test for normally distributed continuous variables and Mann–Whitney or Wilcoxon Ranked Sum test for non-normally distributed variables.

3 | RESULTS

3.1 | Baseline characteristics

Patient characteristics are given in Table 1. Median time from diagnosis to study inclusion in the whole NET patient cohort ($n = 130$) was 10.5 months (interquartile range 4–58.5 months, range 1–373 months). At the time of study inclusion/plasma sampling, 61% (44/72) of SI-NET and 37% (13/35) of pancreatic NET patients presented with metastasis. 26/72 SI-NET patients had symptoms of flushing and/or diarrhea interpreted as CS.

At inclusion, 68/130 patients were curatively operated and had no detectable disease at radiological examination. The patients included in this group ($n = 68$) had radical surgery performed at a median of 6 months (range 1–154) prior to plasma sampling, and 65/68 patients had no signs of residual disease on dedicated computed tomography (CT) or magnetic resonance imaging (MRI) performed within 1 month prior until 12 months after sampling. The remaining 3 patients displayed negative somatostatin receptor imaging (SRI) within 3 months after sampling. In addition, SRI was performed in the majority of curatively operated patients (65/68) at a median of 4 months (range 0–98) prior to sampling. SRI demonstrated either no residual disease, or the primary tumor only in case of preoperative examination.

At the time of inclusion 47/130 patients were treated with somatostatin analogues (SSA). Of these, 38 had distant metastasis, whereas 9 had no detectable tumor (radically operated). No significant difference in tryptophan metabolite levels was found when comparing patients treated with or without SSA in the respective subgroups; distant metastasis ($n = 60$) or no detectable tumor ($n = 68$).

3.2 | Tryptophan metabolite concentrations

The highest levels of serotonin, 5-HIAA, and 5OH-tryptophan were found in SI-NET patients with metastasized disease and in patients with CS. Results of the different tryptophan metabolites in all NET

TABLE 1 Patient clinical characteristics at study inclusion.

Characteristic	Healthy controls, $n = 20$	NET, $n = 130$	Subgroups				
			SI, $n = 72$	Pancreas, $n = 35$	Rectum, $n = 6$	Colon, $n = 4$	Other, $n = 13$
Age in years, median (range)	49.5 (33–75)	60* (20–86)	63 (30–86)	59 (26–73)	48 (26–66)	65 (55–73)	60 (20–69)
Sex, *n* (%) female	15 (75%)	60 (46%)**	26 (36%)	18 (51%)	5 (83%)	2 (50%)	9 (69%)
Distant/regional metastasis, *n* (%)	N/A	60 (46%)	44 (61%)	13 (37%)	1 (17%)	1 (25%)	1 (8%)
Detectable disease (no metastasis), *n* (%)	N/A	2 (2%)	0	1 (3%)	0	0	1 (8%)
Curatively operated, *n* (%)	N/A	68 (52%)	28 (39%)	21 (60%)	5 (83%)	3 (75%)	11 (84%)
Ki67 (%), median (range)	N/A	2% (0.1–60)	2% (0.1–60)	5% (1.0–30)	2% (1.0–5.0)	2% (1.0–2.0)	2% (1.0–10)
NET G1, *n* (%)	N/A	75 (58%)	43 (60%)	13 (37%)	5 (83%)	4 (100%)	10 (77%)
NET G2, *n* (%)		46 (35%)	25 (34.5%)	17 (49%)	1 (17%)	0	3 (23%)
NET G3, *n* (%)		9 (7%)	4 (5.5%)	5 (14%)	0	0	0
CgA, nmol/L median (range)	2.05 (1.6–3.9)	4.0 (1.6–8932.0)***	5.1 (1.6–5418.0)	3.7 (1.6–8932.0)	2.5 (2.0–13.3)	3.4 (2.5–111.3)	3.7 (1.6–19.1)
Missing data (CgA)		7	3	3	0	1	0

Note: The subgroup other ($n = 13$) consists of gastric-NET ($n = 6$), appendix-NET ($n = 5$), and pulmonary carcinoid ($n = 2$). CgA values were missing in seven patients. *Significant difference between pooled NET patients and controls in age* ($p = .005$), gender distribution** ($p = .016$), and CgA levels*** ($p < .001$).

Abbreviations: CgA, chromogranin A; G1–G3, Grades 1–3; NET, neuroendocrine tumor; SI, small intestinal.

patients, as well as subgroups of patients, are summarized in Table 2 and illustrated in Figure 2 (SI-NETs) and Figure S2 (all NET patients). Amongst NET patients with metastasized disease ($n = 60$), we found highest levels of serotonin in G1 tumors compared with G2, and G3 tumors ($p = .20$ and .28, respectively). Contrary, slightly higher 5-HIAA results were found in G3 tumors compared with G1/G2. Focusing only on SI-NET with metastasis ($n = 44$), we found the same pattern with higher 5HIAA, in addition to increased 5OH tryptophan levels, in G3 tumors compared with G1 ($p = .07$ and .32, respectively), Figure S4A and S4B.

In curatively operated NET patients without radiologically detectable disease at plasma sampling, median values of plasma serotonin,5-HIAA, and 5OH-tryptophan were below LLOQ. Although, interestingly, 15/68 patients had levels of serotonin above LLOQ (range 24.5–612.7 nmol/L), 19/68 patients displayed 5-HIAA levels above the LLOQ (range 134.2–1248.3 nmol/L), and 23/68 patients had levels of 5OH-tryptophan above LLOQ (range 11.3–368.3 nmol/L). In comparison, 6/20 healthy controls had increased levels of serotonin (range 6.8–28.0 nmol/L), none had 5-HIAA detectable above the LLOQ, and 2/20 controls had levels of 5OH-tryptophan above LLOQ (range 7.1–12.3 nmol/L).

Plasma samples were drawn before, and 2–3 h after meal, in nine healthy controls. Slightly increased serotonin levels were detected in 3/9 individuals (mean serotonin 6.53 nmol/L before and 8.18 nmol/L after meal), but no significant differences were found in neither plasma serotonin ($p = .11$) nor levels of the other metabolites.

3.3 | Plasma tryptophan metabolites as a diagnostic tool in NET patients

We created a ratio between the sum of quantified serotonin- and kynurenine pathway metabolites (serotonin +5-HIAA +5OH-tryptophan divided by the sum of kynurenine +3-OH kynurenine) and examined its relationship with distinct stages of NET disease. We found significantly higher values of this ratio ($p < .0001$) in patients with metastasized disease and/or CS compared with healthy controls and radically operated patients. Figure 3 illustrates the serotonin/kynurenine pathway ratio in subgroups of SI-NET patients (Figure 3A) as well as in the whole NET patient cohort (Figure 3B).

Further, we assessed the ability of plasma serotonin, 5-HIAA, 5OH-tryptophan, and the serotonin/kynurenine pathway ratio to differentiate NET patients from healthy controls by performing an ROC analysis, Figure 4. Plasma CgA was included in the ROC analysis for comparison. The area under the curve (AUC), ROC derived cutoff values and test performances are summarized in Table 3.

The serotonin/kynurenine pathway ratio displayed a sensitivity of 82% and specificity of 100% in detecting SI-NET patients with metastasis from healthy controls, Figure 4B. Plasma serotonin and 5-HIAA demonstrated lower sensitivities, but high specificity (90%–100%). Performing the same analysis in all NET patients with distant metastasis, Figure 4C, we found slightly lower test attributes. Comparing CS patients to healthy controls, Figure 4A, the serotonin/kynurenine pathway ratio was able to distinguish patients from controls with

TABLE 2 Results of plasma tryptophan metabolites quantified by liquid chromatography-tandem mass spectrometry in NET patients and healthy controls.

		Serotonin	5-HIAA	5OH-tryptophan	Tryptophan	Kynurenine	3-hydroxy-kynurenine	CgA
Curatively operated	All NET, $n = 68$	<2.84[a] IQR: <2.84[a]–<2.84[a]	<26.2[a] IQR: <26.2[a]–141.0	<2.3[a] IQR: <2.3[a]–14.0	326,085 IQR: 228,864–431,512	5449 IQR: 3729–8904	103 IQR: 84–143	3.1 IQR: 2.1–4.3
	SI-NET, $n = 28$	<2.84[a] IQR: <2.84[a]–19.10	<26.2[a] IQR: <26.2[a]–107.2	<2.3[a] IQR: <2.3[a]–18.2	344,573 IQR: 218,861–424,149	5906 IQR: 4148–10,811	92 IQR: 74–143	2.9 IQR: 2.1–4.2
	P-NET, $n = 21$	<2.84[a] IQR: <2.84[a]–17.43	<26.2[a] IQR: <26.2[a]–238.8	<2.3[a] IQR: <2.3[a]–19.3	342,563 IQR: 251,572–1,113,892	7567 IQR: 3486–17,489	121 IQR: 89–142	3.1 IQR: 2.1–4.2
Detectable disease. No metastasis	All NET, $n = 2$	<2.84[a] IQR: <2.84[a]–2.84[a]	<26.2[a] IQR: <26.2[a]–26.2[a]	16.1 IQR: 15.0–17.1	225,231 IQR: 220,333–230,129	2897 IQR: 2156–3637	72 IQR: 58–86	19.1
Regional/distant metastasis	All NET, $n = 60$	54.45 IQR: <2.84[a]–213.74	314.5 IQR: <26.2[a]–1158.3	17.4 IQR: <2.3[a]–37.0	220,335 IQR: 180,414–342,056	5097 IQR: 3414–8179	124 IQR: 82–171	14.35 IQR: 3.9–63.9
	SI-NET, $n = 44$	79.59 IQR: <2.84[a]–256.42	329.8 IQR: <26.2[a]–1527.5	18.3 IQR: <2.3[a]–37.3	217,369 IQR: 179,790–342,056	5199 IQR: 3738–8407	120 IQR: 80–160	16.1 IQR: 4.3–65.2
	P-NET, $n = 13$	<2.84[a] IQR: <2.84[a]–31.44	<26.2[a] IQR: <26.2[a]–315.4	16.5 IQR: <2.3[a]–40.4	220,213 IQR: 148,362–256,082	3798 IQR: 3011–6558	127 IQR: 93–360	9.6 IQR: 4.0–30.6
Regional/distant metastasis: results according to Ki67	G1 (Ki67 < 3%), $n = 31$	90.97 IQR: <2.84[a]–240.84	322.9 IQR: <26.2[a]–1025.0	15.0 IQR: <2.3[a]–37.4	245,961 IQR: 173,755–344,856	5207 IQR: 3734–8473	133 IQR: 94–178	14.5 IQR: 3.9–67.9
	G2 (Ki67 3–20%), $n = 22$	21.48 IQR: <2.84[a]–175.07	142.1 IQR: <26.2[a]–1152.2	18.9 IQR: <2.3[a]–29.2	218,285 IQR: 183,136–404,229	6267 IQR: 3489–7855	126 IQR: 80–183	14.2 IQR: 4.1–62.7
	G3 (Ki67 >20%), $n = 7$	<2.84[a] IQR: <2.84[a]–106.80	478.6 IQR: <26.2[a]–8973.7	<2.3[a] IQR: <2.3[a]–47.8	220,213 IQR: 194,081–270,878	4108 IQR: 3205–4923	115 IQR: 47–146	15.4 IQR: 2.7–162.6
Carcinoid syndrome	$n = 26$	147.0 IQR: 27.58–310.93	1113.9 IQR: 176.6–2019.6	22.8 IQR: <2.3[a]–62.0	244,284 IQR: 166,252–336,457	4653 IQR: 3129–8995	124 IQR: 88–175	36.2 IQR: 3.5–99.9
Healthy cotrols	$n = 20$	<2.84[a] IQR: <2.84[a]–9.48	<26.2[a] IQR: <26.2[a]–<26.2[a]	<2.3[a] IQR: <2.3[a]–<2.3[a]	226,596 IQR: 213,407–238,499	4081 IQR: 3199–4897	85 IQR: 73–112	2.05 IQR: 1.9–2.6

Note: In addition, Chromogranin A (CgA) in plasma analyzed by enzyme-linked immunosorbent assay is included in the table. Results are given in nmol/L, median value, and interquartile range (IQR). CgA values are missing in 7/130 patients.

[a]LLOQ (lower limit of quantification, nmol/L).

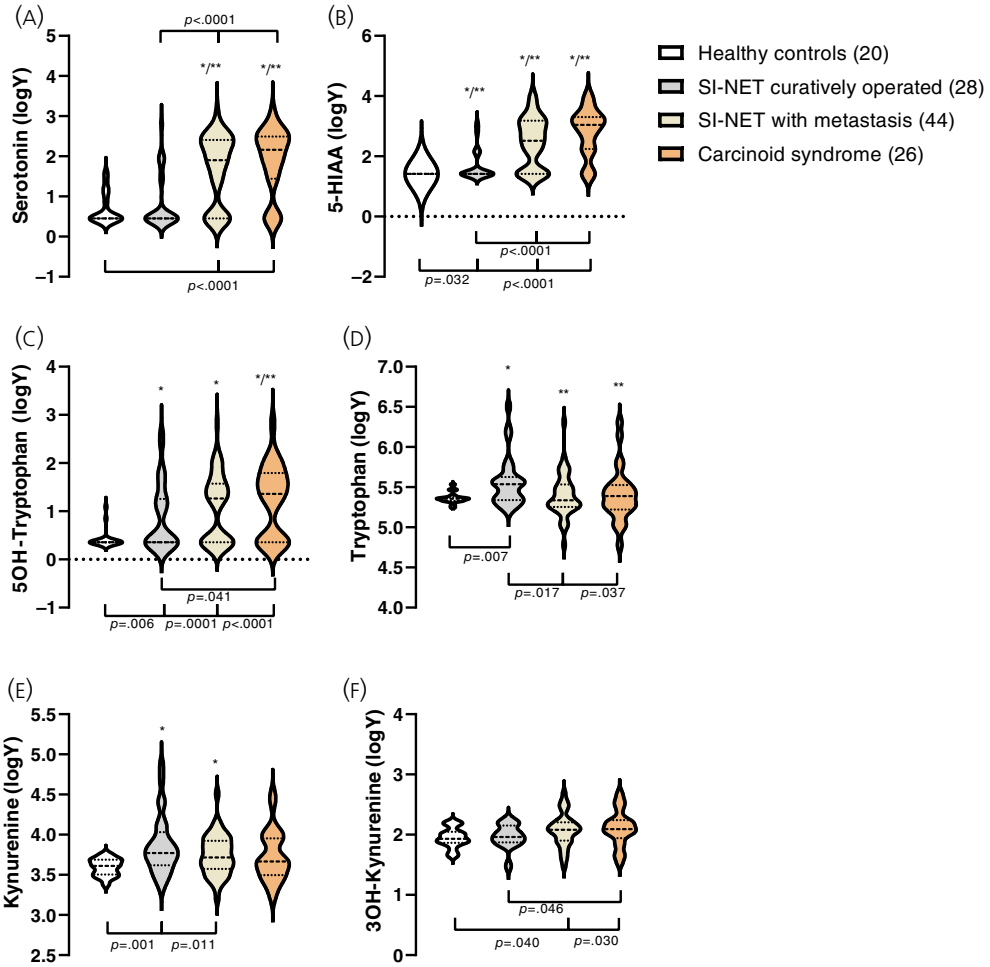


FIGURE 2 Violin plots of the different tryptophan metabolites in subgroups of small intestinal neuroendocrine tumor (SI-NET) patients and healthy controls. Data are presented in 10-logarithmic scale. *Significant difference from healthy controls. **Significant difference from radically operated patients.

92.3% sensitivity and 100% specificity (area under the ROC [AUROC] = 0.93).

Plasma serotonin, 5-HIAA, 5OH-tryptophan, and the serotonin/kynurenine ratio did not significantly differ between curatively operated NET patients and healthy controls.

We further investigated a combined marker based on three metabolites of the serotonin pathway (serotonin, 5-HIAA, and/or 5-OH tryptophan), for simplicity we named this marker Combo1/3. This test was regarded positive if minimum one of these metabolites was elevated above a chosen ROC-derived cutoff value. If none of the metabolites were elevated above cutoff, the test was negative. This test performed better than singular metabolites in distinguishing NET patients with metastasized disease, as well as patients with CS, from healthy controls (Table 3). Further, we found the Combo1/3 test to be positive in a majority of patients curatively operated without radiologically detectable disease at sampling (17/28 SI-NET and 35/68 of all NET patients) compared with only 2/20 of healthy controls ($p < .001$). Figure 5 illustrates the results of the Combo1/3 test in different NET subgroups and healthy controls.

Figure S3A–F illustrates the diagnostic attributes of the plasma serotonin/kynurenine pathway ratio, the Combo1/3 test, plasma-serotonin, 5-HIAA, and 5-OH tryptophan, in addition to CgA, in different NET subgroups and healthy controls.

4 | DISCUSSION

In this study, we quantified plasma serotonin, 5-HIAA, 5OH-tryptophan, and five other metabolites of the tryptophan metabolism in NET patients and healthy controls using LC–MS/MS. Our results showed that using several metabolites simultaneously in a serotonin/kynurenine pathway ratio increased diagnostic sensitivity (92.3%) and specificity (100%) identifying NET patients with CS from healthy controls. In addition, this ratio as well as our Combo1/3 test, identified SI-NET patients with metastasized disease from healthy controls with 82%–88.5% sensitivity and 100%–90% specificity, respectively. In addition, the Combo1/3 test was positive in 61% of curatively operated SI-NET patients compared with only 10% of healthy controls ($p < .001$).

To our knowledge, our study investigating these combinations of tryptophan metabolites in plasma using LC–MS/MS has not earlier been evaluated as a clinical tool in NET patients. Our results indicate that the combination of metabolites is preferred to the use of singular analytes, which is currently used in clinical practice. The diagnostic accuracy of our tests was highest when applying them to the SI-NET patient group, which is not surprising considering SI-NETs are the main source of serotonin secretion in NET patients.

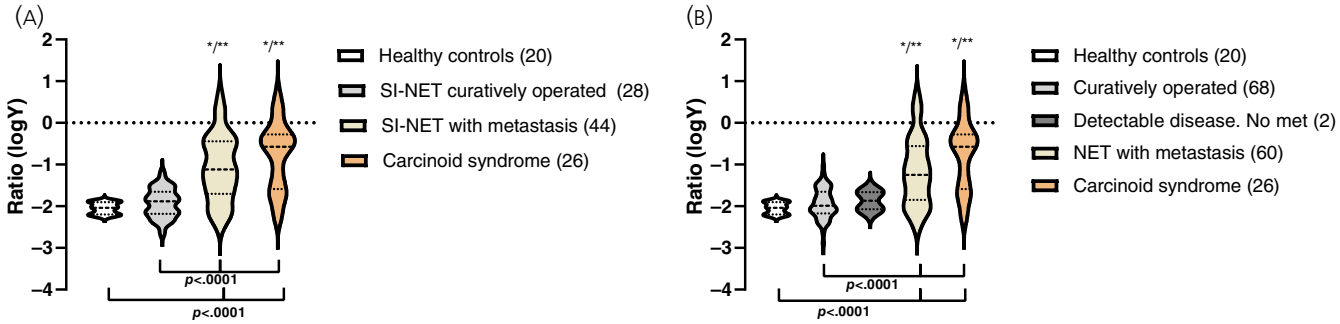
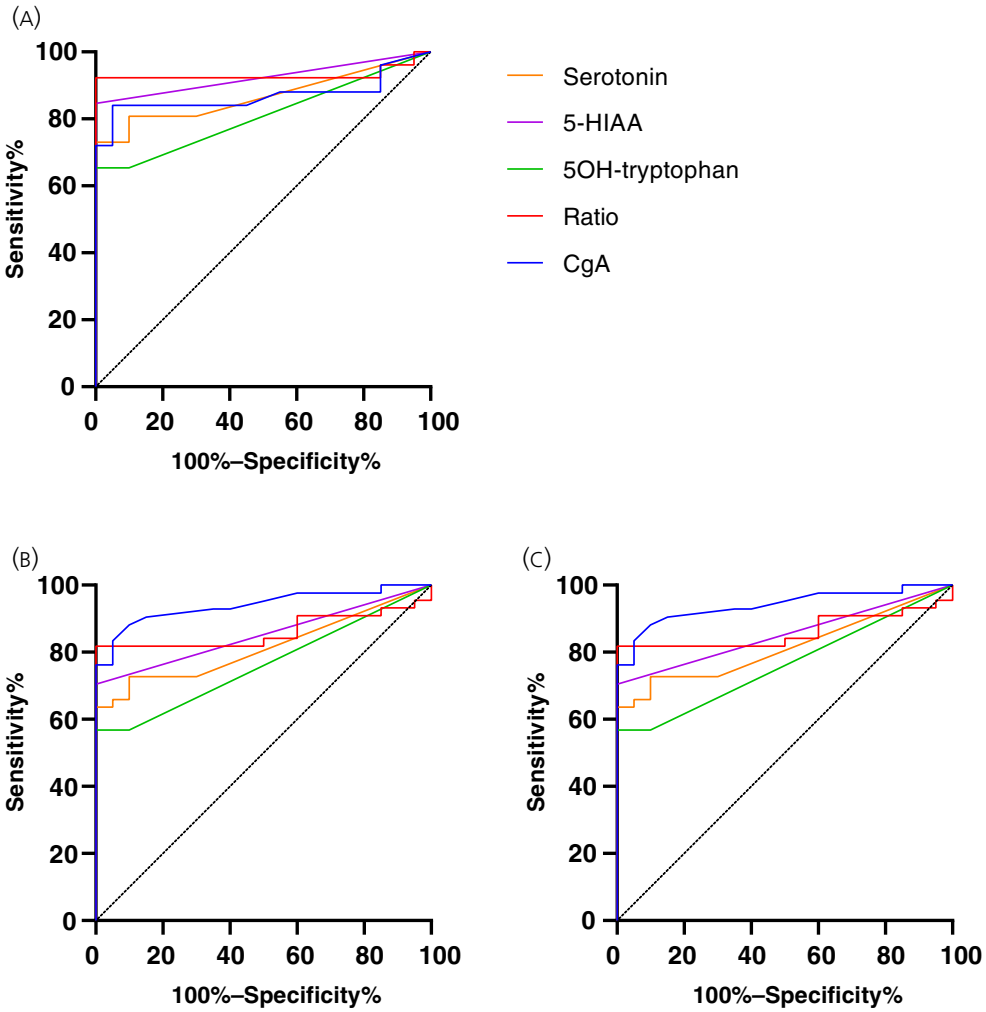


FIGURE 3 Violin plots of the serotonin/kynurenine pathway ratio in subgroups of (A). small intestinal neuroendocrine tumor (SI-NET) patients ($n = 72$) and (B) the whole NET patient cohort ($n = 130$). Data are presented in 10-logarithmic scale. *Significant difference from healthy controls. ** Significant difference from radically operated patients.

FIGURE 4 Receiver operating characteristic (ROC) analysis of plasma serotonin, 5-hydroxyindoleacetic acid (5-HIAA), 5OH-tryptophan, serotonin/kynurenine pathway ratio, and Chromogranin A (CgA) performance differentiating neuroendocrine tumor (NET) patients from healthy controls. (A) NET patients with carcinoid syndrome (CS, $n = 26$), (B) small intestinal NET (SI-NET) patients ($n = 44$) with metastasis. (C) All NET patients with metastasis ($n = 60$).



LC–MS/MS has been shown to give the most reliable results analyzing plasma serotonin.[16] The concentration of free serotonin in plasma is extremely low, and care must be taken to avoid preanalytical leakage of serotonin from platelets.[16,17] Our plasma serotonin results in SI-NET patients with metastasized disease (median 79.6 nmol/L, range <2.84–256.4) are similar to findings by de Jong et al.[18] (mean 60.8 nmol/L, range 3.7–244.7). Our plasma serotonin in healthy controls is also comparable to other studies applying the same methodology.[18–20] Others have investigated the ability of serum serotonin in detecting patients with metastasized SI-NET from healthy controls reporting slightly better, but comparable, diagnostic performance (sensitivity 75%/specificity 97% vs. our 73%/90%).[20] Studies on urine and plasma 5-HIAA have reported similar test performances to ours, with high specificity and lower sensitivities.[9,21–25]

TABLE 3 Plasma serotonin, 5-HIAA, 5OH-tryptophan, serotonin/kynurenine pathway ratio, Chromogranin A, and the Combo1/3 test differentiating patients from healthy controls.

Clinical setting	Metabolite	Cutoff (nmol/L)	AUC (95% CI)	*p*-Value	Sensitivity (95% CI)	Specificity (95% CI)
SI-NET with metastasis versus controls ($n = 44$)	Serotonin	17.1	0.81 (0.71–0.92)	<.0001	72.7% (58.2–83.7)	90% (69.9–98.2)
	5-HIAA	79.3	0.85 (0.76–0.94)	<.0001	70.5% (55.8–81.8)	100% (83.9–100)
	5-OH tryptophan	13.2	0.76 (0.65–0.88)	.0008	56.8% (42.2–70.3)	100% (83.9–100)
	Ratio	0.014	0.86 (0.77–0.95)	<.0001	81.8% (68.0–90.5)	100% (83.9–100)
	CgA ($n = 42$)	3.7	0.94 (0.88–1.0)	<.0001	78.6% (64.1–88.3)	95% (76.9–99.7)
	Combo1/3			<.0001	88.6%	90%
NET with metastasis versus controls ($n = 60$)	Serotonin	17.1	0.75 (0.65–0.85)	.0008	61.7% (49.0–72.9)	90% (69.9–98.2%)
	5-HIAA	79.3	0.83 (0.74–0.91)	<.0001	65.0% (52.4–75.8)	100% (83.9–100)
	5OH-tryptophan	13.2	0.76 (0.66–0.86)	.0005	56.7% (44.1–68.4)	100% (83.9–100)
	Ratio	0.014	0.84 (0.76–0.93)	<.0001	76.7% (64.6–85.6)	100% (83.9–100)
	CgA ($n = 56$)	3.7	0.94 (0.89–0.99)	<.0001	76.8% (64.2–85.9)	95% (76.4–99.7)
	Combo1/3			<.0001	83.2%	90%
NET with carcinoid syndrome versus controls ($n = 26$)	Serotonin	21.0	0.87 (0.76–0.98)	<.0001	80.8% (62.1–91.5)	90% (69.9–98.2)
	5-HIAA	84.6	0.92 (0.84–1.0)	<.0001	84.6% (66.5–93.9)	100% (83.9–100)
	5OH-tryptophan	13.2	0.81 (0.68–0.94)	.0004	65.4% (46.2–80.6)	100% (83.9–100)
	Ratio	0.014	0.93 (0.84–1.0)	<.0001	92.3% (75.9–98.6)	100% (83.9–100)
	CgA ($n = 25$)	3.5	0.87 (0.75–0.99)	<.0001	76.0% (56.6–88.5)	95% (76.4–99.7)
	Combo1/3			<.0001	92.3%	90%

Note: Cutoff values are derived by ROC curve analysis. Respective area under the curve (AUC), sensitivity and specificity with confidence interval (95% CI) and levels of significance (*p*-value) are listed for each metabolite. CgA results were missing in four patients.
Abbreviation: 5-HIAA, 5-hydroxyindoleacetic acid; CgA, chromogranin A; SI-NET, small intestinal neuroendocrine tumor; ROC, receiver operating characteristic.

Although CgA demonstrated the highest AUROC in NET patients with metastasized disease in our study, its diagnostic performance was not superior to our combinational markers when applying cutoff levels used in clinical practice. In differentiating between CS patients and healthy controls, our combined tryptophan metabolite markers performed even better compared with CgA. Our CgA findings were comparable to data from other studies.[6,26,27]

Our median plasma tryptophan concentrations in healthy controls were higher than reported in earlier studies using similar LC–MS/MS methodology and nonfasting plasma samples (226.6 vs. 45–88 μmol/L).[11,28–30] The other analyte concentrations (5OH-tryptophan, kynurenine, 3OH-kynurenine) were comparable to those found by others.[11,26–28]

The presence of CS significantly worsen prognosis in NET patients.[12,31,32] Measurements of urine- or plasma 5-HIAA is recommended in the diagnostics and follow-up of these patients. Our results displayed higher capability detecting CS patients from healthy controls compared with findings by de Mestiere (sensitivity 72.2% and specificity 64.9%) applying the same methodology.[21] Our results may indicate an advantage of the simultaneous quantification of tryptophan pathway metabolites projecting the metabolic profile of the secreting tumor. This should be further validated as a potential clinical tool in CS patients.

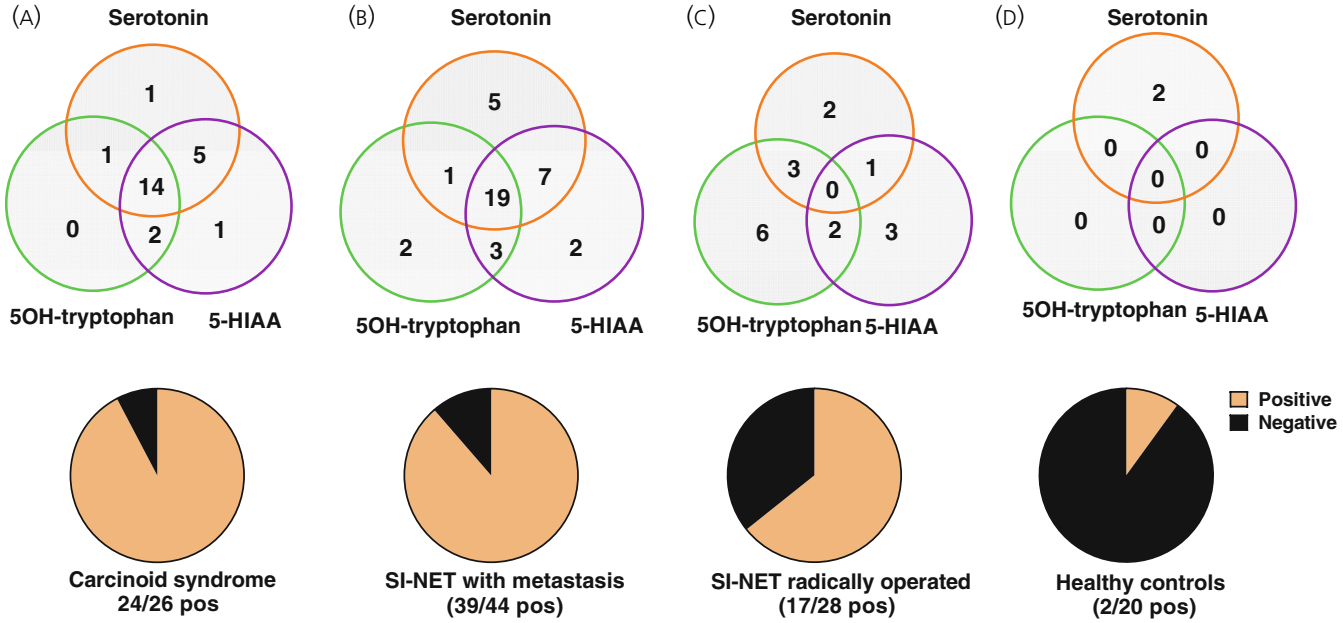


FIGURE 5 Results of a combinational marker based on minimum one out of three (serotonin, 5-hydroxyindoleacetic acid [5-HIAA], and/or 5-OH tryptophan) above cutoff in (A) carcinoid syndrome (CS) patients, (B) small intestinal neuroendocrine tumor (SI-NET) patients with metastasis, (C) SI-NET patients radically operated, and (D) healthy controls. The corresponding Venn-diagrams above illustrate the proportion of positive metabolites in each subgroup.

Despite curative treatment, a significant proportion of radically operated NET patients experience relapse at a later time. Besides liquid biopsies like the NETest, measuring NET gene expression in blood,[33–35] no other good predictive marker is available in presumed radically operated patients. Our Combo1/3 marker was positive in more than half of the radically operated patients, significantly more frequent compared with healthy controls. Whether the simultaneous quantification of several tryptophan pathway metabolites may increase the chance of detecting residual microscopic disease is hypothetical, but would be of interest to investigate prospectively.

The strengths of this study include simultaneous testing and analytical validation of several tryptophan metabolites in a well-stratified NET patient group. We included a subgroup of CS patients where earlier studies have described changes in tryptophan metabolism.[12,13] We found plasma samples to be simple, accessible and can be performed at routine visits at the outpatient clinics without fasting or prior diet restrictions. In addition, plasma sampling compared with 24-h urine 5-HIAA, relieves patients of burdensome urine sampling. The availability of LC–MS/MS is increasing and reduces costs for analysis, increasing the potential for implementing this in routing clinical practice. In addition, and in contrast to other biomarker studies,[26,36] our patient cohort includes a large proportion of radically operated disease where great interest exists to find a marker of future relapse.

Weaknesses of our study are our relatively small patient cohort. This may increase the risk of Type 2 errors missing possible relevant effects in the NET population. The patient cohort is also heterogenous, as to tumor localization and dissemination. Another important issue is the treatment with SSA, which several patients were receiving at study inclusion. This may have resulted in reduced values of tryptophan metabolites in this patient group and further underestimating a more pronounced effect in this patient population.

Future prospective studies should validate the prognostic implications of elevated combinational tryptophan metabolites in CS patients, as well as in other NET subgroups including possible early detection of residual disease in radically operated patients.

5 | CONCLUSION

Our study demonstrates a benefit from simultaneously quantifying several tryptophan metabolites in NET diagnostics, especially in CS, as compared to using 5-HIAA or CgA alone.

AUTHOR CONTRIBUTIONS

S. U. Johansen: Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing—original draft. **T. Hansen:** Investigation; resources; validation; writing—original-draft; writing—review and editing. **A. Nordborg:** Investigation; resources; validation. **R. Meyer:** Data curation; resources. **R. Goll:** Conceptualization; methodology; supervision; writing—review and editing. **J. Florholmen:** Conceptualization; funding acquisition; methodology; project administration; supervision; writing—review and editing. **E. Jensen:** Conceptualization; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have nothing to disclose.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jne.13372.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author based on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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