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Original Research

Metabolomic profile of neuroendocrine tumors identifies methionine, porphyrin, and tryptophan metabolisms as key dysregulated pathways associated with patient survival

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Abstract

Objective: Metabolic profiling is a valuable tool to characterize tumor biology but remains largely unexplored in neuroendocrine tumors (NETs). Our aim was to comprehensively assess the metabolomic profile of NETs and identify novel prognostic biomarkers and dysregulated molecular pathways.

Design and Methods: Multiplatform untargeted metabolomic profiling (GC-MS, CE-MS, and LC-MS) was performed in plasma from 77 patients with G1-2 extra-pancreatic NETs enrolled in the AXINET trial (NCT01744249) (study cohort) and from 68 non-cancer individuals (control). The prognostic value of each differential metabolite ($n = 155$) in NET patients ($P < .05$) was analyzed by univariate and multivariate analyses adjusted for multiple testing and other confounding factors. Related pathways were explored by Metabolite Set Enrichment Analysis (MSEA) and Metabolite Pathway Analysis (MPA).

Results: Thirty-four metabolites were significantly associated with progression-free survival (PFS) ($n = 16$) and/or overall survival (OS) ($n = 27$). Thirteen metabolites remained significant independent prognostic factors in multivariate analysis, 3 of them with a significant impact on both PFS and OS. Unsupervised clustering of these 3 metabolites stratified patients in 3 distinct prognostic groups (1-year PFS of 71.1%, 47.7%, and 15.4% ($P = .012$); 5-year OS of 69.7%, 32.5%, and 27.7% ($P = .003$), respectively). The MSEA and MPA of the 13-metablolite signature identified methionine, porphyrin, and tryptophan metabolisms as the 3 most relevant dysregulated pathways associated with the prognosis of NETs.

Conclusions: We identified a metabolomic signature that improves prognostic stratification of NET patients beyond classical prognostic factors for clinical decisions. The enriched metabolic pathways identified reveal novel tumor vulnerabilities that may foster the development of new therapeutic strategies for these patients.

Keywords: neuroendocrine tumors, metabolomics, plasma metabolites, prognostic biomarker

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Significance

Metabolic profiling is a valuable tool for understanding tumor biology but remains largely unexplored in neuroendocrine tumors (NETs). Here, we performed comprehensive metabolic profiling using a multiplatform untargeted metabolomic approach to prospectively analyze plasma from 77 patients with G1-2 advanced extra-pancreatic NETs compared to a control cohort of 68 non-cancer individuals. The results revealed a distinct metabolomic profile in NETs and identified a 13-metabolite signature that improves the prognostic stratification of NET patients beyond classical prognostic factors used in clinical decision-making. Moreover, we identified dysregulation in methionine, porphyrin, and tryptophan metabolic pathways that were strongly associated with patient survival and have the potential to drive the development of new therapeutic strategies for NET patients. Further independent studies are warranted to validate these encouraging results.

Introduction

Neuroendocrine neoplasms (NENs) are a family of tumors of great biological heterogeneity and challenging clinical management.[1] Although they can arise in any organ, the most common primary tumor sites are the lungs (25%) and the digestive tract (~65%). Neuroendocrine neoplasms are still considered rare tumors but their incidence is increasing, being the second most prevalent tumor in the digestive tract. Globally, NENs are classified based on tumor differentiation and proliferation index, as well-differentiated neuroendocrine tumors (WD NETs) grades 1-3 (80% of all NENs) or poorly differentiated neuroendocrine carcinomas (PD NECs), which are always grade 3 (20% of all NENs).[2,3] Generally, NETs display an indolent clinical behavior compared to their exocrine counterparts and are associated with better prognosis, although their outcome depends on multiple factors, including primary tumor site, proliferative index (ki67 or mitotic index), tumor differentiation, and stage.[1,4,5] In contrast, NECs are highly aggressive tumors with a poor prognosis[6–8]

A singularity of up to 20% of NETs—the so-called functional tumors—is their potential ability to synthesize and secrete a variety of metabolically active compounds, such as serotonin, insulin, glucagon, gastrin, vasoactive intestinal peptide (VIP), and others.[9] This secretion can produce a spectrum of hormone-related symptoms or syndromes that can be specific to the tissue of origin (eutopic secretion) or, less commonly, may be characteristic of other anatomic sites (ectopic secretion).[9]

Survival has improved over time for all NETs, likely reflecting earlier diagnosis and improvements in therapy.[1,10] However, a high proportion of patients are still diagnosed with metastatic disease; systemic treatment options for advanced disease remain limited and eventually all patients experience disease progression and death. The development of new approaches that help understand molecular pathways involved in cancer evolution and clinical outcome may contribute to an earlier detection and more precise monitoring of patients and identify new vulnerabilities that may be suitable targets for cancer therapy.

In this context, particular attention has been paid to reprogramed metabolism in oncology.[11–13] The metabolic shift in cancer cells supports proliferation and survival[14] although, to date, the mechanisms involved in the metabolic plasticity of NENs remain to be elucidated. Very recently, our group performed a comprehensive untargeted metabolomic profiling in plasma samples from patients with advanced G1-2 extra-pancreatic NETs ($n = 77$) by means of a multiplatform untargeted approach (LC-MS, GC-MS, and CE-MS). The integrated metabolic data acquired identified 155 differential compounds between NETs and controls. Plasma of NET patients showed increased levels of bile acids, sugars, oxidized lipids, and oxidized products from arachidonic acid, and decreased levels of carnitine. We identified novel enriched metabolic pathways in NETs related to the tricarboxylic acid (TCA) cycle, and to arginine, pyruvate, or glutathione metabolism, which have distinct implications in oncogenesis. Differential metabolites were also related to classical cancer (apoptosis, cell cycle) and NET-specific pathways (tryptophan metabolism, angiogenesis, or the mTOR pathway). In addition, we identified and validated a reduced set of metabolites ($n = 48$) of high diagnostic accuracy that may improve the specific detection of NETs.[15] To our knowledge, this is the most comprehensive metabolic profiling study performed to date in NETs, and provides valuable information to develop useful biomarkers for the management of these patients in clinical practice. In this context, the aim of the present study was to explore the potential role as prognostic biomarkers of the 155 metabolites previously identified as a metabolic signature of NETs, and to functionally characterize molecular pathways involved in determining patient survival.

Methods

Patient population

The study population included 77 patients with advanced, G1-G2 extra-pancreatic NETs (study cohort) and 68 non-cancer individuals (control cohort) with similar distribution of gender, age, and body mass index (BMI). Main clinical and pathological features of the study population are presented in Table 1. Biochemical parameters are summarized in Table S1.

The NET study population belonged to the first cohort of patients enrolled in the AXINET trial (clinicalTrials.gov Identifier: NCT01744249, EUDRACT 2011-001550-29). AXINET was a phase II-III, prospective, multicenter, randomized (1:1), double-blind study to evaluate the efficacy and tolerability of axitinib and octreotide LAR versus placebo and octreotide LAR in patients with advanced G1-G2 neuroendocrine tumors (WHO 2010) of non-pancreatic origin.[16,17] This study enrolled 256 patients, 106 patients in the first part of the study (phase II), and 150 additional patients in the second part of the study (phase III). Treatment was continued until disease progression, unacceptable toxicity, consent withdrawal, or death, whichever occurred first. The study population included all patients with advanced, G1-G2 extra-pancreatic NETs enrolled in the phase II part of the AXINET trial that provided consent to donate plasma samples for additional translational research ($n = 77$). Baseline plasma samples (before the first dose of study treatment) were taken, processed, and stored as previously reported.[15]

Table 1. Clinicopathological characteristics of the study population.

Features	*n* (%)
Age (years)	
Median value	63 (range: 38-83)
BMI	
Median value	25.9 (range: 17.2-52.5)
BMI	
Underweight (BMI < 18.5)	1 (1.3%)
Normal weight (BMI 18.5-24.9)	28 (36.4%)
Overweight (BMI 25.0-29.9)	26 (33.8%)
Obesity (BMI > 30)	19 (24.7%)
Unknown	3 (3.9%)
Gender	
Female	34 (45.5%)
Male	42 (54.5%)
ECOG PS	
0	49 (63.6%)
1	28 (36.4%)
Localization of primary tumor	
Gastric	1 (1.3%)
Duodenum	1 (1.3%)
Jejunum-ileum	39 (50.6%)
Colon	2 (2.6%)
Rectum	6 (7.8%)
Lung	19 (24.7%)
Unknown primary	7 (9.1%)
Thymus	1 (1.3%)
Parotid	1 (1.3%)
Localization of metastases	
Liver	66 (85.7%)
Lung	11 (14.3%)
Lymph nodes	42 (54.5%)
Other	44 (57.1%)
Grade	
G1 (Ki-67 < 3%)	25 (32.5%)
G2 (Ki-67 3%-20%)	52 (67.5%)
Ki67 (%)	
Median (range)	5 (1-15)
≤5	51 (66.2%)
>5	26 (33.8%)
Functioning tumor	
Yes (carcinoid syndrome)	24 (31.2%)
No	53 (68.8%)
Time from diagnosis to study entry	
≤12 months	33 (40.3%)
>12 months	44 (57.1%)
Prior systemic treatment	
No	31 (40.3%)
1 line	34 (44.2%)
≥2 lines	12 (15.6%)
Prior therapies	
SSA	40 (51.9%)
Chemotherapy	10 (13.0%)
PRRT	0 (0.0%)
Interferon	4 (5.2%)
Everolimus	8 (10.4%)
Radiotherapy	2 (2.6%)
Locoregional therapy	8 (10.4%)
Surgery	34 (44.2%)
Follow-up period	
Median (range)	46 months (2-96)
Exitus letalis	
Yes	39 (50.6%)
No	38 (49.4%)

Abbreviations: BMI, body mass index; ECOG PS, Eastern Cooperative Oncology Group performance status scale; PRRT, peptide receptor radionuclide therapy; SSA, somatostatin analogs.

The study protocol was approved by the institutional review board and ethics committee at each participating institution. The study was conducted in accordance with standards of Good Clinical Practice. All patients provided written informed consent before study entry. An additional optional informed consent was required for translational studies.

Clinical data analysis

Descriptive analysis was used to characterize the most relevant clinical, biochemical, and pathological features of the study population. The association of categorical variables was assessed by the chi-squared test or Fisher's exact test, when appropriate. The distribution of quantitative variables among study groups was evaluated by parametric (Student's *t*-) or non-parametric (Kruskal-Wallis or Mann-Whitney) tests as required for each variable. Overall survival (OS) was calculated from the date of randomization into the study to the date of death from any cause or of last contact in surviving patients. Progression-free survival (PFS) was calculated from the date of treatment initiation within the AXINET trial to the date of disease progression or the last contact in patients without progression. The Kaplan-Meier product limit method was used to estimate PFS and OS, and differences observed among patient subgroups were assessed by the log-rank test. Statistical significance was established at $P \leq .05$.

Analysis of the prognostic impact of metabolites

The prognostic value for PFS and OS was analyzed for each identified metabolite ($n = 155$) with a differential availability in NET patients when compared to non-cancer individuals,[15] considering their expression as a continuous variable, by univariate Cox proportional-hazards regression using R survival package.[18] The adjusted *P*-value for multiple testing was estimated by the false discovery rate (FDR) method.[19,20] We also evaluated the potential prognostic impact of identified metabolites considered as categorical variables, with the median value as the cutoff point (> or ≤median), using the Kaplan-Meier method and log-rank test to assess the statistical significance of observed differences between groups.

Metabolites with a significant impact on PFS or OS were selected for further association analyses. The potential association of selected metabolites ($n = 34$) with most relevant clinicopathological features (gender, age, BMI, grade, primary tumor origin, and tumor functionality) and most common concomitant medications was assessed by the chi-squared or Fisher's exact tests, as appropriate. Drug classes selected for association analyses were those taken by >10% of patients at study entry, and included the following: Antihypertensives, analgesics, nonsteroidal anti-inflammatory drugs, diuretics, antiplatelet agents, anxiolytics, H2-receptor blockers, and lipid-lowering medications. Statistical significance was established at $P < .05$. To evaluate the independent prognostic value of selected metabolites for both PFS and OS, a multivariate analysis was performed using the Cox proportional-hazards method including gender, age, grade, and primary tumor location (gastrointestinal, pulmonary, or other) as covariables. In addition, BMI, tumor functionality, and concomitant drugs were also included as potential confounding factors in the

multivariate model for those metabolites for which a significant association between these additional variables (BMI, functionality, and concomitant drugs) and the specific metabolite was documented in univariate analysis.

All statistical analyses were performed using IBM-SPSS version 25 (IBM Corporation, New York, United States) and R software version. 3.6.1.

Metabolite Pathway Analysis (MPA) and Molecular Set Enrichment Analysis (MSEA)

To identify aberrant molecular pathways involved in NET patient survival, we analyzed prognostic metabolites by Metabolite Pathway Analysis (MPA) and Metabolite Set Enrichment Analysis (MSEA) using MetaboAnalyst 4.0 platform (http://www.metaboanalyst.ca/).[21] The databases of reference employed were KEGG homo sapiens (Oct 2019) and SMPD.

Heatmap and hierarchical clustering

Heatmaps were conducted with the log10 value of plasma levels of selected metabolites. Unsupervised hierarchical clustering was performed for metabolites and patients using Pearson correlation and average as linkage method. Both were conducted using the Morpheus Software[22] (Broad Institute; https://software.broadinstitute.org/morpheus).

Results

Metabolites with differential availability in NET patients that are independent prognostic biomarkers

We assessed the prognostic impact of 155 metabolites with a differential availability in NET patients that we had identified in previous work.[15] Each metabolite was analyzed both as a continuous and as a dichotomic variable (categorized as high or low according to their median value) to explore their potential impact on patient survival. Thirty-four metabolites were significantly associated with PFS ($n = 16$) and/or OS ($n = 27$) (Table S2).

The main characteristics of the 34 prognostic metabolites according to the Human Metabolome Database (HMDB) (www.hmdb.ca) are provided in Table S3. There were 9 metabolites associated with both PFS and OS: Glu-Hyp, Glu-Lys/Ɛ-Glu-Lys 2, suberylglycine, pyranose (glucose/altrose/galactose/talose), eicosapentaenoic acid, 3-hydroxy-5-octenoylcarnitine, LPC (22:1), urocanate nicotinamide N-oxide, and 5-hydroxyindoleacetic acid. Progression-free survival rates at 1 year and OS rates at 5 years are reported by metabolite abundance (classified as "low" or "high" according to their median values) in Table S4.

Multivariate analyses demonstrated 13 of these 34 prognostic metabolites were independently associated with PFS ($n = 5$) and/or OS ($n = 11$) (Table 2). For PFS, Glu-Hyp ($P = .018$), Glu-Lys/Ɛ-Glu-Lys 2 ($P = .006$), methionine S-oxide ($P = .012$), 3-hydroxy-5-octenoylcarnitine ($P = .018$), and PG (28:0) ($P = .050$) were statistically significant in multivariate analysis. For OS, Glu-Hyp ($P = .011$), Glu-Lys/Ɛ-Glu-Lys 2 ($P = .004$), MG (20:0) ($P = .054$), N-palmitoyl glutamic acid ($P = .045$), 3-hydroxy-5-octenoylcarnitine ($P = .001$), 3-hydroxy-5-tetradecenoylcarnitine ($P = .045$), LPC (22:1) ($P = .002$), 5-hydroxyindoleacetic acid ($P = .041$), 1-methyladenosine ($P = .006$), biliverdin ($P = .019$), and ecdysone 25-O-D-glucopyranoside ($P = .051$) retained independent statistical significance in the multivariate model. Three of these metabolites, Glu-Hyp, Glu-Lys/Ɛ-Glu-Lys 2, and 3-hydroxy-5-octenoylcarnitine, showed a significant impact on both PFS and OS. Kaplan-Meier curves for PFS and OS relating to the 13 independent prognostic metabolites (2 for PFS; 8 for OS; and 3 for both) are depicted in Figure 1.

Prognostic metabolites that identify novel dysregulated oncogenic pathways in NET patients

We performed a functional analysis of the 34 prognostic metabolites to identify enriched signaling pathways involved in NET patient prognosis. Metabolite Set Enrichment Analysis showed enrichment of pathways related to alpha linolenic and linoleic acid, porphyrin, methionine, and tryptophan metabolisms (Figure 2A). Metabolite Set Enrichment Analysis restricted to the 13 metabolites with an independent impact on PFS and/or OS showed that porphyrin and tryptophan metabolisms were the 2 main enriched pathways (Figure 2B). Metabolite Pathway Analysis of all 34 prognostic metabolites showed that histidine metabolism, sphingolipid metabolism, porphyrin metabolism, biosynthesis of unsaturated fatty acids, glycerophospholipid metabolism, and tryptophan metabolism were the most commonly dysregulated pathways related to NET prognosis (Figure 2C). Metabolite Pathway Analysis restricted to the 13 selected metabolites confirmed methionine, porphyrin, and tryptophan metabolisms as the most relevant dysregulated pathways (Figure 2D). More specifically, the 5 metabolites with a significant impact on PFS were associated with methionine and tryptophan metabolisms, whereas the 11 metabolites with a significant impact on OS were associated with porphyrin and tryptophan metabolisms. Consistently, pathway analysis performed with only the 3 common metabolites with a significant impact on both PFS and OS identified tryptophan metabolism as the most relevant dysregulated pathway associated with patient prognosis.

Selected metabolites that define prognostic subgroups of NET patients

To identify metabolite profiles that define biological and prognostic subgroups of NET patients, we conducted unsupervised analyses of the 13 selected metabolites with an independent impact on PFS and/or OS.

Unsupervised heatmap cluster plot of the 5 metabolites with an independent impact on PFS identified 2 distinct clusters; a smaller one (cluster 1) encompassing 26 NET patients and a larger one (cluster 2) including the remaining 51 patients (Figure 3A, left side). These clusters did not seem to be related to classical clinical features such as primary tumor site, grade, or function. Remarkably, these 2 clusters presented a significantly different PFS, with a PFS rate at 1 year of 64.7% for cluster 2 versus 38.5% for cluster 1 (HR = 0.56, $P = .045$) (Figure 3A, right side).

Unsupervised heatmap cluster plot of the 11 metabolites with an independent impact on OS identified 4 different clusters (Figure 3A, left side). No clear association was found between metabolic clusters and most relevant clinical features. Cluster 4 was associated with a better OS than the other 3 clusters (Figure 3B, right side) (HR = 0.14, $P = .073$). Overall survival rate at 5 years was 50.0%, 63.6%, 45.5%, and 88.9% for clusters 1, 2, 3, and 4, respectively.

Unsupervised heatmap cluster plot of the 3 selected metabolites with an independent impact both on PFS and OS

Table 2. Metabolites with significant impact on PFS and/or OS (univariate and multivariate analyses).

	PFS						OS					
	Univariate				Multivariate		Univariate				Multivariate	
	Continuous		Median		Median		Continuous		Median		Median	
	P	FDR	*P*	FDR	*P*	HR	*P*	FDR	*P*	FDR	*P*	HR
Amino acids, peptides, and analogs												
Glu-Hyp	.035	0.624	.042	0.561	**.018**	0.486	.033	0.399	X	X	**.011**	0.393
Glu-Lys/Ɛ-Glu-Lys 2	.023	0.586	.002	0.168	**.006**	0.411	X	X	.016	0.365	**.004**	0.353
Methionine S-oxide	X	X	<.000	0.099	**.012**	0.448	X	X	X	X	X	X
Fatty Acyls												
MG (20:0)	X	X	X	X	X	X	.0238	0.399	.024	0.421	**.054**	0.359
N-palmitoyl glutamic acid	X	X	X	X	X	X	X	X	.027	0.421	**.045**	0.493
3-Hydroxy-5-octenoylcarnitine	.026	0.586	.008	0.462	**.018**	0.508	<.000	0.071	.002	0.255	**.001**	0.292
3-Hydroxy-5-tetradecenoylcarnitine	X	X	X	X	X	X	X	X	.027	0.421	**.045**	0.493
Glycerophospholipids												
LPC (22:1)	.010	0.586	.013	0.508	.069	0.546	.002	0.108	.015	0.365	**.002**	0.293
PG (28:0)	X	X	.047	0.561	**.050**	0.560	X	X	X	X	X	X
Imidazoles												
Urocanate nicotinamide N-oxide	X	X	X	X	X	X	.048	0.443	.010	0.352	.071	0.573
Lactones												
N-(4-Coumaroyl)-homoserine lactone	X	X	X	X	X	X	.004	0.124	.004	0.255	.098	0.514
Purine nucleosides												
1-Methyladenosine	X	X	X	X	X	X	<.000	**0.017**	.004	0.255	**.006**	0.348
Indoles and derivatives												
5-Hydroxyindoleacetic acid	X	X	.046	0.561	.170	0.603	X	X	X	X	**.041**	0.471
Steroids and steroid derivatives												
Biliverdin	X	X	X	X	X	X	X	X	X	X	**.019**	0.417
Ecdysone 25-O-D-glucopyranoside	X	X	X	X	X	X	.025	0.399	X	X	**.051**	2.039

P-values ≤ .05 were highlighted in bold. Abbreviations: FDR, false discovery rate; OS, overall survival; PFS, progression-free survival.

stratified patients into 3 prognostic groups (Figure 3C). Clinical outcome was significantly different by metabolic cluster, with cluster 3 associated with the best prognosis in terms of both PFS and OS. Multivariate analysis including age, gender, grade, functionality, primary tumor location, treatment arm, and time from randomization to study entry as covariables confirmed the metabolic signature as a significant independent prognostic factor ($P < .001$ for OS) (Table 3).

Discussion

Reprogramed metabolism to support neoplastic proliferation under hostile conditions is one of the key hallmarks of cancer. Our group recently published the most comprehensive metabolic profiling study performed to date in NETs, which identified a distinctive metabolic signature in plasma of NET patients of potential diagnostic relevance.[15] Based on this metabolic fingerprint, in the present study, we analyzed the potential prognostic value of 155 compounds with differential availability in NET patients. Thirty-four metabolites were found to be significantly associated with PFS ($n = 16$) and/or OS ($n = 27$). Functional analysis of these performed by MSEA revealed enriched pathways involved alpha linolenic and linoleic acid, porphyrin, methionine, and tryptophan metabolisms. Moreover, MPA confirmed porphyrin and tryptophan metabolisms as relevant dysregulated metabolic pathways, and also identified the biosynthesis of unsaturated fatty acids, and histidine, sphingolipid, and glycerophospholipid metabolisms as additional pathways implicated in NET prognosis. The results of this work demonstrate that NET patients have a distinct metabolomic profile that provides relevant information on disease biology of potential clinical application. Indeed, we have identified a set of 34 metabolites that may be used as prognostic biomarkers to improve patient stratification beyond classical clinical and pathological prognostic factors. Furthermore, pathway analysis of this set of prognostic metabolites enabled the identification of dysregulated pathways, which may facilitate the development of new therapeutic strategies in the future.

Notably, 10 of the 34 analyzed metabolites are related to an essential pathway involved in the metabolic shift of cancer cells: The TCA cycle. Tricarboxylic acid is known to be involved in tumorigenesis in different tumor types by sustaining mitochondrial metabolism.[23,24] Cancer cells can adapt to the availability of various fuels, as well as to hostile microenvironmental conditions such as hypoxia and acidosis, because the metabolic versatility provided by this mitochondrial input confers the cancer cell a survival advantage.[25] Consistent with this, in our study higher levels of glutamine, glucose and fatty acids, which represent the main fuel of the TCA cycle, were associated with decreased patient survival. The relevance of TCA cycle dysregulation in NET biology has been reported in other studies conducted in GI NETs[26] and is also well established in the pathogenesis of other types of neuroendocrine tumors, such as pheochromocytomas and paragangliomas.[27]

Our study also identified a characteristic lipidome in NETs, mainly represented by the enrichment of glycerophospholipids (5 of 34 prognostic metabolites), fatty acids (6 of the 34 selected metabolites), and steroids/steroid derivatives (4 of 34 prognostic metabolites). More specifically, oxidized lysoglycerophospholipids (oxLPCs) were more abundant in NETs, indicating a strong oxidative stress in these tumors.[15,28] Increasing evidence for the role of oxidized lipids in cancer metabolism is paving the way for new and exciting therapeutic opportunities, together with a more profound comprehension

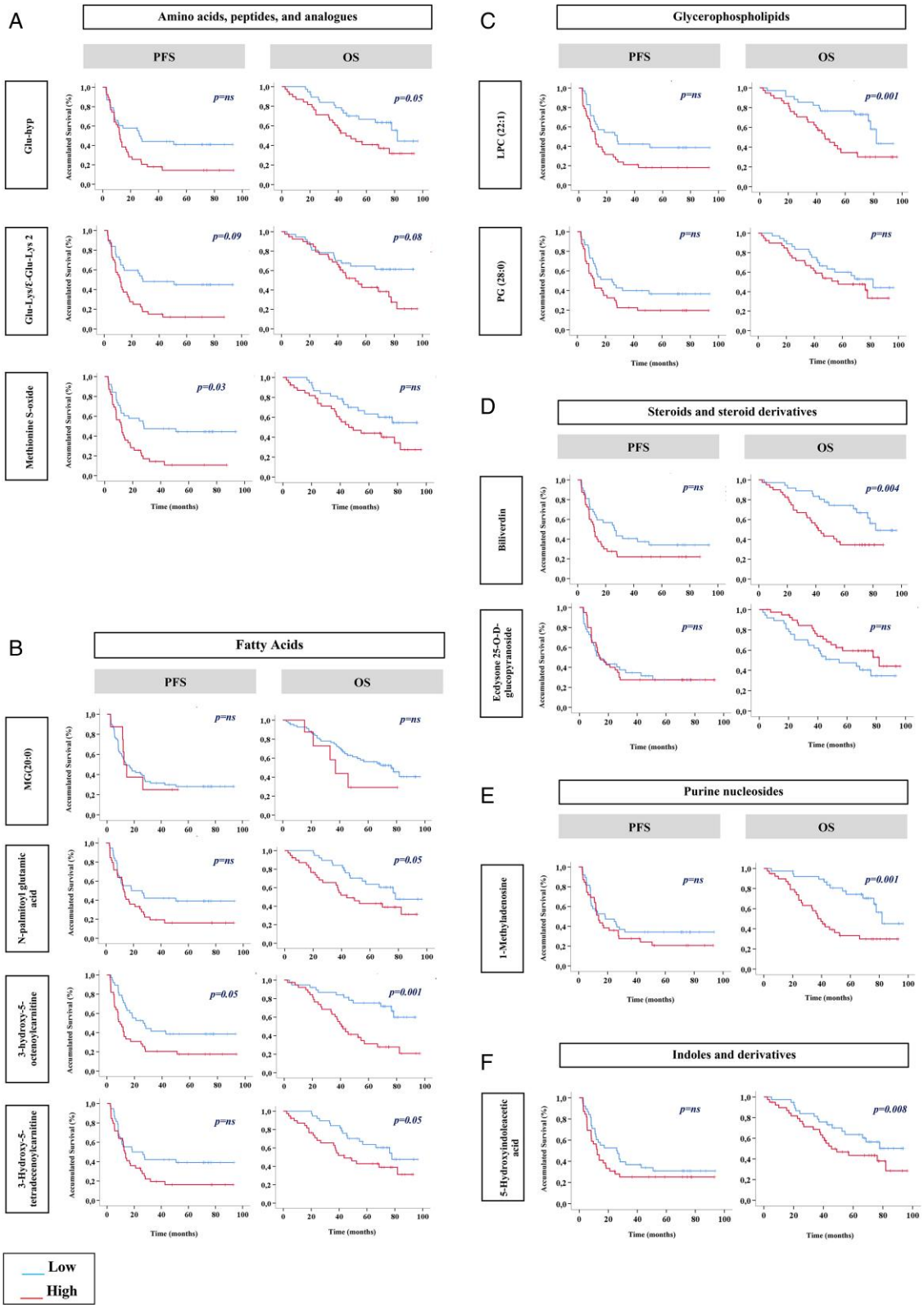

Figure 1. Impact on progression-free survival (PFS) and overall survival (OS) of the 13 independent prognostic metabolites. Kaplan-Meier estimates of PFS and OS in patients with low (≤median) versus high (>median) levels of each metabolite. The selected metabolites are grouped according to their biochemical nature: (A) Amino acids, peptides, and analogs (Glu-Hyp, Glu-Lys/Ɛ-Glu-Lys 2, and methionine S-oxide); (B) fatty acids (MG [20:0], N-palmitoyl glutamic acid, 3-hydroxy-5-octenoylcarnitine and 3-hydroxy-5-tetradecenoylcarnitine); (C) glycerophospholipids (LPC [22:1] and PG [28:0]; (D) steroids and steroid derivatives (biliverdin and ecdysone 25-O-D-glucopyranoside); (E) purine nucleosides (1-methyladenosine); and (F) indoles and derivatives (5-hydroxyindoleacetic acid); $P < .05$ are considered significant.

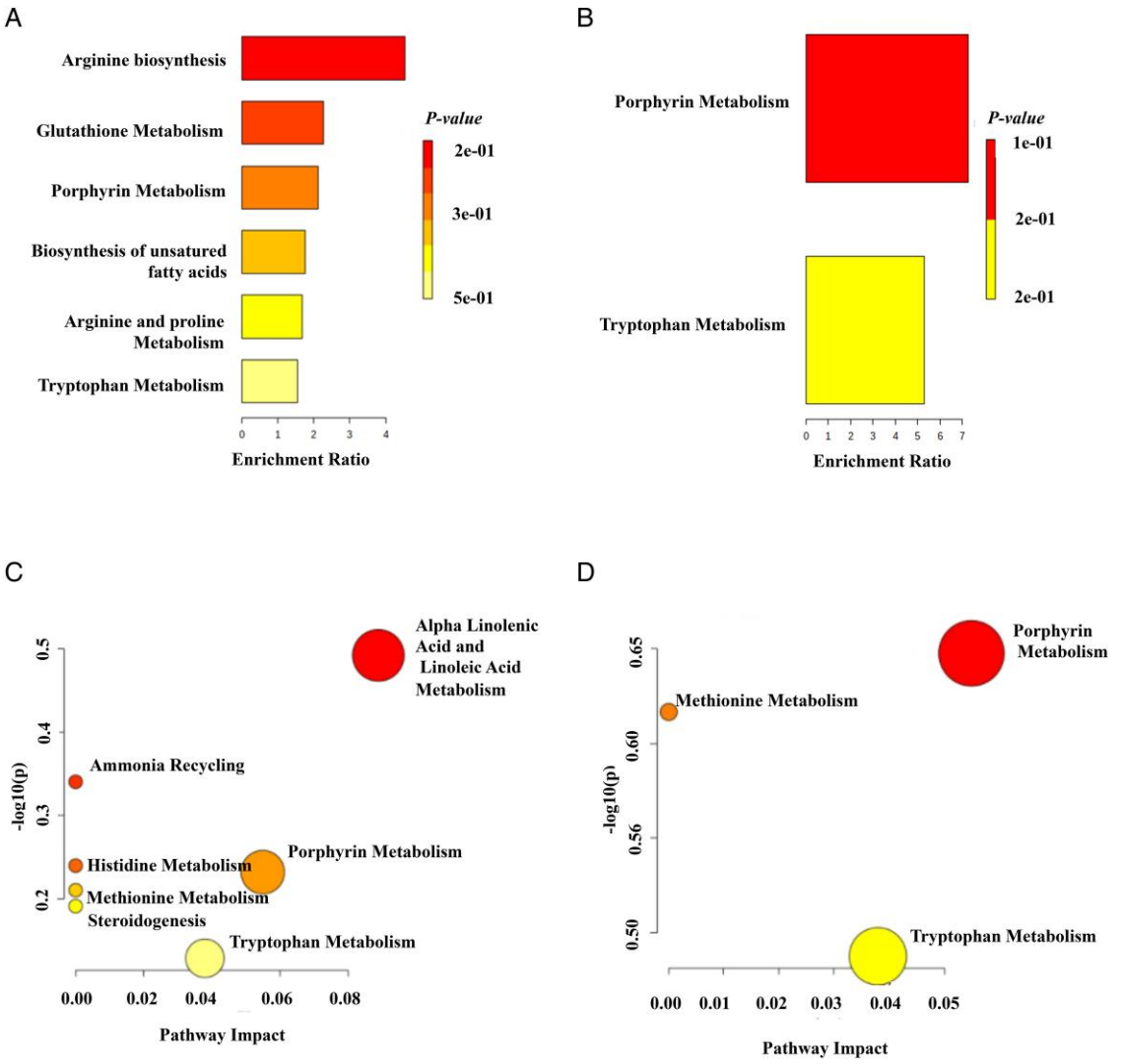

Figure 2. Prognostic metabolites that identify novel dysregulated oncogenic pathways in NET patients. Metabolite Set Enrichment Analysis (MSEA) was performed for 34 (A) and 13 (B) plasma metabolites significantly associated with PFS and/or OS in univariate and multivariate analyses, respectively. The *x*-axis represents the fold enrichment of each metabolite set, and the bar color indicates the raw *P*-value. Additionally, Metabolite Pathway Analysis (MPA) was also performed for these 34 (C) and 13 (D) plasma metabolites, and significantly enriched pathways (FDR < 0.05) are represented. The *x*-axis indicates the impact of matched metabolites from our dataset on the pathway from the topology analysis. The $-\log_{10}$ (*P*-value) is plotted on the *y*-axis and shows the pathway enrichment significance. Circle size represents the impact factor of matched metabolites in the pathway, and circle color denotes the pathway enrichment significance (*P*-value).

of the metabolic wiring of cancer cells.[29–31] However, further knowledge of the dependence of cancer cells on oxidized lipids is necessary, to refine rational approaches toward developing new therapeutic strategies that target lipid catabolism.

Interestingly, 2 of the 34 prognostic metabolites are associated with the angiogenesis switch that is another key hallmark of cancer.[14,32] Neuroendocrine tumors are typically vascularized neoplasms, and angiogenesis dysregulation plays an essential role in the development and progression of these tumors.[33] In our study, greater abundance of arginine correlated with worse survival. Arginine is the main source of nitric oxide, which is deeply involved in the regulation of angiogenesis, cancer initiation, and progression, but also restricts cancer proliferation and invasion, and contributes to the antitumor immune response.[34] Therefore, consistent with the results of our study, regulation of nitric oxide via the synthesis and availability of arginine is strongly linked to cancer biology. Moreover, biliverdin also contributes to angiogenesis through the up-regulation of VEGFA, VEGFC, IL-1β, and IL-8.[35,36] In our series, a greater abundance of biliverdin was associated with a worse prognosis. Overall, these findings support the relevant role that angiogenesis plays in the pathogenesis of NETs.

To further assess the independent prognostic value of the 34 metabolites, we performed a multivariate analysis to adjust for other confounding factors, including those potentially associated with metabolite abundance (BMI, sex, hormonal syndrome, and concomitant medication). Thirteen of the 34 metabolites displayed an independent prognostic value; 5 were associated with PFS, 11 with OS, and 3 with both PFS and OS. Pathway analysis of the 13-metabolite signature identified methionine, porphyrin, and tryptophan metabolisms as the most relevant dysregulated pathways. Unsupervised clustering analysis of the 3 common metabolites stratified patients into 3 distinct prognostic groups associated with good (PFS rate at 1 year 71.1%, OS rate at 5 years 69.7%), intermediate (PFS rate at 1 year 47.4%, OS rate at 5 years 32.5%), and poor prognosis (PFS rate at 1 year 15.4%, OS rate at 5 years 27.7%).

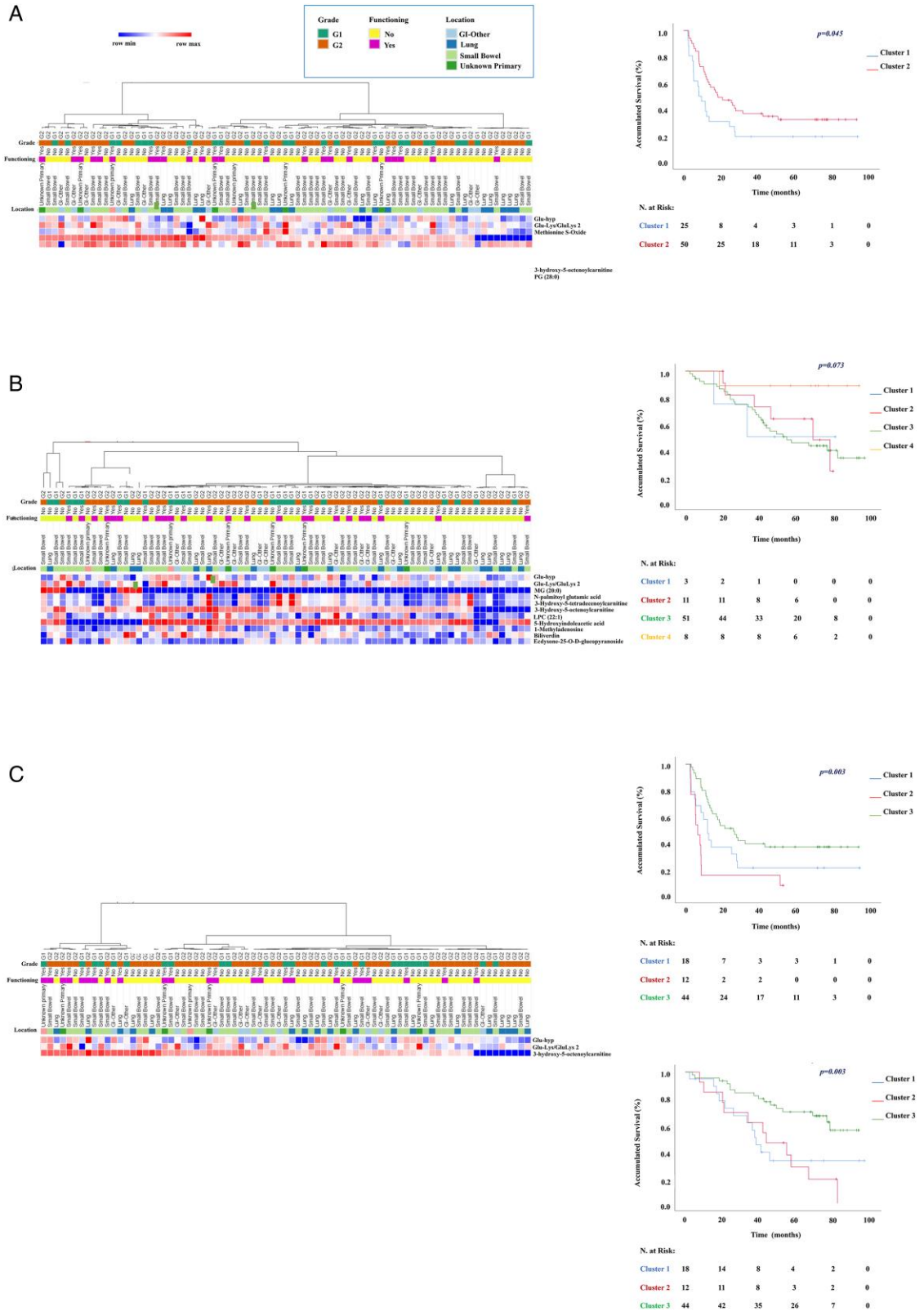

Figure 3. Selected metabolites that define prognostic subgroups of NET patients. Unsupervised hierarchical heatmaps of the 5 metabolites significantly associated with PFS (A), of the 11 metabolites significantly associated with OS (B), and of the 3 metabolites significantly associated with both PFS and OS (C). All samples ($n = 77$) are shown in columns and metabolites in rows. Hierarchical clustering was performed using One minus Pearson correlation metric and average as linkage method. Individual values are coded as colors, ranging from blue (row minimum) to red (row maximum). These heatmaps showed 2, 4, and 3 clusters, respectively, with a significant impact on survival represented by Kaplan-Meier curves. *P*-values of the corresponding univariant Cox regression analyses are reported.

Table 3. Multivariate analysis assessing the impact of the 3-metabolite cluster on PFS and OS.

Feature	PFS		OS	
	HR	*P*	HR	*P*
Metabolic cluster (3 metabolites)		.066		**.000**
Cluster 3 "good prognosis"	REF	—	REF	—
Cluster 1 "intermediate prognosis"	1.568	.197	2.901	**.001**
Cluster 2 "poor prognosis"	3.719	**.001**	3.962	**.021**
Gender (male vs female)	0.626	.132	0.269	**.001**
Age	1.017	.230	1.024	.127
Primary tumor location		.236		.514
Small bowel	REF	—	REF	—
GI other	1.453	.085	1.879	.154
Lung	1.685	.159	1.134	.498
Unknown primary	1.768	.225	0.968	.244
Other	4.548	.300	3.648	.163
Grade (G2 vs G1)	1.209	.551	1.092	.813
Functioning (no vs yes)	0.851	.625	1.332	.475
Time from randomization to study entry	1.005	.195	0.996	.372
Treatment arm (placebo vs axitinib)	0.754	.367	1.016	.964

P-values ≤ .05 were highlated in bold. Abbreviations: G1, grade 1; G2, grade 2; OS, overall survival; PFS, progression-free survival.

An aberrant porphyrin metabolism has been demonstrated in several tumor types[37,38] and is associated with oxidative stress, lipid peroxidation and mitochondrial disfunction,[39,40] with a deep effect on cell survival and cell growth.[41] Specifically, recent evidence has suggested an antiproliferative effect of porphyrins in NETs.[42,43] Two of the prognostic metabolites in our study, Glu-Hyp (glutamyl-hydroxyproline) and biliverdin, are associated with porphyrin metabolism. A higher abundance of Glu-Hyp was associated with worse survival in our cohort. Biliverdin is a heme derivative that is converted to the powerful antioxidant molecule bilirubin by biliverdin reductase-A (BVR-A). Up-regulation of BVR-A occurs as an adaptative response to oxidative stress and inflammation.[44] Therefore, BVR-A has been hypothesized to have a cytoprotective activity.[45] The conversion of biliverdin to bilirubin by BVR has been demonstrated to determine cell protection, due to direct and indirect antioxidant actions of bilirubin.[46] In our study, high levels of biliverdin were associated with worse survival, indirectly suggesting a protective role for BVR.

A second dysregulated pathway identified by the functional analyses in our study was methionine metabolism. Besides its essential role in protein synthesis, methionine is also involved in epigenetic regulation, nucleotide biosynthesis, cell detoxification (glutathione), membrane lipid homeostasis, and several other signaling pathways controlled by methylation. The role of this pathway in the metabolic shift of cancer cells is well established.[47] Interesting preclinical data from breast cancer show that the reduction of methionine sulfoxide reductase A (MsrA), an enzyme responsible for reversing the oxidation of methionine, results in an increase in cell proliferation and extracellular matrix degradation, leading to a more aggressive cellular phenotype both in vivo and in vitro.[48] In accordance with this, we found that higher levels of methionine S-oxide, the oxide derivative of methionine, correlated with a poor outcome of NET patients, suggesting that the regulation of methionine availability could be a useful strategy in limiting cancer growth.[47] Methionine S-oxide has been described as a biomarker of many relevant biological processes such as oxidative stress,[49] inflammation,[50] necrosis,[51] and hypoxia,[52] and has been implicated in a wide spectrum of human diseases including cancer.[53]

Tryptophan metabolism, the third main dysregulated pathway identified in our cohort, has been shown to be involved in different types of cancer.[54] The enzyme indoleamine-2,3-dioxygenase (IDO), expressed in tumor cells or antigen-presenting cells, has a fundamental role in tryptophan catabolism and has been identified as an essential suppressor of antitumor immune responses through tryptophan depletion and accumulation of immunosuppressive tryptophan catabolites. This has been documented in several types of gastrointestinal malignancies,[55] clear cell renal cell carcinoma,[56] and gynecological cancers.[57] More specifically, tryptophan metabolism plays a crucial role in NETs. Serotonin (5-hydroxytryptamine, 5-HT), a biogenic monoamine, is the most common metabolically active substance in functioning NETs and is produced from the essential amino acid tryptophan.[58] Excess tumor production and secretion of serotonin cause carcinoid syndrome (CS), characterized by flushing and diarrhea, and—less commonly—carcinoid heart disease, bronchoconstriction, and pellagra. Approximately 20% of NET patients present CS, most commonly those with small intestinal NETs, and this has a negative impact on patient outcomes and quality of life.[59] The metabolism of tryptophan is altered in patients with NET and CS, with approximately 60% of all dietary tryptophan being consumed by tumor cells for serotonin synthesis.[60] 5-Hydroxyindoleacetic acid is the primary metabolite of serotonin degradation in the liver, and its quantitation in the urine is the most sensitive and specific test for confirming the diagnosis of CS. Higher levels and doubling time of 5-HIAA in NET patients have been associated with a higher risk of progression and mortality.[61–63] Consistent with these observations, in our study, higher levels of 5-HIAA were associated with a worse prognosis. Moreover, higher values of MG (20:0), which have been shown to induce serotonin secretion in preclinical models,[64] were also associated with a worse prognosis in our series.

To date, only 2 pilot studies have investigated the metabolomic profiles of NET patients, but neither used plasma as the primary source for metabolic analysis. One examined urine sample from 28 gastroenteropancreatic NETs using 1H-NMR spectroscopy. This study identified distinct metabolomic phenotypes based on the primary tumor site and function and observed that hippurate metabolism played a significant role in class description. However, study limitations included a small sample size, a substantial age gap between control and tumor populations, and a lack of control for other confounding variables (such as gender, renal function, or concomitant drug therapy). The second study evaluated the metabolomic fingerprints of 46 small intestinal NET primary tumors and 18 liver NET metastases using 1H-NMR spectroscopy, compared to 30 normal small intestine and liver samples. Results suggested alterations in crucial metabolic pathways, such as the TCA cycle.[65,66]

One of the strengths of our study is that it was performed in prospectively collected and analyzed plasma samples from a homogeneous population of 77 patients with G1-2 advanced, uniformly treated, extra-pancreatic NETs. The identification of some metabolites (arginine, glutamine, phenylalanine, among others) across more than 1 analytical platform, and their validation through a target analysis with a different analytical platform in the same cohort,[15] significantly increases the confidence of metabolite identification. Furthermore, robust statistical analysis allowed the reliable identification of 13 metabolites with a significant impact on PFS and/or OS,

independent of other well-established clinical and pathological prognostic factors. This metabolic profile enables the stratification of patients into 3 distinct prognostic groups, to further assist physicians in clinical decision-making.

Metabolic profiling of plasma offers the advantage of dynamically characterizing disease biology. This can be harnessed not only for diagnostic purposes but also for targeted prevention or screening, personalized treatment strategies, therapeutic monitoring, and prediction of patient outcomes. To support this approach, our results should be validated in larger independent patient cohorts including NETs of other primary sites (ie, pancreatic NETs) and patients with exocrine tumors of similar tissue origin. In addition, a comprehensive understanding of the underlying mechanisms in NET development and progression requires complementary *-omic* approaches, such as exome, transcriptome, or methylome analysis of patients. Integrating the metabolomic profile with other plasma-based analytical approaches, such as cell-free nucleic acids profiling, could be particularly beneficial for early diagnosis and patient stratification toward personalized clinical management.

Conclusions

This study is, to our knowledge, the most comprehensive metabolic profiling study performed to date in NETs. Our results demonstrate that NET patients have a distinct metabolomic profile, providing new relevant information on disease biology for potential clinical application. Indeed, we have identified a metabolomic signature that improves the prognostic stratification of patients beyond classical prognostic factors for clinical decisions. In addition, identification of new enriched metabolic pathways may open innovative avenues of clinical research that foster the development of novel therapeutic strategies. Nevertheless, further independent studies are needed to confirm our results and validate these encouraging data.

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Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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Conflict of interest: J.C. has provided scientific advice and/or received honoraria or funding for continuous medical education from AAA, Advanz Pharma, Amgen, Bayer, Esteve, Hutchmed, Ipsen, Merck, Novartis, Roche, Sanofi, Lilly, Eisai, Bayer, and ITM, and has received research support from Pfizer, Novartis, Astrazeneca, Eisai, AAA, Amgen, Bayer, Roche, Gilead, and ITM. M.B. reports Advisory from Novartis, Ipsen, and Pfizer. P.J.-F. has received speaker honoraria or consultant honoraria from Adacap, Astellas, BMS, Lilly, and MSD. D.C. has provided scientific advice and/or received honoraria or funding for continuous medical education from Janssen Oncology, Roche/Genetech, Astellas Pharma, AstraZeneca, Pfizer, Novartis, Ipsen, BMS, MSD, Bayer, Lilly, Sanofi, Pierre Fabre, and Boerhinger. F.S. has received honoraria for medical education and research activity from Ipsen, Novartis, Pfizer, Advanced Accelerator Applications, MSD/Merck, and Hutchmed. A.T. has provided scientific advice and/or received honoraria from ADACAP, Ipsen, Novartis, and Pfizer. R.G.-C. has provided scientific advice and/or received honoraria or funding for continuous medical education from ADACAP, Advanz Pharma, Amgen, Bayer, BMS, Boerhinger, Esteve, Hutchmed, Ipsen, Merck, Midatech Pharma, MSD, Novartis, PharmaMar, Pierre Fabre, Roche, Servier, and Sanofi, and has received research support from Pfizer, BMS, and MSD. The remaining authors declare no conflicts of interest.

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Data availability

The data presented in this study are available from the corresponding author upon reasonable request.

Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of Hospital Universitario 12 de Octubre (Madrid, Spain) (protocol code 19/159, date of approval June 25, 2019).

Informed consent statement

Written informed consent was provided by patients prior to study participation.

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