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Increased Activity of the Immunoregulatory Enzyme Indoleamine-2,3-Dioxygenase with Consecutive Tryptophan Depletion Predicts Death in Patients with Neuroendocrine Neoplasia

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Key Words
Indoleamine-2,3-dioxygenase 1 · Inflammation · Tryptophan metabolites · Catabolites · Sepsis · Immune system · Biomarker

Abstract
Background/Aims: Data from a considerable number of malignancies demonstrate that depletion of the essential amino acid tryptophan via induction of the immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO) serves as an important tumour escape strategy and is of prognostic importance. Here we investigate the predictive value of the activity of IDO as well as levels of tryptophan and respective downstream catabolites in a large cohort of patients with neuroendocrine neoplasms (NEN). ***Methods:*** 142 consecutive Caucasian patients (62 male, aged 60.3 ± 11.9 years) with histologically confirmed NEN were systematically analysed in a retrospective blinded end point analysis. Patients were followed up for a mean period of about 3.9 ± 1.9 years. Clinical outcome, levels of established biomarkers, and tryptophan degradation markers (assessed using tandem mass spectrometry) including estimated IDO activity were recorded. Cox proportional hazards regression models were performed for the assessment of prognostic power. ***Results:*** We found that baseline tryptophan levels were significantly lower and IDO activity was significantly increased in non-survivors. The risk for death inclined stepwise and was highest in patients in the upper tertile of IDO activity. Cox proportional regression models identified IDO activity as an independent predictor of death. ***Conclusions:*** In this retrospective analysis, we observed that baseline activity of the immunoregulatory enzyme IDO was significantly increased in non-survivors. IDO activity was identified as an independent predictor of death in this cohort of NEN patients. Whether IDO activity or tryptophan depletion serves to guide future therapeutic interventions in NEN remains to be established.

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Introduction

Neuroendocrine neoplasms (NEN) are rare epithelial neoplasms most often located in the gastro-enteropancreatic system [1]. Although the initial diagnosis is often established in advanced, i.e. metastatic, stages of the disease, most affected patients have increased survival times when compared to other solid malignancies [2, 3]. This may at least partially be explained by the respective distinct tumour biology that is often characterized by reduced tumour growth [4]. Nevertheless, although survival rates may generally be favourable, a considerable biological heterogeneity can be noted. From a clinical perspective, however, characterization of disease status and assessment of therapeutic efficacy seems pivotal. This underlines the need for novel prognostic markers.

For the assessment of prognosis and guidance of NEN therapy, few prognostic indices were previously identified. Currently established prognostic indices include tumour size and localization, AJCC/UICC TNM classification (i.e. staging), grading based on the proliferative index (PI) via Ki-67 assessment (WHO classification), and, to a lesser extent, serum chromogranin A levels [5, 6]. Nevertheless, the prognostic power of respective indices varies between studies, which may be due to heterogeneous study populations and differing baseline definitions or classifications. The PI is currently regarded as the gold standard for the assessment of prognosis, was shown to independently predict prognosis, and is used for therapeutic stratification. However, it is assessed by immunohistochemical staining of nuclear antigen Ki-67 and requires invasive tumour tissue acquisition [7]. In fact, assessment of the PI (i.e. Ki-67) usually requires needle biopsy or surgical specimens. From a clinical perspective, this implies a major limitation to this gold standard, as most often it cannot be followed up on a routine repetitive pattern and thus allows only limited assessment of the underlying disease kinetics or response to therapy. Therefore, due to the limited availability of prognostic markers and/or the need for invasive assessment, the identification of non-invasive prognostic biomarkers constitutes a high priority.

Besides the necessity of non-invasive prognostic (bio-) markers, further investigations on the underlying molecular mechanisms and pathways for the development of new therapeutic strategies in NEN are needed. When considering novel diagnostic or therapeutic approaches for NEN, it seems important to note that only few data are available on the immune response to NEN. This seems of particular interest as recent data indicate that immunotherapy for NEN, e.g. via targeting of cytotoxic T-lymphocyte antigen 4 or programmed death 1 signalling pathways, may hold promise and may open up novel therapeutic avenues [8].

Tryptophan (Trp) is an essential amino acid in humans and is important for several reasons, including the fact that Trp is catabolized to key neuroendocrine mediators (e.g. serotonin). Moreover, Trp contributes to cellular energy metabolism and regulates local and systemic cell-mediated immunity [9]. Data show that >95% of Trp is catabolized via the conserved 'kynurenine (Kyn) pathway' by induction of the rate-limiting immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO) (fig. 1). IDO induction leads to the generation of a group of catabolites collectively referred to as 'kynurenines' [9]. These catabolites are well known to suppress T-cell function and to induce apoptosis of T-cells [10, 11]. In addition, Trp depletion activates stress mechanisms, e.g. via the kinase general control non-derepressible 2 kinase. This augments T-cell immunosuppressive signalling by induction of T-cell G1 cycle arrest and Fas-mediated apoptosis [12, 13]. Overall, IDO-induced Trp depletion triggers an immunosuppressive environment via depletion, anergy, and apoptosis of T-cells. Thus, IDO-induction plays an important role in the development of immunological tolerance and IDO-induced immunosuppression is used by solid malignancies to protect themselves from immune recognition and cytotoxicity – a fact that is recognized as a key tumour escape mechanism [9, 10, 13–20].

In several solid malignancies and in severe infection, IDO induction serves as a biomarker in the monitoring of disease activity/response to therapy and is associated with an adverse prognosis [15, 17, 19–23, 47]. Thus, induced IDO expression was proposed a novel prognostic indicator in immunohistological studies [21, 24, 25]. As studies on Trp catabolism in neuroendocrine tumours are currently unavailable, we embarked to investigate the role of Trp catabolism and IDO induction in a large cohort of patients with NEN.

Patients and Methods

Study Population and Assessment of Clinical Data

Patient charts of a total of 145 Caucasian patients [62 male, median age 63.5 years (range 23–83)] with histologically confirmed neuroendocrine malignancies consecutively treated between April 2009 until June 2010 at a tertiary care academic centre (Charité, Universitätsmedizin Berlin, Berlin, Germany) were reviewed. Outcome data from 3 (i.e. 2%) of the initial cohort were unavailable

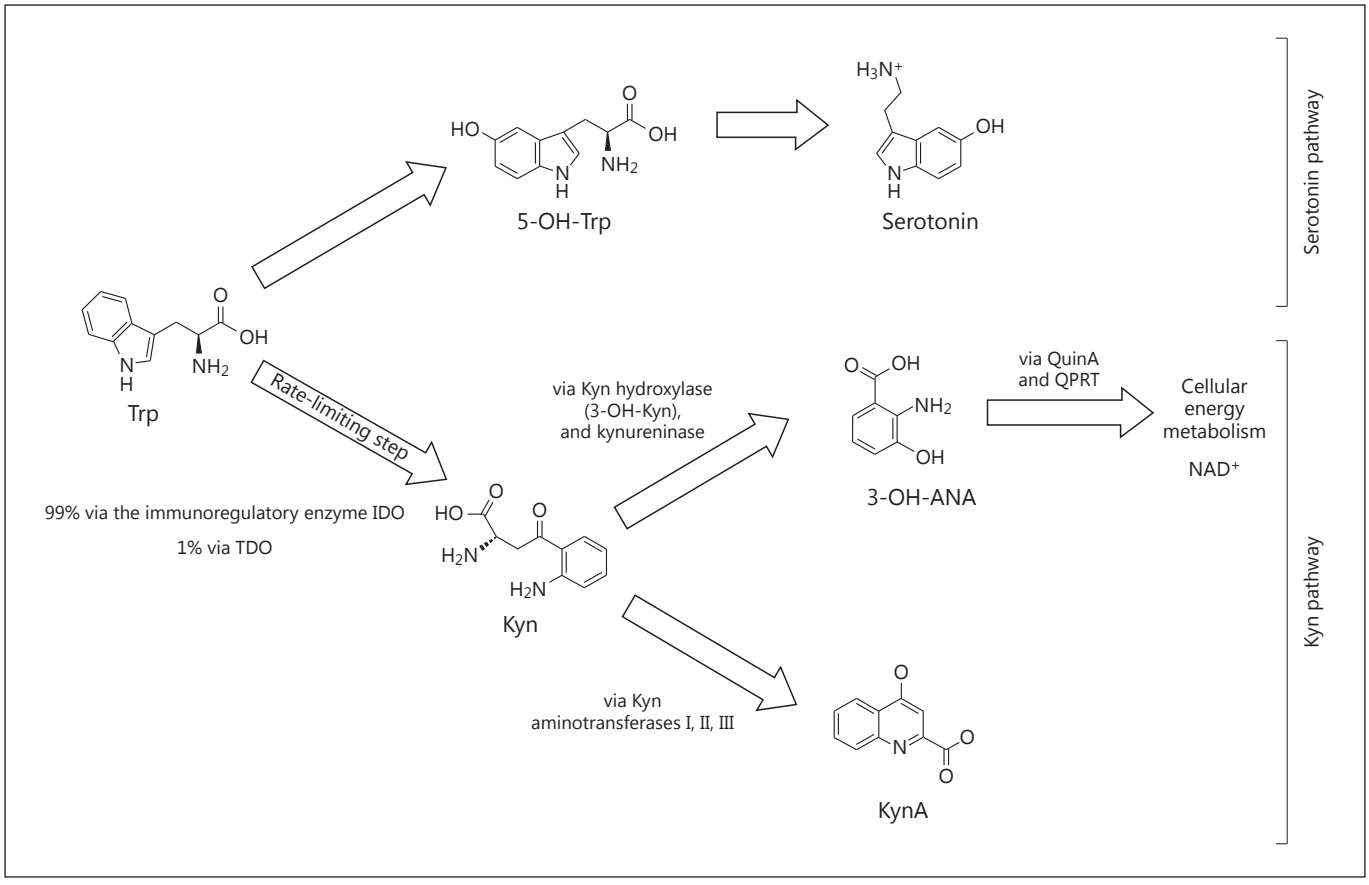

Fig. 1. Overview on Trp catabolism via the serotonin and Kyn pathways. QPRT = Quinolinatephosphoribosyltransferase; NAD^+ = nicotinamide adenosine dinucleotide.

and did thus not enter respective analyses. A retrospective blinded end point analysis design applied. Data recorded at baseline contained, among others, demographic data, date of initial diagnosis, localization of the primary site of cancer, staging (i.e. AJCC/UICC TNM classifications), grading (i.e. proliferation index measured by Ki-67 staining), biomarkers (e.g. Trp catabolism and serum chromogranin A expression in a limited number of patients), tumour functionality (based on clinical symptoms), results of imaging studies and surgical procedures, as well as clinical outcomes. The study was performed in accordance with the Declaration of Helsinki and written informed consent was achieved.

Primary tumour localizations were confirmed by endoscopy, surgery, and/or conclusive imaging studies including computed tomography (CT), magnetic resonance imaging (MRI) and somatostatin receptor imaging. Respective histological diagnoses were based on conventional haematoxylin-eosin staining and immunohistochemistry for neuroendocrine markers. Histological typing of endocrine tumours was applied by using the 2010 WHO classification of gastro-enteropancreatic NEN [26, 27]. CT and MRI were available for all patients. Tumour response evaluation was performed by an independent experienced radiologist. Response to treatment was measured according to the Response Evaluation Criteria in Solid Tumors (RECIST, version 1.0).

Histological Assessment of Tumour Characteristics and Ki-67 Expression

The initial histopathological diagnosis of NEN was established in our tertiary care academic centre in 127 (i.e. 89%) patients. In the remaining 15 patients, the histopathological diagnosis was established in external referring institutions. Histological differentiation grade, immunohistochemistry, mitotic index, and the Ki-67 labelling index were examined, as described previously [6, 27, 28].

Assessment of Blood Samples and Biomarkers for NEN

Blood samples were obtained at baseline using a standard phlebotomy protocol following informed consent. Samples were centrifuged (20,000 *g* for 5 min), and aliquots were stored at –80°C until assessment.

Assessment of Serum Chromogranin A Levels

Chromogranin A serum levels were assessed using a standardized and validated technique in the accredited lab of the Charité, Universitätsmedizin Berlin, Berlin, Germany. A commercially available radioimmunoassay was used for assessment (CisBio, Codolet, France). Limits of normal for this essay were 19–150 µg/l. Serum levels of chromogranin A were available only in 74% of study patients within 90 days from baseline.

Table 1. Patient demographics and prognosis-relevant indices

	Total cohort	Survivors	Non-survivors	Between-subgroup p value
Total number of subjects	142 (100%)	85 (59%)	57 (39%)	–
Male	61 (43%)	35 (25%)	26 (18%)	0.43
Age, years	60.3±11.9	58.3±11.4	63.4±12.0	0.01
Mean follow-up time, days	1,457.1±717.4	1,844.0±479.1	880.1±620.8	<0.0001
Primary origin of malignancy				0.03
Small intestine	58 (41%)	40 (28%)	18 (13%)	–
Pancreatic	37 (26%)	14 (10%)	23 (16%)	–
Duodenal	10 (7%)	10 (7%)	0 (%)	–
Cancer of unknown primary	9 (6%)	4 (3%)	5 (3%)	–
Others	28 (20%)	17 (12%)	11 (8%)	–
Tumor-related symptoms (diarrhoea, flushing, or both)	45 (32%)	25 (18%)	20 (14%)	0.67
Grading based on WHO 2010				0.0007
G1	56 (39%)	43 (30%)	13 (9%)	–
G2	72 (51%)	40 (28%)	32 (23%)	–
G3	14 (10%)	2 (1%)	12 (9%)	–
UICC staging (2009)				0.0006
Stage I	15 (11%)	15 (11%)	0 (0%)	–
Stage II	3 (2%)	2 (1%)	1 (1%)	–
Stage III	20 (14%)	18 (13%)	2 (1%)	–
Stage IV	104 (73%)	50 (35%)	54 (38%)	–
Baseline chromogranin A serum levels (missing data in 25.5% of cases)	1,312.0±5,838.7	1,319.9±7,522.4	1,352.5±2,382.6	0.98
Therapy				0.0001
Complete resection	30 (21%)	28 (20%)	2 (1%)	–
'Wait and watch'	36 (25%)	26 (18%)	10 (7%)	–
Somatostatin analogue	44 (30%)	22 (15%)	22 (15%)	–
Chemotherapy	20 (14%)	3 (2%)	17 (12%)	–
Targeted therapy	6 (4%)	2 (1%)	4 (3%)	–
Other	5 (4%)	4 (3%)	1 (1%)	–
Unknown	1 (1%)	0 (0%)	1 (1%)	–
Disease progression during observational period	77 (54%)	38 (27%)	39 (27%)	0.96
Progression-free survival time, days	1,699.4±585.5	1,749.9±551.5	975.7±696.8	
Survival time/time until death, primary site of cancer, days				
Small intestine	1,655.6±620.4	1,970.8±251.5	955.3±626.3	<0.0001
Pancreas	1,285.8±729.7	1,755.5±608.1	998.3±652.6	0.0013
Others	1,347.7±773.5	1,720.4±599.5	625.5±521.8	<0.0001

Means ± SD, independent-samples t test and χ^2 test, as appropriate.

Assessment of Trp Catabolism and Estimated IDO Activity

In addition to the assessment of established (bio-)markers for NEN, the assessment of Trp and Trp catabolites (an overview on Trp catabolism is provided in fig. 1) was performed from aliquots of plasma samples drawn at baseline. Respective samples were analysed for Trp and major Trp breakdown products in a quantitative fashion using a tandem mass spectrometry technique. The general approach used here was in accordance to the procedure proposed by Zhu et al. [29]. Estimated IDO activity was calculated as Kyn × 100/Trp ratio, as demonstrated earlier [30].

In detail, for performing mass spectrometry measurements, commercially available Trp, Kyn, Kyn acid (KynA), quinolinic acid (QuinA), 5-hydroxy Trp (OH-Trp), 3-hydroxy anthranilic acid (3-OH ANA), serotonin, phenylalanine (Phe) (all Sigma-Aldrich, St. Louis, Mo., USA) deuterium-labelled compounds Kyn-d_6, KynA-d_5, Phe-d_5, Trp-d_5 (all Cambridge Isotope Laboratories, Andover, Mass., USA), water (Optima MS grade; Fisher Scientific, Waltham, Mass., USA) and acetonitrile (Optima grade; Fisher Scientific) were used. Frozen samples were thawed prior to analysis. One hundred microlitres of heparinized plasma was added to a deuterated internal standard mixture (50 µl) of equal volumes of Kyn-d_6, KynA-d_5, Phe-d_5, and Trp-d_5. After shaking the solution for 2 min, 500 µl acetonitrile was added and left over night at –20°C to precipitate the protein. Samples were centrifuged (20,000 *g*, 10 min), and the supernatant was dried under vacuum centrifugation (Savant SpeedVac Plus SC210A and Savant Refrigerated

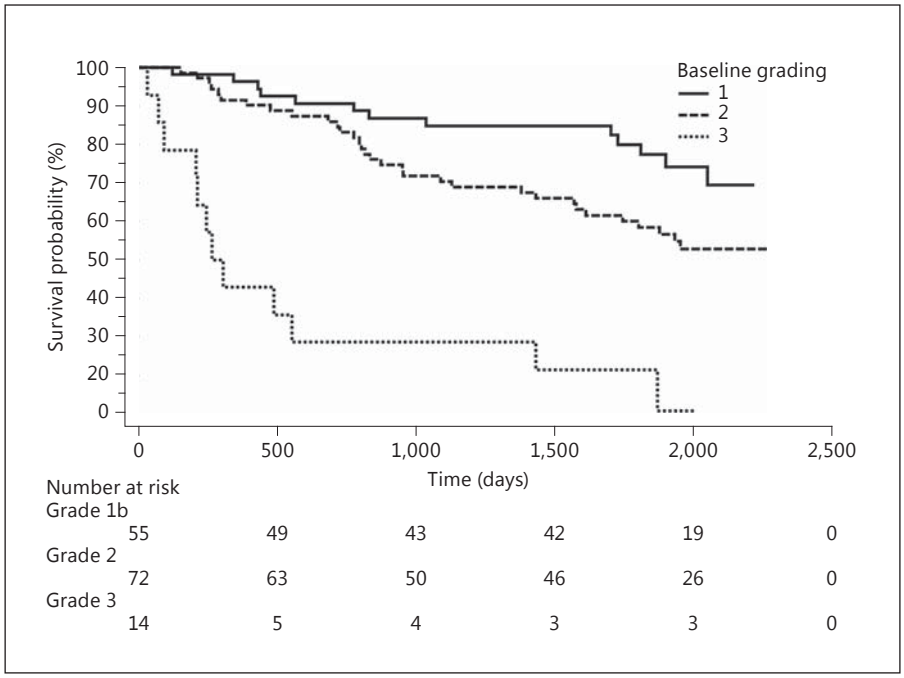

Fig. 2. Kaplan Meier survival estimates of NEN patients grouped for grading (Ki-67 expression) at baseline. Follow-up time and respective numbers at risk are given. Overall model: $\chi^2 = 39.5$, $p < 0.0001$ (log rank).

Vapor Trap RVT 4104). Dried samples were reconstituted with 100 µl H_2O/acetonitrile (95%/5%). A Waters Acquity UPLC-TQD system (Milford, Mass., USA) was equipped with an electrospray ion source using MRM detection in a positive ion mode. The following transitions of mass-to-charge ratios (*m/z*) of 205/188 for Trp, 210/193 for Trp-d_5, 209/192 for Kyn, 215/198 for Kyn-d_6, 168/150 for QuinA, 190/144 for KynA, 195/149 for KynA-d_5, 154/136 for 3HAA, 177/160 for serotonin, 221/204 for OH-Trp, 166/120 for Phe, and 171/125 for Phe-d_5 were detected using argon as a collision gas. For separation of analytes, an Acquity UPLC BEH C18 column (1.7 µm, 100 mm) was used. A gradient of water/acetonitrile was utilized starting at a ratio of 97/3 and ramping up to a ratio of 70/30 in 5 min using a flow rate of 0.35 ml/min. For quantification, calibration curves were performed referring the analytes to appropriate deuterated standards. Calibration curves were fitted by linear least-square regression. Serum (ClinChek® Control, Recipe, Germany) with known Phe and Trp concentrations was used as quality control to ensure the accuracy and precision of both the sample preparation and the measurements produced by the UPLC-MS/MS.

Statistical Analysis

For statistical analysis, StatView 5.0 (SAS Institute, Cary, N.C., USA) and MedCalc 12.0 Software were used (MedCalc Software, Mariakerke, Belgium). For between-group comparisons, either the unpaired t test or the Mann-Whitney U test was used, as appropriate. Data are reported as means ± standard deviations or medians and interquartile range, if not indicated otherwise. All data were checked for normal distribution using the Kolmogorov-Smirnov test. In case of non-normally distributed data, log transformation was performed. Sensitivity/specificity analyses were performed and ROC curves calculated. Between-ROC p values are given. For the assessment of prediction of non-survival, Cox proportional hazards regression models were calculated. Kaplan-Meier cumulative survival estimate curves were calculated for illustrative purposes (Mantel Haenszel log rank p values are given). A p value <0.05 was considered statistically significant.

Results

Characterization of the Study Cohort and Patient Demographics

Data from 142 Caucasian patients (mean age 60.3 years, interquartile range 50–70, range 23–83) with histologically confirmed NEN treated at our institution were analysed in a systematic fashion. In the overall cohort, the gender distribution was comparable (61 male, 43%). Main primary tumour localizations were small intestine (41%) and pancreas (26%). Full details are given in table 1. Metastases were observed in 73% of cases. Forty-five patients (i.e. 32%) were clinically considered to have functional tumours defined as patients with the following symptoms: diarrhoea not otherwise explained, skin flushing, or both. Primary localization of functional tumours was 62.2% ileum and 15.6% pancreas. The most frequent treatment strategies were complete surgical resection, 'wait and watch', or treatment with somatostatin receptor analogues (table 1).

Patients were followed up for a mean period of about 3.9 ± 1.9 years (table 1). The rate of all-cause mortality

Table 2. Trp degradation products and IDO activity at baseline (means ± SD)

	Total cohort	Survivors	Non-survivors	Between-subgroup p value
Trp, μmol/l	56.7±15.4	59.9±13.8	52.5±16.7	0.008
Kyn, μmol/l	2.69±1.21	2.53±1.02	2.93±1.42	0.049
KynA, μmol/l	0.043±0.029	0.038±0.016	0.051±0.039	0.007
3-OH ANA, μmol/l	0.03±0.017	0.03±0.015	0.04±0.02	0.16
Serotonin, μmol/l	2.77±3.13	2.78±3.31	2.77±2.87	0.96
IDO activity	5.10±3.24	4.34±1.85	6.23±4.38	0.0005

Table 3. Univariate and multivariate survival models in patients with NEN

Variable	HR	95% CI	p value	χ^2
Single-predictor model for non-survival				
Age (per 1-year increase)	1.026	1.002–1.051	0.031	4.65
Gender (male)	1.045	0.620–1.760	0.87	0.03
UICC stadium (per 1 increase)	3.639	1.486–8.912	0.005	7.98
Ki-67 expression (per 1 SD increase)	2.055	1.574–2.685	<0.0001	27.96
IDO activity (per 1 SD increase)	1.704	1.363–2.132	<0.0001	21.81
Trp levels (per 1 SD increase)	0.660	0.529–0.824	0.0002	13.521
Chromogranin A levels (per 1 SD increase)	1.487	1.184–1.867	0.0006	11.68
Multivariable model for non-survival				
IDO activity (per 1 SD increase)[1]	1.591	1.250–2.025	0.0002	14.240

[1] After adjustment for age, gender, UICC stadium, and Ki-67 expression.

during the observational interval was 39%. Importantly, the cause of death was considered to be associated with NEN disease (i.e. liver or respiratory failure due to NEN metastases, tumour-induced cachexia or infection) in all patients. We observed that the overall time of survival was dependent on the primary tumour site (table 1), as previously demonstrated [3]. Grading (G1, G2, G3) based on PI Ki-67 (ENETS guidelines, WHO classification 2010) was significantly correlated to the risk of death from NEN (table 1). After grouping of patients in upper, middle, and lower tertiles of Ki-67 levels (i.e. grouping for grading), the following hazard ratios (HR) for non-survival applied (fig. 2): (1) subgroup of patients with G2 tumours: HR 1.98 (95% CI 1.16–3.38), and (2) subgroup of patients with G3 tumours: HR 8.57 (95% CI 2.39–30.73).

Progressive disease was identified in 17 patients; 12 patients (8%) died within the mean follow-up time. During follow-up, progressive disease was noted in 54% of patients (n = 77). At censor date, 27% (n = 39) of patients within the subgroup of patients with progressive disease died. Progression-free survival times are given in table 1.

Baseline Levels of Trp, Trp Catabolites Including Serotonin, and Estimated IDO Activity in Surviving versus Non-Surviving Patients

Levels of Trp were significantly lower (p = 0.008), whereas Kyn and KynA levels were significantly increased in non-surviving patients (p = 0.049 and 0.007, respectively, table 2). Estimated IDO activity was significantly higher in non-survivors when compared to surviving patients (6.23 ± 4.38 vs. 4.34 ± 1.85, respectively; p = 0.0005). Serotonin levels and levels of 3-OH ANA did not statistically differ between survivors and non-survivors. Full details are given in table 2.

Moreover, we analysed the subgroup of patients with (n = 45) versus without (n = 100) functional tumour syndrome (defined as diarrhoea not otherwise explained, skin flushing, or both). When compared to patients without functional syndrome, higher mean serotonin levels were observed in patients with tumour-related (i.e. 'functional') symptoms such as e.g. diarrhoea or flushing (4.05 ± 3.76 vs. 2.18 ± 2.61 μmol/l, p = 0.0007; please also compare to the total cohort data presented in table 2). In the

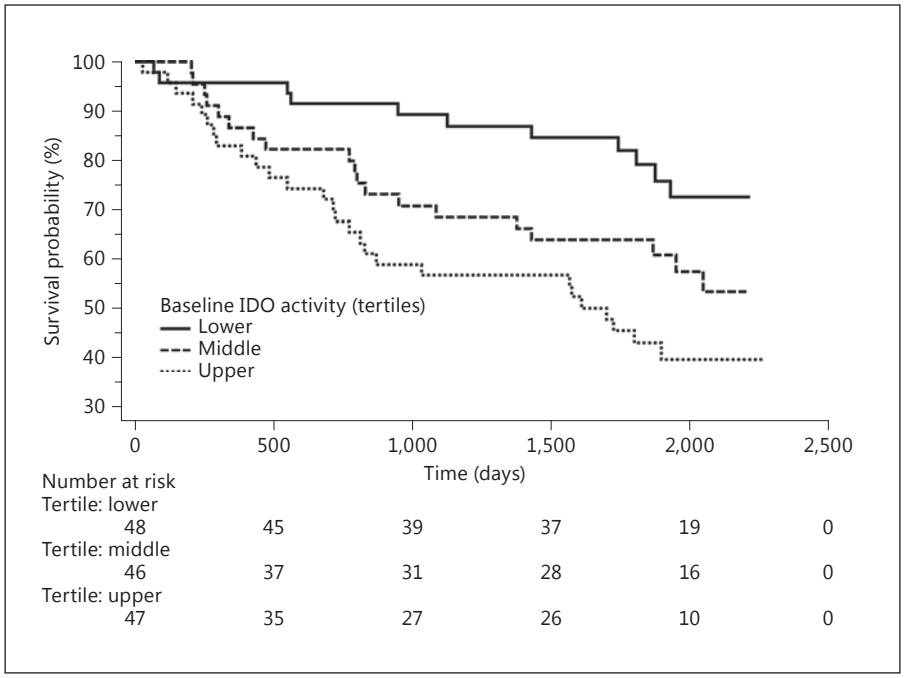

Fig. 3. Kaplan Meier survival estimates of NEN patients grouped for baseline IDO activity (tertiles). Follow-up time and respective numbers at risk are given. Overall model: $\chi^2 = 12.2$, $p = 0.0022$ (log rank).

subgroup of patients with functional syndrome, statistically significant differences were not noted with regard to Trp, Kyn, KynA, 3-OH ANA, or IDO activity (all $p > 0.19$). In addition, IDO activity was investigated in patients with metastatic NEN disease (UICC stage IV). In this subgroup of patients, IDO activity was significantly increased when non-surviving patients were compared to survivors (6.31 ± 4.46 vs. 4.55 ± 2.09, respectively, $p = 0.01$).

Outcome Prognostication in the NEN Cohort under Investigation

In an effort to investigate a potential association of Trp catabolites with clinical outcome, univariate and multivariate outcome models were performed. After adjusting for disease severity and demographics, Cox proportional hazards regression models identified IDO activity as an independent predictor of death in the cohort under investigation (table 3). In detail, the HR for non-survival of IDO activity was 1.59 (95% CI 1.25–2.03; $p = 0.0002$, $\chi^2 = 14.2$). Grouping of patients into upper, middle, and lower tertiles of IDO activity showed the following HRs for non-survival (fig. 3): for patients in the middle tertile, the respective HR was 2.05 (95% CI 1.11–3.81), and for patients in the upper tertile, the HR was 3.27 (95% CI 1.73–6.19). Thus, after grouping for tertiles of IDO activity, a stepwise increase in the risk of death was observed (fig. 3).

Moreover, sensitivity/specificity analyses for non-survival were performed in order to calculate respective AUCs for IDO and Ki-67. ROC analyses demonstrated that the sensitivity/specificity for non-survival were comparable between the two indices. In detail, the following AUCs applied: IDO activity 0.69 (0.61–0.77) and Ki-67 0.72 (0.63–0.79); $p = 0.71$ (between ROCs).

Discussion

Identification of an effective biomarker set and demonstration of a potential novel therapeutically relevant pathway constitutes a high priority in NEN disease. In this study, we investigated the Trp catabolic pathway including IDO activity with regard to its prognostic power in a large cohort of patients with neuroendocrine neoplasms. After inclusion and analysis of 142 patients, we found that estimated IDO activity was significantly higher in non-surviving patients. Cox proportional hazard regression models identified IDO activity as an independent predictor for non-survival in a general population of NEN patients. The power of IDO activity to predict death was comparable to the established (invasive) prognostic marker of histological Ki-67 expression. From a clinical perspective, the subgroup of metastatic NEN disease (UICC stage IV) might benefit most from assessment of

IDO activity, because a change in the treatment regimen often goes along with a need for new PI assessment and thus with an invasive biopsy. In conclusion, assessment of estimated IDO activity may serve as a novel non-invasive biomarker in NEN disease. Nevertheless, whether or not estimated IDO activity serves to guide therapeutic interventions in NEN remains to be established.

Malignant cells often express tumour-specific antigens that trigger immune recognition and consecutive T-cell-mediated destruction [31–33]. This 'tumour surveillance' is a host-protective measure, but it is well known that malignant cells developed escape strategies [31, 34]. The underlying molecular mechanisms are currently under intense research, and mounting data show that IDO plays a key role in tumour escape via T-cell modulation and induction of immunosuppression [19, 20, 25, 35]. Interestingly, early data from animal models suggest that treatment with IDO inhibitors enhances anti-tumour immune response and acts synergistically with major currently used therapeutic interventions [36]. However, to the best of our knowledge, Trp catabolism and systemic IDO activity was not investigated in neuroendocrine malignancies. Our data confirm previous studies of Trp catabolism in that IDO activity may be chronically induced in malignancies and that increased IDO activity is associated with more extensive disease [21, 37–39]. Nevertheless, although we are unable to conclude from our data that NEN may indeed employ tumour escape mechanisms involving IDO, it seems tempting to speculate that IDO induction and respective Trp depletion may indeed have a specific role in NEN progression. Further longitudinal clinical and experimental studies are thus warranted.

Our finding that higher IDO activity is associated with increased mortality is supported by the fact that, when compared to survivors, levels of downstream catabolites such as Kyn and KynA were significantly increased in non-survivors. These catabolites are well known to suppress T-cell proliferation, induce T-cell apoptosis, and negatively affect natural killer cell function [40–42].

Thus, when speculating on the underlying reasons for increased mortality in the cohort under investigation, the increased mortality in patients with higher IDO activity may theoretically also be due to downstream, rather than direct IDO-induced effects. In general, however, IDO levels in malignant cells correlate with increased metastasis and poor patient outcome, and IDO induction was previously linked to tumour cell resistance with regard to immunotherapy, radiation therapy, and chemotherapy [20]. Importantly, the molecular mechanisms by which these compounds exert their immunological effects are only partly understood. In general, IDO can be expressed directly by malignant cells or it can be induced via activation of tumour recognition pathways, e.g. in antigen-presenting cells. However, IDO expression itself induces an immunosuppressive milieu that prevents key anti-tumour cytotoxic immune mechanisms. Interestingly, IDO overexpression may mediate resistance to cancer immunotherapies including e.g. therapeutic approaches using immunomodulatory antibodies targeting cytotoxic T-lymphocyte-associated protein 4 or programmed cell death protein 1, compounds which could play key roles in future NEN therapeutic approaches [8, 43, 44]. Moreover, in a transgenic mouse model of breast cancer, IDO inhibition with 1-methyl Trp was combined with paclitaxel and resulted in tumour regression [45]. Currently, we associate circulating PlGF levels with increased IDO activity in a subset of NEN patients (data not shown). PlGF is a cytokine which modulates immune responses in tumours and is strongly correlated with disease progression in NENs [46]. Awaiting further evaluation, these data suggest interdependence between changes in Try metabolism and expression of growth factors, such as PlGF.

Importantly, our investigation has some limitations that require discussion. First, as mentioned before, the presented data demonstrate associations rather than causal relationships or mechanistic insights. Second, the sample size of our investigation is rather limited. This is due to the fact that NEN per se is a rather rare tumour entity and assessment of large NEN cohorts may be regarded difficult. Limitations in sample size, however, restricts the analysis of the present data to the overall study cohort and prevents meaningful investigations on survival effects in subgroups including analyses in patients with progression of NEN disease. Thus, further studies in larger cohorts are needed to evaluate the prognostic power with regard to clinical disease progression. Nevertheless, we are convinced that our observations derive from a typical cohort of NEN patients as indicated by primary tumour distribution, grading distribution, outcome data, and serotonin levels in carcinoid syndrome patients. Third, in the current analysis, the assessment of the activity and influence of IDO II or the hepatic enzyme Trp-2,3-dioxygenase (TDO) was beyond the scope of this analysis. Fourth, other metabolic pathways and drugs may theoretically interfere with the conversion of Trp to Kyn (e.g. steroids) in the clinical setting of NEN, and this may potentially influence our data. Although we believe that our cohort represents a typical cohort of NEN patients, further investigations should take effects induced by co-

medication and nutritional state into account. Fifth, co-existing medical conditions such as chronic kidney disease are associated with increased IDO activity and production of downstream catabolites [30]. As this was a retrospective investigation, we are by definition unable to clearly exclude a potential bias induced by co-existing morbidities. Sixth, baseline chromogranin A serum levels were not included in the multivariate model as a considerable amount of data was missing due to the retrospective nature of the analysis. Seventh, all-cause mortality was investigated in the present investigation only. We are thus technically unable to exclude that patients may have died e.g. from cardiovascular events, a fact that should be kept in mind when interpreting our data.

In conclusion, our data point to an important role of IDO and respective downstream products in advanced NEN. We speculate from our data that induction of IDO may serve as a tumour escape mechanism in NEN. Moreover, the assessment of IDO activity may hold promise in several ways in that it may serve as a prognostic marker, potential biomarker for specific assessment of respective tumour biology/tumour behaviour, as a biomarker to guide systemic therapies, or even as a potential therapeutic target. Thus, our data may provide the basis for more extensive prospective evaluations of Trp catabolism and IDO activity in NEN and we propose to consider including IDO activity into the portfolio of biomarkers in NEN trials.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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