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Research Article

Neuronal Distribution in Colorectal Cancer: Associations With Clinicopathological Parameters and Survival

Maartje Massen[a], Meike S. Thijssen[a,b], Glenn Rademakers[a], Musa Idris[a,c], Kim A.D. Wouters[a], Jaleesa R.M. van der Meer[a], Nikkie Buekers[a], Desirée Huijgen[a], Iryna V. Samarska[a], Matty P. Weijenberg[e], Piet A. van den Brandt[e], Manon van Engeland[a], Marion J. Gijbels[a,d], Werend Boesmans[a,b], Kim M. Smits[a], Veerle Melotte[a,c,*]

[a] *Department of Pathology, GROW—Research Institute for Oncology and Reproduction, Maastricht University Medical Center, Maastricht, The Netherlands;* [b] *Biomedical Research Institute, Hasselt University, Hasselt, Belgium;* [c] *Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands;* [d] *Department of Medical Biochemistry, Amsterdam Cardiovascular Sciences: Atherosclerosis & Ischemic Syndrome and Amsterdam Infection and Immunity: Inflammatory Diseases, Amsterdam University Medical Center Location, University of Amsterdam, Amsterdam, The Netherlands;* [e] *Department of Epidemiology, GROW—Research Institute for Oncology and Reproduction, Maastricht University Medical Center, Maastricht, The Netherlands*

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ABSTRACT

Over the past years, insights in the cancer neuroscience field increased rapidly, and a potential role for neurons in colorectal carcinogenesis has been recognized. However, knowledge on the neuronal distribution, subtypes, origin, and associations with clinicopathological characteristics in human studies is sparse. In this study, colorectal tumor tissues from the Netherlands Cohort Study on diet and cancer (n = 490) and an in-cohort validation population (n = 529) were immunohistochemically stained for the pan-neuronal markers neurofilament (NF) and protein gene product 9.5 (PGP9.5) to study the association between neuronal marker expression and clinicopathological characteristics. In addition, tumor and healthy colon tissues were stained for neuronal subtype markers, and their immunoreactivity in colorectal cancer (CRC) stroma was analyzed. NF-positive and PGP9.5-positive nerve fibers were found within the tumor stroma and mostly characterized by the neuronal subtype markers vasoactive intestinal peptide and neuronal nitric oxide synthase, suggesting that inhibitory neurons are the most prominent neuronal subtype in CRC. NF and PGP9.5 protein expression were not consistently associated with tumor stage, sublocation, differentiation grade, and median survival. NF immunoreactivity was associated with a worse CRC-specific survival in the study cohort ($P = .025$) independent of other prognostic factors (hazard ratio, 2.31; 95% CI, 1.33-4.03; $P = .003$), but these results were not observed in the in-cohort validation group. PGP9.5, in contrast, was associated with a worse CRC-specific survival in the in-cohort validation ($P = .046$) but not in the study population. This effect disappeared in multivariate analyses (hazard ratio, 0.81; 95% CI, 0.50-1.32; $P = .393$), indicating that this effect was dependent on other prognostic factors. This study demonstrates that the tumor stroma of CRC patients mainly harbors inhibitory neurons and that NF as a single marker is significantly associated with a poorer CRC-specific survival in the study cohort but necessitates future validation.

Introduction

The management of colorectal cancer (CRC) is challenging because of the dynamic environment of the gastrointestinal (GI)

These authors contributed equally to this work: Kim M. Smits and Veerle Melotte.
* Corresponding author.
E-mail address: veerle.melotte@maastrichtuniversity.nl (V. Melotte).

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tract and the intertumor and intratumor diversity.[1,2] The heterogeneity of CRC is also characterized by the tumor microenvironment (TME), which consists of a variation of cell types, blood vessels, the microbiome, and extracellular matrix components. Over the past years, it has been claimed that the nervous system should also be considered a TME component in cancer, including CRC.[3-5] Tumor innervation has been reported as a high-risk pathologic feature and a marker for poor disease outcomes in several cancer types.[3,6] The intestine contains a neural network unique to this organ: the enteric nervous system (ENS), which consists of >200 million neurons and a multitude of glial cells. Although the ENS is highly connected to the central nervous system, it can independently control GI homeostasis and orchestrate vital gut function, such as intestinal motility, secretion, and local blood flow by regulating the activity of other intestinal cell types.[7] In the past years, the role of nerves in CRC was mainly studied in the context of perineural invasion (PNI).[8,9] PNI is defined as the infiltration/spreading of tumor cells alongside the nerves surrounding the tumor. PNI in CRC is associated with more advanced and aggressive disease because it has been shown to occur predominantly in stage III/IV. Furthermore, PNI is associated with reduced 5-year disease-free, cancer-specific, and overall survival.[8,9] In addition to the structural basis of PNI, neurons have also been shown to play a more active role through bidirectional communication with cancer cells. Cancer cells induce neuronal sprouting and promote their own innervation. Nerve cells, in contrast, can stimulate tumor growth through the release of neurotransmitters and other messengers.[5,10,11] We recently demonstrated that enteric neurons communicate with cancer cells by the secretion of extracellular matrix proteins, thereby enhancing colorectal carcinogenesis.[11] However, the neuronal distribution, subtypes, origin, and associations with clinicopathological characteristics or the prognostic value of nerves in large human studies are still missing. Based on the mounting evidence indicating that neurons are important participants in CRC, we here investigated the neuronal subtype distribution and associations with clinicopathological characteristics in the colorectal TME.

Materials and Methods

Study Population and Tissue Samples

Formalin-fixed, paraffin-embedded CRC tissues from the Netherlands Cohort Study (NLCS) on diet and cancer were used for this study.[12] NLCS was initiated in 1986 with the inclusion of 120,852 healthy men and women, between 55 and 69 years old, who completed a self-administered questionnaire on diet, family history of cancer, and other risk factors at baseline. Cancer cases within the cohort were identified by annual record linkage to the Netherlands Cancer Registry and the "Pathologisch-Anatomisch Landelijk Geautomatiseerd Archief," a nationwide database of pathology reports,[12] covering 20.3-year follow-up. Follow-up for vital status of CRC patients was carried out through linkage to the Central Bureau of Genealogy and the municipal population registries. Cause of death was retrieved from Statistics Netherlands. Information regarding tumor localization, staging, differentiation grade, and incidence was made available through the Netherlands Cancer Registry.[13,14]

Tumor tissues of NLCS participants were collected in 2 phases. First, from 1989 to 1994, with the exclusion of the first 2.3-year follow-up, 867 identified cases were histologically confirmed (ICD-O: 153.0-154.1), and formalin-fixed, paraffin-embedded tumor tissues of 773 of these cases were collected from 54 pathology registries throughout the Netherlands after the approval of the Ethical Review Board of the Maastricht University Medical Center (MEC 85-012). For this study, a random subset of 490 patients from this first phase was selected using the Stata random sample command (Fig. 1, Table 1). To expand this initial tumor collection, tumor tissues of incident CRC cases diagnosed within a longer follow-up period (from the start of NLCS to 2007; n = 4597) were collected as part of the Rainbow-Tissue MicroArray project.[14,15] Tumor tissues were available for 2694 CRC cases. For this study, a random subset of 529 patients from the second phase was selected using the Stata random sample command (Fig. 1, Table 1). For the neuronal subtypes vasoactive intestinal protein (VIP) and neuronal nitric oxide synthase (nNOS), a random set of 50 cases from the study cohort described above was selected (Supplementary Table S1).

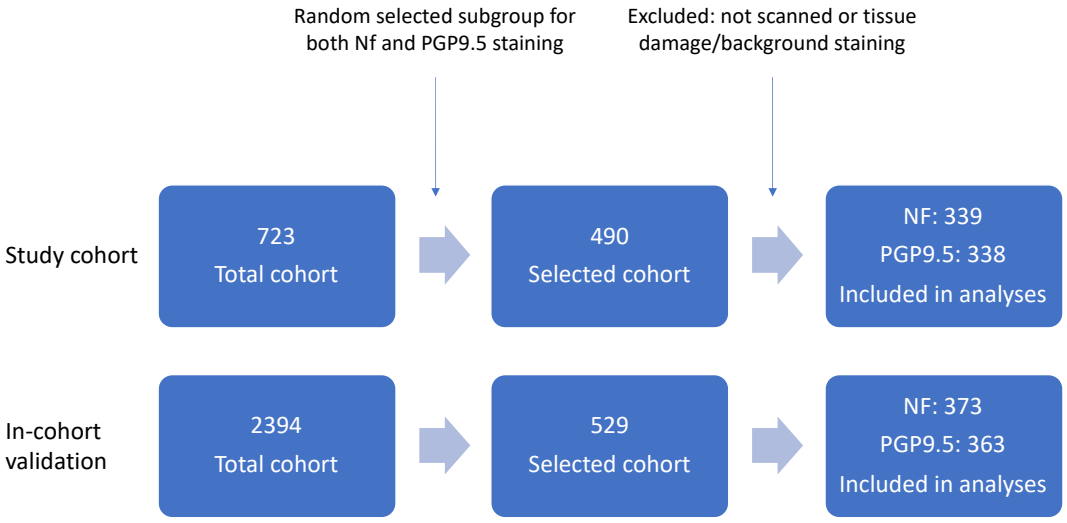

Figure 1.
Flowchart with patient sample numbers in the study cohort and in-cohort validation groups. NF, neurofilament; PGP9.5, protein gene product 9.5.

Table 1
Clinicopathological characteristics of the study cohort (subset NLCS) and the in-cohort validation with *P* values of the comparison of both populations

Patient demographics	Study cohort, n = 490, n (%)	In-cohort validation, n = 529, n (%)	*P*
Sex			
Male	280 (57.1)	283 (53.5)	
Female	210 (42.9)	246 (46.5)	.242
Age at diagnosis (y)			
Mean ± SD	68 ± 4.3	74 ± 5.9	<.0001
Cancer stage (TNM)			
Stage I	127 (27.9)	106 (20.0)	
Stage II	158 (33.6)	214 (40.5)	
Stage III	115 (24.4)	141 (26.7)	
Stage IV	71 (15.1)	59 (11.2)	.009
Sublocation			
Right proximal	149 (30.9)	188 (36.0)	
Left distal	160 (33.1)	182 (34.8)	
Rectosigmoid/rectum	174 (36.0)	153 (29.2)	.058
Differentiation grade			
Undifferentiated	6 (1.2)	1 (0.2)	
Poor	65 (13.3)	78 (14.7)	
Moderate	304 (62.0)	359 (67.9)	
Well	54 (11.0)	44 (8.3)	
Unknown	61 (12.5)	47 (8.9)	.030
CRC death			
Yes	201 (41.3)	205 (38.8)	
No	286 (58.7)	315 (59.5)	.550

CRC, colorectal cancer; NLCS, the Netherlands Cohort Study.

Moreover, 10 patients in whom colorectal tumor tissue and adjacent "healthy" tissue were collected were selected for neuronal subtype analysis. These samples were collected at the Maastricht University Medical Center from CRC resection material.

Immunohistochemistry

Colorectal tumor paraffin sections (3-4 μm) were deparaffinized in xylene, rehydrated, and incubated with 0.3% hydrogen peroxide in methanol for 20 minutes. Antigen retrieval was performed by boiling the sections in target retrieval solution (pH 6.0, Dako) for 20 minutes followed by blocking nonspecific antibody binding with phosphate buffered saline, 20% fetal bovine serum, and 0.1% Tween. Sections were incubated for 60 minutes with the primary antibodies (Table 2) diluted in phosphate buffered saline/1% bovine serum albumin/0.1% Tween. Subsequently, sections were incubated with Poly-HRP-GAM/R/R immunoglobulin (ready to use, VWR International) for 30 minutes and visualized using 3,3′-diaminobenzidine substrate (Dako; protein gene product 9.5 [PGP9.5] for 1 minute, neurofilament [NF] for 3 minutes, VIP for 30 seconds, nNOS for 30 seconds, choline acetyltransferase [ChAT] autostainer, serotonin [5-HT] for 1 minute, and tyrosine hydroxylase [TH] for 7 minutes) as a chromogen. Slides were counterstained with hematoxylin, dehydrated, and mounted.

Table 2
Primary antibody information and dilutions that were used for immunohistochemical staining

Antibody	Dilution	Antibody reference
ChAT	1:750	AMAb91129 (Atlas Antibodies)
TH	1:100	ab112 (Abcam)
nNOS	1:400	18984-1-AP (Proteintech)
5-HT	1:2000	20080 (Immunostar)
VIP	1:250	ab22736 (Abcam)
PGP9.5	1:200	Z5116 (Dako)
NF-H/L	1:50	M0762 (Dako)

5-HT, serotonin; ChAT, choline acetyltransferase; NF-H/L, neurofilament heavy/light chain; nNOS, neuronal nitric oxide synthase; PGP9.5, protein gene product 9.5; TH, tyrosine hydroxylase; VIP, vasoactive intestinal peptide.

Histologic Slide Imaging

Approximately 339 of 490 NF-stained slides (69.2%) and 338/490 of PGP9.5-stained slides (69.0%) could be used for further analyses in the study cohort (Fig. 1). Approximately 373 of 529 (70.5%) for NF and 363 of 529 (68.6%) for PGP9.5 were successfully stained for the in-cohort validation (Fig. 1). Slides were excluded in case of a lack of tumor material on the slide or heavy damage to the tissue. The slides were scanned at ×20 magnification with the Ventana iScan HT (Roche Diagnostics).[16] The digitized slides were then viewed using the Quantitative Pathology & Bioimage Analysis platform (QuPath).

Histologic Assessment

To compare the impact of different immunohistochemistry evaluation methodologies, we compared (1) random sampling vs (2) whole-slide evaluation in a subset of the study population (Supplementary Tables S2 and S3). For the histologic evaluation using random sampling, tumor tissue, including the nonepithelial tumor stroma, was manually outlined by 2 independent observers (M.M. and G.R.) under the supervision of an experienced GI pathologist (I.V.S.). Tumor regions that displayed significant damage or folds and noncancerous regions, including the normal epithelium, mucosa, submucosa, submucosal and myenteric plexuses, and circular and longitudinal muscles, were excluded (Fig. 1). Based on the tumor outline, the QuPath software automatically calculated the area of each outline. Within this area, regions of interest for histologic evaluation were automatically generated through random sampling using rectangular-shaped measurement sites sized 100 × 100 μm. The number of measurement sites was dependent on the tumor area. Thereafter, 2 independent observers (M.M. and G.R.) histologically evaluated each measurement site for NF or PGP9.5 staining within a neuron or nerve-like structure. Slides were positive when ≥1 sites displayed expression of NF and PGP9.5 within nerves or negative when no expression was found. In cases of discordance between observers (ie, disagreement on whether positive staining is observed), cases were reexamined and discussed until consensus was reached. Whole-slide evaluation was based on the tumor area outline from the random sampling method to be able to compare the methods, but no measurement sites were used. Slides were scored for the presence/absence of NF and PGP9.5 in a joint meeting between observers (M.M. and G.R.) in which the presence of positive staining was discussed until consensus was reached.

After the comparison, the whole study cohort and validation cohort were histologically evaluated using the whole-slide evaluation method. All slides were scored for the presence/absence of NF and PGP9.5 within the direct tumor stroma by 2 independent observers (M.M. and K.A.D.W.). Differentially assessed slides were discussed between both observers until consensus was reached. Slides stained for the neuronal subtype markers were evaluated for the number of positive fibers based on the whole slide by 2 independent observers (M.S.T. and D.H.).

Data Analysis

Agreement between the immunohistochemical evaluation methods (random sampling vs whole-slide evaluation) was assessed using the intermethod variability analyses (Cohen kappa). Descriptive statistics and frequency distributions were used to present clinical and pathologic characteristics (ie, age of diagnosis, sex, TNM stage, tumor sublocation, and differentiation grade). Differences between staining subgroups (ie, positive and negative staining of NF or PGP9.5) were analyzed using the Pearson χ^2 test for categorical variables and *t* tests for continuous variables. CRC cause-specific survival was defined as time from cancer diagnosis to CRC-related death or the end of follow-up; all deaths within 2 weeks after surgery were excluded for analyses because these might have been the result of surgical complications. Univariate survival analyses were performed using the Kaplan-Meier curve and the Wilcoxon (Breslow) test. Hazard ratios (HR) and corresponding 95% CI were assessed by the use of Cox proportional hazard models adjusted for a priori selected potential confounders and known prognostic factors (ie, age at diagnosis [continuous], sex, TNM stage, sublocation, and differentiation grade). All statistical analyses were performed using STATA 17.0 (StataCorp LLC).

Results

Characterization of Neuronal Innervation in Human Colorectal Tumors

Immunohistochemistry for the Pan-Neuronal Markers Neurofilament and Protein Gene Product 9.5 Reveals Neuronal Fibers in the Colorectal Cancer Stroma

First, the pan-neuronal markers NF and PGP9.5 were used to characterize the neuronal distribution in CRC. NF and PGP9.5 immunoreactivity were found within the cytoplasm of cell bodies located in ENS ganglia, confirming their neuronal specificity. NF and PGP9.5 expression were observed in both the myenteric plexus (Auerbach plexus) (Fig. 2A, E), which is located between the circular and longitudinal muscle layers, and the submucosal plexus (Meissner plexus), which is located in the intestinal submucosa (Fig. 2B, F). PGP9.5 was also highly expressed in the nerve fibers located in the circular and longitudinal muscle layers, whereas NF immunoreactivity was minimal or not detected in the muscle layers (Fig. 2C, G). Within the tumor area, nerve fibers expressed both NF and PGP9.5 (Fig. 2D, H). In few cases, larger nerve bundles were observed, potentially indicating that some ganglia have been surrounded by tumor tissue. Between tumor samples, a large variety in the amount of pan-neuronal marker expression was observed within the tumor area, with overall more PGP9.5 compared with NF immunoreactivity within the same samples.

Neuronal Fibers in the Colorectal Cancer Stroma Are Mostly Expressing Vasoactive Intestinal Protein and Neuronal Nitric Oxide Synthase

To examine the distribution of different neuronal subtypes in the CRC stroma, colorectal tumor tissue was analyzed for the expression of the neurochemical markers VIP, nNOS, TH, 5-HT, and ChAT. PGP9.5 was used to identify the neuronal fibers in the tumor stroma (Fig. 3A), and the submucosal plexus and myenteric plexus were used as a positive control for the neurochemical marker staining (Supplementary Fig. S1). All neurochemical markers were observed in the stroma of the colorectal tumor tissue (Fig. 3). VIP (8/8 patients, mean of VIP-positive fibers/total PGP9.5 fibers: 75%) and nNOS (8/8, mean of nNOS-positive fibers/total PGP9.5 fibers: 52%) immunoreactive neuronal processes were abundant within the CRC stroma of all patient samples and, in some cases, found to colocalize

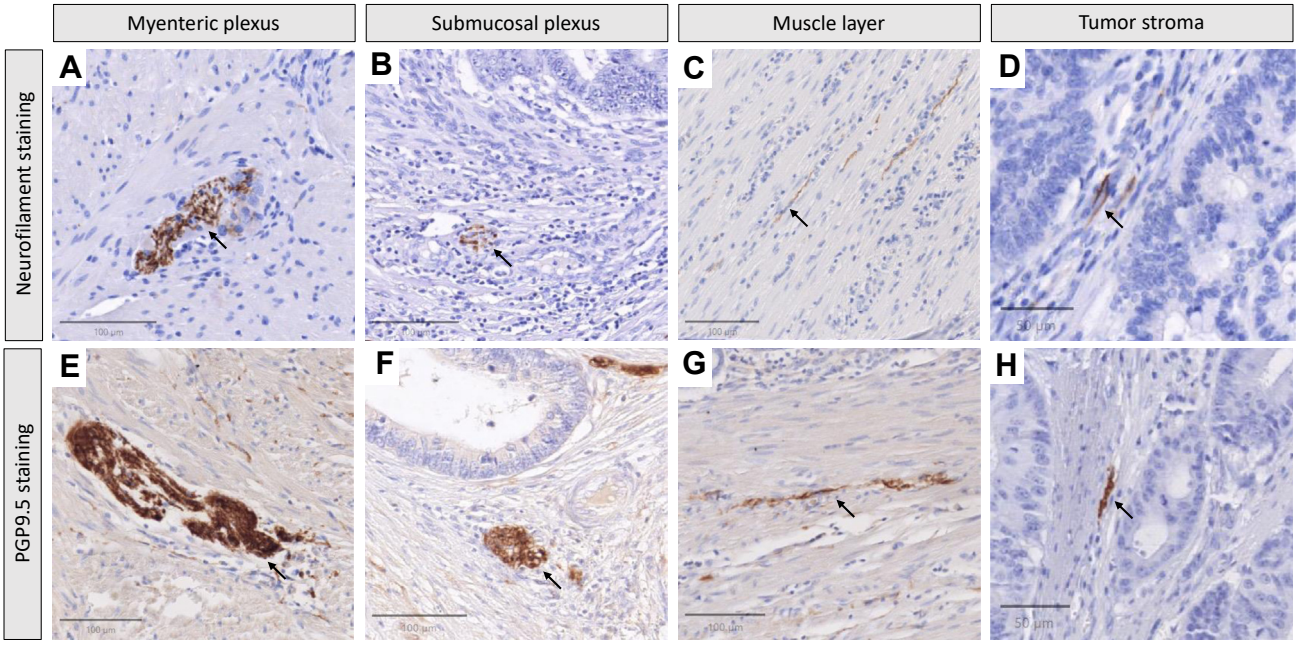

Figure 2.
Representative images showing neurofilament (NF) and protein gene product 9.5 (PGP9.5) immunoreactivity within normal colon and colorectal tumors. (A) NF and (E) PGP9.5 immunohistochemistry show positivity within the myenteric plexus. The submucosal plexus displays positive expression for both (B) NF and (F) PGP9.5. The intestinal muscle layers contain high levels of (G) PGP9.5 but have limited expression of (C) NF. Both (D) NF and (H) PGP9.5 expression can be found within nerve fibers in the stroma of colorectal tumor. (A-H) Black arrows refer to positivity. Scale bar, 100 μm or 50 μm for the tumor stroma.

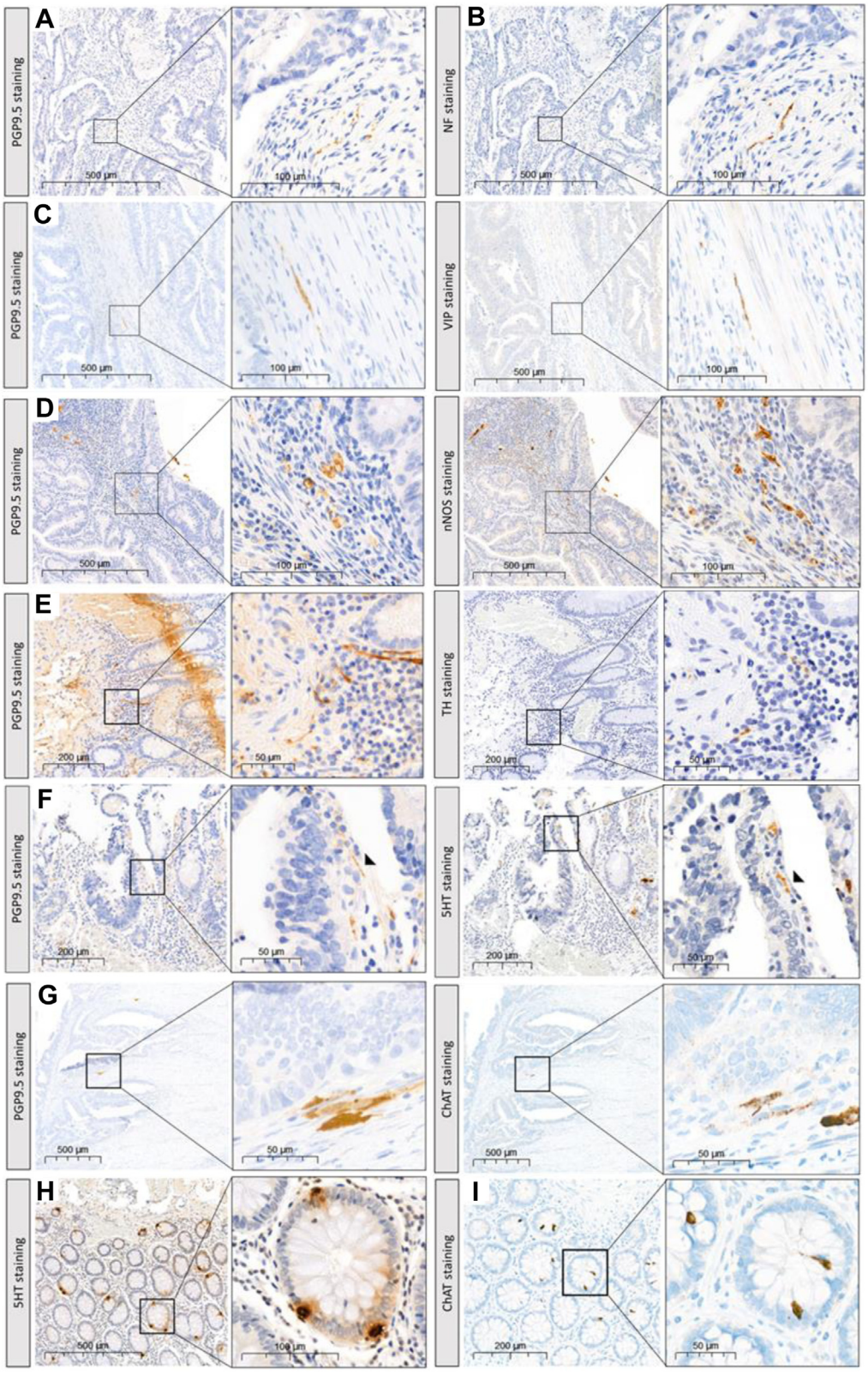

Figure 3.
Representative images of pan-neuronal and neurochemical marker staining within the CRC stroma. Neuronal fibers stained by the pan-neuronal markers (A) PGP9.5 and (B) NF serve as a positive control for the presence of neurons. Fibers immunoreactive for (C) VIP, (D) nNOS, (E) TH, (F) 5-HT, and (G) ChAT are observed in the tumor stroma as verified by PGP9.5 staining at the same location. Arrowheads point to 5-HT positive nerve fibers. (H) 5-HT staining also identifies enterochromaffin cells. (I) Staining by ChAT is observed in other cells as well, potentially tuft cells. 5-HT, serotonin; ChAT, choline acetyltransferase; NF, neurofilament; nNOS, neuronal nitric oxide synthase; PGP9.5, protein gene product 9.5; TH, tyrosine hydroxylase; VIP, vasoactive intestinal peptide.

Table 3
Immunoreactivity for the pan-neuronal marker PGP9.5 and the neurochemical markers VIP, nNOS, TH, 5-HT, and ChAT observed in colorectal tumor tissues of 8 patients

Patient No.	PGP9.5	Neuronal staining observed (% PGP9.5)				
		VIP	nNOS	TH	5-HT	ChAT
Patient 1	37	27 (73)	11 (30)	2 (5)	1 (3)	0 (0)
Patient 2	44	33 (75)	28 (64)	0 (0)	1 (2)	0 (0)
Patient 3	25	20 (80)	25 (100)	1 (4)	0 (0)	0 (0)
Patient 4	10	6 (60)	10 (100)	0 (0)	1 (10)	0 (0)
Patient 5	22	20 (90)	3 (14)	0 (0)	0 (0)	0 (0)
Patient 6	25	11 (44)	2 (8)	1 (4)	0 (0)	0 (0)
Patient 7	27	27 (100)	24 (89)	0 (0)	0 (0)	2 (7)
Patient 8	16	12 (75)	2 (13)	0 (0)	0 (0)	0 (0)

Number of positive neuronal fibers per marker and per patient are quantified together with the percentage of total PGP9.5-positive fibers that are stained for the subtype markers.
5-HT, serotonin; ChAT, choline acetyltransferase; nNOS, neuronal nitric oxide synthase; PGP9.5, protein gene product 9.5; TH, tyrosine hydroxylase; VIP, vasoactive intestinal protein.

(Fig. 3C, D; Table 3). TH-positive, 5-HT−positive, and ChAT-positive nerve fibers were only seen in a few tumors. The TH-stained neuronal fibers were observed at the tumor border near the healthy epithelium (3/8; Fig. 3E, Table 3). For the subtype-specific neuronal marker 5-HT, neuronal staining was mostly observed toward the lumen of the gut (3/8; Fig. 3F, Table 3). Furthermore, 5-HT immunoreactivity was also observed in serotonin-producing enterochromaffin cells, which were not considered in the analyses (Fig. 3H). The ChAT-stained neuronal fibers were observed at the tumor border near the healthy tissue (1/8) (Fig. 3G). Tuft cells in the mucosa were stained by ChAT as well and not further considered for analyses (Fig. 3I). The presence of mostly VIP and nNOS expressing neuronal processes in the CRC stroma suggests that inhibitory neurons are the most prominent neuronal subtype in CRC.

Evaluation of Pan-Neuronal Markers in Population-Based Colorectal Cancer Series

Random Sampling and Whole-Slide Evaluation Show Comparable Results

To develop a methodology for the consistent identification of neuronal fibers within the CRC stroma, a subgroup of patient samples stained for NF and PGP9.5 were analyzed using random sampling and whole-slide evaluation. Using whole-slide evaluation, slightly more NF-positive tumors (n = 93, 64.6%) were observed compared with the random sampling method (n = 87, 60.4%). However, a strong agreement was observed ($\kappa = 0.823$) between both methodologies. For PGP9.5, based on random sampling, positive staining was found in 94 tumors (74.6%), whereas 32 (25.4%) showed no PGP9.5 staining. With the whole-slide evaluation method, more PGP9.5-positive tumors were identified (n = 108, 85.7%) compared with the random sampling method. However, similar to the NF staining, a substantial agreement was observed between the whole-slide evaluation and random sampling for the PGP9.5 staining ($\kappa = 0.608$). Both methodologies show comparable results within the various analyses, hereby indicating that both methods are equally usable for studying the prognostic value of both NF and PGP9.5 (Supplementary Tables S2 and S3). Therefore, whole-slide evaluation was used for further analyses in the whole study cohort and validation cohort.

Association of Immunoreactivity for Neurofilament and Protein Gene Product 9.5 and Clinical Characteristics in Two Patient Series

Although the study cohort and in-cohort validation population were randomly selected to strive toward an equal distribution of clinical characteristics, considerable variation was observed between the 2 populations (Table 1). The study cohort was considerably younger at diagnosis compared with the in-cohort validation (68 ± 4.3 years vs 74 ± 5.9 years, respectively; $P < .0001$). More stage I and IV cases, and less stage II cases, were present in the study population ($P = .009$). In addition, cases in the study population were less often diagnosed with a proximal tumor and more often diagnosed with a tumor in the rectum compared with the in-cohort validation ($P = .058$). Not only more well-differentiated tumors in the study population were diagnosed but also more tumors with an unknown differentiation ($P = .030$). No differences were seen between the 2 populations for sex and CRC-related mortality ($P = .242$ and $P = .550$, respectively) (Table 1).

Both in the study population and in the in-cohort validation, more positively stained tumors were found compared with tumors where staining was absent (NF staining: study cohort: n = 278, 82.0%; in-cohort validation: n = 260, 69.7% and PGP9.5 staining: study cohort: n = 308, 91.1%; in-cohort validation: n = 311, 85.7%) (Tables 4 and 5). Although not statistically significant, more negative NF staining appeared to be present in the right proximal colon compared with the other locations (n = 24, 40.7%; $P = .091$). In contrast, within the in-cohort validation group, more NF-negative tumors seemed to be present in the left distal colon (n = 47, 42%; $P = .259$). In the in-cohort validation population, TNM stage II was more often NF negative (n = 54, 48.7%; $P = .021$); this observation was not found in the study population. No clear association was observed for NF staining with the differentiation grades of the tumors in both patient populations (Table 4). Similar to NF staining in the study population, the in-cohort validation of PGP9.5 staining showed more negative tumors in the right proximal colon (n = 27, 51.9% positive; $P = .010$), and these results were not observed in the study cohort for PGP9.5. PGP9.5 staining also suggested more negative slides in the TNM stage II category in the in-cohort validation (n = 27, 51.9%; $P = .058$). This association was not found in the study population. Compared with the NF staining, PGP9.5 staining showed no clear differences in the differentiation grades in both populations (Table 5).

Association of Immunoreactivity for Vasoactive Intestinal Protein and Neuronal Nitric Oxide Synthase and Clinical Characteristics

To further evaluate the association between nNOS, VIP staining, and patient characteristics, 50 samples for the student cohort were additionally stained for VIP and nNOS (Supplementary Table S1). For VIP, we observed absent staining in 2 cases (4%), low staining in 17 cases (38%), moderate staining in 19 cases (42%), and high staining in 7 cases (16%). For nNOS, we observed absent staining in 7 cases (15%), low staining in 32 cases (70%), and moderate staining in 7 cases (15%). Owing to the low number of cases with absent staining and the large variation in staining, analyses were performed on categories of staining instead of negative vs positive staining. No associations between VIP/nNOS staining and patient or tumor characteristics (age, sex, sublocation, degree of differentiation, TNM stage, or CRC mortality) were observed (data not shown).

Table 4
Association of NF immunohistochemistry with the clinicopathological characteristics in the study and in-cohort validation populations

Patient variable	Patient subgroups	NF immunohistochemistry			
		Study cohort (n = 339)		In-cohort validation (n = 373)	
		Negative, n (%)	Positive, n (%)	Negative, n (%)	Positive, n (%)
		61 (18.0)	278 (82.0)	113 (30.3)	260 (69.7)
Sex	Male	27 (44.3)	165 (59.4)	61 (54.0)	136 (52.3)
	Female	34 (55.7)	113 (40.6)	52 (46.0)	124 (47.7)
		$P = .031$		$P = .766$	
Sublocation	Right proximal	24 (40.7)	73 (26.7)	40 (35.7)	86 (33.6)
	Left distal	19 (32.2)	99 (36.3)	47 (42.0)	92 (36.0)
	Rectosigmoid/rectum	16 (27.1)	101 (37.0)	25 (22.3)	78 (30.5)
		$P = .091$		$P = .259$	
Differentiation grade	Undifferentiated	1 (1.8)	4 (1.7)	—	1 (0.4)
	Poorly	8 (14.0)	37 (15.5)	14 (13.6)	31 (12.9)
	Moderate	42 (73.7)	167 (69.9)	76 (73.8)	191 (79.6)
	Well	6 (10.5)	31 (13.0)	13 (12.6)	17 (7.1)
		$P = .944$		$P = .349$	
Cancer stage	Stage I	16 (28.6)	68 (25.1)	21 (18.9)	53 (20.7)
	Stage II	16 (28.6)	92 (34.0)	54 (48.7)	102 (39.8)
	Stage III	15 (26.8)	68 (25.1)	18 (16.2)	76 (29.7)
	Stage IV	9 (16.1)	43 (15.9)	18 (16.2)	25 (9.8)
		$P = .878$		$P = .021$	
CRC death	Yes	17 (27.9)	146 (52.7)	38 (33.9)	104 (40.8)
	No	44 (72.1)	131 (47.3)	74 (66.1)	151 (59.2)
		$P = .006$		$P = .214$	

CRC, colorectal cancer; NF, neurofilament.

Survival Analysis for the Neuronal Markers Neurofilament, Protein Gene Product 9.5, Vasoactive Intestinal Protein, and Neuronal Nitric Oxide Synthase

In the study population, patients with an NF-positive tumor had a worse CRC-specific survival ($P = .025$) (Fig. 4A), and this was independent of other prognostic factors (ie, age at diagnosis, sex, TNM stage, sublocation, and differentiation grade) (HR, 2.31; 95% CI, 1.33-4.03; $P = .003$). This effect was, however, not seen for PGP9.5-positive tumors in the study cohort ($P = .70$; multivariate HR, 0.63; 95% CI, 0.31-1.28; $P = .20$) (Fig. 4B). The in-cohort validation population showed no CRC-specific survival difference for NF staining ($P = .28$) (Fig. 4C). For PGP9.5, however, positively stained CRC patients showed a shortened survival ($P = .05$) (Fig. 4D). The multivariate analyses in the in-cohort

Table 5
Association of PGP9.5 immunohistochemistry with the clinicopathological characteristics in the study and in-cohort validation populations

Patient variable	Patient subgroups	PGP9.5 immunohistochemistry			
		Study cohort (n = 338)		In-cohort validation (n = 363)	
		Negative, n (%)	Positive, n (%)	Negative, n (%)	Positive, n (%)
		30 (8.9)	308 (91.1)	52 (14.3)	311 (85.7)
Sex	Male	18 (60.0)	172 (55.8)	23 (44.2)	175 (56.3)
	Female	12 (40)	136 (44.2)	29 (55.8)	136 (43.7)
		$P = .661$		$P = .107$	
Sublocation	Right proximal	11 (37.9)	91 (29.8)	27 (51.9)	94 (30.6)
	Left distal	9 (31.0)	111 (36.4)	15 (28.9)	115 (37.5)
	Rectosigmoid/rectum	9 (31.0)	103 (33.8)	10 (19.2)	98 (31.9)
		$P = .657$		$P = .010$	
Differentiation grade	Undifferentiated	—	5 (1.8)	—	1 (0.4)
	Poorly	4 (16.0)	40 (14.4)	8 (17.0)	35 (12.3)
	Moderate	16 (64.0)	204 (73.7)	36 (76.6)	223 (78.3)
	Well	5 (20.0)	28 (10.1)	3 (6.4)	26 (9.1)
		$P = .418$		$P = .747$	
Cancer stage	Stage I	8 (29.6)	78 (25.9)	6 (11.4)	67 (22.0)
	Stage II	8 (29.6)	106 (35.2)	27 (51.9)	117 (38.4)
	Stage III	10 (37.0)	73 (24.3)	10 (19.2)	88 (28.9)
	Stage IV	1 (3.7)	44 (14.6)	9 (17.3)	33 (10.8)
		$P = .250$		$P = .058$	
CRC death	Yes	11 (36.7)	121 (39.4)	25 (50.0)	112 (36.6)
	No	19 (63.3)	186 (60.6)	25 (50.0)	194 (63.4)
		$P = .769$		$P = .071$	

CRC, colorectal cancer; PGP9.5, protein gene product 9.5.

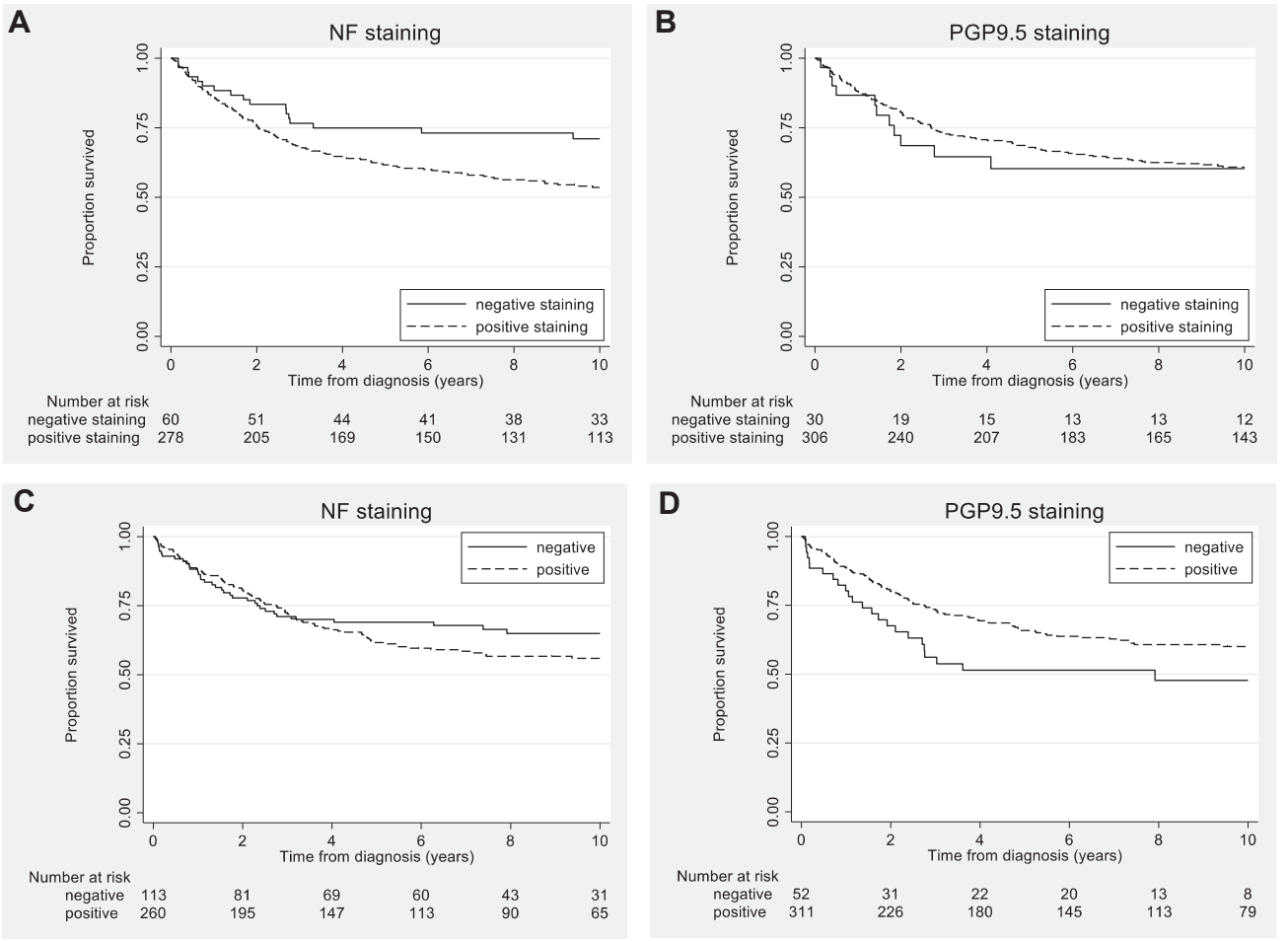

Figure 4.
Ten-year cancer-specific survival curves for NF and PGP9.5 staining within the study cohort and the validation cohort. Kaplan-Meier curves of (A) NF staining in the study cohort ($P = .025$) and (B) PGP9.5 staining in the study cohort ($P = .70$). Kaplan-Meier curves of (C) NF staining in the validation cohort ($P = .280$) and (D) PGP9.5 staining in the validation cohort ($P = .046$). NF, neurofilament; PGP9.5, protein gene product 9.5.

validation group adjusted for age, sex, TNM stage, sublocation, and differentiation grade showed no association with survival for NF or PGP9.5 staining (HR_{NF}, 1.26; 95% CI, 0.85-1.88; $P = .254$; $HR_{PGP9.5}$, 0.81; 95% CI, 0.50-1.32; $P = .393$). For VIP and nNOS, no survival differences were observed in the subset of 50 cases (P_{VIP}, .983; P_{nNOS}, .881).

Discussion

In this study, we explored the innervation of CRC tumors, using the pan-neuronal markers NF and PGP9.5 and the neurochemical markers VIP, nNOS, TH, 5-HT, and ChAT, and evaluated the association with clinicopathological characteristics in 2 CRC patient series. NF and PGP9.5 immunoreactivity were found within the tumor stroma of the majority of patients. In most of the tumor tissues, we observed only single nerve fibers within the tumor area and no cell bodies, suggesting that axons infiltrate and grow into the tumor, possibly to support cancer growth. Interestingly, although the gut wall is highly innervated, the number of nerve fibers (per tumor area) in CRCs was much lower as previously described in other cancers, eg, pancreatic cancer.[17] Larger nerve bundles, which most likely originated from ganglia that became surrounded by tumor tissue, were observed only in a limited amount of cases, whereas most ganglia were excluded from the invasive front of the tumor as previously described by Godlewski and Kmiec.[18]

To identify the origin/subtype of the neuronal processes within the tumor area, we stained the CRC stroma for neuronal subtype markers VIP, nNOS, TH, 5-HT, and ChAT. This study shows that the neuronal fibers in the tumor stroma were mostly immunoreactive for VIP and nNOS, probably representing inhibitory (secreto)motor and interneurons.[19] The role of the neuropeptide VIP has already been described to contribute to cell proliferation, migration, and survival.[20] In human colonic adenocarcinoma cancer cells, VIP also stimulated cell proliferation.[21] In a small study with human tissues, VIP-positive fibers were observed more in the lamina propria of tumor-neighboring mucosa than in remote normal mucosa.[22] In nasopharyngeal carcinoma cells, nitric oxide and nitric oxide synthase activity are shown to promote survival through inhibition of autophagy.[23] Therefore, the presence of nNOS producing neuronal fibers in the CRC stroma shown in this study could be a source of nitric oxide possibly enhancing tumor growth. Based on these results, specific subtype marker panels, including VIP and nNOS, can be potentially better to predict patient prognosis compared with pan-neuronal markers because neuronal subtypes are known to function

differently in response to cancer. Supporting this idea, research showed that the immunoreactivity for VIP in the lamina propria of tumor-neighboring mucosa is marginally greater in more advanced CRC tumor stages.[22] Other research suggested a stage-dependent reduction in the abundance of nNOS in human colon cancer.[24] However, this needs to be validated in larger patient cohorts.

Here, we aimed to associate the neuronal distribution, using pan-neuronal markers, with clinicopathological characteristics using 2 large population-based cohorts. Previously, it has been shown that the methodology to determine marker positivity often differs between studies, hereby affecting the determined value of the results.[9,25-28] To assess the influence of methodology, we compared histologic evaluation based on random sampling with whole-slide evaluation for both markers. This showed similar results, which was confirmed using intermethod variability analyses (Cohen kappa). Whole-slide evaluation through digitized slides was chosen as the preferred method of analysis as evaluation through random sampling is considerably more time consuming because manual assessment of each separate measurement site is required.[29] In contrast, histologic evaluation through random sampling can also be standardized because of the opportunities to create and store digitized measurement sites, hereby improving reproducibility and clinical implementation of the biomarker.[29] This emphasizes the importance of evaluating or discussing the best methodology up front before starting the analysis of a complete study population.[30]

To our knowledge, this is the first study evaluating the expression of NF in CRC. Whole-slide evaluation results from the study cohort suggest a poorer prognosis for patients with positive NF staining independent of other prognostic variables. However, these results could not be confirmed in our in-cohort validation population. Additional validation studies are necessary to study the prognostic potential of NF. The prognostic value of PGP9.5 was already studied by other groups, although using smaller patient cohorts. Albo et al[31] studied PGP9.5 staining in a cohort of 236 CRC patients to evaluate whether neuronal processes were present and whether they had a role in CRC prognosis. In that study, the exact number of nerves in hotspot regions with the highest nerve density was counted, which differs from the 2 methodologies used in our study. In contrast to our study, neuronal fibers were observed in 63% of the 236 patients, and 37% of the patients had no nerves in the primary tumor,[31] which is higher as what we observed in our study cohort and validation cohort. In another study, 46% of patients showed immunoreactivity for PGP9.5 in a total of 77 patient cases.[32] Moreover, both Albo et al[31] and Yamazaki et al[32] found an association between the PGP9.5 expression and the occurrence of more advanced or invasive CRC, and Albo et al[31] also reported a decreased survival for patients with high levels of neuronal fibers. In our study, PGP9.5 also showed an association with a worse CRC-specific survival, but only in the in-cohort validation population. This effect, however, was not seen after adjustment for other prognostic factors, indicating that this is not an independent effect. The other studies often did not include multivariate analyses, raising the question whether those results are dependent on other prognostic factors as observed in our study. A multivariate analysis could confirm the neuronal association with a worse survival in only 1 study that included slightly different adjustment factors compared with our analysis.[31]

Within our study, we could not validate the prognostic value of NF in our in-cohort validation. Differences in the 2 population cohorts could influence the distribution and prognostic outcomes in both populations[33,34] and are also limitations of this study. The age at the time of diagnosis is higher in the in-cohort validation group compared with that in the study cohort, which reflects the difference in the inclusion period of the incident cases between the 2 populations. Moreover, the in-cohort validation group comprises more lower stage cases compared with the study cohort.

To conclude, this is the first study to investigate the distribution and association of 2 pan-neuronal markers, PGP9.5 and NF, in 2 large CRC patient populations. No clear associations with patient characteristics were identified. Nevertheless, positive NF staining does depict a worse CRC-specific survival in the study population independent of other prognostic factors. We observed that most neuronal fibers in the CRC stroma are immunoreactive for VIP and nNOS. However, neither associations with patient or tumor characteristics (age, sex, sublocation, degree of differentiation, TNM stage, or CRC mortality) nor survival was found for these markers in a small cohort of 50 patients. Future studies focusing on specific neuronal subtypes could give more insights on the function and/or prognostic value of neurons in the colorectal TME.

Author Contributions

M.M., G.R., M.v.E., K.M.S., W.B., and V.M. were involved in study design. M.P.W. and P.A.v.d.B. were responsible for cohort and patient selection and patient data availability. M.J.G. and I.V.S. were involved as pathologists and confirmed specificity of staining. M.M., G.R., M.S.T., J.R.M.v.d.M., N.B., K.A.D.W., and D.H. performed the neuronal staining in the patient cohorts. M.M., K.A.D.W., M.S.T., and D.H. analyzed the patient slides for neuronal staining. K.M.S. performed the association and statistical analysis for the patient cohorts. M.M., G.R., M.S.T., K.W., D.H., and K.M.S. created the figures and tables. M.M., G.R., M.S.T., K.M.S., and V.M. wrote the manuscript. All authors edited and approved the manuscript versions.

Data Availability

All data supporting the findings in this study are available in the article and supplementary information files and from the corresponding author upon reasonable request.

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Declaration of Competing Interest

None declared.

Ethics Approval and Consent to Participate

Samples were collected as part of the Netherlands Cohort Study from 54 pathology registries throughout the Netherlands after the approval of the Ethical Review Board of the Maastricht University Medical Center (MEC85-012). These registries previously obtained consent to use the samples for scientific purposes.

Supplementary Material

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