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Unveiling the Prognostic Role of Synaptophysin in Conventional Colorectal Carcinomas

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Keywords
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Abstract

Introduction: Although neuroendocrine differentiation in colorectal carcinomas (CRCs) has been extensively reported, the biological behavior of adenocarcinomas expressing synaptophysin (Syn) but lacking typical neuroendocrine morphology remains unclear. ***Methods:*** We tested 663 conventional CRCs with non-neuroendocrine morphology for Syn expression and correlated the results with clinicopathological and molecular characteristics as well as patient survival (overall survival [OS] and disease-free survival [DFS]). The survival characteristics of Syn expression group were compared to those of conventional CRCs and subsequently to those of 14 minENs. ***Results:*** Syn immunohistochemical expression ≥30% was confirmed in 27 (4.1%) patients and correlated with right colon site, grade 2,

This work is dedicated to the memory of Laura Salvaterra, a courageous woman who battled against cancer. This is an invitation to fight cancer every day in her name, even after she has left us.

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marked intratumoral lymphocyte infiltrate, and *BRAF* p.V600E mutation. At univariate analysis, variables associated with poor OS were 10-year increase in age ($p = 0.001$), stage III–IV ($p = 0.001$), Syn ≥30% ($p = 0.001$), infiltrative growth ($p = 0.04$), and residual tumor R1–2 ($p = 0.03$). At multivariable analysis, Syn expression in ≥30% of gland-forming tumor cells emerged as an independent negative prognostic factor for both OS and DFS. Moreover, 10-year increase in age, stage III–IV, and Syn ≥30% ($p < 0.001$) were associated with poor OS and marked peritumoral lymphocyte infiltrate with longer OS ($p = 0.02$). Comparable results were obtained according to DFS; in addition, right colon site ($p = 0.04$) was associated with longer DFS while *KRAS* mutation ($p = 0.04$) was associated with poor DFS at univariate analysis. MiNEN patients showed a shorter DFS than all conventional adenocarcinomas with or without Syn expression in univariate analyses ($p < 0.001$). ***Conclusions:*** Among conventional CRCs, we provided evidence that Syn expression is associated with worse OS and DFS and contributes to predicting clinical outcome. Future studies should explore the molecular mechanisms underlying the acquisition of the neuroendocrine phenotype to identify new targeted treatment strategies.

Introduction

Mixed tumors composed of non-neuroendocrine epithelial and neuroendocrine elements (mixed neuroendocrine-non-neuroendocrine neoplasms [MiNENs], according to the current WHO guidelines) are rare, accounting for about 1–2% of all colorectal malignancies in the reported literature [1, 2]. In 1924, Cordier [3] showed, for the first time, the coexistence of both a neuroendocrine and an exocrine component in the same intestinal neoplasm. The gray zone, between pure neuroendocrine and non-neuroendocrine neoplasms, covers the spectrum of intestinal tumors having mixed divergent differentiation with variable extension and associated with several morphological patterns of the two components [4]. The molecular landscape of special phenotypes of colorectal carcinoma (CRC) needs to be properly understood and somatic genetic alterations and signaling pathways should be clarified in order to be translated into prognostic markers or new molecularly defined targets for therapy of CRC.

Adenocarcinoma with neuroendocrine differentiation (NED) is a special type of cancer. Because NED is related to nerve and endocrine function, it can be diagnosed by detecting the presence of cytoplasmic neurosecretory granules and immunohistochemical positivity to chromogranin A (CgA) and/or synaptophysin (Syn) proteins as gold standard for the detection of neuroendocrine phenotype [5]. NED has been identified in various solid tumors, with prostate cancer being the most extensively studied. Research in this area has explored not only the adverse impacts of NED on treatment outcomes and prognosis but also the mechanisms by which hormones secreted by NED cells influence neighboring cells increasing their metastatic and invasive potential [6, 7]. The acquisition of the neuroendocrine phenotype has been associated with shorter survival and in advanced disease with therapy resistance [8, 9]. Fassan et al. [10] documented high prevalence of Syn expression in *BRAF* p.V600E-mutated (*BRAF*mt) metastatic CRCs (mCRCs) with worse cancer-specific survival and progression-free survival (PFS). Although NED in CRCs has been extensively clarified, neither strong nor easily reproducible criteria are currently available for colorectal adenocarcinoma expressing Syn neither for its real clinical impact.

Colorectal high-grade neuroendocrine carcinomas (NECs) exhibit highly aggressive behavior with a low reported median survival of 5–11 months [11]. Despite significant advancements in survival rates for colorectal adenocarcinomas over recent decades, these improvements have not extended to NECs [12]. In contrast to colorectal adenocarcinomas, surgery alone is rarely curative for high-grade NECs, and its effectiveness in extending survival has been questioned in prior studies [13]. It is crucial to decipher the prognostic significance of NED in colorectal adenocarcinoma, given its distinct role compared to NECs, mixed forms, and conventional adenocarcinomas. To this end, we focused this study on 663 conventional CRCs with the aim of evaluating incidence, clinicopathological, molecular features and prognostic impact of CRC, according to their Syn expression at immunohistochemistry (IHC).

Methods

Study Design and Case Selection

Overall, 663CRC patients referred to two Italian oncology centers (IRCCS National Cancer Institute Foundation of Milan, ASST Sette Laghi di Varese), between 2009 and 2021, were retrospectively enrolled in the present study (Table 1). An available archival formalin-fixed, paraffin-embedded (FFPE) tissue samples (either of primary tumor and/or of distant metastatic site) and matched clinical and molecular data of these patients were collected. For each patient, the original hematoxylin and eosin stains and the original pathology reports were

Table 1. Characteristics of patients with CRC according to synaptophysin expression ≥30%

	All patients	Conventional CRC	Syn-positive CRC	*p* value[1]
Total	663 (100)	636 (100)	27 (100)	
Gender				
Female	294 (44.3)	282 (44.3)	12 (44.4)	
Male	369 (55.7)	354 (55.7)	15 (55.6)	1.0
Age, median [range]	66 [20–91]	66 [20–91]	67 [33–86]	0.6
Stage				
0	4 (0.6)	4 (0.6)	0 (0.0)	
I	131 (20.3)	129 (20.9)	2 (7.4)	
II	201 (31.3)	192 (31.1)	9 (33.3)	
III	254 (39.4)	241 (39.1)	13 (48.1)	
IV	54 (8.4)	51 (8.3)	3 (11.1)	0.4
Site				
Colon, unknown site	13 (2.0)	12 (1.9)	1 (3.7)	
Total colon	7 (1.1)	7 (1.1)	0 (0.0)	
Right colon	227 (34.2)	216 (34.0)	11 (40.7)	
Transverse colon	7 (1.1)	7 (1.1)	0 (0.0)	
Left colon	97 (14.6)	87 (13.7)	10 (37.0)	
Sigma	19 (2.9)	17 (2.7)	2 (7.4)	
Sigma rectum	70 (10.6)	70 (11.0)	0 (0.0)	
Rectum	223 (33.6)	220 (34.6)	3 (11.1)	**0.003**
Grade				
Grade 1	10 (1.6)	10 (1.7)	0 (0.0)	
Grade 2	434 (69.3)	409 (68.0)	25 (100.0)	
Grade 3	182 (29.1)	182 (30.3)	0 (0.0)	**0.0007**
Vascular invasion				
Absent	178 (28.3)	170 (28.2)	8 (32.0)	
Present	330 (52.6)	313 (51.9)	17 (68.0)	
Only extra	16 (2.5)	16 (2.7)	0 (0.0)	
Only intra	104 (16.5)	104 (17.2)	0 (0.0)	0.07
Perineural invasion				
Absent	275 (44.4)	273 (45.0)	2 (16.7)	
Present	343 (55.6)	333 (55.0)	10 (83.3)	0.08
Intratumoral lymphocyte infiltrate				
Absent	52 (8.3)	45 (7.5)	7 (29.2)	
Mediated	238 (38.2)	235 (39.2)	3 (12.5)	
Mild	282 (45.2)	272 (45.3)	10 (41.7)	
Severe	52 (8.3)	48 (8.0)	4 (16.7)	**0.0006**
Growth edges				
Expansive	233 (37.1)	222 (36.9)	11 (44.0)	
Infiltration	394 (62.9)	380 (63.1)	14 (56.0)	0.5
Crohn's-like lymphoid reaction				
Absent	406 (66.4)	392 (66.7)	14 (60.9)	
Present	205 (33.6)	196 (33.3)	9 (39.1)	0.7
Peritumoral lymphocyte infiltrate				
Absent	34 (5.4)	30 (5.0)	4 (16.0)	
Mediated	318 (50.6)	308 (51.1)	10 (40.0)	
Mild	190 (30.3)	181 (30.0)	9 (36.0)	
Severe	86 (13.7)	84 (13.9)	2 (8.0)	0.1

Table 1 (continued)

	All patients	Conventional CRC	Syn-positive CRC	p value[1]
Residual tumor				
R0	376 (85.6)	370 (85.6)	6 (85.7)	
R1–R2	63 (14.4)	62 (14.4)	1 (14.3)	1.0
Adjuvant therapy				
No	105 (39.0)	102 (38.9)	3 (42.9)	
Yes	164 (61.0)	160 (61.1)	4 (57.1)	1.0
MMR IHC				
Proficient (MMRp)	403 (86.7)	380 (86.2)	23 (95.8)	
Deficient (MMRd)	62 (13.3)	61 (13.8)	1 (4.2)	0.2

CRC, colorectal carcinoma; Syn, synaptophysin; R, residual; MMR, mismatch repair; IHC, immunohistochemistry. [1]p value based on the Fisher's exact for categorical variables and the Wilcoxon rank-sum test for continuous variables.

jointly reviewed by two expert pathologists (M.M. and C.C.); tumor histotype and grading were classified according to the 2019 WHO classification of tumors of the digestive system [2]. Pure NECs and neuroendocrine tumors grade 3 were expressly excluded from the present series. Further available clinicopathological variables were age, sex, primary tumor location, TNM stage, lymphovascular invasion, perineural infiltration, growth pattern, peritumoral and intratumoral inflammatory infiltrate, Crohn's-like lymphoid reaction, time to metastatic presentation, systemic treatment, response, and survival.

Fourteen colorectal MiNENs from patients who underwent surgical resection between 2010 and 2021 at the IRCCS National Cancer Institute Foundation of Milan were further analyzed as control group. Not all patients included in the study had complete information on clinicopathological characteristics and survival data, although fully available tumor tissue was ensured for all cases.

IHC and Histochemistry

Immunohistochemical stainings were automatically performed using the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle Upon Tyne, UK) in the BOND-MAX system (Leica Biosystems) on 4 μm-thick sections. The Syn-positive cells (Leica Biosystems) were counted, and the mean percentage of positive cells was assigned for each tumor. A cytoplasmic Syn expression was considered specific. Only Syn-positive CRCs ≥30% tumors were included in the study and compared to ordinary CRCs. Positive cases were further studied by applying an immunohistochemical/histochemical double staining method [14]: an immunohistochemical staining (Syn, Leica Biosystems; brown, alkaline phosphatase) combined with histochemistry (periodic acid-Schiff [PAS] and/or Alcian blue [AB]). Fourteen colorectal cancers were diagnosed as MiNENs, according to current WHO guidelines [2], and were used as a statistical control group for further analyses, comparing them with survival characteristics of Syn expression groups.

DNA mismatch repair (MMR) complex status was assessed by staining MLH1, PMS2, MSH2, and MSH6 (Dako, Glostrup, Denmark). Nuclear immunostaining for MLH1, PMS2, MSH2, and MSH6 was assessed following the GIPAD-SIAPeC criteria to identify mismatch repair deficiency and mismatch repair proficiency profile [15].

Tumor Infiltrating Lymphocytes

The number of tumor infiltrating lymphocytes was assessed as the mean value of five random observations and counted at high-power fields (40×) of tumor-enriched areas composed of >60% of neoplastic cells, as previously described [16]. Only tumor epithelium infiltrating lymphocytes were retained for scoring and positive cases were classified in mild, moderate, and severe infiltrate. All samples were jointly evaluated by two gastrointestinal pathologists who were unaware of any clinical information.

Targeted NGS

Data on mutations (*KRAS, NRAS, BRAF, TP53, APC*) were extracted exclusively from patients' investigations conducted primarily for therapy decision-making using next-generation sequencing (NGS) analysis. FFPE 5-μm cut sections were manually microdissected to isolate the highest possible percentage of neoplastic cells.

Targeted NGS was performed using the Ion AmpliSeq™ Cancer Hotspot Panel v2 (Thermo Fisher Scientific, #4475346), a commercially available panel widely used for investigation of various tumors: it is easily reproducible and gives a plenty of data particularly targeting most of the mutations described in high-grade gastroenteropancreatic neuroendocrine neoplasms. Raw sequencing data were processed using Torrent Suite™ Software (version 5.12.0); the variant calling from sequencing data was generated by Variant Caller plugin. The variants were annotated using Ensemble Variant Effect Predictor pipeline, Ion Reporter™ analysis software version 5.20, ClinVar database, COSMIC database, and dbSNP database. Variants with a MAF (minor allele frequency) population value greater than 0.01 in 1,000 genomes combined populations were considered as common SNP and thus excluded. All the obtained variants were finally examined with manual curation using the Integrative Genomic Viewer IGV tool.

Statistical Analysis

The primary goal of this study was to explore the prognostic value of Syn status in a large series of CRCs. Syn positive group was compared to study population using the Fisher's exact test for categorical variables and the Wilcoxon rank-sum test for continuous variables. Main endpoints included overall survival (OS) and disease-free survival (DFS). OS was assessed from the date of diagnosis to the date of death or last follow-up. DFS was assessed from the date of diagnosis to the date of first relapse, death, or last follow-up, whichever occurred first. OS and DFS curves were drawn using the Kaplan-Meier method. The log-rank test was used to assess the survival difference between patients' groups. Univariable and multivariable Cox proportional regression models were used to assess the association between clinicopathological characteristics and DFS and OS. Hazard ratio (HR) is presented with 95% confidence interval (CI). Data analysis was performed using the R environment v4.0.3 for statistical computing and graphics. All tests were two sided and p values <0.05 were considered statistically significant.

Results

Clinicopathological Features of the Study Population

Overall, 663 patients were included (369 males and 294 females; mean age 66, range 20–91). Not all patients included in the study had complete information on clinicopathological characteristics and survival data, although fully available tumor tissue was ensured for all cases. Information on all 663 cases is available only for gender and tumor site, while data on tumor grade are available for 626 patients, stage for 644, vascular invasion for 628, perineural invasion for 618, intratumoral lymphocyte infiltration for 624, tumor growth pattern at the invasive front for 627, peritumoral lymphocyte infiltration for 628, residual tumor status for 439, adjuvant therapy for 269, and MMR IHC status for 465 patients. Based on the available clinical data, the main clinicopathological features were right-sidedness of primary tumor (n = 227, 34.2%), stage III disease diagnosis (n = 254, 39.4%), and grade II according to 2019 WHO classification (n = 434, 69.3%). The vast majority of patients showed intra- and extravascular invasion (n = 330, 52.5%), perineural invasion (n = 343, 55.5%), mild intratumoral lymphocyte infiltrate (n = 282, 45.2%), infiltrative growth edges (n = 394, 62.8%), peritumoral lymphocyte infiltrate (n = 318, 50.6%), and residual tumor R0 (n = 376, 85.7%). High percentage of CRC patients (n = 164, 61%) was subjected to adjuvant therapy. A total of 403 (86.7%) cases were MMR stable according to their immunohistochemical profiling. Baseline characteristics and major clinicopathological parameters are summarized in Table 1. Regarding molecular alterations, we included exclusively NGS analyses performed primarily for therapy decision-making purposes, with data available for the following: *KRAS* in 178 patients, *NRAS* in 132, *BRAF* in 170, *TP53* in 74, and *APC* in 73.

Based on available data, among 663 CRC cases, 52 (70.3%) showed *TP53* mutation, 83 (46.6%) showed *KRAS* mutation, only 5 patients (3.8%) were *NRAS* mutated, 26 (15.3%) had *BRAF* mutation, and finally 30 (41.1%) showed *APC* mutation. Patient molecular alterations are summarized in Table 2.

Clinicopathological Features of the Synaptophysin Positive Group

Among our cohort, the Syn expression ranged from single scattered Syn-positive tumor cells (>1%) to a strong and diffuse immunoreactivity (>95%) Fig. 1d–f). To properly describe the clinicopathological landscape of Syn-positive carcinomas, we focused on those cases showing at least 30% of positive neoplastic cells. Overall, 27 patients on the 663 enrolled ordinary CRCs (15 males and 12 females; mean age 67, range 33–86) were characterized by a positivity in at least 30% of tumor cells. In particular, 14 cases (51.85%) showed a positivity in >30% and <69% of tumor cells and 13 cases (48.15%) were characterized by a diffuse Syn staining in more than 70% of the tumor (6 cases resulted 100% Syn positive).

Endoscopic and macroscopic examinations usually revealed ulcerative neoplastic masses with destructive infiltration and extension into the subserosal tissue. From

Table 2. Molecular alterations according to synaptophysin expression ≥30%

	All patients	Conventional CRC	Syn-positive CRC	p value[1]
Total	663 (100)	636 (100)	27 (100)	
TP53				
WT	22 (29.7)	19 (30.6)	3 (25.0)	
Mut	52 (70.3)	43 (69.4)	9 (75.0)	1.0
KRAS				
WT	95 (53.4)	82 (51.9)	13 (65.0)	
Mut	83 (46.6)	76 (48.1)	7 (35.0)	0.3
NRAS				
WT	127 (96.2)	114 (95.8)	13 (100.0)	
Mut	5 (3.8)	5 (4.2)	0 (0.0)	1.0
BRAF				
WT	144 (84.7)	132 (86.8)	12 (66.7)	
Mut	26 (15.3)	20 (13.2)	6 (33.3)	**0.04**
APC				
WT	43 (58.9)	39 (55.7)	9 (75.0)	
Mut	30 (41.1)	27 (44.3)	3 (25.0)	0.3

CRC, colorectal carcinoma; Syn, synaptophysin; WT, wild type; Mut, mutated. [1]p value based on the Fisher's exact for categorical variables.

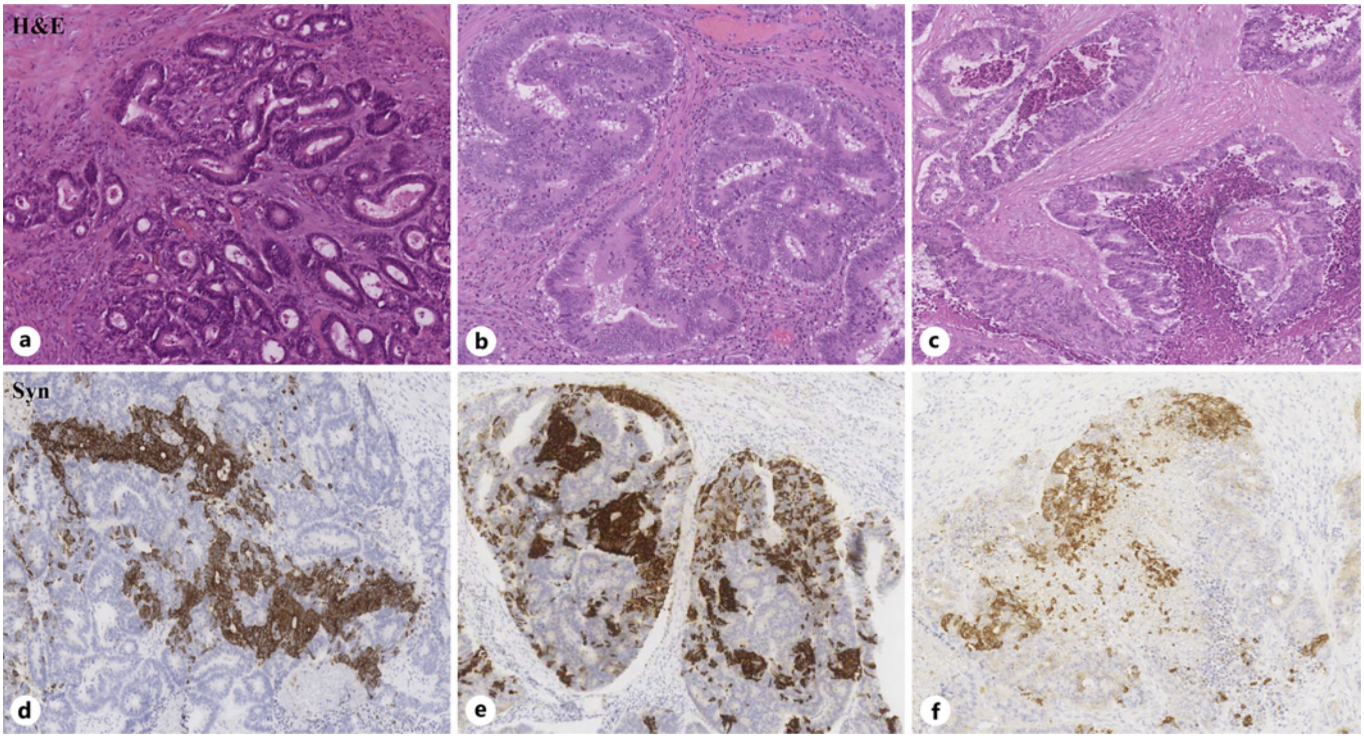

Fig. 1. Scanning magnification of synaptophysin-expressing variable patterns in conventional colorectal cancers with a non-neuroendocrine morphology on hematoxylin and eosin (well-differentiated glandular pattern or cribriform with luminal comedonecrosis pattern). Synaptophysin-positive cells accounting for variable percentage of the whole tumor. **a–c** Hematoxylin and eosin (H&E). **d–f** Synaptophysin (Syn). Magnification, ×20.

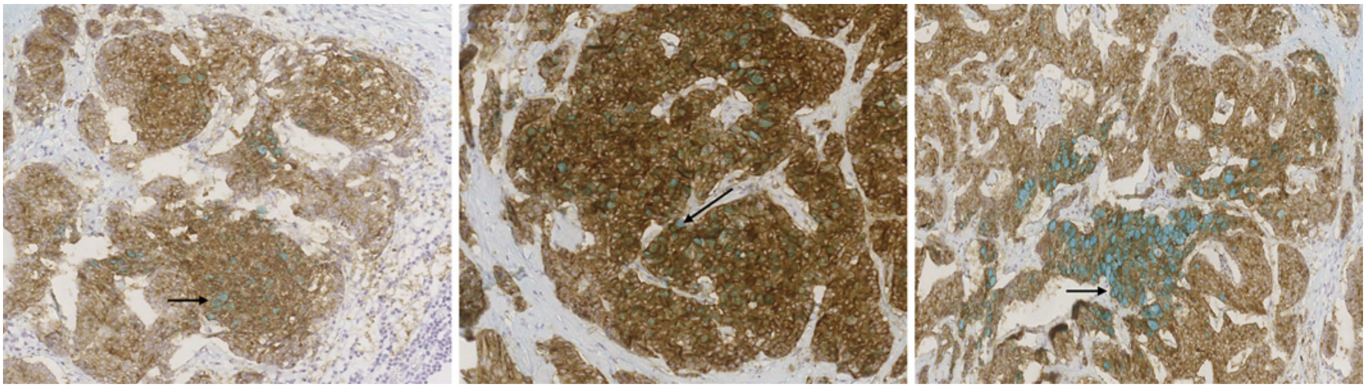

Fig. 2. Coexistence of synaptophysin immunoreactivity (brown) and AB staining (PAS and/or Alcian blue [AB] [pH 2.5], arrows) in the same neoplastic cells documented "amphicrine phenotype." Magnification, ×20.

the histopathological point of view, 23 cases were adenocarcinoma not otherwise specified (85.2%), with prevalent glandular or cribriform and comedonecrosis-associated patterns (Fig. 1a–c), 3 mucinous adenocarcinomas (11.11%), and 1 was adenocarcinoma with special histopathological subtype (signet ring cell carcinoma, 3.7%). Neuroendocrine morphological features could not be suspected at histological examinations.

Subsequently, all cases were stained using a combined immunohistochemical/histochemical method. This approach involved two sequential steps: first, immunohistochemical staining was performed with a specific antibody against Syn, an integral component of the neuroendocrine secretory granule membrane and a widely used marker for neuroendocrine cells. In the second step, histochemical staining (PAS and/or AB pH 2.5) was applied to the same sections previously subjected to IHC. The results of this combined staining confirmed the coexistence of Syn immunoreactivity and PAS/AB staining within the same neoplastic cells in cases where ≥30% of cells were Syn positive (Fig. 2). These findings surprisingly showed that this unexplored neoplastic entity presented also an amphicrine phenotype. Syn-positive group (≥30%), compared to ordinary CRCs, correlated with right and left colon site ($p = 0.003$), grade 2 ($p = 0.0007$), mild intratumoral lymphocyte infiltrate ($p = 0.0006$), and *BRAF* mutation ($p = 0.04$) (Tables 1, 2). As previously described for CRCs with worse prognosis, the vast majority of Syn-positive group examined was microsatellite stable (95.8%). To further study the NED of these tumors, CgA was analyzed in a set of 15 out of 27 Syn-positive cases and resulted negative in all samples. No peculiar Ki-67 staining distribution was observed among the tumors or according to the Syn positivity.

Univariate and Multivariate Analyses

OS and DFS in univariate and multivariate analyses are reported in Tables 3 and 4, respectively, and graphically represented in Figures 3 and 4 for Syn status. At univariate analysis, variables associated with poor OS were 10-year increase ($p = 0.001$), stage IV-III ($p = 0.001$), Syn ≥30% ($p = 0.001$), infiltrative growth edges ($p = 0.04$), and residual tumor R1–2 ($p = 0.03$), while severe peritumoral lymphocyte infiltrate associated with longer OS ($p = 0.04$).

At multivariable analysis, only 10-year increase, stage IV-III, and Syn ≥30% ($p < 0.001$) remained significantly associated with poor OS and severe peritumoral lymphocyte infiltrate with longer OS ($p = 0.02$). Furthermore, comparable results were obtained with the same variables according to DFS, except for the right colon site ($p = 0.04$) associated with longer DFS and *KRAS* mutation ($p = 0.04$) associated with poor DFS. Therefore, in terms of both OS and DFS, Syn immunohistochemical expression ≥30% in the conventional CRC proved to be an independent negative prognostic factor. Regarding molecular analyses, no single gene alterations analyzed using NGS were statistically associated with OS.

Survival of True MiNENs Compared to CRCs and to Synaptophysin-Positive Group

We finally compared the survival characteristics of ordinary CRCs and *Syn-positive group* to MiNENs (summarized in Table 5). MiNENs showed a significantly worse clinical outcome compared to *Syn-positive group* in both univariate (DFS, $p < 0.0001$; Table 6) and multivariate analyses (DFS, $p < 0.0001$; Table 6) (Fig. 5).

Table 3. Univariate and multivariate analysis of OS of patients with CRCs

Variable	Univariate HR (95% CI)	*p* value	Multivariable model HR (95% CI)	*p* value
Sex (male vs. female)	0.84 (0.52–1.33)	0.45		
Age (10-year increase)	1.39 (1.14–1.70)	**0.001**	1.46 (1.18–1.80)	<**0.001**
Stage (IV-III vs. I–II)	2.25 (1.37–3.71)	**0.001**	2.39 (1.43–4.00)	<**0.001**
Site				
Sigma rectum	1.00			
Left colon	0.75 (0.32–1.77)	0.51		
Rectum	0.73 (0.36–1.46)	0.37		
Right colon	0.94 (0.49–1.79)	0.85		
Sigma	0.47 (0.11–2.04)	0.31		
Colon, unknown site	0.94 (0.28–3.26)	0.93		
Syn expression (Syn ≥30% vs. conventional CRC)	4.76 (2.04–11.1)	**0.001**	4.24 (1.78–10.23)	<**0.001**
Vascular invasion				
Absent	1.00			
Both	1.65 (0.91–2.99)	0.10		
Only extra	2.52 (0.57–11.1)	0.22		
Only intra	0.86 (0.37–1.96)	0.71		
Perineural invasion (absent vs. present)	1.53 (0.92–2.55)	0.11		
Intratumoral lymphocyte infiltrate				
Absent	1.00			
Mediated	0.40 (0.17–0.98)	0.05		
Mild	0.76 (0.77–1.73)	0.52		
Severe	0.61 (0.21–1.82)	0.37		
Growth edges (infiltration vs. expansive)	1.94 (1.04–3.65)	**0.04**		
Crohn's-like lymphoid reaction (present vs. absent)	0.97 (0.59–1.61)	0.91		
Peritumoral lymphocyte infiltrate				
Absent	1.00		1.00	
Mediated	0.68 (0.29–1.64)	0.39	0.55 (0.23–1.35)	0.19
Mild	0.75 (0.30–1.89)	0.54	0.58 (0.23–1.48)	0.25
Severe	0.32 (0.11–0.96)	**0.04**	0.26 (0.08–0.81)	**0.02**
Residual tumor (R1–2 vs. R0)	2.31 (1.09–4.91)	**0.03**		
Adjuvant therapy (yes vs. no)	0.70 (0.32–1.49)	0.35		
MMR IHC (proficient vs. deficient)	0.70 (0.21–2.31)	0.56		
TP53 (Mut vs. WT)	1.02 (0.20–5.29)	0.98		
KRAS (Mut vs. WT)	1.31 (0.62–2.78)	0.48		
NRAS (Mut vs. WT)	2.60 (0.47–14.5)	0.28		
BRAF (Mut vs. WT)	2.22 (0.73–6.76)	0.16		
APC (Mut vs. WT)	0.75 (0.14–4.09)	0.74		

HR, hazard ratio; CI, confidence interval; Syn, synaptophysin; CRC, colorectal carcinoma; R, residual; MMR, mismatch repair; IHC, immunohistochemistry; WT, wild type; Mut, mutated.

Discussion

According to the current WHO guidelines, MiNENs are rare, accounting for about 1–2% of all colorectal malignancies in the reported literature [1, 2]. The huge spectrum of mixed neoplastic forms include also unknown categories of amphicrine tumors, characterized by the coexistence of both ordinary glandular and neuroendocrine features within the same neoplastic cell [17, 18]. Despite the NED in CRCs has been previously

Table 4. Univariate and multivariate analysis of DFS of patients with CRCs

Variable	Univariate HR (95% CI)	*p* value	Multivariable model HR (95% CI)	*p* value
Sex (male vs. female)	0.91 (0.62–1.35)	0.65		
Age (10-year increase)	1.24 (1.06–1.46)	**0.009**	1.23 (1.04–1.46)	**0.02**
Stage (IV-III vs. I–II)	1.85 (1.23–2.76)	**0.003**	1.83 (1.22–2.77)	**0.004**
Site				
Sigma rectum	1.00			
Left colon	0.53 (0.26–1.09)	0.09		
Rectum	0.80 (0.47–1.36)	0.41		
Right colon	0.55 (0.32–0.96)	**0.04**		
Sigma	0.15 (0.02–1.10)	0.06		
Colon, unknown site	0.52 (0.16–1.73)	0.29		
Grade (3 vs. 1–2)	1.22 (0.78–1.90)	0.39		
Syn expression (Syn ≥30% vs. ordinary CRC)	3.56 (1.64–7.71)	**0.001**	2.95 (1.33–6.53)	**0.008**
Vascular invasion				
Absent	1.00			
Both	1.34 (0.83–2.16)	0.23		
Only extra	2.51 (0.76–8.34)	0.13		
Only intra	0.90 (0.48–1.72)	0.76		
Perineural invasion (absent vs. present)	1.37 (0.91–2.08)	0.13		
Intratumoral lymphocyte infiltrate				
Absent	1.00			
Mediated	0.49 (0.21–1.17)	0.11		
Mild	1.41 (0.64–3.09)	0.39		
Severe	0.76 (0.27–2.17)	0.61		
Growth edges (infiltration vs. expansive)	1.69 (1.04–2.75)	**0.04**		
Crohn's-like lymphoid reaction (present vs. absent)	0.86 (0.56–1.32)	0.49		
Peritumoral lymphocyte infiltrate				
Absent	1.00		1.00	
Mediated	0.63 (0.30–1.35)	0.24	0.59 (0.27–1.27)	0.18
Mild	0.80 (0.37–1.75)	0.58	0.71 (0.32–1.58)	0.40
Severe	0.30 (0.11–0.77)	**0.01**	0.28 (0.11–0.74)	**0.01**
Residual tumor (R1–2 vs. R0)	2.95 (1.69–5.14)	**0.0001**		
Adjuvant therapy (yes vs. no)	1.18 (0.69–2.04)	0.54		
MMR IHC (proficient vs. deficient)	0.72 (0.28–1.81)	0.48		
TP53 (Mut vs. WT)	1.34 (0.42–4.28)	0.62		
KRAS (Mut vs. WT)	1.85 (1.03–3.30)	**0.04**		
NRAS (Mut vs. WT)	0.43 (0.05–3.47)	0.43		
BRAF (Mut vs. WT)	1.54 (0.67–3.54)	0.31		
APC (Mut vs. WT)	1.56 (0.52–4.71)	0.43		

HR, hazard ratio; CI, confidence interval; Syn, synaptophysin; CRC, colorectal carcinoma; R, residual; MMR, mismatch repair; IHC, immunohistochemistry; WT, wild type; Mut, mutated.

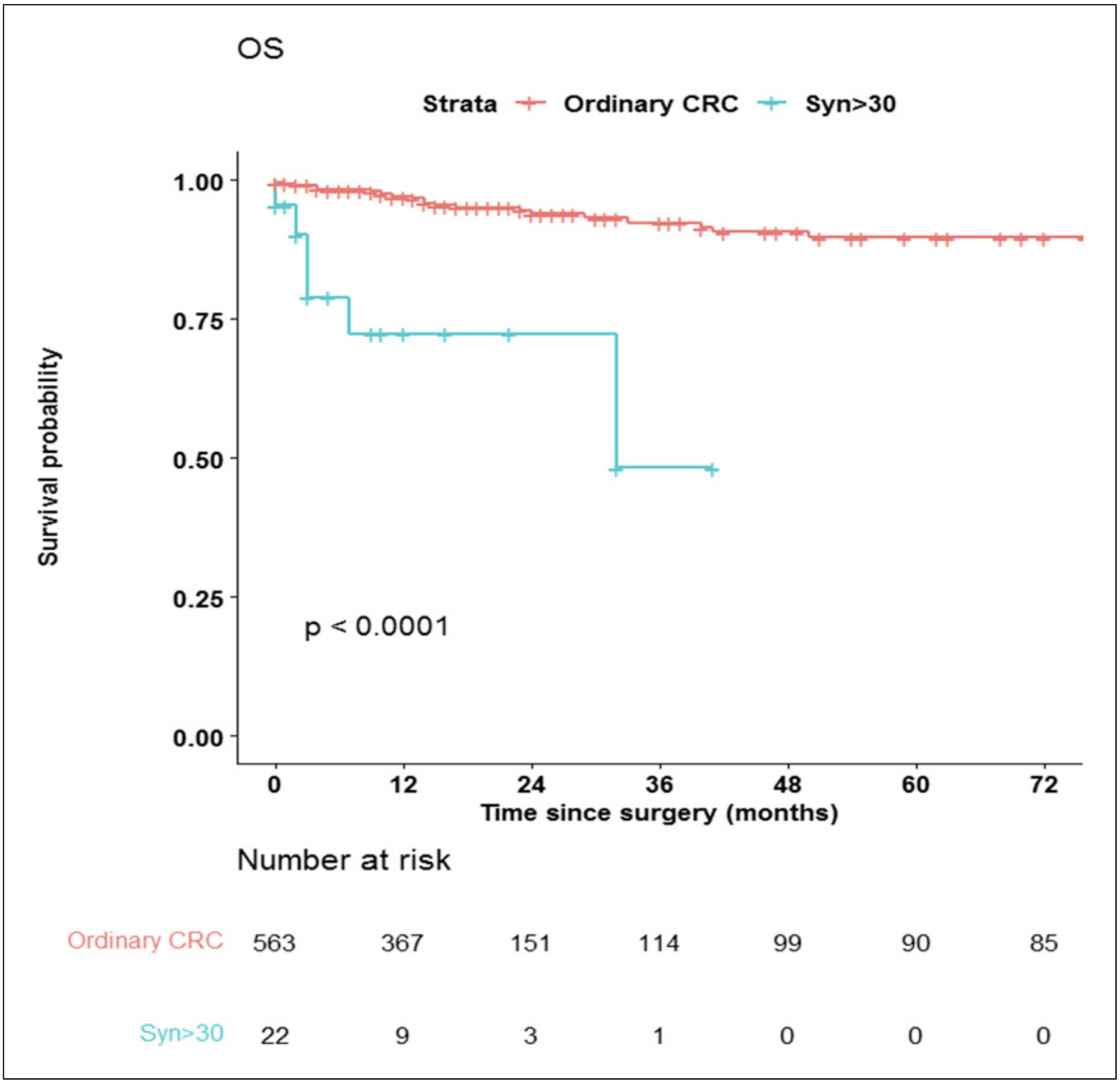

Fig. 3. Overall survival (OS) in colorectal cancers (CRCs) according to synaptophysin (Syn) expression.

assessed [19–21], at present, no clear definitions nor strong and reproducible criteria are available for defining this rare histotype. Since the 90s, Capella et al. [18] showed that the identification of neuroendocrine cells in CRCs ensured a worse prognosis than ordinary ones. Moreover, CRCs with NED showed poorer response to classical chemotherapy regimens [8, 9].

WHO classification of digestive system tumors proposed as general neuroendocrine markers chromogranin A (CgA) and/or Syn, underlining that CgA expression decreases progressively with the loss of a clear neuroendocrine histotype [2]. We focused our study exclusively on Syn staining, which is preserved also in poorly differentiated NECs [20, 22].

Among 663 ordinary CCRs, we defined "*Syn-positive group*" only cases characterized by a ≥30% Syn immunoreactivity. We demonstrated a worse OS and DFS in the 27 Syn ≥30% patients in comparison with conventional CRC patients. Our results further confirm the evidence that the Syn expression in CRCs is associated with an adverse prognosis. In CRC, the presence of neuroendocrine cells in the primary tumor was associated with shorter survival [21]. A total of 151 cases of patients with stage II CRC were assayed for Syn and CgA using IHC, following which patients were classified as NED (+) or NED (−). For the Syn (+) group, the 5-year survival was 60%, whereas it was 90% for the Syn (−) group. Liu et al. [21] demonstrated that NED was revealed to be an

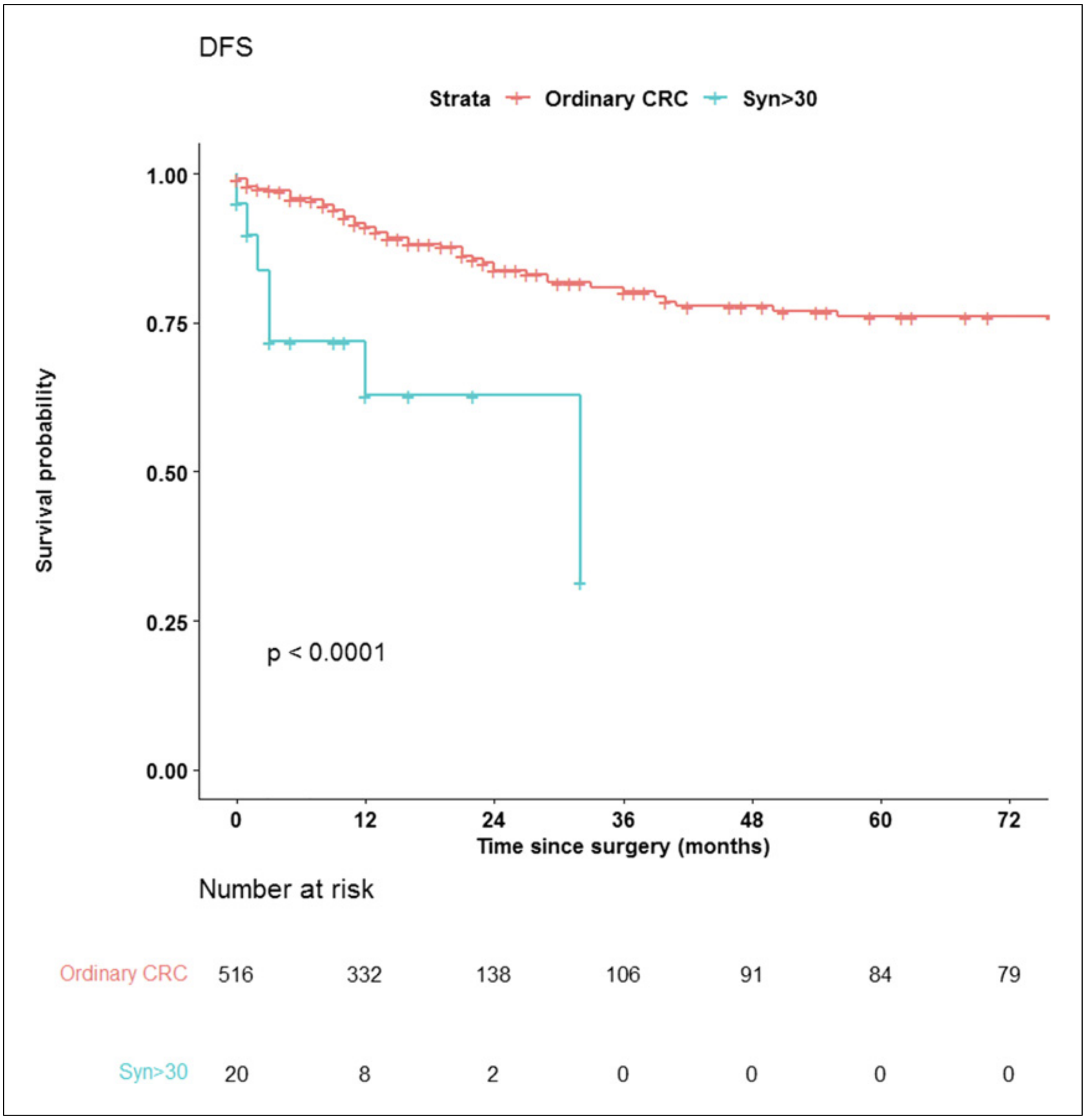

Fig. 4. Disease-free survival (DFS) in colorectal cancers (CRCs) according to synaptophysin (Syn) expression.

independent factor of poor prognosis for patients with stage II CRC.

Of note, NED was historically described in *BRAF*mt CRCs at widely variable frequencies, ranging from 5% to 51.5% [23], and *BRAF* has been described to be frequently mutated in poorly differentiated neuroendocrine CRCs [24–26]. Idrees et al. [26] hypothesized that *BRAF* mutations may be a common oncogenic driver in NECs and therefore sequenced *BRAF* exons 11 and 15, encoding the BRAF kinase domain, in an additional seven colonic NECs and demonstrated that 44% of analyzed tumors harbored *BRAF* mutations. Lee and Sung [27] examined a total of 30 colorectal NECs, using targeted NGS of a 382 gene panel, and showed high rates of *BRAF* mutation (7/30, 23%), predominantly p.V600E (5/7, 71%). Compared with poorly differentiated conventional adenocarcinoma, poorly differentiated NECs frequently harbored *BRAF* mutations (59% vs. 5%; $p < 0.001$) and are associated with poor OS ($p < 0.001$) [25]. These findings suggest that activating *BRAF* mutations are common within gastrointestinal NECs and treating these patients with BRAF/MEK inhibitor therapy is worth further clinical investigation. The series by Klempner et al. [24] constitutes the largest study on genomic profiling of

Table 5. Characteristics of patients with CRC according to synaptophysin expression ≥30% and MiNEN

	All patients	Conventional CRC	Syn-positive CRC	MiNEN	*p* value^1	Pairwise comparisons		
						p value^1 conventional CRC versus Syn ≥30%	*p* value^1 conventional CRC versus MiNEN	*p* value^1 Syn ≥30% versus MiNEN
Total	677 (100)	636 (100)	27 (100)	14 (100)				
Gender								
Female	299 (44.2)	282 (44.3)	12 (44.4)	5 (35.7)				
Male	378 (55.8)	354 (55.7)	15 (55.6)	9 (64.3)	0.85	1.00	0.59	0.74
Years								
Median [range]	66 [20–91]	66 [20–91]	67 [33–86]	64.5 [50–82]	0.66	0.54	0.75	0.53
Stage								
I–II	338 (51.4)	325 (52.7)	11 (40.7)	2 (14.3)				
III–IV	320 (48.6)	292 (47.3)	16 (59.3)	12 (85.7)	**0.007**	0.24	**0.005**	0.16
Grade								
Grade 1	10 (1.6)	10 (1.7)	0 (0.00)	0 (0.00)		G1 versus G2: 1.00	G1 versus G2: 1.00	G1 versus G2: 1.00
Grade 2	435 (68.2)	409 (68.0)	25 (100.0)	1 (8.3)		G1 versus G3: 1.00	G1 versus G3: 1.00	G1 versus G3: 1.00
Grade 3	193 (30.3)	182 (30.3)	0 (0.00)	11 (91.7)	<**0.0001**	G2 versus G3: **0.0002**	G2 versus G3: <**0.0001**	G2 versus G3: <**0.0001**
MMR IHC								
Proficient	410 (86.9)	380 (86.2)	23 (95.8)	7 (100.0)				
Deficient	62 (13.1)	61 (13.8)	1 (4.2)	0 (0.0)	0.32	0.23	0.60	1.00
Mutation								
TP53								
WT	24 (28.6)	19 (30.6)	3 (25.0)	2 (20.0)				
Mut	60 (71.4)	43 (69.4)	9 (75.0)	8 (80.0)	0.86	1.00	0.71	1.00
KRAS								
WT	105 (55.6)	82 (51.9)	13 (65.0)	10 (90.9)				
Mut	84 (44.4)	76 (48.1)	7 (35.0)	1 (9.1)	**0.02**	0.34	**0.01**	0.20
NRAS								
WT	137 (95.8)	114 (95.8)	13 (100.0)	10 (90.9)				
Mut	6 (4.2)	5 (4.2)	0 (0.0)	1 (9.1)	0.45	1.00	0.42	0.46
BRAF								
WT	155 (85.6)	132 (86.8)	12 (66.7)	11 (100.0)				
Mut	26 (14.4)	20 (13.2)	6 (33.3)	0 (0.0)	**0.03**	**0.04**	0.36	0.058
APC								
WT	50 (60.2)	34 (55.7)	9 (75.0)	7 (70.0)				
Mut	33 (39.8)	27 (44.3)	3 (25.0)	3 (30.0)	0.44	0.34	0.50	1.00

CRC, colorectal carcinoma; Syn, synaptophysin; MiNEN, mixed neuroendocrine-non-neuroendocrine neoplasm; MMR, mismatch repair; IHC, immunohistochemistry; WT, wild type; Mut, mutated. ^{1}*p* value based on the Fisher's exact for categorical and *t* test for continues variables.

advanced high-grade colorectal neuroendocrine tumors, offering strong evidence that BRAF(V600E) serves as an oncogenic driver responsive to BRAF-MEK combination therapy in this molecular subset.

Several studies demonstrated that *BRAF*mt CRCs constitute a different clinicopathological subgroup with specific molecular features [28]. The occurrence of *BRAF* mutation is a well-established negative prognostic marker

Table 6. Univariate and multivariate analysis of DFS of patients with CRCs according to synaptophysin expression ≥30% and MiNEN

Variable	Univariate HR (95% CI)	*p* value	Multivariable model HR (95% CI)	*p* value
Sex (male vs. female)	1.00 (0.69–1.46)	0.99		
Age (10-year increase)	1.22 (1.05–1.43)	**0.01**	1.22 (1.04–1.44)	**0.02**
Stage (IV-III vs. I–II)	1.96 (1.33–2.89)	**0.0007**	1.76 (1.18–2.63)	**0.005**
Grade (3 vs. 1–2)	1.22 (0.78–1.90)	0.39		
Morphology				
Conventional CRC	1.00		1.00	
Syn ≥30%	3.58 (1.65–7.77)	**0.001**	3.29 (1.51–7.16)	**0.003**
MiNEN	5.04 (2.43–10.42)	<**0.0001**	4.27 (2.02–9.03)	**0.0001**

HR, hazard ratio; CI, confidence interval; CRC, colorectal carcinoma; Syn, synaptophysin; MiNEN, mixed neuroendocrine-non-neuroendocrine neoplasm.

in mCRC and patients with this alteration typically show limited response to treatment regimens involving multikinase inhibitors [29–31].

Digiacomo and colleagues [23] reported a high prevalence of NED (29.1%), in BRAFmt mCRCs, defined only through Syn immunohistochemical staining from score ≥1. Recently, Fassan et al. [10] evaluated the incidence, clinical, pathological, and molecular features of tumors according to their Syn expression levels and explored the prognostic value of Syn status in a large series of *BRAF*-mutated mCRC in terms of PFS and OS. An available archival FFPE tissue sample (primary tumor or distant metastatic site) and matched clinical and molecular data of 159 p.V600 *BRAF*mt mCRC patients referred to eight Italian oncology units were collected within the frame of the "*BRAF*-Be-Cool" study. Both multivariate and univariate analyses revealed that high Syn expression, among *BRAF*mt mCRCs, was a negative independent prognostic factor for PFS (HR = 2.00, 95% CI 1.21–3.33, $p = 0.006$) and OS (HR = 2.27, 95% CI 1.35–3.85, $p = 0.001$) [10]. Syn expression in p.V600E *BRAF*mt advanced CRCs identifies a new subgroup of tumors with a worse prognosis. In agreement with above molecular analyses, our preliminary molecular results demonstrated that the Syn-positive group correlated with a high prevalence of *BRAF* mutations ($p = 0.04$), in comparison to conventional adenocarcinomas.

Moreover, our results incidentally explored the detection of amphicrine phenotype in 27 selected Syn ≥30% positive cases through a further co-staining with Syn and AB, showing an evident coexistence of neuroendocrine phenotype and mucous secretion and in general with glandular differentiation. Fassan et al. [10] initially described an evident coexistence of Syn positivity and the presence of mucous secretion within the tumor cells, by co-staining a limited part of the cohort tumors with Syn and AB.

We finally compared the survival characteristics of conventional CRCs and Syn expression groups to colorectal MiNENs. According to recent observations of Konukiewitz et al. [32], we demonstrated that true colorectal MiNENs, on the other hand, showed a significantly shorter survival than all conventional adenocarcinomas with or without Syn expression in uni- and multivariate analyses (e.g., multivariate DFS: $p < 0.001$). This result suggests that the neuroendocrine morphology still drives the prognosis in mixed CRCs.

Amphicrine tumors have been described in both digestive and extra-digestive systems and their relationship with MiNENs has long been debated [33, 34]. Recent findings including morphological, immunostaining, and transcriptome investigations suggest that amphicrine phenotype is a specific entity with biological and histological features distinct from those observed either in adenocarcinomas or neuroendocrine neoplasms [35, 36]. Using this recommendation, even the last 2022 WHO classification of neuroendocrine tumors advises us to distinguish amphicrine tumors from MiNENs as it does not fulfill the diagnostic criteria of mixed forms with absence of two neuroendocrine and non-neuroendocrine distinct and recognizable populations of neoplastic cells [37–39].

However, some limitations should be recognized concerning our study. First, the small number of Syn-positive cases that further underline the need for a multicentric retrospective study in order to better explore the characteristics of this rare entity. Second, data concerning mutations were extracted from molecular

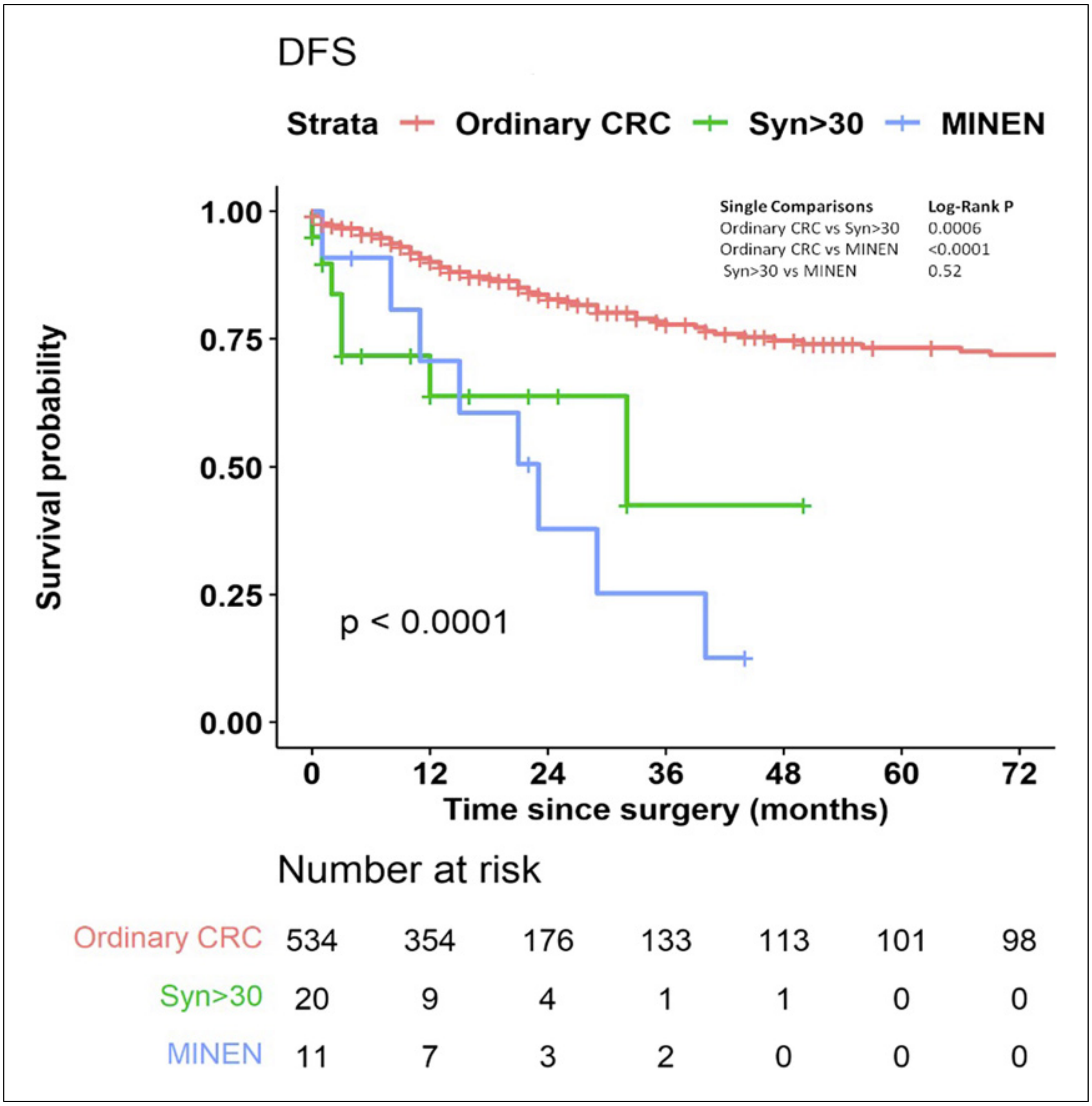

Fig. 5. Disease-free survival (DFS) in MiNEN group compared to conventional colorectal cancers (CRCs) and to "synaptophysin (Syn) positive group."

analyses performed for therapy decision-making. For this reason, further molecular characterization is underway to better dissect this rare unknown entity and to allow a patient-specific targeted therapeutic approach.

Conclusions

Our study explored the real gray zone between pure neuroendocrine and pure non-neuroendocrine neoplasms existing in the literature, investigating the clinicopathological and molecular features of an unexplored neoplastic entity. At multivariable analysis, we provided evidence that Syn expression in conventional colorectal adenocarcinomas is associated with worse OS and DFS and contributes to predicting clinical outcomes of CRCs. Our findings differ from those reported by Konukiewitz et al. [32], who demonstrated that Syn expression in this context was associated with only reduced DFS ($p = 0.037$). However, they found no significant association with OS or disease-specific survival in univariate analyses, nor any impact on survival outcomes in multivariate analyses. On the contrary, according to observations of Konukiewitz et al. [32], we demonstrated that patients with "true"

MANECs, on the other hand, showed a significantly shorter survival than all conventional adenocarcinomas with or without Syn expression. Our preliminary data suggest that neuroendocrine morphology in CRCs remains a key determinant of prognosis. Additionally, we underscore the importance of neuroendocrine markers detection in conventional CRC without neuroendocrine morphology, in order to provide detailed information on CRCs prognosis. Therefore, this heterogeneous group deserves further molecular characterization to allow a patient-specific targeted therapeutic approach.

Statement of Ethics

This study was performed according to the clinical standards of the 1975 and 1983 Declaration of Helsinki and was approved by the Ethical Committee of Fondazione IRCCS Istituto Nazionale dei Tumori (INT), Milan, Italy (No. INT 21/16). All patients had signed written informed consent for using their data for research purposes.

Conflict of Interest Statement

Stefano La Rosa was a member of the journal's Editorial Board at the time of submission. The other authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

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Author Contributions

Study concept and design: Carlo Capella, Massimo Milione, Giovanna Sabella, and Giovanni Centonze. Acquisition of data: Giovanni Centonze, Giovanna Sabella, Vincenzo Lagano, Carlotta Pardo, Giovanna Garzone, Daniela Galbiati, and Stefania Gobba. Analysis and interpretation of data: Giovanna Sabella, Giovanni Centonze, Carlo Capella, Massimo Milione, and Vincenzo Lagano. Drafting of manuscript: Giovanna Sabella, Giovanni Centonze, and Alessandro Mangogna. Critical revision of the manuscript for important intellectual content: Massimo Milione, Carlo Capella, Stefano La Rosa, Silvia Uccella, Matteo Fassan, Alessandro Mangogna, Andrea Scardino, Filiberto Belli, Valentina Angerilli, Fausto Sessa, Andrea Vingiani, Sara Pusceddu, Luca Agnelli, and Giancarlo Pruneri. Statistical analysis: Giovanni Centonze. Study supervision: Massimo Milione and Carlo Capella.

Data Availability Statement

The data supporting this study's findings are not publicly available because they contain information that could compromise the privacy of research participants. Still, they are available on request from the corresponding author, Massimo Milione.

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