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TUMOR STAGING BUT NOT GRADING IS ASSOCIATED TO ADVERSE CLINICAL OUTCOME IN NEUROENDOCRINE TUMORS OF THE APPENDIX: A RETROSPECTIVE CLINICAL PATHOLOGICAL ANALYSIS OF 138 CASES.

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ABSTRACT

Appendiceal neuroendocrine neoplasms are rare and usually incidentally discovered. Most cases are clinically indolent although the rare aggressive ones are poorly predictable. The aim of this study was to test the applicability and prognostic significance of the new WHO classification, as well as several pathological features and TNM staging systems (American Joint Committee on Cancer -AJCC, and European NEuroendocrine Tumor Society - ENETS) in these tumors. A multi-institutional retrospective series of 138 appendiceal neuroendocrine neoplasms was selected based on the availability of both pathological material and clinical information, including follow up. All cases were reviewed to record pathological features and to apply year 2000 and 2010 WHO classifications, as well as ENETS and AJCC TNM stages. Clinical and pathological characteristics were compared to disease outcome by contingency, univariate and multivariate survival analyses. Although up to one third of cases presented several malignancy-associated pathological features, only four patients died of the disease. Adverse outcome was significantly associated with extra-mural extension (including mesoappendix), well-differentiated carcinoma diagnosis (2000 WHO classification), pT3-4 stage, older age and presence of positive resection margins, but not with tumor size, mitotic or proliferative indexes, and - consequently - 2010 WHO grading. In the appendix, at variance with midgut/hindgut neuroendocrine neoplasms, the 2000 WHO classification performs better than the grading-based 2010 WHO scheme, and together with tumor stage, is the most relevant parameter associated to clinical aggressiveness.

KEY WORDS: Appendix; carcinoid; neuroendocrine tumor; prognosis; staging.

INTRODUCTION

Neuroendocrine neoplasms of the appendix are rare and in general incidentally found in the tip or body of the appendix after appendectomy for inflammatory processes. Data from Surveillance, Epidemiology and End Results (SEER) databases in the US set their incidence in the general population at 0.1-0.2/100.000 per year, a figure stable along the last 30 years, with a slight female predominance and a mean age of 48 years.[1,2] Similarly, recent data from European registries reported an incidence of 0.08/100.000 with a median age of 38 years.[3] In surgical case series, the occurrence of neuroendocrine neoplasms is encountered in less than 0.5% of all appendectomies, although this tumor type represents the most frequent neoplasm in this location[4-9] and the second most frequent neuroendocrine neoplasm within the gastrointestinal tract.[3,10]

The prognosis is generally good with a reported 5 year survival varying from 88% to 94% in patients with localized disease, 78% to 84% in patients with regional dissemination, and 25% to 26% in patients with distant metastases.[1,2,11] However, survival data from SEER databases probably over-estimate the clinical aggressiveness of appendiceal neuroendocrine neoplasms due to their malignancy-based selection criteria. On the other hand, the rare cases that follow an aggressive course with local recurrence and/or distant metastases are poorly predictable at time of diagnosis. Tumor size is generally considered a major predictor of prognosis, since cases less than 1 cm almost invariably have favorable outcome, whereas those with a diameter exceeding 2 cm are associated to a 5-year mortality of 29%.[11] More controversial prognostic factors include lympho-vascular invasion, subserosal invasion, extension to the mesoappendix and infiltration of the appendiceal base. Even the presence of lymph node metastases does not seem to be associated to a different 10 year survival rate, irrespective of the tumor size.[12]

On the search for the most appropriate pathological characterization and prognostication of appendiceal neuroendocrine neoplasms, minimal requirements for pathological reporting in these tumors have been proposed, but the real impact of all such information is far from being established.[11,13] The evaluation of mitotic and proliferative indexes has been recommended[11,13,14] and, as a matter of fact, is now part of the WHO classification scheme of gastrointestinal neuroendocrine neoplasms[15], but their clinical usefulness in appendiceal neuroendocrine neoplasms has not been tested, yet. Moreover, two different staging systems exist, one proposed by the European NEuroendocrine Tumor Society (ENETS) in 2007[16] and the other recently proposed by the American Joint Committee on Cancer (AJCC)[17] and incorporated in the 2010 WHO classification.[15] Although in both classifications the "T" category is defined by tumor size and depth of invasion, the two systems differ considerably in the cut-off values and classification rules with a major shift between "organ confined" and "locally advanced" stages in a relevant number of case.

As a consequence, the best therapeutic strategy for these tumors is not well established. In fact, in pediatric populations the rate of patients cured by appendectomy is approaching 100%[18,19], thus questioning, according to some authors, the real need of routinely examining appendiceal specimens[20]. Conversely, the recent consensus guidelines from ENETS[21] and North American NEuroendocrine Tumor Society (NANETS)[11] claimed the need for right hemicolectomy in a relevant proportion of cases (i.e. those with a tumor size above 2 cm, accounting for up to 17% of all cases).

Based on the foregoing, a retrospective study was designed with the aim of testing the applicability and prognostic significance of the previous[22] and current[15] WHO classification schemes, of different pathological parameters and TNM staging systems (2010 WHO/AJCC and ENETS 2007), to determine the most clinically relevant parameters for the pathological characterization of appendiceal neuroendocrine neoplasms.

MATERIALS AND METHODS

Case selection.

A database search including topography "appendix" and Systematized Nomenclature of Medicine (SNOMED) diagnoses "carcinoid" and "neuroendocrine carcinoma" was performed in seven Pathology Units of a North-Western Italian Province in Piedmont. For all cases the availability of representative histological material was a major criterion for entering the study. A diagnosis of primary appendiceal neuroendocrine neoplasm was done in 168 unselected cases from year 1988 to 2009. Case distribution was as follows: 45 cases from the Molinette Hospital, Turin; 25 cases from the Alba Hospital; 25 cases from the Santa Croce Hospital, Moncalieri; 24 cases from the Giovanni Bosco Hospital, Turin; 19 cases from the Santa Croce and Carle Hospital, Cuneo; 15 cases from the San Luigi Hospital, Orbassano; 15 cases from the Mauriziano Hospital, Turin. In all but 21 cases, detailed clinical and pathological information including follow up data were obtained from pathological reports, clinical charts and/or register offices.

Pathological revision and immunohistochemistry.

One-hundred-forty-seven cases were entered in the study and were reviewed by the local pathologists and then re-classified centrally by two of us (LD, MV) according to the 2000 and 2010 WHO classification schemes.[15,22] The neuroendocrine phenotype was assessed at the time of original diagnosis or upon revision by means of chromogranin A (clone LK2H10, diluted 1:800; NeoMarkers, Fremont, CA, USA) and/or synaptophysin (clone Sy38, diluted 1:100, DakoCytomation, Glostrup, Denmark) immunostainings. Mitotic index was expressed as number of mitoses per 10 HPF (approximately 2 square mm) evaluating at least 50 high power fields (HPF) (or the entire tumor area in the case of small tumors). Ki-67 proliferative index was assessed in all but

19 cases (clone MIB-1, diluted 1:150, Dako) following 2010 WHO recommendations.[15] The following pathological parameters were recorded following recently proposed protocols and checklists:[13] size of the lesion, location within the appendix, percentage of appendix wall involvement, depth of invasion, extent of subserosal invasion, presence of mesoappendix invasion, presence of vascular and perineural invasion, status of the resection margin, growth pattern and pathological T stage, according to both ENETS[16] and 2010 WHO/AJCC[15,17] staging systems.

Statistical analysis.

Dichotomic clinical and pathological variables were compared with 2000 and 2010 WHO classifications and disease status using Chi-square and Fisher's exact tests, whereas continuous variables were compared using the *t* or ANOVA tests. Spearman's correlation was used to correlate tumor grade evaluated as mitotic or proliferative indexes. Disease-related survival curves were computed using the Kaplan-Meier method and compared using the Log-rank test in univariate analysis. All parameters with a borderline or significant impact on survival at univariate analysis were considered for multivariate analysis using the Cox proportional hazard model. Statistical significance was set at $p<0.05$. All tests were performed using GraphPad version 5.0 and STATISTICA version 7.0 softwares.

RESULTS

Classification and clinical data.

Nine cases with goblet cell carcinoid features were re-classified as mixed adeno-neuroendocrine carcinomas, and according to the new 2010 WHO classification were excluded from further analysis.

Of 138 remaining cases, 59 were classified as well-differentiated endocrine tumors - benign, 34 as well-differentiated endocrine tumors - uncertain behavior and 45 as well-differentiated endocrine carcinomas according to the 2000 WHO nomenclature. Moreover, based on the new 2010 WHO classification, 120 cases were graded as neuroendocrine tumor - G1 and 18 cases as neuroendocrine tumor - G2. A comparison of case distribution within the two classification schemes is illustrated in **Figure 1**. No case was diagnosed as poorly-differentiated/neuroendocrine carcinoma - G3.

In 17 cases, a predominant tubular growth was observed with occasionally associated Paneth-like cells (**Figure 2a**), whereas in all other cases the growth pattern was typically organoid, with tumor cells arranged in islets or ribbons growing in a variably desmoplastic stroma (**Figure 2b**), irrespective of the depth of invasion (**Figure 2c**).

By sex distribution, 61 males and 77 females were included, having a mean age of 38 years (range 8-86, median 34). Appendectomy was the primary intervention in all but 15 cases that directly underwent hemicolectomy (10 cases), or appendectomy concurrent to hysterectomy with oophorectomy (4 cases) or cystectomy (1 case) for other diseases. Right hemicolectomy followed appendectomy in another four patients, all having an original diagnosis of well-differentiated endocrine carcinoma according to 2000 WHO classification. In four patients, information of surgical procedures possibly performed after appendectomy were not available. Specific nodal status was assessed in the 14 patients treated with hemicolectomy, and all were staged pN0. Concurrent or subsequent associated neoplastic diseases included adenocarcinoma originated from colon (10 cases), endometrium (2 cases), biliary tract (2 cases) and stomach (1 case), squamous cell carcinomas from lung and uterine cervix (1 case, each), bladder urothelial carcinoma (1 case) and serous papillary ovarian carcinoma (1 case). Mean follow up time was 86.5 months (range 1-267). Overall, 24 patients had died, but only four patients died of their

appendiceal tumor. Specific surgical procedures for these cases were appendectomy only in two cases, primary hemicolectomy in one case, appendectomy and subsequent hemicolectomy in the latter case. Due to the incompleteness of clinical information regarding tumor relapse and disease status in two/thirds the patients alive at last follow up, disease-free and time to progression survival analyses could not be performed.

Clinical pathological correlations and survival analysis.

Clinical and pathological parameters were compared with the two different WHO classification systems (**Table 1**). Tumor groups defined according to the 2000 WHO classification were different in terms of tumor size, extent of appendiceal wall involved, presence of positive resection margins, mean proliferation index and tumor grade. The groups of neuroendocrine tumor - G1 and neuroendocrine tumor - G2, as defined by the 2010 WHO classification, differed in terms of age, tumor size, percentage of appendiceal wall involved, depth of invasion, presence of mesoappendix infiltration, presence of vascular and perineural invasion.

Disease-related survival analysis was mostly limited by the small number of cases dead of disease (responsible for the extremely wide 95% confidential intervals), although this limitation emphasizes that fatal outcome is a very rare event in appendiceal neuroendocrine neoplasms, even in advanced stages. Univariate survival analysis (**Table 2**) showed that the invasion of or beyond subserosa and of the mesoappendix impacted on survival, whereas tumor size *per se* did not, either grouped according to the mean value or using 1 cm as the cut off. Cases having a size of >2 cm were all alive at the time of follow up. Moreover, the presence of positive resection margins and age above the median value were associated to a shorter survival. A trend to significance was also observed for female sex and presence of vascular invasion. By contrast, neither mitotic count nor proliferation index were associated to the risk of disease-specific death. As a sub-analysis of

these latter parameters, we separately compared the 2010 WHO tumor grade as defined by mitotic count or Ki-67 index (the distribution of these parameters is illustrated in **Figure 3**): although significantly correlated ($p<0.0001$), the Spearman's Rho coefficient was only moderate (0.394) due to 18 discordant cases.

When comparing WHO classification schemes at contingency and univariate survival analyses, only the 2000 WHO classification was associated to prognosis in our series (**Table 3**) (**Figure 4, left**). However, the most significant prognostic indicator was represented by pT stage, according to both ENETS and 2010 WHO/AJCC systems (**Figure 4, right**). Due to the very small number of patients dead of the disease, the value of median survival was not reached for any of the parameters considered and multivariate analysis was limited. In fact, among variables significant at univariate analysis, age, 2000 WHO classification, depth of invasion including mesoappendix and ENETS pT stage were not computable, since the considered parameter was present in all four patients dead of the disease. At Cox regression analysis including 2010 WHO/AJCC pT stage, status of the resection margins and size, 2010 WHO/AJCC pT stage only was statistically significant ($p=0.007$).

DISCUSSION

To our knowledge, this is the first study specifically designed on appendiceal neuroendocrine tumors to test the applicability of the novel WHO and TNM classifications of gastrointestinal neuroendocrine neoplasms, and to compare detailed pathological features with clinical outcome. Herein, we present the evidence that, despite a relatively high frequency of malignancy-related parameters, appendiceal neuroendocrine tumors are almost invariably indolent, and that pT stage but not the new 2010 WHO grading system seems to be the best predictor of adverse outcome in the small proportion of clinically aggressive cases here investigated.

The summary of clinical and pathological findings of the whole series is in line with the available literature. The slight female predominance and the mean age <40 years were comparable to other series,[2,3] as well as the distribution of size and depth of invasion.[7,12] No case of neuroendocrine carcinoma - G3 was detected in our series, to confirm the extreme rarity of poorly-differentiated cases in this location, at variance with neuroendocrine neoplasms developing in the right colon.

The study was designed first to verify the practical applicability of a wide set of parameters in the pathological characterization of appendiceal neuroendocrine neoplasms, following recent recommendations,[11,13,14] and then to test their distribution in a large retrospective case series classified according to both 2000 and 2010 WHO classifications, as compared to clinical outcome. All these features are variably included in (old) classification or staging schemes or considered of clinical value, although their distribution is poorly detailed in the literature, which is mostly based on cancer registry series without pathological (re)evaluation. The unselected nature of the present series (with the exclusion of cases lacking pathological material available for review) allowed to define the impact of their recognition and association with disease outcome more closely to the real diagnostic practice. By univariate survival analysis, extramural invasion, invasion of the mesoappendix and presence of positive resection margins were statistically associated with shorter disease-related survival. As expected, a strong impact on survival was observed also for 2000 WHO classification which is basically based on the above parameters. By contrast, neither Ki-67/mitotic indexes *per se* nor 2010 WHO grading-based classification were associated to a specific survival. In fact, three out of four cases dead of the disease were G1 tumors. It is worth to notice that mean mitotic and proliferation indexes in the whole series were quite low (0.7 and 1.9, respectively) and only 13% of cases were G2, as compared to recent data on midgut and hindgut NENs, detecting up to 32% of G2 tumors.[23] Apart from biological considerations on the low proliferative properties of appendiceal neuroendocrine neoplasms, this observation suggests that

cut off levels proposed for grading are inadequate for this particular location, as also supported by the poor correlation of individual mitotic and Ki-67 indexes in the present series, as opposed to other gastrointestinal locations.[24]

Pathological T stage according to both ENETS and AJCC was the most significant feature associated to a shorter survival: this finding is of special relevance since the two schemes are quite different and based on divergent rules for classification, being generally the former more invasion-oriented and the latter more size-oriented. However, ENETS pT stage seemed to be less specific: in fact, although all patients dead of disease were staged pT3 or 4, high tumor stage (including seven pT4 cases) was observed also in up to 25% of patients alive at the time of follow up. Moreover, though limited by the small number of patients dead of the disease, the prognostic strength of 2010 WHO/AJCC pT stage was confirmed by multivariate analysis, as compared to status of the resection margins and size. It should be outlined, however, that stage-specific analysis in this paper was limited to "T" stage due to the absence of cases with positive lymph nodes (among those that underwent node dissection). In addition, age above the median was also slightly associated with a shorter disease-related survival, a finding previously not specifically reported in the literature. Finally, we could not confirm the major prognostic significance of size, as proposed in the literature, since none of the four patients dead of their disease had a tumor size $\geq$ 2 cm.

The clinical meaning of the present findings is difficult to assess, but in general it can be suggested that the diagnostic procedures, according to the currently proposed pathological datasets for the appendiceal location, may overestimate the real clinical aggressiveness of the lesion. It is stated in both European[21] and American[11] guidelines that size >2 cm, positive resection margins, deep invasion of the mesoappendix, presence of lympho-vascular invasion and intermediate grade (G2) are all features supporting right hemicolectomy. A recent retrospective study supports this recommendation since 4 of 12 patients who underwent hemicolectomy for tumors larger than 2

cm, or with Ki-67 >2% and/or extended to mesoappendix or appendiceal base, had residual disease or nodal metastases.[25] However, our data claim that at least some of the above (namely size, vascular invasion and tumor grade) might be reconsidered in future guidelines to refine criteria for right hemicolectomy and prevent overtreatment in these patients.

In conclusion, this retrospective study describes a spectrum of appendiceal neuroendocrine neoplasms having a low grade, a variable stage but an indolent course in the vast majority of cases. In this specific location, mesoappendix invasion (and the 2000 WHO classification), positive resection margins and tumor stage were the most relevant features associated to clinical aggressiveness, whereas, at variance with midgut/hindgut tumors, the 2010 WHO grading system did not perform so well. With regard to tumor stage, it seemed that the ENETS system produced an overestimation, whereas the 2010 WHO/AJCC TNM system was more accurate and specific in selecting the very few aggressive and fatal cases.

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FIGURE LEGENDS

Figure 1. Comparison of WHO classification schemes proposed in 2000 (Solcia 2000) and 2010 (Bosman 2010). WDT-B: well differentiated endocrine tumor, benign; WDT-UB: well differentiated endocrine tumor, uncertain behavior; WDC: well differentiated endocrine carcinoma; NET: neuroendocrine tumor.

Figure 2. Morphological features of tubular (**a**) and classic appendiceal neuroendocrine tumors, the latter either when confined to the muscolar wall (**b**) or invading the mesoappendix (**c**). Hematoxylin-Eosin stains; a: original magnification 200x, b,c: original magnification 100x.

Figure 3. Distribution of mitoses (mean value in 10 HPF: 0.7) and Ki-67 (mean %: 1.9) in our series of appendiceal neuroendocrine neoplasms.

Figure 4. Disease-related survival of 138 appendiceal neuroendocrine neoplasms according to 2000 and 2010 WHO classifications and available staging systems.

Table 1. Clinical and pathological characteristics of 138 appendiceal neuroendocrine neoplasms, as compared to WHO classifications.

	WHO 2000			p	WHO 2010		p
Parameter	**WDT-B #59**	**WDT-UB #34**	**WDC #45**		**NET G1 #120**	**NET G2 #18**	
M/F ratio	28/31	10/24	23/22	0.126	53/67	8/10	1.00
Age: mean (range)	42 (8-86)	34 (8-80)	39 (10-85)	0.256	41 (8-86)	27 (8-65)	0.011
Size mm: mean (range)	4.3 (1-20)	8.7 (4-15)	12 (4-35)	<0.0001	6.7 (1-35)	13.1 (4-22)	<0.0001
Location tip	49	24	35	0.372	93	15	0.76
body	10	10	10		27	3	
Mean % of appendiceal wall involved (range)	5.6 (0.5-18)	12.2 (6-30)	17.8 (2-50)	<0.0001	9.6 (0.5-50)	20.2 (8-42)	<0.0001
Growth pattern:							
organoid	51	29	41	0.686	105	16	1.00
tubular	8	5	4		15	2	
Depth of invasion:							
mucosa/submucosa	20	0	0	//	19	1	0.045
muscular	39	4	0		41	2	
subserosa	0	30	35		53	12	
serosa	0	0	10		7	3	
Presence of mesoappendix invasion	//	//	45	//	34	11	0.013
Extension of subserosal/ mesoappendix invasion*							
≤3	//	17	17	0.223	26	8	1.00
>3		11	21		25	7	
na		5	4		6	3	
Presence of vascular invasion	//	18	29	0.358	35	12	0.003
Presence of perineural invasion	//	9	15	0.204	17	7	0.017
Presence of positive resection margins	0	1	11	<0.0001	11	1	1.00
Mean mitotic index (range)	0.43 (0-2)	0.71 (0-6)	0.95 (0-6)	0.052	0.42 (0-2)	2.27 (0-6)	//
Mean proliferation index (range)	1.29 (1-5)	1.87 (1-10)	2.45 (1-15)	0.021	1.26 (1-2)	5.28 (1-15)	//
Grade G1	58	29	33	0.0008	//	//	//
G2	1	5	12				

Legend. M: male; F: female; WDT-B: well differentiated endocrine tumor, benign; WDT-UB: well differentiated endocrine tumor, uncertain behavior; WDC: well differentiated endocrine carcinoma; NET: neuroendocrine tumor; *: numbers refer to the 75 cases only with presence of subserosal or mesoappendix invasion.

Table 2. Univariate disease-related survival analysis.

Clinical or pathological parameter	**Univariate analysis HR (95% CI)**	**p**
Female Sex	5.705 (0.786-41.41)	0.086
Age above median (34 years)	7.498 (1.055-53.28)	**0.044**
Tumor size above mean (7 mm)	3.197 (0.447-22.84)	0.247
Tumor size above 1 cm	7.566 (0.916-62.51)	0.060
Subserosal/serosal invasion	8.851 (1.238-63.3)	**0.030**
Invasion of the mesoappendix	32.74 (3.748-286)	**0.002**
Subserosa/mesoappendix invasion >3 mm	9.541 (0.978-93.09)	0.052
Presence of vascular invasion	7.303 (0.89-59.87)	0.064
Presence of perineural invasion	1.716 (0.128-22.93)	0.683
Positive resection margins	377.6 (9.6-14844)	**0.002**
Mitotic index ≥ 1	0.501 (0.069-3.656)	0.495
Proliferative index ≥2	0.626 (0.081-4.845)	0.654

Table 3. Correlation between WHO classifications and available T stages with disease status and disease-related overall survival.

Parameter	Disease status		p	Univariate analysis HR (95% CI)	p
	alive/DOC #134	DOD #4			
WHO 2000					
WDT (B/UB)	93	0		/	
WDC	41	4	**0.01**	25.24 (3.031-210.2)	**0.003**
WHO 2010					
G1	117	3		/	
G2	17	1	0.432	2.865 (0.156-52.55)	0.479
ENETS pT stage					
1-2	100	0		/	
3-4	34	4	**<0.0001**	60.91 (6.203-598.1)	**0.0004**
AJCC pT stage					
1-2	130	1		/	
3-4	4	3	**<0.0001**	$9.535x10^6$ ($88293-1.03x10^9$)	**<0.0001**

Legend. WDT: well differentiated endocrine tumor; B: benign; UB: uncertain behavior; WDC: well differentiated endocrine carcinoma; DOC: dead of other causes; DOD: dead of disease.

Figure 1

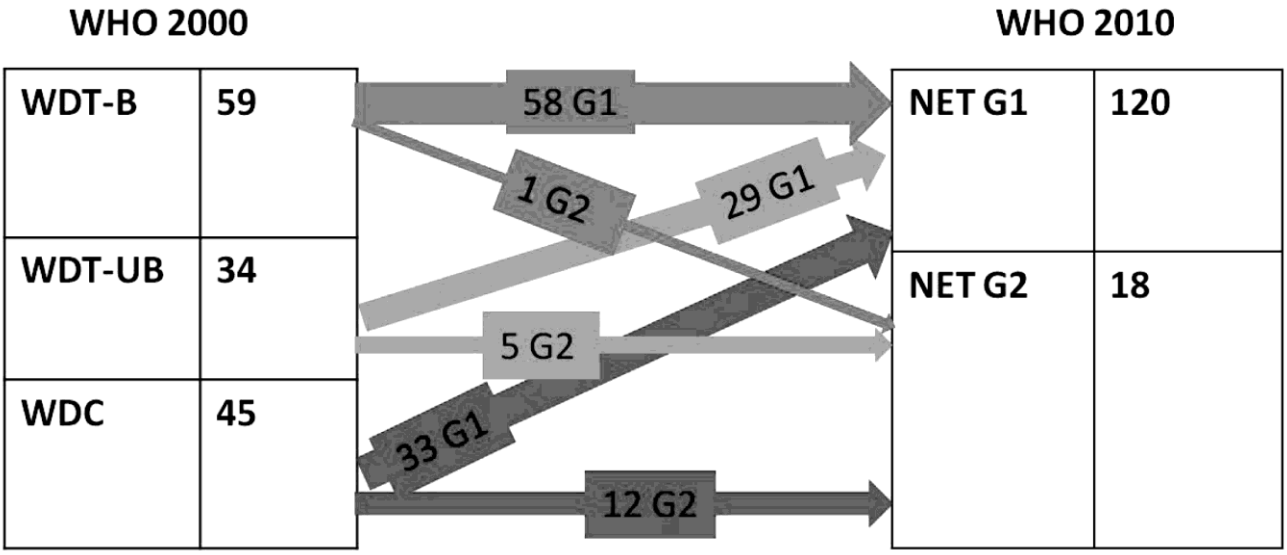

WHO 2000
WHO 2010
WDT-B
59
WDT-UB
34
WDC
45
NET G1
120
NET G2
18
58 G1
1 G2
29 G1
5 G2
33 G1
12 G2

Figure 2

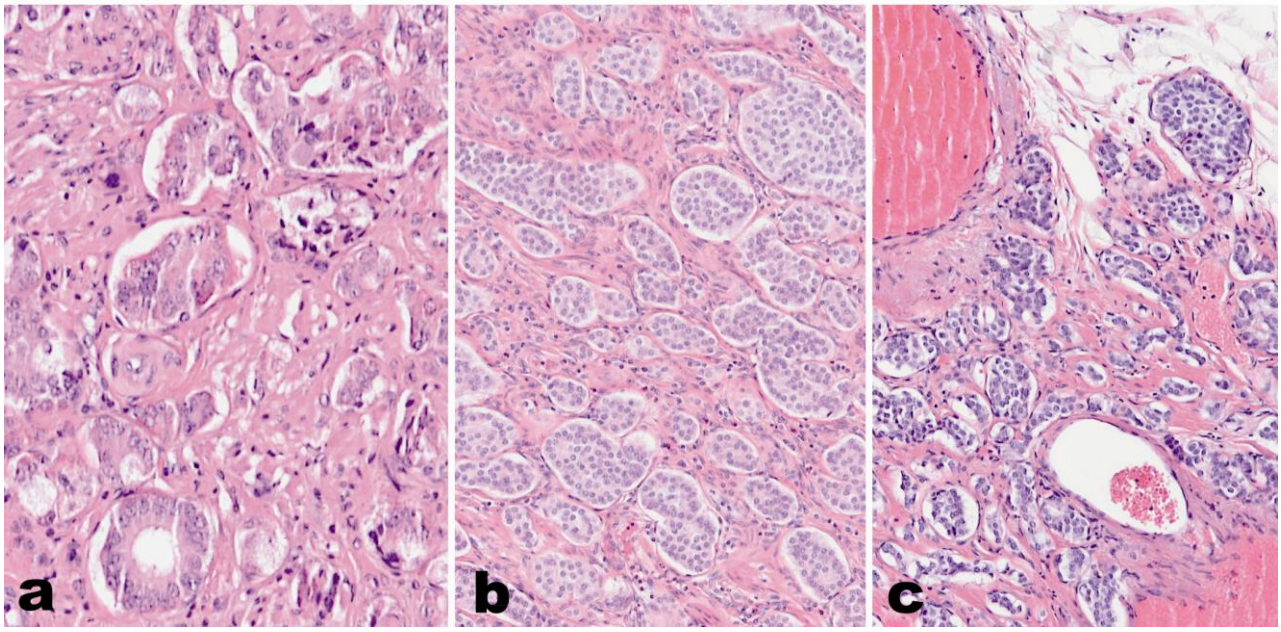


Figure 3

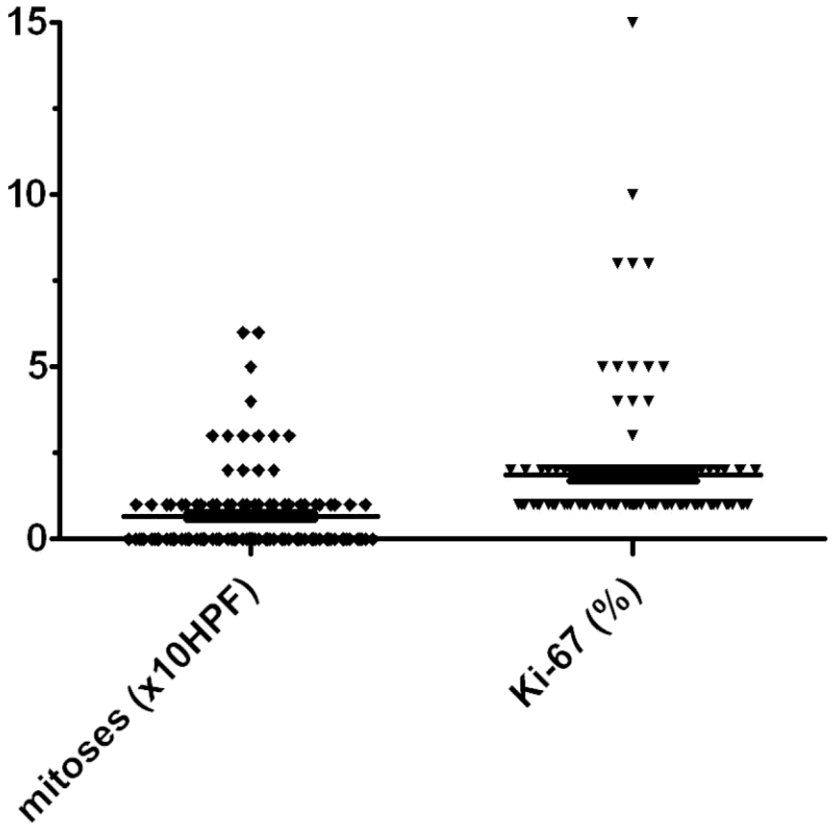
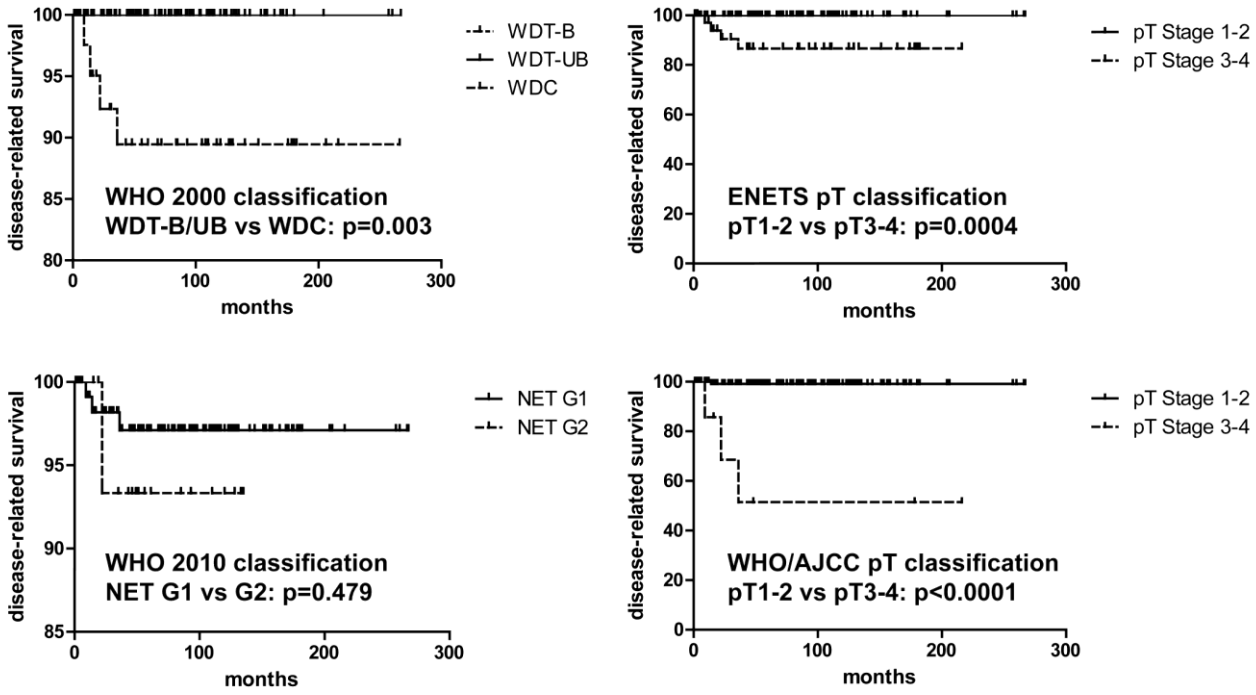


Figure 4


disease-related survival
months
WDT-B
WDT-UB
WDC
WHO 2000 classification
WDT-B/UB vs WDC: p=0.003
pT Stage 1-2
pT Stage 3-4
ENETS pT classification
pT1-2 vs pT3-4: p=0.0004
NET G1
NET G2
WHO 2010 classification
NET G1 vs G2: p=0.479
WHO/AJCC pT classification
pT1-2 vs pT3-4: p<0.0001