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Circulating Tumor Cells As Prognostic Markers in Neuroendocrine Tumors

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ABSTRACT

Purpose
To determine the prognostic significance of circulating tumor cells (CTCs) in patients with neuroendocrine cancer.

Patients and Methods
In this single-center prospective study, 176 patients with measurable metastatic neuroendocrine tumors (NETs) were recruited. CTCs were measured using a semiautomated technique based on immunomagnetic separation of epithelial cell adhesion molecule–expressing cells.

Results
Overall, 49% patients had ≥ one CTC, 42% had ≥ two CTCs, and 30% had ≥ five CTCs in 7.5 mL blood. Presence of CTCs was associated with increased burden, increased tumor grade, and elevated serum chromogranin A (CgA). Using a 90-patient training set and 85-patient validation set, we defined a cutoff of < one or ≥ one as the optimal prognostic threshold with respect to progression-free survival (PFS). Applying this threshold, the presence of ≥ one CTC was associated with worse PFS and overall survival (OS; hazard ratios [HRs], 6.6 and 8.0, respectively; both $P < .001$). In multivariate analysis, CTCs remained significant when other prognostic markers, grade, tumor burden, and CgA were included. Within grades, presence of CTCs was able to define a poor prognostic subgroup. For grade 1, HRs were 5.0 for PFS ($P = .017$) and 7.2 for OS ($P = .023$); for grade 2, HRs were 3.5 for PFS ($P = .018$) and 5.2 for OS ($P = .036$).

Conclusion
CTCs are a promising prognostic marker for patients with NETs and should be assessed in the context of clinical trials with defined tumor subtypes and therapy.

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INTRODUCTION

Neuroendocrine tumors (NETs) comprise a heterogeneous group of tumors that arise most commonly from the GI tract. Incidence is approximately five per 100,000, but because of prolonged survival in some groups, the prevalence exceeds those of pancreatic, gastric, and esophageal cancers in the United States.[1] In the past few years, there have been a number of important advances in therapy, including the approval of sunitinib[2] and everolimus[3] for well-differentiated pancreatic NETs.

Currently, tumor grade provides the best method of defining prognosis,[4,5] but this is usually based on a small biopsy sample that may not reflect the heterogeneity present in advanced tumors.[6] Furthermore, the sample may have been taken years before a treatment decision needs to be made and may not be representative of the tumor that has evolved. Additionally, there are no prospectively validated biomarkers for predicting outcome from treatment. Response to treatment is a poor surrogate for survival benefit because the best response is often stable disease, and if there is a response, it can be delayed by up to 1 year.[7,8] Therefore, there is a pressing need to develop robust biomarkers for NETs that can inform patient management and facilitate drug development.

There has been increasing interest in circulating tumor cells (CTCs) as biomarkers since the development of technology that can detect CTCs in small volumes of blood. The CellSearch platform detects CTCs with high sensitivity, specificity, and reproducibility and is the only system approved by the US Food and Drug Administration. Large studies have reported that the number

of CTCs in patients with metastatic breast cancer is an independent predictor of progression-free (PFS) and overall survival (OS),[9,10] with similar results reported in metastatic colorectal and prostate cancers.[11,12]

The CellSearch platform requires the cellular expression of the epithelial cell adhesion molecule (EpCAM), and we recently reported that a majority of NETs have strong heterogeneous membranous expression of EpCAM and that CTCs can be detected in patients with NETs.[13] Therefore, we sought to define the prognostic relevance of CTCs in a large population of patients with NETs. To our knowledge, this is the first such report.

PATIENTS AND METHODS

Patients

This was a prospective single-institution study conducted at the Royal Free Hospital, London, United Kingdom. The study was approved by the local ethics committee, and all patients provided written informed consent. Eligible participants were age > 18 years and had histologically proven NETs and metastatic disease measurable by RECIST 1.1.[14] Patients who had undergone systemic anticancer therapy or embolization within the previous 2 months were excluded because recent treatment has been shown to affect CTC count in other tumors.[11,15,16] Patients receiving long-term somatostatin (SST) analogs were permitted. Data were collected on primary site, previous treatment,

Table 1. Demographic and Clinical Characteristics of Sample of Patients With NETs

Characteristic	Primary Site					Total
	Pancreatic	Midgut	Bronchopulmonary	Unknown Primary	Hindgut	
No. of patients	42	101	17	12	3	175
Age, years						
Median	53	63	55	62.5	74	63
Range	23-87	34-85	30-80	31-78	43-75	23-87
Sex						
Male	25	54	8	4	1	92
Female	17	47	9	8	2	83
Grade						
Low	17	59	6	1	0	83
Intermediate	10	36	8	7	2	63
High	15	6	3	4	1	29
Burden of liver metastases, %						
≤ 25	19	48	11	4	1	83
25 to ≤ 50	15	36	4	4	0	59
50 to ≤ 75	3	11	2	3	1	20
> 75	5	6	0	1	1	13
Duration of diagnosis, months						
Median	41	30	22	13	18	26
Range	2-166	1-134	9-287	1-67	5-22	1-287
ECOG PS						
0	29	60	12	6	1	108
1	13	34	5	5	2	59
2	0	6	0	0	0	6
3	0	0	0	1	0	1
4	0	1	0	0	0	1
Previous treatment						
Resection of primary	18	50	6	0	1	75
SST	13	59	3	3	1	79
Chemotherapy	18	10	4	6	1	39
TAE	3	13	0	2	0	18
PRRT	5	16	1	1	0	23
Interferon	3	2	0	0	0	5
Liver resection	7	10	0	0	0	17
Subsequent treatment						
SST	3	26	3	2	0	34
Chemotherapy	13	5	3	7	1	29
TAE	4	10	2	1	1	18
PRRT	6	30	2	1	1	40
Interferon	0	4	0	0	0	4
Sunitinib	4	0	0	0	0	4
Surgery	0	5	2	0	0	7
RFA	1	1	0	0	0	2

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; NET, neuroendocrine tumor; PRRT, peptide receptor radionuclide therapy; RFA, radiofrequency ablation; SST, somatostatin analogue; TAE, transarterial embolization.

and Eastern Cooperative Oncology Group performance status, and grade of tumor based on Ki-67 proliferation index was recorded according to European Neuroendocrine Tumor Society guidelines.[4,5]

Baseline assessments included hepatic tumor burden from four to six slices of a computed tomography/magnetic resonance imaging scan selected at the level of greatest relative disease and categorized as ≤ 25%, 25 to ≤ 50%, 50% to ≤ 75%, or > 75% based on the relative area of tumor to normal liver. Baseline chromogranin A (CgA) analysis was performed on plasma samples at the same time point as CTC sampling using a radioimmuno assay kit (Roche; Basel, Switzerland).

CTC Analysis

CTC enumeration was performed using the CellSearch (Veridex, Raritan, NJ) system as described previously.[13] Blood samples were collected in 10-mL CellSave tubes (Veridex), stored at room temperature, and analyzed within 96 hours according to manufacturer instructions. All evaluations were performed without knowledge of the clinical status of the patients by two independent operators (M.S.K., T.T.). Data regarding reproducibility and results from healthy controls were previously reported.[17]

Statistical Analysis

Previously, we demonstrated a median PFS of 9.1 months in a series of patients with NETs attending our clinic to start chemotherapy.[7] Assuming a power of at least 90% and two-sided α of 0.05, a sample size of 138 would be needed to detect a hazard ratio (HR) of at least 2, corresponding to a median PFS of 6 months in the good prognosis group and 12 months in the bad prognosis group, as defined by CTC cutoff. Because we were unsure of the proportion of patients falling into each group, we increased the sample size to 168 to allow for a ratio (good prognosis to bad prognosis) as low as 0.4 or as high as 2.5 (bad prognosis to good prognosis). To define a prognostic cutoff with respect to the number of CTCs detected, we applied the internal validation method described by Altman et al[18] and divided the whole data set into a training set and validation set. Results for the first 90 patients enrolled (training set) were used to select a cutoff level of CTCs. This cutoff level was then validated with the 85 patients subsequently enrolled onto the study (validation set).

Analysis was performed using SPSS for Windows (SPSS, Chicago, IL) and GraphPad Prism (GraphPad Software, San Diego, CA), where *P* values < .05 were considered significant. Differences in baseline characteristics between progressors and nonprogressors were analyzed using Fisher's exact, Mann-Whitney, and *t* tests. Correlations between CTCs and continuous or ordinal clinicopathologic data were assessed using Spearman's rank test. Associations between level of CTCs and dichotomous variables were evaluated with the Mann-Whitney U test. Association between presence or absence of CTCs and clinicopathologic data was analyzed using χ^2 (or Fishers) or Mann-Whitney test. Because CgA was not normally distributed (even when transformed onto a logarithmic scale), this was analyzed in two groups: > and < 2× the upper limit of normal (ULN; 120 pmol/L), consistent with previous studies.[19] Age was analyzed as a continuous variable, and HRs are presented for 10-year intervals.

PFS and OS were estimated using Kaplan-Meier methods from date of baseline sample to date of radiologic progression (RECIST 1.1) and date of death resulting from neuroendocrine cancer or last follow-up. Radiologic progression was assessed by an independent radiologist blind to study. Survival curves were compared using log-rank testing. Cox proportional hazards regression analysis was used to obtain univariate HRs for PFS or OS. Factors found to be significant on univariate analysis were included in multivariate analysis in addition to age. The study design met the REMARK (REporting recommendations for tumor MARKer prognostic studies) criteria.[20]

RESULTS

Between August 2009 and June 2011, 176 patients with metastatic NETs were recruited. One sample was discarded because of hemolysis, leaving 175 evaluable patients. Baseline characteristics are listed in

Table 2. Baseline CTC Counts

Characteristic	Patients With CTCs (%)					Range of CTC Counts
	≥ 1	≥ 2	≥ 5	≥ 10	≥ 50	
Primary site						
All NETs (n = 175)	49	42	30	22	9	0-3,731
Midgut (n = 101)	51	47	32	24	6	0-294
Pancreatic (n = 42)	36	24	19	17	12	0-430
Bronchial (n = 17)	41	29	24	18	12	0-452
Unknown (n = 12)	92	92	67	33	17	0-3,731
Tumor burden, %						
≤ 25	33	24	17	10	2	
> 25	64	59	42	34	14	
P	< .001	< .001	< .001	< .001	.005	
Tumor grade						
1	40	31	24	22	4	
2	54	49	27	13	6	
3	66	59	55	45	28	
P	.036	.014	.006	.003	< .001	
ECOG PS						
0	49	43	30	22	7	
> 1	50	38	38	38	1	
P	1.0	1.0	.70	.379	.519	
CgA, pmol/L						
≤ 120	29	23	19	16	5	
> 120	64	57	39	27	11	
P	< .001	< .001	.005	.1	.275	

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; ECOG PS, Eastern Cooperative Oncology Group performance status; NET, neuroendocrine tumor.

Table 1. All patients had liver metastases, and midgut NETs represented the biggest subgroup. The majority had grade 1 or 2 tumors, with only 17% having grade 3 tumors. Of the 175 patients analyzed, 102 had received prior anticancer therapy, and 70 were receiving long-term SST. The median time between last anticancer therapy and CTC sampling was 20 months (range, 5 to 144 months), and 138 patients embarked on additional therapies within 4 weeks of CTC sampling, as listed in Table 1. Patients had been diagnosed a median of 26 months (range, 1 to 134 months) before sampling, and at the time of analysis, the median follow-up was 12.6 months (range, 5 to 28 months).

CTC Count in Relation to Clinicopathologic Characteristics

Enumeration of CTCs according to primary NET is listed in Table 2. Because of the low number of hindgut NETs, the data are not shown in the figure, but one of these three patients had CTCs (10/7.5 mL). Overall, for all 175 patients, the mean number of CTCs was 45 (standard deviation, 300); 49% had at least one CTC; 42%, ≥ two; and 30%, ≥ five.

For all NETs, the liver burden categories (ie, 25% to 50%, 50% to 75%, and > 75%) were combined because of the small number of patients in these categories (n = 59, 20, and 13, respectively) compared with those in the < 25% category (n = 83). CTC levels were higher in patients with a greater burden (Mann-Whitney $P < .001$). There was a weak correlation between CTC level and grade ($r = 0.224$; $P = .003$) and CgA ($r = 0.29$; $P < .001$). However, applying presence or absence of CTCs as a dichotomous variable, there was a significant association between grade ($P = .036$) and CTC presence and between CgA and CTC presence ($P < .001$). There was no association between SST therapy and presence of CTCs (χ^2 $P = .508$), nor between SST therapy and number of CTCs (Mann Whitney $P = .359$).

Establishing Prognostic Threshold for CTCs

To establish the optimal prognostic CTC threshold, we systematically evaluated CTC number and PFS using a training set comprising 90 patients. Kaplan-Meier survival curves were plotted comparing patient groups above and below each threshold and log-rank testing performed to obtain significance levels of difference across groups. Thresholds were tested commencing at CTCs of ≥ one, increasing by

Table 3. Univariate and Multivariate Analyses for Prognostic Markers (n = 175)

		PFS			OS		
Risk Factor	No. of Patients	HR	95% CI	*P*	HR	95% CI	*P*
Univariate analysis							
CTC count							
< 1	89	1.0			1.0		
≥ 1	86	6.6	3.2 to 13.6	< .001	8.0	3.1 to 21	< .001
CgA, pmol/L							
≤ 120	75	1.0			1.0		
> 120	100	1.8	0.9 to 3.3	.057	2.5	1.2 to 5.3	.018
Grade (Ki67)				< .001*			.001*
1	83	1.0			1.0		
2	63	2.4	1.1 to 5		1.6	0.6 to 3.7	
3	29	6.4	3.0 to 14.0		4.3	1.8 to 10.2	
Burden, %							
< 25	83	1.0			1.0		
≥ 25	92	2.5	1.3 to 4.6	.004	3.6	1.6 to 7.9	.002
ECOG PS							
0-1	167	1.0			1.0		
≥ 2	8	1.7	0.5 to 5.4	.385	1.3	0.8 to 5	.401
Age for every 10 years		0.8	0.6 to 1	.075	1.01	0.8 to 1.4	.921
Multivariate analysis							
CTC count							
< 1	89	1.0			1.0		
≥ 1	86	3.3	1.6 to 6.6	.001	3.7	1.6 to 8.9	.003
CgA, pmol/L							
≤ 120	75	1.0			1.0		
> 120	100	1.1	0.5 to 2.2	.844	1.5	0.6 to 3.7	.402
Grade (Ki67)				< .001*			.001*
1	83	1.0			1.0		
2	63	2.0	0.9 to 4.2		1.2	0.5 to 3.1	
3	29	5.5	2.4 to 12.3		3.4	1.3 to 8.3	
Burden, %							
< 25	83	1.0			1.0		
≥ 25	92	1.3	0.6 to 2.6	.484	1.9	0.8 to 4.6	.126
Age for every 10 years		1.3	1.1 to 2.1	.034	1.1	0.8 to 1.4	.543

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.
*Overall *P* value.

one CTC until 50 CTCs. Thereafter, CTC thresholds between 50 and 1,000 were tested at increments to include the next CTC count sequentially. Both HRs and difference in 1-year survival were greatest comparing groups without CTCs (CTC = 0) and those with CTCs (CTC ≥ one; Appendix Table A1, online only). The same cutoff was also defined when OS was analyzed instead of PFS. Thus, a cutoff of ≥ one CTC per 7.5 mL was chosen to distinguish patients with an unfavorable prognosis from patients with a favorable prognosis.

The CTC threshold was tested in an 85-patient validation set. The distributions of patients above and below the cutoff level in the training and validation sets were compared with the use of Fisher's exact tests, and median PFS and median OS in the two sets were compared with nonparametric *k*-sample test for equality of the medians. All *P* values were two sided. Neither PFS nor OS was significantly different between the validation and training sets ($P = .32$; $P = .56$). The distribution of patients with CTC levels above the cutoff of ≥ one CTC per 7.5 mL blood did not differ between the training and validation sets ($P = .41$). The cutoff level from the training set was confirmed as separating two significantly different prognostic groups in this validation set. Kaplan-Meier curves for the training and validation sets are shown in Appendix Figure A1 (online only), and patient characteristics for the two sets are listed in Appendix Table A2 (online only).

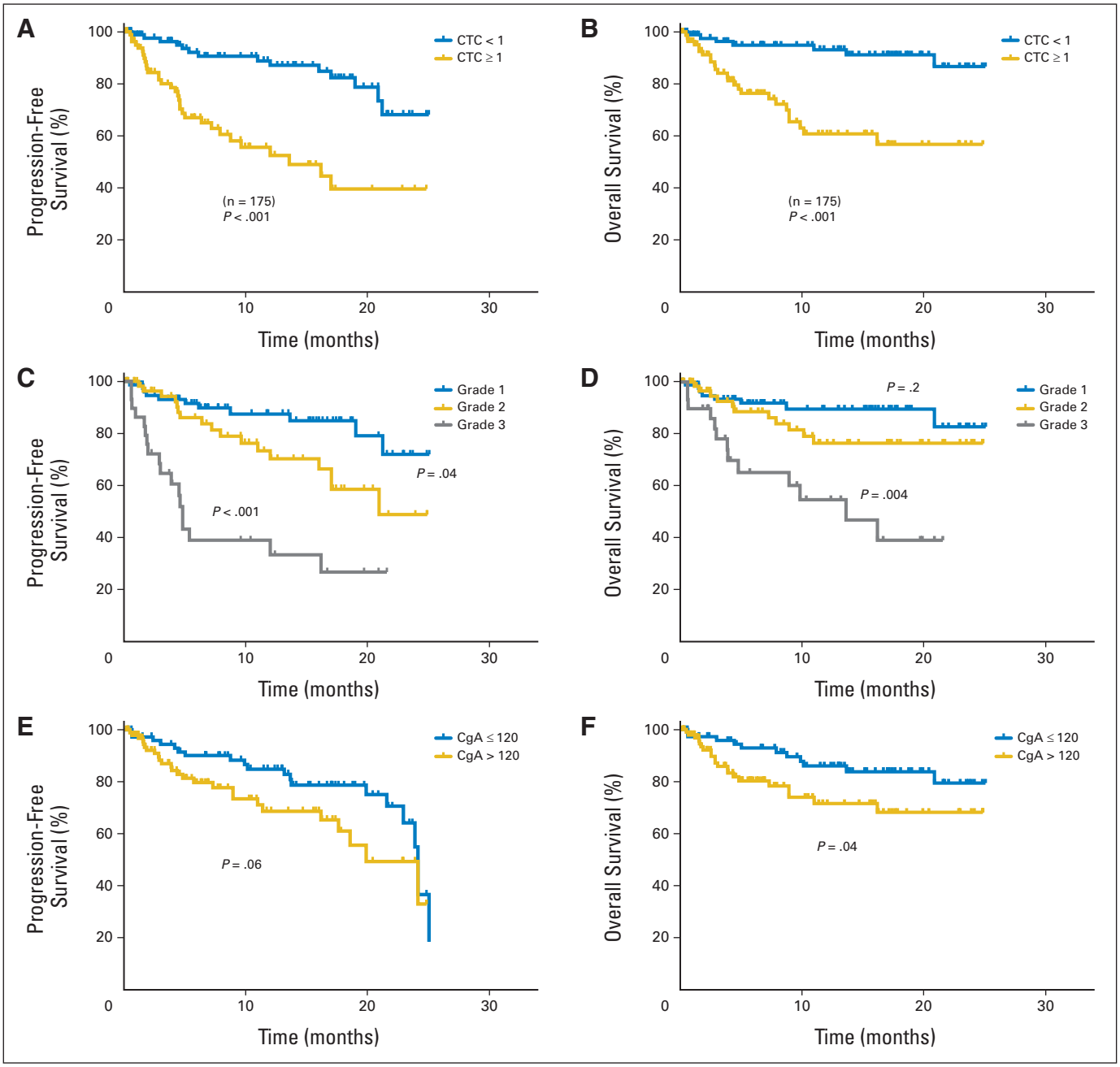

Fig 1. Survival curves according to (A, B) presence of circulating tumor cells (CTCs), (C, D) grade, and (E, F) chromogranin A (CgA) demonstrating differences in (A, C, E) progression-free and (B, D, F) overall survival.

CTCs As Prognostic Markers

Overall, 1- and 2-year OS were 79% (SE, 3) and 73% (SE, 4), and 1- and 2-year PFS were 72% (SE, 4) and 49% (SE, 6), respectively. In univariate analysis, we confirmed that grade, CgA, and tumor burden were prognostic (Table 3). However, the highest hazard ratios for both PFS and OS were defined by CTC < one or ≥ one. Kaplan-Meier survival curves of PFS and OS are shown in Figure 1, and the results are consistent with the univariate analysis demonstrating a significant difference in outcome between those with and without CTCs. Applying multivariate analysis, CTC presence or absence remained a highly significant discriminator in terms of PFS and OS (Table 3). Of the other factors, grade retained significance for PFS and OS, and age was significant for PFS alone. When the midgut cohort was analyzed separately, presence of CTCs was a significant prognostic factor for OS and PFS, whereas for the pancreatic cohort, there was a similar trend, but this was nonsignificant, possibly because of the smaller number in this subgroup (Appendix Tables A3 and A4, online only).

Because grade 1 and 2 tumors constitute a large and potentially heterogeneous subgroup, we also examined the prognostic significance of CTCs within these subgroups. Applying univariate analysis, only CTC presence or absence was significant for PFS and OS among the 83 patients with grade 1 and 63 patients with grade 2 tumors (Tables 4 and 5).

Table 5. Univariate Analysis for Prognostic Factors in Patients With Grade 2 NETs (n = 63)

Risk Factor	No. of Patients	PFS HR	PFS 95% CI	PFS *P*	OS HR	OS 95% CI	OS *P*
Baseline CTC count							
< 1	29	1.0			1.0		
≥ 1	34	3.5	1.2 to 10.1	.018	5.2	1.1 to 24	.036
Baseline CgA, pmol/L							
≤ 120	29	1.0			1.0		
> 120	34	1.7	0.6 to 4.4	.293	3.6	0.9 to 13.6	.060
Burden, %							
< 25	31	1.0			1.0		
≥ 25	32	0.9	0.3 to 2.4	.890	1.7	0.5 to 5.7	.419
ECOG PS							
0-1	61	1.0			1.0		
≥ 2	2	1.3	0.2 to 10	.805	2.9	0.4 to 22.9	.308
Age for every 10 years		0.9	0.6 to 1.4	.684	1.5	0.9 to 2.6	.139

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio; NET, neuroendocrine tumor; OS, overall survival; PFS, progression-free survival.

DISCUSSION

In this study, the number of CTCs detected in patients with NETs was comparable to other tumors in which CTCs have been shown to have prognostic relevance. Overall, 42% of patients with NETs had ≥ two detectable CTCs, as compared with 57% for prostate, 37% for breast, 30% for colorectal, and 20% for non–small-cell lung cancers.[17] In our study, the greatest number of patients had midgut (n = 101) or pancreatic (n = 42) tumors, and in these subgroups, 47% and 24%, respectively, had ≥ two CTCs detected. The reason for the high detection rate may be the high expression of EpCAM, which we and others have demonstrated in NETs.[13,21,22]

Using a training set, we found that the optimum threshold for prognostication using CTCs was ≥ one. For metastatic breast, prostate, and non–small-cell lung cancers, a CTC count of ≥ five has been used to define an unfavourable group,[10,12,23] whereas for colorectal cancer, ≥ three has been used.[24] In early breast cancer, presence of ≥ one CTC defines a poor prognostic group.[25] Applying a cutoff or three or five cells in our training set gave rise to a lower HR and difference in 1-year survival compared with a threshold of ≥ one. In a study of 145 healthy female volunteers, eight patients (5.5%) were found to have ≥ one, whereas none had ≥ two.[17] Hence, there is a possibility that using a threshold of ≥ one will incorrectly assign a patient to a poor prognostic group, but this must be balanced against the risk of wrongly assigning a patient to a good prognostic group if a cutoff of ≥ two is used, and a patient has only one CTC detected.

Presence of CTCs was clearly associated with increasing tumor burden and grade. Despite this, CTCs were independently associated with PFS and OS in multivariate analysis, with a 3.3-fold increase risk of progression and 3.7-fold increase risk of death in those patients with ≥ one CTC at baseline. The only other factor to retain significance was grade, and this was mainly because of the higher risk associated with grade 3 tumors. However, in clinical practice, high-grade tumors represent the minority of NETs and are usually treated at diagnosis with chemotherapy. Therefore, it is important to understand the implications of CTCs in grade 1 and 2 tumors, which constitute the majority. In both groups, presence of CTCs was still able to define a poor prognostic group for PFS and OS in univariate analysis. However, a longer period of follow-up and more events are needed to produce more accurate estimates of the effect sizes with regard to OS, particularly in patients with grade 1 disease.

Grade is usually assigned on a baseline biopsy that may not be representative of a heterogeneous tumor and might have been performed years before treatment decisions are made. CTCs, on the contrary, provide information about the tumor and prognosis at the

Table 4. Univariate Analysis for Prognostic Factors in Patients With Grade 1 NETs (n = 83)

Risk Factor	No. of Patients	PFS HR	PFS 95% CI	PFS *P*	OS HR	OS 95% CI	OS *P*
Baseline CTC count							
< 1	50	1.0			1.0		
≥ 1	33	5.0	1.3 to 18.5	.017	7.2	1.3 to 39.4	.023
Baseline CgA, pmol/L							
≤ 120	29	1.0			1.0		
> 120	54	2.4	0.6 to 9.4	.200	1.3	0.3 to 5.6	.724
Burden, %							
< 25	44	1.0			1.0		
≥ 25	39	2.8	0.8 to 9.8	.098	2.6	0.6 to 10.8	.197
ECOG PS							
0-1	78	1.0			1.0		
≥ 2	5	2.2	0.3 to 18.0	.449	3.0	0.4 to 25.0	.311
Age for every 10 years		1.2	0.7 to 2.0	.562	1.2	0.7 to 2.2	.559

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio; NET, neuroendocrine tumor; OS, overall survival; PFS, progression-free survival.

time a management decision needs to be made and seem to provide useful information over and above that provided by grade alone. The only other prognostic marker widely used in NETs is CgA, an acid glycoprotein and component of dense core secretory granules in neuroendocrine cells. The absolute CgA levels measured in serum or plasma vary depending on the assay used, and levels can be elevated in both benign and malignant conditions. Therefore, CgA is not a specific marker for NETs.[26] As a prognostic marker, the results in clinical studies are conflicting, and there are few prospective studies.[7,8] In the PROMID study of octreotide LAR in metastatic midgut NETs, CgA levels above the ULN were not prognostic for time to progression or tumor-related death,[27] whereas in studies of everolimus, elevation of CgA $> 2\times$ ULN has been associated with reduced PFS.[19] Our prospective data do support CgA as a prognostic marker, but as a dichotomous variable $> 2\times$ ULN, it is a poor discriminator in univariate analysis and not significant in multivariate analysis.

There are some limitations to our study. First, we have looked at a heterogeneous population in terms of primary site, in which midgut tumors were highly represented, and few data were available for bronchial, hindgut, and unknown primary. In the limited number that were included, CTCs were detected, and these subgroups will require further analysis in multicenter prospective studies. Second, although the follow-up for PFS was sufficient, the follow-up time for OS was relatively short, and median survival was not reached in patients with grade 1 and 2 tumors. Therefore, a further period of follow-up is required to define the role of CTCs with respect to OS.

In the past few years, there have significant advances in the treatment of NETs with the approval of both sunitinib and everolimus in pancreatic NETs. Because of the indolent nature of some NETs, many trials mandate documented progression within 12 months, which may require a period of observation off treatment, which is not always appropriate or acceptable. The ability of CTCs to define a population of patients with well-differentiated tumors that are likely to progress may provide a means of stratifying a high-risk group for early intervention. The evaluation of CTC-based clinical decision making is being addressed in ongoing trials in other cancers (such as SWOG [Southwest Oncology Group] S0500) and requires assessment in NETs.

The identification of CTCs in NETs also presents a number of other opportunities. Their role as predictive biomarkers of response to treatment needs to be explored; several NET trials have incorporated CTCs as an exploratory end point. Moreover, the capacity to capture and characterize CTCs may further facilitate the stratification of patients for therapy.[28-30] Outstanding challenges for cancer therapy include tumor heterogeneity and the emergence of drug resistance; the ability to readily sample tumor cells in the form of CTCs during the course of the disease may help inform treatment choices. Therapeutic targets including human epidermal growth factor receptor 2 and epidermal growth factor receptor have been identified in CTCs, and recent studies have demonstrated the ability to undertake single-cell sequencing.[31-33] New methods to isolate CTCs for molecular characterization are being developed, and these innovations present exciting opportunities to interrogate CTCs as an alternative to invasive biopsies.[34,35]

In summary, this is the first study to our knowledge in NETs demonstrating the prognostic significance of CTCs as detected by the CellSearch system. The findings expand on those in other epithelial cancers and open the way for further evaluation of CTCs as liquid biopsies in this tumor type.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Mohid S. Khan, Martyn E. Caplin, Tim Meyer
Collection and assembly of data: Mohid S. Khan, Thoedora Tsigani, Tim Meyer
Data analysis and interpretation: Mohid S. Khan, Amy Kirkwood, Jorge Garcia-Hernandez, John A. Hartley, Tim Meyer
Manuscript writing: All authors
Final approval of manuscript: All authors

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Appendix

Table A1. Establishment of Cutoff for CTCs in Training Set*

Groups (CTC cutoff)	No. in Each Group	12-Month PFS (%)	Survival Difference	HR	*P*
0	35	88			
≥ 1	50	50	38	4.3	.001
0-1	40	87			
≥ 2	50	50	37	4.3	.002
0-2	46	85			
≥ 3	44	55	30	3.3	.002
0-3	49	78			
≥ 4	41	52	26	3.3	.002
0-4	54	77			
≥ 5	36	49	28	3.4	.001
0-5	55	76			
≥ 6	35	45	31	3.1	.002
0-6	58	74			
≥ 7	32	45	29	2.8	.004
0-7	60	70			
≥ 8	30	51	18	2.4	.013
0-8	62	71			
≥ 9	28	47	24	2.4	.012
0-9	64	71			
≥ 10	26	54	17	2.4	.015
0-10	66	71			
≥ 11	24	51	20	2.8	.005
0-11	68	71			
≥ 12	22	50	21	2.5	.015
0-12	69	71			
≥ 13	21	48	23	2.3	.023
0-13	69	71			
≥ 14	21	48	23	2.3	.023
0-14	69	71			
≥ 15	21	48	23	2.3	.023
0-15	69	71			
≥ 16	21	48	23	2.3	.023
0-16	70	71			
≥ 17	20	48	23	2.3	.023
0-17	71	72			
≥ 18	19	45	27	3.0	.004
0-18	71	72			
≥ 19	19	45	27	3.0	.004
0-19	71	72			
≥ 20	19	45	27	3.0	.004
0-20	72	73			
≥ 21	18	41	32	3.3	.001
0-21	73	73			
≥ 22	17	37	36	3.7	.001
0-22	73	73			
≥ 23	17	37	36	3.7	.001
0-23	73	73			
≥ 24	17	37	36	3.7	.001
0-24	73	73			
≥ 25	17	37	36	3.7	.001
0-25	73	73			
≥ 26	17	37	36	3.7	.001
0-26	74	72			
≥ 27	16	39	33	3.3	.002
0-27	74	72			
≥ 28	16	39	33	3.3	.002
0-28	74	72			
≥ 29	16	39	33	3.3	.002
0-29	74	72			
≥ 30	16	39	33	3.3	.002
0-30	74	72			
≥ 31	16	37	35	3.6	.001

(continued on following page)

Table A1. Establishment of Cutoff for CTCs in Training Set* (continued)

Groups (CTC cutoff)	No. in Each Group	12-Month PFS (%)	Survival Difference	HR	*P*
0-31	74	72			
≥ 32	16	37	35	3.6	.001
0-32	74	72			
≥ 33	16	37	35	3.6	.001
0-33	74	72			
≥ 34	16	37	35	3.6	.001
0-34	74	72			
≥ 35	16	37	35	3.6	.001
0-35	74	72			
≥ 36	16	37	35	3.6	.001
0-36	74	72			
≥ 37	16	37	35	3.6	.001
0-37	76	71			
≥ 38	14	34	37	3.9	.001
0-38	76	69			
≥ 39	14	31.5	37.5	3.9	.001
0-39	76	69			
≥ 40	14	31.5	37.5	3.9	.001
0-40	76	69			
≥ 41	14	31.5	37.5	3.9	.001
0-41	76	69			
≥ 42	14	31.5	37.5	3.9	.001
0-42	76	69			
≥ 43	14	31.5	37.5	3.9	.001
0-43	76	69			
≥ 44	14	31.5	37.5	3.9	.001
0-44	76	69			
≥ 45	14	31.5	37.5	3.9	.001
0-45	76	69			
≥ 46	14	31.5	37.5	3.9	.001
0-46	77	69			
≥ 47	13	31.5	37.5	3.9	.001
0-47	77	69			
≥ 48	13	31.5	37.5	3.9	.001
0-48	77	69			
≥ 49	13	31.5	37.5	3.9	.001
0-49	77	69			
≥ 50	13	31.5	37.5	3.9	.001
0-50	79	63			
≥ 51	11	27	36	4.1	.001
0-53	80	63			
≥ 54	10	30	33	3.9	.001
0-58	81	63			
≥ 59	9	33	30	3.8	.002
0-59	83	61			
≥ 60	7	29	32	4.1	.002
0-70	84	65			
≥ 71	6	33	32	3.7	.008
0-110	85	65			
≥ 111	5	40	25	3.0	.040
0-270	86	65			
≥ 271	4	25	40	4.8	.011
0-430	87	71			
≥ 431	3	0	71	11.2	.001
0-542	88	70			
≥ 543	2	0	70	7.9	.006
0-1,150	89	70			
≥ 1,151	1	0	70	21.5	.006

Abbreviations: CTC, circulating tumor cell; HR, hazard ratio; PFS, progression-free survival.
*Groups were split into below and above different CTC cutoffs in rows.

Table A2. Demographic and Clinical Characteristics of Training and Validation Sets

Characteristic	Training Set	Validation Set
No. of patients	90	85
Primary site		
Pancreatic	20	22
Midgut	51	50
Bronchopulmonary	9	8
Unknown	8	4
Hindgut	2	1
Age, years		
Median	59	64
Range	30-80	23-87
Sex		
Male	49	43
Female	41	42
Grade		
Low	38	45
Intermediate	32	31
High	20	9
Burden of liver metastases, %		
≤ 25	40	43
25 to ≤ 50	31	28
50 to ≤ 75	13	7
> 75	6	7
Duration of diagnosis, months		
Median	25	41
Range	1-150	1-287
ECOG PS		
0	55	53
1	31	28
2	2	4
3	1	0
4	1	0
Previous treatment		
Resection of primary	47	28
SST	44	35
Chemotherapy	23	16
TAE	9	9
PRRT	8	15
Interferon	2	3
Liver resection	12	5
Subsequent treatment		
SST	15	19
Chemotherapy	18	11
TAE	13	5
PRRT	30	10
Interferon	4	0
Sunitinib	1	3
Surgery	4	3
RFA	1	1

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; PRRT, peptide receptor radionuclide therapy; RFA, radiofrequency ablation; SST, somatostatin analogue; TAE, transarterial embolization.

Table A3. Univariate Analysis of Prognostic Factors for Midgut Cohort (n = 101)

Risk Factor	No. of Patients	PFS			OS		
		HR	95% CI	*P*	HR	95% CI	*P*
CTC count							
< 1	49	1.0			1.0		
≥ 1	52	5.1	1.6 to 16.4	.006	4.98	1.1 to 22.6	.038
CgA, pmol/L							
≤ 120	32	1.0			1.0		
> 120	69	1.3	0.47 to 3.4	.655	1.3	0.35 to 4.4	.730
Grade (Ki67)							
1	59	1.0			1.0		
2	36	1.6	0.68 to 3.7	.290	1.9	0.68 to 5.3	.224
3	6	1.6	0.34 to 7.5	.549	0.91	0.11 to 7.8	.934
Burden, %							
< 25	48	1.0			1.0		
≥ 25	53	0.62	0.25 to 1.56	.309	1.1	0.34 to 3.5	.888
ECOG PS							
0-1	94	1.0			1.0		
≥ 2	7	2.3	0.44 to 12.2	.322	2.6	0.47 to 14.6	.888
Age for every 10 years		1.02	0.63 to 1.6	.948	1.6	0.86 to 2.9	.140

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.

Table A4. Univariate Analysis of Prognostic Factors for Pancreatic Cohort (n = 42)

Risk Factor	No. of Patients	PFS			OS		
		HR	95% CI	*P*	HR	95% CI	*P*
CTC count							
< 1	27	1.0			1.0		
≥ 1	15	2.8	0.43 to 18.5	.277	4.3	0.34 to 53.9	.263
CgA, pmol/L							
≤ 120	30	1.0			1.0		
> 120	12	1.2	0.23 to 6.5	.817	1.8	0.19 to 17.9	.603
Grade (Ki67)							
1	17	1.0			1.0		
2	10	1.9	0.25 to 14.0	.542	1.7	0.09 to 31.0	.711
3	15	30.9	4.03 to 236	.001	27.2	1.9 to 399	.016
Burden, %							
< 25	19	1.0			1.0		
≥ 25	23	8.1	1.4 to 45.3	.017	8.7	0.67 to 112	.098
ECOG PS							
0-1*	42						
≥ 2	0						
Age for every 10 years		1.8	0.84 to 3.8	.132	2.3	0.72 to 7.1	.160

Abbreviations: CgA, chromogranin A; CTC, circulating tumor cell; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.
*ECOG PS 0-1 for all patients.

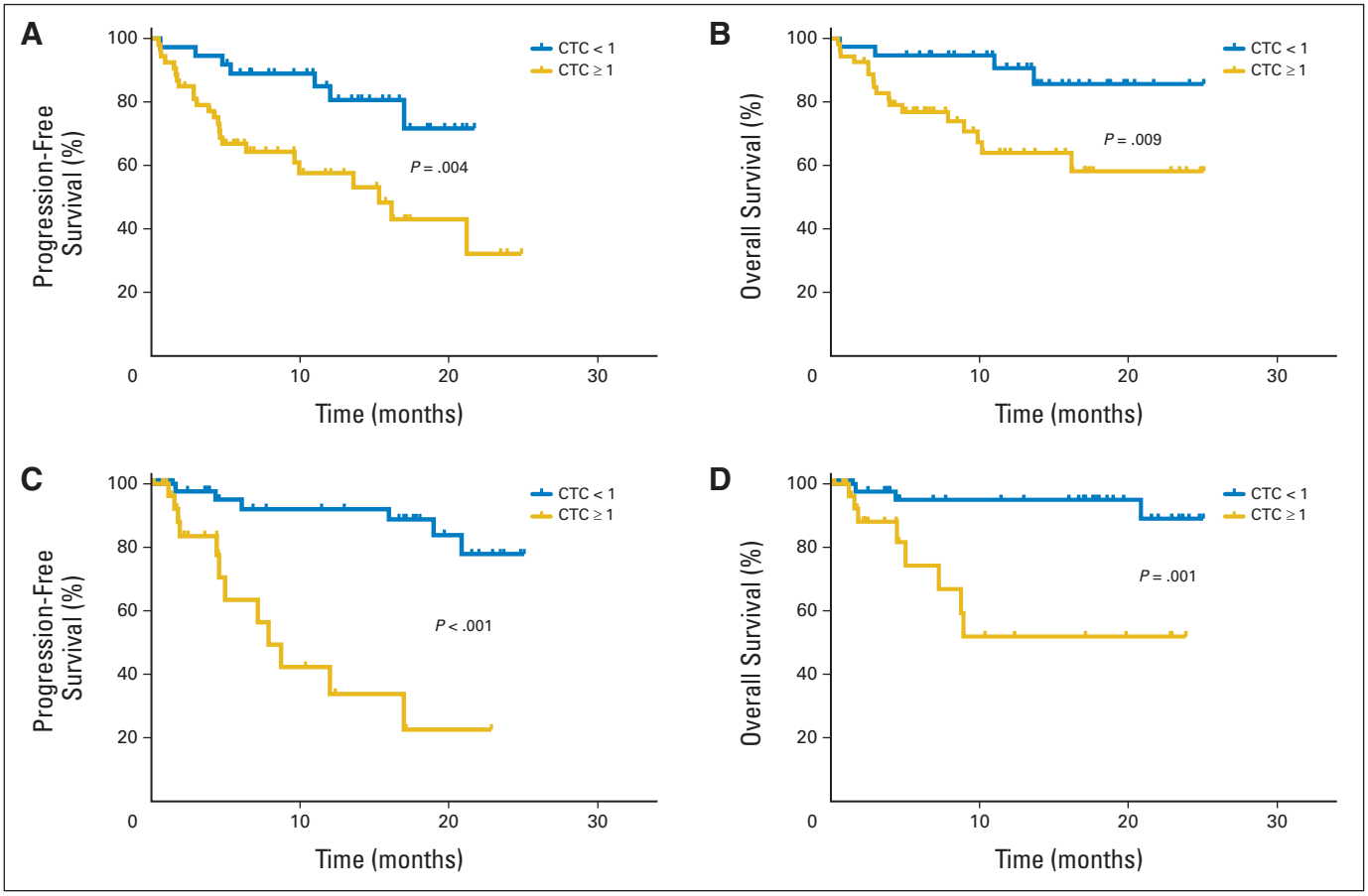

Fig A1. Kaplan-Meier curves of progression-free and overall survival in (A, B) training and (C, D) validation sets. CTC, circulating tumor cell.