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Diagnostic and prognostic role of plasma levels of two forms of cytokeratin 18 in patients with non-small-cell lung cancer

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ABSTRACT

Purpose: Cytokeratin 18 (CK18) can be used as a serum biomarker for carcinoma cell death, whereas caspase-cleaved (ccCK18) fragments reflect tumour apoptosis. We explored the potential diagnostic and prognostic role of circulating CK18 and ccCK18 in patients with non-small-cell lung cancer (NSCLC) in comparison with Cyfra 21.1, a fragment of cytokeratin 19.

Methods: Subject cohorts consisted of 200 healthy blood donors (HBD), 113 patients with benign lung diseases (BLD) and 179 NSCLC cases. Plasma levels of ccCK18, total CK18 and Cyfra 21.1 were determined with ELISA assays.

Results: Plasma levels of ccCK18 and total CK18 were higher in the NSCLC group compared to the HBD and BLD cohorts ($p < 0.0001$). Using a cut-off of 104 U/L for ccCK18 and 302 U/L for total CK18 (95% specificity in the HBD group) the diagnostic accuracy of both CK18 forms to distinguish between NSCLC and BLD cases was 56%, whereas it was 94% for Cyfra 21.1. Multivariate survival analysis showed that total CK18 was a stronger prognostic factor than both ccCK18 and Cyfra 21.1 (HR 0.64 for low versus high total CK18 levels, 95% confidence interval (CI) 0.50–0.82; $p = 0.0004$) in the entire NSCLC cohort and in 78 patients with locally advanced or metastatic disease treated with chemoradiotherapy or first-line chemotherapy (HR 0.70 95% CI 0.52–0.94; $p = 0.018$).

Conclusions: Cyfra 21.1 is a useful diagnostic biomarker for NSCLC. Total CK18 shows a promising potential as prognostic marker in NSCLC patients, independently of the therapeutical intervention. In contrast, ccCK18 was not of prognostic value in NSCLC, suggesting that tumour necrosis is of particular importance in this disease.

1. Introduction

Lung cancer is a major cause of cancer-related mortality.[1] Non-small-cell lung cancer (NSCLC) represents approximately 85% of lung cancer cases and comprises several histological phenotypes, the most common being adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma.

Physicians face a number of clinical problems when dealing with NSCLC. At the time of diagnosis, only a third of NSCLC cases are considered technically and oncologically

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treatable with a radical surgical intervention, whereas most patients have locally advanced or metastatic disease[2]; NSCLC exhibits high resistance to anticancer treatment and the overall prognosis is poor, with an overall 5-year survival rate of approximately 15%[1]; the screening of the population at risk to diagnose tumours at a potentially curable early stage is still an experimental procedure.

A promising breakthrough to improve the outcome for NSCLC patients is the introduction of validated biomarkers into clinical management. These may be crucial not only for early diagnosis but also to assist treatment choice for the most optimal therapeutic interventions.

NSCLC is a tumour type characterised by elevated cell death rate, which has shown prognostic implications in the advanced disease setting.[3] The possibility to detect circulating cell death products in NSCLC patients could eventually be used to detect small asymptomatic tumours or to function as surrogate markers of worse prognosis.

Cytokeratin 18 (CK18) is an acidic cytokeratin member of the protein family of the cytoskeletal intermediate filaments. It is widely expressed in normal epithelial and endothelial cells and in a variety of tumours of epithelial origin.[4] The observation that CK18 is released in the bloodstream as a consequence of cell death gave rise to great interest regarding the potential use of circulating CK18 as a tumour biomarker. In addition, during apoptosis CK18 is cleaved by caspases and specific fragments are generated.[5]

The ability to distinguish and quantitatively measure the caspase-cleaved and total CK18 forms by means of specific assays has prompted several investigators to explore the differential expression of CK18 in the blood in diverse tumour forms, and its kinetic in response to chemotherapy or radiotherapy.

In the present study we measured baseline plasma levels of both caspase-cleaved CK18 (ccCK18) and total CK18 in patients with NSCLC or benign lung diseases and in healthy blood donors. We sought to determine the diagnostic and prognostic potential of these proteins in NSCLC in comparison with the circulating levels of cytokeratin 19 fragment (Cyfra 21.1), an established biomarker in lung cancer.[6]

2. Patients and methods

2.1. Patient characteristics

In the present study three separate patient cohorts were included.

The first cohort consisted of 179 patients with stages I–IV NSCLC. Cases were selected from a large biobank on the basis of availability of plasma sample and adequate follow-up for survival analyses. Median age was 67 years (range 39–93 years). On data analysis, patients were grouped by age in elderly (aged > 70 years, 73 cases) or adults (<70 years, 106 cases). The distribution of clinical characteristics of patients from this cohort is shown in Table 1. In summary, the majority of cases (71%) had a non-squamous histology, including adenocarcinoma and large-cell carcinoma. Patients were diagnosed with early stage disease (stage I-resectable IIIA) in 38% of cases. Of these, 64 patients received curative surgical resection followed or not by adjuvant chemotherapy, as appropriate, whereas 4 patients received stereotactic body radiotherapy. The remaining 62% of patients had locally advanced or advanced disease (stage IIIAbulky-IIIB or IV); 31 cases received a combination of chemo- and radiotherapy, 50 subjects were treated with palliative chemotherapy that consisted of a combination of carboplatin and gemcitabine in the majority of cases (70%) and in 27 patients the only suitable treatment option was best supportive care. Three cases with stage IIIB or IV NSCLC initially received surgery, followed by adjuvant chemotherapy in two cases. Survival of NSCLC patient was calculated from the date of sample collection until death from any cause (events) or last follow-up (censors). Median follow-up of 65 censor cases (36%) was 33 months (range 19–51 months). In 111 subjects with locally advanced or advanced NSCLC, the median follow-up of 14 censor cases (12%) was 37 months (range 29–48 months).

The second cohort consisted of 113 consecutive cases with benign lung diseases (BLD). This patient group was matched by age and gender with the NSCLC group. The most common diagnoses were pulmonary or pleural infections (51 cases) and benign lung infiltrates or lung nodules (30 cases) including 6 subjects with hamartoma. In this cohort the median age was 67 years (range 16–86), and 51% of cases were males.

Finally, the third patient cohort consisted of 200 healthy blood donors (HBD). Plasma samples from this subject group were purchased from Visby Lasarett, Gotland, Sweden.

The study was performed according to the ethical regulations in Sweden and was approved by the ethics committee at the Karolinska University Hospital, Stockholm. Written informed consent was provided by all patients.

2.2. Sample collection and ELISA assays

Sample collection and storage was performed as suggested in line with guidelines for banking biological fluids for studies on disease-related biomarkers.[7] In patients from the NSCLC and BLD groups, peripheral venous blood was collected in EDTA tubes and kept at 4 °C until preparation to prevent coagulation and minimise protein degradation, as previously described.[8] EDTA tubes were centrifuged at 1500*g* at 4 °C for 10 min. Plasma was transferred to a new tube and centrifuged at 3000*g* at 4 °C for 10 min. Supernatant was aliquoted and kept in –80 °C until analysis. All samples used in this study were prepared within 2 h of sample collection and showed no signs of haemolysis. Plasma from NSCLC patients was obtained at diagnosis, prior to any anticancer treatment. ccCK18 was measured using the sandwich enzyme-linked immunosorbent assay (ELISA) M30-Apoptosense® ELISA (Peviva AB, Bromma, Sweden), whereas total CK18 was measured with the M65 EpiDeath® ELISA (Peviva AB, Bromma, Sweden). This novel assay (product # 10040) is a modified version of the more commonly used M65® ELISA (Peviva AB, Bromma, Sweden). The M65® assay is based on two antibodies (Abs), M5 and M6, directed against two different epitopes of CK18, and recognises total CK18, in both its intact and caspase-cleaved forms. The M6 Ab (capture Ab) is coated to the well whereas the M5 Ab (detection Ab) is conjugated to horseradish-peroxidase (HRP). However, the M65® ELISA was not originally developed for applications intended for disease detection and generates significant signals (median levels ranging from

Table 1 – Plasma levels of ccCK18 and total CK18 in the three patient cohorts and in detail in the NSCLC group according to patient characteristics.

Group	ccCK18				Total CK18			
	# (%)	Median	IQR	P	#	Median	IQR	P
Blood donors	200	47	38–62	<0.0001	200	94	62–146	<0.0001
Benign diseases	113	35	21–57		112	131	87–190	
NSCLC	179	51	34–91		176	234	129–452	
Clinical characteristics in the NSCLC Group								
Gender				0.05				0.08
Male	81 (45)	64	37–94		79	245	146–502	
Female	98 (55)	47	30–81		97	202	113–413	
Age				0.9				0.8
⩽70	109 (61)	49	35–92		107	241	128–457	
>70	70 (39)	56	32–90		69	209	129–453	
Stage				0.007				<0.0001
I-IIIA	68 (38)	44	32–66		67	158	109–270	
IIIAbulky-IIIB	57 (32)	64	33–88		56	233	132–498	
IV	54 (30)	75	37–139		53	318	221–690	
Smoking history				0.4				0.3
Current	87 (48)	48	31–90		86	233	130–337	
Former	77 (42)	60	35–92		75	226	115–502	
Never	15 (8)	75	35–136		15	270	152–913	
Performance status				0.006				0.0002
0	76 (42)	43	29–75		75	162	92–298	
1	70 (38)	56	34–96		69	242	145–473	
2–3	33 (18)	69	40–130		32	427	190–704	
Histology				0.1				0.3
Non-squamous	128 (71)	49	32–89		126	225	118–400	
Squamous	51 (29)	64	34–123		50	249	136–535	

IQR, inter-quartile range.

200 U/L to 300 U/L in healthy donors[9–11]). The M65 EpiDeath® ELISA implements the same Abs as the M65® ELISA, but conversely to the latter, the capture Ab is M5 and the detection HPR-conjugated Ab is M6. This results in the generation of lower signal levels in the control groups compared to what is obtained with the M65® ELISA. In addition, the M65 EpiDeath® ELISA uses the same capture antibody (M5) as the M30-Apoptosense® ELISA, resulting in improved compatibility between these two assays for the determination of the fraction (ratio) of CK18 which is caspase-cleaved. For these reasons in the present study the M65 EpiDeath® assay was preferred over the M65® ELISA.

Cyfra 21.1 was measured using a sandwich ELISA (Demeditec Diagnostics, Kiel-Wellsee, Germany) according to manufacturer' instructions.

All experiments were performed blindly to clinical correlates.

2.3. Statistical analyses

Levels of ccCK18 and total CK18 are expressed in medians and interquartile range. The distribution of these parameters was non-normal in each of the three patient groups. Hence, the non-parametric tests Kruskal–Wallis and Mann–Whitney *U* were used, as appropriate, to correlate the plasma levels of the analytes with clinicopathological characteristics. Spearman's correlation analysis was used to examine the relationship between continuous variables. To determine the diagnostic accuracy of ccCK18, total CK18 and Cyfra 21.1 receiver operating characteristic (ROC) curves were retrieved from logistic regression analysis and the area under the curve (AUC) was calculated. Univariate survival analyses were performed using the Kaplan–Meier method, and curves were examined using the logrank test. To determine which factors had an independent impact on survival multivariate analysis was performed using the Cox proportional hazard method. A p value < 0.05 was considered statistically significant. Statistical analyses were conducted using the JMP software 5.1.2 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Plasma levels of ccCK18 and total CK18 are elevated in the NSCLC group

M30-Apoptosense® ELISA was successful in all 492 cases, M65 EpiDeath® ELISA was not successful in four cases (three NSCLC and one BLD) and Cyfra 21.1 was not successful in four cases (two NSCLC and two BLD).

Median levels of circulating CK18 were significantly higher in the NSCLC group compared with the BLD and the HBD groups ($p < 0.0001$) (Table 1). However, as depicted by the box plots presented in Fig. 1, the concentration of both ccCK18

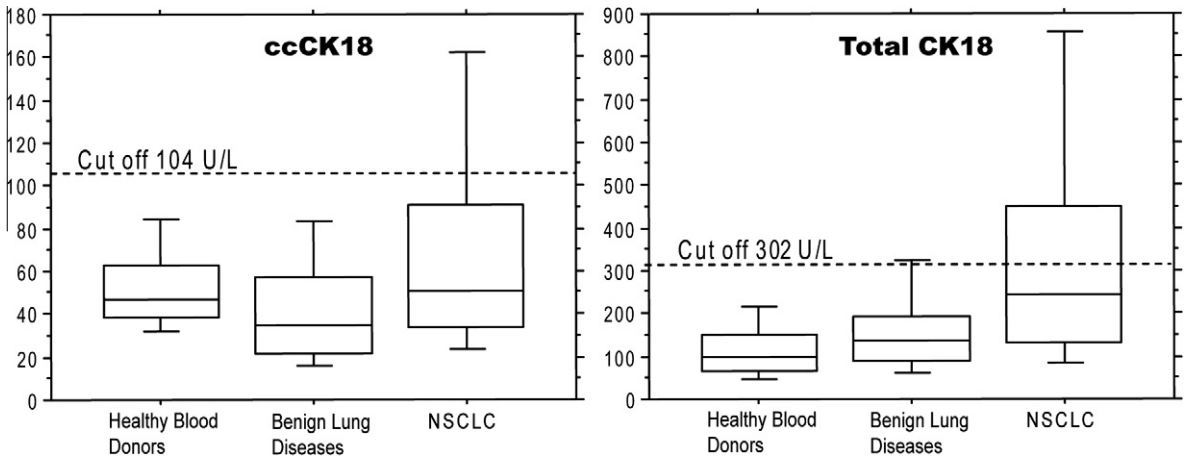

Fig. 1 – Box plots of caspase-cleaved CK18 (ccCK18) and total CK18 (displays 10%, 25%, median, 75%, 90%).

and total CK18 levels showed wide ranges of distribution in all the three groups.

In the NSCLC cohort, plasma levels of ccCK18 and total CK18 were significantly associated with stage and performance status (PS; World Health Organization criteria). Interestingly, in early-stage disease, plasma levels of both ccCK18 and total CK18 were similar to those obtained in the two control groups. No correlation was observed between ccCK18 and total CK18 plasma levels and patient age, gender and smoking history or tumour histology. Spearman's rank correlation analyses showed that there was a significant correlation between plasma levels of ccCK18 and total CK18 (Rho = 0.54, $p < 0.0001$) and between each form of CK18 and Cyfra 21.1 (Rho = 0.49 and 0.72 for ccCK18 and total CK18, respectively; $p < 0.0001$ in both cases).

Finally, in order to determine whether the storage time of plasma samples could have had an impact on the concentration of ccCK18 or total CK18, as suggested by Greystoke et al.,[12] samples from the NSCLC and BLD groups were ranked according to the date of sample collection. Median storage time was 31 months (range 18–43 months). No statistically significant correlation between the storage time and the biomarker levels was observed (p value > 0.5 in both cases).

3.2. *Diagnostic accuracy of M30-Apoptosense® and M65 EpiDeath®*

To determine the diagnostic potential of the M30-Apoptosense® and M65 EpiDeath® assays, we first calculated which concentration corresponded to 95% specificity in the healthy blood donors group. This level was found to be 104 U/L for ccCK18 and 302 U/L for total CK18 and was used to estimate the sensitivity, specificity, negative and positive predictive values of ccCK18 and total CK18 in the NSCLC and BLD groups. Similarly, the diagnostic accuracy of Cyfra 21.1 was evaluated using the established cutoff of 3.6 ng/ml.[6]

The diagnostic performance of the three biomarkers is summarised in table 2. Some studies have suggested that the determination of circulating Cyfra 21.1 levels is more useful in the diagnosis of squamous tumours than adenocarcinomas.[13] In our cohort, Cyfra 21.1 performed equally well in both histologies. Conversely, the sensitivity of the two CK18 forms was slightly better in squamous than non-squamous tumours (40% versus 32% for total CK18, respectively and 49% versus 35% for ccCK18, respectively).

Table 2 – Diagnostic performance of total CK18, ccCK18 and Cyfra 21.1 to discriminate between cases with NSCLC or benign lung diseases.

	Total CK18	ccCK18	Cyfra 21.1
Sensitivity	34%	39%	97%
Specificity	90%	83%	90%
Negative predictive value	47%	46%	94%
Positive predictive value	85%	85%	94%
Accuracy	56%	56%	94%
AUC-ROC	0.70	0.67	0.98

3.3. *Survival analyses*

We determined whether the baseline plasma concentration of ccCK18 or total CK18 would be of any prognostic relevance in NSCLC. For this purpose NSCLC patients were dichotomized using the same cutoff below which the 95% of healthy subjects in the blood donors group were comprised.

Median survival of patients with ccCK18 > 104 U/L ($n = 70$) was 11.5 months, compared with 28 months in patients with ccCK18 ⩽ 104 U/L ($n = 109$) (log rank $p = 0.0005$). Similarly, NSCLC cases with a baseline plasma concentration of total CK18 > 302 U/L ($n = 70$) had a median survival time of 7.5 months, compared with 36 months in subjects with total CK18 levels below or equal to the cut-off ($n = 106$) (log rank $p < 0.0001$) (Fig. 2).

Cyfra 21.1 was also a prognostic factor in NSCLC (log rank $p < 0.0001$). However, due to the high sensitivity of this analyte, almost all NSCLC cases had a plasma level >3.6 ng/ml. Hence, for prognostic purposes, the NSCLC cohort was dichotomized at the median Cyfra 21.1 level, which resulted to be 6 ng/ml. Multivariate Cox regression analysis was adjusted by gender, age, PS, histology, stage (I-IIIA versus IIIAbulky-IIIB versus IV), smoking history and whether or not patients received chemotherapy. As presented in Table 3, total CK18

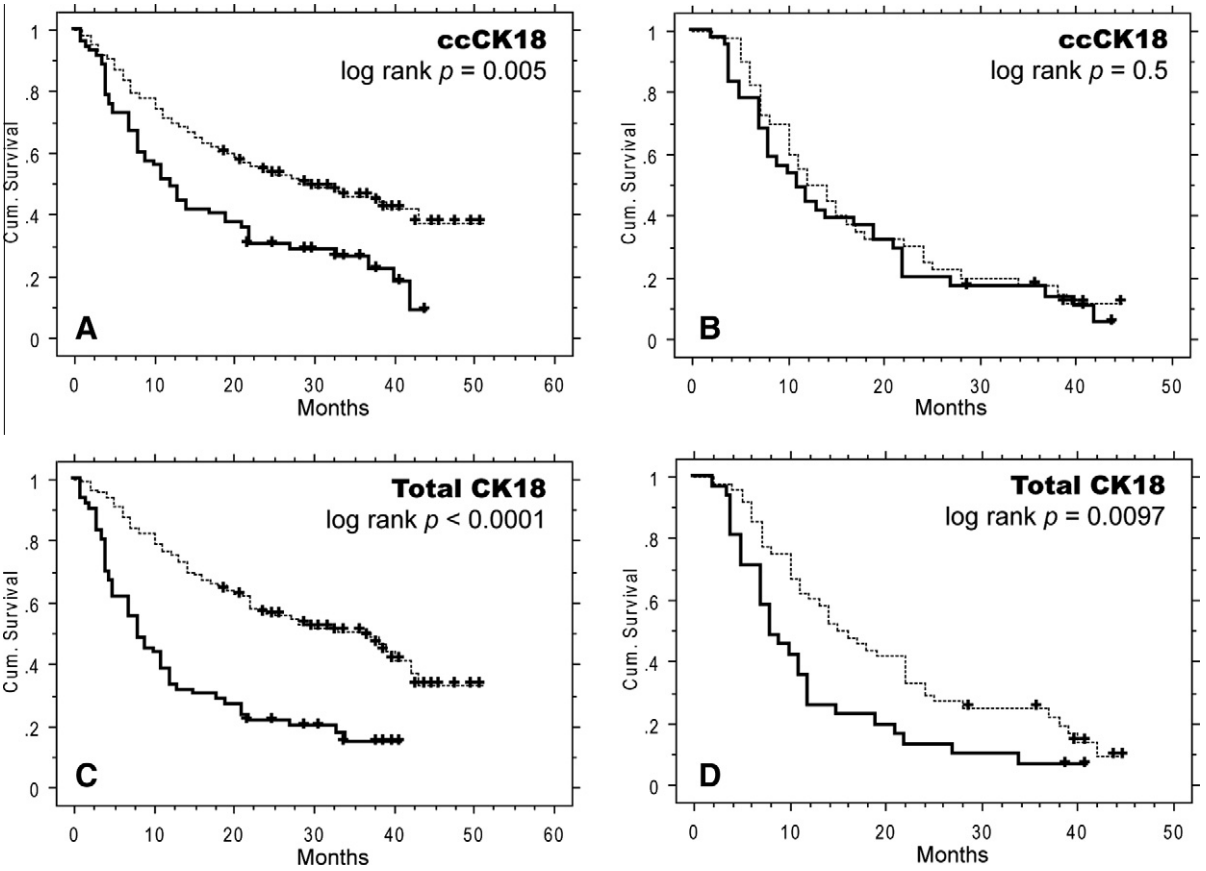

Fig. 2 – Kaplan–Meier curves for caspase-cleaved CK18 (ccCK18) and total CK18 in all NSCLC cases (A and C) and in the subgroup of patients with stages III and IV NSCLC receiving either combination chemoradiotherapy or first-line palliative chemotherapy (B and D). (High total CK18 or ccCK18 levels straight lines; low levels dotted lines).

Table 3 – Multivariate survival analysis.

	All NSCLC cases ($n = 174$)			NSCLC receiving chemoradiotherapy or 1st-line chemotherapy ($n = 78$)		
	HR	95% CI	P	HR	95% CI	P
ccCK18	1.16	0.92–1.47	0.19			
Total CK18	0.64	0.50–0.82	0.0004	0.70	0.52–0.94	0.018
Cyfra 21.1	0.80	0.63–1.01	0.061	0.98	0.73–1.32	0.90

HR, hazard ratio; 95% CI, 95% confidence interval.

was a stronger prognostic factor than Cyfra 21.1, which showed only a statistical trend, whereas ccCK18 was not confirmed as an independent prognostic variable, maybe as a result of the co-expression with total CK18. In order to exclude whether this result would have been biased by the chosen cut-off value, CK18 and Cyfra 21.1 were included in the multivariate analysis as continuous variables. Hazard ratio (HR) was 1.001 [95% confidence interval (CI) 1.000–1.002; $p = 0.02$] for total CK18 and 1.007 (95% CI 0.992–1.019; $p = 0.33$) for Cyfra 21.1.

We further sought to explore whether the baseline plasma concentration of CK18 would effectively predict overall survival in the selected population of 81 patients with locally advanced or advanced/metastatic NSCLC receiving an active treatment for their disease. ccCK18 showed no prognostic potential (log rank $p = 0.5$) in this patient cohort and was not included into the multivariate model. Conversely, patients with total CK18 levels $\leqslant$ 302 U/L had a median survival time of 15 months, whereas patients with a concentration above the cutoff had a median survival time of 7.5 months (log rank $p = 0.0097$) (Fig. 2). In addition, Cyfra 21.1 plasma levels, dichotomized at the median value of 8 ng/ml in this cohort, were also significantly associated to patient survival (log rank $p = 0.018$). On multivariate analysis, adjusted by gender, age, PS, smoking history, histology, stage (III versus IV) and treatment setting (curative chemoradiotherapy versus 1st-line palliative chemotherapy), total CK18 resulted to be the only independent prognostic factor (Table 3).

4. Discussion

We have here determined the plasma levels of caspase-cleaved and total CK18 in a relatively large patient population with detailed information regarding clinical parameters and follow-up and evaluated the clinical role of these markers. We show that circulating levels of ccCK18 and total CK18 were elevated in patients with NSCLC compared to patients with BLD. However, at the cutoff established on the healthy control cohort neither marker was sufficiently accurate to be used in diagnostic tests, due to poor sensitivity and negative predictive values. Conversely, in this cohort Cyfra 21.1 showed an excellent diagnostic performance.

Interestingly, baseline total CK18, but not the apoptotic product, was an independent prognostic factor in NSCLC and in the subgroup of patients who received active treatment for locally-advanced or metastatic disease. In addition, total CK18 was a stronger prognostic factor than Cyfra 21.1. Although even a large meta-analysis on more than 2000 cases confirmed that NSCLC patients with high circulating Cyfra 21.1 levels have a worse outcome,[6] our data suggest that in this disease a marker of tumour cell death (i.e. CK18) is a more accurate prognosticator than a marker of epithelial differentiation (i.e. Cyfra 21.1).

The utility of CK19 and CK18 as lung cancer biomarkers was already explored in some previous reports, where CK18 levels were determined measuring the well known tissue polypeptide specific (TPS) antigen. In terms of diagnostic performance, also previous studies showed that Cyfra 21.1 is a more accurate marker for detecting lung cancer than CK18, especially due to the low sensitivity of this latter analyte.[13,14] In addition, one study exploring the prognostic implications of these biomarkers showed that both are independent prognostic factors in NSCLC.[15] However, it must be noted that compared to the present report, the multivariate survival analysis conducted in the cited study included only stage as a clinical variable, and as such the independent prognostic implications of the biomarkers may have been overestimated.

The main strengths of this study are the following: (i) samples were collected from all cases according to strict standard operation procedures; (ii) the diagnostic accuracy of the CK18 biomarkers was determined comparing NSCLC patients with a proper control group, namely patients with non-tumoural lung diseases; (iii) the cut-off that functioned as an adequate prognostic discriminator in NSCLC was not developed in the selected tumour cohort, but was the same cut-off that was retrieved from the blood donors as representing the threshold of normality in a healthy population; and (iv) the direct comparison with Cyfra 21.1 confirms the limitation of CK18 as a diagnostic biomarker in NSCLC but strengthens its role in prognosis. We believe that these features make our results particularly reliable.

The finding that circulating total CK18, but not ccCK18, is a prognostic factor in NSCLC suggests that total cell death is a more important parameter than apoptotic cell death in this disease, consistent with previous histopathological findings.[3] This histological feature cannot be evaluated in all cases with advanced disease, since in this clinical setting the diagnosis is mainly done by analysis of cytological specimens. A complimentary test performed in blood samples that are easy to collect might add information in this setting.

Plasma levels of either ccCK18 or total CK18 have been found significantly elevated in diverse tumour forms when compared with control groups, including colorectal cancer,[16] head and neck tumours,[10] breast cancer[18] and lung cancer.[9,19] However, in none of these reports the determination of circulating CK18 reached sufficient accuracy to be proposed as a potential diagnostic assay. As also showed in our study, the main limitation to the applicability of testing for CK18 plasma levels in tumour diagnosis is that more than 50% of patients have values below the proposed cutoff, drastically reducing the sensitivity of the test. Nevertheless, plasma CK18 is a surrogate marker of cell death activity in tumours, and its determination can be used for other clinically relevant purposes. For instance, in colorectal cancer, CK18 has been measured before and after surgery, with post-operative high plasma levels being correlated with tumour recurrence and presence of residual disease.[17,20] A positive correlation between baseline circulating CK18 levels and patient outcome has been suggested in several tumour diseases such as colorectal cancer,[16,20] gastrointestinal tumours,[21] testis tumours,[22] lung cancer[9,19] and pancreas cancer.[23] The prognostic role of ccCK18 in lung cancer was evaluated in an earlier publication.[19] However, in that study, both NSCLC and small-cell lung cancer (SCLC) patients were included and serum levels of total CK18 were not measured. In our study we found that ccCK18 lost its statistical significance as prognostic factor in NSCLC when total CK18 was included into the multivariate model. In addition, ccCK18 was not a prognostic factor on univariate analysis in the subset of patients with stages III and IV disease. This suggests that in advanced NSCLC the relative amount of apoptosis, as assessed by a circulating biomarker, may not *per se* predict the natural history of the disease.

In a more recent study including only SCLC cases, ccCK18 and total CK18 serum concentrations were prognostic of patient survival at baseline, and related to treatment response. The caspase-cleaved form increased early after therapy, reflecting the induction of tumour apoptosis by treatment, and both forms decreased later on, in correspondence with tumour shrinkage.[9] SCLC is a tumour type characterised by the presence of large necrotic areas, and by a remarkable sensitivity to firstline treatment. Both these variables make the evaluation of circulating cell death products particularly relevant. Most of the patients with SCLC have high plasma levels of CK18 at the time of diagnosis, which decrease soon in concomitance with response to anticancer drugs. The kinetics of either ccCK18 or total CK18 levels in successive measurements before and after the administration of chemotherapy has been evaluated as an early predictor of response in breast cancer[24] and in other tumour types.[25] However, the predictive role of CK18 forms seems to vary as related to tumour histology and type of chemotherapy. In NSCLC this issue should be prospectively and more extensively explored.

Another potential application of testing for CK18 is the determination of the ratio between the ccCK18 and the total CK18 plasma levels, as a surrogate marker of the relative amount of apoptosis versus total cell death. In our study the

ccCK18/total CK18 ratio did not provide any additional information compared to what was assessed with the evaluation of the single biomarkers (data not shown).

In conclusion, the main limitation of this study is its retrospective nature. The prognostic cut-off of CK18, as well as the correlation with diverse types of treatment interventions across disease stages and settings in NSCLC warrants further prospective validation.

Conflict of interest statement

R. Herrmann is an employee of Peviva AB. S. Linder is a consultant and shares holder of Peviva AB.

Role of the funding source

The funding sources had no role in the study design, collection, analysis and interpretation of data.

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