Prognostic utility of pre-operative circulating osteopontin, carbonic anhydrase IX and CRP in renal cell carcinoma

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BACKGROUND: Objectively measured circulating biomarkers of prognosis complementing existing clinicopathological models are needed in renal cell carcinoma (RCC).

METHODS: Blood samples collected from 216 RCC patients in Leeds before nephrectomy (median follow-up 7 years) were analysed for C-reactive protein (CRP), osteopontin (OPN) and carbonic anhydrase IX (CA9) and prognostic significance determined.

RESULTS: CA9, OPN and CRP were univariately prognostic for overall survival (OS), cancer-specific survival (CSS) and disease-free survival (DFS) with CRP and CA9 being independently prognostic for OS/CSS and OS, respectively. Including CA9, OPN and CRP with other conventional prognostic factors gave a superior predictive capacity when compared with a previously published pre-operative clinical nomogram (Karakiewicz et al, 2009). Osteopontin outperformed this nomogram and the post-operative SSIGN score for OS but not for CSS, being significantly predictive for non-cancer deaths. Osteopontin, CRP and CA9 outperformed stage (c-index 76% compared with 70% for stage) and OPN or CA9 identified several subsets of poor prognosis patients including in T1 patients, who may benefit from adjuvant therapy and increased surveillance.

CONCLUSION: Circulating CA9, OPN and CRP add value to existing clinicopathological prognostic factors/models and support further studies to investigate their potential use in improving the clinical management of RCC.

For patients with metastatic renal cell carcinoma (RCC), tyrosine kinase inhibitors, principally targeting vascular endothelial growth factor receptor form the standard of care (Escudier and Albiges, 2011). Large, randomised phase III adjuvant studies (e.g., SORCE; S-TRAC) are currently underway and the safety and efficacy of neo-adjuvant therapy (e.g., NCT00480935) is being explored. Early and accurate risk stratification for individual patients thus becomes paramount with patients destined to do well potentially being spared years of toxic and costly adjuvant therapy. Others, with poor risk disease, may be directed towards early systemic therapy, delaying or avoiding a nephrectomy they may not benefit from.

Various prognostic models based on clinicopathological factors have been proposed in RCC (Zisman et al, 2002; Leibovich et al, 2003; Sorbellini et al, 2005; Ficarra et al, 2006; Karakiewicz et al, 2007a, 2009) with predictive accuracies of up to 89% being achieved in external validation (Ficarra et al, 2006; Karakiewicz et al, 2007a). Improved prognostic nomograms could lead to more optimal selection of surveillance regimens and risk stratification for therapy and/or clinical trials and a recent nomogram generating a score derived solely from pre-operatively available factors (age, gender, symptom score, CT-derived size and T stage

and presence or absence of metastatic disease) may enable prognostication in the neo-adjuvant setting even (Karakiewicz et al, 2009). The inclusion of molecular markers can enhance the performance of prognostic models, as illustrated by the improvement of the UISS model with the inclusion of tissue-based markers p53, vimentin and CA9 (Kim et al, 2004). Several protein markers of potential prognostic value are emerging, mainly tissue based, but increasingly in biological fluids such as serum and plasma. Such fluids have the advantage of being easily accessible, available pre-operatively and providing an objective measurement through standardised assays.

Several circulating markers reflecting different aspects of tumour biology have been shown to have potential prognostic value. The acute phase protein C-reactive protein (CRP) that may in part arise as a systemic response but additionally has been shown to be produced by RCC cells (Jabs et al, 2005) has independent prognostic value, outperforming or enhancing various prognostic models (Karakiewicz et al, 2007b; Jagdev et al, 2010). Both the soluble form and tissue expression of the hypoxia-inducible marker carbonic anhydrase IX (CA9) which is one of the proteins upregulated in RCC tissue as a consequence of inactivation of the Von Hippel Lindau tumour suppressor gene may also have potential prognostic utility, although results are conflicting (Li et al, 2008; Papworth et al, 2010; Peña et al, 2010; Stillebroer et al, 2010). A single small study involving 80 patients including all histological subtypes and with some patients following nephrectomy (Ramankulov et al, 2007) has also reported

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independent prognostic significance for osteopontin (OPN), a member of the SIBLING (small integrin binding ligand, N-linked glycoprotein) family that has been found to have prognostic utility in several cancers (Anborgh *et al*, 2010).

The aims of the current study were to evaluate in a large well-characterised population of RCC patients, the prognostic significance of CA9, OPN and CRP alone or in combination, as objectively measured biomarkers reflecting different aspects of RCC tumour biology, in terms of (a) improving upon existing prognostic clinicopathologic factors and (b) whether incorporation into existing pre-operative (Karakiewicz *et al*, 2009) or post-operative (Leibovich *et al*, 2003) models would result in improved prognostic accuracy.

MATERIALS AND METHODS

Patient population

Blood samples were prospectively collected between 1999 and 2006 in Leeds from untreated patients with renal cancer, before nephrectomy. Exclusion criteria included histological subtype other than conventional (clear cell) RCC (ccRCC), familial VHL disease or polycystic kidney disease. The project was approved by the Local Research Ethics Committee and informed consent obtained. Samples were processed and stored at $\leqslant -70\,^{\circ}$C according to standard protocols (Wind *et al*, 2011). For histological assessment, Fuhrmann grading was used and pre-operative TNM staging (UICC/AJCC, 2002) was assessed using CT of the chest, abdomen and pelvis.

Measurement of CRP, OPN and CA9

Carbonic anhydrase IX and OPN concentrations were quantified in EDTA plasma using commercially available enzyme-linked immunosorbent assays (ELISA) Quantikine kits (R&D Systems, Minneapolis, MN, USA). All samples were measured in duplicate and QC samples included on each plate. We have previously validated the CA9 assay (Wind *et al*, 2011) and the OPN assay was similarly validated before use. In brief, a significant reduction in measured OPN was observed in serum *vs* paired plasma samples ($P < 0.05$, $n = 6$) due to cleavage of OPN during clotting and accordingly EDTA plasma was used. The intra- and inter-assay precision was $< 10\%$; parallelism was $< 15\%$; recovery in EDTA plasma samples was between 80% and 120%; the LoQ was $< 312\,\text{pg ml}^{-1}$ and no hook effect or interference from bilirubin, haemolysis, triglycerides or rheumatoid factor was observed. Serum CRP was measured by the NHS Clinical Biochemistry labs using the wide range CRP assay (Siemens Healthcare Diagnostics Inc, Tarrytown, NY, USA).

Study design and statistical analysis

The design and analysis of this retrospective study was conducted according to ReMARK guidelines (McShane *et al*, 2005). Sample size was pre-determined with the study having at least 90% power to detect hazard ratios of 2 or more for the markers, for division above and below a cutpoint, with a significance level of 0.01 rather than 0.05 because of the number of different variables being considered. The published pre-operative nomogram (Karakiewicz *et al*, 2009) did not include T4 disease so these patients were excluded from such analyses. Survival curves were constructed using the Kaplan–Meier method, with significance determined using the log-rank test. Multivariate analysis used the proportional hazards model with a forward stepwise approach using a *P*-value for inclusion of 0.01. Harrell's c-index (Harrell *et al*, 1982) was used to quantify the predictive accuracy of survival models. We included both stage and T stage separately in the multivariate models to ensure that the significance of the new variables made

the maximum allowance for stage. Since they are correlated, only one of these variables was ever included in the multivariate model.

Disease-free survival (DFS) was calculated to the date of disease recurrence or death from any cause. Patients still alive or lost to follow-up were censored, as were patients who died from causes other than RCC in the cancer-specific survival (CSS) analysis. To find any continuous relationship between variables and overall survival (OS)/CSS/DFS, fractional polynomials were calculated using pre-defined transformations of predictor variables (Royston and Altman, 1994; Altman and Royston, 2006). These were of relevance for OPN, CRP and CA9. For example, in the latter variable for DFS an inverse square root transformation captured both a benefit in patients with low CA9 values and a detriment in patients with high CA9 values. An additional degree of freedom was added when evaluating the test statistics for these variables (Hosmer and Lemeshow, 1999). Optimum cutpoints were chosen to depict the effects of these variables on OS, CSS and DFS graphically but significance levels should be taken from Table 2 and Supplementary Table 1 based on their consideration as continuous variables.

RESULTS

Patient details are shown in Table 1. Significant associations between CRP, OPN and CA9 and several clinicopathological factors were seen such as TNM stage and nuclear grade (Spearman's $\rho = 0.39/0.38$, $0.51/0.38$, $0.34/0.21$ comparing stage/grade, respectively, with CRP, OPN and CA9, all *P*-values < 0.01). The three markers are intercorrelated, with OPN values being correlated both with CRP values (log(OPN) *vs* sqrt(CRP) $r = 0.53$, $P < 0.001$), and with CA9 values (log(OPN) *vs* log(CA9) $r = 0.38$, $P < 0.001$), but clearly not to a degree that precludes them having independent prognostic significance (see below). CA9 was not significantly correlated with CRP (log(CA9) *vs* sqrt(CRP) $r = 0.10$, $P = 0.06$). Highly significant differences ($P < 0.0001$; Mann–Whitney) were seen between concentrations of these variables in patients with metastatic disease compared with localised disease (Figure 1) with median values (IQR) respectively of 22.2 (4.2–111) *vs* 5.7 (0.6–11.5) mg l^{-1} for CRP, 189.2 (114.8–303.4) *vs* 82.3 (575.5–131.1) μg l^{-1} for OPN and 186.4 (89.6–336.2) *vs* 102.5 (61.6–153.9) μg l^{-1} for CA9.

Univariate analysis of biomarkers and survival

Carbonic anhydrase IX, OPN and CRP were all found to be significant prognostic factors for DFS, CSS and OS (Table 2; Figure 2A–C), with an inverse relationship with survival. Furthermore, OPN was strongly predictive of non-cancer survival (c-index 70%, Figure 2D), especially within early stage patients (stage $<$IV and T stage $<$3b, c-index 76%, $\chi^2 = 19.6$, $P < 0.0001$) (Figure 2E). This interaction with stage was highly significant ($P < 0.0001$) and so unlikely to be a chance effect. Log transformation of OPN was used for the Cox model but cutoffs of 135 and 230 μg l^{-1} were used to depict differences in the Kaplan–Meier survival curves as OPN appeared to separate out a very poor group and an intermediate group. Similarly cutoffs of 225 ng l^{-1} and 15 mg l^{-1} were used for CA9 and CRP, respectively.

Multivariate analysis of biomarkers and survival

The multivariate Cox model results are presented in Table 2. In addition to the circulating factors examined here, other factors initially included in the analysis were the recognised clinicopathological factors and sodium which we have previously reported to be prognostic (Vasudev *et al*, 2008). Multivariate analysis showed that sqrtCRP ($\chi^2 = 27.8$, $P < 0.0001$) and log(CA9) ($\chi^2 = 8.4$, $P = 0.01$, 2 df) were independent prognostic factors for OS (Table 2). Osteopontin was not significant in the multivariate

Table 1 Characteristics of the 216 conventional (clear cell) renal cancer patients included in the study. Median follow-up time was 7 years 1 month

Characteristic	n (%)	Characteristic	Divisions	Median (range)
Age (years)		Hb (g dl^{-1})		13.6 (7–20)
Median	64			
Range	29–85			
Gender		RBC ($\times 10^{12}$ l^{-1})		4.6 (2.8–14.7)
Male	130 (60)			
Female	86 (40)			
Grade		WBC ($\times 10^{9}$ l^{-1})		7.7 (3.2–607)
1	4 (2)			
2	48 (22)			
3	105 (49)			
4	58 (27)			
Unknown	1 (0)			
Max tumour size (cm)		Platelets ($\times 10^{9}$ l^{-1})		284 (87–884)
Median	6.5			
Range	0.7–18			
pT stage		Sodium (mmol l^{-1})		140 (127–147)
1	82 (38)			
2	20 (9)			
3	111 (51.5)			
4	2 (1)			
Unknown	1 (0.5)			
N stage		Potassium (mmol l^{-1})		4.4 (3.1–6.0)
0	171 (79)			
1+	40 (19)		Unknown	4 (2)
Unknown	5 (2)			
M stage		Creatinine (μmol l^{-1})		95 (52–1106)
0	164 (76)			
1	52 (24)			
TNM stage		CRP (mg l^{-1})		7.6 (<5–266)
I	75 (35)		$\leqslant$15	152 (70%)
II	14 (6)		>15	64 (30%)
III[a]	74 (34.5)			
IV	52 (24)			
Unknown	1 (0.5)			
CT T stage		Osteopontin (ng ml^{-1})		98 (6.6–1231)
1a	47 (22)		$\leqslant$135	140 (65%)
1b	62 (28.5)		>135–230	39 (18%)
2	52 (24)		>230	37 (17%)
3	39 (18)			
4	10 (5)			
Unknown	6 (2.5)			
CT tumour size (cm)		CA9 (pg ml^{-1})		109 (12–1049)
Median	5.9		$\leqslant$225	175 (81%)
Range	2–21		>225	41 (19%)
Unknown	16 (7)			
Symptom score[b]		Urea (mmol l^{-1})		5.7 (2–28.8)
1	90 (42)			
2	72 (33)			
3	52 (24)			
Unknown	2 (1)			

Abbreviations: CA9 = carbonic anhydrase IX; CRP = C-reactive protein; RBC = red blood cell; TNM = tumour-nodes-metastasis; WBC = white blood cell. [a]Includes six patients classified as at least stage III. [b]Symptom score used in the published preoperative prognostic model (Karakiewicz *et al*, 2009).

analysis, correlating with many factors including CRP and CA9. The combined c-index of all factors in the final Cox model for OS (Table 2a) was 78%, with Log$_{10}$OPN being the most predictive single (univariate) factor for OS (c-index 75%). SqrtCRP was independently prognostic for CSS together with N, M and T stages. Overall, the combined c-index for all variables included in the final Cox model for CSS (Table 2b) was 87% with stage proving to be the most predictive factor (univariate c-index of 79%). T stage was the only independently prognostic factor for DFS. SqrtCRP was just above the 0.01 significance level for DFS since including the additional degree of freedom for the square root transformation

yielded a χ^2 of 7.7, $P = 0.02$. Note that the analysis of DFS included four patients with resected adrenal metastases. Additional analyses excluding these four patients did not change the results.

In addition to the results when all patients were considered, the analysis was also undertaken solely on the patients with non-metastatic disease (M0), with similar results being obtained (Supplementary Table 1) with the exception that CA9 was not independently significant for OS. Significance levels tend to be smaller with the reduced patient numbers – which would exclude some factors since they would not therefore reach the 0.01 significance level.

Pre-operative nomogram

Good separation of the survival curves for CSS was achieved using the pre-operative nomogram score based on age, gender, symptom score, CT-derived size and T stage and presence or absence of metastatic disease (Karakiewicz *et al*, 2009). The CSS actually observed in this patient population, excluding T4 patients since these were not included by Karakiewicz *et al*, was consistently slightly better than that predicted by the nomogram, with 1-, 2-, 5- and 10-year survivals of 93%, 86%, 74% and 62%, respectively, compared with nomogram predictions of 86%, 78%, 72% and 62%. This may reflect the inclusion of only the conventional clear cell subtype of RCC in our study with the nomogram being developed for all RCC cases. SqrtCRP ($\chi^2 = 7.8$, $P = 0.005$) added significantly to the nomogram prediction. Osteopontin appeared to add additional predictive capacity for CSS within the patient population with nomogram scores of >150 ($P = 0.02$) (Supplementary Figure 1a and b).

Post-operative SSIGN score and patients with non-metastatic disease

Focussing on patients with non-metastatic disease only, the post-operative SSIGN score (Frank *et al*, 2002; Leibovich *et al*, 2003), separated patients at high risk but patients defined as low risk had only marginally better survival to the intermediate risk group (Supplementary Figure 2). Osteopontin was more predictive for OS than the SSIGN score, with a predictive accuracy of 70% compared with only 64% for the SSIGN score. However, the SSIGN score was more predictive for CSS, with a predictive accuracy of 77% (compared with 70% for OPN alone), and with neither OPN, CA9, nor CRP adding to this. This difference is explained by the fact that the SSIGN score had no predictive capacity for non-cancer death. Note that while the pre-operative nomogram score was highly correlated with the post-operative SSIGN score ($r^2 = 0.61$), OPN was poorly correlated with both ($r^2 = 0.17$ and 0.15 respectively), and therefore appears to be representing a different effect or mechanism.

Relevance of the new markers as an addition to, or replacing, stage and identification of poor prognosis subgroups

Stage has a c-index of 70%, increasing to 77% when OPN, CRP and CA9 are added. However, using just the three markers gives a c-index of 76%. In addition, within patients with stage I/II disease, CA9 identifies a poor prognosis group. Osteopontin also identifies a poor prognosis group within the stage III group and a very small number of stage IV patients with especially bad prognosis (Figure 3A–C). Focusing on the 76 patients with T1N0M0 disease, high OPN (>135) or high CA9 (>225) together identify 10 patients with very poor outcome where all 8 deaths were non-cancer related (Figure 3D).

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Table 2 Cox model predictors of (a) OS, (b) CSS and (c) DFS.

Variable	Univariate				Multivariate			
	χ^2	HR	HR CI[a]	P-value	χ^2	HR	HR CI[a]	P-value
(a) OS								
Gender[b]	6.0	1.7	1.1–2.7	0.015				NS
Age (continuous)	0.5	1.01	0.99–1.03	0.50				NS
Stage (I+II vs III vs IV)	50.7	2.7	2.0–3.5	<0.0001				NS
Grade	29.6	2.3	1.7–3.1	<0.0001				NS
T stage (1 vs 2+3a vs 3b vs 4)	42.1	2.1	1.7–2.6	<0.0001				NS
N stage (−ve vs +ve)	54.2	6.1	4.0–9.5	<0.0001	18.1	2.9	1.8–4.7	<0.0001
M stage (−ve vs +ve)	53.6	5.7	3.7–8.8	<0.0001	18.6	2.9	1.8–4.5	<0.0001
Log_{10} (osteopontin)	66.9	18.1	8.8–37.0	<0.0001[c]				NS
Na (continuous)	8.2	1.12	1.04–1.20	0.004				NS
Sqrt(CRP)	50.4	1.2	1.15–1.26	<0.0001[c]	30.0	1.17	1.11–1.23	<0.0001[c]
Log(CA9)	20.2	3.6	2.1–6.1	<0.0001[c]	8.4	2.4	1.4–4.4	0.01[c]
(b) CSS								
Gender[b]	5.1	1.8	1.1–3.1	0.025				NS
Age (continuous)	0.9	0.99	0.97–1.01	0.34				NS
Stage (I+II vs III vs IV)	73.6	4.2	2.9–6.0	<0.0001				NS
Grade	43.4	3.5	2.4–5.2	<0.0001				NS
T stage (1 vs 2+3a vs 3b vs 4)	67.1	3.0	2.3–4.0	<0.0001	11.9	1.7	1.3–2.3	0.002
N stage (−ve vs +ve)	50.0	7.4	4.5–12.2	<0.0001	14.1	2.9	1.7–5.0	0.0001
M stage (−ve vs +ve)	65.5	9.0	5.4–15.0	<0.0001	20.2	3.5	2.0–6.1	<0.0001
Log_{10} (osteopontin)	52.9	22.7	9.5–54.1	<0.0001[c]				NS
Na (continuous)	11.4	1.16	1.06–1.27	0.0008				NS
Sqrt(CRP)	49.2	1.23	1.17–1.30	<0.0001[c]	14.5	1.14	1.07–1.21	0.0007[c]
Log(CA9)	13.2	3.5	1.8–6.6	0.001[c]				NS
(c) DFS								
Gender[b]	5.5	2.0	1.1–3.8	0.019				NS
Age (continuous)	0.6	0.99	0.97–1.02	0.46				NS
Stage (I+II vs III vs IV)	14.3	2.4	1.6–3.7	0.0002				NS
Grade	22.9	2.9	1.9–4.5	<0.0001				NS
T stage (1 vs 2+3a vs 3b vs 4)	34.5	2.7	1.9–3.7	<0.0001	34.5	2.7	1.9–3.7	<0.0001
N stage (−ve vs +ve)	14.5	5.5	2.6–11.5	0.0001				NS
M stage (−ve vs +ve)	0.8	2.01	0.5–8.3	0.38				NS
Log_{10} (osteopontin)	23.2	15.4	4.9–48.5	<0.0001[c]				NS
Na (continuous)	0.9	1.05	0.94–1.18	0.35				NS
Sqrt(CRP)	20.6	1.23	1.14–1.33	<0.0001[c]				NS
FP(CA9)[d]	11.5	1.3[e]	1.1–1.5[e]	0.003[c]				NS

Abbreviations: CA9 = carbonic anhydrase IX; CI = 95% confidence interval; CRP = C-reactive protein; CSS = cancer-specific survival; DFS = disease-free survival; FP = fractional polynomial; HR = hazard ratio; NS = not significant; OS = overall survival. Units are as in Table 1. [a]95% Confidence interval; HR. [b]Worse survival in male patients. [c]χ^2 with 2 degrees of freedom because of a 1-degree fractional polynomial. In the DFS analysis, M is included as a variable and refers to four patients with metastatic adrenal disease which was surgically resected. [d]Fractional polynomial: inverse square root for DFS. [e]HR comparing lower and upper quartiles for variable coded with fractional polynomial transformation.

DISCUSSION

Improved prognostic biomarkers can direct clinicians towards more appropriate treatment and surveillance strategies which in turn will have an impact on patient outcomes, quality of life and health economics. Markers available pre-operatively offer the opportunity to contribute to emerging neo-adjuvant strategies and also to clinical decisions regarding surgical options, complementing imaging findings whereas those available post-operatively can contribute similarly but complementing the additional information available from pathological investigations. The enhancement of the UISS model with p53, vimentin and CA9 (Kim *et al*, 2004), and the Bioscore model based on B7-H1, survivin and Ki-67 (Parker *et al*, 2009) indicate the potential utility of combining tissue markers with clinicopathological models. Circulating markers have the advantage of being objectively and reproducibly measured but studies examining these are few, generally confined to a single marker and often relatively small and/or include heterogeneous patient groups. We clearly show in this study of 216 patients with all stages of ccRCC that CRP and CA9 are independently prognostic for OS/CSS and OS, respectively, and that CRP, CA9 and OPN all either add value to a pre-operative prognostic nomogram (Karakiewicz *et al*, 2009) or provide greater prognostic

discrimination than stage. Moreover, OPN and/or CA9 identify specific poor prognosis subsets of patients, including for OPN those with non-cancer-related risk who may be best served by adopting an expectant approach to their management. Interestingly, CA9 was not independently significant for OS when only data from the patients with non-metastatic disease were examined.

Low tissue CA9 expression has been associated with poor prognosis (Bui *et al*, 2003; Sandlund *et al*, 2007; Patard *et al*, 2008) although confirmed only on univariate but not on multivariate analysis in a subsequent larger study (Leibovich *et al*, 2007), possibly due to differences in patient groups and analysis methods, for example, the proportion of patients with metastatic disease differed considerably and immunohistochemical analysis was undertaken either on whole sections or on tissue microarrays and CA9 expression is heterogeneous.

Since a soluble form of CA9 was first described (Zavada *et al*, 2000), serum CA9 has also been significantly associated with stage, grade and tumour size on univariate analysis (Li *et al*, 2008; Zhou *et al*, 2010) with higher levels in the 12 out of 91 ccRCC patients with recurrent disease (Li *et al*, 2008). A recent larger study involving 361 RCC patients including 287 with clear cell histology found a positive trend between serum and tissue CA9 and a

significant correlation between serum CA9 and stage but not grade. Patients with serum concentrations higher than the median tended to have shorter survival (mean of 48 vs 79 months) although not reaching significance (Papworth et al, 2010). In contrast, we found correlations with both stage and grade and independent prognostic significance for CA9 for OS with higher serum concentrations being associated with poorer outcome. In agreement with the above study, CA9 was not independently significant for CSS. However, there was a similar trend, and the lack of significance perhaps merely results from there being less CSS events. Patient populations appear to be similar between the two studies in terms of stage and being untreated at the time of sampling. In our study, all patients underwent nephrectomy but that may not be the case in the earlier study (Papworth et al, 2010), samples had been stored for a considerably shorter period although no effect of storage has been noted and in contrast to

examining outcome on the basis of median CA9 concentration ($149 \, pg \, ml^{-1}$) we treated CA9 as a continuous variable and analysed it using fractional polynomial transformations. However, this difference between studies was still apparent when we reanalysed our data using either our median value ($109.5 \, pg \, ml^{-1}$) or their median value of $149 \, pg \, ml^{-1}$ as the cutpoint for CSS (e.g., in the latter case, $\chi^2 = 17.1$, $P = 0.00004$, HR $= 2.65$, 95% CI on HR: 1.64–4.27). Clearly, this difference in studies warrants further investigation – median serum concentrations seem to be about 30–50% higher and far higher maximum concentrations were seen in the previous study (Papworth et al, 2010) although the same assay was used which we have recently extensively validated (Wind et al, 2011). Interestingly, CA9 has been reported as being associated with survival on univariate analysis in patients receiving sorafenib (Peña et al, 2010) but this is difficult to interpret given that EDTA plasma was used and we have previously identified metal ion interference with the specific immunoassay used in that study (Wind et al, 2011).

Osteopontin is an integrin-binding glycophosphoprotein implicated in processes such as invasion and angiogenesis and is overexpressed in many tumour types and associated with prognosis (Bellahcene et al, 2008; Anborgh et al, 2010). In ccRCC ($n = 171$), 61% of samples were completely negative for OPN in tumour cells with varying degrees of positivity in the remaining cases. Positivity was reported to be strongly correlated with tumour size, grade and stage, and was associated with poor survival but only at the univariate level (Matusan et al, 2006). In a study involving 80 RCC (55 ccRCC) patients, plasma OPN correlated with T stage and grade and achieved independent prognostic significance although interestingly tumour stage and grade failed to do this (Ramankulov et al, 2007). This is in contrast to our study although we also found significantly higher concentrations in patients with metastatic disease which they report. This difference may reflect sample size or type with 55 out of 80 patients being ccRCC compared with our study which was completely ccRCC subtype and almost 50% having metastatic disease (some of whom had previously undergone nephrectomy) compared with 24% in our study. Additionally in our study the close correlation of OPN with stage and CRP that were
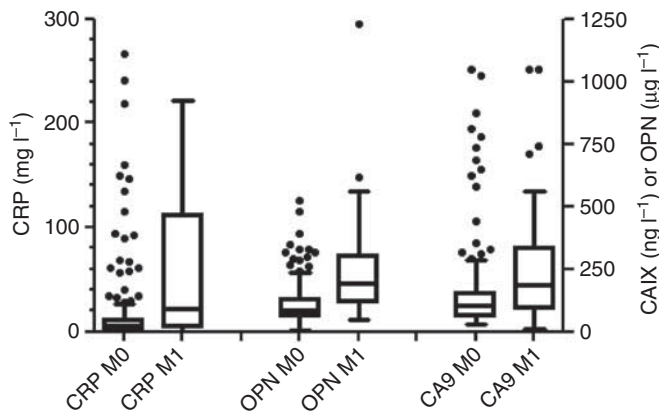

Figure 1 Tukey's box and whisker plots for each of CRP, OPN and CA9 in M1 and M0 ccRCC patients. Values were median (IQR), respectively, of 22.2 (4.2–111) vs 5.7 (0.6–11.5) $mg \, l^{-1}$ for CRP, 189.2 (114.8–303.4) vs 82.3 (57.5–131.1) $\mu g \, l^{-1}$ for OPN and 186.4 (89.6–336.2) vs 102.5 (61.6–153.9) $ng \, l^{-1}$ for CA9. Concentrations of all analytes were significantly different ($P < 0.0001$; Mann–Whitney) between M0 and M1 groups.
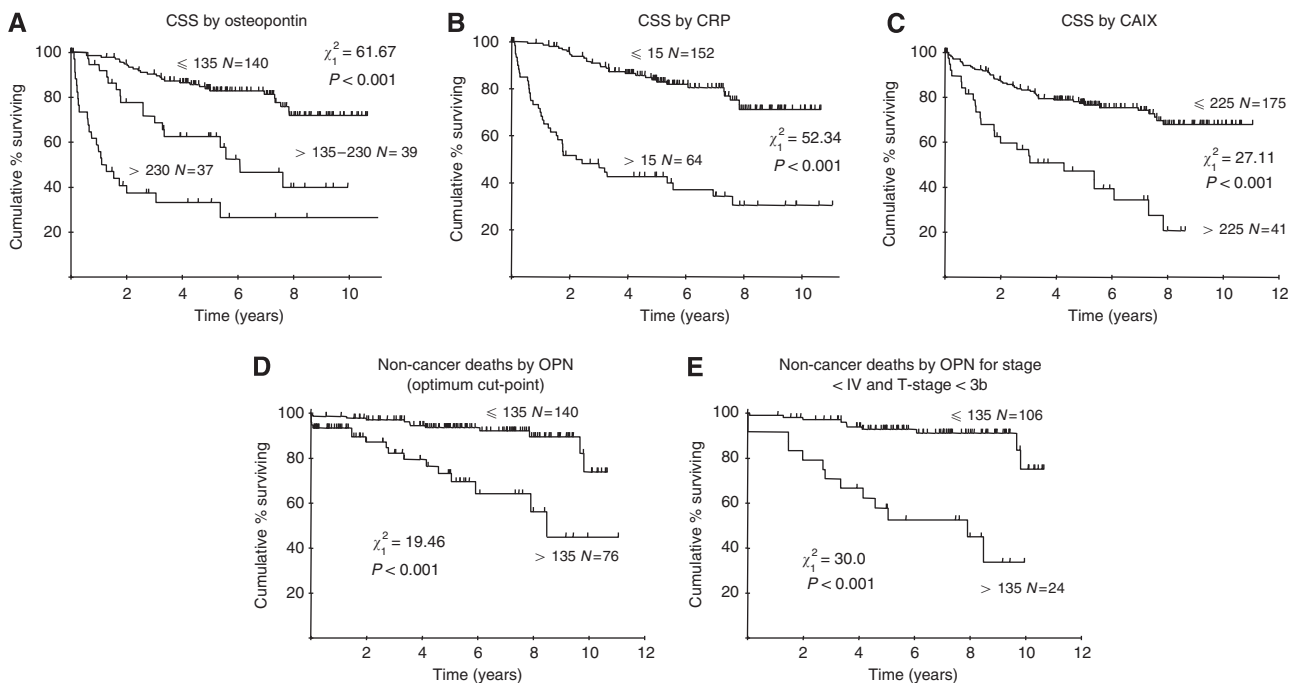

Figure 2 Kaplan–Meier curves for CSS based on circulating concentrations of (**A**) OPN, (**B**) CRP and (**C**) CA9; and for non-cancer deaths based on OPN in (**D**) all patients in the study and (**E**) early stage patients (stage <IV and T stage <3b).
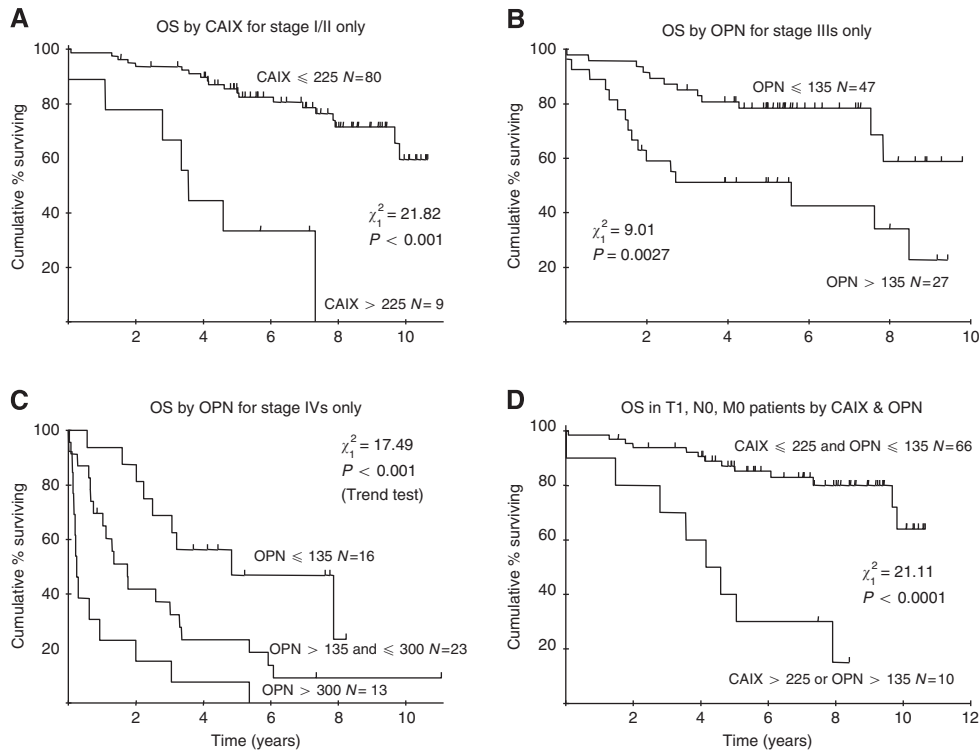
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Figure 3 Kaplan–Meier curves for OS showing the prognostic utility of CA9 or OPN in distinguishing specific poor prognosis subgroups of RCC patients. (**A**) CA9 in stage I/II patients; (**B**) OPN in stage III patients; (**C**) OPN in stage IV patients; (**D**) CA9 and OPN combined in patients with T1, N0, M0 disease.

independently significant may have contributed to this. In our study, OPN was the most predictive factor for OS, outperforming the pre-operative OS nomogram, and adding to the CSS nomogram by identifying a poor prognosis group of patients with a pre-operative nomogram score >150, suggesting that elevated OPN may be predictive of both cancer and non-cancer survival.

The detection of small incidental renal masses has increased due to the widespread use of imaging modalities. Imaging alone is often inadequate to characterise the malignant potential of renal masses and percutaneous biopsy of solid enhancing renal masses has a limited role in the diagnosis of RCC and its variants. Guidelines on assessing risk and appropriate surveillance frequency are needed and for elderly patients and patients with significant comorbidities, competing health risks may influence OS more significantly than an untreated small renal mass. High levels of CA9 or OPN identified a high-risk group of stage I patients, who mostly died from non-cancer-related causes. Osteopontin levels are elevated in vascular and inflammatory disorders (Scatena *et al*, 2007), which may explain this and which may potentially influence some previously reported cancer studies examining OS in the context of OPN concentrations. Carbonic anhydrase IX, being hypoxia driven, may also be similarly affected in vascular and inflammatory disorders. This needs further validation but may prove useful in identifying high-risk patients who should not undergo needless surgery for their small renal mass.

This study has clearly shown the potential prognostic utility of the circulating markers CRP, CA9 and OPN in RCC prognosis, outperforming stage alone, with possible uses including

identification of poorer prognosis subgroups pre-operatively to allow appropriate neo-adjuvant intervention or influence surgical decisions, or post-operatively for stratification in terms of treatment or surveillance. Further larger scale multicentre studies are now warranted to confirm and extend these findings and to determine their role in complementing or being incorporated into existing clinicopathological models, either for ccRCC or for additional histological subtypes.

Disclaimer

The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

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