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## **Presence of tumoural C-reactive protein correlates with progressive prostate cancer**

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## **Abstract**

C-reactive protein (CRP) is an acute phase protein implicated in the progression of cancer. A positive correlation between tumour stage and plasma CRP levels was demonstrated in prostate cancer, indicating a relationship between raised CRP levels and more aggressive disease suggesting a role for inflammatory response in tumour progression.

Aim of this study was to assess the tumoural presence and cellular location of CRP and establish if these are linked to clinicopathological features of the cohort and patient survival.

Tissue microarray technology was employed to analyse 50 matched pairs of hormone sensitive and refractory prostate cancers. Immunohistochemistry was performed using antibody to CRP. CRP was assessed using the weighted histoscore method.

CRP presence was observed in the cytoplasm and nucleus of selected tumours. Cytoplasmic CRP correlated positively with metastases at diagnosis ( $p=0.039$ ), whereas nuclear CRP presence correlated with metastases at relapse ( $p=0.006$ ). A trend towards an increase in cytoplasmic and nuclear CRP presence from hormone sensitive to hormone refractory tumours was noticed. No significant association between tumoural CRP presence, time to biochemical relapse or disease specific survival was observed.

Tumoural CRP is likely to play a role in progression of prostate cancer, as it is associated with increased presence of metastases at time of diagnosis and time of relapse. A larger powered study is necessary to establish if CRP presence is associated with disease specific survival.

## Introduction

Prostate cancer has the highest incidence and the second highest mortality of any cancer affecting UK males. Whilst the 5 year relative survival for organ confined prostate cancer is over 90%, for locally advanced or metastatic disease it is only around 30% <sup>1</sup>. The mainstay of treatment for locally advanced or metastatic prostate cancer is androgen deprivation therapy <sup>2</sup>. Unfortunately this therapy is only effective for a median of 18 months, following which the patients relapse and the tumours are able to grow in a castrate environment <sup>3</sup>. At this stage they are termed castrate resistant (CRPC). Currently there is no definitive curative treatment for castrate resistant prostate cancers <sup>2</sup>, however taxane therapy can extend survival for a median time period of 3 months.

C-reactive protein (CRP) is an acute phase protein implicated in the progression of prostate cancer. CRP is a member of the pentraxin family and composed of five identical polypeptide units which confer a very high stability to the molecule. Plasma CRP is mainly produced by hepatocytes in response to inflammatory cytokines, particularly IL-6 and IL-1. Due to its high stability, CRP has a relatively long plasma half life of 19 hours, and is a naive systemic marker of inflammation <sup>4,5</sup>. Elevated plasma CRP levels are found in many disease processes including infection, myocardial infarction and malignancies <sup>5</sup>.

Elevated circulating CRP levels have been shown to be linked to poor prognosis in several cancers including multiple myeloma, melanoma, lymphoma, ovarian, renal, pancreatic, lung and gastrointestinal tumours <sup>6</sup>. In metastatic and castrate resistant prostate cancer elevated plasma CRP levels have been recorded and shown to be independent predictors of poor survival and to be associated with a lower probability of response to chemotherapy <sup>7,8</sup>.

A positive correlation between tumour stage and plasma CRP levels has been demonstrated in both renal cell carcinoma and prostate cancer <sup>7,8</sup>. This indicates a relationship between

raised CRP levels and more aggressive disease suggesting a role for inflammatory response in tumour progression. But as yet the mechanism for this is unexplained.

It is hypothesised that CRP exerts its effect via activation of NF- $\kappa$ B pathway. NF- $\kappa$ B presence has been linked with invasion and metastasis in prostate cancer and has been shown to be upregulated in castrate resistant prostate cancer <sup>9,3</sup>. NF- $\kappa$ B is also believed to have a proinflammatory role; its activation in tumour associated inflammatory cells has been shown to contribute to tumour growth by inducing synthesis of tumour promoting proinflammatory mediators <sup>10</sup>. In vitro evidence from human hepatoma cell lines also suggests a link between NF- $\kappa$ B and CRP, suggesting that NF- $\kappa$ B participates in C-reactive protein production. Other work done in hepatoma cell lines and coronary artery smooth muscle cells looking at IL-17 stimulation of C-reactive protein production again showed this relationship between NF- $\kappa$ B and CRP <sup>11</sup>.

Recent work has shown that CRP levels are not only increased systemically but also locally within the tumour. Jabs *et al* showed that circulating CRP levels correlated with CRP mRNA expression in renal cell carcinoma and Nozoe has provided immunohistochemical evidence of CRP production by squamous cell carcinoma of the oesophagus <sup>8,12</sup>. The origin of this “tumour C-reactive protein” is poorly explained.

This study seeks to determine the relationship between tumoural CRP presence, interrelationship to clinicopathological features of the patient cohort and cancer specific survival in the progression of prostate cancer from hormone naive to castrate resistant tumours.

## **Material and Methods:**

### *Patient Cohort*

Ethics approval was granted to this study by the Scottish multi-centre research ethics committee.

Patients with matched hormone naive and castrate resistant tumour samples were retrospectively selected for analysis. To be selected for the study, patients had to have initially responded to hormone deprivation therapy, defined as a decrease of PSA level of 50%, and then gone on to relapse, described as two consecutive rises in PSA level of over 10%. A pre and post sample had to be available for analysis. Pre-hormone naive samples were obtained from patients diagnosed with locally advanced or metastatic prostate cancer who received surgery followed by androgen deprivation or maximum androgen blockade therapy. The hormone naive samples were obtained from a TURP or TRUS guided biopsy. Patients then had to relapse. Post-hormone samples could only be collected if patients required TURP to relieve clinical symptoms (e.g. bladder outflow obstruction). This tissue was then utilised to construct a tissue microarray (TMA).

#### Tissue microarray construction

Tissue microarrays were already available for use in the current study. Two 0.6mm<sup>2</sup> cores of lowest and highest Gleason grade prostate cancer tissue, identified by the pathologist, were removed from representative areas of the tumour taken from prostate cancer patients. All TMA blocks were constructed in duplicate. For missing samples and samples not on the TMA due to insufficient tissue 4 µm sections taken from the original paraffin blocks were used.

#### Immunohistochemistry- anti C-Reactive Protein

Sections were dewaxed and rehydrated through a series of xylene and alcohol washes. Heat mediated antigen retrieval was performed in Tris EDTA buffer (pH 8) for 20 minutes in a DAKO microwave. Sections were incubated in 5% blocking solution at 25°C for 20 minutes before being incubated in 1:100 dilution of primary antibody at 25°C for 2 hours. The primary antibody for detecting C- reactive protein was rabbit monoclonal [Y284] to C-reactive protein

(Abcam plc., Cambridge, UK). Antibody antigen binding was detected using the peroxidase-based Envision (Dako, Cambridgeshire, UK) technique using a diaminobenzidine chromogenic substrate system. Sections were then counterstained using Haematoxylin, dehydrated and mounted in DPX. In each run a positive and negative isotype matched control was included to ensure no false positive staining.

#### Immunohistochemistry- Scoring

Protein presence of each core (4 per tumour specimen) was assessed using the weighted histoscore method (H score method). The weighted histoscore method grades staining intensity as negative (0), weak (1), moderate (2) and strong (3) and then multiplies the percentage of tumour cells within each category by the number assigned to that category (0, 1, 2 or 3 respectively)<sup>13</sup>. The histoscore ranges from zero (minimum) to 300 (maximum). All TMAs were scored independently by two observers for their nuclear and cytoplasmic protein staining within each tumour core. Agreement between observers was good and measured in interclass correlation coefficient (ICCC). All ICCC scores were above 0.7.

#### Western Blot

Western blots were performed to ensure the specificity of the antibody used in the immunohistochemistry study.

Purified Heat-Shock Protein (HSP) 90 (Abcam plc, Cambridge), C-Reactive Protein (Abcam plc, Cambridge), and colorectal cell lysates (Abnova, Taiwan) were resolved by 4-12% gradient Bis-Tris gel electrophoresis (Invitrogen, UK); proteins were transferred to nitrocellulose membranes (Millipore, UK), blocked for 1 hour in 5% Marvel in TTBS and probed with primary antibody, anti-CRP (1:1000 Abcam, UK) or anti- HSP90 (1:750 AbCam, UK) at 4°C overnight. Membranes were incubated with secondary antibodies (anti-rabbit 1:2000, Cell Signalling Technologies, UK) and 10ul of anti-biotin (Amersham, UK) and visualized with ECL kit (Amersham, UK). The membranes were stripped by incubating with Re-Blot Plus stripping buffer (Chemicon, UK) before re-probing with primary antibody, anti-

HSP-90 (1:25 Abcam, UK) at 4°C overnight. Membranes were incubated with secondary antibodies (anti-mouse 1:10000, Cell Signalling Technologies, UK) and 10ul of anti-biotin (Amersham, UK) and visualized with ECL kit (Amersham, UK) (figure 1).

### Statistical Analysis

Wilcoxon Signed Rank Tests were used to compare CRP presence between androgen naive and androgen innaive tumours. Kaplan-Meier curves generated time to relapse, time to death from relapse and disease specific survival rates. The log rank test was used to compare significant differences between subgroups using univariate analysis. A p-value of 0.05 was deemed to be significant. Data are expressed as median and range. The statistical analyses were performed using a statistical software package (SPSS 15.0 Inc., Chicago, IL, USA).

### **Results:**

#### Clinicopathological details of patient cohort

The cohort consisted of 50 patients. Matched data pairs were available for 42 patients. At initial diagnosis the median age of patients was 70 years (Interquartile range (IQR) 65.5-73.0 years). Median Gleason score was 7.0 (IQR 6.5-9.0) and 15 patients (35.7%) had metastases. Median time to biochemical relapse from diagnosis was 2.59 years (IQR 1.67-4.05 years) and median overall survival was 4.39 years (IQR 3.34-6.74). Patients with locally advanced (66%) or metastatic prostate cancer (34%) subsequently received surgery and androgen deprivation therapy (14 sub capsular bilateral orchidectomy, 27 GnRH analogue, 1 had both). Twenty-nine of the 42 patients also received anti androgen therapy and this included all those who received GnRH analogues. On univariate analysis tumoural PSA expression ( $p < 0.001$ ) was associated with shorter time to biochemical relapse and overall survival was associated with presence of metastases at relapse ( $p = 0.013$ ).



During the period of follow up, 36 patients died of their cancer, a further 10 patients died of intercurrent disease, 1 patient was lost to follow. At the end of the follow up period 3 patients were still alive. Correlations between the clinicopathological characteristics of this cohort are shown in table 1a and 1b.

#### CRP presence in hormone naive prostate tumours

Due to tissue limitation CRP presence was successfully assessed in 86% of hormone naive tumour samples (43/50). From this 86%, 79.1% (34/43) exhibited cytoplasmic and 27.9% (12/43) nuclear CRP presence (figure 2a). Tumours were subdivided into high or low presence of CRP according to median values of the protein being assessed. Chi square analysis was then performed to assess correlation between clinical parameters and protein presence (table 1a). Presence of CRP in the cytoplasm correlated positively with metastases at diagnosis ( $p=0.039$ , table 1a). Interestingly, cytoplasmic CRP presence was not shown to correlate with nuclear CRP presence.

#### CRP presence in castrate resistant prostate tumours

Due to tissue limitation CRP presence was successfully assessed in 82% of castrate resistant tumour samples (41/50). From this 82%, 75.6% (31/41) displayed cytoplasmic and 42.9% (18/42) nuclear CRP presence (figure 2b). Again tumours were subdivided into high or low CRP presence according to median values of the protein being assessed. Chi square analysis was then performed to assess correlation between clinical parameters and protein presence (table 1b). Nuclear CRP presence correlated with metastases at relapse ( $p=0.006$ , table 1b).

#### Change in CRP presence in the transition from hormone naive to castrate resistant disease

In order to determine if levels of CRP presence changed in the transition from hormone naive prostate cancer to castrate resistant disease, boxplots were constructed and levels

were compared hormone naive tumours to castrate resistant tumours using the Wilcoxon Signed Ranks Tests. Whilst these results did not reach significance, there was a trend towards an increase in presence of cytoplasmic and nuclear C-reactive protein noted (figure 3a and 3b).

As the hormone naive and castrate resistant tumour samples are matched in this cohort, it was possible to analyse changes of CRP levels in pre and post hormone escape on an individual patient basis. In order for a change in CRP presence to be deemed significant, the difference had to be greater than 50. This value was chosen as it is the threshold used to judge acceptable levels of interobserver variability and is unlikely to be a chance finding. This information can then be used to divide patients into those with a decrease in CRP presence, those with no change and those with an increase.

### Survival Analysis

Kaplan-Meier survival curves were generated and Log rank tests performed to assess if presence of CRP within the tumour was related to time to relapse or disease specific survival in hormone naive prostate cancer or time to death from relapse or disease specific survival in castrate resistant prostate cancer. Again, CRP presence was divided into high or low according to median values. No significant association was seen in the hormone naïve prostate cancer patient cohort between CRP presence, time to biochemical relapse (cyto CRP  $p=0.392$ , nuc CRP  $p=0.998$ ; figure 4a and 4b) and disease specific survival (cyto CRP  $p=0.0.830$ , nuc CRP  $p=0.0.481$ ; figure 4c and 4d). In the castrate resistant patient cohort no significant associations with time to death from relapse (cyto CRP  $p=0.946$ , nuc CRP  $p=0.783$ ; figure 4e and 4f) and disease specific survival (cyto CRP  $p=0.917$ , nuc CRP  $p=0.139$ ; figure 4g and 4h) was observed either.

Increased CRP presence in the cytoplasm was noticed in 4 patients, whereas 2 patients showed a decrease in cytoplasmic CRP. However the majority of patients ( $n=26$ ) had no significant changes CRP presence in the cytoplasm. None of patients showed a significant change in presence of CRP in the nucleus. Therefore due to the low patient numbers

demonstrating significant changes in tumoural CRP presence, survival analysis was not performed.

### **Discussion:**

Currently it is hypothesised that CRP plays an important role in the progression of prostate cancer and is associated with a more aggressive and invasive phenotype. The results of the current study demonstrate that cytoplasmic CRP in hormone naive tumours significantly correlates with presence of metastases at cancer diagnosis. Serum levels of CRP have previously been linked with metastases in prostate cancer <sup>14</sup>. This present study also established a correlation between metastases at relapse and nuclear CRP presence.

To our knowledge, there is currently no literature available examining the cellular location of CRP in prostate tumour specimens. It is not established if cellular location of CRP influences clinical outcome of prostate cancer patients. Multiple proteins (Akt, NF-kappa-B, IKK alpha) are known to have diverse roles in different locations of the cell. Src kinase, when activated at tyrosine site 419, and highly expressed in breast cancer tissue at cellular membrane is associated with shorter disease specific survival <sup>15</sup>.

In order to study the role of CRP in the progression of prostate cancer, CRP presence was assessed before and after progression of the tumour. There was no statistically significant difference between CRP presence before and after castrate resistance. However there was a trend towards increased CRP presence in castrate resistant prostate cancer specimens noted. This could be likely due to low patient numbers and warrants further work in expanding the patient cohort.

There are limitations of examining the biology of the primary tumour versus its metastases. It would be of interest to investigate CRP presence and expression profile of primary tumours with matched metastasis to provide insight regarding change of CRP. Recent literature suggests that the cancer's biology at its metastatic sites differs from the primary tumour. Erin

*et al.* found altered gene expression in breast cancer liver metastasis <sup>16</sup> , whereas Koo *et al.* established different immunohistochemical breast cancer phenotypes expression according to metastatic site <sup>17</sup>. Not just the potential dissimilar biology of the primary to the metastasis needs to be considered, but also the possibility that metastatic sites may be even more predictive of prostate cancer patients' clinical outcome than primary tumours.

No significant association was seen between CRP presence within the tumour and disease specific survival. This is most likely due to this study being underpowered to detect significant associations with survival. The majority of available literature relating high C-reactive protein expression with decreased survival uses serum levels of CRP to correlate with patient survival <sup>18</sup>.

Recent work has shown that C-reactive protein levels are not only increased systemically but also locally within the tumour. Jabs *et al* showed that circulating C-reactive protein levels correlated with CRP mRNA expression in renal cell carcinoma and Nozoe has provided immunohistochemical evidence of CRP production by squamous cell carcinoma of the oesophagus <sup>8,12</sup>. The origin of this "tumour C-reactive protein" is unclear. It has been suggested that the tumour is responsible for increased IL-6 production which correlates with CRP production <sup>18,19</sup>. However McArdle *et al* propose an alternative explanation, suggesting that the source of the IL-6 is the host inflammatory cells rather than the tumour <sup>19</sup>. Indeed, increased CD4+ T-lymphocyte infiltration in prostate cancer has been associated with poor cancer specific survival, independent of stage <sup>10</sup>. We have performed RT-PCR for CRP in 10 clinical prostate specimens and have used liver tissue as a positive control in this study mRNA CRP was undetectable in prostate tumour samples (unpublished results). This therefore suggests that the tumour cells are taking up CRP from their surrounding environment rather producing it.

The role of tumoural presence of CRP or even tumour expression of CRP, which was predicted to be linked to survival, is a relatively novel concept. Low patient numbers and

small range of histoscores made classification of patients into sufficiently large subgroups for statistically analysis difficult and could be possible reasons why this study did not show a significant association between relapse, survival and CRP presence in prostate tumours.

### **Conclusions:**

This study demonstrates that tumoural CRP presence is likely to play a role in progression of prostate cancer, being associated with presence of metastases at time of diagnosis and time of relapse. However this study does not show a significant association between tumoural CRP presence, biochemical relapse and patient survival. CRP presence in this cohort fails to predict time to relapse or disease specific survival. Despite of this, CRP remains key potential target as driver of cancer progression. Further work is required in a larger cohort to definitively determine the association of tumoural presence and location of CRP, biochemical relapse and survival, to explore the relationship between serum CRP and presence of CRP within the tumour itself and to elucidate the possible roles of involved signalling pathways.

### **Acknowledgement:**

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**Table 1a: The interrelationships between the clinicopathological characteristics of patients with hormone naive prostate cancer**

<b>Variables</b>	Gleason score <7 / ≥7	Metastases at Diagnosis Yes/no	Metastases at Relapse Yes/No	PSA at Relapse ≤4 / >4	CRP cyto High/Low	CRP nuclear High/Low
Age <70yrs / >70yrs	NS	NS	<b>0.012</b>	<b>0.010</b>	NS	NS
Gleason score <7 / ≥7		<b>0.005</b>	NS	NS	NS	NS
Metastases at Diagnosis Yes/No			<b>0.041</b>	NS	<b>0.039</b>	NS
Metastases at Relapse Yes/No				NS	NS	NS
PSA at Relapse ≤4 / >4					NS	NS
CRP cyto High/Low						NS

**Table 1b: The interrelationships between the clinicopathological characteristics of patients with hormone castrate resistant prostate cancer**

<b>Variables</b>	Gleason score <7 / ≥7	Metastases at Diagnosis Yes/No	Metastases at Relapse Yes/No	PSA at Relapse ≤4 / >4	CRP cyto High/Low	CRP nuclear High/Low
Age <70yrs / >70yrs	NS	NS	<b>0.012</b>	<b>0.010</b>	NS	NS
Gleason score <7 / ≥7		<b>0.005</b>	NS	NS	NS	NS
Metastases at Diagnosis Yes/No			NS	NS	NS	NS
Metastases at Relapse Yes/No				NS	NS	<b>0.006</b>
PSA at Relapse ≤4 / >4					NS	NS
CRP cyto High/Low						NS

**Table 1a** shows the interrelationship between CRP presence at different cellular levels and clinicopathological characteristics of patients with hormone naive prostate cancer; **Table 1b** demonstrates the interrelationship between CRP presence at different cellular levels and clinicopathological characteristics of patients with hormone castrate resistant prostate cancer; CRP cyto= cytoplasmic CRP presence. p-values are deemed non-significant (NS) if above 0.05

## Figure legend

### Figure 1: Western Blots for CRP and HSP90 antibody specificity

Figure 1a: Panel 1 shows a western blot probed with antibody to C-reactive protein, against purified HSP 90 lane 1 and purified C-reactive protein. Panel 2 shows a western blot probed with antibody to HSP-90, against purified HSP 90 lane 1 and purified C-reactive protein. These western blots show that the C-reactive protein antibody only detects C-reactive protein and does not cross react with HSP 90.

Figure 1b: Panel 1 shows a western blot probed with antibody to C-reactive protein, against; lane 1 purified C-reactive protein, lane 2 nuclear fraction of cell lysates extracted from colon tumour cells, lane 3 cytoplasmic fraction of cell lysates extracted from colon tumour cells and lane 4 whole cell lysates extracted from colon tumour cells. Panel 2 shows a western blot probed with anti body to HSP-90, against; lane 1 purified C-reactive protein, lane 2 nuclear fraction of cell lysates extracted from colon tumour cells, lane 3 cytoplasmic fraction of cell lysates extracted from colon tumour cells and lane 4 whole cell lysates extracted from colon tumour cells. These western blots show that the C-reactive protein antibody is not expressed in colon tumour cells.

**Figure 2:** IHC pictures demonstrating the distribution of CRP in hormone naive (Fig 2a) and hormone castrate resistant (Fig 2b) prostate cancer samples.

**Figure 3:** Boxplots illustrating the difference of cytoplasmic CRP (Fig 3a) and nuclear CRP (Fig 3b) in hormone naive and hormone castrate resistant prostate cancer specimens.

**Figure 4:** Recurrence and Survival graphs for hormone naive and castrate resistant prostate cancer cohorts. Bold line: patients with high CRP presence; dotted line: patients with low CRP presence; cyto CRP= cytoplasmic CRP; nuc CRP= nuclear CRP.

Figure 4a and 4b: No significant association noted between CRP presence in the cytoplasm and nucleus and time to biochemical relapse in hormone naive prostate cancer patients.

Figure 4c and 4d: No significant association observed between CRP presence in the cytoplasm and nucleus and disease specific survival of hormone naïve prostate cancer patients.

Figure 4e and 4f: No significant association was seen between CRP presence in the cytoplasm and nucleus of castrate resistant prostate cancer specimens and time to death from biochemical relapse.

Figure 4g and 4h: No significant association was observed between CRP presence in the cytoplasm and nucleus of castrate resistant prostate cancer specimens and disease specific survival.

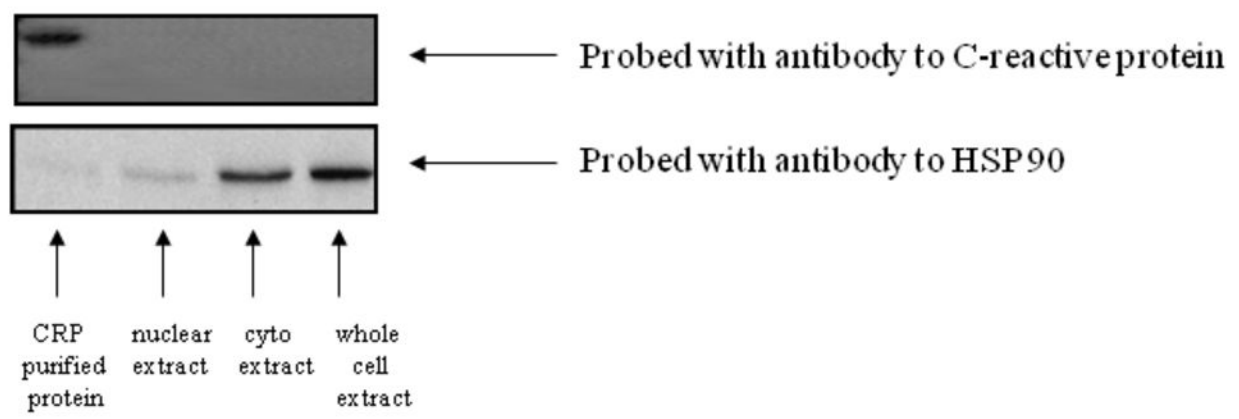
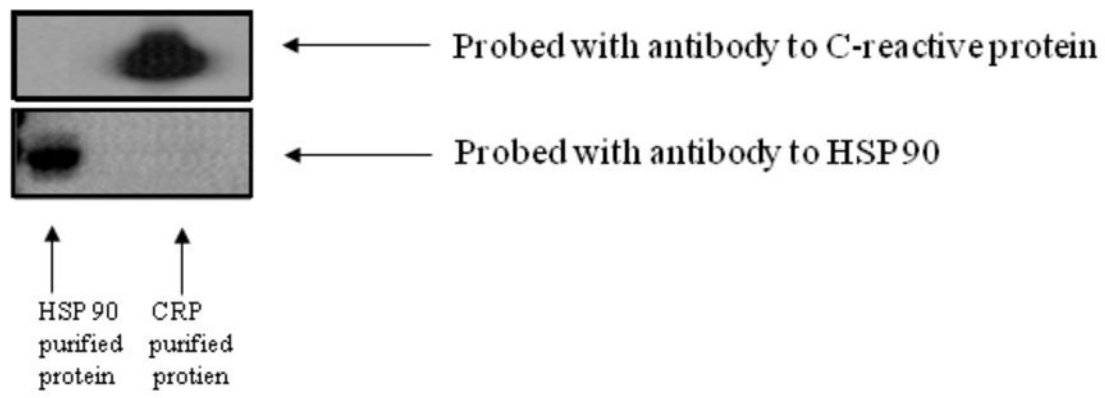


Fig 2a

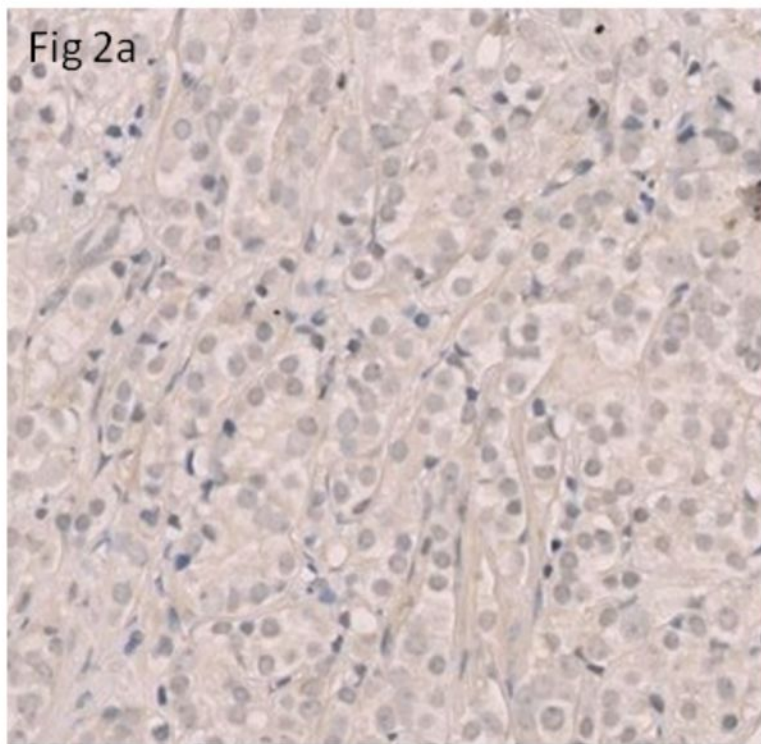


Fig 2b

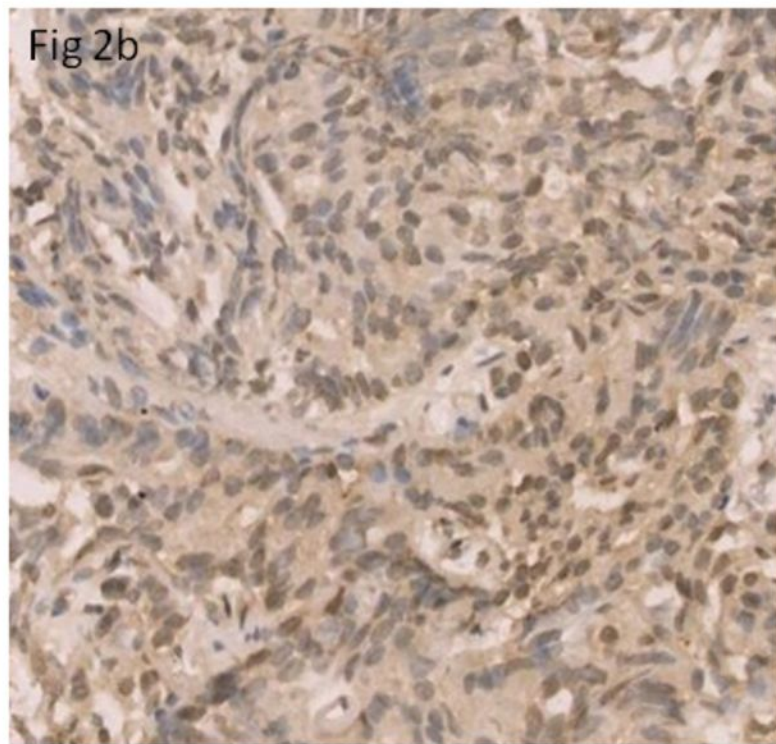


Figure 3a

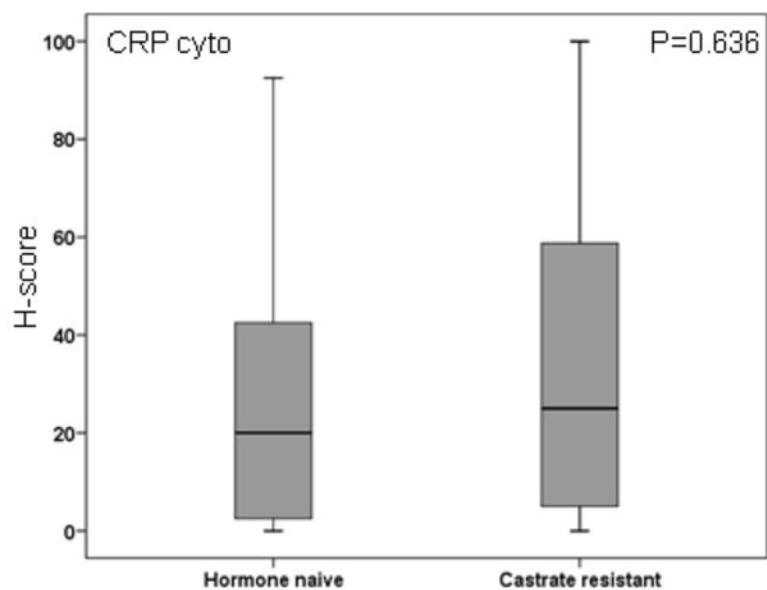
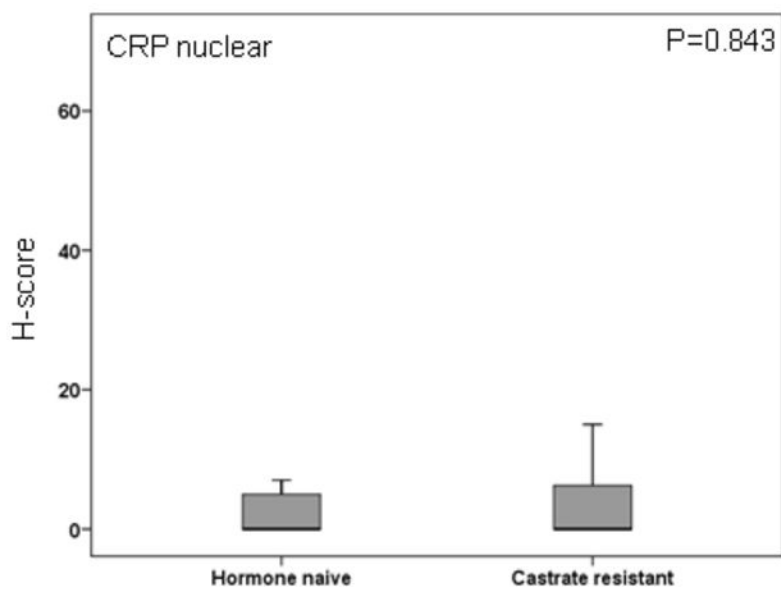
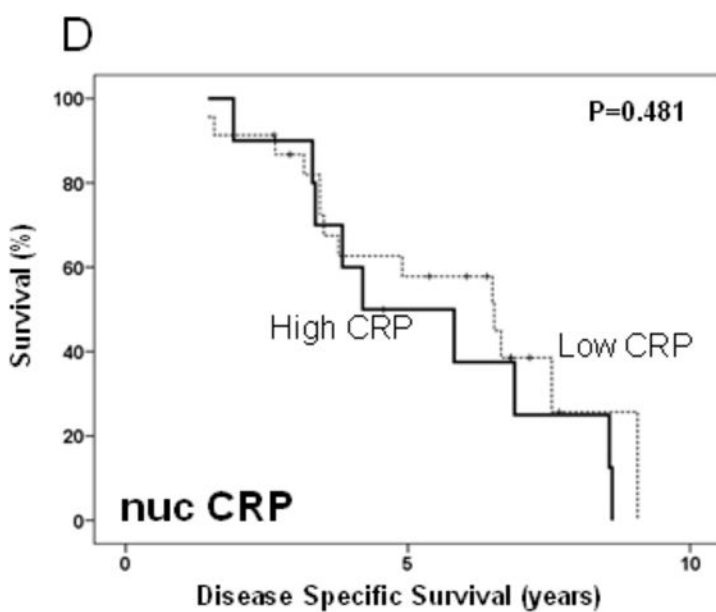
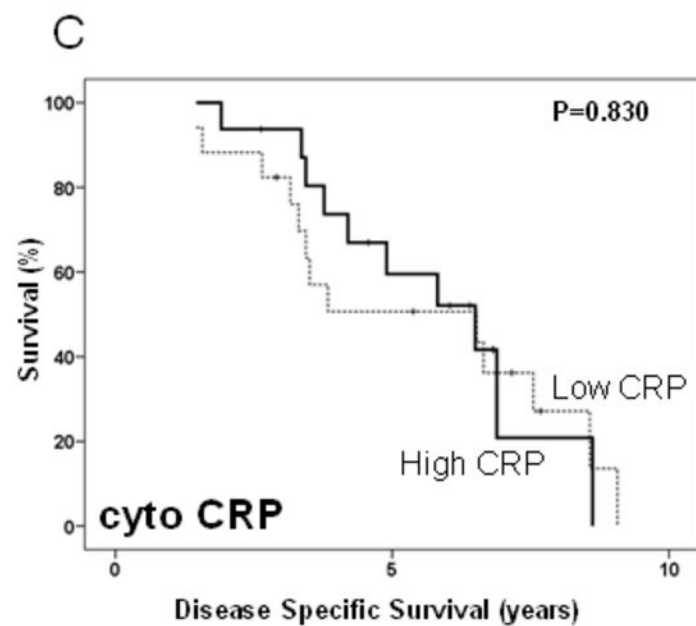
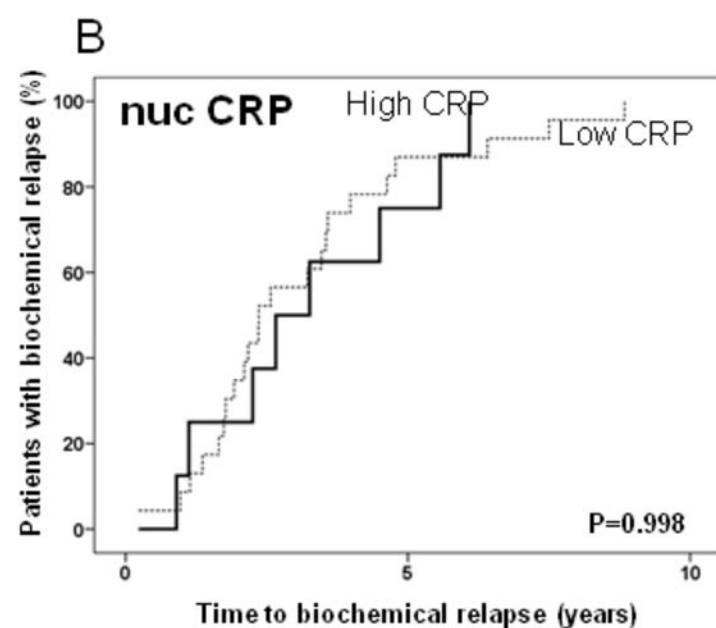
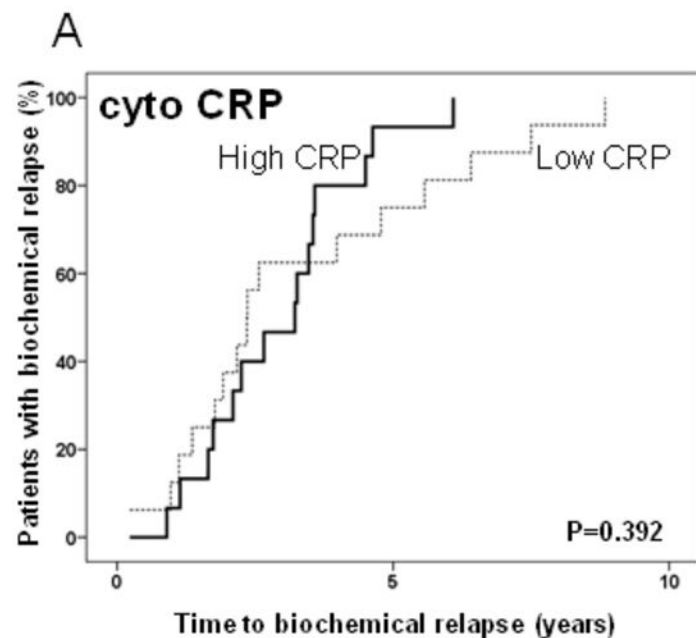


Figure 3b



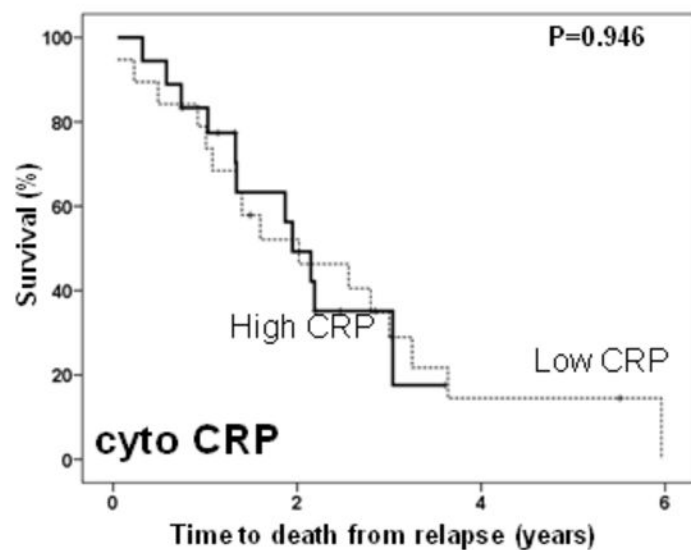


# Hormone naïve prostate cancer patients

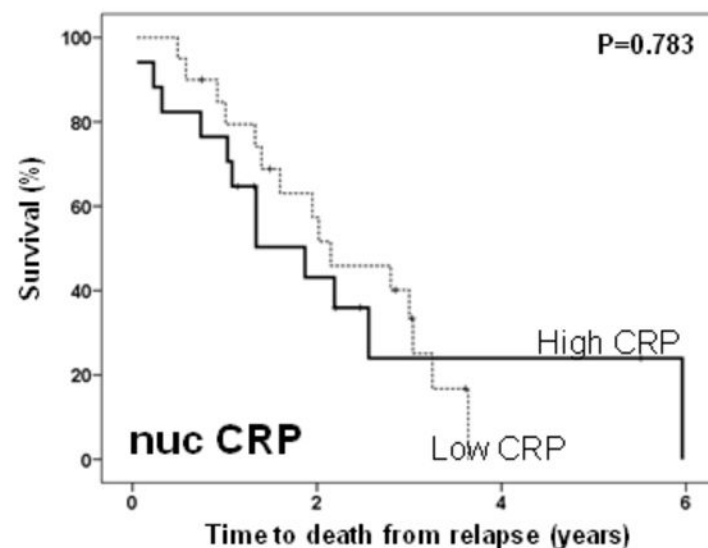


# Castrate resistant prostate cancer patients

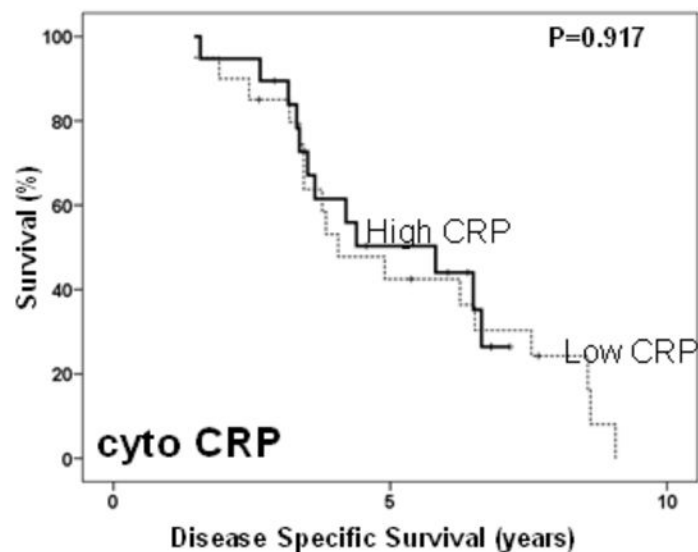
E



F



G



H

