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ETS-Related Gene (ERG) Expression as a Predictor of Oncological Outcomes in Patients with High-grade Prostate Cancer treated with Primary Androgen Deprivation Therapy: a cohort study

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ETS-Related Gene (ERG) Expression as a Predictor of Oncological Outcomes in Patients with High-grade Prostate Cancer treated with Primary Androgen Deprivation Therapy: a cohort study

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ABSTRACT

Objectives: To determine whether *ETS-related gene* (ERG) expression can be utilised as a biomarker to predict biochemical recurrence and prostate cancer-specific death in patients with high Gleason grade prostate cancer treated with androgen deprivation therapy (ADT) as monotherapy.

Methods: A multicentre retrospective cohort study identifying 149 patients treated with primary ADT for metastatic or non-metastatic prostate cancer with Gleason score 8-10 between 1999 and 2006. Patients planned for adjuvant radiotherapy at diagnosis were excluded. Age at diagnosis, ethnicity, prostate-specific antigen, and Charlson-comorbidity score were recorded. Prostatic tissue acquired at biopsy or transurethral resection surgery was assessed for immunohistochemical expression of ERG. Failure of ADT defined as PSA nadir+2. Vital status and death certification data determined using the National Cancer Registry. Primary outcome measures were overall survival and prostate cancer specific survival. Secondary outcome was biochemical recurrence-free survival.

Results: The median overall survival of our cohort was 60.2 months (C.I. 52.0-68.3). ERG expression observed in 51/149 cases (34%). Univariate analysis showed significant association of ERG positivity with increased age at diagnosis (p=0.03) and Caucasian ethnicity (p=0.04). Cox regression analysis showed Gleason score (p=0.003) and metastatic status ($p<1x10^{-5}$) to be the only significant predictors of prostate cancer specific survival. Age (p=0.02) was an additional predictor of overall survival.

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Conclusions: No significant association was found between ERG status and any of our outcome measures. ERG does not appear to be a useful biomarker in predicting response to ADT in patients with high risk prostate cancer.

Key Words: Prostatic Neoplasms; Androgen Deprivation Therapy; ERG protein; Castrate Resistance

Strengths and limitations of this study

- This observational study consists of a large cohort of solely high-risk cancers treated with initial ADT as monotherapy with subsequent vital status determination through a national death certification registry.
- The association between ERG expression and oncological survival is explored for the first time in patients on ADT as monotherapy.
- Determination of ERG status is limited to immunohistochemical detection of the protein without classification of its mutation at a genomic level.

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INTRODUCTION

The development of castration resistance is a major clinical hurdle in patients with advanced prostate cancer and is taken as a marker of impending mortality. Early identification of patients who develop castrate resistant prostate cancer can be clinically useful in enabling early aggressive treatment and therefore in reducing cancer-related deaths.

A recurrent gene fusion event involving the 5' end of ERG (ETS-related gene) to 3' TMPRSS2 (transmembrane protease, serine 2) [1] is one of the most frequently occurring genetic mutations in prostate cancer[2] but its prognostic value is under debate[3]. A meta-analysis evaluating the role of TMPRSS2:ERG fusion protein in patients undergoing radical prostatectomy found no association with biochemical recurrence or lethal disease[4].

Given that the TMPRSS2:ERG fusion protein is androgen regulated[5], its association with oncological outcomes in patients treated with androgen deprivation therapy (ADT) is possible. ERG expression inversely correlates with the levels of androgen receptor protein in the cell and may exert a selective pressure for the development of a castrate-resistant state[6]. Furthermore, ERG expression appears to be re-established in ERG fusion-positive prostate cancer after the development of castration resistance[7].

In vivo validation of ERG's metastatic influence has been controversial. Scheble VJ et al. had shown a greater proportion of castration resistant metastatic prostate cancer driven by ERG negative tumours[8], whilst Perner S et al. had observed a greater predilection to metastases in fusion positive foci[9].

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The aim of this study is to establish an association between ERG expression status and oncologic outcomes in high grade and advanced prostate cancer patients treated by ADT as monotherapy. The primary end points are overall survival (OS) and prostate cancer specific survival (PCSS). The secondary end point is biochemical recurrence-free survival (BRFS).

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PATIENTS, MATERIALS AND METHODS

Data Collection, Study Inclusion and Exclusion Criteria

Institutional approval was granted prior to the study. Patients were identified from the pathology databases at two large neighbouring hospitals, Guy's and St Thomas' hospitals NHS Foundation Trust and King's College Hospital NHS Foundation Trust in London, UK, between January 1999 and August 2006. All patients treated with primary ADT were included in the study. Those were identified among patients with a total Gleason score of 8-10. For each patient, the initial assigned treatment was identified using electronic and paper records. Patients with both metastatic and non-metastatic disease were included in the study. Clinical data collected included the age at diagnosis, the assigned treatment at diagnosis, ethnicity (Caucasian, Afro-Caribbean, or other), Charlson comorbidity score[10], date of diagnosis, total modified International Society of Urologic Pathology 2005 Gleason Score, radiological evidence of metastasis at diagnosis, history of previous prostate cancer treatment, and serial prostate specific antigen (PSA) values (ng/ml). Patients were excluded from the study for any missing data, if they did not receive ADT or were planned to receive other adjuvant therapies such as radiotherapy. The primary end points were OS and PCSS. The secondary end point was BRFS.

Vital status and death certification data

Patient vital status data were retrieved from the National Cancer Registry in Public Health England[11]. Following institutional approval, unique patient NHS numbers were linked to vital

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status, dates of death, and ICD-10 codes on the immediate cause of death (cause 1a), other diseases or conditions leading to 1a (causes 1b & 1c), underlying cause of death, and other significant conditions not directly related to death (cause 2)[12]. A prostate cancer death was defined as any death stating 'Prostate Cancer' in any of causes 1a, 1b, 1c, or an underlying cause. Biochemical recurrence was defined as an increase of more than 2ng/ml from the PSA nadir value with censoring on the date when PSA rose more than 2ng/ml above nadir [13].

Prostate cancer sample collection, tissue processing and immunohistochemical (IHC) staining

Prior to retrieval of archived prostate tissue samples, available haematoxylin and eosin-stained slides were examined by two consultant histopathologists to select one tissue block for each patient based on the largest cancer volume. Specimen numbers were used to retrieve the corresponding paraffin-embedded blocks from the archives. 3µm sections were cut from each block using the Rotary Microtome HM 32S. Immunohistochemistry was performed in batches using the Ventana BenchMark ULTRA IHC/ISH automated stainer (Ventana Medical Systems). Deparaffinization of the sections was carried by warming up the slides at 72°C in Ventana EZ Prep solution. Endogenous peroxidase activity was blocked using the Ventana inhibition kit and antigen retrieval was carried out by incubating the slides in Cell Conditioning solution-1 and subsequently heating at 100°C for 8 minutes. 100µl of Anti-ERG (EPR3864) Rabbit Monoclonal Primary Antibody was applied on each slide for 32 minutes. Visualisation was performed using anti-rabbit horseradish peroxidase (HRP)-labelled secondary antibody and 3,3'-diaminobenzidine tetrahydrochloride (DAB)

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chromogen (Roche/Ventana Ultra View DAB kit). The slides were washed and counterstained with Ventana Haematoxylin and Ventana Bluing Solution.

The IHC nuclear reactivity for ERG protein expression in the vascular endothelial cells was used as positive internal controls[14]. Tests were repeated when endothelial cells failed to stain with ERG antibody **(see supplementary figure 1)**.

H-scoring

Semi-quantitative IHC analysis of ERG expression was conducted by the H-scoring system[14]. Percentages of prostate cancer cells with positive and negative nuclear ERG staining were assessed at high magnification for each sample by two consultant histopathologists. The H-score was calculated as: 3x percentage cells with strong ERG expression + 2x percentage of cells with intermediate ERG expression + 1x percentage of cells with weak ERG expression [15]. The total H-score per sample therefore ranged from 0 to 300. H-scores were classified as negative (0-50), weakly positive (51-100), moderately positive (101-200) or strongly positive (201-300) **(see supplementary figure 2)**.

Validation of antibody clone against an alternative anti-ERG antibody

Alternative ERG staining was carried out on selected cancer tissue samples using an alternative monoclonal ERG antibody (clone 9FY, ab139431). The results are depicted on the photomicrographs shown in **supplementary figure 3**.

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Statistical Methods

OS and PCSS were determined using the Kaplan-Meier method. Multivariable Cox proportional hazards analysis was used to determine the association of clinico-pathological parameters with survival. Statistical analyses were conducted using SPSS ver 22, Graphpad Prism 5.0, and Microsoft Excel software.

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RESULTS

Cohort characteristics

527 patients with high Gleason score prostate cancer were diagnosed on biopsy, of which 169 patients were assigned to primary ADT as monotherapy. Exclusion of patients was due to tissue samples being unavailable (n=4), lack of vital status data output from the National Cancer Registry (n=4), or one or more missing clinical parameters (n=12). Complete data was available for 149 patients which formed the study population.

Mean follow-up was 46.5 (±25.2) months. 59 patients (40%) had metastatic disease at presentation. The clinical characteristics of the cohort are shown in **(Table 1)**.

ERG expression was observed in 51 cases (34%), of which nearly all demonstrated high ERG expression (92%) **(Figure 1)**, (intensity distribution of ERG staining shown in **supplementary figure 4**). No ERG expression was found in incidental benign acini within samples. ERG positivity was associated with older age, and Caucasian ethnicity, but not Gleason score, initial PSA level, or presence of metastatic disease at presentation **(Table 1)**.

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TABLE 1	ERG negative (N=98)	%	ERG positive (N=51)	%	P-value
Mean age (±SD), years	72.3 (±8.3)		75.5 (±8.6)		**0.03***
Ethnicity					
Caucasian	51	52	37	73	**0.04**
Afro-Caribbean	41	42	11	22	
Other	6	6	3	6	
Gleason score					
8	22	52	13	25	0.88
9	71	42	36	71	
10	5	6	2	4	
PSA (±SD), ng/ml	1378 (±10849)		283 (±1203)		0.48*
<10.00	4	4	4	8	0.38
10-19	17	18	7	14	
20-49	24	25	18	36	
50-99	13	13	10	20	
≥100	39	40	11	22	
Metastasis					
No	60	61	30	59	0.78
Yes	38	39	21	41	
Charlson Comorbidity					
0	43	44	25	49	0.05
1	30	31	6	12	
2	16	16	11	22	
≥3	9	9	9	18	
Follow-up (±SD), mths	47.9 (±25.5)		43.7 (±24.9)		0.34

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Table 1. Clinical characteristics of the study population stratified by ERG expression status (*p*-values obtained by χ^2 or *T-tests)

National Cancer Registry-linked oncological survival outcomes following primary androgen deprivation therapy in metastatic and non-metastatic high Gleason-grade prostate cancer

The National Cancer Registry was used to determine the vital status and death certification details for each patient. 75 patients (50%) had died during follow-up, of whom 55 had died as a result of prostate cancer. Median overall survival for the cohort was 60.2 months. OS, PCSS, and BRFS for the cohort are shown (**Figure 2)**.

Presence of metastatic disease at diagnosis significantly affected OS (p=0.001), PCSS ($p<1x10^{-7}$) and BRFS ($p<1x10^{-6}$). Gleason score significantly affected OS (p=0.004) and PCSS (p=0.004) but not BRFS (p=072). PSA at presentation only affected BRFS ($p=1x10^{-5}$). Those associations were calculated using logrank analysis.

Association of ERG expression and oncological outcomes in high risk cases treated by primary androgen deprivation therapy

Logrank analysis was first conducted to determine whether ERG expression predicted oncological outcomes in the high-risk cohort stratified by ERG expression status **(Figure 3)**.

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No statistically significant association was observed between ERG expression and OS, PCSS, or BRFS.

Cox proportional hazards regression analysis was conducted to determine independent predictors of oncological outcomes. Mutual adjustments were made for ERG expression, age, ethnicity, Gleason score, PSA, presence of metastasis at presentation, and Charlson co-morbidity **(Table 2)**. The presence of metastatic disease was significantly associated with OS (HR 2.60, 95% C.I. 1.54-4.40), PCSS (HR 4.51, 95% C.I. 2.36-8.60), and BRFS (HR 3.15, 95% C.I. 1.93-5.16). Total Gleason score was significantly associated with OS (Gleason 9; HR 2.33, 95% C.I. 1.2-4.53 and Gleason 10; HR 5.81, 95% C.I. 2.04-16.52, reference group Gleason 8) and PCSS (Gleason 9; HR 2.56, 95% C.I. 1.13-5.83 and Gleason 10; HR 6.45, 95% C.I. 2.04-16.52, reference group Gleason 8) but not BRFS. Age was significantly associated with OS only. We found no statistically significant association between ERG expression and OS, PCSS or BRFS. The results did not change when ERG expression status was replaced with the H-score (results not shown).

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TABLE 2	OS				CSS				BRFS			
	p	HR	95% C.I.		*p*	HR	95% C.I.		*p*	HR	95% C.I.	
			Lower	Upper			Lower	Upper			Lower	Upper
ERG expression	0.41	1.24	0.74	2.05	0.92	1.03	0.57	1.87	0.31	0.78	0.47	1.27
Age	**0.02**	1.04	1.01	1.07	0.19	1.02	0.99	1.06	0.82	1.00	0.97	1.03
Ethnicity												
Caucasian (ref)	0.75				0.98				0.44			
Afro-Caribbean	0.70	0.90	0.53	1.53	0.86	0.94	0.51	1.75	0.52	0.85	0.52	1.38
Other	0.57	1.35	0.47	3.86	0.93	0.94	0.22	4.10	0.24	0.49	0.15	1.59
Gleason score												
8 (ref)	**0.003**				**0.01**				0.79			
9	**0.01**	2.33	1.20	4.53	**0.02**	2.56	1.13	5.83	0.50	1.20	0.71	2.01
10	**<0.001**	5.81	2.04	16.52	**<0.01**	6.45	1.92	21.71	0.73	1.24	0.35	4.38
PSA	0.92	1.00	1.00	1.00	0.96	1.00	1.00	1.00	0.88	1.00	1.00	1.00
Metastasis	**<0.001**	2.60	1.54	4.40	**<1x10^{-5}**	4.51	2.36	8.60	**<1x10^{-5}**	3.15	1.93	5.16
Charlson Comorbidity												
0 (ref)	0.15				0.48				0.83			
1	0.19	1.52	0.81	2.87	0.85	1.08	0.50	2.35	0.56	1.18	0.68	2.07
2	0.06	1.81	0.97	3.39	0.67	1.18	0.56	2.47	0.75	0.90	0.49	1.67
≥3	0.09	2.00	0.89	4.47	0.12	2.17	0.81	5.84	0.51	1.29	0.60	2.75

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Table 2. Multivariate Cox proportional hazards analysis of ERG expression with other known oncological outcome parameters. Reference groups are indicated for categorical variables. OS = overall survival, CSS = cancer-specific survival, BRFS = biochemical recurrence-free survival, HR = hazard ratio, C.I. = confidence interval.

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DISCUSSION

This study includes a historical cohort of patients treated solely on ADT as initial therapy for high-risk metastatic or non-metastatic therapy. Advances in adjuvant treatments for high-risk prostate cancer such as radiotherapy or chemotherapy confounds the assessment of biomarkers in patients receiving ADT in more recent cohorts.

ERG is commonly described as an oncogene although its ubiquitous expression in endothelial and haematopoietic stem cells suggest an essential role in angiogenesis, endothelial cell function and haematopoiesis[16, 17]. Since the discovery of the ERG and androgen regulated TMPRSS2 genetic fusion in prostate cancer[1], its role as a sensitive and prevalent marker for prostate cancer has been shown to be highly replicable[4]. Recent whole genome sequencing studies have revealed it to be the most frequent genetic mutation in prostate cancer within the entire genome[2]. Its high prevalence amongst all grades of disease[4, 18-21] however, supports its significance to be a marker of cancer per se rather than a marker for prognosis. ERG overexpression in animal models produces prostate intra-epithelial neoplasia (PIN) but not invasive cancer, suggesting it to be an early event in the natural history of prostate cancer[22].

In organ confined prostate cancer, ERG expression and its association with clinical outcomes has been the subject of numerous studies with conflicting outcomes[3, 23]. A meta-analysis describing ERG fusion positive cancer and its associated outcomes in post-prostatectomy showed ERG fusion events to be associated with a higher clinical stage at diagnosis of T3 over T2 with a risk ratio of 1.23 , yet no association was found for cancer specific survival or disease recurrence[4].

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In this cohort, we examined the association of ERG-expression with survival endpoints in patients treated by primary ADT. Linkage of clinical data was made with the National Cancer Registry which provided an up-to-date vital status on all patients residing in England. Following multivariate analysis, we found no association of ERG expression with OS, PCSS or BRFS (**Table 2**). To the best of our knowledge, only a few studies have determined the effect of primary ADT on ERG positive cancer with varying conclusions[24-26]. Our study is the largest cohort of solely high-risk cancers homogeneously treated with initial ADT as monotherapy with subsequent high quality vital status determination through a national registry.

Although we used IHC to estimate TMPRSS2:ERG gene fusion status, studies have shown very high concordance between more accurate FISH techniques[27-30]**.** The reproducibility of the technique was assessed using an additional antibody clone, as well as determining technical success for each sample using endothelial cell expression as internal controls **(supplementary figures 1 and 3)**. The prevalence (34%) of ERG expression is in line with previous studies[4]. The association between Caucasian ethnicity and ERG expression agrees with a previous study evaluating TMPRSS2:ERG fusion events[31]. Higher age was significantly associated with ERG expression in our cohort (p=0.03), in contrast to other studies that showed a higher proportional expression in the younger men[32] or no correlation at all[4, 14]. It is possible that this is an effect seen only in high grade prostate cancer cases.

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Limitations

The retrospective nature of the study is one limitation. In addition, a subset of patients within the cohort received unplanned adjuvant therapy in addition to androgen deprivation monotherapy which may have influenced the overall survival although this was not assessed as a covariate due to the heterogeneous nature of the treatment and small patient numbers.

Both quantitative[33] and qualitative differences in the ERG mutation have been implicated in prognostication of prostate cancer. Patients with cancer cells exhibiting an aberration consisting of both a duplication and deletion of the 5' end of ERG (known as 2+ "Edel") were predisposed to a poorer disease specific survival[34, 35]. In this context, the use of IHC was a limitation as it cannot detect the genomic fusion quantitatively or qualitatively but only isolated expression of the ERG protein[7, 27]. It is important to note that the H score does not provide a quantitative measurement of the ERG mutation[27]. With this knowledge at hand, sub-classifications of ERG mutations when assessing prognostic indicators is recommended in future clinical studies. IHC may be controversial as a detection method of the TMPRSS2:ERG fusion gene. Sung JY et al. expressed caution to its use for its false positive rate[36] whilst Gsponer and colleagues identified a subgroup of ERG genetic alterations that are undetectable at a protein level[37] .

We did not examine all cores from each prostate biopsy sample, nor did we look at expression at metastatic sites and hence heterogeneity was not factored in. This is a limitation to our study. A future study using a large number of core samples can minimise this phenomenon.

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CONCLUSION

Whilst the evidence remains that ERG has adverse effects on cancer cell characteristics, controversy remains into whether its expression is significant at a clinical level. A future prospective cohort study investigating the mechanism of ERG mutation in patients with prostate cancer will be a novel reliable method to determine its predictive significance. Despite its limitations, this cohort study shows that ERG expression does not offer predictive value in prostate cancer patients treated with primary ADT.

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FIGURE LEGENDS

Figure 1. H-score distribution of ERG positive cases. 47/51 (92%) had a strongly positive H-score.

Figure 2. Oncologic outcomes of high-risk prostate cancer following primary androgen deprivation therapy. Significant associations shown in bold. BSPositive = bone scan positive.

Figure 3. Kaplan-Meier survival curves stratified by ERG expression status for OS, PCSS, and BRFS**.**

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FOOTNOTES

AUTHOR CONTRIBUTIONS: HY led the study design, analysed data and was involved in revision, drafting and approval of the final manuscript. MR was involved in sample and data collection, data analysis and drafting of the manuscript. AC and DA were both involved in analysis of the prostate cancer tissue. HM, MY and PD provided critical review of the study design and manuscript.

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SUPPLEMENTARY INFORMATION: Supplementary information is available online at *BMJ Open's* website

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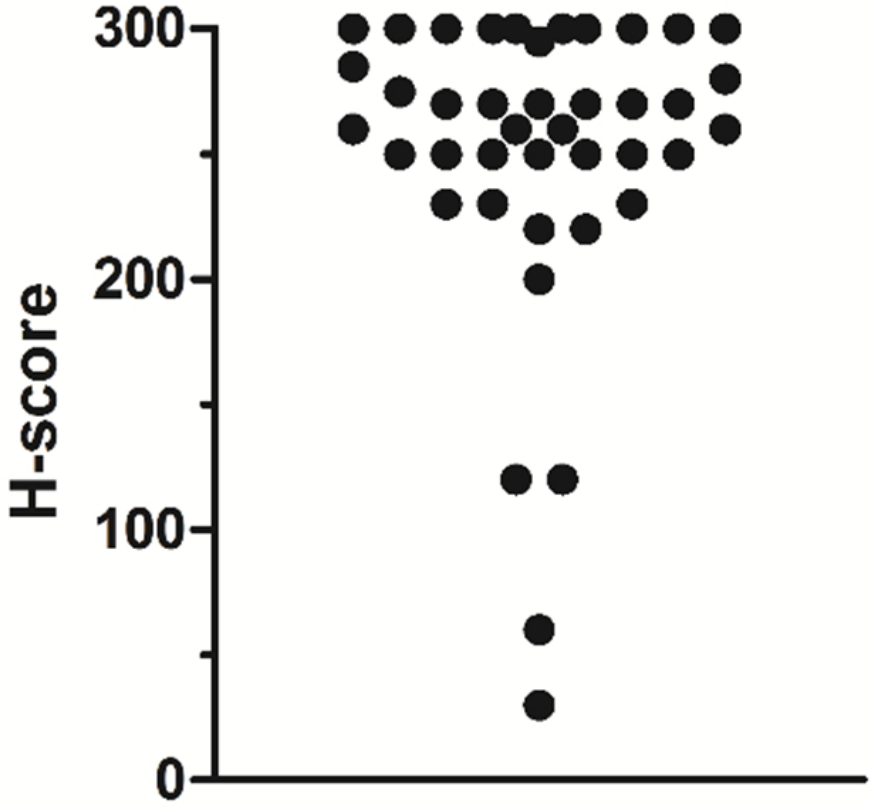

Figure 1. H-score distribution of ERG positive cases. 47/51 (92%) had a strongly positive H-score.

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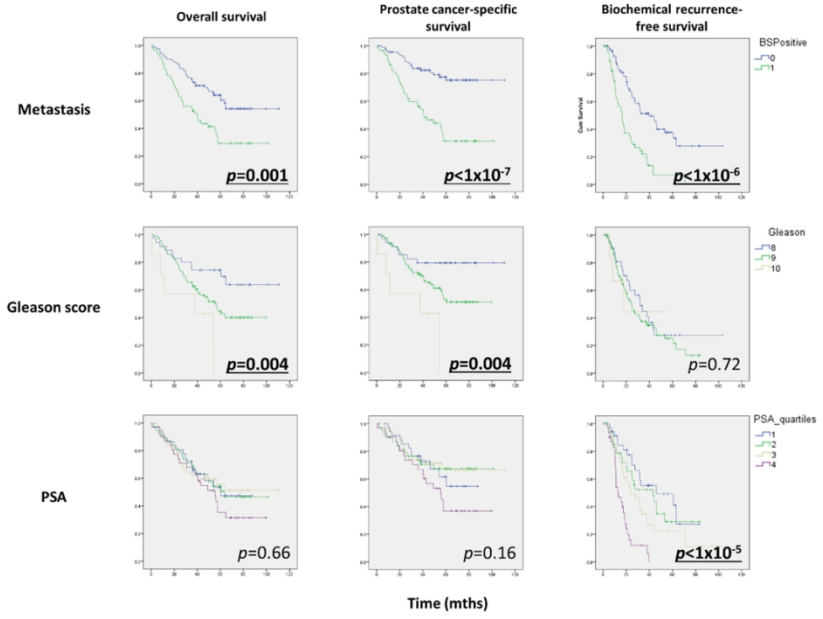

Figure 2. Oncologic outcomes of high-risk prostate cancer following primary androgen deprivation therapy. Significant associations shown in bold. BSPositive = bone scan positive.

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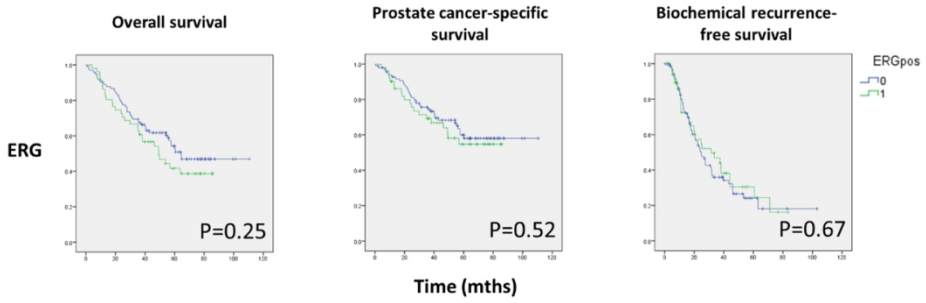
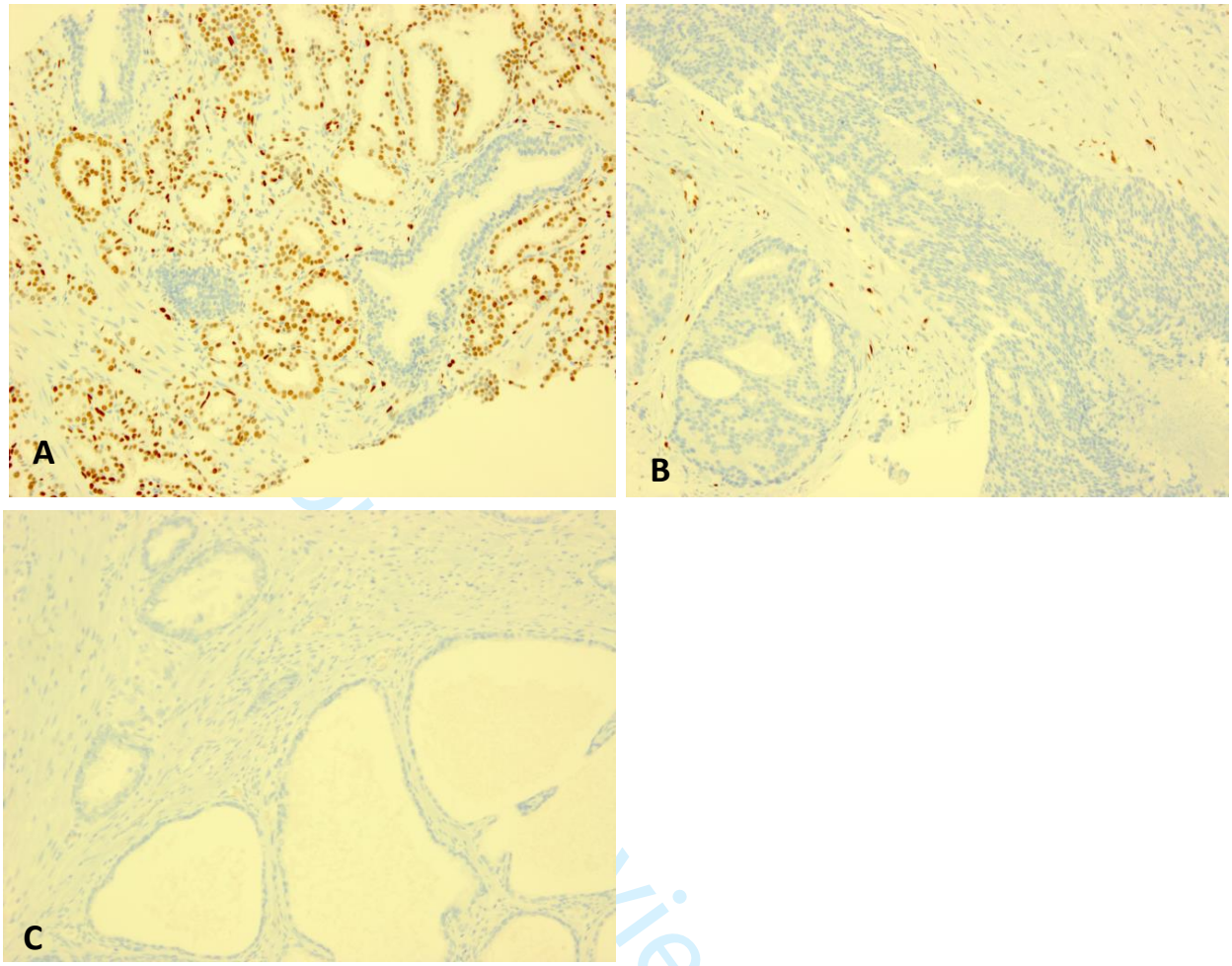


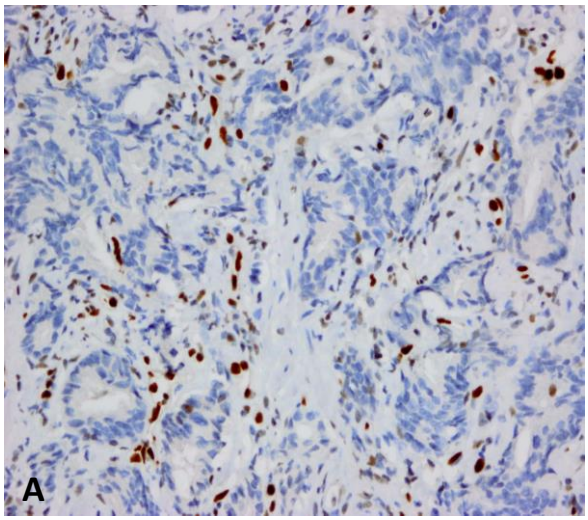
Figure 3. Kaplan-Meier survival curves stratified by ERG expression status for OS, PCSS, and BRFS.

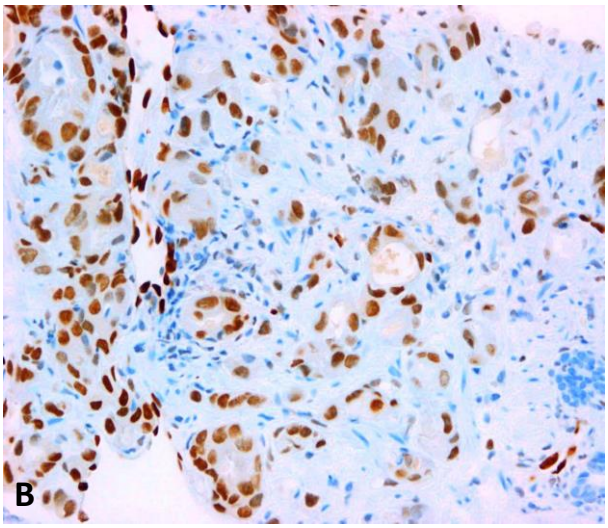
362x131mm (96 x 96 DPI)

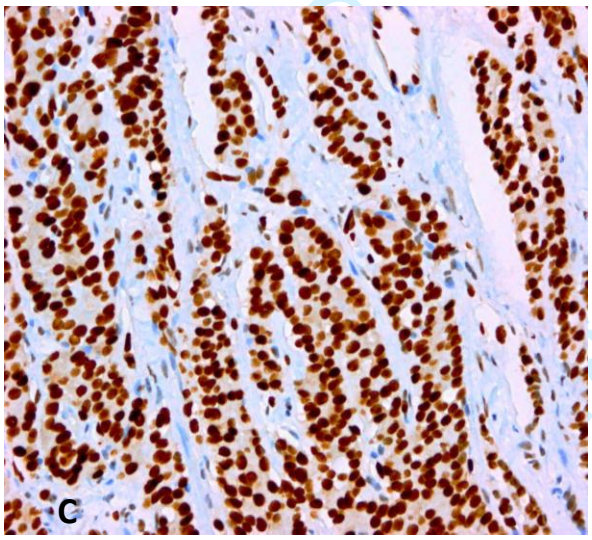
Supplementary figures



Supplementary Figure 1 – Immunohistochemistry of prostate core biopsy samples depicting failure of internal control, stained with anti-ERG (EPR3864) rabbit monoclonal antibody 5ml (23µg/ml) staining kit. ERG stains brown upon detection and small amounts are normally detected in cells. **A)** Prostate Cancer staining positive for ERG overexpression, **B)** Prostate Cancer from a patient that is negative for ERG overexpression with positive internal control endothelial staining and **C)** No brown colour is detected as the staining on this control slide was unsuccessful.







Supplementary Figure 2: Range of staining using the ERG-antibody (EPR3864 clone). **A)** ERG expression is negative in tumour cells, but positive in nuclei of lymphocytes and endothelial cells (X200). **B)** Moderate ERG expression is seen in tumour epithelial cell nuclei (X200). **C)** A strongly positive ERG expression in seen showing fused acinar prostate cancer (X200).

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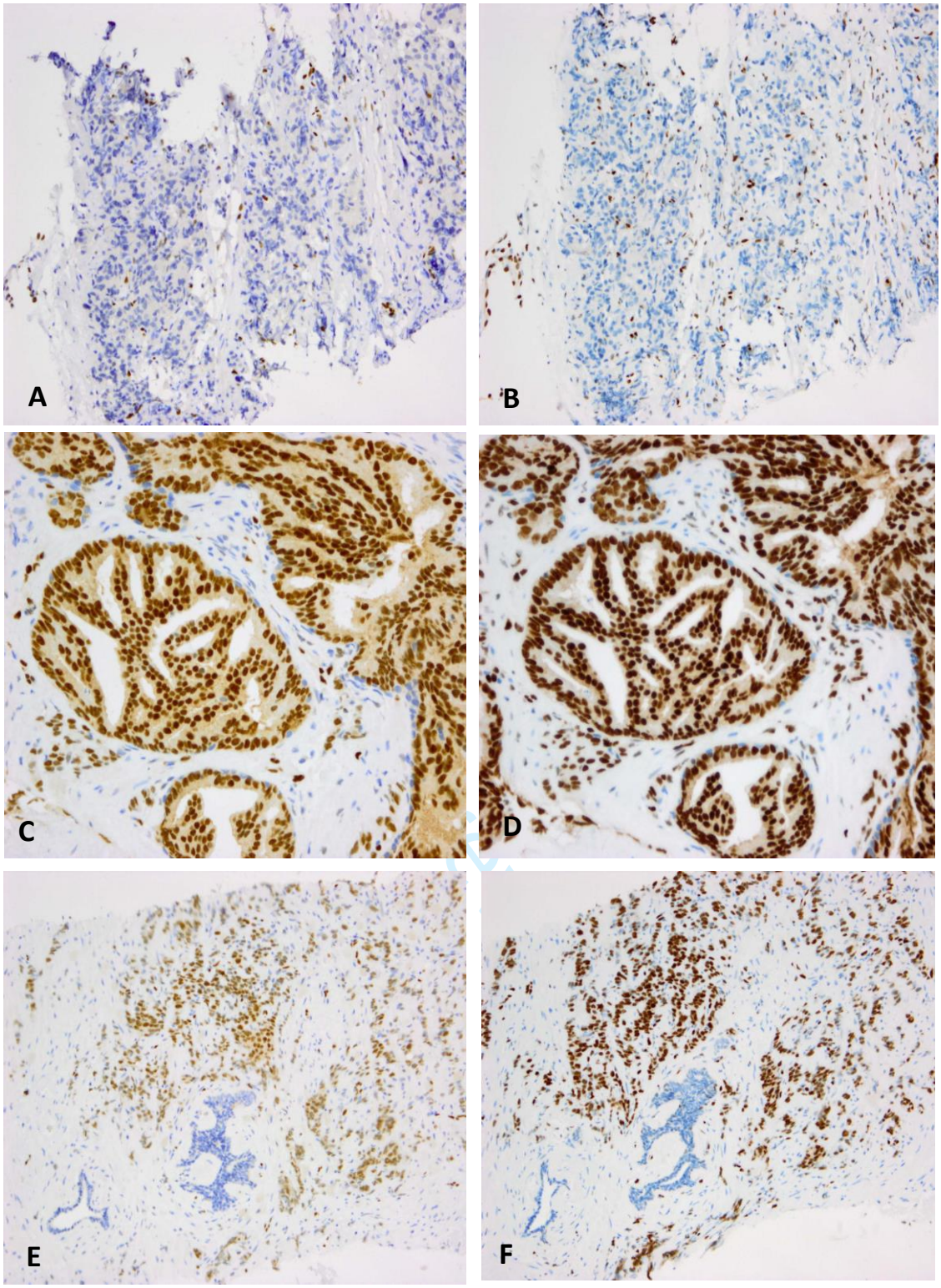
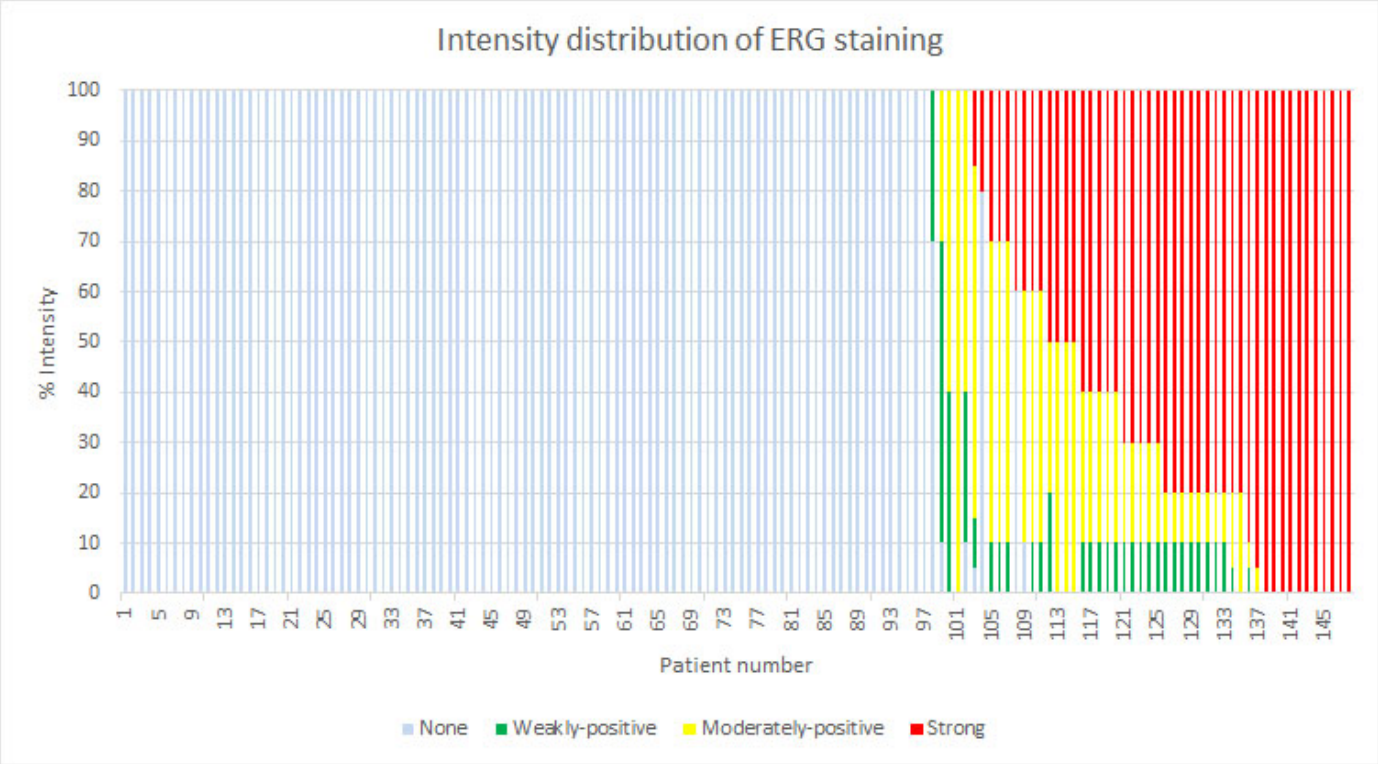


Supplementary Figure 3: Comparison of IHC of Abcam anti-ERG antibody clone [9FY] (ab139431) with Roche monoclonal anti-ERG antibody (EPR3864). **A)** and **B)** ERG negative prostate cancer staining with anti-ERG antibody clone [9FY] (ab13943) and anti-ERG antibody (EPR3864) respectively (X100). **C)** and **D)** Strongly positive (+3) ERG staining showing cribriform formation, which is characteristic feature of high grade prostate cancer (X200) with anti-ERG antibody clone [9FY] (ab13943) and anti-ERG antibody (EPR3864) respectively. **E)** and **F)** Strongly positive (+3) ERG staining with anti-ERG antibody [9FY] (ab139431) and anti-ERG antibody (EPR3864) respectively, demonstrating a sheet of cancer cells in prostate cancer with Gleason score 4.



Supplementary Figure 4: Intensity distribution of ERG staining across the study population. This is the raw data used for calculating the H-score.

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1, 2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6, 7
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6, 7
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	7, 8
Bias	9	Describe any efforts to address potential sources of bias	6, 7, 8
Study size	10	Explain how the study size was arrived at	10
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	9, 6 (Charlson), 12-13
		(*b*) Describe any methods used to examine subgroups and interactions	9, 13
		(*c*) Explain how missing data were addressed	10
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	9, 13

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Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	10
		(b) Give reasons for non-participation at each stage	10
		(c) Consider use of a flow diagram	N/A
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	10-12
		(b) Indicate number of participants with missing data for each variable of interest	N/A see p10
		(c) Summarise follow-up time (eg, average and total amount)	10,11
Outcome data	15*	Report numbers of outcome events or summary measures over time	12
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	13-15
		(*b*) Report category boundaries when continuous variables were categorized	13-15
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	13-15
Discussion			
Key results	18	Summarise key results with reference to study objectives	17
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	18
Generalisability	21	Discuss the generalisability (external validity) of the study results	18
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	25

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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ETS-Related Gene (ERG) Expression as a Predictor of Oncological Outcomes in Patients with High-grade Prostate Cancer treated with Primary Androgen Deprivation Therapy: a cohort study

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Primary Subject Heading:	Oncology
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Keywords:	Prostate disease < UROLOGY, Cancer genetics < GENETICS, THERAPEUTICS

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ETS-Related Gene (*ERG*) Expression as a Predictor of Oncological Outcomes in Patients with High-grade Prostate Cancer treated with Primary Androgen Deprivation Therapy: a cohort study

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ABSTRACT

Objectives: To determine whether *ETS-related gene* (*ERG*) expression can be utilised as a biomarker to predict biochemical recurrence and prostate cancer-specific death in patients with high Gleason grade prostate cancer treated with androgen deprivation therapy (ADT) as monotherapy.

Methods: A multicentre retrospective cohort study identifying 149 patients treated with primary ADT for metastatic or non-metastatic prostate cancer with Gleason score 8-10 between 1999 and 2006. Patients planned for adjuvant radiotherapy at diagnosis were excluded. Age at diagnosis, ethnicity, prostate-specific antigen, and Charlson-comorbidity score were recorded. Prostatic tissue acquired at biopsy or transurethral resection surgery was assessed for immunohistochemical expression of *ERG*. Failure of ADT defined as PSA nadir+2. Vital status and death certification data determined using the UK National Cancer Registry. Primary outcome measures were overall survival (OS) and prostate cancer specific survival (CSS). Secondary outcome was biochemical recurrence-free survival (BRFS).

Results: The median overall survival of our cohort was 60.2 months (C.I. 52.0-68.3). *ERG* expression observed in 51/149 cases (34%). Multivariate Cox proportional hazards analysis showed no significant association between *ERG* expression and OS (p=0.41), CSS (p=0.92) and BRFS (p=0.31). Cox regression analysis showed Gleason score (p=0.003) and metastatic status ($p<1x10^{-5}$) to be the only significant predictors of prostate cancer specific survival.

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Conclusions: No significant association was found between *ERG* status and any of our outcome measures. Despite a limited sample size, our results suggest that *ERG* does not appear to be a useful biomarker in predicting response to ADT in patients with high risk prostate cancer.

Key Words: Prostatic Neoplasms; Androgen Deprivation Therapy; ERG protein; Castrate Resistance

Strengths and limitations of this study

- This observational study consists of a large cohort of solely high-risk cancers treated with initial ADT as monotherapy with subsequent vital status determination through a UK national death certification registry.
- The association between *ERG* expression and oncological survival is explored for the first time in patients on ADT as monotherapy.
- Our study population is of limited sample size. Accuracy of results may be reduced from lack of covariates gained through retrospective data collection.
- Determination of *ERG* status is limited to immunohistochemical detection of the protein without classification of its mutation at a genomic level.

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INTRODUCTION

The development of castration resistance is a major clinical hurdle in patients with advanced prostate cancer and is taken as a marker of impending mortality. Early identification of patients who develop castrate resistant prostate cancer can be clinically useful in enabling early aggressive treatment and therefore in reducing cancer-related deaths.

A recurrent gene fusion event involving the 3' end of *ERG* (*ETS-related gene*) to 5' *TMPRSS2* (*transmembrane protease, serine 2*) [1] is one of the most frequently occurring genetic aberrations in prostate cancer[2] but its prognostic value is still being explored[3]. A meta-analysis evaluating the role of *TMPRSS2:ERG* fusion protein in patients undergoing radical prostatectomy found no association with biochemical recurrence or lethal disease[4].

Given that *TMPRSS2:ERG* is androgen regulated[5], its association with oncological outcomes in patients treated with androgen deprivation therapy (ADT) is possible. *ERG* expression inversely correlates with the levels of androgen receptor protein in the cell and may exert a selective pressure for the development of a castrate-resistant state[6]. Furthermore, androgen-regulated *ERG* expression appears to persist following the development of castration resistance[7].

In vivo validation of *ERG*'s metastatic influence has been controversial. Scheble VJ et al. had shown a greater proportion of castration resistant metastatic prostate cancer driven by *ERG* negative tumours[8], whilst Perner S et al. had observed a greater predilection to metastases in fusion positive foci[9].

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The aim of this study is to explore a possible association between *ERG* expression status and oncologic outcomes in high grade and advanced prostate cancer patients treated by ADT as monotherapy. The primary end points are overall survival (OS) and prostate cancer specific survival (CSS). The secondary end point is biochemical recurrence-free survival (BRFS).

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PATIENTS, MATERIALS AND METHODS

Data Collection, Study Inclusion and Exclusion Criteria

Institutional approval was granted prior to the study. Patients were identified from the pathology databases at two large neighbouring hospitals, Guy's and St Thomas' hospitals NHS Foundation Trust and King's College Hospital NHS Foundation Trust in London, UK, between January 1999 and August 2006. All patients treated with primary ADT were included in the study. Those were identified among patients with a total Gleason score of 8-10. For each patient, the initial assigned treatment was identified using electronic and paper records. Patients with both metastatic and non-metastatic disease were included in the study. Clinical data collected included the age at diagnosis, the assigned treatment at diagnosis, ethnicity (Caucasian, Afro-Caribbean, or other), Charlson comorbidity score[10], date of diagnosis, total modified International Society of Urologic Pathology 2005 Gleason Score, radiological evidence of metastasis at diagnosis, history of previous prostate cancer treatment, and serial prostate specific antigen (PSA) values (ng/ml). Patients were excluded from the study for any missing data, if they did not receive ADT or were planned to receive other adjuvant therapies such as radiotherapy. Data on unplanned adjuvant therapy following ADT was not collected due to incomplete follow-up data. The primary end points were OS and CSS. The secondary end point was BRFS.

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Vital status and death certification data

Patient vital status data were retrieved from the National Cancer Registry in Public Health England[11]. Following institutional approval, unique patient NHS numbers were linked to vital status, dates of death, and ICD-10 codes on the immediate cause of death (cause 1a), other diseases or conditions leading to 1a (causes 1b & 1c), underlying cause of death, and other significant conditions not directly related to death (cause 2)[12]. A prostate cancer death was defined as any death stating 'Prostate Cancer' in any of causes 1a, 1b, 1c, or an underlying cause. Biochemical recurrence was defined as an increase of more than 2ng/ml from the PSA nadir value with censoring on the date when PSA rose more than 2ng/ml above nadir [13].

Prostate cancer sample collection, tissue processing and immunohistochemical (IHC) staining

Prior to retrieval of archived prostate tissue samples, available haematoxylin and eosin-stained slides were examined by two consultant histopathologists to select one tissue block for each patient based on the largest cancer volume. Specimen numbers were used to retrieve the corresponding paraffin-embedded blocks from the archives. 3µm sections were cut from each block using the Rotary Microtome HM 32S. Immunohistochemistry was performed in batches using the Ventana BenchMark ULTRA IHC/ISH automated stainer (Ventana Medical Systems). Deparaffinization of the sections was carried by warming up the slides at 72°C in Ventana EZ Prep solution. Endogenous peroxidase activity was blocked using the Ventana inhibition kit and

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antigen retrieval was carried out by incubating the slides in Cell Conditioning solution-1 and subsequently heating at 100°C for 8 minutes. 100µl of Anti-ERG (EPR3864) Rabbit Monoclonal Primary Antibody was applied on each slide for 32 minutes. Visualisation was performed using anti-rabbit horseradish peroxidase (HRP)-labelled secondary antibody and 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen (Roche/Ventana Ultra View DAB kit). The slides were washed and counterstained with Ventana Haematoxylin and Ventana Bluing Solution.

The IHC nuclear reactivity for *ERG* protein expression in the vascular endothelial cells was used as positive internal controls[14]. Tests were repeated when endothelial cells failed to stain with ERG antibody **(see supplementary figure 1)**.

H-scoring

Semi-quantitative IHC analysis of *ERG* expression was conducted by the H-scoring system[14]. Percentages of prostate cancer cells with positive and negative nuclear *ERG* staining were assessed at high magnification for each sample by two consultant histopathologists. The H-score was calculated as: 3x percentage cells with strong *ERG* expression + 2x percentage of cells with intermediate *ERG* expression + 1x percentage of cells with weak *ERG* expression [15]. The total H-score per sample therefore ranged from 0 to 300. H-scores were classified as negative (0-50), weakly positive (51-100), moderately positive (101-200) or strongly positive (201-300) **(see supplementary figure 2)**.

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Validation of antibody clone against an alternative anti-ERG antibody

Alternative *ERG* staining was carried out on selected cancer tissue samples using an alternative monoclonal ERG antibody (clone 9FY, ab139431). The results are depicted on the photomicrographs shown in **supplementary figure 3**.

Statistical Methods

OS and CSS were determined using the Kaplan-Meier method. Univariate analysis of survival was performed using the Log-rank method. Multivariable Cox proportional hazards analysis was used to assess OS, CSS, and BRFS with adjustments for *ERG* expression, age, ethnicity, Gleason score, PSA, presence of metastasis at presentation and Charlson co-morbidity score. Statistical analyses were conducted using SPSS ver 22, Graphpad Prism 5.0, and Microsoft Excel software.

Patient and Public Involvement

No patients and public persons were involved in the commencement of this research.

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RESULTS

Cohort characteristics

527 patients with high Gleason score prostate cancer were diagnosed on biopsy, of which 169 patients were assigned to primary ADT as monotherapy. Exclusion of patients was due to tissue samples being unavailable (n=4), lack of vital status data output from the National Cancer Registry (n=4), or one or more missing clinical parameters (n=12). Complete data was available for 149 patients which formed the study population.

Mean follow-up was 46.5 (±25.2) months. 59 patients (40%) had metastatic disease at presentation. The clinical characteristics of the cohort are shown in **(Table 1)**.

ERG expression was observed in 51 cases (34%), of which nearly all demonstrated strong *ERG* expression (92%) **(Figure 1)**, (intensity distribution of *ERG* staining shown in **supplementary figure 4**). No *ERG* expression was found in incidental benign acini within samples. *ERG* positivity was associated with older age, and Caucasian ethnicity (when compared to Afro-Carribean and Other ethnic groups), but not Gleason score, initial PSA level, or presence of metastatic disease at presentation **(Table 1)**.

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TABLE 1	*ERG* negative (N=98)	%	*ERG* positive (N=51)	%	P-value
Mean age (±SD), years	72.3 (±8.3)		75.5 (±8.6)		**0.03***
Ethnicity					
Caucasian	51	52	37	73	**0.04**
Afro-Caribbean	41	42	11	22	
Other	6	6	3	6	
Gleason score					
8	22	52	13	25	0.88
9	71	42	36	71	
10	5	6	2	4	
PSA (±SD), ng/ml	1378 (±10849)		283 (±1203)		0.48*
<10.00	4	4	4	8	0.38
10-19	17	18	7	14	
20-49	24	25	18	36	
50-99	13	13	10	20	
≥100	39	40	11	22	
Metastasis					
No	60	61	30	59	0.78
Yes	38	39	21	41	
Charlson Comorbidity					
0	43	44	25	49	0.05
1	30	31	6	12	
2	16	16	11	22	
≥3	9	9	9	18	
Follow-up (±SD), mths	47.9 (±25.5)		43.7 (±24.9)		0.34

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Deaths					
All causes	46	47	28	55	0.39
Prostate Cancer Specific	29	30	17	33	0.71

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Table 1. Clinical characteristics of the study population stratified by *ERG* expression status (*p*-values obtained by χ^2 or *T-tests)

National Cancer Registry-linked oncological survival outcomes following primary androgen deprivation therapy in metastatic and non-metastatic high Gleason-grade prostate cancer

The National Cancer Registry was used to determine the vital status and death certification details for each patient. 75 patients (50%) had died during follow-up, of whom 55 had died as a result of prostate cancer. Median overall survival for the cohort was 60.2 months. OS, CSS, and BRFS for the cohort are shown (**Figure 2)**.

Presence of metastatic disease at diagnosis significantly affected OS (p=0.001), CSS ($p<1x10^{-7}$) and BRFS ($p<1x10^{-6}$). Gleason score significantly affected OS (p=0.004) and CSS (p=0.004) but not BRFS (p=072). PSA at presentation only affected BRFS ($p=1x10^{-5}$). Those associations were calculated using Log-rank analysis.

Association of ERG expression and oncological outcomes in high risk cases treated by primary androgen deprivation therapy

Log-rank analysis was first conducted to determine whether *ERG* expression predicted oncological outcomes in the high-risk cohort stratified by *ERG* expression status **(Figure 3)**. No statistically significant association was observed between *ERG* expression and OS, CSS, or BRFS.

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Cox proportional hazards regression analysis was conducted to determine independent predictors of oncological outcomes. Mutual adjustments were made for *ERG* expression, age, ethnicity, Gleason score, PSA, presence of metastasis at presentation, and Charlson co-morbidity **(Table 2)**. The presence of metastatic disease was significantly associated with OS (HR 2.60, 95% C.I. 1.54-4.40), CSS (HR 4.51, 95% C.I. 2.36-8.60), and BRFS (HR 3.15, 95% C.I. 1.93-5.16). Total Gleason score was significantly associated with OS (Gleason 9; HR 2.33, 95% C.I. 1.2-4.53 and Gleason 10; HR 5.81, 95% C.I. 2.04-16.52, reference group Gleason 8) and CSS (Gleason 9; HR 2.56, 95% C.I. 1.13-5.83 and Gleason 10; HR 6.45, 95% C.I. 2.04-16.52, reference group Gleason 8) but not BRFS. Age was significantly associated with OS only. We found no statistically significant association between *ERG* expression and OS, CSS or BRFS. The results did not change when *ERG* expression status was replaced with the H-score (results not shown).

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TABLE 2	OS				CSS				BRFS			
	p	HR	95% C.I.		*p*	HR	95% C.I.		*p*	HR	95% C.I.	
			Lower	Upper			Lower	Upper			Lower	Upper
***ERG* expression**	0.41	1.24	0.74	2.05	0.92	1.03	0.57	1.87	0.31	0.78	0.47	1.27
Age	**0.02**	1.04	1.01	1.07	0.19	1.02	0.99	1.06	0.82	1.00	0.97	1.03
Ethnicity												
Caucasian (ref)	0.75				0.98				0.44			
Afro-Caribbean	0.70	0.90	0.53	1.53	0.86	0.94	0.51	1.75	0.52	0.85	0.52	1.38
Other	0.57	1.35	0.47	3.86	0.93	0.94	0.22	4.10	0.24	0.49	0.15	1.59
Gleason score												
8 (ref)	**0.003**				**0.01**				0.79			
9	**0.01**	2.33	1.20	4.53	**0.02**	2.56	1.13	5.83	0.50	1.20	0.71	2.01
10	**<0.001**	5.81	2.04	16.52	**<0.01**	6.45	1.92	21.71	0.73	1.24	0.35	4.38
PSA	0.92	1.00	1.00	1.00	0.96	1.00	1.00	1.00	0.88	1.00	1.00	1.00
Metastasis	**<0.001**	2.60	1.54	4.40	**<1x10^{-5}**	4.51	2.36	8.60	**<1x10^{-5}**	3.15	1.93	5.16
Charlson Comorbidity												
0 (ref)	0.15				0.48				0.83			
1	0.19	1.52	0.81	2.87	0.85	1.08	0.50	2.35	0.56	1.18	0.68	2.07
2	0.06	1.81	0.97	3.39	0.67	1.18	0.56	2.47	0.75	0.90	0.49	1.67
≥3	0.09	2.00	0.89	4.47	0.12	2.17	0.81	5.84	0.51	1.29	0.60	2.75

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Table 2. Multivariate Cox proportional hazards analysis of *ERG* expression with other known oncological outcome parameters.

Reference groups are indicated for categorical variables. OS = overall survival, CSS = cancer-specific survival, BRFS = biochemical recurrence-free survival, HR = hazard ratio, C.I. = confidence interval.

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DISCUSSION

In this cohort, we examined the association of *ERG*-expression with survival endpoints in patients treated by primary ADT. Following multivariate analysis, we found no association of *ERG* expression with OS, CSS or BRFS (**Table 2**). Advances in planned adjuvant treatments for high-risk prostate cancer such as radiotherapy or chemotherapy confounds the assessment of biomarkers in patients receiving ADT in more recent cohorts. Linkage of clinical data was made with the National Cancer Registry which provided an up-to-date vital status on all patients residing in England.

ERG is commonly described as an oncogene although its ubiquitous expression in endothelial and haematopoietic stem cells suggest an essential role in angiogenesis, endothelial cell function and haematopoiesis[16, 17]. Since the discovery of the *ERG* and androgen regulated *TMPRSS2* genetic fusion in prostate cancer[1], its role as a sensitive and prevalent marker for prostate cancer has been shown to be highly replicable[4]. Recent whole genome sequencing studies have revealed it to be the most frequent genetic aberration in prostate cancer within the entire genome[2]. Its high prevalence amongst all grades of disease[4, 18-21] however, supports its significance to be a marker of cancer per se rather than a marker for prognosis. *ERG* overexpression in animal models produces prostate intra-epithelial neoplasia (PIN) but not invasive cancer, suggesting it to be an early event in the natural history of prostate cancer[22].

Androgen receptor is known to play a role in the development of castrate resistance in prostate cancer[5] and its levels have been shown to correlate with *ERG* expression[6]. To the best of our

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knowledge, only a few studies have looked into the possible association of primary ADT on *ERG* positive cancer with varying conclusions[23-25]. Similar to the findings of our study, Berg et al suggest no association between *ERG* expression and the development of castrate resistance in patients treated with primary ADT[24]. Huang et al had shown that combined *ERG* and androgen receptor status was significant in its association with a worsened survival in prostate cancer[23]. However, sole expression of *ERG* had not conferred worsened survival outcomes in patients with prostate cancer. Graff et al suggest a protective benefit in managing *ERG* positive prostate cancer with ADT[25].

Our study is the largest cohort of solely high-risk cancers homogeneously treated with initial ADT as planned monotherapy with subsequent high-quality vital status determination through a national registry.

In organ confined prostate cancer, ERG expression and its association with clinical outcomes has been the subject of numerous studies with conflicting outcomes[3, 26]. A meta-analysis describing ERG fusion positive cancer and its associated outcomes in post-prostatectomy showed ERG fusion events to be associated with a higher clinical stage at diagnosis of T3 over T2 with a risk ratio of 1.23 , yet no association was found for cancer specific survival or disease recurrence[4].

Although we used IHC to estimate *TMPRSS2:ERG* gene fusion status, studies have shown very high concordance between more accurate FISH techniques[27-30]**.** The reproducibility of the technique was assessed using an additional antibody clone, as well as determining technical success for each sample using endothelial cell expression as internal controls **(supplementary**

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figures 1 and 3). The prevalence (34%) of *ERG* expression is in line with previous studies[4]. The association between Caucasian ethnicity and *ERG* expression agrees with a previous study evaluating *TMPRSS2:ERG* fusion events[31]. Higher age was significantly associated with *ERG* expression in our cohort (p=0.03), in contrast to other studies that showed a higher proportional expression in the younger men[32] or no correlation at all[4, 14]. It is possible that this is an association seen only in high grade prostate cancer cases.

Limitations

The retrospective nature of the study had resulted in a reduction in the collection of other covariates such as stage at diagnosis[4].

Moreover, a subset of patients within the cohort received unplanned adjuvant therapy in addition to androgen deprivation monotherapy which may have influenced the overall survival. This was not assessed as a covariate due to the heterogeneous nature of the treatment and small patient numbers.

Patients who did not have a complete dataset were excluded from the study although this had represented a small proportion of patients (20/169). In addition, despite being the largest cohort of patients solely treated with primary ADT, the sample size remains small reducing the power of this study population.

Both quantitative[33] and qualitative differences in the *ERG* mutation have been implicated in prognostication of prostate cancer. Patients with cancer cells exhibiting an aberration consisting

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of both a duplication and deletion of the 5' end of *ERG* (known as 2+ "Edel") were predisposed to a poorer disease specific survival[34, 35]. In this context, the use of IHC was a limitation as it cannot detect the genomic fusion quantitatively or qualitatively but only isolated expression of the ERG protein[7, 27]. It is important to note that the H score does not provide a quantitative measurement of the *ERG* aberration[27]. With this knowledge at hand, sub-classifications of *ERG* mutations when assessing prognostic indicators is recommended in future clinical studies. IHC may be controversial as a detection method of the *TMPRSS2:ERG* fusion gene. Sung JY et al. expressed caution to its use for its false positive rate[36] whilst Gsponer and colleagues identified a subgroup of *ERG* genetic alterations that are undetectable at a protein level[37] .

CONCLUSION

Whilst *ERG* expression is known to be strongly associated with oncogenesis, we show that *ERG* expression did not predict oncological survival in prostate cancer patients treated with ADT. Our findings are in line with other studies showing a lack of association between *ERG* expression and prostate cancer treatment outcomes.

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FIGURE LEGENDS

Figure 1. H-score distribution of ERG positive cases. 47/51 (92%) had a strongly positive H-score.

Figure 2. Oncologic outcomes of high-risk prostate cancer following primary androgen deprivation therapy. Significant associations shown in bold. BSPositive = bone scan positive.

Figure 3. Kaplan-Meier survival curves stratified by ERG expression status for OS, CSS, and BRFS**.**

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FOOTNOTES

AUTHOR CONTRIBUTIONS: HY led the study design, analysed data and was involved in revision, drafting and approval of the final manuscript. MR was involved in sample and data collection, data analysis and drafting of the manuscript. AC and DA were both involved in analysis of the prostate cancer tissue. HM, MY and PD provided critical review of the study design and manuscript.

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DATA SHARING STATEMENT: Due to the confidential nature of our data which may identify individuals, even following anonymisation, we have chosen not to make our data publicly available.

DISCLOSURE/DUALITY OF INTEREST: The authors declare no conflict of interest

SUPPLEMENTARY INFORMATION: Supplementary information is available online at *BMJ Open's* website

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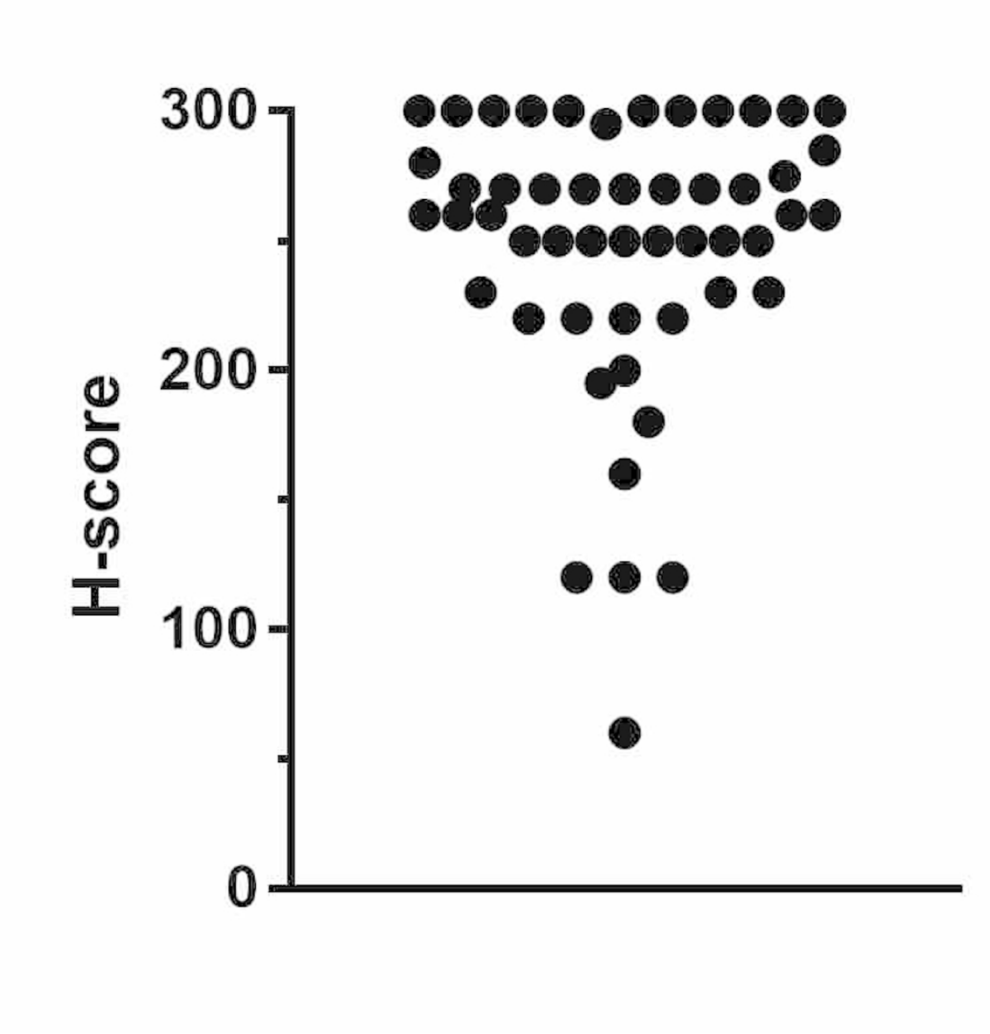

Figure 1. H-score distribution of ERG positive cases. 47/51 (92%) had a strongly positive H-score.

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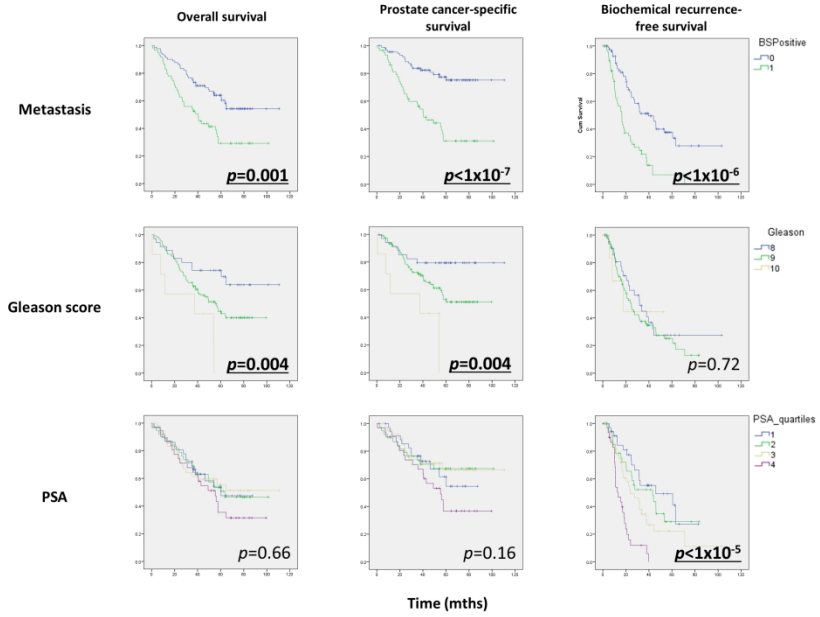

Figure 2. Oncologic outcomes of high-risk prostate cancer following primary androgen deprivation therapy. Significant associations shown in bold. BSPositive = bone scan positive.

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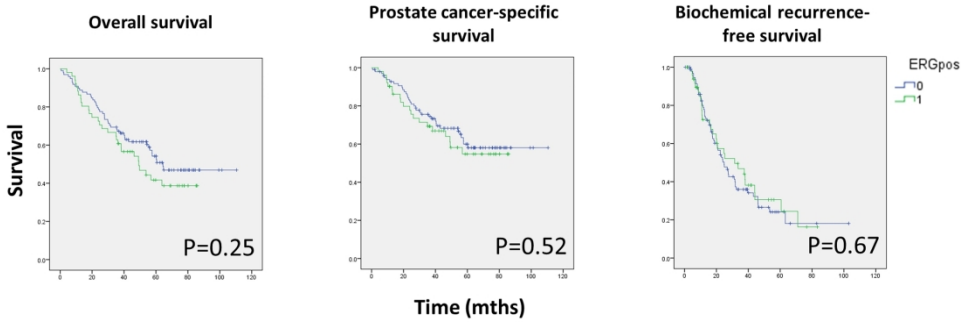

Figure 3. Kaplan-Meier survival curves stratified by ERG expression status for OS, CSS, and BRFS.

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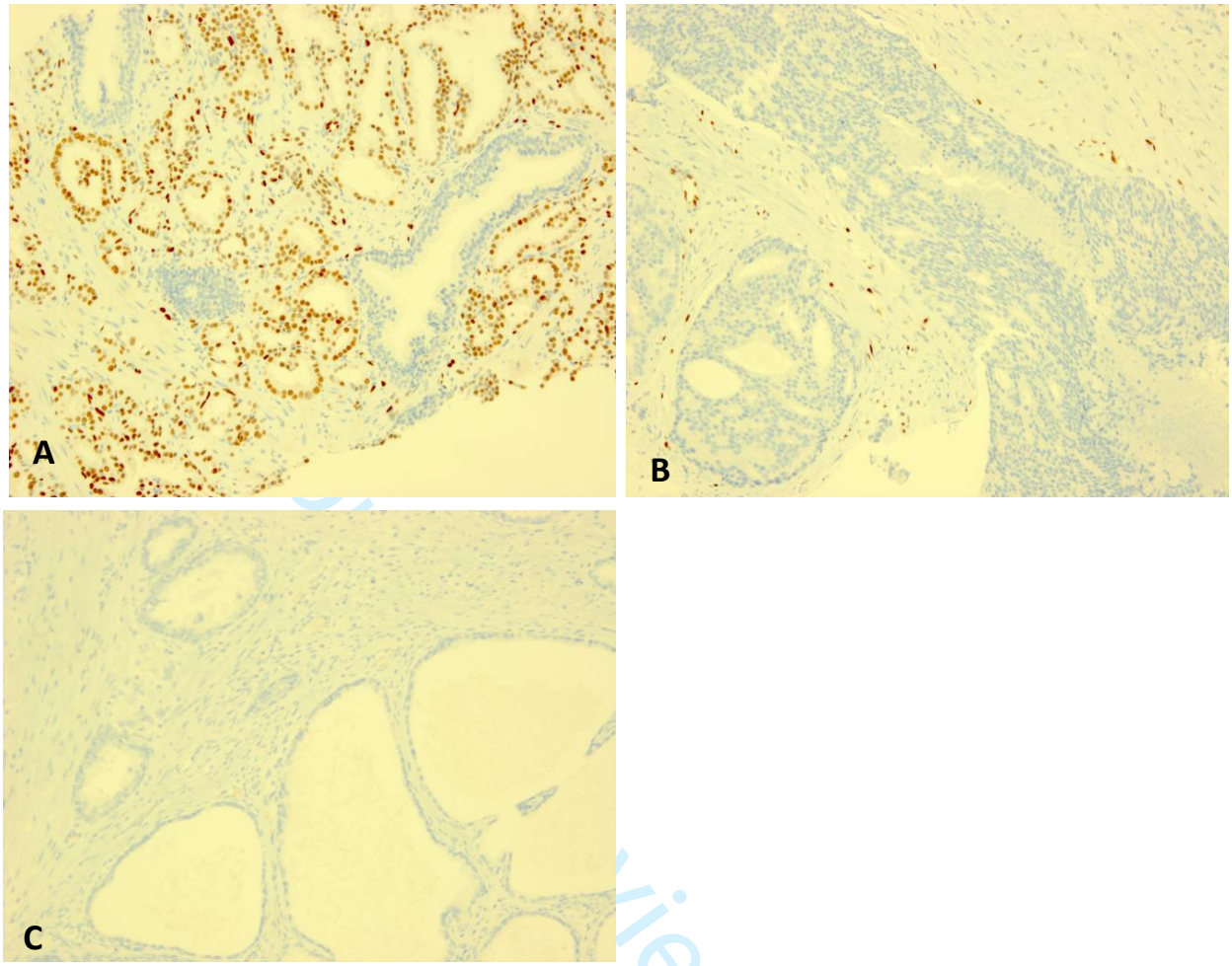
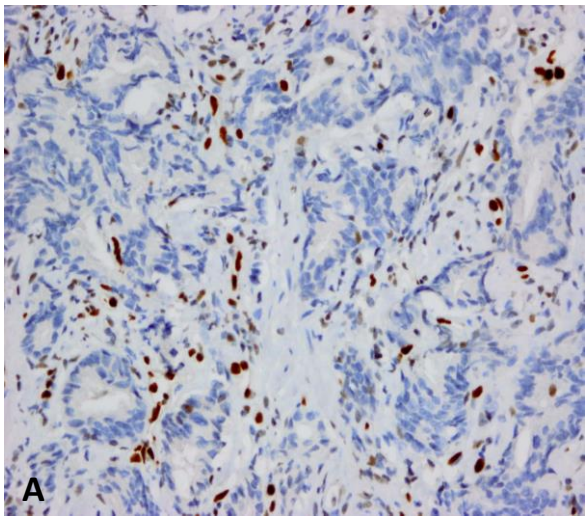
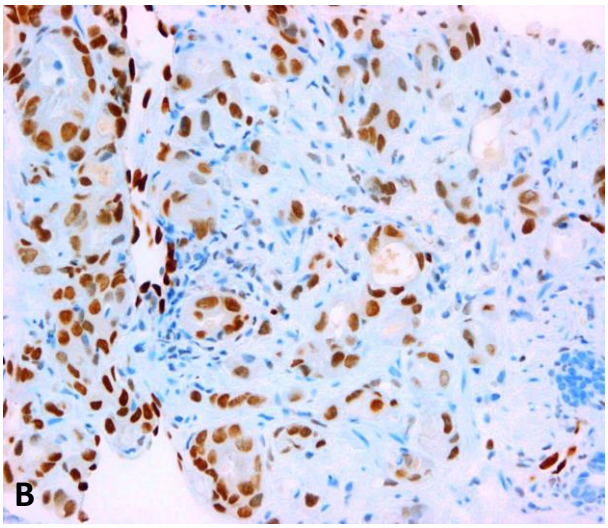
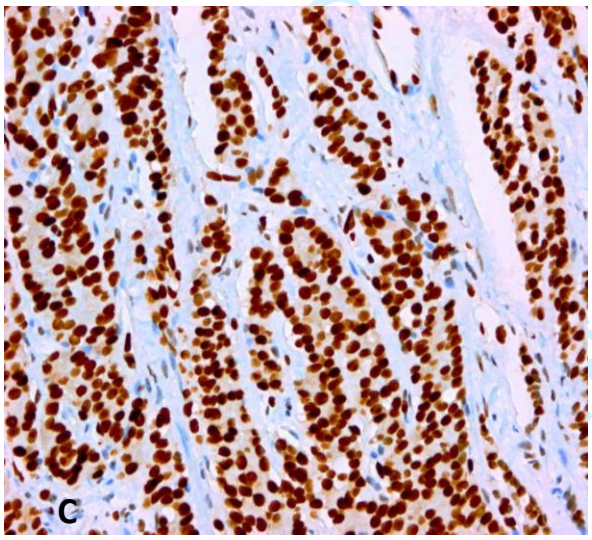
Supplementary figures



Supplementary Figure 1 – Immunohistochemistry of prostate core biopsy samples depicting failure of internal control, stained with anti-ERG (EPR3864) rabbit monoclonal antibody 5ml (23µg/ml) staining kit. ERG stains brown upon detection and small amounts are normally detected in cells. **A)** Prostate Cancer staining positive for ERG overexpression, **B)** Prostate Cancer from a patient that is negative for ERG overexpression with positive internal control endothelial staining and **C)** No brown colour is detected as the staining on this control slide was unsuccessful.







Supplementary Figure 2: Range of staining using the ERG-antibody (EPR3864 clone). **A)** ERG expression is negative in tumour cells, but positive in nuclei of lymphocytes and endothelial cells (X200). **B)** Moderate ERG expression is seen in tumour epithelial cell nuclei (X200). **C)** A strongly positive ERG expression in seen showing fused acinar prostate cancer (X200).

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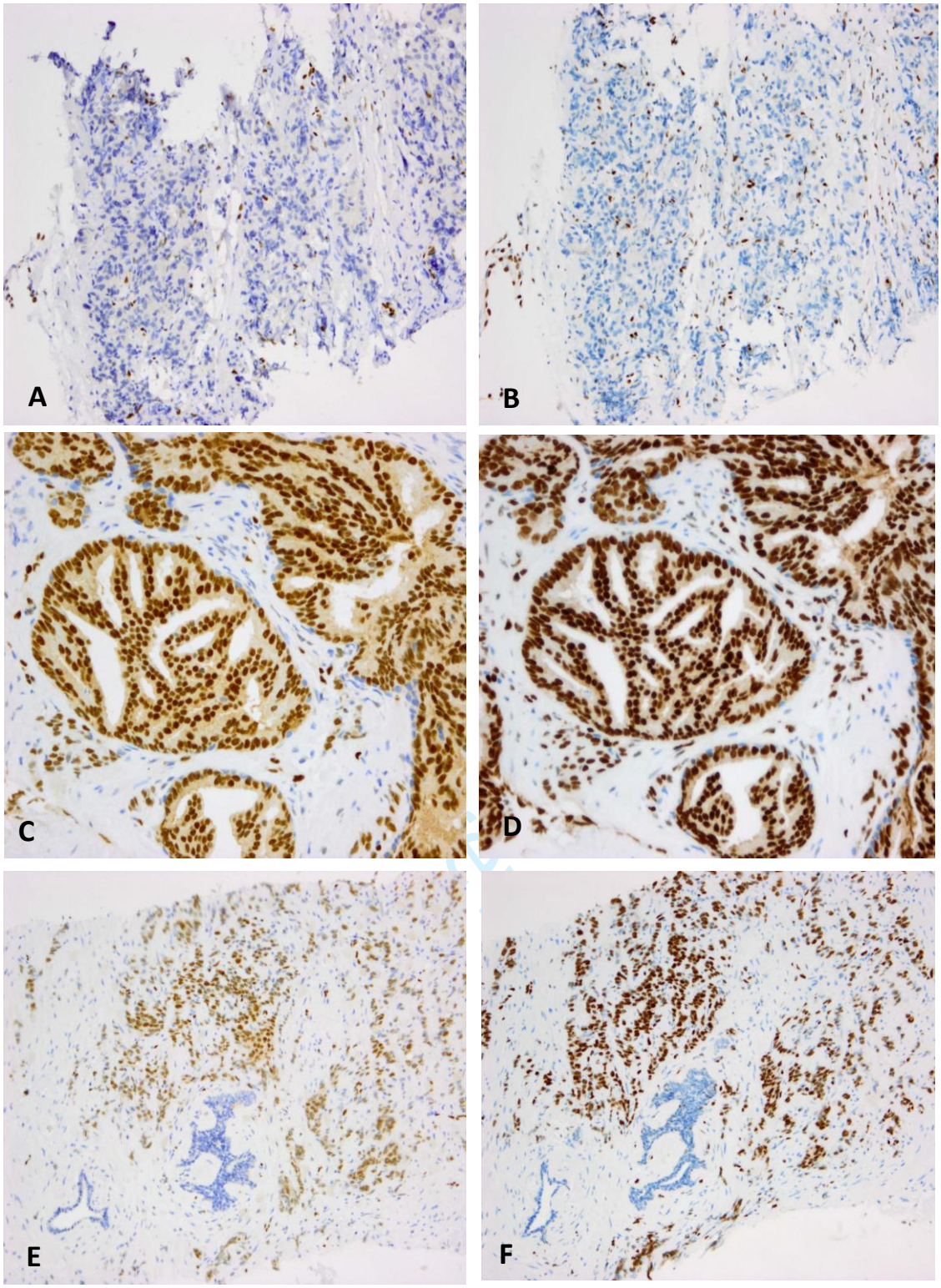
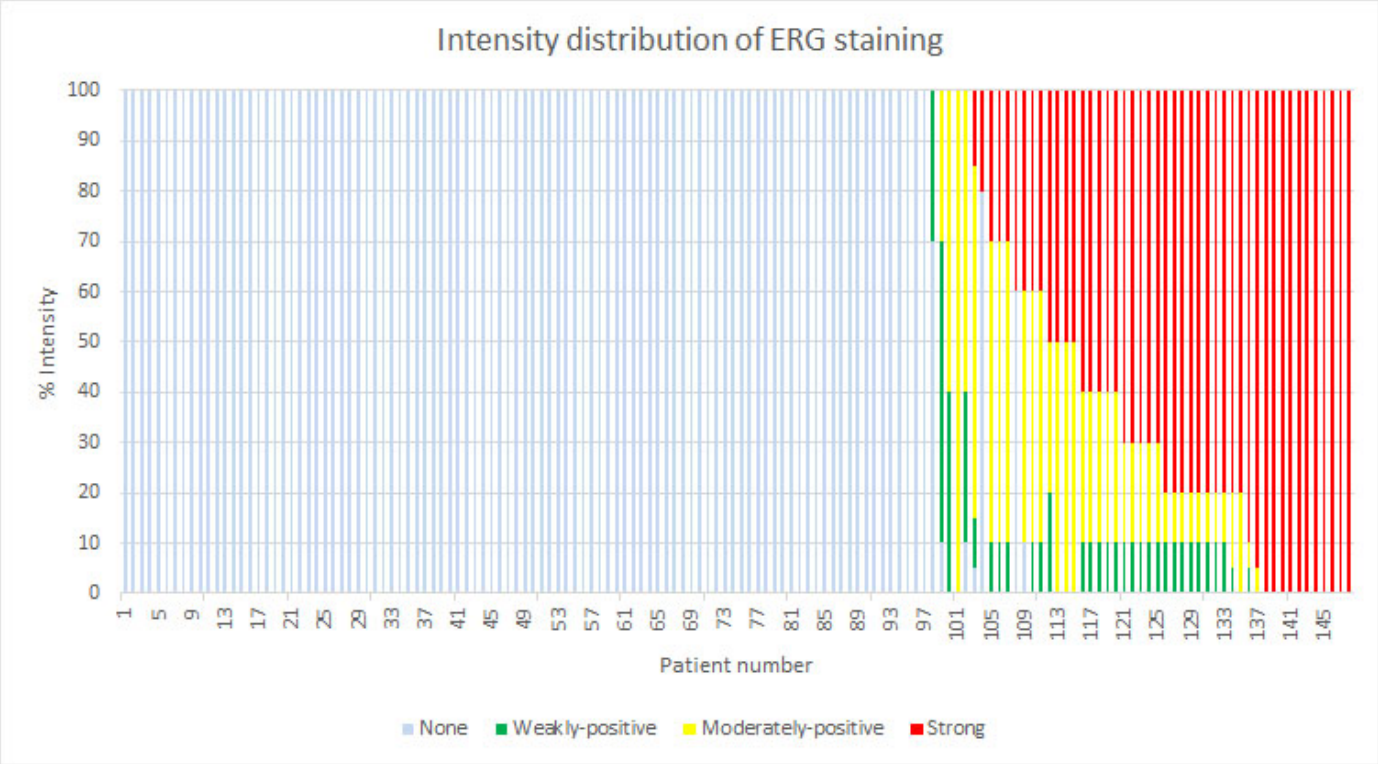


Supplementary Figure 3: Comparison of IHC of Abcam anti-ERG antibody clone [9FY] (ab139431) with Roche monoclonal anti-ERG antibody (EPR3864). **A)** and **B)** ERG negative prostate cancer staining with anti-ERG antibody clone [9FY] (ab13943) and anti-ERG antibody (EPR3864) respectively (X100). **C)** and **D)** Strongly positive (+3) ERG staining showing cribriform formation, which is characteristic feature of high grade prostate cancer (X200) with anti-ERG antibody clone [9FY] (ab13943) and anti-ERG antibody (EPR3864) respectively. **E)** and **F)** Strongly positive (+3) ERG staining with anti-ERG antibody [9FY] (ab139431) and anti-ERG antibody (EPR3864) respectively, demonstrating a sheet of cancer cells in prostate cancer with Gleason score 4.



Supplementary Figure 4: Intensity distribution of ERG staining across the study population. This is the raw data used for calculating the H-score.

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1, 2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6, 7
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6, 7
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	7, 8
Bias	9	Describe any efforts to address potential sources of bias	6, 7, 8
Study size	10	Explain how the study size was arrived at	10
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	9, 6 (Charlson), 12-13
		(*b*) Describe any methods used to examine subgroups and interactions	9, 13
		(*c*) Explain how missing data were addressed	10
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	9, 13

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Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	10
		(b) Give reasons for non-participation at each stage	10
		(c) Consider use of a flow diagram	N/A
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	10-12
		(b) Indicate number of participants with missing data for each variable of interest	N/A see p10
		(c) Summarise follow-up time (eg, average and total amount)	10,11
Outcome data	15*	Report numbers of outcome events or summary measures over time	12
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	13-15
		(*b*) Report category boundaries when continuous variables were categorized	13-15
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	13-15
Discussion			
Key results	18	Summarise key results with reference to study objectives	17
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	18
Generalisability	21	Discuss the generalisability (external validity) of the study results	18
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	25

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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