Inflammatory infiltration is associated with AR expression and poor prognosis in hormone naïve prostate cancer

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Abstract

Background: Tumor microenvironment inflammatory infiltration is proposed as a protumorigenic mechanism for prostate cancer with proinflammatory cytokines stimulating androgen receptor (AR) activity. However, association with patient prognosis remains unclear. This study derives an inflammatory gene signature associated with AR expression and investigates CD3+ and CD8+ T-lymphocyte infiltration association with AR and prognosis.

Methods: Gene profiling of inflammatory related genes was performed on 71 prostate biopsies. Immunohistochemistry on 243 hormone-naïve prostate cancers was performed for CD3, CD8, AR, and phosphorylated AR tumor expression.

Results: Multiple proinflammatory genes were differentially expressed in association with high AR expression compared with low AR expression including PI3KCA and MAKP8 (adjusted \( P < .05 \)). High CD3+ and high CD8+ infiltration associated with reduced cancer-specific survival (\( P = .018 \) and \( P = .020 \), respectively). High CD3+ infiltration correlated with high tumor cytoplasmic AR expression and if assessed together, they associated with reduced cancer-specific and 5-year survival from 90% to 56% (\( P = .000179 \)). High CD8+ cytotoxic infiltration associated with high androgen-independent tumor nuclear AR serine 213 phosphorylation (correlation coefficient = 0.227; \( P = .003 \)) and when assessed together associated with poor clinico-pathological features including perineural invasion (\( P = .001 \)). Multiple genes involved in proinflammatory signaling pathways are upregulated in high AR expressing prostate samples.

Conclusion: T-lymphocyte infiltration in hormone-naïve disease associates with androgen-independent driven disease and provides possible therapeutic targets to reduce transformation from hormone-naïve to castrate-resistant disease.

Keywords
androgen receptor, inflammation, phosphorylation, prostate cancer, T-lymphocytes

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1 | INTRODUCTION

Androgens activating the androgen receptor (AR) play a crucial role in the development of 80% to 90% of prostate cancers (CaP). AR activation via induction of AR phosphorylation at serine 81 (pARser81) promotes cell proliferation and reduces cell death, therefore current therapies aim to inhibit AR activation directly or via depletion of androgens by androgen deprivation therapy (ADT). However, despite 10-year survival rates tripling over the past 40 years in the UK, over 300,000 men worldwide still die each year from CaP due to late stage diagnosis and development of castrate-resistant disease. This highlights the need to understand the pathogenesis and progression of CaP further. In addition to classical AR activation, the AR can be activated independent of androgens through numerous pathways including phosphatidylinositol-3-kinase (PI3K)/AKT, onterleukin-6 (IL-6)/Janus kinase (JAK)/signal transducer and activator of transcription (STAT), and Ras/Raf/mitogen-activated protein kinase (MAPK) which result in AR phosphorylation to promote transcriptional activity, protein stability and the development of castrate-resistant disease. For example, phosphorylation of the AR at Serine 213 via PI3K/AKT signaling results in activation of the AR via an androgen independent mechanism, the PI3K/AKT pathway through loss of phosphatase and tensin homolog (PTEN) is upregulated in 40% to 70% of CaPs.

The prostate is an immune-competent organ composed of infiltrating immune cells including macrophages, dendritic cells, B-lymphocytes, and T-lymphocytes. Inflammatory changes observed in CaP include increased inflammatory cell infiltration and proinflammatory cytokine expression, which promote multiple signaling pathways linked with CaP development, such as PI3K/AKT and IL-6/JAK/STAT. It is hypothesized that these proliferative conditions promote the dedifferentiation of prostate epithelial cells, activation of AR signaling, and ultimately CaP development.

In CaP, the adaptive immune response is highly active and prominent including cytotoxic T-lymphocytes, helper T-lymphocytes, and regulatory T-lymphocytes. CD4+ helper T-lymphocytes induce (TNF)-related apoptosis, with a reduction in CD4+ infiltration in CaP, when compared with benign prostate hyperplasia and prostatic intraepithelial neoplasia, associating with poor clinicopathological features including high Gleason sum and elevated prostate specific antigen (PSA) concentration. Additionally, regulatory T-cells (Tregs) suppress and regulate T-lymphocytes, with elevated Tregs within the tumor microenvironment reducing the antitumor effects of T-lymphocytes. However, in CaP elevated Treg infiltration associates with reduced cancer specific survival (CSS) in CaP. One cell type regulated by Tregs are CD8+ cytotoxic cells which destroy microbes and cancer cells via cytokine release, Fas signaling, or the perforin pathway. In colorectal cancer, high CD8+ infiltration is associated with favorable clinical outcomes and can be modulated to increase its cytotoxic functions via immune checkpoint inhibitors which target programmed cell death protein 1 or anti-cytotoxic-T-lymphocyte-associated protein 4 (CTLA4). However, in CaP these immune checkpoint inhibitors have shown little effect with high immune checkpoint blockade resistance, with over expression of AR significantly reducing CD8+ infiltration, PC-1 and CTLA-4 expression, an correlating with tumor progression, increased serum PSA concentration, and the presence of metastases. Interestingly, loss of PTEN, a common feature in CaP, is associated with increased CD8+ infiltration and reduced time to biochemical relapse, highlighting interesting links between inflammation and AR. However, contradictory reports have been published for each inflammatory cell type depending on disease stage, tissue location, AR expression and cytokine expression. Ultimately, further research is required to identify important players of inflammatory driven CaP and potential therapeutic targets or synergistic drug combinations. Therefore, we aimed to derive an inflammatory gene expression signature associated with high AR expression within a prostate biopsy and establish if this signature is associated with more aggressive disease. Furthermore, we aimed to determine the relationship between local adaptive immune cell infiltration in hormone naive CaP and AR phosphorylated AR expression.

2 | METHODS

2.1 | Patient biopsy cohort

Prostate biopsies from 71 patients were collected between 2007 and 2012 via trans-rectal ultrasound (TRUS) guided biopsies and snap frozen upon extraction. Ethical approval was gained from the Multicentre Research Ethics Committee for Scotland (MREC/01/0/36) and Local Research and Ethics Committees. An anonymized database was available containing clinical information with all patient identifiers removed and included age at biopsy, diagnosis, Gleason sum, serum PSA concentration, and presence of metastases.

2.2 | Gene expression analysis

RNA was extracted from frozen TRUS biopsies using the RNease Mini kit (Qiagen, UK). Biopsies were minced in RNA lysis buffer lysis buffer and centrifuged in QiaShredder columns (Qiagen, UK) for 2 minutes. Samples were transferred to a RNeasy mini column with 70% ethanol and centrifuged for 30 seconds. Samples were washed in RW1 buffer followed by RNA wash buffer with ethanol buffer via centrifugation for 30 seconds and eluted in RNase free water. Each sample RNA was reverse-transcribed by adding 1 µL oligo (dT) and 1 µL dNTPmix for 5 minutes at 65°C. Each sample was then added to 4 µL 5X first strand buffer, 0.5 µL water, 0.5 µL SuperScript III reverse transcriptase, 1 µL 0.1 M DTT, and 1 µL RNaseOUT and incubated for 30 minutes at 50°C followed by 15 minutes at 70°C (Invitrogen, UK). Ninety-six primer pairs (Table S1) were pooled together and 0.5 µL primer mix was added to each sample along with 2.5 µL TaqMan PreAmp Master Mix (Thermo Fisher Scientific, UK), and 0.75 µL water. These primers were pre-selected based on their applicability across a range of cancers, prostatic diseases, and inflammatory processes. Samples were vortexed, centrifuged, and placed in a
thermocycler for 10 minutes at 95°C, 10 to 14 cycles for 15 seconds at 
95°C, and 4 minutes at 60°C. Samples underwent exonuclease treatment 
by adding 0.2 µL exonuclease I reaction buffer, 0.4 µL 
exonuclease I, and 1.4 µL water and incubating for 30 minutes at 
37°C and 15 minutes at 80°C (New England Biolabs, UK). Each 
sample was diluted in five-fold in TE buffer, added to 3 µL 2X SoFast 
EvaGreen Supermix (Bio-Rad, UK) and 0.3 µL 20X DNA Binding Dye 
sample loading reagent (Fluidigm, UK), and vortexed and centrifuged. 
Individual primer pairs were added to 2.5 µL 2X assay loading re-
agent and 2.25 µL 1X DNA suspension buffer. Each individual primer 
pair and sample was loaded into a 96.96 Dynamic Array IFC (Flu-
digm) and run on the Fluidigm Biomark HD system (Table S2).

2.3 Immunohistochemistry

In total, 243 patients diagnosed with CaP were recruited from the 
Greater Glasgow and Clyde Health Board between 2008 and 2009 
(Biobank ethical approval for use of tissue, GG&C health board ethics 
number 10/50704/60, Safe Haven ethical approval for use of clinical 
information, GG&C health board ethics number 12/WS/0142). An 
anonymized database was available containing clinical informa-
tion with all patient identifiers removed and included Gleason sum, 
surface PSA concentration, presence of metastases, perineural 
invansion, serum albumin concentration, C-reactive protein 
concentration, hormonal therapy, time to biochemical relapse, and 
time to cancer specific death. All samples were gathered via 
trans-ultrasound guided biopsied.

Immunohistochemistry was performed for AR, pARser81, AR 213 
phosphorylation (pARser213), Ki67 proliferation index, CD3, and CD8. 
Sections were dewaxed in histoclear, rehydrated through graded 
ethylenediaminetetraacetic acid (Tris EDTA, pH8 or 9) for antigen retrieval. Sections were placed in 3% H2O2 
for 30 minutes and blocked for 30 minutes in 1.5% or 5% horse serum in 
foam Tris-buffered saline. Antibodies for AR (M3562; Dako, UK), pARser81 
(07-1375; Millipore, UK), Ki67 (Clone MIB-1; Dako), CD3 (RM-9107-S; 
Thermo Fisher Scientific, UK), and CD8 (Clone C8/114b; Dako) were 
incubated overnight at 4°C diluted at 1:100, 1:1000, 1:1000, 1:200, 
and 1:200 respectively. Antibody for pARser213 (IMG-561; Imagenex, UK) 
was incubated for 1 hour at room temperature diluted at 1:100. EnVi-
vision (Dako) or ImmPRESS detection kit (Vector Laboratories, UK) was 
used to detect bound antibodies followed by 3,3-diaminobenzidine 
tetrahydrochloride (DAB, Dako). Samples were counterstained and de-
hydrated before being mounted with Di-N-butylphthalate in xylene 
(DPX). Slides were scanned and visualized using Hamamatsu Nano-
Zoomer (Welwyn Garden City, UK) and Slidepath Digital Image Hub, 
version 4.0.1 (Leica Biosystems, UK). Staining intensity for AR, pARser81, 
and pARser213 was performed using a weighted histo-score method for 
both nuclear and cytoplasmic expression. In brief, the score was cal-
culated by sum of (1X % cells staining weakly positive) + (2X % cells 
staining moderately positive) + (3X % cells staining strongly positive) 
with a maximum of 300 (100% strongly stained) and a minimum of 0 
(100% with no staining). Ki67, CD3, and CD8 expression was performed 
by counting positive cells within three representative 0.6 × 0.6 mm in-
tratumoural areas. Tissue staining intensity and positive cell counts 
were scored by two independent observers.

2.4 Statistical analysis

Statistical analysis was performed using SPSS version 22 for Windows. 
Interclass correlation coefficients confirmed the weighted histoscores 
and positive cell counts were consistent between two independent 
observers. Receiver operator characteristic (ROC) curve analysis 
was employed to determine high and low thresholds. CSS from diagnosis 
was analyzed using Kaplan Meier log-rank analysis from date of diag-
nosis to date of death from CaP. Cox-regression, χ2 analysis, Pearson’s 
rank correlation, and Mann-Whitney U tests were performed. Gene 
expression analysis performed using R. Delta cycle thresholds values for 
each biopsied sample were quantile normalized using the R package 
lmm.13 Hierarchical clustering (Ward.D2) of the normalized expression 
data was then performed identifying two broad clusters. Genes differ-
entially expressed (DE) between clusters were then identified using the 
R package limma.13 Heatmaps visualizing DE genes were generated 
using the R/Bioconductor package ComplexHeatmap.14 Gene set 
enrichment analysis was performed using the R/Bioconductor packages 
clusterProfiler15 and ReactomePA.16 Regression analysis was per-
formed using the lm function form R package stats and the results 
plotted using R package ggplot2. Boxplots were generated using the R 
package ggpubr with significant difference between distributions cal-
culated using the Kruskal-Wallis test.

3 RESULTS

3.1 Inflammatory gene expression in prostate cancer

Inflammatory gene expression profiling was performed on 71 
frozen TRUS prostate biopsies taken to diagnose a patient’s pro-
static disease to determine an inflammatory gene signature and 
whether this associates with high AR expression (Table S3). Un-
supervised hierachal clustering separated the 71 prostate biop-
sies into two distinct classes (Figure 1A). Class 1 contained 11 
patients of which 45.5% were CaP, 45.5% were benign, and 9% 
were prostate intraepithelial neoplasia (PIN). Patients in class 1 
were diagnosed with CaP at a median age of 69 years (inter-
quartile range [IQR]: 67-71 years) with a median Gleason sum of 7 
(IQR: 6-7) and a median PSA concentration of 8.9 ng/mL (IQR: 5.8-
17.1 ng/mL) at time of biopsy. Class 2 contained 60 patients of 
which 23.3% were CaP, 31.7% were benign, and 5% were PIN. 
Patients in class 2 were diagnosed with CaP at a median age of 
69.5 years (IQR: 62.3-74 years) with a median Gleason sum of 7 
(IQR: 7-7) and a median PSA concentration of 12.7 ng/mL (IQR: 
9.1-27.8 ng/mL) at time of biopsy.
AR gene expression was trending towards being expressed higher in class 1 biopsies compared with class 2 biopsies, however this was not significant (Kruskal-Wallis $P = .051$). Interestingly, the gene for AR regulated PSA, KLK3, was significantly highly expressed in class 1 samples versus class 2 (Kruskal-Wallis $P = 2.2 \times 10^{-5}$). In total, 54 out of 96 genes were computed as DE in class 1 versus class 2 biopsies, with the majority of upregulated genes being proinflammatory and protumorigenic including KLK3, EGFR, CASP1, TGFB1, PI3CA, MAPK14, and NF-$\kappa$B (adjusted $P$ value [padj] < .05) (Figure 1). Furthermore, gene set enrichment analysis highlighted multiple pathways significantly enriched in class 1 versus class 2 samples. Among the enriched pathways, those relating to signaling by IL (20 genes) and IL-4 and IL-13 signaling (11 genes) were the most significant (padj < $1 \times 10^{-12}$) (Figure 2).

Subsequently, when the 19 CaP samples were selected for, these samples fell into the same two classes as identified following gene clustering analysis (Table S4). 26 out of the 96 genes were significantly DE in cancer class 1 versus cancer class 2 of which 25 were upregulated (Figure 1B). All genes DE in the cancer class 1 overlap with the genes identified in all class 1 samples (Table S5). Despite AR gene expression not being statistically different between cancer class 1 compared with cancer class 2 (Kruskal-Wallis $P = .15$) (Figure S1A), KLK3 was significantly higher in cancer class 1 versus cancer class 2 (Kruskal-Wallis $P = .0016$) (Figure S1B). Interestingly, 6 out of the 26 gene expression profiles identified in both all class 1 samples and cancer class 1 samples significantly correlated with higher AR expression including PI3CA, ERB3, IL1R, MAPK1, MAPK8, and PSMA ($P < .05$) (Figure S1C-H).
3.2 | Clinico-pathological characteristics

Analysis was performed on 243 hormone-naïve CaP patients, with clinico-pathological features and associations with CSS and the development of biochemical relapse seen in Table 1. Patient characteristics included age, Gleason sum, serum PSA concentration, presence of metastases, perineural invasion, serum albumin concentration, C-reactive protein concentration, time to biochemical relapse, hormonal therapy receive, and time to cancer specific death.

At diagnosis, high Gleason sum (>7) (P < .0001), high serum PSA concentration (>20 ng/mL) (P = .000419), presence of metastasis (P < .0001), perineural invasion (P = .006), and high albumin concentration (P = .001) was associated with decreased CSS. Thirty-eight patients developed biochemical relapse with a mean time to biochemical relapse of 2.20 years (IQR: 1.08–2.90 years). The development of biochemical relapse significantly associated with reduced CSS (P = .000067). Eighty-one patients died from CaP with a mean time to death of 4.90 years (IQR: 4.50–5.73 years).

**FIGURE 2** Gene set enrichment analysis between class 1 and class 2 samples from the frozen TRUS biopsy cohort by Fluidigm array analysis. Dot plot demonstrates the enriched pathways in class 1 samples compared with class 2 samples with color chart representing significance of enriched pathways and circumference of dot representing the gene ratio. MAP, mitogen-activated protein; NF-κB, nuclear factor κB; TRUS, trans-rectal ultrasound.
3.3 | Androgen receptor and AR phosphorylation expression analysis

As AR expression at the messenger RNA level associated with expressions of inflammatory genes, we investigated if AR expression and phosphorylation status associated with inflammatory cell infiltrate in the tumor and microenvironment. Tumor cytoplasmic (TC) AR expression ranged from 0 to 233 weighted histoscore units (WHU) with a median of 76 WHU (IQR: 100). ROC curve analysis determined a threshold of 83 WHU that separated TC AR expression into high and low. TC AR expression significantly associated with reduced CSS (P = .001; hazard ratio [HR] = 7.135 [95% confidence interval [CI]: 2.304-22.091]) and stratified 5-year survival from 69% (low expression) to 54% (high expression) (Figure 3A,B,C,D). CD3+ T-lymphocyte infiltration count ranged from 0 to 274 positive cells with a median of 68 (IQR: 44-105). ROC curve analysis determined a threshold of 60 positive cells that separate CD3+ infiltration into high and low (Figure 4A). CD3+ infiltration was associated with reduced CSS (P = .018; HR = 1.383 [95% CI, 0.591-3.236]) and stratified 5-year survival from 69% (low infiltration) to 54% (high infiltration) (Figure 5A). CD3+ T-lymphocyte infiltration was associated with Gleason sum and perineural invasion at diagnosis following χ² analysis and positively correlated with elevated TC AR expression (Pearson’s correlation coefficient (cc) = 0.278, P = .000232) (Table 2 and Figure S2D). High CD3+ infiltration, when combined with high TC AR expression associated with reduced CSS when compared with low expression. 

| TABLE 1 | Clinico-pathological cohort characteristics and relationship with clinical outcome measures in 243 patients diagnosed with prostate cancer and recruited from the Greater Glasgow and Clyde Health Board between 2008 and 2009; the number of patients in each group are described along with the significance to cancer specific survival (CSS) and time to biochemical relapse (R); Kaplan Meier survival curves with log-rank tests were considered significant if P < .05 |
| Clinico-pathological characteristics | Patient numbers n (%), total n = 243 | Clinical outcome significance |
|-----------------------------------|----------------------------------------|-------------------------------|
| Gleason sum (≤7/7/>7/missing) | 76 (31.3%), 85 (35.0%), 60 (24.7%), 22 (9.1%) | CSS: P < .00001 |
| Serum PSA concentration (ng/mL) (<10/10-20/>20/missing) | 76 (31.3%), 53 (21.8%), 101 (41.6%), 13 (5.3%) | CSS: P = .00419 |
| Metastases (No/yes/missing) | 132 (54.3%), 62 (25.5%), 49 (20.2%) | CSS: P < .00001 |
| Biochemical relapse (No/yes/missing) | 135 (55.6%), 38 (15.6%), 70 (28.8%) | CSS: P = .000067 |
| Hormonal therapy (No/yes/missing) | 67 (27.6%), 134 (55.1%), 42 (17.3%) | CSS: P = .047 |
| Perineural invasion (No/yes/missing) | 115 (47.3%), 99 (40.7%), 29 (11.9%) | CSS: P = .006 |
| Albumin, g/L (<35, >35, missing) | 19 (7.8%), 170 (70%), 54 (22.2%) | CSS: P = .001 |
| C-reactive protein, mg/L (<10, >10, missing) | 61 (25.1%), 41 (16.9%), 141 (58.0%) | CSS: P = .113 |
| Abbreviation: PSA, prostate specific antigen. | | |
Androgen Receptor

(A) Low Expression

High Expression

(B)

\[ P = 0.001 \]

\[ \text{High Expression: } n=104, \text{ Mean Survival: 5.56 years} \]

\[ \text{Low Expression: } n=111, \text{ Mean Survival: 6.82 years} \]

\( P = .000179; \text{ HR } = 2.118 [95\% \ CI, 1.427-3.144] \)

and reduced 5-year survival from 90% (low expression) to 56% (high expression), and associated with poor clinic-pathological features including Gleason sum, PSA concentration, metastases, perineural invasion, development of biochemical relapse, and highly proliferative Ki67+ tumors (\( P = .000026, P = .025, P = .001, P = .000043, P = .022, \) and \( P = .000349, \) respectively) (Figure 5B).

Furthermore, cytotoxic CD8+ T-lymphocyte infiltration count ranged from 3 to 36 positive cells with a median of 15 (IQR:11-19). ROC curve analysis determined a threshold of 13 positive-cells that separated CD8+ infiltration into high and low (Figure 4B). CD8+ infiltration was associated with reduced CSS (\( P = .020; \text{ HR } = 2.121 [95\% \ CI, 0.773-5.817] \) and stratified 5-year survival from 66% (low infiltration) to 53% (high infiltration) (Figure 5C). CD8+ T-lymphocyte infiltration was associated with Gleason sum, serum PSA concentration, and perineural invasion at diagnosis and positively correlated with TC AR expression (\( c.c = 0.247, P = .001 \) (Table 2 and Figure S2A). High CD8+ infiltration, when combined with high TC AR expression associated with reduced CSS when compared with low expression (\( P = .0000257; \text{ HR } = 1.945 [95\% \ CI, 1.356-2.790] \) and 5-year survival was reduced from 81% (low expression) to 57% (high expression). Additionally, high CD8+ infiltration combined with high TC AR expression was associated with Gleason sum, PSA concentration, metastases, perineural invasion, development of biochemical relapse, and highly proliferative Ki67+ tumors (\( P = .000208, P = .000226, P = .016, P = .000017, P = .002, \) and \( P = .000263, \) respectively) (Figure 5D). Interestingly, cytotoxic CD8+ T-lymphocyte infiltration did not
correlate with androgen driven pARser81 within the cytoplasm or nucleus of tumor cells (c.c = 0.152, P = .054 and c.c = 0.024, P = .762, respectively) (Table 2). However, CD8+ T-lymphocyte infiltration significantly correlated with androgen-independent AKT driven pARser213 within the cytoplasm and nucleus (c.c = 0.185, P = .015 and c.c = 0.227, P = .003, respectively) (Table 2 and Figure S2B,C). High CD8+ T-lymphocyte infiltration combined with high tumor nuclear pARser213 expression is significantly associated with poor clinicopathological features including perineural invasion following chi-squared analysis (P = .001).

4 | DISCUSSION

Elevated inflammatory cells within the peripheral zone of the prostate are linked with the development of CaP (relative risk = 1.3, 95% confidence interval = 1.10-1.54).8,17 With this in mind, we investigated how T-lymphocyte infiltration associated with cancer specific survival and the AR and the prognostic use of this for patients with Ca. Additionally, we aimed to investigate if a single TRUS biopsy could be used to identify aggressive characteristics in CaP. We found multiple proinflammatory genes DE in prostate biopsies including NFκB which has previously been reported to be upregulated in CaP and seen to promote increased cellular proliferation, ADT resistance, and induce antiapoptotic signals.18-21 Furthermore, we observed significant T-lymphocyte infiltration in hormone-naïve CaP and a reduction in cancer specific survival which associated with high AR expression and androgen-independent AR serine 213 phosphorylation.

In contrast to our findings, many claim that inflammatory cell infiltration delivers a protective environment and reduces the risk of CaP.22,23 This is further reflected in various other cancers including colorectal cancer and renal cell carcinoma whereby studies into modulating the immune response to provoke an antitumour effects.
have proved successful.\textsuperscript{24,25} However, contradictory studies, in conjunction with our own, have identified a protumorigenic response associated with local inflammatory infiltration within the tumor microenvironment. Chronic inflammation within the prostate is linked with the dedifferentiation of the prostatic epithelium and protumourigenesis with multiple pathways dysregulated that alter the tumor microenvironment and provide a highly proliferative environment for CaP.\textsuperscript{26,27} Interestingly, in CaP it is hypothesized that it
is the pro-inflammatory cytokines secreted by local inflammatory infiltrate, such as IL-6, IL-8, and TNF-α that stimulates AR signaling independent of androgens for example via JAK-STAT, RAS-RAF-MAPK, PI3K-AKT, and nuclear factor B (NF-xB) pathways. Following unsupervised hierarchical clustering of the frozen TRUS prostate biopsies, two classes separated the cohort into patients expressing significantly higher KLK3 expression in class 1 and lower KLK3 expression in class 2 (P = .016). We discovered 54 genes were DE in class 1 samples versus class 2 samples including the overexpression of MAPK14, a key mediator of MAPK signaling implicated in controlling cell death and survival. Uregulation of MAPK signaling as a result of IL-6 is involved in the development of castrate resistant CaP. Furthermore, Wegiel et al also identified IL-6 activated PI3K signaling in CaP, in which we interestingly found a key member of this signaling pathway, PI3KCA, differentially and over expressed in class 1 high KLK3 expressing biopsies. Additionally, overexpression of NFkB was observed in high KLK3 expressing biopsies, a gene involved in regulating IL-8 expression and promoting chemotaxis. Uregulation of this gene and NF-xB/RELA signaling is observed following androgen stimulation of androgen-dependent LNCaP cells. However, contradictory to our findings of CASP-1 overexpression, downregulation of caspase-1 is observed in breast cancer, and when knocked out provided a highly proliferative environment for colon epithelial cells and the development of colon tumors. Interestingly, when we analyzed only those patients who were diagnosed with CaP, all DE genes in cancer class 1 were also DE in all samples in class 1 including EGFR, AKT1, CASP-1, and NFkB. Furthermore, we found multiple genes including PI3CA, PSMA, ERB3, and IL1R all to significantly correlate with increased AR expression, a poor prognostic marker of CaP. Therefore, we decided to explore how T-Lymphocyte infiltration within the tumor microenvironment was associated with CaP specific survival and if it associated with AR expression.

Steiner et al reported a significant increase in infiltrating CD3+ cells in benign prostatic hyperplasia when compared with normal to the normal prostate. However, contradictory to this, high CD3+ infiltrating T-cells have a positive prognostic effect in many cancers. We observed high CD3+ T-lymphocyte infiltration significantly reduced cancer specific survival in hormone-naïve CaP (P = .018). Furthermore, this correlated with high TC AR expression and discovered high CD8+ infiltration and high AR expression, cancer specific survival reduced significantly. However, Patnaik et al identified that following blocking AR with a DNA vaccine in androgen sensitive mice, CD3+ T-cell infiltration increased and prolonged CaP recurrence. Additionally, we explored how CD8+ cytotoxic T-cells effects survival and discovered high CD8+ infiltration reduced cancer specific survival and associated with high TC AR expression. High CD8+ and high AR expression significantly reduced survival from 7.06 to 5.17 years. However, conflicting results have been observed in triple negative breast cancer, with high CD8+ T-cell infiltration reducing breast cancer mortality. In many cancers, high CD8+ infiltration is a favorable prognostic marker, with many immunotherapies aiming at stimulating CD8+ anti-tumoural behavior via anti-CTLA4 or anti-PDL1 immunotherapies. As such, in some cancers the lack of an intra-tumoural immune niches of stem-like CD8 T-cells is associated with more progressive disease. Interestingly, high CD8+ infiltration also associated with high pARser213 expression in hormone-naïve tumors. Serine 213 is a known site for AR binding and becomes activated via the PI3K/AKT cascade which is regulated by PTEN, a tumor suppressor commonly deleted or mutated in 30% of primary and 100% of metastatic CaP. When patients express a loss of PTEN this associates with elevated CD8+ infiltration in the prostate tumor microenvironment and significantly reduces time to recurrence from diagnosis. However, the mechanism though which loss of PTEN stimulates CD8+ infiltration remains unclear.

Together, our results have provided evidence that levels of T-cells in CaP microenvironment are associated with poor prognosis. Controversial results have been demonstrated here suggesting a poor prognostic effect of T-lymphocyte infiltration in hormone-naïve disease and a strong association between AR expression as well as androgen-independent AR activation. However, how this is reflected in castrate resistant disease is critical to provide prevention and further therapeutic options for this lethal form of CaP.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS
MM performed experiments, analyzed data and wrote manuscript.
VC performed immunohistochemistry for CD3. SP collected all patient information and developed the cohort of samples used for immunohistochemistry analysis within this study. HG assisted with immunohistochemistry. PB assisted with bioinformatic analysis.
HW helped design Fluidigm experiment. MU aided in project design and the gathering of prostate biopsy material. HL aided in project design. JE helped with all aspects of the manuscript including project design and execution.

ETHICS STATEMENT
Multicentre Research Ethics Committee for Scotland (MREC/01/0/36) and Local Research and Ethics Committees.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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