Primary Mutational Landscape Linked with Pre-docetaxel Lactate Dehydrogenase Levels Predicts Docetaxel Response in Metastatic Castrate-resistant Prostate Cancer

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**Primary mutational landscape linked with pre-docetaxel LDH levels predicts
docetaxel response in mCRPC**

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Abstract:**Background**

Docetaxel chemotherapy is a standard of care for metastatic castrate resistant prostate cancer (mCRPC): 40-50% of patients achieve a biochemical response. However, there is a lack of response predictive biomarkers.

Objective

Assess LDH as a docetaxel response biomarker in mCRPC. Examine LDH association with genomic alterations in *primary* diagnostic biopsies.

Design, Setting and Participants

Clinical and associated primary tumour targeted next-generation sequencing data from matched training (n=150) and test (n=120) cohorts of progressive mCRPC patients receiving docetaxel therapy were analysed. Data was correlated with large scale prostate cancer genomic datasets.

Outcome measurements and Statistical Analysis

Prostate-specific antigen (PSA) response, radiographic response, biochemical progression-free (PFS), overall (OS) survival, genomic analysis of primary biopsies and genomic datasets (MSKCC, SUCF/PCF).

Results and Limitations

Serum LDH ≥ 450 U/L is a reliable prognostic biomarker [AUC:0.757 (SD 0.054 95% CI 0.650-0.864; $p < 0.001$)] in progressive mCRPC, predicting PFS at 3 months. Patients with LDH ≥ 450 U/L were poorer PSA responders, with shorter PFS (213 vs 372 days, HR 1.876, 95% CI 1.289-2.7300) and OS (362 vs 563 days, HR 1.630, 95% CI 1.127-2.357. High LDH is an independent surrogate marker for survival following docetaxel and predicts poor radiologic response ($p = 0.043$). Of the 14 patients with LDH ≥ 450 U/L available for NGS 9/14 (64.3%) were more likely to have

66 DNA repair gene mutation(s) (BRCA1/2, ATM, CHEK2, Fanconi's anaemia gene) in
67 their *primary* biopsy. Cross-correlation with MSKCC and SUCF/PCF databases
68 revealed a positive correlation between LDHA, PARP1 ($r=0.667$; $p<0.01$) and other
69 DNA repair genes.

70 **Conclusions**

71 Genomic abnormalities of LDHA and DNA repair in primary biopsies link to high pre-
72 treatment LDH and poor response to docetaxel in mCRPC.

73 **Patient Summary**

74 The presence of mutations of the LDH and DNA repair pathways are associated with
75 aggressive prostate cancer and poor response to chemotherapy later in the disease.

76

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Introduction:

Docetaxel chemotherapy improves survival in men with mCRPC[1,2]. It remains a valuable treatment for such patients. However, docetaxel resistance occurs in approximately 50%, who have no biochemical or clinical response to therapy[2]. Some patients also respond to treatment initially but develop early therapeutic resistance. This creates uncertainty when deciding on the sequencing of treatments at this stage of the disease. There is therefore a need to explore biomarkers in relation to treatment outcomes that may help guide patient management. Lactate dehydrogenase (LDH) plays an important role in normal cellular glycolytic and gluconeogenesis pathways, it is dysregulated in cancer metabolism. Serum LDH level has clinical implications as a surrogate for overall survival and treatment response in several cancers[3-6]. Understanding the molecular mechanisms that govern the development of high serum LDH levels prior to docetaxel chemotherapy may provide critical insight to cancer biology and treatment.

Genomic profiling is also used increasingly to guide cancer treatment e.g. colorectal cancer (30-50% tumours with KRAS mutation), lung cancer (50% tumours with EGFR mutation), and melanoma (25% tumours with BRAF mutation [7-9]. In prostate cancer (PCa) this has been problematic due to tumour heterogeneity but more recently, DNA repair linked genes have proved to be important in identifying response to DNA repair modulating drugs [10].

Herein we demonstrate the prognostic potential of serum LDH levels in mCRPC and develop this finding to link, for the first-time, LDH predicted response to docetaxel in

102 mCRPC with genomic LDHA and DNA repair linked gene alterations present in the
103 primary biopsy at first diagnosis.

104

Methods:**Cohort**

A classical cohort (N=270) of docetaxel treated, progressing mCRPC (2005-2012) were studied (REC#07/H1003/161+5 10_NOCL_02). Archival FFPE primary diagnostic prostate biopsy tissues were split into training (n=150) and test (n=120) cohorts. High Gleason grade tumour regions were defined by a uro-pathologist from fresh H&E sections. Blocks with incomplete clinical data, poor tissue quality, lack of PCa related mortality were excluded on secondary screening. Clinic-pathological data included age, Gleason score, M stage at initiation of docetaxel , alkaline phosphatase, lactate dehydrogenase, albumin, PSA, and %PSA fall after 6 cycles of docetaxel. PSA progression was defined as per PCWG3 criteria. Radiographic progression was defined as a >20% increase in the sum of the diameters of soft-tissue target lesions on CT/MRI or >2 new lesions on bone scanning.

Statistics

Clinic-pathological characteristics at the time of initiation of docetaxel therapy were recorded and cut-off points for LDH, ALP, and best PSA response were determined by receiver operating characteristics (ROC) analysis, based on 3 month progression free survival. The clinic-pathological associations were assessed by student t-test, Wilcoxon rank sum and Chi-square tests. Cox regression analysis was used to assess biomarker variables in univariate and multivariate analysis. Chi square test assessed the predictive value of LDH in best PSA and radiologic response. Time to event outcomes was evaluated by Kaplan-Meier. All statistical analyses were 2-sided and performed at 95% significance level using SPSS 20.1 software.

Genomic Analysis

Regions of tumour were macro-dissected and DNA extracted using a proteinase K buffer for 12-24h followed by Promega Maxwell 16 Tissue LEV DNA kit purification as previously described[11]. Extracted DNA was quantified by standardised PicoGreen fluorescence assay. Sequencing libraries were constructed from 200ng of sheared DNA/total DNA (if <200ng was recovered). To correlate the NGS results, the distribution of genetic alterations were dichotomised by pre-treatment LDH levels. Total mutational burden/megabase of the 118 genes assessed was determined[12] (Supplementary Methods). To calculate copy number alterations (CNA) burden, the copy number of gains/losses was summed and calculated as a percentage of the size of the coding region territory (Supplementary Methods).

Genomic Meta-Analysis

CBioPortal for Cancer Genomics (<http://ciportal.org>)[13] was used to explore genetic alterations between LDHA and DNA repair genes in MSKCC[14], TCGA[15] and SUCF/PCF[16] cohorts. Volcano plots were constructed with Plotly (<https://plot.ly>). Findings were compared with the findings from primary tissue genomic analysis.

Results:

Clinical Characteristics

270 patients receiving docetaxel with prednisolone at standard dose in 3-weekly cycles between August 2005 and February 2012 were evaluated. Baseline characteristics of patients responding to docetaxel were correlated with LDH and ALP thresholds. Poor responders had significantly elevated LDH ($p=0.007$) and ALP ($p=0.048$) levels (Table 1). Higher and lower LDH cohorts had similar baseline characteristic regarding age, treatment modalities, blood-based parameters, and therapies following docetaxel treatment (Table 2).

Pre-docetaxel serum LDH correlates with progression free survival (PFS)

The median value for pre-treatment LDH was 517U/L (upper limit normal =550U/L). In Figure 1, we compared LDH with ALP and best PSA response (%fall of PSA post 6 cycles of docetaxel)[17] as predictors of 3 month PFS in the combined cohort. Pre-treatment serum LDH was the strongest biomarker for this (ROC 0.757 SD 0.054; 95% CI 0.650–0.864, $p<0.001$). Reducing the cut-off to 450U/L increased sensitivity and specificity to 86.4% and 63.3% respectively compared with 81.8% and 50.2% at 517U/L (Table 3). Focusing on the endpoint of 3 months PFS, LDH was the most accurate tool for predicting PSA response in both training and test sets (Supplementary Figure 1).

High mCRPC LDH correlates with adverse clinic-pathological characteristics

and docetaxel response. Pre-docetaxel clinic-pathological characteristics showed that patients with LDH ≥ 450 U/L had a higher PSA (median 383ng/L; range 2.3–5325ng/L vs 166ng/L; range 6.5–4453ng/L) and ALP (median 322U/L; 60–3112U/L vs

139U/L; range 37-1736U/L) at initiation compared to those with LDH $\leq$ 449U/L (Table 4). Pre-treatment LDH $\geq$ 450U/L patients had poorer PFS (median 255 vs 373 days, $p < 0.001$ and 213 vs 372 days, $p < 0.001$ for training and tests sets respectively), and OS (median 356 vs 514 days, $p = 0.016$ and 362 vs 563 days, $p = 0.011$) (Figure 3). The prognostic potential of pre-treatment LDH was also seen in multivariate analysis with the reported clinic-pathological markers (Supplementary Table 2).

LDH $\geq$ 450U/L correlated with a poor docetaxel response based on both %fall of PSA post docetaxel and radiological progression. Pre-treatment serum LDH $\geq$ 450U/L correlated with progressive disease, as defined by any PSA rise after 6 cycles of docetaxel (43.6% vs. 21.3%; $p = 0.0004$). However, pre-treatment serum LDH does not predict a positive response to docetaxel. No significant difference was seen between the two LDH cohorts in stable disease (20.4% vs. 44.9%; $p = 0.8482$) or in partial responders (35.9% vs. 33.7%; $p = 0.7868$) (Figure 2 & Table 5).

Radiological response was assessed in 71 (26.3%) patients who had RECIST 1.1 measurable disease at baseline. Radiologic disease progression was seen in 32 (45%), of whom 23 (72%) had pre-treatment LDH levels $\geq$ 450U/L (Table 5). Chi square testing showed that LDH was prognostic of poor radiologic response ($p = 0.043$) unlike %PSA or ALP falls ($p = 0.755$, 0.838 respectively). However pre-treatment LDH did not correlate with stable disease (SD) or partial response (PR) (47.4% vs. 52.6%, $p = 0.499$ and 55% vs. 45%, $p = 0.129$ respectively).

LDH $\geq$ 450U/L is associated with higher frequency of genomic alterations in primary biopsies

Using LDH thresholds as a marker for poor response to docetaxel we dichotomised our cohort and performed targeted next generation sequencing (NGS) on primary diagnostic biopsy tissue to characterise the genomic landscape at the point of diagnosis to ascertain whether the molecular profile *at diagnosis* was associated with LDH levels and response to docetaxel treatment in mCRPC (Figure 4). Analysis was limited to previously described functional alterations and excluded variants of unknown significance (VUS). 21 samples with available primary diagnostic tissue (Supplementary Table 1) were analysed, of which 14 were linked with pre-treatment LDH ≥ 450 U/L and 7 had LDH ≤ 450 U/L. LDH ≥ 450 U/L samples had a higher tumour mutational load (TMB) (mean 46.2 vs 6.2 mut/MB; $p=0.129$ Supplementary Figure 2A) and higher Copy Number Alteration (CNA) (mean, 1.7% vs 0.7%; $p=0.3747$, Supplementary Figure 2B) at the point of primary biopsy.

Correlations between LDHA mRNA expression and DNA repair mRNA gene

transcription. Genomic analysis of primary biopsies from pre-treatment LDH ≥ 450 U/L patients showed mutations throughout the genomic regions tested. Of note we observed a differential cluster within the DNA repair pathway. Tumour aberrations in DNA-repair genes were observed in (9/14, 64%) LDH ≥ 450 U/L patients including 2 with somatic homozygous deletion of BRCA1, 1 somatic homozygous deletion of BRCA2, 3 with ATM aberrations (1 truncation of the ATM gene, 2 missense mutations) and 2 patients with homozygous deletions of CHEK2. Mutations within DNA repair genes, such as Fanconi anaemia, ATR, MRE11 were observed more commonly in the LDH ≥ 450 U/L cohort (6/14, 42.8%) along with mutations in TP53 (10/14, 71%), a gatekeeper for response to DNA damage. Finally,

8/14 (57%) LDH ≥ 450 U/L patients had tumour aberrations in PI3K pathway signalling genes.

To strengthen this association between mutations in the DNA repair pathway in primary biopsy with pre-docetaxel serum LDH ≥ 450 U/L, we performed a meta-analysis of the localised PCa MSKCC cohort. Tumour specific expression of the LDHA gene, which gives rise to the main monomer of the LDH enzyme, has a positive correlation with PARP1 gene expression ($r=0.667$, $p<0.001$, Figure 5). The DNA repair genes, ATR, MLH1, and ATM also correlated with LDHA ($r=0.632$, $r=0.463$, $r=0.464$, $p<0.001$, Figure 5). This association was further examined in the metastatic mCRPC SUCF/PCF cohort. Volcano plot analysis showed that transcription of several DNA repair genes, including BRCA1 and BRCA2 was upregulated in LDHA overexpressed samples (Figure 6A & 6B, Table 6). Lastly, LDHA positively correlated with PARP1 ($r=0.27$, $p<0.001$), BRCA1/2, ($r=0.319$, $r=0.26$; $p<0.001$) and CHEK2 ($r=0.412$, $p<0.001$) gene expression in the TCGA database (Supplementary Figure 4).

Discussion:

The link between cancer metabolism and DNA damage is established and the Warburg effect remains a key component of the dysregulation of energy metabolism in this condition[18]. LDH becomes dysregulated in cancer cells and energy is produced via glycolysis, rather than oxidative phosphorylation, resulting in increased lactate production. The reasons underlying this metabolic shift are incompletely understood but there is a clear linkage to activation of oncogenes and loss of tumour suppressor genes, with associated genetic instability and compromised DNA repair.

The results of this study suggest that a subgroup of highly aggressive late stage mCRPC patients, whose response to docetaxel in late stage disease is limited can be identified by stratification according to the absolute level of serum LDH in late stage failure, and that the genotype of these patients is identifiable in the genomic profile at *primary* diagnosis. The subset of mCRPC patients with unfavourable LDH $\geq$ 450U/L had a higher primary tumour mutational burden (TMB), with greater incidence of defects in DNA repair and LDH-linked genes within their primary diagnostic biopsy and this correlated with a poorer response to subsequent docetaxel therapy on progression in the castrate resistant state. The frequencies of somatic mutations of TP53 gene were also seen more commonly in this subset. Our findings suggest that analyses of primary diagnostic tumour biopsy samples using next generation sequencing methods can increase our understanding not only of the biological aggression of a tumour at presentation, but also to its association with treatment response to castration resistant progression .

Our sample size for NGS analysis was small, but the data generated therefrom was consolidated through meta-analysis of large-scale cohorts, whose data support our NGS findings. The independent MSKCC cohort found expression of tumour specific LDHA, a dominant subunit of the LDH family, was positively associated with expression of the PARP1 gene, and several other DNA repair genes (ATR, MLH1, and ATM) at the mRNA level. A volcano plot analysis of the SUCF/PCF cohort, containing metastatic mCRPC samples also confirmed that DNA repair genes were associated with samples with overexpression of LDHA. Together, these data emphasise a congruent link between LDH, associated with the Warburg switch, and DNA repair genes as seen in our targeted next generation sequencing data, which is identifiable when the patient first presents.

Other studies[19-21] have shown that LDH alone is a surrogate for survival in the management of individual patients with mCRPC. Our data support this, adding further information which may be of clinical utility in relation to the use of docetaxel. Patients with an LDH threshold beyond 450U/L generally failed in their response to treatment and when LDH levels are in excess of this threshold consideration might be given alternative therapeutic approaches as response to docetaxel in this setting is generally poor. However, this finding, whilst supportive of other studies is not new. By contrast, our identification of a high frequency of DNA repair gene mutations in the primary biopsy and their association with LDHA genes linked to high LDH levels and response to docetaxel treatment in later stages of their disease is novel. Whilst it might be argued that the high LDH levels observed in our poorly responding patients was a manifestation of lead time and disease burden, it is hard to reconcile this with the fact that the DNA repair and LDH linked genetic aberrations were present at the

initial diagnosis in the majority of these patients. This suggests that the association is true and that the mechanistic link is worth exploring further. It may be of value to screen men with high risk localised and de-novo metastatic PCa to test the hypothesis that this group of patients might be sub-selected for a different treatment pathway rather than involving conventional Taxane based approaches.

There are a number of limitations in this study, the relatively modest size and classical pre-abiraterone/enzalutamide cohort nature. The higher overall incidence of genomic defects in DNA repair genes in our cohort (42.8% (9/21)) compared to previous reports[16,22,23] reflects the aggressive nature of the final disease of this cohort. It is unclear whether this enrichment is secondary to a tumour evolution process from localised disease to metastasis, or small sampling size. However, this weakness arising from the relatively small numbers is mitigated by the consistency of data when comparing large genomic data banks from patients with a spectrum of clinically indolent and clinically aggressive disease whose natural history is established. We believe this consistency in the data trend and the presence in pre-treatment biopsies adds weight to the notion that our findings show a true effect.

Conclusions

Serum LDH level at the point of initiation of docetaxel therapy in progressive mCRPC is a strong prognostic marker for PFS and OS and levels ≥ 450 , indicative of poor response, may be of use in therapy selection in these patients. Late failure with high serum LDH is associated with a high frequency of genomic alterations in LDHA and DNA linked repair genes in primary biopsies. This group of patients have an aggressive phenotype and exhibited a poor response to docetaxel based chemotherapy in the castrate resistant state. Our data adds evidence to the notion that there is a primary metabolic, gene associated linkage to a high energy driven glycolytic status within PCa cells which drives a biologically aggressive phenotype. Further interrogation of this process may help to refine predictive signatures of aggression in clinically localised disease, sub-select patients who may not respond to docetaxel based chemotherapy and also, further our understanding of the inter-relationship between dysfunctional energy handling in cancer cells and the resultant progression through the metastatic process.

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Table Legends

Table 1: Baseline descriptive characteristics of chemoresponsive and chemoresistant patients in the training set (n=150). The association of clinic-pathological characteristics of cohorts were assessed by student t-test, Wilcoxon rank sum test and Chi-square test for continuous (Age, PSA at initiation of docetaxel, LDH, ALP, Hb, Albumin) and categorical (Presence of bone metastases, and presence of visceral metastases) variables respectively.

Table 2: Baseline descriptive characteristics of the training (n=150) and test (n=120) sets. The association of clinic-pathological characteristics of both cohorts were assessed by student t-test, Wilcoxon rank sum test and Chi-square test for continuous (age, LDH, ALP, Hb, albumin, PSA at initiation of docetaxel) and categorical [treatment modalities at M0/M1 stage, metastatic stage (M1a/b/c), post docetaxel treatment, and PSA responders] data respectively.

Table 3: Progression Free Survival sensitivity and specificity using pre-treatment serum LDH. The upper limit normal of LDH value used at the Christie is 550 U/L. The median value of training, test and combined cohort is 546, 493 and 517 respectively. The sensitivity and specificity of LDH >450U/L as a prognostic biomarker is reported at the bottom row for each cohort. The sensitivity and specificity were calculated based on AUC analysis, using SPSS.

Table 4: Clinic-pathological characteristics of mCRPC patients who underwent docetaxel therapy dichotomised by pretreatment LDH levels. Correlation

statistics between LDH expression and selected clinic-pathological characteristics among men treated with docetaxel chemotherapy at castrate level in combined cohort. Unpaired student t test and Wilcoxon rank sum test was used for continuous variables. Chi square test was used for categorical variables. Abbreviation: LN: lymph node.

Table 5: Pre-treatment LDH $\geq$ 450U/L correlates with poor docetaxel response.

Docetaxel response was based on %fall in PSA, or by radiological progression in patients who had RECIST (v1.1) measurable disease at baseline. Progressive disease was defined as any rise in PSA after 6 cycles of treatment or a >20% increase in the sum of the diameters of the lesion on CT/MRI or >2 new bone lesions.

Table 6: LDHA expression correlates with DNA repair genes in the SU2C/PCF cohort. Expression of DNA repair genes (RAD18, BRCA1, EZH2, PARP1, BRCA2, CHEK2, ATM, MLH1, ATR) within the metastatic prostate samples (n=118) from the SU2C/PCF database were correlated with altered LDHA expression. Data presented as mean mRNA expression $\pm$ standard deviation (SD)

Figure Legends

Figure 1: LDH is the most accurate prognostic indicator. ROC Curve analysis comparing LDH, ALP and Best PSA response as a predictor of 3 month PFS. Best PSA response is defined as % fall of PSA post 6 cycle of docetaxel.

Figure 2: Pre-treatment serum LDH of ≥ 450 U/L predicts docetaxel treatment failure. Waterfall plots depicting best Prostate Specific Antigen (PSA) response dichotomised by < 450 U/L (red) or ≥ 450 U/L (blue) LDH levels. Best PSA response was measured based on percentage changes from PSA level at 1st cycle of docetaxel chemotherapy to the end of 6th cycle of docetaxel chemotherapy.

Figure 3: Serum LDH is prognostic for progression free and overall survival in mCRPC patients. Kaplan Meier Progression free survival (PFS) analysis dichotomised by pre-treatment serum LDH levels < 450 or ≥ 450 U/L for training (A) and test (B) sets. Kaplan Meier Overall Survival (OS) analysis dichotomised by pre-treatment serum LDH levels < 450 or ≥ 450 U/L for training (C) and Test (D) sets.

Figure 4: The genomic landscape of primary prostate cancer patients. The genomic landscape of primary diagnostic biopsies from patients (n=21) who later went on to be treated with docetaxel for mCRPC, dichotomised by pre-treatment LDH levels (< 450 vs. ≥ 450 U/L), was determined on the FoundationOne platform. Genes were grouped according to function: 1. Tumour suppressor genes; 2. AR; 3. Metabolism; 4. DNA repair; 5. PIK3CA/AKT pathway; 6. Transcription; 7: Chemotaxis; 8. Apoptosis; 9. Oncogene; 10. Wnt signalling; 10. TGF signalling.

Figure 5: Meta-analysis of the MSKCC cohort shows a link between LDHA and

DNA repair genes. Scatterplot analysis of normalised LDHA mRNA expression vs

A) PARP1, B MLH1, C)ATM, D) ATR mRNA expression. Expression shown as log2

whole transcript mRNA expression (Affymetrix Human Exon 1.0 ST arrays). Pearson

correlation statistical analysis was used to compare the linear association of specific

genes.

Figure 6: Meta-analysis of the metastatic CRPC SUCF/PCF cohort shows a link

between LDHA and DNA repair genes. Volcano plot of SUCF/PCF cohort showing

the differential expressed genes, dichotomised by the LDHA gene. The spots above

the horizontal line represent genes with statistically significant expression differences

with respect to controls. A total of 14660 genes were analysed. A mean value was

deduced following the deduction of mean mRNA expression of the altered group and

non-altered group. Positive mean changes indicate up-regulation compared with the

unaltered group, while negative mean changes indicate down-regulation. High –

log10pvalue indicates an increased statistical significance in the studied gene

expression in relation to the LDHA altered/unaltered groups.

Take Home Message

Pre-treatment serum LDH levels are prognostic for docetaxel therapy failure within the metastatic castrate resistant prostate cancer setting, and is linked to a genomic subtype present in a high proportion of these patients at the point of diagnostic biopsy.

Table 1

Patient characteristics	Chemosensitive (n=93)	Chemoresistant (n=57)	p value
Age (mean $\pm$ SD)	66 $\pm$ 8	67 $\pm$ 7	0.127
PSA at initiation of docetaxel	576.9	462.1	0.610
LDH median (range)	522 (245 - 3000)	625 (359 – 3710)	0.007
ALP median (range)	232 (49 - 1451)	304 (70 – 3112)	0.048
Hb median (range)	11.8 (8.5 - 15.6)	11.5 (8.6 - 14.3)	0.239
Albumin median (range)	43 (32 – 49)	42 (34 – 51)	0.112
M stage			
Bone metastases, n (%)			
YES	68 (73.1%)	47 (82.5%)	0.208
NO	25 (26.9%)	10 (17.5%)	
Visceral metastases, n (%)			
YES	7 (7.5%)	13 (22.8%)	0.028
NO	86 (92.5%)	44 (77.2%)	

Table 2

Patient Characteristics	training set	test set	p value
Number of patients	150	120	
Age, median (range), y	67 (49 -81)	68 (54 -80)	0.056
M0 at diagnosis	n=92	n=75	
M1 at diagnosis	n=58	N=45	
Primary treatment, No. (%)			
Prostatectomy	5 (5.4)	6 (8.0)	0.498
Radiotherapy	48 (52.1)	45 (60.0)	0.143
Brachytherapy	4 (6.5)	6 (8.0)	0.318
Primary ADT	35 (38.0)	18 (24.0)	0.008
ADT for M1 Disease	58 (100.0)	45 (100.0)	
Metastatic disease status at initiation of docetaxel chemotherapy, No. (%)			
LN only (M1a)	34 (22.7)	30 (25.0)	0.676
Bone +/- LN (M1b)	96 (64.0)	85 (70.8)	0.266
Visceral +/- Bone +/- LN (M1c)	20(13.3)	5 (4.2)	0.009
Laboratory measures, median (range)			
LDH U/L	546 (245 -3710)	493 (248 -4668)	0.005
Haemoglobin g/DL	11.6 (8.5 – 15.6)	11.6 (7.5 -16.3)	0.456
ALP U/L	213 (49 -3112)	215 (37 -2439)	0.707
Albumin, g/DL	43 (32 – 51)	42 (32 -50)	0.236
PSA, ng/mL	252 (1.6 – 4453)	244 (3.9 – 5325)	0.887
Postdocetaxel treatment (%)			
AR therapies- arbitraterone/enzalutamide only	7 (4.7)	12 (10.0)	0.0988
Taxanes- rechallenge docetaxel, cabazitaxel	1 (0.7)	2 (1.7)	0.5866
AR and Taxanes	2 (1.3)	5 (4.2)	0.2474
Palliative care	134 (89.3)	95 (79.2)	0.0262
Others, radium 223, clinical trials etc.	6 (4.0)	6 (5.0)	0.7708
PSA response (%)			
Partial response	52 (34.7)	46 (38.3)	0.6106
Stable disease	58 (38.7)	43 (35.8)	0.7044
Progressive disease	40 (26.7)	31 (25.8)	0.8904

Table 3

Sensitivity and Specificity for predicting 3 months PFS, by cutoffs of LDH						
		Training set (n=150)		Test set (n=120)		Combined cohort (n=270)
LDH, U/L		Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity Specificity
550 (upper limit normal)		81.8	45.3%	63.6%	29.7%	72.7 40.4
Median	546	81.8%	47.4%	63.6	31.2	72.7 42.4
	517	90.9	53.3	72.7	39.9	81.8 50.2
	493	90.9%	59.9	72.7	44.9%	86.4 55.1
	450	95.2%	68.6%	81.1%	50.7%	86.4 63.3

Table 4

Characteristics	Overall cohorts (n =270)		p value
	High (≥450)	Low (<449)	
Patients, n (%)	181	89	
Age at initiation of docetaxel, median (range)	68 (52 -83)	68 (49 – 83)	0.183
PSA at initiation of treatment, median (range)	383 (2.3 – 5325)	166 (6.5 -4453)	<0.001
Bone only	94 (51.9)	41 (46.1)	
LN only	18 (9.9)	9 (10.1)	0.158
LN + Bone only	40 (22.1)	30 (33.7)	
LN + Bone + visceral only	29 (16.1)	9 (10.1)	
Haemoglobin	11.3 (7.5 – 16.3)	12.1 (8.3 – 15.6)	<0.001
ALP	321.5 (60 -3112)	139 (37 -1736)	<0.001
Albumin	42 (34 – 51)	43 (32 -49)	0.253

Table 5

	<449 LDH	≥450 LDH	p value
PSA responder, n (%)			
Partial responder (>50% fall in PSA)	30 (33.7)	65 (35.9)	0.7868
Stable disease (<49% fall PSA)	40 (44.9)	37 (20.4)	0.8482
Progressive disease (any PSA rise)	19 (21.3)	79 (43.6)	0.0004
Best radiologic response, n (%)			
Partial responder	11 (55.0)	9 (45.0)	0.129
Stable disease	9 (47.4)	10 (52.6)	0.499
Progressive disease	9 (28.1)	23 (71.9)	0.048

Table 6

Gene	Cytoband	Mean mRNA expression ±SD		p value	q value
		Altered group	Un-altered group		
RAD18	3p25.3	2.75±0.40	1.94±0.95	2.64x10 ⁻⁶	2.852x10 ⁻³
BRCA1	17q21	3.06±1.06	1.53±0.99	1.831x10 ⁻⁴	0.0153
EZH2	7q35-q36	4.19±0.97	2.74±0.94	1.307x10 ⁻⁴	0.0139
PARP1	1q41-q42	6.20±0.85	5.44±0.79	8.096x10 ⁻³	0.0807
BRCA2	13q12.3	0.81±1.87	-0.74±1.39	0.0121	0.0982
CHEK2	22q12.1	2.67±0.96	2.05±0.78	0.0415	0.185
ATM	11q22-q23	2.84±0.74	2.45±0.82	0.0982	0.293
MLH1	3p21.3	4.02±0.68	3.82±0.61	0.336	0.572
ATR	3q23	3.50±0.76	3.57±0.73	0.766	0.878

Figure 1
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Figure 2
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Figure 3
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Supplementary Methods

Patient samples

270 mCRPC patients who underwent docetaxel therapy for progressive disease between 2005 and 2012. The cohort was dichotomised into a training (n=150) and test (n=120) set. The study was conducted with full ethical approval. Archived formalin fixed paraffin embedded (FFPE) diagnostic transrectal ultrasound (TRUS)-guided prostate or Transurethral resection of prostate (TURP) tissue was retrieved for each case and clinically significant regions of tumour defined by an uro-pathologist from H&E sections. Blocks with incomplete clinical data, poor tissue quality, and lack of PCa related mortality were excluded on secondary screening. Clinic-pathological data included age, Gleason score, M stage(M1a/b/c) at initiation of docetaxel chemotherapy, alkaline phosphatase, lactate dehydrogenase, albumin, PSA, and % PSA fall post 6 cycles of docetaxel.

DNA extraction, library preparation and sequencing.

Targeted next-generation sequencing studies (NGS) were conducted by Foundation Medicine as previously described [1]. Regions of tumour were macro-dissected and DNA extracted using a proteinase K buffer for 12-24h followed by purification with the Promega Maxwell 16 Tissue LEV DNA kit as previously described [1]. Extracted DNA was quantified by standardised PicoGreen fluorescence assay. 200ng of sheared DNA was recovered from samples or total DNA from samples with less than 200ng was used to construct from sequencing libraries. Libraries were constructed with the use with custom biotinylated RNA oligo pools (custom SureSelect kit, Agilent) representing 3167 exons of 118 cancer-related genes plus 18 introns of 6 genes often rearranged in cancer (Supplementary Table 3). Pair end sequencing (49 x49 cycles) was performed using the HiSeq 2000 (Illumina). Sequence data from genomic DNA and cDNA were mapped to the human genome as previously described [1, 2].

Microarray data analysis:

We explored 3 microarray data sets to analyse the association of genes with LDHA, the main monomer of LDH.

1. MSKCC Prostate Cancer Oncogenome Group dataset consists of 218 prostate tumours (181 primaries, 37 metastases) and 12 prostate cancer cell lines and xenografts, using Agilent Human Exon 1.0 ST array [3]. The expression array data were downloaded from MSKCC cbioportal under accession number GSE21032 (cbio.mskcc.org/cancergenomics/prostate/data/). Primary tumours with complete mRNA data (n=131) were included; all metastatic samples were excluded. To examine the association of genes involved in DNA repair pathway and LDHA in MSKCC cohort, log2 whole transcript mRNA expression values of each gene from downloaded process data (Affymetrix Human Exon 1.0 ST array) were used as scatterplots. Pearson correlation coefficient was used to measure the linear correlation between LDHA and individual genes in DNA repair pathway.
2. The Cancer Genomics Atlas (TCGA) consortium dataset consists of 383 samples: 50 benign samples and 333 primary tumours, using IlluminaGA RNA Seq [4]. The expression array data were downloaded from UCSC Cancer Genome Browser (<https://genome-cancer.ucsc.edu/>) - TCGA_PRAD_exp_HiSeqV2-2014-05-02.tgz. Associated clinical data were downloaded from the TCGA Data Portal (<https://tcga-data.nci.nih.gov/tcga/>). Primary tumours with complete mRNA data (n=333) were included. To examine the association of genes involved in DNA repair pathway and LDHA in TCGA cohort, log2 whole transcript mRNA expression values of each gene from downloaded process data (RNA Seq V2

RSEM) were used as scatterplots. Pearson correlation coefficient was used to measure the linear correlation between LDHA and individual genes in DNA repair pathway.

3. SU2C/PCF Dream Team dataset consists of 150 metastatic samples derived from several anatomical locations, such as lymph node (42%), bone (28.7%), liver (12.7%) and other soft tissues (16.7%), using TruSeq RNA protocol (Illumina) [5]. The expression array data were downloaded from cBioPortal for cancer genomics under accession number dbGap: phs000915.v1.p1 (www.cbioportal.org/study?id=prad_su2c_2015\summary). Tumour samples with complete mRNA data (n=118) were included. The mRNA expression of LDHA correlates with DNA repair genes in both MSKCC and TCGA datasets. We conducted an unbiased volcano plot analysis to examine the differential expressed transcript between mean high and low LDHA/BRCA1 in SU2C/PCF Dream Team cohort, using Plotly (<https://plot.ly>). Data were filtered based on processed total RNA from poly-A RNA sequencing data. In this cohort, gene expression were calculated as fragments per kilobase of exon per million fragments mapped (FPKM; normalised measure of gene expression), as published [15].

Bioinformatics

Tumour Mutational Burden (TMB): To calculate total mutational burden, we calculated the number of somatic, coding, base substitution, and indel mutations per megabase of the examined genome [6]. All base substitutions and indels in the coding region of targeted genes, including synonymous alterations, are initially counted before filtering as described below. Non-coding alterations were not counted. Alterations listed as known somatic alterations in COSMIC and truncations in tumor suppressor genes were not counted. This correction was performed to avoid upward skewing of mutational load, because FoundationOne preferentially profiles genes known to be recurrently mutated in cancer. Alterations predicted to be germline by the somatic –germline-zygosity algorithm were not counted. To calculate the mutation load per MB, the total number of mutations counted was divided by the coding region target territory, covering 1.09 MB for the 124 genes.

Copy number alterations (CNA): To calculate copy number alterations (CNA), the total genomic territory spanned by gain, amplification, loss and deletion segments was summed and a percentage was generated using the size of the examined total genomic territory [7].

Supplementary Figure 1: LDH remains the best accurate tool for prognostic indicator, among ALP and Best PSA response in (A) training set, (B) test set of the Christie cohorts focusing on the endpoints of 3 months PFS. Best PSA response is defined as % fall of PSA post 6 cycle of docetaxel.

Characteristics	Patients with available NGS data		P value
	High (≥450)	Low (<449)	
Patients, n (%)	14	7	NA
Age at initiation of docetaxel, median (range)	64 (60 – 75)	68 (53 -75)	0.279
PSA at initiation of treatment, median (range)	409.1 (74.1 – 1789)	116.1 (8 – 221.4)	<0.001
M stage			
Bone only (M1b)	11 (78.6)	5 (71.4)	0.771
LN only (M1a)	0 (0.0)	0 (0.0)	
LN + Bone only (M1a/b)	2 (14.3)	1 (14.3)	
LN + Bone + visceral only (M1a/b/c)	1 (7.1)	1 (14.3)	
Best PSA response			
Partial responder (>50% fall in PSA)	2 (14.3)	4 (57.1)	0.094
Stable disease (<49% fall PSA)	6 (42.9)	2 (28.6)	
Progressive disease (any PSA rise)	6 (42.9)	1 (14.2)	
Blood-based parameters			
Haemoglobin	11.4 (8.8 -13.1)	11 (9.7 -13.5)	<0.001
ALP	462 (95 – 1451)	263 (112 -540)	<0.001
Albumin	45 (36 -51)	42 (36 -44)	0.26

Supplementary Table 1: Baseline characteristics of patients with tissue available for NGS characterisation by Foundation Medicine. Age between high/low LDH groups were compared using Student's *t* test; PSA level at initiation of docetaxel, haemoglobin, ALP and albumin was compared using the Wilcoxon rank-sum test; M stage and Best PSA response was compared using Chi-square test.

	Univariate analysis				Multivariate analysis			
	Progression free survival (PFS)		Overall Survival (OS)		Progression free survival (PFS)		Overall Survival (OS)	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
LDH (<450 vs. >451)	0.535 (0.366 – 0.781)	0.001	0.645 (0.451 – 0.921)	0.016	0.598 (0.401- 0.892)	0.012	0.776 (0.528- 1.140)	0.003
ALP	0.448 (0.305 – 0.698)	0.002	0.410 (0.304 – 0.684)	<0.0001	0.545 (0.389 - 0.833)	0.038	0.452 (0.303 - 0.691)	0.054
PSA at initiation of docetaxel	0.804 (0.565 – 1.145)	0.066	0.620 (0.422 – 0.938)	0.139	1.211 (0.856 - 1.701)	0.180	0.701 (0.501- 1.103)	0.201
Hb	0.825 (0.731- 0.932)	0.002	0.748 (0.656- 0.853)	0.001	0.895 (0.783-1.022)	0.895	0.806 (0.699- 0.930)	0.197
Age	0.989 (0.966 - 1.012)	0.342	0.995 (0.972 - 1.018)	0.654	0.996 (0.973-1.019)	0.996	1.001 (0.977 - 1.026)	0.932
PSA responder (Any fall vs. Any rise)	1.228 (0.874 - 1.727)	0.237	0.643 (0.456- 0.908)	0.012	0.827 (0.577-1.184)	0.299	0.675 (0.472 - 0.964)	0.30

Supplementary Table 2:

Supplementary Figure 2: Tumour Mutational Burden (TMB) and Copy Number Alteration (CNA) associated with patients with high and low LDH prior to docetaxel chemotherapy.

TMB (mean, 46.2 vs 6.2 mut/MB; Mann Whitney $p=0.1319$) and CNA (mean, 1.7% vs 0.7%; Mann Whitney $p=0.3747$) in tumours from patient with high LDH ($n=14$) and low LDH ($n=7$), dichotomised by 450U/L LDH.

Supplementary Figure 3: Distribution of alteration counts in samples by high/low LDH in the contemporary cohort. For the panel, somatic mutations in the targeted sequenced genes were taken into account. The samples were subdivided into high (blue bar chart) or low (red bar chart) LDH.

Supplementary Figure 4: Meta-analysis of the TCGA database shows a link between LDHA and DNA repair genes. Scatterplot analysis of normalised LDHA mRNA expression vs A) PARP1, B) BRCA1, C) BRCA2, D) CHEK2 mRNA expression. Expression shown as log2 whole transcript mRNA expression (Affymetrix Human Exon 1.0 ST arrays). Pearson correlation statistical analysis was used to compare the linear association of specific genes.

Common genes rearranged in cancer	Cancer related genes			
BRAF	ABL1	DDR2	KRAS	RAD51
ETV1	ABL2	DICER1	LRP1B	RB1
ETV4	AKT1	EIF4E	MAP3K1	RBM10
MLL	AKT1S1	EIF4EBP1	MAPKAP1	RHEB
TEMPRSS2	AKT3	EPHB1	MCL1	RICTOR
ERG	APC	ERBB3	MDM2	RNF43
	AR	ERBB4	MDM4	RPS6
	ARID1A	FANCL	MED12	RPS6KB2
	ASXL1	FGF12	MET	RUNX1T1
	ATM	FGF19	MLL2	SDHD
	ATR	FGF23	MLST8	SF3B1
	AXIN1	FGF3	MSH2	SLIT2
	BCL2L2	FGF4	MTOR	SMAD4
	BCL6	FGFR1	MUTYH	SOX2
	BCOR	FGFR3	MYC	SPEN
	BRCA2	FGFR4	NBN	SPOP
	BRIP1	FH	NF2	STAT3
	C11orf30	FLT1	PARK2	TBX3
	CARD11	FLT4	PIK3C2B	TERC
	CCND1	FOXO3	PIK3CA	TP53
	CDH1	FOXP1	PIK3CB	TSC1
	CDK12	GATA2	PIK3CG	TSC2
	CDK4	GSK3B	PIK3R1	TSHR
	CDK6	HGF	PREX2	U2AF1
	CDKN1B	HRAS	PRKCI	VEGFA
	CDKN2A	IL7R	PRKDC	VHL
	CHEK2	IRS2	PRR5L	WISP3
	CREBBP	JAK1	PTCH1	ZNF703
	CTNNB1	KDM6A	PTEN	
	CUL3	KLHL6	PTPN11	

Supplementary Table 3: Foundation Medicine ligation-based sequencing libraries representing 3167 exons of 118 cancer-related genes plus 18 introns of 6 genes often rearranged in cancer.

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The authors listed below have made substantial contributions to the intellectual content of the paper in the various sections described below.

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_ conception and design	Brown, Clarke, Elliott
_ acquisition of data	Hiew, Hart
_ analysis and interpretation of data	Hiew, Brown, Clarke, Ali, Hart
_ drafting of the manuscript	Hiew, Brown Clarke
_ critical revision of the manuscript for important intellectual content	Brown, Clarke
_ statistical analysis	Hiew, Brown, Ali
_ obtaining funding	Clarke
_ administrative, technical, or material support	Lau, Maddineni, Ramani, Sangar
_ supervision	Clarke, Brown
_ other (specify)	

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☒ None of the contributing authors have any conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript.

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- ☐ Preparation
- ☐ Review
- ☐ Approval of the manuscript

OR

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This corresponding author certifies that:

- all persons who have made substantial contributions to the work reported in this manuscript (eg, data collection, analysis, or writing or editing assistance) but who do not fulfill the authorship criteria are named with their specific contributions in an Acknowledgment in the manuscript.
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M.D. Brown

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Dear Professor Gratzke and Professor Catto

Thank you for giving us the opportunity to transfer this manuscript entitled “Primary mutational landscape linked with pre-docetaxel LDH levels predicts docetaxel response in mCRPC” to European Urology Focus. We are grateful to the reviewers for their comments following our original submission to European Urology. We have endeavoured to address the issues raised as follows:

Reviewer #1:

1. Has the study been approved by an ethical committee?

Response: The study was carried out with full ethical approval. We have added a statement to this effect in the main text at line 108

Reviewer #2:

1. The authors have not paid full attention to the Author instructions for European urology. For example, automatic page and line numbering have not been activated (these greatly facilitate the review process); double line spacing etc. Do please make a better effort to read the author instructions for manuscript preparation.

Response: We thank the reviewer for pointing this out. The format has been revised to reflect the author instructions

2. Abstract; results and limitations - it is and dated in the author instructions that limitations are also included in this section. Please do so

Response: We have stated that only 21 patients underwent NGS in this study in the main text (Line 206), 14 of which had LDH $\geq$ 450U/L within the abstract (Line 64-65) and that the NGS findings were cross correlated through meta-analysis of the MSKCC and SUCF/PCF databases

3. It is striking how few patients in the M0 dataset who underwent surgery (5%) as primary treatment, versus those who underwent RT (52%) and even primary ADT in the M0 setting (38%, odd management). Do the authors have any comments on this?

Response: We note the views of Reviewer#2 in this regard. However, the majority of the patients who were M0 at diagnosis in our training and test sets were risk-stratified as “high-risk” based on D’Amico classification. Radiotherapy combined with ADT is currently a standard curative treatment for patients with this disease type, as is reflected in the EAU Prostate Cancer Guidelines (Section

6.2.4). At the time of sampling most UK patients were treated in this way and indeed, in contemporary UK management of high risk localised disease most patients still receive radiotherapy and ADT as their standard of care (National Prostate Cancer Audit report 2017: www.npca.uk.com). As far as we are aware there is no level 1 evidence to show that treatment of high risk localised prostate cancer in this way is inferior to treatment with radical surgery.

Regarding the use of ADT monotherapy, evidence regarding benefit by combining this with local radiotherapy was not published until 2013 (Warde P et al J Clin Oncology 2013), predating our sample collection. The survival improvement from the combined therapy in that study was 8% and all of our patients in this analysis had failed treatment, developing metastases. We therefore feel that the differential treatment effect is likely to be negligible in the context of this study.

4. Were all primary biopsies done on prostate biopsy, or were some on metastases? Please comment

Response: All of the samples were taken from the primary prostate biopsies (Line 108-109).

5. Please explain how the training and test cohorts were selected. Please explain the rationale for this

Response: The training and test cohorts were dichotomised based on year of biopsy, pre and post 2006. As described in the manuscript the cohorts have broadly similar demographics and can therefore be described as matched.

6. Statistics - I defer to comments from statistical reviewer

Response: Statistical analysis was conducted by the lead and co-authors and all analyses were reviewed separately by a professional medical statistician (Hitesh Mistry): his contribution is highlighted in the acknowledgements.

7. The incidence of DRGs is extraordinarily high for what (I presume) is a primary biopsy cohort with no prior therapy. Please explain further; this is much higher than one would expect

Response: We thank the reviewer for this observation. However, we emphasise (Line 250) that this cohort of men were in late stage, with mCRPC and undergoing docetaxel therapy. They are therefore a selected group of patients with aggressive disease at the outset or with aggressive localised disease which had progressed with metastatic development. In this cohort type we would expect a higher incidence of DRGs in their primary biopsy. In support of this notion, the published literature shows that prostate cancer patients with DRGs reported ranges from 13% (TCGA) to 40% (SU2C/PCF), reflecting the stages of disease when the analysis was carried out. We feel that our patients, being late stage and all progressive would likely have a high rate of DRG disruption. In acknowledgment of this, we have added a line of explanation in the discussion section (Line 297-300).

8. Further, what was the incidence in the M0 vs M1 at presentation groups

Response: The incidence of M0 and M1 disease at presentation is presented in table 2. To further clarify this we have edited the table to highlight the number of patients within each cohort presenting with either M0 or M1 disease

Reviewer#3

The cohort should be considered a classical cohort (2005-2012), i.e. pre-abi/enza and the overall survival is short. This is a limitation with respect to the clinical implication of CRPC AD 2018! This should be made clear in the discussion.

Response: We thank the reviewer for this observation, with which we agree completely. We have added a rider in the patients / methods section to highlight this (Line 107) and we have further emphasised this fact with an explanatory line in the discussion (Line 297-300).

Overall, the paper is not well written;

It takes the reader (and the reviewer) a lot of effort to judge the significance of the results Eg Table 4 LDH cut of in training and tests sets; the sens and spec in the test cohort is much lower; when AUC's are compared, how are 95% CIs?

Genomic changes; TMB and CAN have a p-value > p.05, i.e. not significantly different.

Response: We thank the reviewer for this helpful observation and agree that both the TMB and CAN do not reach 95% confidence limit ($P < 0.05$). However, there is a trend towards an increase in both in mCRPC patients with high serum LDH levels. We have not overstated these finding in the manuscript. We would also plead in mitigation that a topic of this complexity can be “dense” and take time to read. However, we maintain that it is important to add in detail to facilitate understanding of the message conveyed. We hope that this does not detract from the main focus of the manuscript and the resulting discussion ie: that high LDH predicts poor response to docetaxel in the mCRPC setting and that mutations, particularly within the DNA repair pathway found within the *primary* biopsy of this associated phenotype.

Fig 1. AUCs; are these of the combined cohort? If so that is not justified, training and test should be plotted separately.

Response: We acknowledge that the AUC in figure 1 relates to the combined cohort (Line 163). However, the AUCs for both the training and test cohorts were supplied as figure 1 in the supplementary material. We have added words to the text to direct the reader (Line 169).

These are just a few of the unclarities in the paper; In this large cohort only few tumors were molecularly classified. Again a significant limitation of the paper

Response: We agree with reviewer 3 that a limitation of this study was the small numbers of patients with sufficient tissue available for genomic analysis. This is, in part, because of the prohibitively costly nature of undertaking genomic sequencing of this nature in such patient cohorts, and more importantly, the paucity of patient cohorts with matched primary biopsies internationally with follow up to death and fully annotated treatment outcome throughout their disease journey. To mitigate this fact and strengthen our hypothesis and conclusions undertook a meta-analysis of the larger TCGA (333 localised prostatectomies), SU2C/PCF (150 mCRPC biopsies) and MSKCC (181 localised prostatectomies) cohorts. These demonstrated that the link between genomic abnormalities within the DNA repair pathway, subsequent high LDH in the mCRPC setting and the poor response to docetaxel treatment. We feel this consistency with our primary biopsy results is sufficiently strong for us to draw our conclusions as set out in the manuscript.

We hope that our responses to the helpful comments and observations of the reviewers have been comprehensive and satisfactory. We thank you again for reconsidering this manuscript for publication in European Urology Focus.

Yours faithfully

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