1   **Dynamics of the cell-free DNA methylome of metastatic castration resistant prostate cancer**

2   **during androgen-targeting treatment**

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25

26   **Abstract:**

27   **Background:** Identifying treatment resistance in metastatic castration resistant prostate cancer

28   (mCRPC) prior to initiating therapy would be a major asset in the clinical management of this

29   highly aggressive and lethal form of the disease. As biopsy of metastatic lesions in this disease

30   state is often not feasible, circulating cell free DNA (cfDNA) is an emerging tool to detect

31   molecular drivers of resistance. While several studies have implicated the potential of mutation

32   detection in cfDNA, DNA methylation changes have also shown promise as diagnostic and

33   prognostic biomarkers in all stages of prostate cancer. Indeed, these tumor-derived DNA

34   methylation patterns are detectable in circulation. However, the dynamics of the methylome in

35   cfDNA has not been extensively studied, especially during treatment with current androgen-

36   targeting agents.

37   **Results:** In this study, we performed genome-wide methylation sequencing analysis of cfDNA

38   derived from mCRPC patients undergoing treatment with either Abiraterone acetate or

39   Enzalutamide. Analysis of sequentially collected plasma cfDNA samples was performed,

40   examining changes in the cfDNA methylome from prior to starting treatment (baseline), 12-

41   weeks during treatment and upon clinical progression. We developed a comprehensive analysis

42   pipeline to identify differentially methylated regions within each patient during treatment. We

43   examined the frequency of genome wide cfDNA methylation changes across all patients and

44   correlated these patterns with time to clinical progression. Overall, changes in well-established

45   prostate cancer methylation markers and alterations in key pathways, such as Wnt and neuronal

46   development, were detectable in cfDNA. Patients that maintained methylation changes from

47   baseline to week-12 and until progression tended to have a longer time to clinical progression

48   (TTP). Importantly, we observed that markers associated with a highly aggressive form of the

49   disease, Neuroendocrine-CRPC (NE-CRPC), could be detected prior to initiating treatment and

50   were associated with a faster TTP.

51   **Conclusions:** Treatment-related methylation changes associated with TTP highlights the

52   potential of monitoring cfDNA methylome during therapy in mCRPC. Our findings also suggest

53   that detection of NE-CRPC associated methylation signatures in earlier stages of treatment may

54   serve as predictive markers of response to androgen-targeting agents.

55   **Key words:**   metastatic castration resistant prostate cancer, circulating cell free DNA, DNA

56   methylation, epigenetic modifications, genome-wide methylation analysis

57   **__Background__**:

58   Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer-

59   related deaths among males (1, 2). Androgen deprivation therapy (ADT) to reduce systemic

60   androgen levels continues to be the major treatment for aggressive PCa (3). While ADT is

61   initially beneficial, treatment resistance leads to the most lethal form of PCa, metastatic

62   castration resistant prostate cancer (mCRPC) (4). Despite castrate levels of systemic androgens,

63   these tumors can continue to rely on the androgen receptor (AR) pathway to promote tumor

64   growth and metastasis (5). Indeed, androgen-targeting agents, Enzalutamide and Abiraterone

65   acetate, can improve overall and progression-free survival in both the pre- and post-

66   chemotherapy settings (6-9).

67

68 Enzalutamide is an antiandrogen that directly inhibits full-length AR. Abiraterone

69 inhibits CYP17A1, which catalyzes extragonadal androgen production (10, 11). Both treatments

70 perform similarly to suppress the androgen pathway, and choice of treatment is often dependent

71 on comorbidities (12). However, primary and eventual secondary resistance to Abiraterone or

72 Enzalutamide remains an ongoing challenge. As a result, the treatment landscape of mCRPC is

73 complex and continues to focus primarily on the order/sequencing of therapies. For instance,

74 recent trials demonstrated early treatment with Abiraterone or Enzalutamide in the hormone-

75 sensitive state could be beneficial (13-15). However, there is a diverse array of molecular drivers

76 contributing to disease heterogeneity amongst mCRPC patients (16), leading to variable response

77 to current treatment strategies. Therefore, reliable biomarkers are needed to facilitate optimal

78 therapy sequences for each patient prior to starting treatments.

79

80 Liquid biopsies are emerging as a minimally invasive source of biomarkers, reflecting

81 tumor turnover (17). This is particularly advantageous for mCRPC, as most patients have had

82 prior radical prostatectomy and bone metastases are often inaccessible. Numerous studies have

83 explored the potential of circulating tumor cell (CTC) or circulating nucleic acid markers in

84 mCRPC (18). Counting the number of CTCs and/or characterization of molecular alterations (i.e.

85 genomic and transcriptomic) are promising markers that capture tumor heterogeneity (19). For

86 instance, increased CTC number is associated with poor prognosis, and expression of the ligand-

87 independent AR-V7 splice variant by CTCs is associated with resistance to Enzalutamide and

88 Abiraterone (20-22). However, low CTC counts prior to first-line treatment and the need for

89 robust CTC surface markers for their optimal detection remain an ongoing challenge (23, 24).

90 Furthermore, some AR-V7 positive patients may still benefit from androgen therapies, while

4

91   certain AR-V7 negative patients show variability in treatment response (25, 26). In addition,

92   highly aggressive androgen-independent Neuroendocrine-CRPC (NE-CRPC) tumors express

93   lower levels of AR-V7 than Adenocarcinoma-CRPC patients (27). Therefore, additional markers

94   are needed to identify this subset of patients.

95

96   Circulating cell free DNA (cfDNA) can harbor tumor-specific somatic mutations and

97   capture tumor heterogeneity (28). There is a high concordance (>90%) of tumor mutations (i.e.

98   *AR, BRCA2*) between matched cfDNA and mCRPC biopsies (17). Although the proportion of

99   circulating tumor DNA (ctDNA) can vary from 1-2% to ~30% of cfDNA in mCRPC, the

100 sequencing throughput of massively parallel sequencing platforms can sample cfDNA with

101 sufficient coverage (29). For instance, targeted sequencing of *AR* in cfDNA can detect mutations

102 associated with resistance to Enzalutamide or Abiraterone (30, 31). However, genomic

103 aberrations are not the only contributors to molecular and phenotypic heterogeneity in mCRPC.

104 Aberrations in the epigenome are a hallmark of all stages of PCa, including DNA methylation

105 alterations (32, 33). Hypermethylation in promoter regions of several genes, such as *GSTP1*,

106 *APC*, *HOXD3* and *TBX15*, are currently being investigated as diagnostic and prognostic markers

107 in earlier stages of PCa (33, 34). Furthermore, in biopsy tissue from metastatic lesions, distinct

108 DNA methylation patterns were observed between NE-CRPC and Adenocarcinoma-CRPC (27).

109

110 CpG methylation can be detected from DNA extracted from tissue and various biofluids

111 (35-37). Presence of promoter methylation in *GSTP1* and *APC* in cfDNA from mCRPC patients

112 is prognostic of overall survival (35, 38). Using an array-based platform, differential cfDNA

113 methylation patterns were observed between Abiraterone responsive versus resistant patients

114   (36). While these observations are promising, extensive genome wide analysis of the mCRPC

115   cfDNA methylome during Enzalutamide or Abiraterone treatment has not been performed.

116   Specifically, the changes/dynamics in the cfDNA methylome at various stages of treatment could

117   help further delineate and predict response to current mCRPC treatments. Recently, genome-

118   wide analysis using cell-free methylated DNA immunoprecipitation and high-throughput

119   sequencing (cfMeDIP-seq) revealed tumor-specific methylation changes in cfDNA of patients

120   with various cancers (39).

121

122   To identify circulating DNA methylation changes associated with response to either

123   Enzalutamide or Abiraterone treatment, we monitored sequentially collected cfDNA samples

124   from mCRPC patients, starting from prior to treatment initiation to eventual clinical progression,

125   and performed comprehensive methylome assessment /analyses. We identified changes in key

126   genes known to have altered methylation state in PCa. Pathway analysis of cfDNA methylome

127   changes also reflected alterations in several oncogenic pathways, such as Wnt and neuronal

128   development related genes. Importantly, we observed that methylation patterns in genes

129   associated with NE-CRPC were correlated with time to clinical progression.

130

131   **Results:**

132   **Study design and patient cohort**

133   Tumor methylation signatures can be identified through comparison of cancer patients'

134   cfDNA with either matched tumor tissue or healthy control cfDNA (39). In the case of mCRPC,

135   biopsy of metastatic lesions in bone is not routinely feasible, and there is considerable molecular

136   heterogeneity in this clinical state. Therefore, to identify methylation markers associated with

6

137   response to therapy, we opted for within/intra-patient differential methylation analysis. We

138   hypothesized that sequential collection of cfDNA from visit A (baseline/pre-treatment) to visit B

139   (week-12) to visit C (clinical progression) would reveal changes in the cfDNA methylome that

140   reflect tumor responses, with each patient serving as their own internal control. As outlined in

141   **Figure 1**, intra-patient comparison could potentially detect changes in abundance of methylated

142   DNA fragments related to tumor response. For instance, loss of methylated fragments may

143   reflect loss/reduction of tumor cells sensitive to treatment. In contrast, gains in methylated

144   fragments could be associated with gains in treatment resistant tumor cells, since changes in

145   methylome could occur due to non-tumor/treatment related changes. Therefore, our analysis

146   entailed: (1) identification of intra-patient differentially methylated regions (**DMRs**) and (2)

147   finding common genes/pathways with differential methylation, and establishing the frequency of

148   these changes across all patients in this study.

149

150   We prospectively collected plasma from mCRPC patients receiving either Enzalutamide

151   (n=12) or Abiraterone acetate (n=4) treatment. In total, 45 blood samples were collected and

152   summarized in **Figure 2a**. Two Enzalutamide-treated patients were unable to provide Visit C

153   samples (P2 and P26) and one Abiraterone-treated patient progressed prior to Visit B (P36). The

154   majority of patients (14/16) presented with bone metastatic lesions upon mCRPC diagnosis, with

155   a subset showing lymph node and soft tissue metastases (**Figure 2b**). Twelve patients

156   demonstrated an initial favorable prostate-specific antigen (PSA) response ($\geq$ 50% decline from

157   baseline, **Figure 2c**), and most patients exhibited maximal PSA response (favorable or plateau)

158   around Visit B (**Supplementary Figure 1**). Overall time to clinical progression (**TTP**) varied for

159 each patient, ranging from rapid/primary resistance to therapy to treatment-driven resistance

160 following initial favorable response (**Figure 2d**).

161

162 **Performance of cfMeDIP-seq strategy with PCa cell line DNA and cfDNA samples**

163 We utilized an established cfMeDIP-seq approach, which was developed for low amounts

164 of cfDNA and capable of ctDNA fractions as low as 0.001% (39). This technique was able to

165 distinguish methylation patterns between various cancer types, including pancreatic, breast and

166 colorectal cancer (39). Briefly, methylated cfDNA is enriched using an antibody specific for

167 methylated CpG sites, followed by amplification of adapter-ligated libraries. For each sample,

168 10% of the DNA was reserved as an input control (without immunoprecipitation).

169

170 To benchmark this methodology for PCa samples, we generated a control from genomic

171 DNA derived from PCa cell line, 22Rv1, sheared to the same size as cfDNA (**Supplementary**

172 **Figure 2**). As an additional control, we spiked all samples with methylated and unmethylated

173 *Arabidopsis thaliana* (AT) DNA, and confirmed increased recovery of methylated AT DNA

174 (~80%) compared to unmethylated DNA (<0.06%) (**Supplementary Figure 2a**). Spiked AT

175 DNA was added following sequencing adapter ligation to cfDNA and not detectable during

176 sequencing. Increased enrichment of known methylated genes/regions in the genome of 22Rv1

177 cells, (40) including the *CDKN2A* gene body and the first exon of *TGFB2,* compared to the

178 unmethylated promoter of *HOXD8* was confirmed (**Supplementary Figure 2b**).

179

180 We performed cfMeDIP-seq on all 45 samples collected. The average starting amount of

181 dsDNA was 27 ng, and ranged from 9 to 50 ng, with no significant differences in cfDNA

182   amounts across visits and treatments (**Supplementary Figure 2c**). Following alignment and base

183   quality filtering, 48-62% of the cfMeDIP reads and 80-85% of input control reads remained for

184   DMR analysis (**Supplementary Figure 2d-e**). A majority of cfMeDIP-seq sequence reads

185   aligned within gene bodies (major transcript starting from transcriptional start site/TSS to

186   transcription termination site/TTS) and intergenic regions, as well as CpG Island (CGI), CGI

187   shores, and TSS/promoter regions (TSS +/-1.5Kb), together representing the entire genome

188   (**Supplementary Figure 2f**). As expected, enrichment of methylated cfDNA in the MeDIP

189   fraction over input control fraction was observed, especially within CpG rich regions. Similar

190   distribution of regions was observed between Enzalutamide and Abiraterone treated patients

191   (**Supplementary Figure 2 g-h**).

192

193   **Intra-patient differential methylation analysis**

194   To identify treatment-related differentially methylated regions (DMRs), we developed a

195   DMR calling strategy for intra-patient analysis termed "cfDNA DMRHunter" (See

196   **Supplemental methods** and **Supplementary Figure 3**). As location-specific cfDNA recovery

197   sensitivity is unknown *a priori*, we used input control libraries to ensure uniform cfDNA read

198   coverage across the whole genome. DMRHunter also uses the input controls to remove

199   alignment artifacts from analysis. After filtering low quality and PCR duplicated reads,

200   DMRHunter builds a background model and identifies differentially methylated CpGs (DMCs)

201   across the genome. Adjacent DMCs with the same differential methylation state (UP or DOWN)

202   were merged to identify DMRs with stringent z-score (>1.5), average read count ($\geq$10) and false

203   discovery rate (FDR = 0.01) cut-offs. There were a large number of DMRs across all

204   comparisons and treatments, ranging from 93 000 to 154 000 DMRs (**Figure 3a**) with no

205 significant difference in median number of DMRs between comparisons and treatment (**Figure**

206 **3b-c**). The overall median DMR size was 74 bp, ranging from 2bp (single CpG sites) up to 4163

207 bp.

208

209 We next examined the directionality of these changes across comparisons.

210 Conventionally, hypermethylation or hypomethylation is the typical terminology for methylation

211 changes. However, for cfDNA, it is unclear whether these changes reflect a gain/loss of

212 methylated CpGs at these specific genomic sites or changes in DNA fragment abundance,

213 reflecting an altered cellular abundance contributing to the cfDNA pool. For our analysis we

214 opted to define these DMRs as gains (UP) or loss (DOWN) in methylated fragments. There was

215 no significant difference in the overall proportion of regions with increased or decreased

216 methylation in all visit comparisons (**Figure 3d-f**) and between the treatment types for B vs A

217 and C vs B (**Supplementary Figure 4a-d**). However, Enzalutamide-treated patients showed

218 gains in the overall proportion of methylated cfDNA fragments in visit C compared to A

219 (**Supplementary Figure 4e-f**). The majority of DMRs were found within gene bodies (protein

220 coding and pseudogenes) and intergenic regions (**Figure 3g**). DMRs near/within transcription

221 start sites (TSS)/promoters and CpG Islands (CGI), as well as non-coding RNA (ncRNA), were

222 found, but with lower abundance. We chose to focus on DMRs within promoter and gene body

223 regions, as CpG methylation changes in these locations are known to be associated with gene

224 expression changes (41).

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226

227

Methylation changes within promoter and gene body regions

The majority of DMRs within promoters (TSS +/- 1.5 Kb) were associated with protein coding (PC) genes, followed by long non-coding RNA (lncRNA), pseudogenes (Pseudo) and short non-coding RNA (sncRNA) regions (**Supplementary Figure 5a**). We observed minimal overlap among promoters of different gene/ncRNA categories, and overall distribution of DMRs in these regions did not vary between patients and visits (**Supplementary Figure 5b**). For all patients combined, the distribution of methylation changes within promoters (UP vs DOWN) did not vary significantly across most regions, with the exception of a slight increase in overall methylation in pseudogenes for C vs A (**Supplementary Figure 5c**). We focused on protein coding genes with at least one promoter DMR. Analyzing recurrence of these genes, we found that many of these genes were found in less than 5 patients for each comparison (**Supplementary Figure 5d-f**). We examined the most common protein coding genes (with DMRs in $\geq$ 5 patients) and found many genes overlapping across visit comparisons (**Figure 3h**), with the majority shared between Abiraterone and Enzalutamide groups. The most common genes showing either increases or decreases in promoter methylation are in **Supplementary Figure 6**, with certain genes implicated in PCa or other cancers. For instance, pyrroline-5-carboxylate reductase 1 (*PYCR1*), which is involved in proline biosynthesis and can promote tumor cell growth (42), often demonstrated gain of methylation at visit C.

We next performed pathway enrichment analysis for gene sets, which showed increased or decreased methylation in promoters during treatment (**Supplementary Figure 7 and 8 respectively**). Variability in the types and frequency of pathways identified was observed across patients; however, cancer-related pathways such as Wnt and PI3K signaling were observed, with

11

251   neuronal pathways being the most frequent. Several promoter associated DMRs were only found

252   among Enzalutamide-treated patients (**Figure 3h and Supplementary table 2)**. The most

253   frequently (6-7 patients, 20 comparisons) differentially methylated promoter region within the

254   Enzalutamide cohort was *FGFR1*, which is overexpressed in PCa and implicated in metastasis

255   (43).

256

257   We also examined the distribution of DMRs within gene bodies/known transcripts. The

258   majority of these DMRs were associated with protein coding gene bodies across

259   (**Supplementary Figure 9a-b**). Overall changes in methylation levels (UP vs DOWN) for these

260   key regions did not change across comparisons (**Supplementary Figure 9c**). In contrast to

261   promoter DMRs, many genes with body DMRs were shared across patients (**Supplementary**

262   **Figure 9d-f**). Similarly, we focused on commonly altered gene bodies and found extensive

263   overlap between visit comparisons and treatments (**Figure 3i**). We further examined net

264   methylation change across these genes by scoring the ratio of increased to decreased methylation

265   within each gene body, excluding genes that had no net change (i.e. equal number of UP and

266   DOWN trends). We found 91 genes that were commonly altered across all comparisons

267   (**Supplementary Figure 10**). In most cases, regardless of visit comparison, the methylation

268   pattern for these genes clustered closely for the same patients. One such group of genes with

269   increases in methylation at Visit C included *NFATC1*, which can promote tumorigenesis (44),

270   and a histone deacetylase, *HDAC4*. While several Enzalutamide-specific genes were found

271   (**Supplementary table 3**), common pathways including several differentiation-related and

272   neuronal pathways were found among gene-body associated DMRs (**Supplementary Figures 11**

273   **& 12**),

PCa associated methylation changes in cfDNA

274

275   Aberrations in the DNA methylome is a hallmark of both androgen dependent PCa and

276   mCRPC. Genomic DNA extracted from primary PCa tumors can harbor recurrent

277   hypermethylation in gene promoters, including *GSTP1*, *TBX15*, *AOX1* and members of the HOX

278   family of transcription factors (33). We assessed the overall promoter methylation status of

279   genes that have been established as differentially methylated in PCa (**Figure 4a**). Among the

280   most common differentially methylated gene promoters were genes involved in tumorigenic

281   processes, such as *RUNX3*, *RGS12* and *FBP1*, with several differentially expressed in NE-CRPC

282   (27), including the neuronal transcription factor *PHOX2A* (45) and a regulator of apoptosis,

283   *CTBP2* (46). Similarly, several differentially methylated and cancer-related genes were found

284   (**Supplementary Figure 13**), such as a master regulator of AR and driver of NE-CRPC,

285   *ONECUT2* (47).

286

287   DMRs in genes associated with diagnosis and prognosis could reflect tumor cell response

288   to treatments. While differential promoter methylation events in the well-established PCa-

289   specific hypermethylated gene, *GSTP1*, were infrequent (**Figure 4b**), decreased gene body

290   methylation coinciding with treatment response (i.e. PSA reduction) was shown in some patients

291   (i.e. P3). Similar methylation regions were also observed in the PCa cell line LNCaP (48).

292   Similarly, changes in *TBX15* methylation (1st intron) were associated with response to treatment

293   (i.e. PSA) in certain patients (**Figure 4c**). While promising, there were no consistent DMRs in

294   these genes that could classify patients by androgen-targeting treatment response. A caveat is

295   that many of these methylated genes were reported in earlier stages of PCa and may not reflect

296   the changes in mCRPC.

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297

298   To find potential "treatment sensitive" CpG sites in the genome, we next examined

299   differentially methylated CpG sites (DMCs) among patients that completed all study visits,

300   specifically CpG sites that fluctuated in ≥2 visit comparisons. DMCs were analyzed as DMR size

301   for similar genomic regions varied within and across patients. We stratified the proportion of

302   DMCs that were uniquely found in single visit comparisons and those shared across all visits

303   (**Figure 5a**). We identified DMCs present in two or more visit comparisons for each patient and

304   associated them with gene promoters and bodies. Analysis of gene promoters containing DMCs

305   revealed minimal overlap amongst patients, with P4, P31, P38 and P3 showing the highest

306   overlap of 8 shared genes: *NPBWR1*, *ZSCAN12*, *PCDHGA11*, *PHOX2A*, *TBX10*, *TEX28*, *TKTL1*

307   and *TSPAN32* (**Supplementary Figure 14a**). In contrast, we observed a high degree of overlap

308   amongst patients when looking at DMCs within gene bodies, with the exception of P3

309   (**Supplementary Figure 14b**). Interestingly, we also noted a positive correlation between the

310   proportion of DMCs shared across all visits and TTP (**Figure 5a**).

311

312   We examined 3 major combined scenarios observed among 14 total: (1) scenarios 7 and

313   8, increase/decrease in methylation in visit C vs B or A (**Figure 5a**); (2) scenarios 9 and 10,

314   increase/decrease in B and C vs A; (3) those that go up or down in B vs A and then return to

315   same levels in C (13 &14). The proportion of these three combined scenarios was correlated with

316   time to progression (TTP), and we found that those patients with more DMCs that

317   increased/decreased at visit B and maintained at visit C (scenarios 9 and 10) had a longer TTP

318   (**Figure 5b**). Although scenarios 7 & 8 did not correlate with TTP, those patients with a higher

319   proportion of scenarios 13 & 14 had a possible trend towards faster TTP (**Supplementary figure**

14

320   **14 c-d**). This suggests that genome-wide cfDNA methylation dynamics can reflect response to

321   treatment in mCRPC patients.

322

323   **Neuroendocrine-CRPC related cfDNA DMRs**

324   As many of the DMRs were associated with genes implicated in several neuronal

325   pathways and differentiation, we integrated cfDNA DMRs with those identified from biopsy

326   samples from NE-CRPC versus Adenocarcinoma-CRPC in an independent mCRPC patient

327   series (27). While this published dataset utilized reduced representation bisulfite sequencing

328   (RRBS), which primarily covers CpG rich regions (i.e. CGIs), several of these DMCs were

329   found within cfDNA DMRs (29 355 CpG sites out of 84 930 in NE-CRPC). In order to assess

330   whether changes in these regions at earlier time points are associated with overall response to

331   androgen-targeting agents, we examined the overlap between DMRs from the A vs B

332   comparison and NE-associated DMRs. As P36 progressed prior to visit B (**Figure 2d**), we

333   included this patient in the analysis (examining A vs C). In this case, we examined the changes

334   in methylation from the perspective of visit A. That is, increased methylation in visit A vs B/C

335   (UP in A) or decreased methylation in visit A (DOWN in A). We proposed that the abundance of

336   methylated cfDNA fragments from certain regions may reflect key genes/regions to monitor

337   prior to initiating therapy. The overall proportion of these DMRs that were increased or

338   decreased in methylation at A vs B (or C for ID 36) varied across the patients (**Figure 6a**).

339   Interestingly, we noted that these DMRs (A vs B) appeared to be related to TTP. We examined

340   the ratio of DMRs containing NE-CRPC associated CpG sites with more methylated fragments

341   in Visit A to those with less methylation. In order to further delineate key regions, we applied

342   various FDR thresholds to these DMRs to examine the effect on correlation with TTP (**Figure

15

343   **6b**). We found an optimal set of regions that were negatively correlated with TTP: that is, the

344   more methylated cfDNA fragments containing these NE-CRPC associated CpGs, the

345   faster/shorter the TTP (**Figure 6c**). To determine whether the amount of NE-CRPC related

346   methylation patterns at visit A was associated with a faster TTP, we performed ROC analysis for

347   various TTP cut-offs (ranging from ≤20 to 35 weeks). We found that an increased ratio of NE-

348   CRPC DMRs UP in visit A can stratify patients that progressed as early as 25-weeks (area under

349   the curve, AUC: 0.927, CI: 0.773-1) (**Figure 6d**). These sites may serve as potential predictive

350   markers of resistance to androgen-targeting agents.

351

352   **Discussion**

353   In this study, we showed that methylome analysis of the cfDNA from mCRPC patients

354   can capture treatment-related epigenetic alterations. We found changes in well-established PCa

355   methylation markers associated with response to treatment. Importantly, we demonstrated that

356   cfDNA contains NE-CRPC related methylation signals, which have the potential to be utilized as

357   predictive markers of treatment response. Methylation of several oncogenic driving pathways

358   were detected in cfDNA across time points, suggesting that methylome analysis of patients'

359   liquid biopsies can serve as a monitoring tool during treatment, as well as highlight potential

360   targets for future therapies.

361

362   A key advantage of liquid biopsy-based versus tissue-biopsy approaches is the ability to

363   capture tumor heterogeneity. To date, much focus has been placed on genomic alterations, which

364   can help identify *AR* mutations related to resistance to Abiraterone/Enzalutamide (30, 31).

365   However, few studies have highlighted that cfDNA methylation markers may help further

16

366   stratify patients (35, 36). In a recent study of 600 mCRPC patients receiving docetaxel, increased

367   methylated *GSTP1* at baseline was associated with longer overall survival, and loss of

368   methylation after two cycles of treatment corresponded with longer time to PSA progression

369   (49). Furthermore, patients that exhibited loss in methylated *GSTP1* after two cycles of treatment

370   had better overall survival. In this study, we observed some cases in which there were losses of

371   *GSTP1* methylation in certain treatment responsive patients (PSA reduction). We also examined

372   other tissue-based methylation markers (promoter and gene body), including *TBX15* and *RUNX3*

373   (33), and similar heterogeneity amongst patients was observed. This in part could be explained

374   by differing mechanisms between docetaxel and androgen-targeting agents. Due to intra- and

375   inter-tumor heterogeneity (50, 51), androgen targeting agents may not target all androgen-

376   independent tumor cells which could also harbor these methylation markers. Furthermore, most

377   well-studied methylation markers were derived from earlier stages of PCa, whereas further

378   epigenomic instability is known to occur with the mCRPC state (52).

379

380   A unique strength of our study is the sequential analysis using a genome-wide approach,

381   which highlights progressive changes in genes involved in tumorigenic processes in PCa and

382   other cancers. Commonly altered genes in the promoter region included *PYCR1* and *SHARPIN,*

383   which are known to be upregulated in PCa tissue and involved in processes such as proliferation

384   and angiogenesis (53). Gene body methylation was observed in *NFATC1, HDAC4, PHOX2A,*

385   *CTBP2*, and master regulator of NE-CRPC, *ONECUT2* (44-47). Pathway analysis also further

386   highlighted key mechanisms that have been established in prostate tumor tissue studies,

387   including the wnt pathway (16). Interestingly, the methylation patterns amongst these genes

388   demonstrated extensive variability across patients and visits. In order to further delineate key

17

389   regions, we further assessed common regions that changed during treatment. These changes

390   appeared to reflect dynamics of treatment response. For instance, patients that demonstrated CpG

391   changes (up or down) in visit B and sustained the changes by visit C appeared to have a longer

392   TTP.   In contrast, there was a trend towards shorter TTP amongst patients that did not

393   demonstrate sustained methylation changes. Among the genes included was Neuropeptides B/W

394   receptor 1 (*NPBWR1*), which was shown to be upregulated in basal cells from prostate tissue

395   (54), thus highlighting that genome-wide cfDNA methylation analysis is able to capture tumor

396   dynamics and may further have additive potential to mutation analysis.

397

398   We also observed that many of the differentially methylated genes overlapped between

399   Enzalutamide- and Abiraterone-treated patients. Although there are ongoing studies to compare

400   efficacy of either treatment (55), the current consensus is that both perform similarly, and

401   deciding between treatments in a patient-specific manner remains an ongoing challenge. We

402   identified sets of genes altered in the promoter and gene body locations, including one large set

403   amongst Enzalutamide treated patients. We were limited by the number of Abiraterone-treated

404   patients, which may have limited heterogeneity, as seen in the larger Enzalutamide cohort.

405   Further studies are needed to examine if the methylome is different between these treatment

406   types. However, similar pathways were observed between the treatments, with neuronal

407   pathways being the most prominent.

408

409   Given these neuronal-related genes/pathways, we wanted to examine the utility of these

410   methylation changes in helping to predict treatment outcome, especially in identifying

411   Neuroendocrine-CRPC. With our unique study design of sequential sampling, we could detect

18

412   these methylation trends at earlier time points, demonstrating that the proportion of

413   Neuroendocrine-like DMRs correlated with faster/shorter time to progression. In particular, the

414   higher the proportion of certain DMRs with these NE-CRPC associated methylation patterns, the

415   faster the time to progression. While we did not have confirmatory biopsies or other markers of

416   NE disease (i.e. chromogranin A), increased methylation at Visit A could be utilized as

417   predictive markers of response to androgen-targeting agents. Although we are currently limited

418   to a single cohort, we now have a set of candidate regions that could be validated in additional

419   cohorts.

420

421   **Conclusions**

422   Our genome-wide sequencing protocol and analysis strategy has demonstrated the utility

423   of intra-patient monitoring of cfDNA methylation. If changes in the methylome during treatment

424   were sustained until clinical progression, these patients appeared to have a better prognosis with

425   androgen-targeting treatment. Interestingly, methylation patterns associated with a highly

426   aggressive form of the disease may serve as potential predictive markers. Further independent

427   validation of these markers is needed to assess this predictive potential.

428

429   **Methods**

430   **Patient cohort**

431   All patients in this study were recruited from the Princess Margaret Cancer Centre

432   Genitourinary Clinic, and informed written consent was obtained in accordance with approved

433   institutional Research Ethics Board protocols from University Health Network (UHN) and Sinai

434   Health System (SHS). The UHN Genitourinary Biobank performed patient recruitment, blood

435   collection and clinical follow-up. All patients were chemotherapy naïve and developed resistance

436   following initial PCa treatments (local therapies and ADT) (**Supplementary Table 1**). A total of

437   12 Enzalutamide-treated (160 mg daily) and 4 Abiraterone-treated patients (1000 mg daily +

438   Prednisone/Dexamethasone) completed study visits for cfDNA methylome analysis. Blood was

439   collected at three time points: prior to starting treatment (**Visit A**), at 12-weeks during treatment

440   (**Visit B**) and upon clinical progression/treatment change (**Visit C**). While we aimed to collect

441   Visit B around week-12 (+/- 2 weeks), average time from baseline for Visit B was 12.7 weeks

442   (ranging 9 - 17 weeks). Serum PSA levels were obtained throughout treatment, and the lowest

443   PSA level (nadir) was used to determine PSA progression. Clinical progression was determined

444   by several factors, including radiological evidence of additional/worsening metastases and

445   symptomatic changes as assessed by the treating physician.

446

447   **Blood sample processing and cfDNA isolation**

448   Blood was collected at each time point in citrate cell preparation tubes (BD Biosciences,

449   San Jose, CA, USA) and samples were processed within 2 hours from collection. Plasma was

450   separated from peripheral blood mononuclear cells (PBMCs) by centrifugation at 1800 x g for 20

451   minutes. Isolated plasma was divided into 1 mL aliquots and stored at -80°C. Prior to cfDNA

452   isolation, frozen plasma samples were rapidly thawed at 37°C and spun at 16 000 x g for 5

453   minutes. For each visit, we utilized 4 mL of plasma and cfDNA was isolated using the QIAamp

454   Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). The concentration of cfDNA was

455   determined using the Qubit dsDNA High Sensitivity Assay Kit (ThermoFisher Scientific,

456   Waltham, MA, USA) and cfDNA fragment distibrution for subset of samples was obtained using

457   the Agilent 2100 Bioanalyzer System. To avoid within-patient batch effects, we isolated plasma

458   cfDNA and performed methylation analysis for all visits from each patient together.

459

460   **cfMeDIP-seq protocol**

461   The cfMeDIP-seq protocol utilized in this study was adapted from a previously published

462   protocol optimized for cfDNA (39). Isolated cfDNA was first end-repaired and A-tailed using

463   the KAPA HyperPrep kit (Roche, Mannheim, Germany), followed by adapter ligation with index

464   adapters (Integrated DNA technologies). Each adapter-ligated cfDNA sample was then spiked

465   with λ phage DNA (ThermoFisher Scientific) as filler DNA, as well as methylated and

466   unmethylated control *Arabidopsis thaliana* (AT) DNA (Diagenode, Denville, NJ, USA) to bring

467   the total DNA amount to 100ng. Prior to immunoprecipitation, 10% of this DNA is saved as

468   input control. The Diagenode MagMeDIP and IPure v2 kits were utilized to enrich and purify

469   methylated cfDNA. Prior to library amplification, qPCR quality checks were performed to

470   ensure: (1) high specificity (>95%) for enrichment of methylated AT DNA over unmethylated

471   AT DNA, and (2) appropriate adapter-ligation. This was followed by adapter-specific

472   amplification of each MeDIP and input control library and gel size selection. All sequencing was

473   performed on the Illumina HiSeq 2500 platform (50-bp single end reads).

474

475   **Sequencing data pre-processing and differential methylation analysis**

476   Raw sequencing data was aligned to hg19 reference genome using Burrows-Wheeler

477   alignment (BWA version 0.7.6a, using 'mem' option with default settings, except using –M

478   flag). Duplicated reads were marked by Picard tools (https://broadinstitute.github.io/picard/) and

479   collapsed to allow only one copy of the reads from reads that have the same alignment position.

480   Reads mapped to the multiple locations were removed by applying alignment quality threshold

481   (QA>5). As the sequencing data was single-end, the average DNA fragment length was

482   evaluated for both cfMeDIP and input controls using self-correlation technique (56). For each

483   library, the fragment coverage genomic profile was calculated using directional extension of all

484   aligned reads with estimated average fragment length (175bp in our case) using an in-house

485   BAM2WIG custom tool (M. Bilenky, unpublished, see http://www.epigenomes.ca/tools-and-

486   software). Within-patient differential methylation analysis for all comparisons was performed

487   using DMRHunter pipeline developed for cfDNA samples (**Supplemental Methods Section**).

488

489   **Statistical analysis of mCRPC-associated DMRs**

490   Differences in regions with overall increase (UP) versus decrease (DOWN) in

491   methylation across all comparisons were analyzed using the Wilcoxon rank-sum test as part of

492   the base package of R (v3.5.3). Spearman correlation analysis was performed using similar base

493   packages. Receiver operating characteristic (ROC) curve analysis was performed using the

494   pROC package (1.14.0). All data was visualized using the ggplots2 package (v 3.1) and

495   heatmaps from the package ComplexHeatmap (1.2). Pathway analysis is described in

496   **Supplemental Methods Section**.

497

498   **<u>List of abbreviations</u>**

499   Androgen deprivation therapy (ADT)

500   Androgen receptor (AR)

501   Circulating cell free DNA (cfDNA)

502   Cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP-seq)

22

503   Differentially methylated CpG sites (DMCs)

504   Differentially methylated regions (DMRs)

505   Metastatic castration resistant prostate cancer (mCRPC)

506   Neuroendocrine-CRPC (NE-CRPC)

507   Prostate cancer (PCa)

508   Prostate-specific antigen (PSA)

509   Time to progression (TTP)

510

511   **Declarations**

512   **Ethics approval and consent to participate:** All patients in this study provided informed

513   written consent in accordance with approved institutional Research Ethics Board protocols from

514   University Health Network (UHN) and Sinai Health System (SHS). Progress updates were

515   submitted and approved annually by respective Research Ethics Boards. The study was

516   performed in accordance with the Declaration of Helsinki.

517

518   **Consent for publication:**   No personal information for any patient is presented in this

519   manuscript. According to REB approvals, each patient was assigned unique study identifiers,

520   which are not linked to personal health information.

521

522   **Availability of data and materials:** All FASTQ and QC files will be made available in a public

523   repository upon publication of this manuscript. Additional supplementary data files have also

524   been included.

525

549   **References**

550   1.   Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, and Jemal A. Global cancer

551   statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in

552   185 countries. CA Cancer J Clin. 2018;68(6):394-424.

553   2.   Global Burden of Disease Cancer C, Fitzmaurice C, Allen C, Barber RM, Barregard L,

554   Bhutta ZA, Brenner H, Dicker DJ, Chimed-Orchir O, Dandona R, et al. Global, Regional, and

555   National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and

556   Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015: A Systematic Analysis for

557   the Global Burden of Disease Study. JAMA Oncology. 2017;3(4):524-48.

558   3.   Wong YN, Ferraldeschi R, Attard G, and de Bono J. Evolution of androgen receptor

559   targeted therapy for advanced prostate cancer. Nat Rev Clin Oncol. 2014;11(6):365-76.

560   4.   Sridhar SS, Freedland SJ, Gleave ME, Higano C, Mulders P, Parker C, Sartor O, and

561   Saad F. Castration-resistant prostate cancer: from new pathophysiology to new treatment. Eur

562   Urol. 2014;65(2):289-99.

563   5.   Crawford ED, Petrylak D, and Sartor O. Navigating the evolving therapeutic landscape in

564   advanced prostate cancer. Urol Oncol. 2017;35S(S1-S13.

565   6.   Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, de Wit R, Mulders P,

566   Chi KN, Shore ND, et al. Increased survival with enzalutamide in prostate cancer after

567   chemotherapy. N Engl J Med. 2012;367(13):1187-97.

568   7.   Loriot Y, Miller K, Sternberg CN, Fizazi K, De Bono JS, Chowdhury S, Higano CS,

569   Noonberg S, Holmstrom S, Mansbach H, et al. Effect of enzalutamide on health-related quality

570   of life, pain, and skeletal-related events in asymptomatic and minimally symptomatic,

571   chemotherapy-naive patients with metastatic castration-resistant prostate cancer (PREVAIL):

572   results from a randomised, phase 3 trial. Lancet Oncol. 2015;16(5):509-21.

573   8.   de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones RJ,

574   Goodman OB, Jr., Saad F, et al. Abiraterone and increased survival in metastatic prostate cancer.

575   N Engl J Med. 2011;364(21):1995-2005.

576   9.   Ryan CJ, Smith MR, Fizazi K, Saad F, Mulders PF, Sternberg CN, Miller K, Logothetis

577   CJ, Shore ND, Small EJ, et al. Abiraterone acetate plus prednisone versus placebo plus

578   prednisone in chemotherapy-naive men with metastatic castration-resistant prostate cancer

579   (COU-AA-302): final overall survival analysis of a randomised, double-blind, placebo-

580   controlled phase 3 study. Lancet Oncol. 2015;16(2):152-60.

581   10.   Bambury RM, and Scher HI. Enzalutamide: The story of its development from bench to

582   bedside. Urol Oncol. 2015.

583   11.   Grist E, de Bono JS, and Attard G. Targeting extra-gonadal androgens in castration-

584   resistant prostate cancer. J Steroid Biochem Mol Biol. 2015;145(157-63.

585   12.   Sumanasuriya S, and De Bono J. Treatment of Advanced Prostate Cancer-A Review of

586   Current Therapies and Future Promise. Cold Spring Harb Perspect Med. 2018;8(6).

587   13.   Fizazi K, Tran N, Fein L, Matsubara N, Rodriguez-Antolin A, Alekseev BY, Ozguroglu

588   M, Ye D, Feyerabend S, Protheroe A, et al. Abiraterone plus Prednisone in Metastatic,

589   Castration-Sensitive Prostate Cancer. N Engl J Med. 2017;377(4):352-60.

590   14.   James ND, de Bono JS, Spears MR, Clarke NW, Mason MD, Dearnaley DP, Ritchie

591   AWS, Amos CL, Gilson C, Jones RJ, et al. Abiraterone for Prostate Cancer Not Previously

592   Treated with Hormone Therapy. N Engl J Med. 2017;377(4):338-51.

593   15.   Armstrong AJ, Szmulewitz RZ, Petrylak DP, Holzbeierlein J, Villers A, Azad A, Alcaraz

594   A, Alekseev B, Iguchi T, Shore ND, et al. ARCHES: A Randomized, Phase III Study of

595   Androgen Deprivation Therapy With Enzalutamide or Placebo in Men With Metastatic

596   Hormone-Sensitive Prostate Cancer. J Clin Oncol. 2019:JCO1900799.

597   16.   Robinson D, Van Allen EM, Wu YM, Schultz N, Lonigro RJ, Mosquera JM,

598   Montgomery B, Taplin ME, Pritchard CC, Attard G, et al. Integrative clinical genomics of

599   advanced prostate cancer. Cell. 2015;161(5):1215-28.

600   17.   Wyatt AW, Annala M, Aggarwal R, Beja K, Feng F, Youngren J, Foye A, Lloyd P,

601   Nykter M, Beer TM, et al. Concordance of Circulating Tumor DNA and Matched Metastatic

602   Tissue Biopsy in Prostate Cancer. J Natl Cancer Inst. 2017;109(12).

603   18.   Morrison GJ, and Goldkorn A. Development and Application of Liquid Biopsies in

604   Metastatic Prostate Cancer. Curr Oncol Rep. 2018;20(4):35.

605   19.   Liu W, Yin B, Wang X, Yu P, Duan X, Liu C, Wang B, and Tao Z. Circulating tumor

606   cells in prostate cancer: Precision diagnosis and therapy. Oncol Lett. 2017;14(2):1223-32.

607   20.   Heller G, McCormack R, Kheoh T, Molina A, Smith MR, Dreicer R, Saad F, de Wit R,

608   Aftab DT, Hirmand M, et al. Circulating Tumor Cell Number as a Response Measure of

609   Prolonged Survival for Metastatic Castration-Resistant Prostate Cancer: A Comparison With

610   Prostate-Specific Antigen Across Five Randomized Phase III Clinical Trials. J Clin Oncol.

611   2018;36(6):572-80.

612   21.   Bastos DA, and Antonarakis ES. CTC-derived AR-V7 detection as a prognostic and

613   predictive biomarker in advanced prostate cancer. Expert Rev Mol Diagn. 2018;18(2):155-63.

614   22.   Scher HI, Graf RP, Schreiber NA, Jayaram A, Winquist E, McLaughlin B, Lu D, Fleisher

615   M, Orr S, Lowes L, et al. Assessment of the Validity of Nuclear-Localized Androgen Receptor

616 Splice Variant 7 in Circulating Tumor Cells as a Predictive Biomarker for Castration-Resistant

617 Prostate Cancer. JAMA Oncol. 2018.

618 23.   Antonarakis ES, Lu C, Luber B, Wang H, Chen Y, Zhu Y, Silberstein JL, Taylor MN,

619 Maughan BL, Denmeade SR, et al. Clinical Significance of Androgen Receptor Splice Variant-7

620 mRNA Detection in Circulating Tumor Cells of Men With Metastatic Castration-Resistant

621 Prostate Cancer Treated With First- and Second-Line Abiraterone and Enzalutamide. J Clin

622 Oncol. 2017;35(19):2149-56.

623 24.   Millner LM, Linder MW, and Valdes R, Jr. Circulating tumor cells: a review of present

624 methods and the need to identify heterogeneous phenotypes. Ann Clin Lab Sci. 2013;43(3):295-

625 304.

626 25.   Bernemann C, Schnoeller TJ, Luedeke M, Steinestel K, Boegemann M, Schrader AJ, and

627 Steinestel J. Expression of AR-V7 in Circulating Tumour Cells Does Not Preclude Response to

628 Next Generation Androgen Deprivation Therapy in Patients with Castration Resistant Prostate

629 Cancer. Eur Urol. 2016.

630 26.   Takeuchi T, Okuno Y, Hattori-Kato M, Zaitsu M, and Mikami K. Detection of AR-V7

631 mRNA in whole blood may not predict the effectiveness of novel endocrine drugs for castration-

632 resistant prostate cancer. Res Rep Urol. 2016;8(21-5.

633 27.   Beltran H, Prandi D, Mosquera JM, Benelli M, Puca L, Cyrta J, Marotz C, Giannopoulou

634 E, Chakravarthi BV, Varambally S, et al. Divergent clonal evolution of castration-resistant

635 neuroendocrine prostate cancer. Nat Med. 2016;22(3):298-305.

636 28.   Corcoran RB, and Chabner BA. Application of Cell-free DNA Analysis to Cancer

637 Treatment. N Engl J Med. 2018;379(18):1754-65.

638   29.   Ritch E, and Wyatt AW. Predicting therapy response and resistance in metastatic prostate

639   cancer with circulating tumor DNA. Urol Oncol. 2017.

640   30.   Romanel A, Tandefelt DG, Conteduca V, Jayaram A, Casiraghi N, Wetterskog D, Salvi

641   S, Amadori D, Zafeiriou Z, Rescigno P, et al. Plasma AR and abiraterone-resistant prostate

642   cancer. Sci Transl Med. 2015;7(312):312re10.

643   31.   Azad AA, Volik SV, Wyatt AW, Haegert A, Le Bihan S, Bell RH, Anderson SA,

644   McConeghy B, Shukin R, Bazov J, et al. Androgen Receptor Gene Aberrations in Circulating

645   Cell-Free DNA: Biomarkers of Therapeutic Resistance in Castration-Resistant Prostate Cancer.

646   Clin Cancer Res. 2015;21(10):2315-24.

647   32.   Yegnasubramanian S. Prostate cancer epigenetics and its clinical implications. Asian

648   Journal of Andrology. 2016;18(4):549-58.

649   33.   Massie CE, Mills IG, and Lynch AG. The importance of DNA methylation in prostate

650   cancer development. J Steroid Biochem Mol Biol. 2017;166(1-15.

651   34.   Kron K, Liu L, Trudel D, Pethe V, Trachtenberg J, Fleshner N, Bapat B, and van der

652   Kwast T. Correlation of ERG expression and DNA methylation biomarkers with adverse

653   clinicopathologic features of prostate cancer. Clin Cancer Res. 2012;18(10):2896-904.

654   35.   Mahon KL, Qu W, Devaney J, Paul C, Castillo L, Wykes RJ, Chatfield MD, Boyer MJ,

655   Stockler MR, Marx G, et al. Methylated Glutathione S-transferase 1 (mGSTP1) is a potential

656   plasma free DNA epigenetic marker of prognosis and response to chemotherapy in castrate-

657   resistant prostate cancer. Br J Cancer. 2014;111(9):1802-9.

658   36.   Gordevicius J, Krisciunas A, Groot DE, Yip SM, Susic M, Kwan A, Kustra R, Joshua

659   AM, Chi KN, Petronis A, et al. Cell-Free DNA Modification Dynamics in Abiraterone Acetate-

660   Treated Prostate Cancer Patients. Clin Cancer Res. 2018;24(14):3317-24.

661   37.   Zhao F, Olkhov-Mitsel E, van der Kwast T, Sykes J, Zdravic D, Venkateswaran V, Zlotta

662   AR, Loblaw A, Fleshner NE, Klotz L, et al. Urinary DNA Methylation Biomarkers for

663   Noninvasive Prediction of Aggressive Disease in Patients with Prostate Cancer on Active

664   Surveillance. J Urol. 2017;197(2):335-41.

665   38.   Hendriks RJ, Dijkstra S, Smit FP, Vandersmissen J, Van de Voorde H, Mulders PFA, van

666   Oort IM, Van Criekinge W, and Schalken JA. Epigenetic markers in circulating cell-free DNA as

667   prognostic markers for survival of castration-resistant prostate cancer patients. Prostate. 2018.

668   39.   Shen SY, Singhania R, Fehringer G, Chakravarthy A, Roehrl MHA, Chadwick D,

669   Zuzarte PC, Borgida A, Wang TT, Li T, et al. Sensitive tumour detection and classification using

670   plasma cell-free DNA methylomes. Nature. 2018;563(7732):579-83.

671   40.   Kamdar SN, Ho LT, Kron KJ, Isserlin R, van der Kwast T, Zlotta AR, Fleshner NE,

672   Bader G, and Bapat B. Dynamic interplay between locus-specific DNA methylation and

673   hydroxymethylation regulates distinct biological pathways in prostate carcinogenesis. Clin

674   Epigenetics. 2016;8(32.

675   41.   Baylin SB, and Jones PA. Epigenetic Determinants of Cancer. Cold Spring Harb Perspect

676   Biol. 2016;8(9).

677   42.   Zeng T, Zhu L, Liao M, Zhuo W, Yang S, Wu W, and Wang D. Knockdown of PYCR1

678   inhibits cell proliferation and colony formation via cell cycle arrest and apoptosis in prostate

679   cancer. Med Oncol. 2017;34(2):27.

680   43.   Yang F, Zhang Y, Ressler SJ, Ittmann MM, Ayala GE, Dang TD, Wang F, and Rowley

681   DR. FGFR1 is essential for prostate cancer progression and metastasis. Cancer Res.

682   2013;73(12):3716-24.

683   44.   Manda KR, Tripathi P, Hsi AC, Ning J, Ruzinova MB, Liapis H, Bailey M, Zhang H,

684   Maher CA, Humphrey PA, et al. NFATc1 promotes prostate tumorigenesis and overcomes

685   PTEN loss-induced senescence. Oncogene. 2016;35(25):3282-92.

686   45.   Wang R, Chen X, Xu T, Xia R, Han L, Chen W, De W, and Shu Y. MiR-326 regulates

687   cell proliferation and migration in lung cancer by targeting phox2a and is regulated by HOTAIR.

688   Am J Cancer Res. 2016;6(2):173-86.

689   46.   Takayama K, Suzuki T, Fujimura T, Urano T, Takahashi S, Homma Y, and Inoue S.

690   CtBP2 modulates the androgen receptor to promote prostate cancer progression. Cancer Res.

691   2014;74(22):6542-53.

692   47.   Guo H, Ci X, Ahmed M, Hua JT, Soares F, Lin D, Puca L, Vosoughi A, Xue H, Li E, et

693   al. ONECUT2 is a driver of neuroendocrine prostate cancer. Nat Commun. 2019;10(1):278.

694   48.   Yang YA, Zhao JC, Fong KW, Kim J, Li S, Song C, Song B, Zheng B, He C, and Yu J.

695   FOXA1 potentiates lineage-specific enhancer activation through modulating TET1 expression

696   and function. Nucleic Acids Res. 2016;44(17):8153-64.

697   49.   Mahon KL, Qu W, Lin HM, Spielman C, Cain D, Jacobs C, Stockler MR, Higano CS, de

698   Bono JS, Chi KN, et al. Serum Free Methylated Glutathione S-transferase 1 DNA Levels,

699   Survival, and Response to Docetaxel in Metastatic, Castration-resistant Prostate Cancer: Post

700   Hoc Analyses of Data from a Phase 3 Trial. Eur Urol. 2018.

701   50.   Brocks D, Assenov Y, Minner S, Bogatyrova O, Simon R, Koop C, Oakes C, Zucknick

702   M, Lipka DB, Weischenfeldt J, et al. Intratumor DNA methylation heterogeneity reflects clonal

703   evolution in aggressive prostate cancer. Cell Reports. 2014;8(3):798-806.

704   51.   Aryee MJ, Liu W, Engelmann JC, Nuhn P, Gurel M, Haffner MC, Esopi D, Irizarry RA,

705   Getzenberg RH, Nelson WG, et al. DNA methylation alterations exhibit intraindividual stability

31

706 and interindividual heterogeneity in prostate cancer metastases. Sci Transl Med.

707 2013;5(169):169ra10.

708 52. Friedlander TW, Roy R, Tomlins SA, Ngo VT, Kobayashi Y, Azameera A, Rubin MA,

709 Pienta KJ, Chinnaiyan A, Ittmann MM, et al. Common structural and epigenetic changes in the

710 genome of castration-resistant prostate cancer. Cancer Res. 2012;72(3):616-25.

711 53. Huang H, Du T, Zhang Y, Lai Y, Li K, Fan X, Zhu D, Lin T, Xu K, Huang J, et al.

712 Elevation of SHARPIN Protein Levels in Prostate Adenocarcinomas Promotes Metastasis and

713 Impairs Patient Survivals. Prostate. 2017;77(7):718-28.

714 54. Zhang D, Park D, Zhong Y, Lu Y, Rycaj K, Gong S, Chen X, Liu X, Chao HP, Whitney

715 P, et al. Stem cell and neurogenic gene-expression profiles link prostate basal cells to aggressive

716 prostate cancer. Nat Commun. 2016;7(10798.

717 55. Hara I, Yamashita S, Nishizawa S, Kikkawa K, Shimokawa T, and Kohjimoto Y.

718 Enzalutamide Versus Abiraterone as a First-Line Endocrine Therapy for Castration-Resistant

719 Prostate Cancer: Protocol for a Multicenter Randomized Phase 3 Trial. JMIR Res Protoc.

720 2018;7(7):e11191.

721 56. Ramachandran P, Palidwor GA, Porter CJ, and Perkins TJ. MaSC: mappability-sensitive

722 cross-correlation for estimating mean fragment length of single-end short-read sequencing data.

723 Bioinformatics. 2013;29(4):444-50.

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728

729 **Figure Legends**

730 **Figure 1. Within patient differential methylation analysis strategy to monitor temporal**

731 **changes in cfDNA.**

732 In order to help identify methylation changes associated with treatment in cfDNA, we opted to

733 perform within-patient methylation analysis to identify differentially methylated regions (DMRs)

734 associated with tumor response and/or resistance to current treatments. Each patient in this study

735 received either Enzalutamide or Abiraterone acetate and blood was collected prior to initiating

736 therapy (**Visit A**), at 12-weeks during treatment (**Visit B**) and upon clinical

737 progression/treatment change (**Visit C**). We applied an established genome-wide method to

738 detect methylated cfDNA fragments followed by extensive analysis to identify DMRs associated

739 with treatment response. We compared all study visits available (Visit B vs A, C vs B, and C vs

740 A) to find losses or gains in methylated cfDNA fragments. For instance, loss of treatment

741 responsive tumor cells (green methylated DNA fragments at visit B) or gains in treatment

742 insensitive/resistant tumor cells (blue DNA fragments at visit C) could be detected with this

743 strategy.

744

745 **Figure 2. Sample collection and patient clinical follow-up overview.**

746 (**a**) Plasma samples from mCRPC patients receiving Enzalutamide (12 patients) or Abiraterone

747 acetate (4 patients) were collected at baseline (Visit A), at week-12 (+/- 2 weeks, Visit B) and

748 upon clinical progression (Visit C). The total number of samples collected at each visit is shown.

749 2 Enzalutamide-treated patients (P2 and P26) were unable to provide samples for Visit C and 1

750 Abiraterone-treated patient (P36) progressed prior to Visit B. For these patients, the date of

751 treatment change/clinical progression was recorded for data analysis. (**b**) Pie chart summarizes

33

752 the distribution of metastasis locations at mCRPC diagnosis. (**c**) Bar plot shows best PSA

753 response for each patient during treatment (nadir), expressed as a percentage of Baseline/Visit A

754 PSA. Dotted line indicates ≥ 50% decline in PSA from Visit A. (**d**) Overall timeline of study

755 follow-up starting from Visit A to Visit C/Final visit is shown for all patients. Green dots

756 represent Visit B, red dots are Visit C or final study visit (if Visit C was not collected), yellow

757 indicates lowest PSA level (nadir) and blue dots show first PSA rise during treatment.

758

759 **Figure 3. Summary of intra-patient DMR analysis.**

760 (**a**) The total number of DMRs for all patients, visit comparison and treatment. (**b**) Boxplot

761 summarizing median, first and third quartile of all comparison DMRs. Whiskers represent the

762 highest and lowest number of DMRs. (**c**) DMR totals were further stratified by treatment type.

763 Boxplots illustrate percentage of DMRs that increased or decreased in methylation in (**d**) B vs A,

764 (**e**) C vs B and (**f**) C vs A. (**g**) The overall proportion of DMRs that were identified near/within

765 CGI, shore, TSS/promoter, ncRNA, intergenic and gene body regions is shown for all visit

766 comparisons (In order of B vs A, C vs B and C vs A). (**h**) Venn diagrams showing the overlap of

767 protein coding genes with DMRs near their promoters is shown for all visit comparisons and

768 between Enzalutamide- and Abiraterone-treated patients. (**i**) Similarly, the overlap of protein

769 coding regions with DMRs within gene bodies is shown.

770

771 **Figure 4. Key prostate cancer related methylation changes observed in cfDNA**

772 (**a**) Heatmap shows methylation trends in promoter regions for all patients and comparisons of

773 the most frequently methylated genes in PCa. For each gene, the proportion of DMRs with

774 increased (blue) and decreased (red) methylation was calculated and the net change is shown.
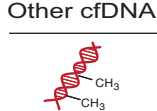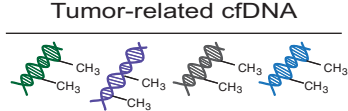
775   Grey indicates no net change in methylation. Representative peaks show methylation levels

776   (normalized read counts) of (**b**) *GSTP1* (black dotted lines indicating gene body) and (**c**) *TBX15*.

777   LNCaP cell line MeDIP-seq data is also shown.

778

779   **Figure 5. Common differentially methylated CpGs (DMCs).**

780   (**a**) For patients that were able to provide samples for all three visits, the proportion of CpG sites

781   that were differentially methylated in a single comparison and shared across 2 or more

782   comparisons is shown. 14 possible scenarios are represented by different colors. TTP in weeks

783   are also indicated for each patient. (**b**) Spearman correlation analysis of the proportion of DMCs

784   that were found to increase or decrease in B/C vs A (scenarios 9 & 10) against time to

785   progression (Spearman rho and p values are shown).

786

787   **Figure 6. NE-CRPC related DMRs in cfDNA.**

788   (**a**) For each patient, the proportion of DMRs that demonstrated methylation differences in NE-

789   CRPC associated regions in visit A vs B. (**b**) For DMRs that contained NE-CRPC associated

790   CpGs, various FDR thresholds were applied to assess for optimal regions that correlated with

791   TTP. (**c**) Using the optimal FDR cut-off, the ratio of NE-associated DMRs with increased

792   methylated fragments in visit A vs B to decreased fragments was correlated with TTP (Spearman

793   rho and p value is shown). For P36, A vs C comparison was used as this patient progressed prior

794   to visit B. (d) ROC analysis shows optimal AUC (0.927, 95% CI: 0.773-1) that stratifies patients
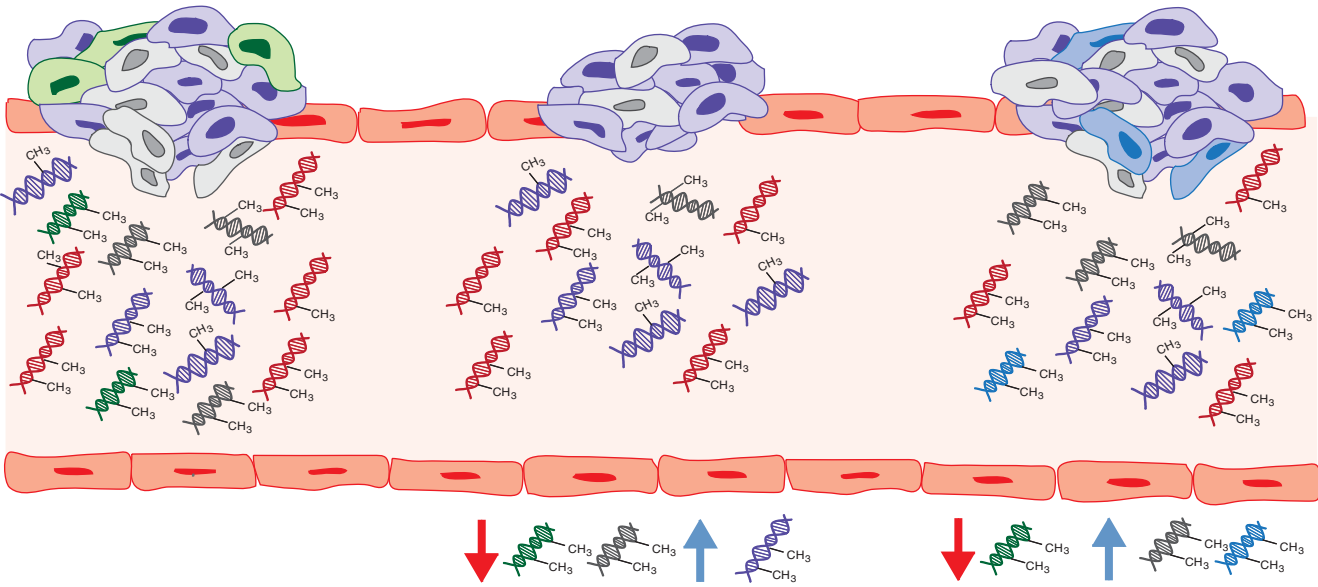
795   according to TTP (<25 weeks).

35

a)

	Visit A: Baseline/Prior to treatment	Visit B: Week-12 (+/- 2 weeks)	Visit C: Clinical Progression/Change in treatment
Enzalutamide (No. visits)	12	12	10
Abiraterone (No. visits)	4	3	4

b) Metastasis sites at mCRPC diagnosis

- Bone
- Bone + Lymph node
- Bone + Lymph node + Lung
- Lymph node

c)

Best PSA response (% of Visit A)

ABI
ENZA

Patient: P3 P7 P1 P2 P4 P8 P9 P15 P31 P12 P6 P38 P26 P37 P36 P28

d)

Visit B
Visit C/Final visit
PSA Nadir
First PSA rise

Weeks from Visit A

a)

b) chr11 region showing GSTP1 gene with CG islands and methylation tracks: 1A_M, 1B_M, 1C_M, 3A_M, 3B_M, 3C_M, LNCaP

c) chr1 region showing TBX15 gene with CG islands and methylation tracks: 31A_M, 31B_M, 31C_M, 38A_M, 38B_M, 38C_M, LNCaP

Net methylation trend: −1 (DN) to 1 (UP)

B vs A | C vs B | C vs A | ABI | ENZA