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TITLE: A Changing Landscape of Advanced Prostate Cancer: Understanding Mechanisms of Resistance to Potent Hormonal Therapies

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14. ABSTRACT:

Transition to a neuroendocrine prostate cancer (NEPC) phenotype has emerged as an important mechanism of treatment resistance to androgen receptor (AR) therapies for patients with metastatic prostate cancer. During the course of this Award, I have performed extensive, first –in-field molecular characterization of metastatic tumor biopsies from patients with castration resistant adenocarcinoma and neuroendocrine prostate cancer. Whole exome, transcriptome, and DNA methylation integrative analyses pointed to key drivers of NEPC including loss of RB1 and TP53, gain of MYCN, overexpression of BRN2, and epigenetic changes. Clonality analysis of serial tumor biopsies in individual patients provides new insights into mechanisms of progression, favoring a model most consistent with divergent clonal evolution of NEPC from an adenocarcinoma precursor. Through preclinical studies including the recent development of patient derived preclinical models of NEPC by our lab, we have better characterized mechanisms of trans-differentiation and have identified new drug targets. I have initiated investigator initiated clinical studies based directly on this work. Also as part of this Award, I have evaluated circulating tumor cells (CTCs) from patients treated with abiraterone and enzalutamide for emergence of NEPC CTC characteristics and found that up to 10% harbor NEPC-like CTCs (characterized by low AR, smaller morphology, loss of CK), and the presence of NEPC CTCs was associated with poor prognostic features. In the current reporting period, I have been using CTCs to detect new biomarkers/ therapeutic targets such as DLL3. We have also been using ctDNA for the early detection of NEPC and to chacterize tumor heterogeneity. I have also started to look even earlier in prostate cancer progression to understand how and when NEPC-assocaited changes occur, evaluating high risk prostate cancers and patients treated with neoadjvuant therapy on the CALGB90203 Phase 3 trial for emergence of NEPC features and harbingers of early resistance. Collectively, results from this Award have multiple clinical implications for early detection, prognostication, and identification of patients less likely to respond to subsequent AR-targeted therapies and more likely to benefit from NEPC-targeted approaches. This Physician Research Training Award has significantly facilitated my career development and has helped establish myself and our lab as leaders in the area of NEPC.

15. SUBJECT TERMS: Prostate Cancer, AR independence, Neuroendocrine prostate cancer, Treatment resistance, Circulating tumor cells, Biomarkers

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1. **INTRODUCTION:** Most castration resistant prostate tumors remain dependent on androgen receptor (AR) signaling, and several highly potent AR-targeted therapies are in widespread clinical use for the treatment of men with metastatic castration resistant prostate cancer (CRPC). These drugs have recently moved earlier in the disease for the treatment of non-metastatic CRPC (Small et al NEJM 2018; Hussain et al NEJM 2018) as well as in combination with androgen deprivation therapy for metastatic castration sensitive disease (CSPC) (Fizazi et al, NEJM 2017; James et al, Lancet Oncol 2017). While these drugs have significantly improved outcomes for patients, they are not curative, and all patients ultimately develop resistance. In most cases, the AR continues to remain active. However, a subgroup of patients treated with AR therapies develop rapid progression and clinical features suggestive AR independence including low PSA progression. Metastatic biopsies in this subgroup have revealed an emergence of tumor morphologic characteristics consistent with small cell carcinoma/neuroendocrine prostate cancer (NEPC). In recent years, NEPC has emerged as a mechanism of resistance in up to 10-20% of patients with CRPC (Bluemn et al, Cancer Cell, 2017, Aggarwal et al JCO 2018, Beltran et al, Nature Med 2016). There is concern with the more potent and earlier targeting of the AR, the incidence of NEPC will continue to rise. There are no approved therapies and prognosis remains poor. The goal of this Award is to systematically evaluate mechanisms of NEPC progression using deep sequencing techniques of metastatic tumor biopsies and non-invasively using liquid biopsies including circulating tumor cells (CTCs).

2. **KEYWORDS:** advanced prostate cancer, androgen receptor, androgen indifference, resistance, neuroendocrine prostate cancer, circulating tumor cells, genomics, biomarkers

3. PROJECT SUMMARY:

Aim 1. To identify molecular determinants of acquired resistance to potent AR targeted therapies. The working hypothesis of this Aim is that advanced prostate tumors acquire genetic alterations in response to newer potent AR targeted therapies that enable them to continue to grow and proliferate. We will perform massively parallel whole exome sequencing of tumor tissue from abiraterone resistant prostate cancers to determine the spectrum of mutations associated with resistance to AR targeted therapies.

Aim 2. To prospectively evaluate circulating tumor cells (CTCs) from patients receiving potent hormonal therapies for acquisition of gene alterations in response to therapy. The working hypothesis of this Aim is that evaluation of CTCs may provide a non-invasive method to detect genomic alterations of key genes that occur before or may be acquired on therapy that predict response or resistance. We will analyze CTCs from patients prior to starting abiraterone, during treatment, and at progression for gene amplification of AR, Aurora kinase A, and N-myc, and correlate with clinical response to therapy.

Aim 3. To evaluate high-risk primary prostate tumors for mutations that may predispose to resistance to AR targeted therapy with comparison to matched metastatic tumors. The working hypothesis of this Aim is that specific genetic alterations occur early and predispose to the development of treatment resistance to AR targeted therapies, and these may be detected at the time of initial diagnosis in high-risk primary prostate tumors.

Aim 1 Progress: I continue to systematically evaluate patients at different time points during treatment with potent AR targeted therapy and during progression from a hormone naive prostate adenocarcinoma to an AR-driven castration resistant adenocarcinoma (CRPC-Adeno) and/or AR –independent castration resistant neuroendocrine prostate cancer (CRPC-NE). Metastatic biopsies have not been considered standard of care for patients with advanced disease; therefore this effort has required prospective enrollment of patients on a research protocol with informed consent. I developed an IRB protocol to perform metastatic biopsies and whole exome sequencing (WES) and other molecular analyses of tumor and germline DNA from patients with advanced disease and to follow patients prospectively to evaluate for response to subsequent therapies, optional re-biopsy at progression, long term follow up for PFS and OS endpoints, and optional participation in a rapid autopsy program. The design and initial results of this protocol were reported in Beltran et al, JAMA Oncology 2015 and our rapid autopsy protocol in Psiapia el, Journal of Clinical Oncology Precision Oncology 2017. Somatic and germline results of enrolled CRPC patients pre and post abiraterone or enzalutamide were also included as part of the

International SU2C-PCF Prostate Dream Team and with initial results published in Robinson et al, Cell 2015 and Pritchard et al, NEJM 2016.

In addition, as study chair of a multi-center Phase 2 trial of the aurora kinase A inhibitor MLN8237 for patients with neuroendocrine prostate cancer, I have obtained 60 pre-treatment metastatic tissue biopsies and blood samples from NEPC patients and have recently reported initial clinical trial results and correlative studies (Beltran at al, *Clin Cancer Res* 2018). During this grant period, I performed WES and RNA-seq on pre-treatment biopsies (Figure 1) and have characterized exceptional responders on the trial. Data from this trial was also incorporated into our extended SU2C-PCF Dream Team study which has associated genomics and clinical response and outcomes in 500 men with advanced prostate cancer (Abida et al, manuscript in review).

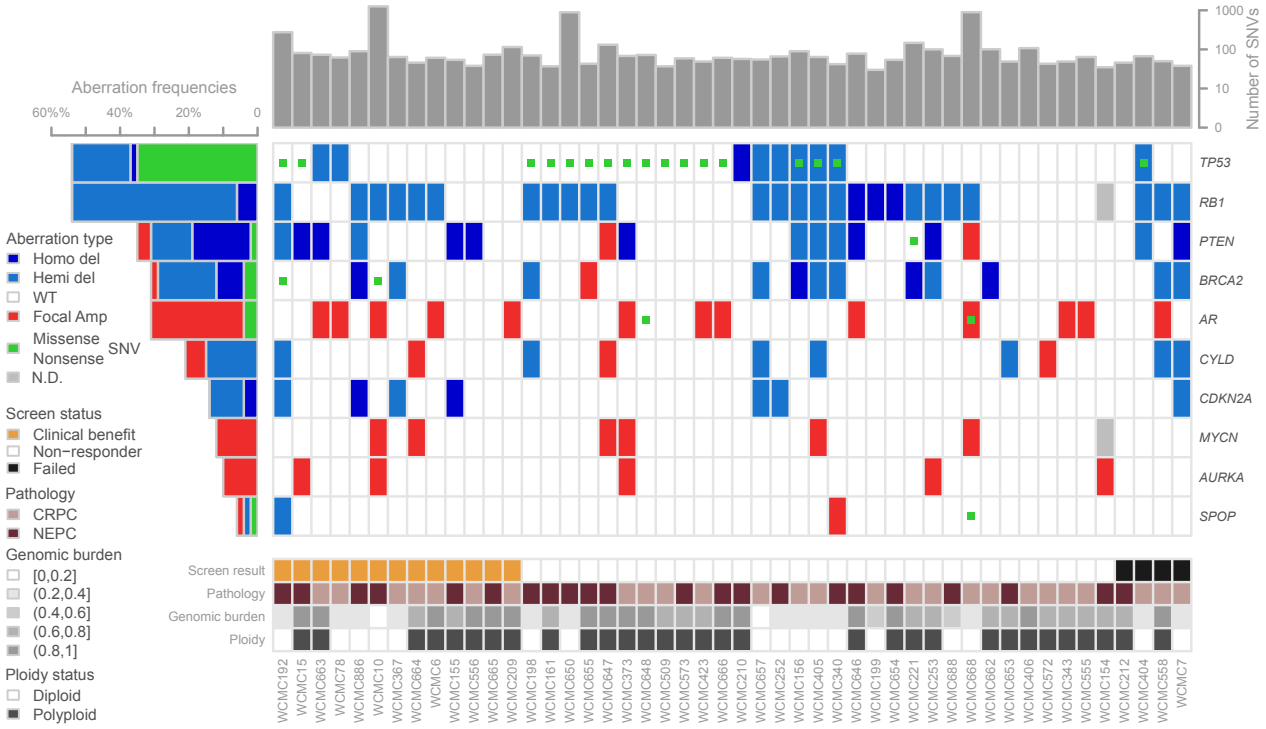


Figure 1: Whole exome sequencing analysis of pre-treatment metastatic biopsies from patients treated on a phase 2 trial of alisertib and annotated by pathology subtype (CRPC-Adeno= pink; NEPC= purple). Plot shows total number of SNVs, aberrations in relevant genes, including non-sense and missense SNVs, copy number deletions and focal amplifications. Copy number calls derive from log2 ratio (tumor/normal) adjusted by ploidy and tumor purity. Deletions and amplifications are defined using the thresholds on log2 ratio.

As part of this Award, we also previously reported an integrative analysis of 114 metastatic tumor specimens from 81 patients including 51 patients with clinical and histologic features of castration resistant adenocarcinoma (CRPC-Adeno), 30 with castration resistant neuroendocrine prostate cancer (CRPC-NE) as confirmed by pathologic consensus criteria, and 17 patients with multiple tumor biopsies (Beltran et al, Nature Medicine 2016). Biopsies were obtained from a wide range of metastatic sites, with a predominance of bone biopsies in CRPC-Adeno compared to CRPC-NE**.** As expected, CRPC-NE demonstrated on average lower protein expression of the AR by immunohistochemistry**.** We also quantified AR signaling status by mRNA and observed overall lower AR signaling in CRPC-NE compared to CRPC-Adeno; however, there was significant overlap with a wide range of values observed within each subtype. The mutational landscape of CRPC-NE was similar to CRPC-Adeno**,** but also consistent with published studies of CRPC-NE including enrichment of *RB1* loss *(*deleted in 70% of CRPC-NE and 32% of CRPC-Adeno, p=0.003) and mutation or deletion of *TP53* (66.7% CRPC-NE versus 31.4% CRPC-Adeno, p=0.0043). Loss of *RB1* is common in primary small cell prostate and lung carcinomas, and promotes small cell carcinoma pathogenesis when concurrent with *TP53* mutation; in our series, concurrent *RB1*

and *TP53* loss was present in 53.3% of CRPC-NE vs.13.7% of CRPC-Adeno ($p<0.0004$). In collaboration with Charles Sawyer lab (MSKCC), we helped further elucidate the role of RB1 and TP53 in patient cohorts and their contribution towards driving AR independent resistance to AR therapies in CRPC (Mu et al, Science, 2017).

During this current grant period, we compared the molecular features of treatment related CRPC-NE to *de novo* small cell carcinoma of the prostate, which is a rare diagnosis. Interestingly we found similar genomic features of not only TP53 and RB1 losses, but also the presence of early prostate cancer lesions TMPRSS2-ERG and FOXA1 mutations in de novo small cell prostate cancer , suggesting possibly a same cell of origin as prostate adenocarcinoma and CRPC-NE. These data were published recently (Chedgy et al, J Pathol 2018)

Another distinguishing feature of CRPC-NE compared to CRPC-Adeno was a paucity of somatic alterations involving the AR gene ($p<0.0001$). Genomic amplification, activating point mutations, and splice variants involving the AR are commonly observed in CRPC-Adeno and associated with treatment resistance to AR-directed therapies (Robinson et al, Cell 2014). This observation was confirmed in our cohort; 29 cases showed *AR* focal amplification or point mutation and 21 cases had alterations in known AR co-activators (*FOXA1, NCOR1/2, ZBTB16*). Although potentially affected by differences in prior therapies, we speculate that the absence of AR genomic alterations in CRPC-NE may be due to clonal selection of non-amplified CRPC-Adeno tumor subpopulations through selective pressure (in the context of AR-directed therapies). As a follow-up to this observation, in the current reporting period, I have been working on circulating tumor DNA evaluation of this same cohort and extended to more patients (n=100) and observed enrichment of AR alterations in CRPC-Adeno vs. CRPC –NE (also relevant to Aim 2) (Beltran et al, manuscript in review).

While informative, the observed DNA changes did not appear to fully explain the clinical aggressiveness of CRPC-NE. We therefore posited that this phenotype may also be mediated by epigenetic changes. Towards this end, we generated data to evaluate CpG-rich methylation genome wide by single cytosine resolution DNA methylation (eRRBS). In contrast to the largely similar genomic data, the CRPC-NE and CRPC-Adeno subtypes showed strong epigenetic segregation by unsupervised analysis using unselected methylation sites**.** In fact, the epigenetic signal comprised an even stronger classifier than standard pathologic classification, as evidenced by the fact that it encompassed three cases that were initially binned as adenocarcinoma based on standard pathology**.** All three of these patients demonstrated radiographic progression in the setting of a stable or low serum level of the androgen-regulated protein prostate specific antigen (PSA). These data suggest that clustering prediction based on DNA methylation may provide additional information associated with AR independence and CRPC-NE that improves on tumor morphology. In 2018, my lab has been extending upon these observations to assess the functional role of DNA methylation changes in driving AR cistrome changes and downstream CRPC-NE features and evaluating intra-patient tumoral DNA methylation heterogeneity across metastatic sites at time of rapid autopsy (ongoing work, see Aim 2).

During this Award period year, in collaboration with West Coast Stand Up To Cancer- PCF Dream Team, I helped to integrate the transcriptome results of our study with a new cohort of metastatic biopsies taken primarily post-abiraterone /enzalutamide (Aggarwal et al, JCO 2018). In that study, the 17% of patients prospectively enrolled had small cell/NE features, and the presence of these pathologic features conferred worse prognosis. Small cell/NEPC in this new study had a similar transcriptome program/gene signature as our initial cohort. These data support distinct molecular features underlying small cell/NEPC.

We found that the DNA methyltransferase EZH2 was significantly overexpressed in CRPC-NE compared to CRPC-Adeno ($p<10^{-6}$, Wilcoxon test). Furthermore, EZH2 target genes are also downregulated in CRPC-NE. Treatment of cell lines with the EZH2 inhibitor GSK126 resulted in a preferential decrease in cellular viability in NCI-H660 compared to other prostate cancer cell lines with significant down-regulation of CRPC-NE associated genes after treatment including CD56, MYCN, and PEG10. During this grant period, I have since been exploring the role of EZH2 as a potential target for patients with CRPC-NE by using preclinical models in my lab including patient derived organoids that we developed and recently reported (Puca et al, Nature Comm 2018). We also performed high throughput drug screening of single agent and combination therapies (with GSK126)**.** Through this unbiased approach, we observed significant activity with the combination of GSK126 and the aurora kinase

A alisertib. This fit well with our preclinical observations linking N-myc, aurora A, EZH2 as cooperators in driving the NEPC phenotype**.** These data have served as rationale for our DOD impact award focused on N-myc (Co-PI Beltran and Rickman).

Since reporting these discoveries, I have continued to delve deeper into the mechanisms underlying NEPC trans-differentiation and have worked in my laboratory and through collaborative efforts to identify the neuronal transcription factor BRN2 as an important driver (Bishop et al, Cancer Discovery 2017), elucidate the combined role of p53 and Rb1 (Mu et al, Science, 2017), and the role of N-myc in promoting NEPC in cooperation with EZH2 (Dardenne, Beltran (co-first author) et al, Cancer Cell, 2016), and more recently the identification of ONECUT2 (Guo et al Nature Comm, in press). I have also used metastatic biopsies from patients enrolled as part of this Aim to develop patient derived organoid models of NEPC and we published the characterization of these models earlier this year (Puca et al Nat Comm 2018). These organoids recapitulate the metastatic tumor biopsy genomics **(Figure 2)** and retain therapeutic drug response as the patients. Overall, this Aim has fueled my lab's basic and translational research in NEPC and has facilitated a number of academic collaborations and publications in 2018.

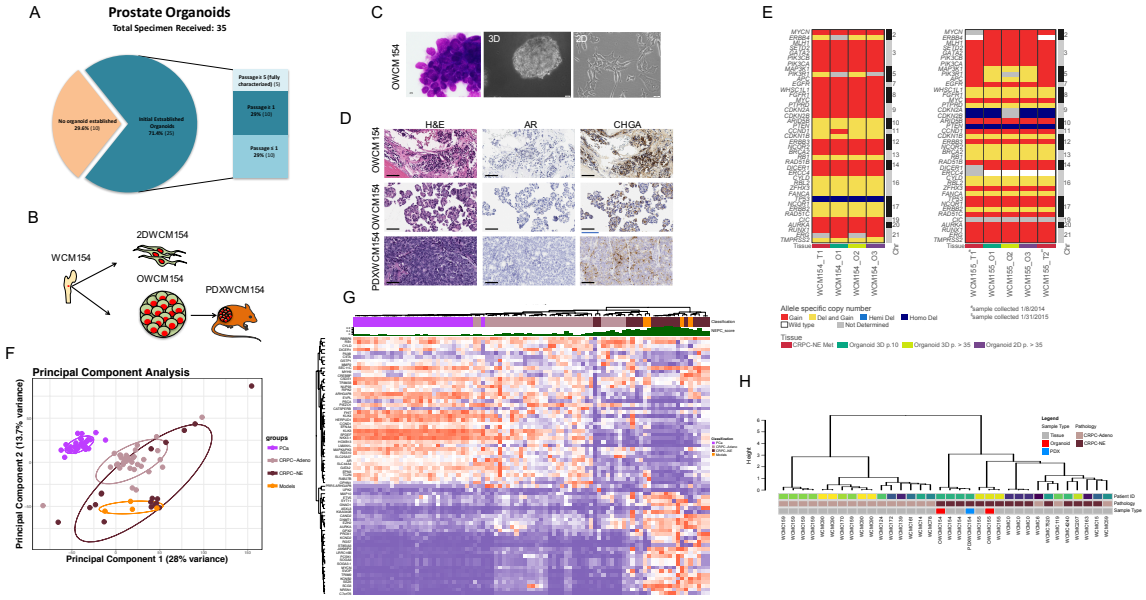


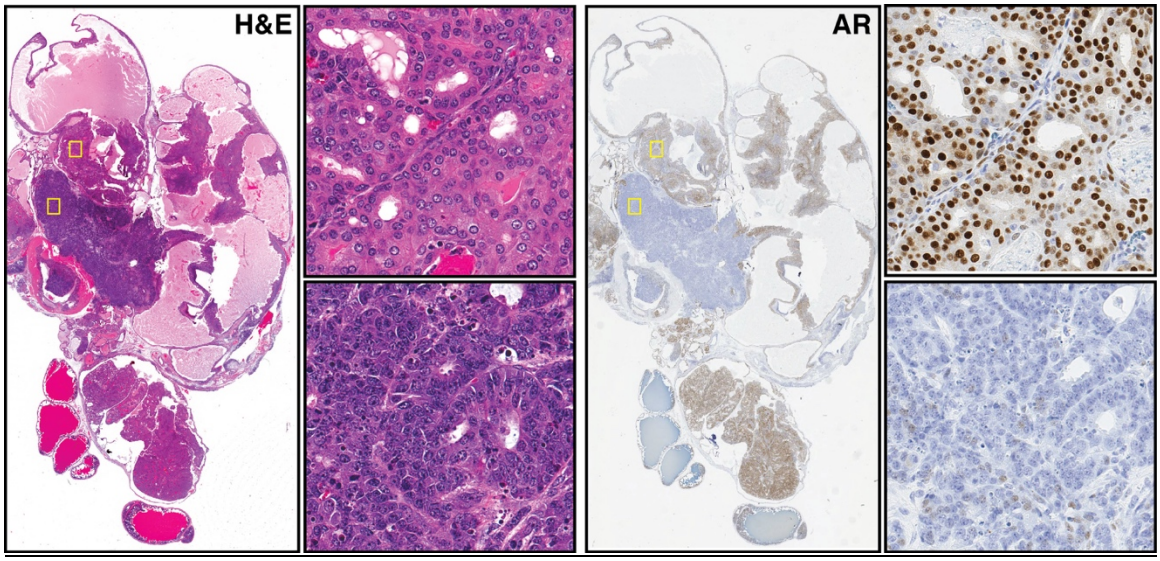
Figure 1

Figure 2: Molecular characterization of prostate cancer organoids A: Pie chart of prostate cancer needle biopsies received in WCM/IPM. B: Schematic view of models we generated from needle biopsies. C: Air dried Diff-Quick stained smears from neuroendocrine prostate carcinoma organoids (40X). D: Histology of native tumor tissue WCM154 compared to corresponding 3D organoid cultures and patient derived xenograft tissue. E: Whole exome sequencing of organoids at different passages over time. F: Principle component analysis including 26 PCA, 33 CRPC 13 NEPC patient samples and NEPC models (NCI-H660, OWCM154, OWCM155, PDXWCM154). G: Unsupervised cluster analysis of genes involved in NEPC phenotype using a cohort of PCA (bright pink), CPRC (light pink) and NEPC (brown) patients (Beltran et el., 2016) and the NEPC models generated (yellow). H: Genome-wide DNA methylation cluster analysis using a cohort of CPRC (light pink) and NEPC (brown) patients (Beltran et el., 2016) together with some of the models generated: organoids (red) and PDX (blue).

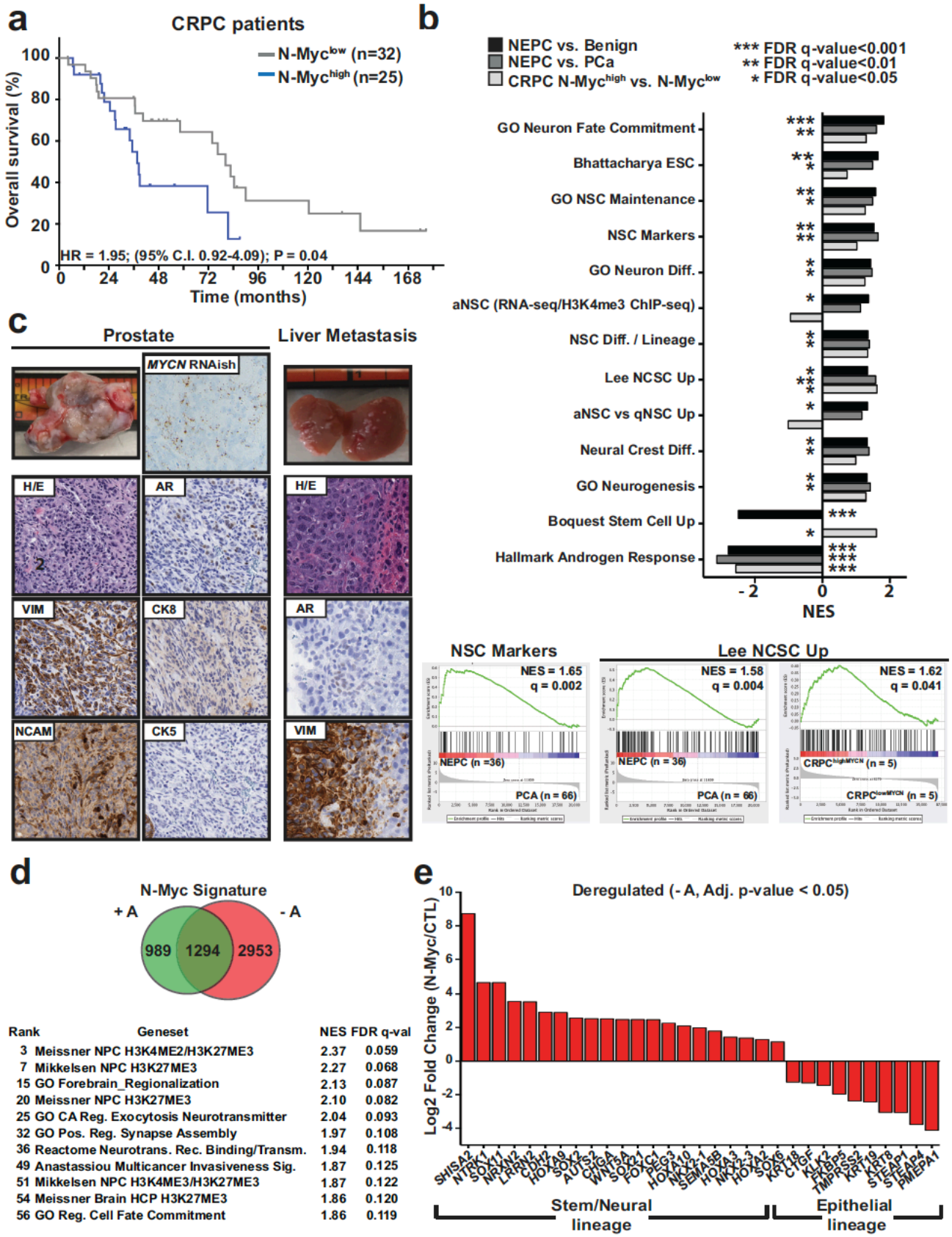
<u>BRN2 in NEPC.</u> In collaboration with Amina Zoubeidi's lab at Vancouver Prostate Cancer who developed multiple enzalutamide –resistant preclinical models, one of which was associated with NEPC features including suppressed AR signaling and upregulation of NEPC markers (called 42D), BRN2 was identified as the most highly upregulated genes in 42D (Fig 6-7). BRN2 is a neuronal transcription factor and master regulator. Through a series of experiments combined with clinical samples BRN2 was identified as a key regulator of NEPC transdifferentiation (Bishop et al, Cancer Discovery 2017). We are now working to develop therapeutic approaches to target BRN2.

<u>N-myc drives NEPC</u> In collaboration with D Rickman at Weill Cornell, we developed preclinical models of N-myc driven prostate cancer and NEPC including GEMM model *Pb-Cre+/-; Ptenf/f; LSL-MYCN+/+* that developed AR-negative NEPC and liver mets that was accelerated by castration. (**Figure 3,** below). Photomicrograph images of H&E stained or AR IHC prostate tissue below show invasive, AR-positive

adenocarcinoma foci and AR-negative NEPC foci.



To look further at the clinical impact of N-myc expression in prostate cancer, we assessed overall survival (OS) in 81 patients (57 CRPC-Adeno, 24 NEPC) with outcomes data. CRPC-Adeno patients with metastatic biopsies harboring high N-Myc expression showed a significantly worse OS compared to those with low N-Myc expression (37.7 versus 80.3 months, hazard ratio (HR) 1.95, 95% confidence interval (CI) 0.92-4.09, p-value=0.040, **Fig.4a**), and this was also significant when also taking into consideration patients with NEPC (34.0 versus 76.5 months, HR 2.27, 95% CI 1.24-4.11, p-value=0.002. Whole transcriptome analyses on an expanded cohort of patients both NEPC (n=36) and CRPC-Adeno (n = 73), as well as localized prostate adenocarcinoma (PCa) (n = 66) and benign prostate (n= 29) from prostatectomy specimens revealed that relative to benign or PCa, NEPC tumors demonstrated a significant enrichment for stem cell genes associated not only with normal neuroendocrine cell precursors (neural crest stem cells (NCSC)), but also with activated neural stem cells (NSC) and embryonic stem (ES) cells **(Fig. 4).** We purified murine NSC from the sub-ventricular zone and performed RNA-seq and histone-3 lysine-4 tri-methylation (H3K4me3) ChIP-seq. From these studies, we combined the upregulated genes marked by H3K4me3 with publicly available single-cell RNA-seq data from activated NSC[36] and revealed a similar enrichment of NSC pathways in NEPC patients samples versus benign prostate tissue samples (NES=1.37, FDR q-value=0.039, Fig. 1b) and versus PCa samples (NES=1.13, FDR q-value=0.23). Unsupervised clustering analysis of the NEPC versus PCa leading-edge genes from ESC, NCSC or NSC gene sets segregated patients according to their tumor histological classification (PCa, CRPC-Adeno or NEPC). MYCN expression correlated with expression of neural lineage genes and inversely with AR signaling. These data are unpublished and are being incorporated into a manuscript in development. Using our N-myc models including a new inducible system with lineage tracing, combined with patient data, we are currently exploring the timing of N-myc and how this influences phenotype and in what genomic context (eg., TP53, RB1) as part of our DOD IMPACT Award (Co-PI Beltran and Rickman).



Rank	Geneset	NES	FDR q-val
3	Meissner NPC H3K4ME2/H3K27ME3	2.37	0.059
7	Mikkelsen NPC H3K27ME3	2.27	0.068
15	GO Forebrain_Regionalization	2.13	0.087
20	Meissner NPC H3K27ME3	2.10	0.082
25	GO CA Reg. Exocytosis Neurotransmitter	2.04	0.093
32	GO Pos. Reg. Synapse Assembly	1.97	0.108
36	Reactome Neurotrans. Rec. Binding/Transm.	1.94	0.118
49	Anastassiou Multicancer Invasiveness Sig.	1.87	0.125
51	Mikkelsen NPC H3K4ME3/H3K27ME3	1.87	0.122
54	Meissner Brain HCP H3K27ME3	1.86	0.120
56	GO Reg. Cell Fate Commitment	1.86	0.119

Figure 4. Clinical NEPC is associated with neural lineage and AR signaling alters the N-Myc transcriptome in vivo. (a) Kaplan–Meier plots of CRPC (n = 57) patients stratified into two categories according to the median value of MYCN mRNA expression. (b) Top: targeted GSEA on NEPC (n=36) patient samples versus benign (n=29) or PCa (n=66) patient samples and on the five CRPC with the highest level of MYCN expression versus the five lowest. Bottom: examples of enrichment plots for indicated gene sets. (c) Photomicrograph images of Hematoxylin and Eosin (H/E) staining, or vimentin (VIM), NCAM1, AR, cytokeratin 8 (CK8) and 5 (CK5) immunohistochemistry on primary prostate tumor region enriched with sarcomatoid differentiation (top) or liver metastatic lesion (bottom) from Pb-Cre+/-; Ptenf/f; LSL-MYCN+/+mice 6 months after castration. (d) Top: N-Myc signature defined from 22Rv1-N-Myc xenografts versus 22Rv1 control (CTL) xenografts

(-1< log2(fold change difference in gene expression) <1, adjusted p-value<0.05, n=3 biological replicates per condition). Bottom: GSEA analysis results comparing N-Myc castrated tumors versus the other 3 groups of tumors. (e) Log2 fold change (N-Myc vs CTL xenografts from castrated mice) of indicated genes.

Aim 2 Progress: The diagnosis of NEPC remains challenging and currently relies on pathologic features. There are no reliable blood biomarkers to consistently diagnose patients transforming to the NEPC phenotype. Detection of NEPC has clinical implications, as NEPC patients would not be expected to respond well to currently approved AR-targeted therapies for CRPC and may be better served by therapies specifically directed to NEPC. As part of this Aim, I have been using liquid biopsies to identify NEPC patients. I published initial work using circulating tumor cells (CTCs) during the last grant period (Beltran et al, Clinical Cancer Research 2017). I am currently investigating ctDNA approaches as well using the molecular classifier developed in Aim 1 (in progress). Preliminary data from this Aim have served as rationale for our recently funded SPORE project focused on ctDNA (Co-PI Beltran and Demichelis).

CTCs in NEPC: I characterized CTCs from patients with CRPC and NEPC utilizing the Epic Sciences platform (Epic Sciences, Inc, La Jolla, CA) and correlated results with patient-matched tumor biopsies and clinical features. Under an IRB approved protocol, patients with metastatic CRPC including those with pure or mixed NEPC were prospectively enrolled. NEPC was defined by the presence of either a pure or mixed small cell high-grade neuroendocrine carcinoma histology in a metastatic tumor biopsy and confirmed by at least 20% positive immunohistochemical staining for a neuroendocrine marker (synaptophysin, chromogranin). CRPC was defined clinically, with or without a metastatic biopsy confirming prostate adenocarcinoma. CRPC patients were sub-classified as atypical CRPC if the biopsy showed adenocarcinoma and the patient had clinical features suggestive of an AR independent transition which included radiographic progression in the setting of a low PSA <1 ng/ml, visceral progression in the absence of PSA progression (defined by Prostate Cancer Working Group 2 criteria and/or elevated serum chromogranin A >3X upper limit of normal.

CTCs from 27 patients with metastatic prostate cancer were evaluated. Overall, bone metastases were present in 24/27 (88.9%) of patients, and liver metastases were present in 8/12 (66.7%) of NEPC and 5/15 (33.3%) of CRPC of whom 4 had atypical clinical features. Median serum PSA level was 1.9 ng/ml in NEPC, 2.8 ng/ml in atypical CRPC, and 53.4 ng/ml in other CRPC patients. Serum neuroendocrine marker levels varied considerably within the NEPC subgroup and were also elevated in cases of CRPC. Two slides from each patient were evaluated by immunofluorescence (IF) using antibodies targeting cytokeratins (CK), CD45, AR, and 4',6-diamidino-2-phenylindole (DAPI) counterstain. Slides were imaged using a platform that captures all 3 million cells per slide in less than 15 minutes, and analyzed by a proprietary software that characterizes each cell by parameters including cell size, shape, nuclear area, presence of macronucleoli, CK and AR expression, uniformity and cellular localization. CK+/CD45- cells with intact, DAPI+ nuclei exhibiting tumor-associated morphologies were classified as traditional CTCs. CTCs with non-traditional characteristics were recorded, such as CK- /CD45- cells with morphological distinction and/or AR positivity, CK+/CD45- small cells, CTC clusters, CTCs with multiple marconucleoli and apoptotic CTCs (with nuclear or cytoplasmic fragmentation).

Enumeration of CTCs using both the CellSearch and Epic platforms was performed**.** Of note, 6/13 evaluated NEPC and atypical CRPC patients had CellSearch® CTC count of <5 CTC/7.5 mL (range 0-384, with 5 of these 13 patients having a CellSearch® CTC count of 0). In contrast, all 17 NEPC and atypical CRPC patients had CTCs ≥5 CTC/7.5mL using the Epic platform. Further characterization of the detected CTCs revealed heterogeneity of cytokeratin (CK) and AR expression in both NEPC and CRPC, with a significantly greater proportion of CK-negative and AR-negative CTCs in NEPC compared to CRPC**.** CTCs in NEPC patients overall had lower AR expression, higher cytoplasmic circularity, and higher nuclear to cytoplasmic ratio. The prevalence of CK-negative CTC subpopulations in NEPC patients is potentially consistent with a proposed epithelial-mesenchymal-transition (EMT). Based on the observed differences in CTCs between groups, we sought to identify CTC characteristics specific to NEPC. KDE analysis of the patient groups' CTCs in aggregate revealed significant differences in CK, AR and morphological characteristics when compared to CRPC. As a validation cohort, we evaluated baseline CTCs from 159 CRPC patients prospectively enrolled in an independent patient cohort at MSKCC for the presence of NEPC+ CTCs**.** NEPC+ CTC subpopulations were identified in 17 of 159 (10.7%) cases. A significantly higher proportion of CRPC patients with visceral metastases harbored NEPC+

CTCs compared to those that were NEPC- (35% versus 15%, respectively; p=0.04). Patients with NEPC+ CTCs also had an overall higher CTC burden (median CTC count 64.6 versus 4.2; p<0.01). In this proof of principle CTC Aim, I demonstrated that CTCs from patients with NEPC have distinct characteristics and thus their detection may potentially help identify patients that are developing NEPC-associated resistance. The results presented here indicate the feasibility of analyzing CTCs using the Epic platform and support the development of further studies to validate the clinical utility of CTCs for the early detection of patients transforming towards NEPC and the prognostic and potential predictive impact of CTC characteristics in predicting response to AR-directed therapies in CRPC.

During this grant period, I continue to collect CTCs and am currently using this platform to investigate the ability of CTCs to serve a non-invasive means to identify an emerging drug target for NEPC (DLL3). I have tested the antibody-drug conjugate against DLL3 called Rova-T in preclinical models and observed encouraging results with complete durable responses seen in NEPC xenografts **(Fig 5)**.

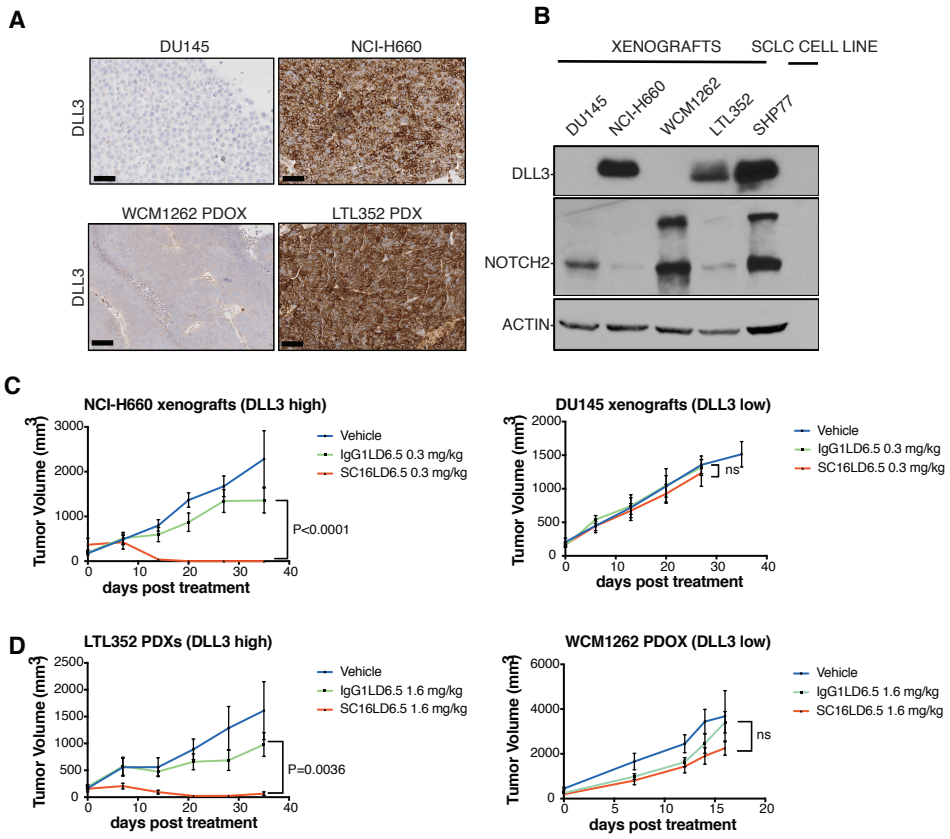


Fig. 4. **DLL3 antibody drug-conjugate activity in DLL3-expressing neuroendocrine prostate tumors *in vivo*. A.** Representative DLL3 immunohistochemistry images of DU145 cells and WCM1262 PDOX (DLL3-negative) and NCI-H660 cells and LTL352 PDX (DLL3-positive) (scale bars: 100um). **B.** Western blot analysis of tissue derived from DU145, NCI H660 WCM1262 and LTL352 xenografts using DLL3 and Notch2 antibodies. Actin is used as a loading control. The SHP77 cell line is used as a control for DLL3 expression. **C.** DU145 and NCI-H660 tumor volume (mm3) measurements after single dose treatment of vehicle, IgG1LD6.5 (0.3mg/Kg) and SC16LD6.5 (0.3mg/Kg) in NU/J mice evaluated for 35 days post treatment. Two-way ANOVA test is performed (p<0.0001). **D.** LTL352 and WCM1262 PDOX tumor volume (mm3) measurements after single dose treatment of vehicle, IgG1LD6.5 (1.6 mg/Kg), and SC16LD6.5 (1.6 mg/Kg) in NOD scid gamma mice evaluated for 35 days post treatment. Two-way ANOVA test is performed (p<0.0001).

We evaluated the expression of DLL3 across patient cohorts and found that DLL3 is expressed in the majority of NEPC (>70%), a subset of CRPC (10-15%), and not expressed in benign prostate or localized prostate cancer. These data suggest that it is a promising biomarker and therapeutic target. As DLL3 testing currently requires metastatic biopsy and IHC assessment as inclusion criteria for enrollment in the Rova-T trial, I have been exploring CTCs as a means to assess DLL3 expression. We found 87% concordance of DLL3 expression between

CTCs and matched tissue biopsies **(Fig 6).** I am currently one of the principal investigators on the phase 1 basket trial of Rova-T and have enrolled NEPC patients on the prostate arm (ESMO 2017) and am planning a Phase 2 study (concept in review) with more extensive correlative tissue and CTC analyses. My lab is also functionally characterizing the Notch signaling pathway during lineage plasticity and trying to understand how and why DLL3 becomes expressed in NEPC (ongoing work).

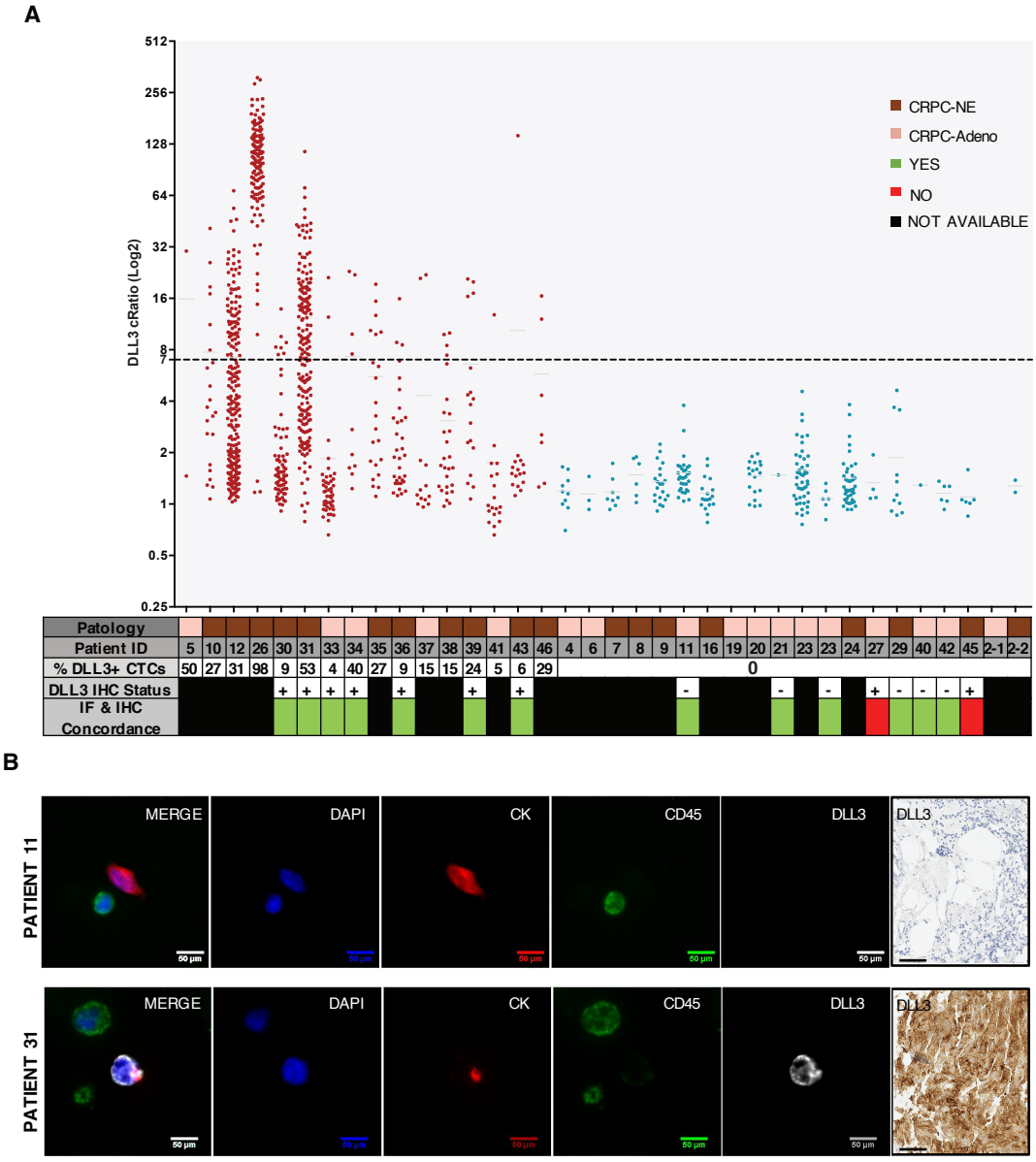


Fig. 5. **DLL3 detection in circulating tumor cells. A.** Graph showing DLL3 positivity in CTCs of 36 CRPC-Adeno and CRPC-NE patients tested with the DLL3 Epic 4-color immunofluorescence (IF) assay. DLL3 cRatio (signal-to-noise ratio) is plotted along the y-axis, and patient ID is plotted along the x-axis. The x-axis also includes a data table with additional patient specific information including pathology (CRPC-NE-brown or CRPC-Adeno-light brown), percentage of DLL3-positive CTCs, DLL3 IHC status, and IF/IHC concordance. Samples expressing DLL3 are indicated in red, samples negative for DLL3 are indicated in blue. Each dot represents a detected cell, and the dotted line at 7 along the y-axis indicates the analytical threshold of positivity for DLL3. **B.** Representative images of Patient 11 (DLL3-negative) and Patient 31 (DLL3-positive) CTCs. In the 4-color composite image, blue represents DAPI, red - cytokeratin (CK), green - CD45, and white - DLL3. Other

images show the independent images for each channel used to create the four-color composite (DAPI, CK, CD45 and DLL3). Corresponding DLL3 IHC image is also included for both patients.

Circulating tumor DNA (ctDNA) analysis in NEPC.

During this grant period we also completed ctDNA analysis of 62 individuals with prostate cancer metastatic to bone (84%), lymph node (52%), liver (32%), lung (16%), brain (6%) prior to their next line of systemic therapy, including 10 hormone naive (mPCA), 36 castration resistant prostate adenocarcinoma (CRPC-Adeno), 17 CRPC-NE patients with 24 having multiple tissue or plasma time-points, for a total of 69 plasma samples and 98 metastatic tissues**.** Computational methods were optimized to adjust for cell free DNA (cfDNA) tumor content (TC) and ploidy and to detect low frequency alterations**.** Median cfDNA TC was 22% [3%-94%] and did not associate with CRPC-NE or adenocarcinoma histology, site or number of metastatic sites, serum prostate specific antigen (PSA) or neuroendocrine markers, or number of prior therapies. Though mutation rates were similar across subgroups, the degree of ploidy and fraction of genome aberrant (CNAF) detected in plasma was highest in CRPC-NE. Prior chemotherapy was associated with higher cfDNA TC, CNAF and mutation rate .

Across tumor tissues, there was greater inter-patient similarity amongst CRPC-NE, irrespective of metastatic biopsy site. We evaluated ctDNA and six sites of metastases from CRPC-NE patient WCM0 at the time of autopsy. In this case, spatially distinct metastases demonstrated average 74% genomic similarity with one another. However, there was differential representation of metastatic lesions in the circulation, with liver metastases contributing more ctDNA compared to other sites. When comparing ctDNA and matched biopsies in individuals with CRPC-Adeno or CRPC-NE, we observed higher genomic similarity rates with respect to both SCNA and SNVs and lower clonality divergence in CRPC-NE. If a plasma sample represents the genomics of all or most of an individual's metastases released into the circulation, a higher concordance between ctDNA and single site biopsy suggests less intra-individual tumoral heterogeneity in CRPC-NE.

Alterations involving *RB1, TP53, PTEN,* and *CYLD* were more common in the ctDNA of CRPC-NE patients, *AR* more common in CRPC-Adeno, and there were no significant differences in DNA repair gene ctDNA aberrations between the subtypes **(Figure 6)**. *SPOP* mutations were absent in CRPC-NE. A cfDNA TC $\geq$50% was significantly associated with worse prognosis across all patients **(**p=0.033**)**. As observed in prior studies, *AR* aberrations were significantly associated with worse OS (p=0.009) in CRPC-Adeno, even when corrected for TC **(**multivariate p= 0.028**)**. Of note, *AR* focal gains could be persistent or heterogeneous when comparing multiple samples in individuals with CRPC-Adeno. The presence of *TP53* and/or *RB1* loss of function alterations in ctDNA of patients with CRPC-Adeno (but not CRPC-NE) associated with worse overall survival (p=0.006) upon correction for TC **(**multivariate p= 0.006**).** Somatic alterations involving *BRCA1, BRCA2,* or *ATM* associated with worse overall survival across the cohort (p= 0.008 multivariate).

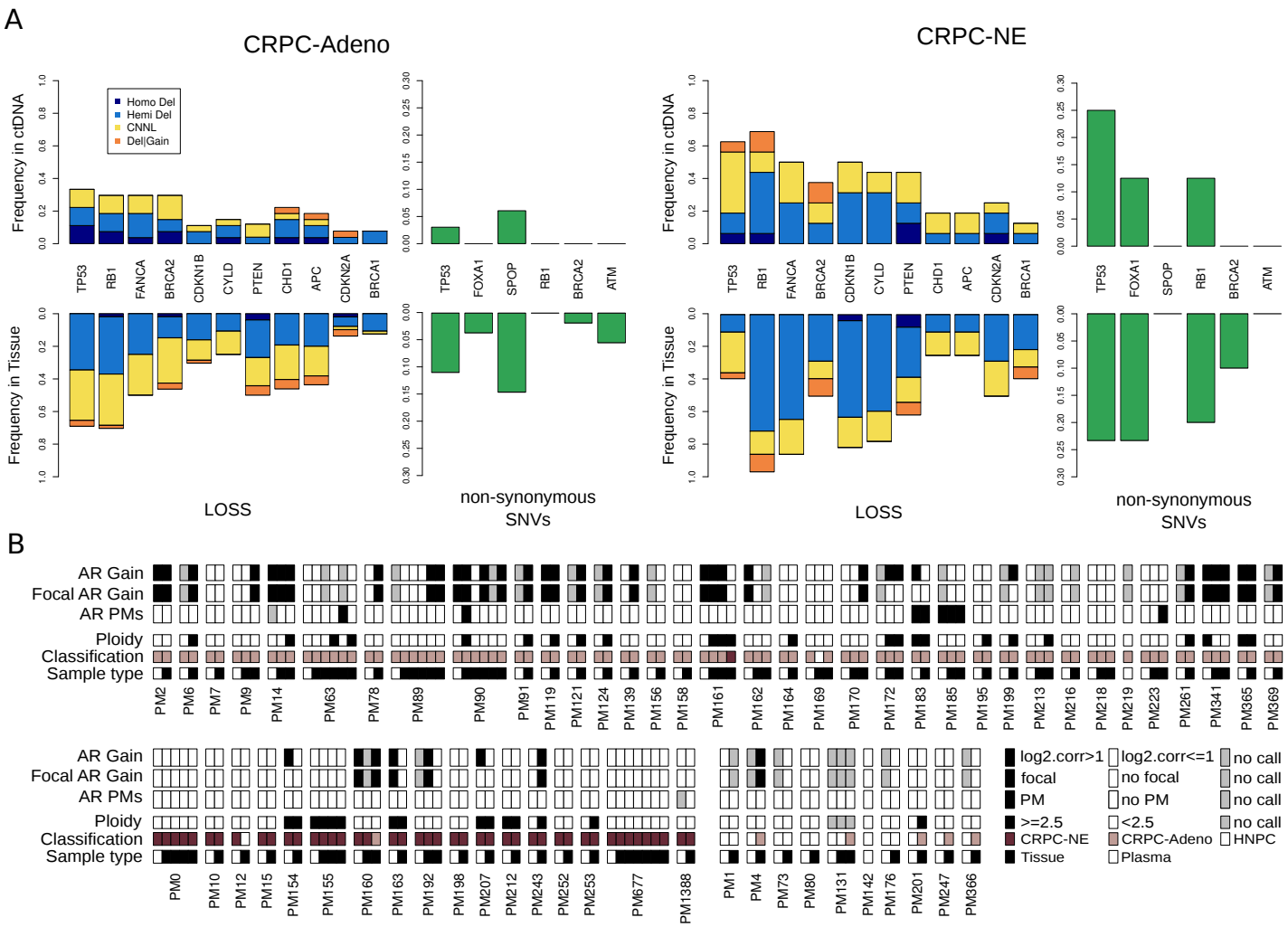


Figure 6: Frequencies of somatic aberrations in advanced prostate cancer driver genes. A) Distribution of somatic copy number loss and SNVs in CRPC-Adeno and CRPC-NE ctDNA and tumor tissue samples. Loss events include homozygous deletions (HomDel), heterozygous deletions (HetDel), copy number neutral losses (CNNL) and events defined by loss of one allele and gain of the other allele (Del|Gain). B) *AR* somatic aberrations status in CRPC-Adeno, CRPC-NE and HNPC plasma and tumor tissue samples. *AR* gain, focal gain and SNV (L702H and T878A positional pileup calls) are shown together with sample ploidy and tumor class.

In order to gain further resolution into the relative contribution of tissue alterations in the plasma, we exploited allele-specific copy number comparison of matched tumor biopsies and ctDNA. In some individuals, these profiles were identical**.** This suggests either the same genomics of all metastases contributing to the circulation, or a predominant release in the circulation from one or more metastases harboring the same profile**.** In other patients, significant differences between allele-specific copy number profiles were observed, suggesting intra-individual metastatic heterogeneity; for instance the liver metastasis of patient WCM183 harbors lesions that are not evident in the plasma sample including extra copies of a set of genes (coordinates (3,2) in the allele-specific space) and different DNA levels of *NKX3.1*. Similarly, the detection of biologically relevant alterations such as *RB1* mutation in WCM163 ctDNA was not observed in the matched analyzed biopsy.

In patient WCM161, we tracked metastatic biopsy time-points during his clinical progression from CRPC-Adeno to CRPC-NE. Interestingly, the plasma sample obtained at time of CRPC-Adeno with only lymph node and bone metastases present displayed a genomic ctDNA profile most similar to the CRPC-NE liver metastasis observed on imaging and biopsied three months later at the time of progression on abiraterone, suggesting the presence of resistant clones in the circulation prior to the development of clinical and pathologic features of CRPC-NE. To delve deeper into tumor dynamics, we assessed serial short-interval time-points in two patients with metastatic castration resistant prostate cancer progressing after multiple lines of systemic therapy. Patient WCM185 is a patient with a rising PSA >3000 ng/ml, bone-only metastases, a clinical picture suggestive of AR-driven disease. Patient WCM14 developed significant progression including visceral metastases despite a non-rising PSA, supportive of AR-independent disease.

We evaluated six serial tumor/plasma time-points for WCM185 collected over three years, with the last three plasma samples obtained approximately weekly during progression. When corrected for ploidy and TC, distinct clones were identified including those with and without *AR* mutations and *TP53* deletions that changed with time, suggesting multiple clones within the circulation with competing frequencies. This fast evolution in tumor clones even in the absence of therapy is consistent with clonal disequilibrium. Notably, his ctDNA demonstrated a high mutation rate not observed in tumor tissue, and he had previously responded to ipilumimab immunotherapy (PSA 72.3 ng/ml to 1.4 ng/ml) on a clinical trial. On the other hand, five serial samples of WCM14 over five years showed consistent alterations including *MYC* gain and *RB1*, *TP53*, *PTEN*, and *BRCA2* losses across all time-points. Notably, loss of *RB1, TP53, PTEN* have been associated with lineage plasticity and CRPC-NE, and were detectable before he had developed any clinical features of AR-independence.

Overall, these data suggest that ctDNA may be dominated by a clone that arises early; in other cases, subclones evolve and also contribute to treatment resistance. By comparing clones with a selective advantage with those clones that are less fit, we may better understand tumor dynamics during prostate cancer progression. In the setting of CRPC-NE, intrinsic drug resistance is likely a major factor that contributes to the development of clonal dominance. However, other biological factors, including a proliferative advantage, the local microenvironment, and epigenetic alterations likely play a concomitant role.

Epigenetic variability contributes to the diversity of phenotypes observed in cancer and other diseases and has been shown to play a key role in differentiation and phenotypic plasticity. We had previously identified significant changes in DNA methylation in CRPC-NE compared to CRPC-Adeno. In order to see if these changes are also captured by ctDNA, we performed whole genome bisulfite sequencing of ctDNA from six patients with CRPC-Adeno and five patients with CRPC-NE and compared these methylation profiles with matched tumor biopsies **(Fig 7).** Overall there was concordance between plasma and tissue methylation genome-wide **(**including differentially methylated regions in CRPC-NE (hypo-methylated and hyper-methylated sites) as well as methylation of specific NEPC classifier genes[2] such as *ASXL3* and *SPDEF***.** Differentially methylated regions in ctDNA were able to distinguish disease subtypes within the cohort regardless of site of matched metastatic biopsy or the number of spatially distinct metastases. When combining the presence or absence of six CRPC-NE associated features that include genomic impairment of *TP53*, *RB1*, *CYLD*, SNV or focal gain of *AR*, and aggregated hypo- and hyper-methylation of previously identified differential sites, these features in the circulation were also capable of identifying individuals with CRPC-NE (P=0.000838).

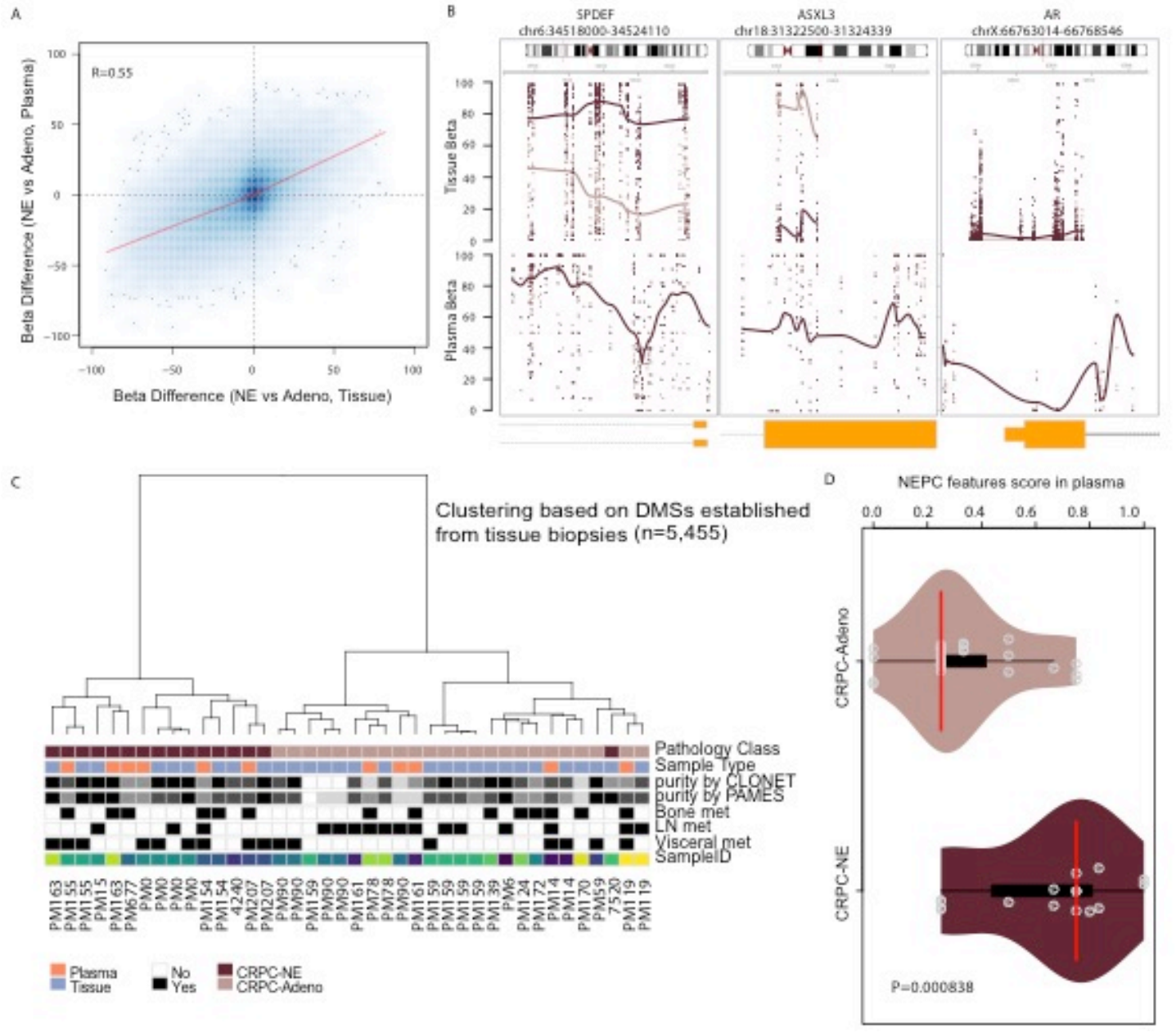


Figure 7: Differential methylation signal is detected in the circulation of patients. A) Genome-wide comparison of methylation patterns in CRPC-NE and CRPC-Adeno as measured in plasma and in tissue samples. The differences of averaged betas are plotted. Red line shows interpolation by the lowess function; R is the Pearson's correlation coefficient. B) Beta values in plasma and tissue samples across portions of three genes of interest. ASXL3 and SPDEF were included in the NEPC classifier from Beltran and Prandi et al[2]. Lines are fitted to CRPC-Adeno and CRPC-NE samples medians of single CpG sites using loess function. Plasma CpG sites were filtered keeping only those presenting at least 2 measurements for each class. C) Ward's hierarchical clustering based on DMRs established from tissue biopsies (Beltran et al, Nat Med 2016) of 39 samples using '1-Pearson's correlation coefficient' as distance measure. Annotation tracks include information on the presence or absence of lymph node, bone or visceral metastases and sample tumor purity. D) NEPC feature scores are plotted as assessed in plasma data of CRPC-Adeno and CRPC-NE patients.

In summary, our ctDNA studies to date have shown that genome-wide analysis of ctDNA is overall concordant with biopsy tumor tissue, but clonal differences between metastases or with time may be appreciated through WES allele-specific analysis. ctDNA was capable of capturing the spectrum of CRPC-NE genomic and epigenomic alterations in patients. We found that CRPC-NE associated alterations may be detectable early during progression.

Aim 3 Progress: I have been evaluating high-risk primary prostate tumors for mutations or other alterations that may predispose to resistance to AR targeted therapy including the development of NEPC (ongoing work). For instance, we compared multiple primaries (high grade adenocarcinoma) and multiple metastases in an individual patient who died from NEPC and had a rapid autopsy case. We reconstructed a phylogenetic tree of all samples and elucidated the clonal evolution. Further, we also evaluated ctDNA from plasma sample obtained before death and performed WES and compared to each metastatic site. On average approximately 50% of mutations found in tumor tissue was present and detectable in ctDNA and higher for liver vs. lymph node. This was performed in order to gain insights into the contribution of each site in the circulation.

In collaboration with Drs. Gleave and Wyatt (Vancouver), we have been evaluating localized prostate cancer samples collected through the Phase 3 CALGB 90203 Trial, "Immediate prostatectomy vs. neoadjuvant docetaxel and androgen deprivation therapy for men with high risk, localized prostate cancer" to assess the impact of therapy on modulation of gene expression of a panel of neuroendocrine prostate cancer (NEPC) pathway signature genes. This includes a gene panel developed and validated by my lab, including AR regulated genes, neural and neuroendocrine marker genes, epithelial mesenchymal genes, cell cycle genes, and others credentialed to distinguish NEPC from prostate adenocarcinoma. This data will be correlated with clinical and pathologic characteristics including AR and neuroendocrine marker immunohistochemistry (IHC), other correlative studies as part of this trial (including ETS fusions status and genomic mutation and copy number profiles), and clinical outcomes (data expected to read out 2018). Strengths of this project include utilization of a novel NEPC signature Nanostring assay that can be performed using limited material from formalin fixed paraffin embedded (FFPE) tissues. RNA expression analyses of neoadjuvant-treated prostatectomy specimens have been challenged in the past due to microscopic residual foci and the necessity for fresh/frozen material. Our NEPC signature Nanostring assay demonstrates significant discrimination between NEPC and adenocarcinoma and has shown high correlation with RNA-seq data(Spearman coefficient 0.9), and therefore represents a significant strength to this study. We evaluated 50 untreated and post-treatment FFPE specimens as well as patient-matched pre-treated needle biopsies and baseline clinical data (Beltran et al, *Clinical Cancer Research 2017*). There was significant upregulation of AR and the ARv7 expression following treatment, as well as a subset of NEPC and EMT genes; three high chromogranin A outlier cases were identified in the treatment arm. There was an overall higher AR score in treated cases (based on expression of 30 AR signaling genes) compared to untreated, along the spectrum of CRPC. These data support the feasibility of quantifying gene expression in neoadjuvant-treated high risk localized PCA cases with limited FFPE tissue requirement. Extensive characterization of AR status and NE/EMT genes identifies molecular outliers that can arise post-treatment and provides new insight into the heterogeneity of treatment response and potential early markers of resistance. We have extended this analysis to 200 samples, integrated RNA data with DNA, and expect to report these results in 2017. The detection and determination of frequency of early NEPC-associated alterations may have significant prognostic and treatment implications in helping identify high-risk, clinically localized prostate cancers as harbingers of resistant disease. During this grant period, we have performed immune profiling pre and post therapy in the same patients (data pending) as well as continue to run cases from this phase 3 trial using the same targeted mRNA panel (data pending).

KEY ACCOMPLISHMENTS:

- Establishment the largest tissue Biorepository of neuroendocrine prostate cancer and patient derived organoids of NEPC (Puca et al, Nature Comm 2018).
- Extensive molecular analysis including whole exome, methylome, and transcriptome sequencing of CRPC-Adeno and CRPC-NE metastatic tumors (and matched primaries) with clinical correlation
- First in field CTC and circulating tumor DNA analysis of NEPC
- Correlative analysis of neoadjuvant treated specimens in the Phase 3 trial CALGB90203 (Beltran et al, GU ASCO 2016) and metastatic samples from the Phase 2 alisertib trial for NEPC

5. **CONCLUSION:** This Award has allowed me to extensively study mechanisms of prostate cancer resistance to AR targeted therapies by performing integrative genomic and epigenomic analyses of metastatic tumors. I have focused on the development of an AR indifferent neuroendocrine phenotype, as this has recently emerged as an aggressive phenotype that is challenging to diagnose and treat. I am using this knowledge to develop biomarkers to improve diagnosis and early detection of patients developing NEPC. I have evaluated CTCs and more recently cell-free DNA in plasma of treated patients at different time points for the emergence of subsets of cells with resistance-associated alterations, as this may serve as a noninvasive method to detect altered genes in an individual patient. I am also looking at high risk localized prostate tumors with and without neoadjuvant therapy for the presence or emergence of NEPC features. With continued work, this project has high potential for further validation and clinical development of biomarkers and could directly influence patient care by identifying patients less likely to respond to subsequent AR –directed therapy and who could be selected for alternative NEPC directed therapeutic approaches. This data has also identified novel drivers of treatment resistance and has nominated therapeutic targets for further preclinical development. This Physician Training Award has greatly facilitated my career development, directly resulting in several academic collaborations, grants, and manuscripts.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS DURING THIS GRANT PERIOD:

(1) Lay Press:

1. Beltran, H. Neuroendocrine Prostate Cancer, *Prostatepedia,* August 2017

(2) Peer-Reviewed Scientific Journals:

Davies AH, **Beltran H**, Zoubeidi A**,** Cellular plasticity and the neuroendocrine phenotype in prostate cancer, Nature Rev Urol, 2018. doi: 10.1038/nrurol.2018.22. [Epub ahead of print]

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(3) Invited Articles:

Ku S, Vlachostergios PJ, **Beltran H***. Genomic Landscape of Prostate Cancer and Opportunities for Precision Oncology. In: Scientific American Urology. Hamilton (ON); 2018. DOI: 10.2310/7900.11093. *corresponding author

(4) Abstracts: List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

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2. Gillessen S, Schmid S, **Beltran H**, de Almeida DVP, Mehra N, Pernelle L, Morales R, Pignatore D, Castro E, Efstathiou E, Le ML, Pezaro C, Suzuki H, Zivi A, Klingbiel D, Omlin A, Platinum-based therapy in men with metastatic castration resistant prostate (mCRPC) with or without DNA repair defects – a multicentre retrospective analysis, ESMO Annual Meeting, Munich Germany, October 2018
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7. **INVENTIONS, PATENTS AND LICENSES:** Nothing to report

8. **REPORTABLE OUTCOMES:** Nothing to report

9. **OTHER ACHIEVEMENTS:**

Recruited to Dana Farber Cancer Institute, Harvard Medical School as Associate Professor of Medicine and Director of Translational Research in Medical Oncology, started September 10, 2018

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11. **APPENDICES:** N/A