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DE DOCTEUR EN MÉDECINE

Suivi longitudinal de l'évolution dynamique de sous-types cellulaires de cancer de la prostate résistant à la castration à partir d'ARN de sang total

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Au Pôle Recherche

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AVERTISSEMENT

La Faculté n'entend donner aucune approbation aux opinions émises dans les thèses : celles-ci sont propres à leurs auteurs.

Table des matières

Résumé

Introduction : Le cancer de prostate (PCa) est une maladie hétérogène. Les décès en résultant sont surtout liés à la phase de résistance à la castration. L'antigène de prostate spécifique (PSA), marqueur utilisé aujourd'hui en routine du diagnostic jusqu'aux stades avancés présente des limites. Il ne reflète pas les modifications tumorales au cours de l'évolution de la maladie et l'impact des différentes lignées thérapeutiques. D'autres marqueurs, comme l'ARN de sang total, sont prometteurs pour répondre à ces limites. Nous avons étudié à partir de prélèvements sanguins l'évolution de sous-types de gènes de cellules épithéliales prostatiques (luminale, neuroendocrine et cellules souches) et leur dynamique au cours de la maladie.

Méthodes : Une étude prospective monocentrique a été réalisée à partir d'ARN de sang total de patients métastatiques et résistants à la castration (mCRPC) prélevé au cours de leur suivi en oncologie. 9 patients avec 2, 3 ou 4 visites de suivi ont été inclus, après une première série de 15 patients avec prélèvement unique, ainsi que 11 volontaires sains. 14 gènes ont été étudiés par qPCR en temps réel (TaqMan). Une régression logistique univariée a été réalisée pour relever la corrélation de ces résultats avec les données cliniques des patients.

Résultats : 7 de ces patients ont présenté une évolution de phénotype au cours de la progression de la maladie. Le phénotype le plus représenté en fin de trajectoire est le neuroendocrinien (55%). La progression sous chimiothérapie était associée négativement avec la surexpression de marqueurs indifférenciées (OR=0,12 p<0,005).

Conclusion : Le renouvellement dynamique de sous-ensemble de gènes épithéliaux de cellules prostatiques issus des tumeurs ou métastases est visualisé à partir de biopsies liquides. Leur utilisation pourrait aider au suivi de la progression de la maladie et au choix du traitements adapté au patient à chaque séquence de la maladie.

Listes des Figures

Liste des Tableaux

Matériel Supplémentaire

Liste des Abréviations

ADT : Hormonothérapie (Androgen Deprivation Therapy)
ADN/DNA : Acide désoxyribonucléique
ADNc/cDNA : ADN complémentaire
AR : Récepteur aux androgènes
ARN/RNA : Acide ribo-nucléique
ARSI : Hormonothérapie de nouvelle génération (Androgen receptor signaling inhibitors)
CK : Cytokératines
CN : Nombre de copies
CRPC : Cancer de prostate résistant à la castration
CTCs : Cellules circulantes tumorales
EMT : Transition épithélio-mésenchymateuse
EpCAM : Molécules d'adhésion cellulaire spécifiques à l'épithélium
FC : X fois plus (Fold change)
L : Luminal
NE : Neuro-endocrine
PCa : Cancer de la prostate (Prostate Cancer)
PSA : Antigène prostatique spécifique
PSMA : Antigène membranaire spécifique de la prostate
RT-qPCR : Réaction de polymérase en chaîne quantitative en temps réel (real-time quantitative Polymerase Chain Reaction)
SC : Cellules souches (Stem-Cell)
SD : Ecart-type (Standard deviation)
UNG : Uracil-DNA glycosylase
V : Visite

Introduction

Épidémiologie

Le cancer de prostate (CaP) est le cancer masculin le plus fréquent en France avec 28% des cas incidents de l'ensemble des cancers. Il occupe le cinquième rang des décès par cancer tous sexes confondus, et le troisième chez l'homme derrière le cancer du poumon et le cancer colorectal (1). Depuis 30 ans, sa mortalité diminue (18,1 pour 100000 habitants à 8,9) (2). Ceci s'explique par l'amélioration de son dépistage précoce et l'amélioration des thérapeutiques. L'âge moyen au diagnostic est actuellement de 70 ans. 1 homme sur 8 recevra un diagnostic de CaP au cours de sa vie. Le CaP pose donc un problème de santé publique de par le vieillissement de la population française.

Histologie

La prostate est une glande exocrine qui fait partie du système uro-génital mâle. Elle entoure l'urètre à la base de la vessie. Elle a une fonction nourricière et de protection du sperme avec une sécrétion de 30% du liquide séminal.

Les acini prostatiques sont constitués de deux couches de cellules épithéliales, les cellules basales et luminales reposant sur une membrane basale et entourées d'un réseau fibromusculaire stromal (**Figure 1**). La grande majorité des cellules luminales expriment le AR (récepteur aux androgènes), sont sensibles à la voie de signalisation des androgènes. Ces cellules sécrètent des protéines spécifiques, tel l'antigène de prostate spécifique (PSA) (3).

Les cellules basales sont alignées entre la membrane basale et la rangée de cellules luminales. Cette couche de cellules abrite également un contingent de cellules souches prostatiques multipotentes capables de prolifération et différenciation (3,4).

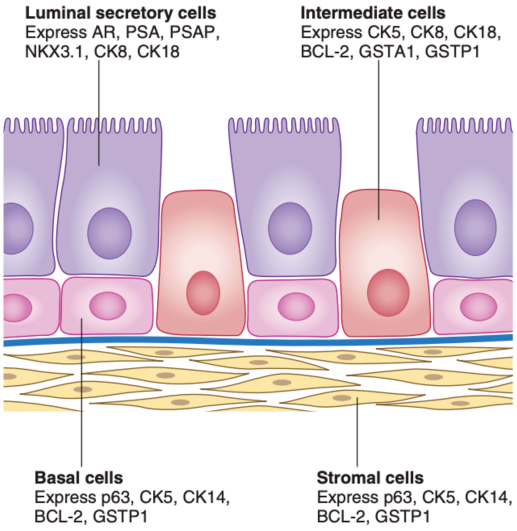

Figure 1. Épithélium prostatique. *D'après Abeloff's Clinical Oncology (2020)*

Les cellules basales et souches survivent en l'absence d'androgènes.

Les cellules neuroendocrines (NE), population la moins nombreuse (<1%) et dispersée entre les cellules épithéliales, sont également androgéno-indépendantes.

Hormonodépendance

Le tissu prostatique est hormono-dépendant. Les hormones stéroïdiennes de la famille des androgènes sont nécessaires au développement de la prostate chez l'embryon ainsi qu'à sa croissance à la puberté. L'androgène le plus abondant est la testostérone synthétisée par les cellules de Leydig dans les testicules, cellules stimulées par l'hormone lutéinisante (LH). La testostérone est convertie en un métabolite plus actif, la 5α dihydrotestostérone (DHT), dans le tissu prostatique par l'activité de la 5α-réductase (3).

La prostate atteint son volume final vers l'âge de 20 ans et reste sous la dépendance de la voie des androgènes toute sa vie.

En effet, la castration ou privation androgénique va entraîner une régression du tissu prostatique. Une apoptose cellulaire rapide se produit conduisant à une involution de

la glande et des cellules androgéno-dépendantes. Mais certaines cellules vont y résister.

Histoire naturelle du CaP

Au stade localisé, le traitement repose sur la surveillance active ou un traitement curatif par chirurgie ou radiothérapie.

En cas de métastases ganglionnaires, osseuses ou viscérales, le paradigme change, et le traitement devient palliatif. Depuis les travaux de Huggins en 1941 (Prix Nobel 1966) sur la sensibilité du tissu prostatique aux androgènes, le traitement repose sur l'hormonothérapie par privation androgénique (5).

La phase métastatique comporte deux périodes successives. Initialement hormono-sensible, la privation androgénique entraîne un contrôle de la maladie dans 90% des cas. Mais dans un délai de 12 à 36 mois, le cancer évoluera de manière quasi-constante vers un stade de cancer de prostate résistant à la castration (CRPC) (malgré un taux de testostérone <50 ng/dL) caractérisé par la reprise évolutive de la maladie et l'élévation du taux de PSA (3).

Hétérogénéité du cancer et mécanisme de résistance

Bien que les CaP présentent une hétérogénéité phénotypique importante, dans plus de 95% des cas il s'agit d'un adénocarcinome de phénotype luminal. Même après transformation maligne, des attributs phénotypiques caractéristiques des cellules luminales sont conservés, incluant notamment l'expression de AR, PSA, PSMA.

L'autre phénotype le plus fréquent est le CaP neuroendocrine représentant moins de 2% du total. Ce dernier, très agressif, est rarement diagnostiqué de novo, ce sous-type histologique est généralement associé à une maladie avancée résistante à la castration (3).

Malgré la réponse initiale, les cellules AR+ sensibles aux androgènes survivant à la castration vont, après quelques années, s'adapter à cette situation de privation androgénique. Il s'agit de la phase de résistance à la castration.

La voie de signalisation AR-dépendante se réactive, malgré l'absence d'androgènes. Plusieurs phénomènes sont proposés comme le montrent la **Figure 4** (6–8):

- Surexpression ou amplification du AR avec activation par de faibles niveaux d'androgènes (**C**) ;

- Expression de variants d'épissages du gène AR (AR-Vs), sans domaine de liaison du ligand dont le mieux caractérisé est AR-V7 (**D**) ;

- Mutation du domaine de liaison du ligand rendant AR constitutivement actif (**D**) ;

- Activation aberrante ligand-indépendante du AR (**E**) ;

- Synthèse endogène d'androgènes (**F**) ;

- Contournement de AR en passant par le récepteur des glucocorticoïdes (GR).

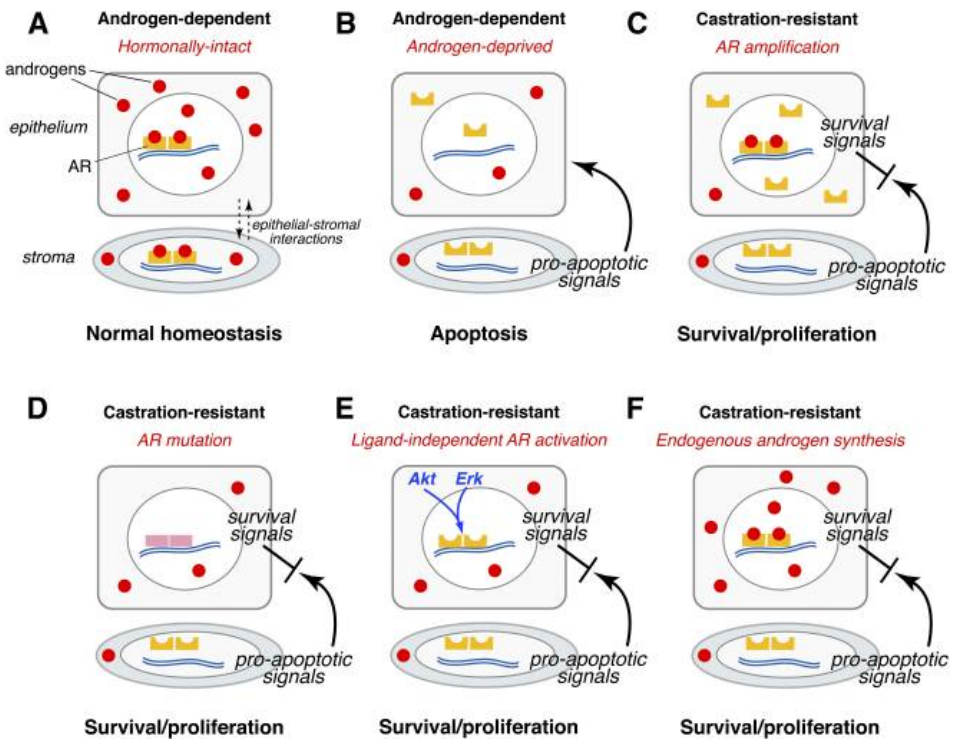

***Figure 2. Mécanismes de résistance à la castration**. D'après Shen et Abate-Shen (2007)*

Cette résistance à la castration peut également être expliquée par la sélection de cellules androgéno-indépendantes échappant à cette privation. Le rôle des cellules neuroendocrines est indubitable dans le cadre d'une différenciation neuroendocrine. Dans certains adénocarcinomes de prostate, on observe des foyers de différenciation neuroendocrine de même que quelques cellules neuroendocrines éparses dans un foyer tumoral (9), plus particulièrement lors de récidive. Il pourrait donc constituer une forme évolutive agressive d'adénocarcinome prostatique par l'intermédiaire d'un mécanisme de transdifférenciation épithélio-neuroendocrine (phénomène de plasticité cellulaire (10).

Par définition, les cellules souches cancéreuses sont indifférenciées et n'expriment pas le AR. Certaines cellules souches seraient impliquées dans la transition épithélio-mésenchymateuse (EMT) (11), un processus complexe menant à une augmentation de mobilité cellulaire via des réarrangements des jonctions ou contacts intercellulaires allant jusqu'à la perte de l'adhésion cellulaire. Durant cette transition, les cellules passent d'un phénotype épithélial à un phénotype mésenchymateux permettant leur migration à distance des foyers cancéreux et leur pénétration dans les vaisseaux sanguins, facilitant ainsi le développement de métastases à distance. L'EMT serait associée à une agressivité du cancer et l'augmentation de la capacité des cellules à migrer (11,12).

Thérapeutique et biomarqueurs

La prévalence des altérations de AR dans le CRPC et la production d'androgènes par les cellules cancéreuses ont conduit au développement d'hormonothérapie de seconde génération ciblant la synthèse des stéroïdes par l'Acétate d'Abiratérone et l'inhibition de la voie de signalisation du AR par l'Enzalutamide (13–16).

Cependant certains patients ne répondent pas à ces hormonothérapies de nouvelles générations, soulignant le caractère hétérogène du CaP et la nécessité de trouver de nouveaux outils afin d'individualiser les traitements.

Le biomarqueur utilisé en routine aujourd'hui est le PSA (1), utilisé tout au long de la maladie du dépistage jusqu'aux stades métastatiques. Cependant son taux ne reflète pas toujours l'évolution de la maladie (17), ni l'hétérogénéité cellulaire prostatique comme peuvent le faire d'autres protéines telles que la Neuro Specific Enolase (NSE) ou Chromogranine A (CGA) (18), marqueurs de différenciation neuroendocrine mais peu utilisé dû à leur manque de sensibilité.

Des travaux ont été entrepris récemment afin de trouver, améliorer et valider des marqueurs moléculaires basés sur des échantillons sanguins et non plus tissulaires. Il s'agit des biopsies liquides (**Figure 3)**, le sang étant le plus fréquemment utilisé pour y dénombrer les cellules tumorales circulantes (CTCs) et les acides nucléiques libres circulants (cfNA) (19,20). Elles ont deux avantages majeurs. Elles permettent de contourner l'aspect invasif de biopsie de métastases de CaP (souvent osseuses) et d'éventuelles complications associées.

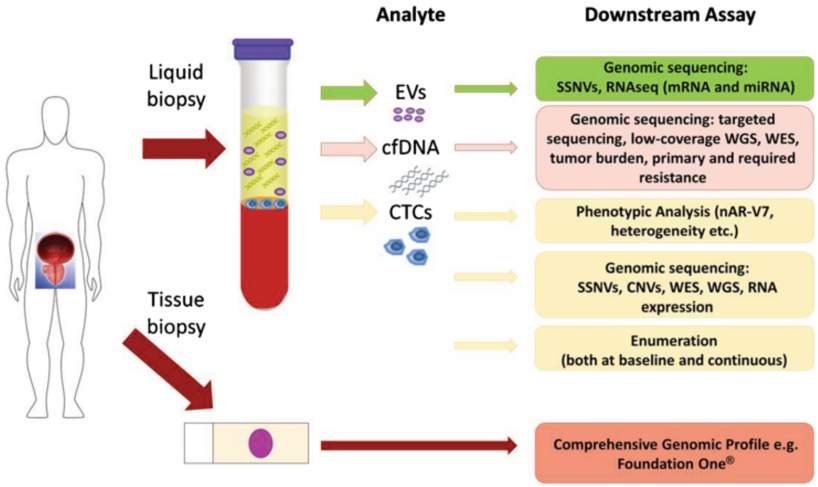

Figure 3. Biopsies Liquides. *D'après Morrison, Goldkorn, Curr. Onc. Rep. (2018)*

Encore peu utilisées en pratique médicale courante, les intérêts majeurs de la recherche sur les biopsies liquides regroupent :

- l'évaluation du risque des rechutes métastatiques ou progression métastatique
- la stratification et la surveillance en temps réel des thérapies,
- l'identification de cibles thérapeutiques et de mécanismes de résistance aux traitements,
- et la compréhension du développement des métastases chez des patients présentant une récurrence.

Nous avons progressivement basculé de l'avènement des thérapeutiques cytotoxiques vers les thérapeutiques ciblées et nous assistons maintenant au développement de la médecine personnalisée. Celle-ci repose sur le postulat que le traitement doit être régulièrement adapté au profil tumoral du patient en se basant sur ses caractéristiques moléculaires.

Les CTCs se détachent activement de la tumeur primaire ou des métastases selon le stade du cancer pour se retrouver dans la circulation sanguine. Leur nombre est lié au pronostic de la maladie métastatique avec une survie totale, de manière plus précise que le suivi par PSA (21). L'intérêt prédictif des CTCs est étudié en se focalisant, non sur leur nombre, mais sur des caractéristiques moléculaires. Cependant, leur identification correcte est souvent discutée et les technologies actuelles pourraient ainsi ne pas dénombrer une importante sous-population de CTCs (22).

L'ADN libre circulant (cell-free DNA ou cfDNA) et ARN libre circulant correspondent à de petits fragments d'acides nucléiques (circulating tumor NA ou ctNA) relargués dans la circulation par des cellules malignes (23). Comparé à des contrôles sains, il existe donc un taux significativement plus élevé de cfDNA chez des patients atteints de cancers (24).

La mesure de l'expression de gènes dans l'ARN de sang total est également utilisée notamment dans le cancer colorectal (25,26), le sein (27) ou le poumon (28) et désormais la prostate (29).

Hypothèses

La résistance à la castration est due à la sélection de cellules androgéno-indépendantes (AR-) telles que les cellules neuroendocrines ou cellules souches indifférenciées et l'adaptation de cellules de type luminal AR+ résistantes à la privation androgénique. L'agressivité tumorale augmente et le phénotype tumoral évolue au cours de la maladie et peut même être différé d'un site tumoral à l'autre chez un même patient.

L'analyse tissulaire du site primitif devient donc peu représentative du stade de la maladie avancée du patient.

Une étude pilote a donc été réalisée à l'institut de recherche du centre universitaire de santé de McGill (IR-CUSM) à Montréal au Canada pour valider l'utilisation de 14 transcrits circulants de 3 sous-types cellulaires d'épithélium de prostate auprès de 40 patients mCRPC permettant de dénombrer 8 phénotypes d'expression génique (Vesval et al).

Objectif

L'objectif de ce travail est d'évaluer l'évolution du profil de cellules cancéreuses (ou leur proportion) libérées des différentes métastases du patient dans le sang, en lien avec la réponse au traitement et la progression de la maladie et non avec l'âge.

Article

Longitudinal study tracks dynamic changes in circulating transcripts representing diverse prostate cell subtypes during prostate cancer progression

(projet de publication)

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Abstract

Background: Prostate Cancer (PCa) is clinically highly heterogeneous. Death is attributed to a metastatic and castration resistance disease. However, prostate-specific antigen (PSA) routinely used today, from diagnosis to advanced stages, has its limitations. It does not reflect changes in the tumor over the course of the disease and the impact of therapies. Other markers, such as whole blood RNA, show promise in responding to this. We have studied genes representing the prostatic epithelial cell subtypes (luminal, neuroendocrine, and stem-cell) in blood drawn from patients and followed dynamic changes in gene patterns longitudinally during disease trajectories as well as the impact of successive therapies.

Methods: Whole blood RNA from metastatic and castration resistant patients (mCRPC) was collected during their oncology follow-up. 14 genes were studied by real-time qPCR (TaqMan). Univariate logistic regression was performed to find the correlation of these results with the clinical data of the patients.

Results: A first series of 15 patients with a single sample was analyzed and then 9 patients with 2, 3 or 4 follow-up visits. 11 healthy volunteers were also included. 7 of these patients showed a change in phenotype during disease progression. The most represented phenotype at the end of the trajectory is the neuroendocrine (55%). Progression on chemotherapy was negatively associated with overexpression of undifferentiated markers (OR = 0.12 p <0.005)

Conclusion: The dynamic turnover of epithelial gene subsets derived from prostate cell subtypes in tumors and metastases is traceable through liquid biopsies. Their use as markers could help to track disease progression and to choose optimal patient-tailored therapies at each step of the disease.

Introduction

In the Western World, Prostate Cancer (PCa) is the most common malignancy in men (30). Despite a fall in mortality in recent decades, PCa remains one of the four most common cause of cancer-related deaths (17). This is largely attributed to the inability to control the metastatic disease. Androgen-Deprivation Therapy (ADT) has been the standard care for advanced cases since the 1940s (5). However, the hormone-sensitivity/dependency of tumor cells is transient and soon or later patients progress to the castration resistant stage (CRPC). The first mechanism responsible of this resistance is the reactivation of the androgenic pathway (7). Accordingly, the development of androgen/androgen receptor (AR) signaling inhibitors (ARSIs) such as Abiraterone and Enzalutamide, together with chemotherapy using taxane derivatives have led to an increase in survival of metastatic castration resistant (mCRPC) patients (14,16). However, some patients do not respond to ARSIs. This underscores the clinical heterogeneity of PCa and highlights the challenges of finding new tools to individualize treatments.

The most widely used biomarker to monitor progression at all stages of disease remains prostate specific antigen (PSA), a protein produced and released in the blood by AR-positive cancer cells with luminal characteristics (31). PSA is reliable to follow patient response to ADT upon recurrence after curative therapies and to some extent, their response to ARSIs in metastatic cases (32). However, levels fluctuate, according to patient response and/or resistance to treatments. As such, PSA may reach very high levels or be barely detectable in the terminal stage of disease (17). This emphasizes the need of new biomarkers, especially at the stage of resistance to castration.

In the last decade, the AR variant V7 (*ARV7*) which lacks the androgen (ligand) binding domain has received great attention. Indeed, the transcript and/or the protein

in circulating tumour cells (CTCs) is predictive of resistance to ARSI therapies (33,34). However, discrepancy (35) suggests that inter-patient heterogeneity may be in cause to explain response to ARSIs.

Another promising candidate is *FOLH1* which encodes for prostate specific membrane antigen (PSMA), a luminal cell surface protein (36) overexpressed in prostatic adenocarcinomas and found at highest levels in advanced cases (37). This luminal marker predicts therapeutic response (38) and outcome (39). Its traceability with radioactive ligands allows for molecular PET imaging, with the detection of metastatic lesions earlier after BCR (40). The signal intensity evolves differently, according to hormone sensitivity and castration resistance (41). Therefore, PSMA imaging has predictive value and holds the promise of diagnosing and staging patients with advanced cases more accurately than conventional imaging. A major limitation is the inter-patient and intra-patient *FOLH1* heterogeneity in metastases (42).

Among promising tools are circulating tumor cells (CTCs), which were first identified in 1869 by microscopy (43). They are predictive biomarkers for tracking advanced PCa cancer by their presence or number in blood (21,44) and by the expression of different nucleic acids, transcripts and/or proteins such as ARV7 (34), TWIST1 and vimentin as markers of epithelial to mesenchymal transition (EMT) (45,46), synaptophysin identifying neuroendocrine differentiation (47), and ABCG2, PROM1/CD133, ALDH1A1, PSCA for cancer stem cells (45). While these observations as a whole underline the heterogeneity of CTCs in advanced PCa, their use in the clinic has been delayed due to technological limitations. Indeed, CTC-enrichment procedures mainly rely on expression of epithelial-specific cell adhesion molecules (EpCAM), some cytokeratins (CK8, CK9, CK19) (48), PSMA (34) and may miss certain cell categories, particularly some small neuroendocrine cells (49). Consequently,

several authors elected to use whole blood RNA as an alternative to measure gene expression in diverse cancers, colorectal (26), breast (27), lung (28) and prostate (29). We opted for this technology to validate the use of 14 specific transcripts to represent the three prostatic epithelial cell subypes (luminal, neuroendocrine and stem-cells) in the blood of patients with advanced disease (Vesval, Wissing, Derderian et al; under revision). We demonstrated that the overexpression of several genes correlates with clinical data. Moreover, bioinformatic analyses of publicly available transcriptomic datasets confirmed that the genes we had selected are preferentially overexpressed in prostatic metastases and to a lesser extent in primary tumors, in comparison to benign tissues.

The present investigation confirms earlier findings in additional mCRPC patients and establishes that cancer but not age matters to detect the overexpression of these luminal, neuroendocrine and stem-cell genes in their blood. Furthermore, substantial changes in overexpression patterns occur in most mCRPC patients followed over time, evolving with further progression. These findings are in favor of a dynamic turnover of cancer cell subtypes and/or proportions released from tumors/metastases into the blood, according to treatment response/resistance and stages of disease. Their monitoring through specific genes in liquid biopsies may help to select the best therapeutic options for a better management of advanced disease.

Materials and Methods

Controls and patients

The study was approved by the Ethics Review Board of the McGill University Health Center (MP-37-2017-3158). Of the 40 patients initially studied, we traced 9 of them who had donated blood in subsequent follow-up visits. A new series of 15 mCRPC patients at 2nd and 3rd lines of systemic treatments was also included along with 11 healthy volunteers with no prostatic disease, nor androgen-based treatment in their lifetime. All participants voluntarily signed an informed consent to donate blood. Clinical data collected from medical records comprise characteristics of primary tumors and metastases, previous and current treatments, clinical and radiological evolution. The identification of samples as patients or controls and patients' characteristics were unknown to the experimenters at the time of assays and revealed only after normalized gene expression had been calculated. Patients' names were anonymized to preserve confidentiality at all times.

Human PCa cell lines

5 human PCa cell lines were used as positive controls. The NCI-H660 (ATCC CRL-124 5813; neuroendocrine, low AR) and 22Rv1 (ATCC CRL-2505; expressing AR and AR-V7) were generous gifts from Dr. A. Zoubeidi (Prostate Centre, Vancouver, BC, 126 CDN) and Dr. M. Tremblay (McGill Goodman Cancer Centre, Montreal, QC, CDN), respectively. The androgen-sensitive/AR-positive LNCaP (ATCC CRL-1740) and androgen-independent/AR negative DU145 and PC-3 (ATCC HTB-81; ATCC CRL-1435) were purchased from ATCC (Manassas, VA, USA). Cells were cultured and passaged according to standard procedures and recommendations.

RNA isolation

Blood was collected at each visit in PAXgene® tubes (Qiagen; Germantown, MD, 138 USA). They were kept at room temperature for 2 hours to allow for cell lysis prior to their transfer at -20°C overnight and storage at -80°C until processing. RNA was extracted automatically with a Qiacube®, using a PAXgene RNA extraction kit as per the manufacturer's protocol, after 2 hours of thawing at room temperature. The RNA integrity number (RIN) and concentrations were determined using a BioAnalyser 2100 (Agilent, Millcreek, ON, Canada) and a Nanodrop ND-1000 (Thermo Scientific, Waltham, MA, USA), respectively. RNA aliquots (70μL) were stored at -80°C. An equal number of the five cell lines was mixed to extract the RNA, using the same procedure.

First Strand cDNA Synthesis

500 ng of total RNA blood, or mix of the 5 cell lines, was reverse transcribed using Maxima H Minus First Strand cDNA Synthesis Kit, with dsDNase (Thermo Fisher Scientific) by following the manufacturer's protocol. Enzyme mediated dsDNA digestion was first performed on each RNA sample (incubated for 2 min at 37°C with dsDNase supplied with the kit in a 10 µL total reaction volume) to ensure elimination of any contaminating genomic DNA. This step was followed by heat-inactivation of the enzyme for 15 min at 65°C. To the same reaction tube, 10 mM dNTP mix, oligo$(dT)_{18}$ primers (25 pmol), and nuclease free water were added for a total reaction volume of 15 μL. Tubes were next incubated for 5 min at 65°C to remove RNA secondary structures. After the incubation, 4 µL 5X RT buffer and 1 µL Maxima H minus enzyme mix were added to the same tubes for a final reaction volume of 20 µL. Reverse transcription was then carried out by incubation for 10 min at 25°C, followed by 15 min at 50°C, and termination by heating for 5 min at 85°C. The synthesized cDNA was stored at -80°C until further use.

TaqMan PCR assays

TaqMan qPCR assays (vs. SyberGreen in the pilot study) were optimized for the 14 selected genes, using RNA from the mix of five PCa human cell lines in line with earlier findings in each cell line.

For each qPCR reaction, 1 μL of cDNA, 5 μL of 2X TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific), 0.5μL of 20X target specific TaqMan assay (Thermo Fisher Scientific) and 3.5μL of nuclease free water were combined for a final volume of 10 μL. Reactions were run in triplicates on 384-well opaque plates (Thermo Fisher Scientific), using CFX384 Touch® qPCR machine (Bio-Rad). After the initial Uracil-DNA glycosylase (UNG) incubation for 2 min at 50°C followed by polymerase activation for 20 seconds at 95°C, the reaction mixtures were subjected to 65 cycles of amplification using the following sequence: 95°C for 3 seconds and 60°C for 30 seconds. Fluorescence levels were registered at the end of each cycle. CFX Maestro 1.1 software (v4.1.2433.1219) was used to quantify gene expression. Three reference genes, *PGK1* (Phosphoglycerate Kinase 1), *RPLP0* (Ribosomal Protein Lateral Stalk Subunit P0) and *PPIB* (Peptidylprolyl Isomerase B) were tested for each sample.

A 4-fold RNA serial dilution series from the mixture of PCa cell lines was used to generate positive control cDNA. This also served as an inter-run calibrator of the qPCR assays on each plate. Water was used for no-template negative controls. Standard dilution curves from known amounts of genes were generated to calculate absolute transcript copy numbers (CN) for each gene. Geometric mean of CN from the three reference genes was used to calculate normalized-CN of target genes in each sample, including healthy controls. Relative fold change (FC) of target gene expression in each sample was then calculated by a ratio of normalized CN in the sample versus average of normalized CN from all the healthy controls.

Statistical analyses

A gene was considered overexpressed in a patient blood if expression was higher than 2.58 times standard deviation from the average gene expression (mean+2.58 SD) in blood RNA samples from volunteers. This would ensure with 99.5% certainty that expression of target genes in patients is significantly higher than that of healthy controls.

To study correlation between age and expression, the coefficient of determination R^2 and a Pearson's correlation were used.

To detect whether patient's or treatment characteristics were associated with overexpression of individual markers, all characteristics were dichotomized, and differences were evaluated using Chi-square tests in 2x2 tables. PSA levels were compared between patients that did and did not overexpress *KLK3* using the Wilcoxon rank-sum test, due to the non-normal distribution of PSA levels (as evaluated using a Shapiro-Wilk test). Multi-level, mixed effects logistic regression was used to identify variables that were associated with overexpression of luminal, neuroendocrine and stem cell markers, in order to adjust for repeated measurements within individuals. Survival analyses for disease progression and death were performed using the Cox proportional hazard model. If no event had occurred, patients were censored at the last date they were known to be alive and/or without disease progression, either clinical (worsening of symptoms: fractures, pain), biological (PSA rising in two subsequent measurements), or radiological (new lesion or increased size of existing lesions). Results with $p<0.005$ were considered significant. All statistical analyses were performed using Stata v16.1.

Results

Prostate cancer but not age matters to detect circulating genes representing prostatic epithelial cell subtypes in mCRPC patients

Prior to studying longitudinal gene patterns representing prostate cell subtypes during PCa progression, we included a new series of 15 mCRPC patients and 11 healthy men as controls to validate the overexpression of the same 14 circulating genes by the TaqMan technology. The age varied from 52 to 85 years old for patients and ranged between 24 to 71 years old for volunteers. The blood RNA of all blood samples was of suitable quality for RT-qPCR, with a mean RIN of 8.96+/- 0.67 for samples from patients (n=37: series of 15 new patients and 9 others followed at different time points) and 8.3 +/- 0.72 (n=11) for controls. There was no significant difference between the groups (p=0.07). Calibration curves (positive controls of RT-qPCR assays) were generated in parallel for every gene (listed in **Table 1**) using RNA from a mix of LNCaP, 22Rv1, DU145, PC-3, and NCI-H660 cells.

Table 1. List of genes selected to represent prostate cell subtypes.

Name of Gene	Encoded Protein	Function
Luminal		
AR (wild-type)	Androgen-receptor full length	Transcription Factor (TF)
ARV7	Androgen receptor variant 7	TF
KLK3	Prostate Specific Antigen (PSA)	Protease/Kallikrein
FOLH1	Prostate Specific Membrane Antigen (PSMA)	Cell surface protein
Neuroendocrine		
ENO2	Neuron Specific Enolase (NSE)	Marker neuronal destruction
SYP	Synaptophysin	Transmembrane proteine of synaptic vesicles
VEGFA	Vascular Endothelial Growth Factor-A	Angiogenesis, PCa cell motility
NCAM1	Neural Cell Adhesion Molecule	Cellular surface glycoprotein
Stem cells		
ALDH1A1	Aldehyde Deshydrogenase	Catalyzes the oxidation of aldehydes
EZH2	Enhancer of zeste homolog 2	Histone methyltransferase
CD44	Cluster of differenciation 44	Stem cell-surface glycoprotein
POU5F1	Octamer-binding Transcription factor (OCT4)	TF in germinal cells
NANOG	Nanog Homebox	TF of pluripotency of stem cells
SOX2	SRY/Sex Determining region Y-box 2	TF for self-renewal of embryonic stem cell

The lack of correlation between age and expression of the circulating 14 genes representing each cell subtype is shown in **Figure 4** and **Supplemental (S) Figure 1** for controls and patients. As expected, there was no signal for *FOLH1* and *KLK3* (**Fig.4** and **S1**) in all controls and in 9 of them for *ARV7* (the 2 *ARV7*+ controls were < 45years old) (**Fig. 4**). A low signal was detected for the remaining 11 genes in all controls.

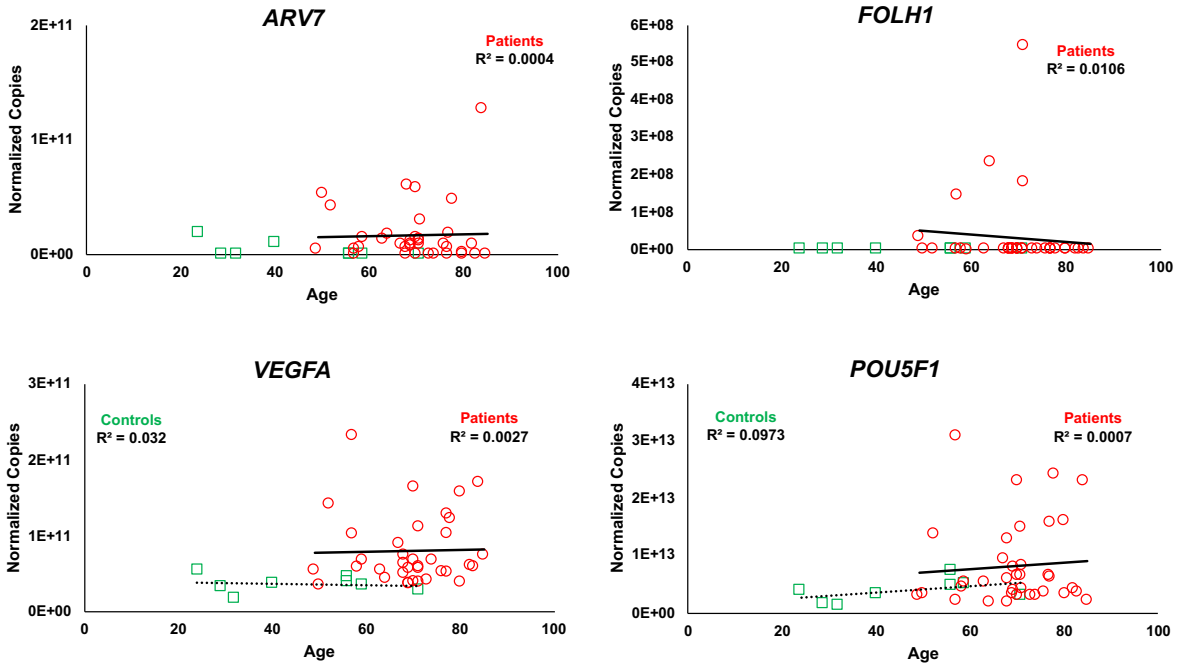
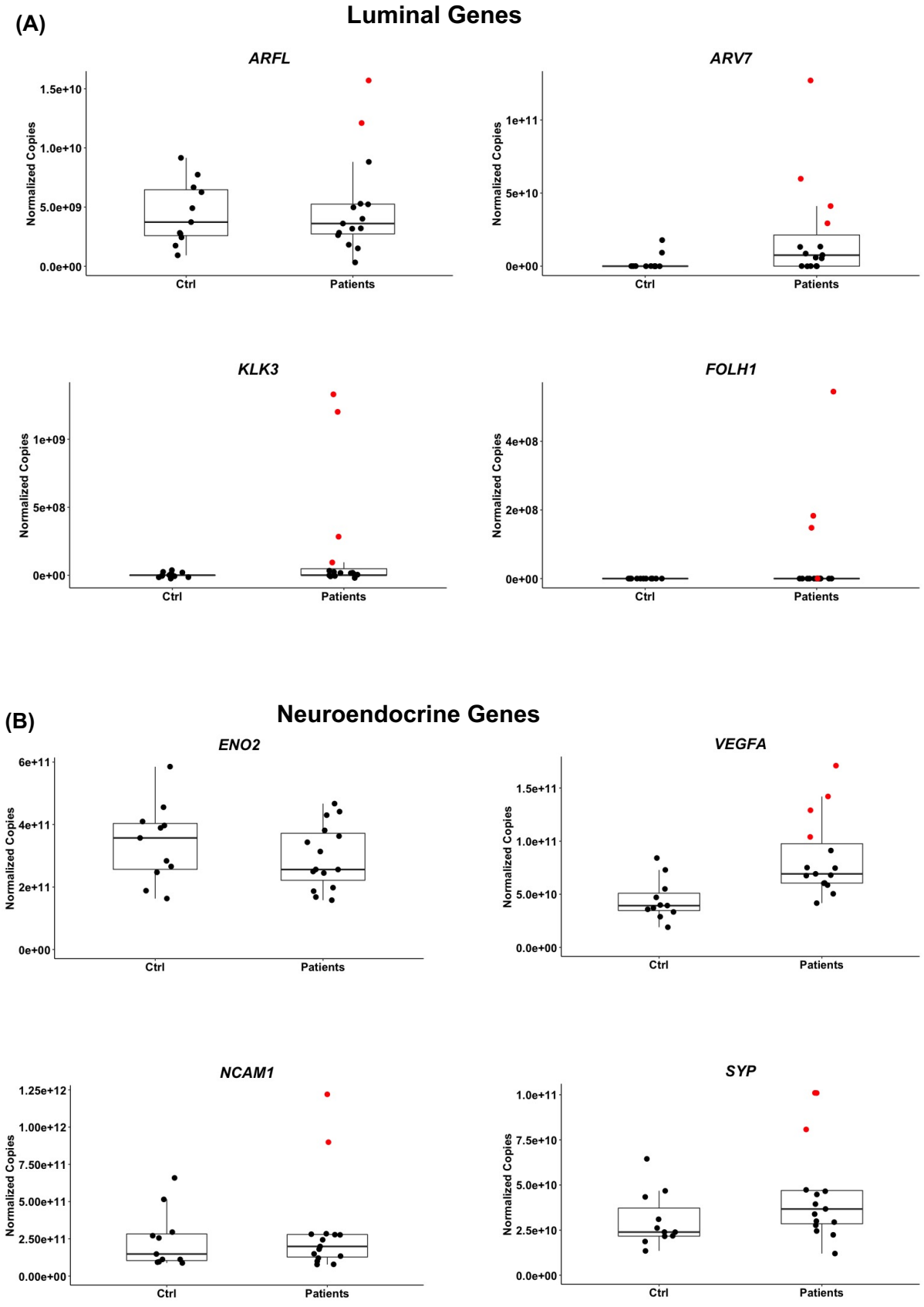


Figure 4. Expression of genes by age.

The 14 gene panel was assayed in the blood RNA of mCRPC patients and healthy males as controls. The normalized copy number of genes is shown for 4 representative genes, with red dots for patients and green squares for controls. Regression lines show lack of correlation between data, with their R^2 coefficients.

Results on overexpression of circulating genes in patients are shown by box plots in **Figure 5 (A-C)**, based on thresholds established for each gene in controls (Supplementary Table 1). All luminal genes were overexpressed, with *FOLH1*, *KLK3* and *ARV7* in 4 (26.7%) patients each, and *ARFL* in 2 cases **(Fig. 5 A)**. Of the NE transcripts, *VEGFA* was the most frequently overexpressed gene in 4 patients (26.7%),

followed by *SYP* and *NCAM1* in 3 (20%) and 2 (13.4%) patients, respectively. Circulating *ENO2* was not overexpressed in any patient **(Fig. 5 B)**.



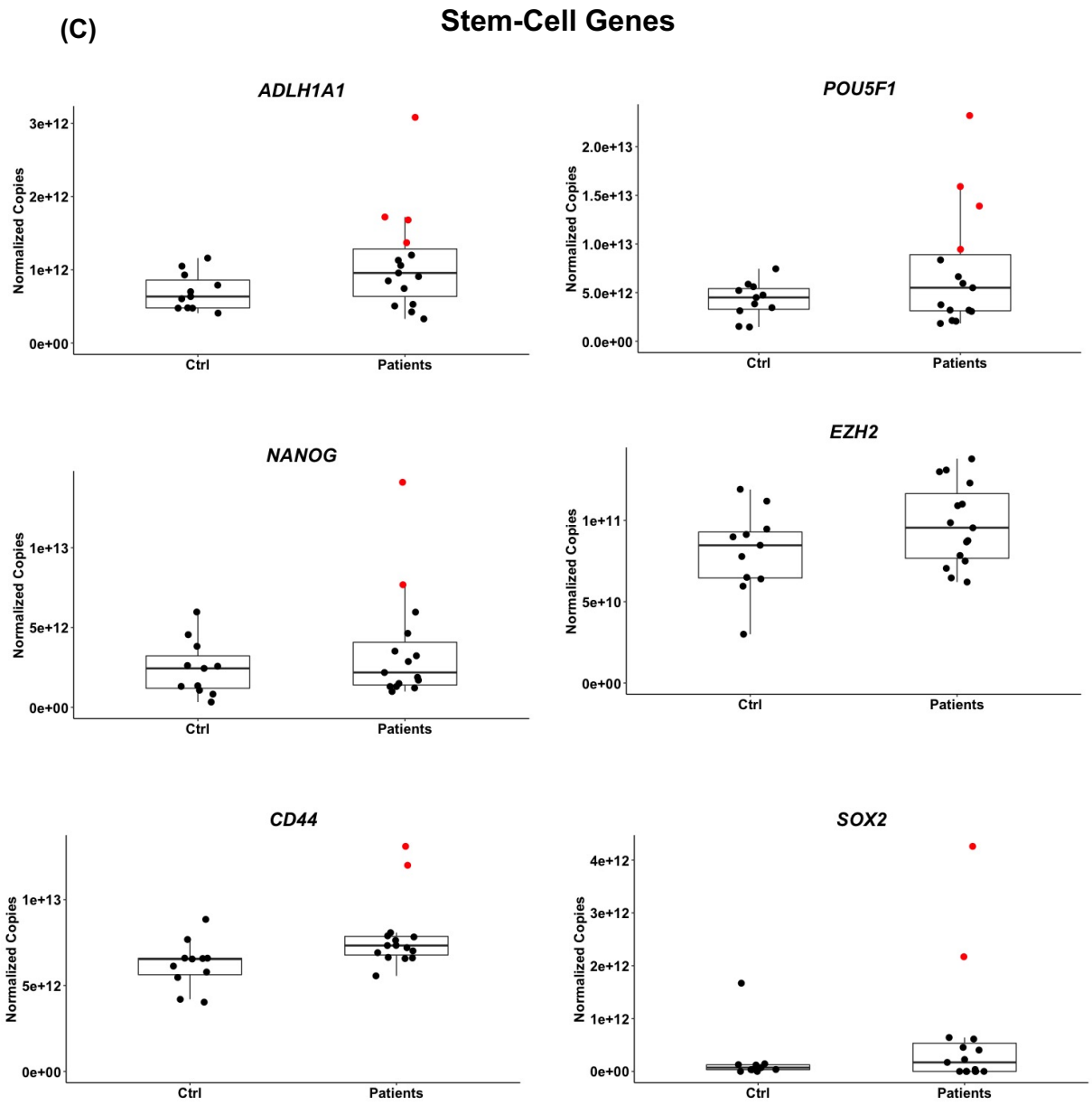


Figure 5. Expression of selected transcripts in liquid biopsies of mCRPC patients.

*The 14 gene panel was assayed in the blood RNA of mCRPC patients and healthy males as controls. The normalized copy number of Luminal (**A**), Neuroendocrine (**B**) and Stem cell (**C**) genes is shown in box plots for patients and controls. Red dots represent the overexpression of specific genes in certain patients, based on the threshold defined for each gene by values greater than means of controls + 2.58SD (Table S1) at a confidence interval of 99.5%; black dots represent expression below the cut-off values for each gene. Some genes (KLK3 and FOLH1) are not detected in controls (no signal) and in subsets of patients.*

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Regarding stem-cell genes, *POU5F1* and *ALDH1A1* were the most overexpressed, with 4 patients each (26.7%), followed by *SOX2*, *CD44* and *NANOG* with 2 cases each (13.3%). *EZH2* was not overexpressed in these patients **(Fig. 5 C)**. Blood PSA is associated with the expression of the *KLK3* genes in the blood RNA, as shown in **Figure 6**. Altogether, 12 of the selected 14 genes were overexpressed at least once in the blood of the newly enrolled 15 mCRPC patients.

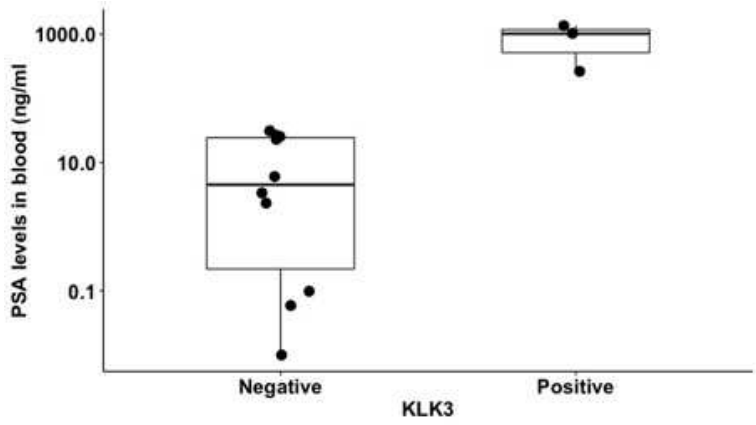


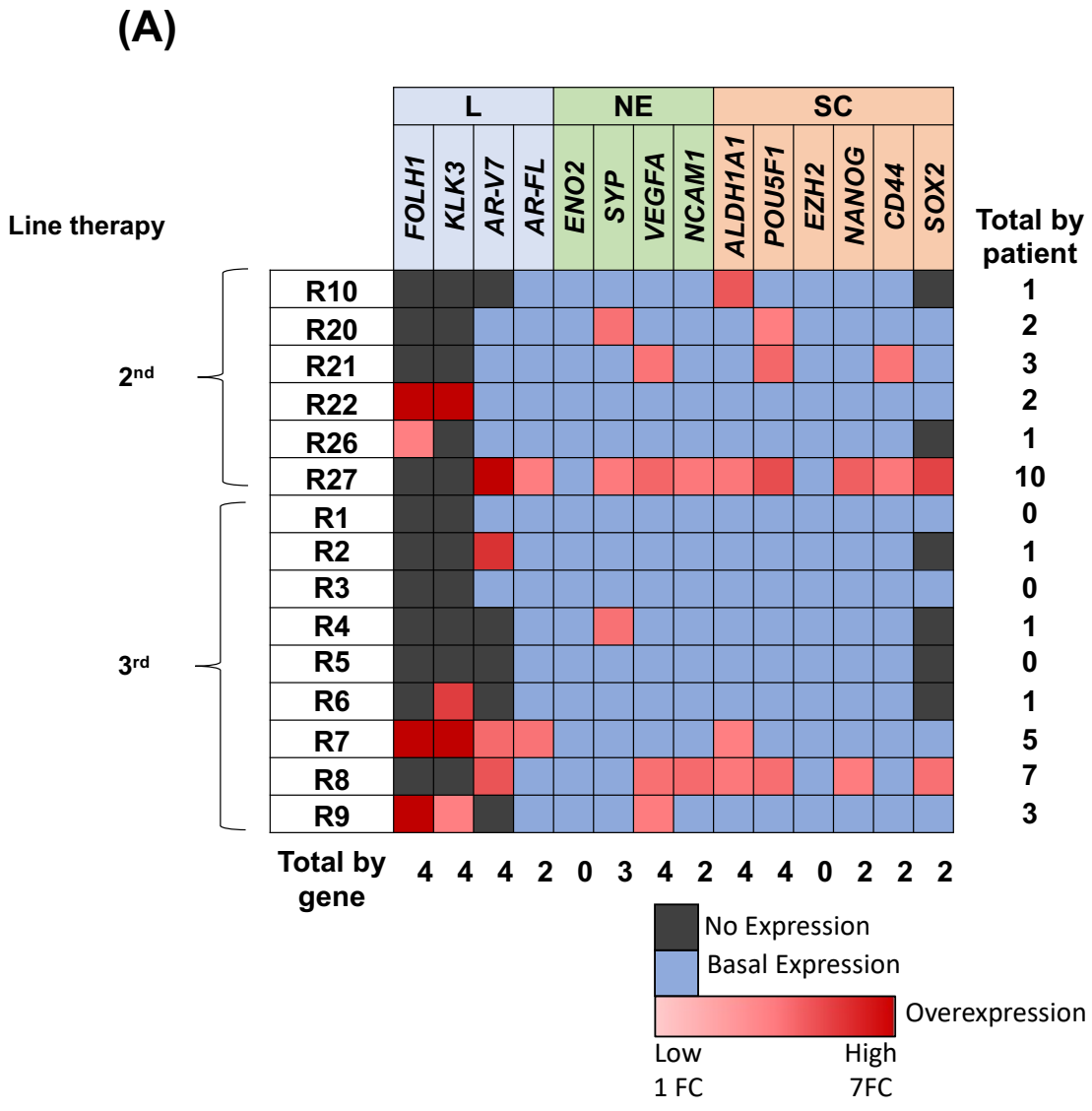
Figure 6. Association between the circulating KLK3 transcript and PSA levels (logarithmic scale) at time of blood draw.

Median 636.5 ng/mL (interquartile range 193.5-1100.5 ng/mL) vs. 6.1ng/mL (interquartile range 1.2-25.5 ng/mL), p<0.005. The p-value was calculated using a Wilcoxon rank-sum test.

The intensity and frequency of overexpression is illustrated by the heatmap in **Figure 7A**. 12 patients (80%) overexpressed at least one of the 14 genes tested in their blood. Luminal genes were the most common, with at least one in 8 patients (53.3%). Neuroendocrine and stem-cell transcripts were overexpressed in 6 patients (40%) each. No patient overexpressed all markers of a cell subtype. 2 patients overexpressed the highest number of circulating genes (7 or 10) distributed among the 3 cell subtypes. A total of 12 unique patterns was observed.

Phenotypic diversity is represented by a pie chart (**Fig. 7B**), with patients forming 8 subcategories. The largest group consisted of patients overexpressing circulating genes of the luminal subtype (27%), followed by a mixed phenotype of all 3 subtypes and a combination of stem cell/neuroendocrine genes with 13% of patients each. The next categories involved the stem cell and neuroendocrine subtypes, as well as their combination with the luminal subtype in one patient each (7%).

The 3 patients not overexpressing any of the 14 selected genes (20%) are under the none-category.



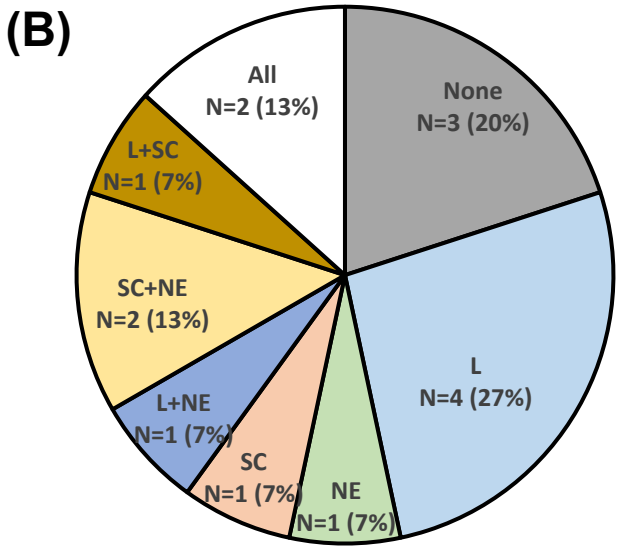


Figure 7. Heterogeneity of circulating PCa cell-derived transcripts in mCRPC patients. *Each gene was analyzed and represented for the 15 enrolled CRPC patients. (**A**) Heat map with black squares representing no signal, blue squares for baseline expression, and white to dark red squares for overexpression, defined by expression greater than means of controls + 2.58SD. Results are presented by fold change from average in patients for the luminal (KLK3, FOLH1) genes which are completely negative in control samples. For the remaining 12 genes, results are presented as fold change from the threshold for overexpression. (**B**) Pie chart representing phenotypic diversity by cell subtypes and combinations thereof (L: Luminal, NE: NeuroEndocrine, SC: Stem Cells), and the percentage of patients overexpressing at least one of the 14 genes for each category.*

Statistical analyses revealed that patients previously treated with taxanes were less likely to overexpress neuroendocrine and stem-cell markers (odds ratio (OR)=0.13 and p<0.005) (**Table S3**). No other significant association was found between the overexpression of a particular gene with patient's characteristics (listed in **Table S2**), lines of treatment, response/resistance and outcome.

The expression of transcripts representing prostate cell subtypes in the blood is stable over time in healthy donors

To further substantiate the above results in patients compared to controls, we assayed the 14 genes set in blood collected and banked at different time-points as a series for each individual, not knowing the sample origin (volunteers or patients) and clinical characteristics. **Figure 8** shows the results obtained in a control who has donated blood over a period of 16 months. As expected, no signal for *KLK3*, *ARV7* and *FOLH1* was observed at all time-points. The levels of each of the remaining 11 circulating genes did not overlap the 2-fold change of the mean of other controls. Similar results were obtained in 2 other volunteers. As a whole, these findings indicate that basal levels of circulating genes reflecting prostate cell subtypes are stable over time in healthy donors.

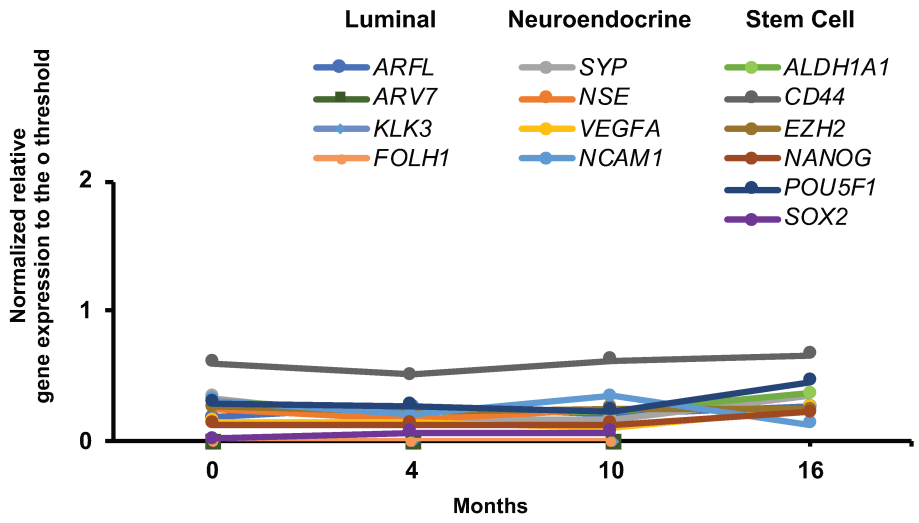


Figure 8. Trajectory of gene expression in a control followed longitudinally.

Each gene was analyzed at four time points in a control over a period of 16 months. The normalized relative gene expression to the threshold for overexpression of each gene of Luminal, Neuroendocrine and Stem cell genes is shown in line graphs for control over time. Each color is corresponding to a particular gene

The pattern of circulating genes overexpressed in mCRPC patients evolves during disease progression

Table 2. Patients' characteristic of the longitudinal series.

	Patients	Ctrl
Number	9	11
Age at inclusion, median (range), years	69 (49-80)	56 (24-71) p<0,02
PSA at V1, median (IQR)	5,13 (0,01-83,12)	
PSA at V2, median (IQR)	2,38 (0,01-97,65)	
Gleason at biopsy		
6	1 (11%)	
7	3 (33%)	
8	3 (33%)	
9	2 (22%)	
Initital treatment at diagnosis		
curative treatment	5 (56%)	
surgery	2 (40%)	
radiotherapy	3 (60%)	
initially metastatic disease	4 (44%)	
ADT	2 (50%)	
ADT + RT	0 (0%)	
ADT + CT	2 (50%)	
Time, median, IQR (month)		
Time between dg and CRPC	20 (2-58)	
Time between CRPC and inclusion	98 (10-216)	
Metastatic sites		
None	0 (0%)	
Bone only	5 (56%)	
Visceral only	1 (11%)	
Both	3 (33%)	
Line of CRPC therapy		
1	4 (44%)	
2	2 (22%)	
3	3 (33%)	
Previous treatment		
ADT only	0 (0%)	
AR-inhibitor	9 (100%)	
Abiraterone	7 (78%)	
Enzalutamide	2 (22%)	
Chemotherapy	5 (56%)	
Docetaxel	5 (100%)	
Cabazitaxel	0 (0%)	
Dead	5 (56%)	

ADT: Androgen-Deprivation Therapy; AR: Androgen Receptor; CRPC: Castrate-Resistant Prostate Cancer; IQR: Inter Quartile Range (Q25-Q75); PSA: Prostate Specific Antigen; V: Visit

The characteristics of the 9 mCRPC patients followed over time are displayed **in Table 2**. Briefly, 5 received curative therapies for a localized disease and four were metastatic at diagnosis. As of now, five had died from PCa, irrespectively of initial therapies. Given the extensive heterogeneity among patients when reaching advanced stages of disease, we draw the clinical trajectories of the 9 mCRPC prospectively followed over time, indicated time-points of blood collections (visits) and presented the genes detected above the threshold in such blood samples by prostate cell subtypes. **Figure 9** shows the results obtained for 4 of them, chosen to illustrate the diversity and unique features of these patients. Patients 2 (**Fig. 9A**) and 5 (**Fig. 9B**) are classic cases of low/intermediate-risk PCa undergoing radical prostatectomy and whose cancers recurred after a certain number of years, became resistant to ADT and metastatic, further progressed and died from PCa. Their blood samples were obtained at 2 late visits in trajectories that spanned over 18 and 24 years, respectively. Patient 2 (**Fig. 9A**) was under Abiraterone when his blood was collected at his 2 visits (V)/blood draws. At V1, one luminal gene *KLK3* and one stem-cell gene *ALDH1A1* were overexpressed. One year later at V2, changes were seen in his luminal genes, with *KLK3* levels being increased by 5-fold and detection of *FOLH1* expression, while the stem-cell gene *ALDH1A1* remained significantly overexpressed. He died 20 months later with a blood PSA at 60ng/mL. Consequently, Patient 2 belongs to the mixed Luminal/Stem Cell category with an enrichment in luminal genes (predominance of *KLK3)* as his cancer further progressed.

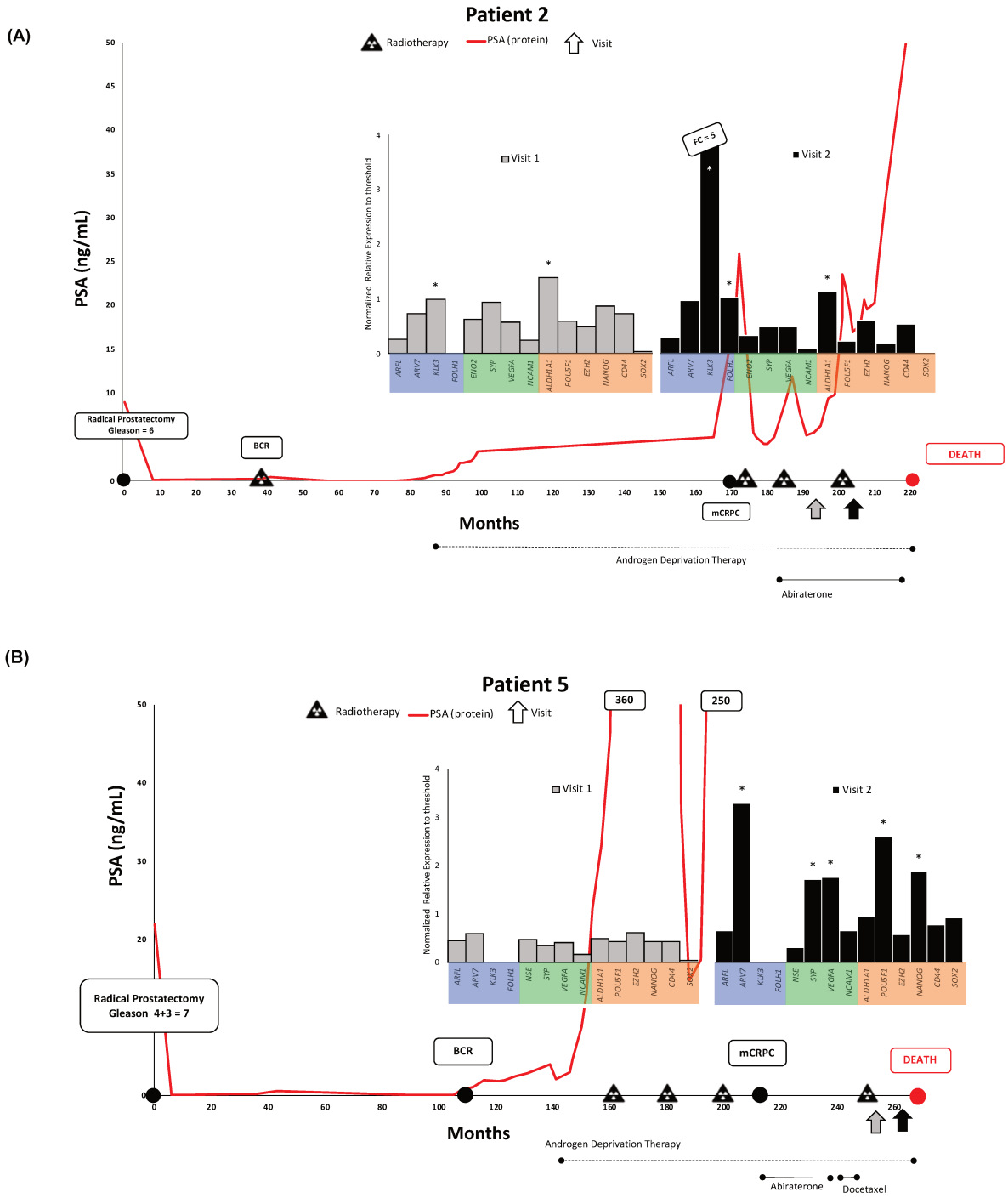


Figure 9. Clinico-biological trajectories of patients.

Figure showing the disease trajectory for patient 2 (A), patient 5 (B), patient 6 (C) and patient 4 (D). The line graph representing PSA kinetics. Histograms representing the normalized relative expression at the threshold of each gene at each visit; stars corresponding to an overexpressed gene. Black dots on trajectories represent diagnosis, BCR or CRPC; red dots indicate death; radiotherapy icon for irradiation of the prostate or metastasis; arrows for visit; black line under the graph for the treatment sequences.

Patient 5 was at a later stage in his trajectory when his blood samples were collected, 10 months apart. He had received radiotherapy of metastatic lesions just before the first draw. The second sample was drawn at a time-point closer to death. His blood PSA was then at 250 ng/mL. At V1, *ARV7* was detected. It was overexpressed by several folds in V2 (**Fig. 9B**). At this time-point, genes of both the NE (*SYP, VEGFA*) and SC (*POU5F1*, *NANOG*) subtypes had become significantly overexpressed. Thus Patient 5 belongs to the mixed Luminal/Neuroendocrine/Stem-Cell category, with circulating transcripts of all prostate cell subtypes being observed with a predominance of *ARV7*.

Patient 6 also had a curative treatment, notably radiotherapy (**Fig. 9C**). Three blood draws were obtained. He was on Enzalutamide for the first two samples, at times when his blood PSA remained near 15 ng/mL. Only *ARV7* and *EZH2* were overexpressed at V1. One year later, the *ARV7* overexpression had not only increased by 2 folds, but additional transcripts of the NE (*SYP*, *VEGFA*, *NCAM1*) and SC (*POU5F1*, *NANOG*, *SOX2*) subtypes were overexpressed at elevated levels. 2 years later at V3, the levels of all genes had drastically decreased to levels even lower than at V1, except for *SYP* which remained the sole overexpressed gene and we observed no more *ARV7* signal. Therefore, the circulating gene patterns of this still alive mCRPC Patient 6 changed over time, being at first in the mixed of Luminal/Stem-Cell category, which evolved towards a mix category where more stem-cell genes were overexpressed and included the Neuroendocrine subtype represented by several overexpressed genes. The patient had been on a Docetaxel chemotherapy for 4 months when the last draw was obtained. The sole overexpressed circulating gene in this sample was of the NE category.

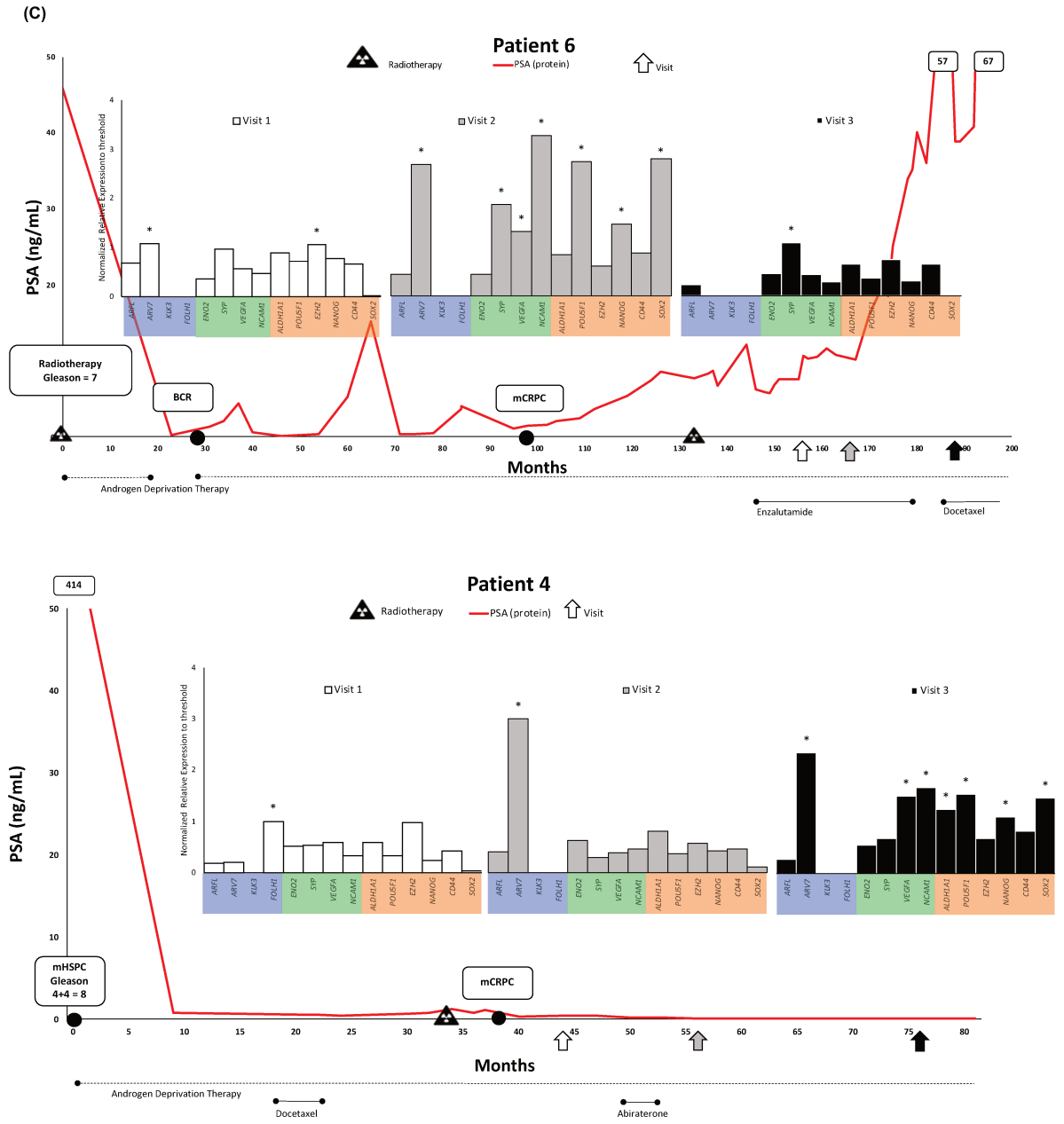


Figure 9. Clinico-biological trajectories of patients.

Finally, Patient 4 was metastatic at diagnosis. His trajectory and circulating gene patterns observed in his three blood samples are shown in **Figure 9D.** He was on ADT with a low blood PSA and had received Docetaxel chemotherapy 8 months before his first blood draw. *FOLH1* (PSMA) was the only overexpressed transcript in V1. 6 months later and after 2 months of Abiraterone prior to the second blood draw and a

low PSA, a marked increase in *ARV7* expression with no *FOLH1* signal were observed. In V3 (20 months later), without any change in therapy and a PSA close to zero, the overexpression of *ARV7* was still observed along with several transcripts of the NE (*VEGFA*, *NCAM*1) and SC (*ALDH1A1*, *POU5F1*, *NANOG*, *SOX2*) subtypes. Therefore, the circulating transcripts of this still alive patient changed from the Luminal category to become a mixed category with the appearance of Neuroendocrine and Stem-Cell genes, despite no apparent clinical signs of disease worsening.

Circulating gene patterns of mCRPC patients become enriched in neuroendocrine and stem-cell genes during progression

The circulating gene patterns of mCRPC patients tested after their enrolment (40 cases of pilot study and **Fig.7** the above 15 new cases) were enriched in luminal transcripts. However, the situation appears to differ in the 4 mCRPC patients presented above and tested at 2 or more visits during their trajectories. A heat map was generated to combine the frequency and intensity of each transcript overexpressed in the blood RNA samples of the 9 mCRPC patients followed over time (**Fig. 10A**) and a swimmer plot to integrate treatments and cell subtypes at different time-points during progression (**Fig.10B**). The heat map shows that the sole case not overexpressing any of the genes of interest was Patient 1 (11%; 1/9), who was already metastatic at diagnosis and had a short trajectory (**Fig.10B**). With this data set, it was not possible to ascribe him one of the categories obtained by combining genes of the 3 cell subtypes. 2 genes, *ARFL* and *ENO2*, were not overexpressed in any blood sample of the other 8 patients. The remaining 12 genes were overexpressed at least once, and no patient overexpressed all genes. Up to 7 genes were overexpressed in a patient at a given time.

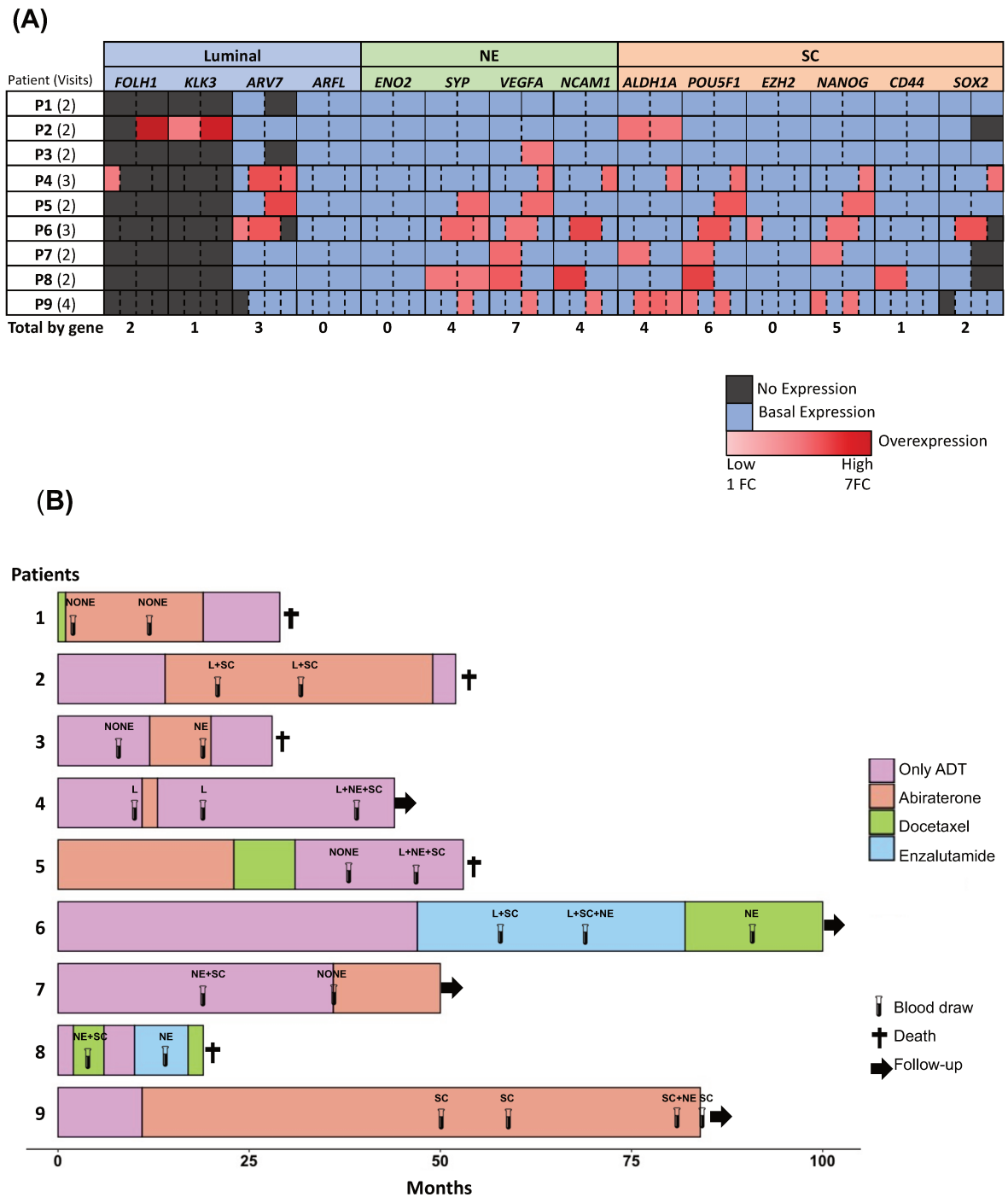


Figure 10. Heterogeneity of circulating PCa cell-derived transcripts in the longitudinal series. *Each gene was analyzed and represented for the 9 followed CRPC patients at each visit. (**A**) Heat map with black squares representing no signal, blue squares for baseline expression, and white to dark red squares for overexpression, defined by expression greater than means of controls + 2.58SD. Results are presented by fold change from average in patients for the luminal (KLK3, FOLH1) genes which are completely negative in control samples. For the remaining 12 genes, results are presented as fold change from the threshold for overexpression of each gene. (**B**) Swimmer plot indicating treatments that patients received, along with timing (and phenotype status according to overexpressed genes) at each blood draw since the date of CRPC to end of follow-up. L: Luminal; NE: Neuroendocrine; SC: Stem-Cell*

The most overexpressed transcripts were *VEGFA* and *POU5F1* as neuroendocrine and stem-cell genes, respectively, and observed in the same 6 patients.

The heatmap and swimmer plot show changing profiles over time, gene by gene in patients and categories or phenotypes emerging when combining genes of the three cell subtypes over time. Indeed, the exact same level of a particular transcript was rarely observed during consecutive visits. As a whole, luminal markers were overexpressed less often than neuroendocrine and stem-cell genes. They were detected in 4 patients during follow-up. *ARV7* ranked first, it was observed in all patients, overexpressed in 3 patients, the overexpressed signal being detected in consecutive visits of 2 cases and the 2nd visit in the third one. *FOLH1* came next, being observed at both visits in 2 patients. *KLK3* was detected in a single patient at his 2 blood draws. Beside *ENO2*, all other NE transcripts, *SYP*, *VEGFA* and *NCAM1* were overexpressed in 87.5% of patients (n=7). *VEGFA* was overexpressed in 7 of them. Stem-cell transcripts were also overexpressed in 87.5% (n=7) of patients. *POU5F1* was the most frequent (in 6 cases) followed by *NANOG* (in 5 cases), with similar patterns over time, twice at the first or last visits and fluctuating in overexpression between visits in one case. *ALDH1A1* was overexpressed in 4 patients, and in 2 of them it was the only or the predominant stem-cell gene observed at 2 or 3 visits. *SOX2* was overexpressed once in 2 patients, at the last visit in 1 and the second of 3 visits in the other. *CD44* and *EZH2* were overexpressed at the first visit of 2 patients.

The swimmer plot depicts the evolving phenotype of 77.8% (n=7) of this series of 9 mCRPC patients during their follow-up. The different gene combinations fitted into 7 phenotypic categories. The most represented was the mixed phenotype with several genes of the 3 cell subtypes in 33% (n=3) of patients, at certain time-points and maintained at death in one case (P5). The appearance of some circulating genes

during a patient trajectory did not necessarily changed the overall phenotype if it was already represented by another transcript of the same cell subtype (P2). In several instances, a phenotype emerged during a trajectory, as observed for genes of the stem-cell subtype in 33% of cases (n=3) and particularly for those of the neuroendocrine subtype in 55.5% of cases (n=5). Fluctuations over time in some patients led to the neuroendocrine phenotype in 3 cases, of which 2 died from the disease. Like the first series, statistical analyses only revealed that patients previously treated with docetaxel were less likely to overexpress stem-cell markers (odds ratio (OR) 0.12) (**Table 3**).

Table 3. Clinical relevance of circulating genes overexpressed in the longitudinal series

		Luminal	Neuroendocrine	Stem-Cell
Age[1]		0,32	3,51	1,78
		(0.05-1.88)	(0.28-44.34)	(0.42-7.58)
PSA[1]		2,48	3,36	1,19
		(0.28-21.88)	(0.32-35.14)	(0.23-6.11)
Bone metastases		NA	1,2	NA
			(0.02-69-61)	
Visceral metastases		0,82	0,79	0,2
		(0.07-9.86)	(0.06-10.92)	(0.03-1.49)
Line of therapy		0,96	0,48	0,33
		(0.22-4.31)	(0.08-2.92)	(0.10-1.13)
Current mCRPC therapy				
	Any AR-I	1,63	0,33	1,64
		(0.24-11.26)	(0.03-4.03)	(0.31-8.78)
	Abiraterone	0,84	0,15	1
		(0.13-5.57)	(0.02-1.59)	(0.22-4.54)
	Enzalutamide	3,4	5,43	1,95
		(0.22-51.47)	(0.25-118.91)	(0.29-12.91)
	Docetaxel	NA	3,74	NA
			(0.02-651.51)	
Past mCRPC therapy				
	Abiraterone	1,69	2,47	1,26
		(0.05-55.73)	(0.06-104.59)	(0.11-14.94)
	Docetaxel	0,76	0,41	**0,12**
		(0.09-6.60)	(0.04-4.41)	**(0.02-0.61)**
Initial therapy	Surgery	5,57	0,52	1,39
		(0.64-48.44)	(0.02-13.01)	(0.21-9.22)
	Radiotherapy	0,5	2,53	1,19
		(0.04-5.68)	(0.20-31.72)	(0.25-5.79)
	Non-curative therapy	0,37	0,61	0,68
		(0.04-3.76)	(0.06-6.73)	(0.14-3.32)

Odds ratios and 99.5% confidence intervals identifying variables associated with luminal, neuroendocrine and stem cell markers.[1] Patients were stratified below and above the median. Multi-level, mixed effects logistic regression models were used to calculate odds ratios and their respective 99% confidence intervals, in order to adjust for repeated measurements within individuals. Bold values are significant (p<0.005). NA: Non-Applicable.

Discussion

The present investigation confirms our earlier findings on the overexpression of 14 circulating transcripts representative of prostate epithelial cell subtypes (luminal, neuroendocrine and stem-cell) being traceable in the blood of mCRPC patients and demonstrates for the first time, dynamic changes in the overexpression of this gene panel occurring during disease progression.

These observations deserve consideration as the close monitoring of several representative genes in liquid biopsies could guide clinicians to choose or adapt therapies for a better management of mCRPC patients reaching more advanced and late stages of the disease.

For the present study, we opted to use the TaqMan vs. the SYBR qPCR method because of its higher specificity and sensibility and the requirement of less blood RNA (50). Results were similar to the pilot study and proved that the same genes were overexpressed in liquid biopsies of a new series of 15 mCRPC patients. The fact that these genes were minimally or not expressed in the blood of healthy men of increasing age adds to the specificity of the circulating genes overexpressed in mCRPC patients and also implies that hyperplastic changes occurring in the prostate (BPH) of older donors and/or else, early stage and low-risk prostate cancer (not yet diagnosed) do not contribute significantly to the overexpression of the 14 gene panel observed in the blood of patients with advanced disease. In any case, there was no *KLK3* and *FOLH1* signals in control men, a finding in agreement with the prostate specificity, which included *ARV7*, shown in the 15 initial volunteers (Vesval, et al). Hence, an *ARV7* signal was detected in the blood of 2 new controls, which is consistent with the recent literature (51,52). A lack of signal for *KLK3*, *FOLH1*, and *ARV7* and a minimal expression of the 11 other genes was observed in the blood of healthy women of

varying ages (data not shown), thereby speaking for basal levels being intrinsic to assays. Consequently, threshold levels were defined to correct for basal gene expression and state on circulating gene overexpression in mCRPC patients at a high confidence interval (99.5%). That the levels of overexpressed genes in mCRPC patients was prostate cancer specific was further supported by the lack of association with the number of WBC of different subtypes present in their blood samples, except for *CD44* and *SYP* also found in monocytes and neutrophils, respectively. Yet, this did not affect the overall phenotypic diversity of corresponding patients, as other transcripts of the same cell subtypes (stem and neuroendocrine) were co-overexpressed (Vesval et al,). As a whole, these data on circulating transcripts are supportive of distinct prostate cell subtypes being present in CTCs or extracellular vesicles released from tumor foci and contributing to progression.

As anticipated, results on the same gene panel in the blood of new mCRPC cases validate the initial findings of the pilot study. 20% of the new patients did not overexpress any of the selected 14 genes compared to 15% in the early series. This could be explained by the limited number of genes tested or the particular time-point in the disease history. The testing of additional genes could thus change their classification from the “none” category. In this study, *ARV7* was detected in the blood of 10 (68%) patients, and 4 of them (27%) reached overexpression levels. These values are in line with the reported 11 to 68% range for circulating *ARV7* (29,34). As expected, patients who expressed *KLK3* had higher PSA levels at the time of blood draw as observed in the pilot study. This finding suggests that *KLK3* detection mirrors the presence of CTCs, whereas the PSA protein released in blood also reflects the overall tumor burden of mCRPC patients. More studies are needed to demonstrate the adequation between the circulating gene and protein. In this series, only 1 patient

(6.7%) displayed a neuroendocrine phenotype compared to 2 (5%) in the initial study. It is admitted that pure neuroendocrine tumors are rare, with less than 2% during the initial diagnosis (10). The most overexpressed transcript of this cell subtype was *VEGFA*, in 27% (4/15) of patients compared to 15% (6/40) in the pilot study. The *VEGFA* transcript was more frequently overexpressed in the longitudinal series of patients and detected in 7/9 or 78% of them. VEGFA is a valuable target for colorectal (53) and prostate cancer (54), and most promising within the context of metastasis as it enhances PCa cell motility (55). The frequency of its association with stem cell markers such as *POU5F1*, *NANOG* or *SOX2* questions the role of these genes in the same pathway (56) or in the promotion of lineage plasticity (57).

We classified the 15 new cases into 8 subgroups, based on overexpression of at least one circulating transcript of a particular cell subtype, defining a category for each of the 3 cell subtypes, diverse combination among them, or none of them. The resemblance between the pie-charts of the new series of patients and the 40 first patients is validating our results. For instance, the percentage of mCRPC patients overexpressing at least a gene representing one of the cell subtypes was 80% (12/15) compared to 85% (34/40). The luminal category was predominant (27% vs. 32%), followed by all cell subtypes (13% each), the combination of stem-cell + neuroendocrine subtypes (13% vs. 8%). 15% were in the none-category vs 20% in our study. Therefore, the gene patterns in patients' liquid biopsies are best represented initially by the luminal cell subtype alone or in combination with the stem and neuroendocrine subtypes. Hence, these findings portrait the situation prevailing at a given time in a patient trajectory.

The key message of this study is the changing gene overexpression patterns during progression. Indeed, clear modifications were detected in 8 of the 9 patients followed

for up 28 months (ranging from 16 to 39 months), while no significant changes were observed in blood samples of healthy controls collected over the same time period. The stability of such gene patterns was also exemplified by a patient (P1) who never displayed any overexpression of the selected genes in the blood drawn at the two time-points (under Abiraterone) during his short trajectory, although *ARV7* was expressed at low levels. Nonetheless, our observations in the remaining mCRPC cases revealed fluctuations in expression vs. overexpression levels between genes of the 3 cell subtypes over time. Analyses of trajectories showed an evolving phenotype in 7 of 9 patients (77.8%). All cell subtypes were represented in 3 patients (33%) during follow-up. An enhanced expression of luminal markers was noticed in 2 patients and such overexpression was reduced in another one. All patients expressed *ARV7* at least once during their trajectories, and 3 of them (33%) overexpressed high levels before a loss of signal (P6) or decreased levels (P4), in line with the literature (58). Neuroendocrine markers were overexpressed in 5 patients (55.6%) and levels decreased in 2 (22.2%); this includes one patient whose phenotype was neuroendocrine earlier in his trajectory. The gene patterns of 3 patients evolved from or towards the “none” phenotype at a certain time points in their trajectory, meaning baseline levels or no signal. This could be associated with treatments. For instance, in P6 several neuroendocrine and stem-cell genes were overexpressed along with high *ARV7* levels with time on Enzalutamide but were next reduced to baseline levels 4 months after the start of Docetaxel chemotherapy, except for one neuroendocrine gene which remained overexpressed. The converse situation was seen in P5, with no gene overexpressed post-Docetaxel therapy, and after irradiation of metastases, whereas in the following months and last draw prior to death, several neuroendocrine and stem-cell genes were overexpressed in addition to elevated levels of *ARV7*. Variations/fluctuations in levels of circulating

transcripts have been reported for *FOLH1* and *ARV7* between 2 time-points (58). ARSIs have changed the standard of care for advanced cases (14,16) by their action on the androgen/AR pathway, which applies to cells of the luminal subtype. Resistance to these therapies for *ARV7* (33) is noted in our series with an increase in the expression of *ARV7* under Abiraterone or Enzalutamide for P4, P6, P8 and P9 and for P6 levels reduced to threshold at the last visit under taxanes. With respect to *FOLH1* (PSMA), it was expressed in only 2 cases (P2, P4) at specific time-points in their trajectory. The landscape of biomarkers and targeted therapies is open for PSMA, to detect recurrent disease non-invasively by molecular imaging (59) and as a theranostic to treat metastatic lesions using PSMA-targeted radiolabeled agents (60). However, a close monitoring of circulating *FOLH1* or of PSMA in CTCs may be a prerequisite to the use of this type of tools. The evolving phenotypes observed in this series of patients shows that the luminal phenotype did not persist with further progression such as in P4, P7, P8 and P9 with a PSA close to 0. The exception was P4 who strongly expressed *ARV7*. These findings are consistent with the literature reporting cases of low PSA levels in advanced disease, characterized by a marked histological dedifferentiation (61). As such, a more pronounced and common overexpression of genes of only one subtype was noticed late in the trajectory of 4 cases (last draw or terminal stage); 1 patient (P9) displayed a stem cell pattern at his last blood draw, whereas 3 patients (33%) (P3, P6, P8) had a neuroendocrine phenotype, thus reinforcing the concept of neuroendocrine differentiation after ADT (62) especially resulting from a luminal phenotype (63). Indeed, P3 to P6 displayed a luminal subtype or expressed *ARV7* before ending their trajectories with a neuroendocrine or mixed neuroendocrine subtype. At present, no drugs are available to target these cell subtypes. Hence, some responses to systemic taxane therapies were observed in the

trajectories of P6, and P8, which resulted in a marked reduction in overexpressed neuroendocrine and stem-cell genes to threshold levels or below. In P8, only one neuroendocrine gene of the 5 overexpressed neuroendocrine and stem-cell genes at his first draw remained overexpressed at his second draw, 10 months after Docetaxel and during the course of the Enzalutamide therapy. The *VEGFA* transcript was overexpressed in 7 of the 9 patients at certain time-points of this longitudinal series. It represents an interesting target as drugs targeting this pathway already exist. Yet they have not shown clinical benefits as inhibitors of angiogenesis. Anti-metastatic drugs may be more suitable and explored as VEGF-A triggers PCa cell motiliy (55).

The overall significance of these findings is unknown but changes in genes patterns during progression may reflect further molecular changes taking place in cells of certain subtypes, according to stage of disease as well as a complete or partial response and/or resistance to therapies. Additional studies, with a closer monitoring of these types of genes, are needed to personalize treatments. For instance, the combined use of ligands binding proteins expressed at the surface of neuroendocrine or stem cells along luminal markers like PSMA-radiolabeled ligands represent promising avenues to detect the diverse cell subsets present in metastatic lesions or tumor foci, at earlier time-points during progression.

Taken together, it is conceivable that genes representing prostate cell subtypes in CTCs or vesicles and released from malignant cells during the trajectory of mCRPC patients reflect the dynamic turnover of certain cell subsets in tumors and metastases of each patient. As changes are traceable through liquid biopsies and could be monitored closely, therapeutic options tailored to patients may be offered to patients in a near future to impact on this lethal disease. The main limitations of our study are the small size of studied cohorts and time-points of the blood draws. Samples collection

along the entire trajectory from diagnosis to death and for each stage of the disease (recurrence, change of treatment, appearance of metastasis) are necessary to confirm our observations.

Conclusion

This investigation confirms the clinical and molecular heterogeneity of PCa as evidenced by circulating genes representing the three cell subtypes in mCRPC patients. Our observations also supporting the concept of a dynamic turnover of distinct prostate cell subsets present in prostate tumors/metastases contributing to progression and being traceable by testing specific genes in liquid biopsies. Sequential samples drawn along patient trajectories could help track disease progression better than currently used biomarkers and determine which treatments fit best and when. The clinical significance of the dynamics of a gene panel from whole blood RNA will be elucidated by follow-up studies at key disease dates.

Conclusion Générale

Cette étude confirme que l'hétérogénéité tumorale prostatique peut être évaluée à partir de biopsies liquides sanguines représentant les trois sous-types de lignées de cellules épithéliales interdépendantes, présentes dans les tumeurs et ciblées différemment par les thérapies hormonales.

Ces observations soutiennent également le concept d'un renouvellement dynamique de sous-ensembles distincts de cellules prostatiques issues des tumeurs ou métastases prostatiques et contribuant à la progression de la maladie.

Une stratégie similaire pourrait également s'appliquer à d'autres cancers après sélection de gènes d'intérêts adéquats.

Des échantillons séquentiels le long des trajectoires pourraient aider à mieux suivre la progression de la maladie que les biomarqueurs couramment utilisés et à déterminer quels traitements conviennent et quand. Des études supplémentaires avec des échantillons prélevés à des dates clés de la maladie permettraient de mettre en lumière la signification clinique de cette dynamique de biomarqueurs et orienter vers une médecine personnalisée de ces cancers.

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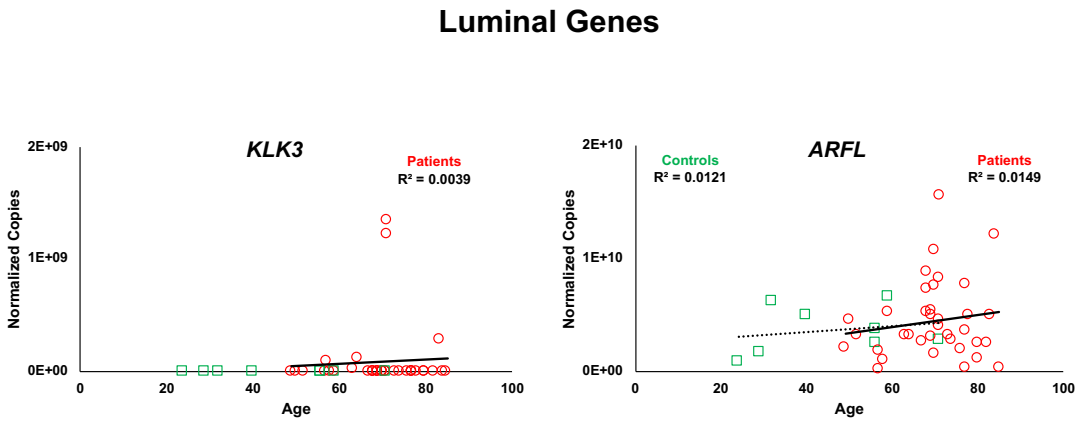
Matériel Supplémentaire

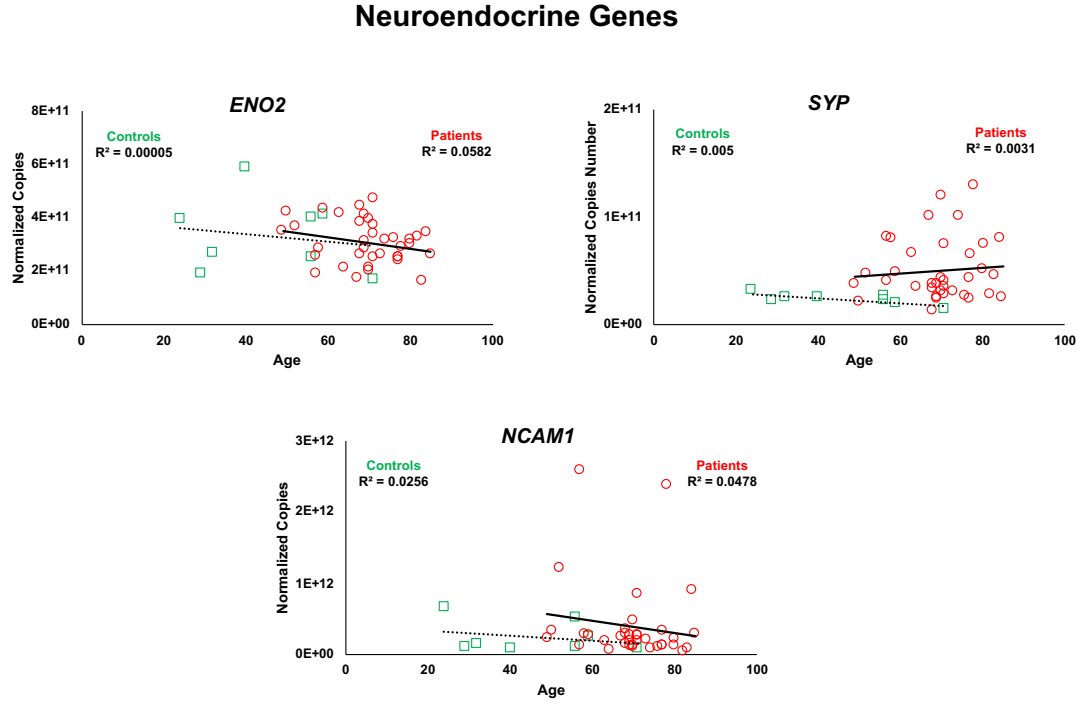
Figure S1. Expression of genes by age

The 14 genes panel was assayed in the blood RNA of mCRPC patients and healthy males as controls. The normalized copy number of remaining Luminal **(A)**, Neuroendocrine **(B)** and Stem-cell **(C)** genes is shown, with red dots for patients and green squares for controls. Regression lines show lack of correlation between data, with their R^2 coefficients.

(A)



(B)



(C) **Stem Cell Genes**

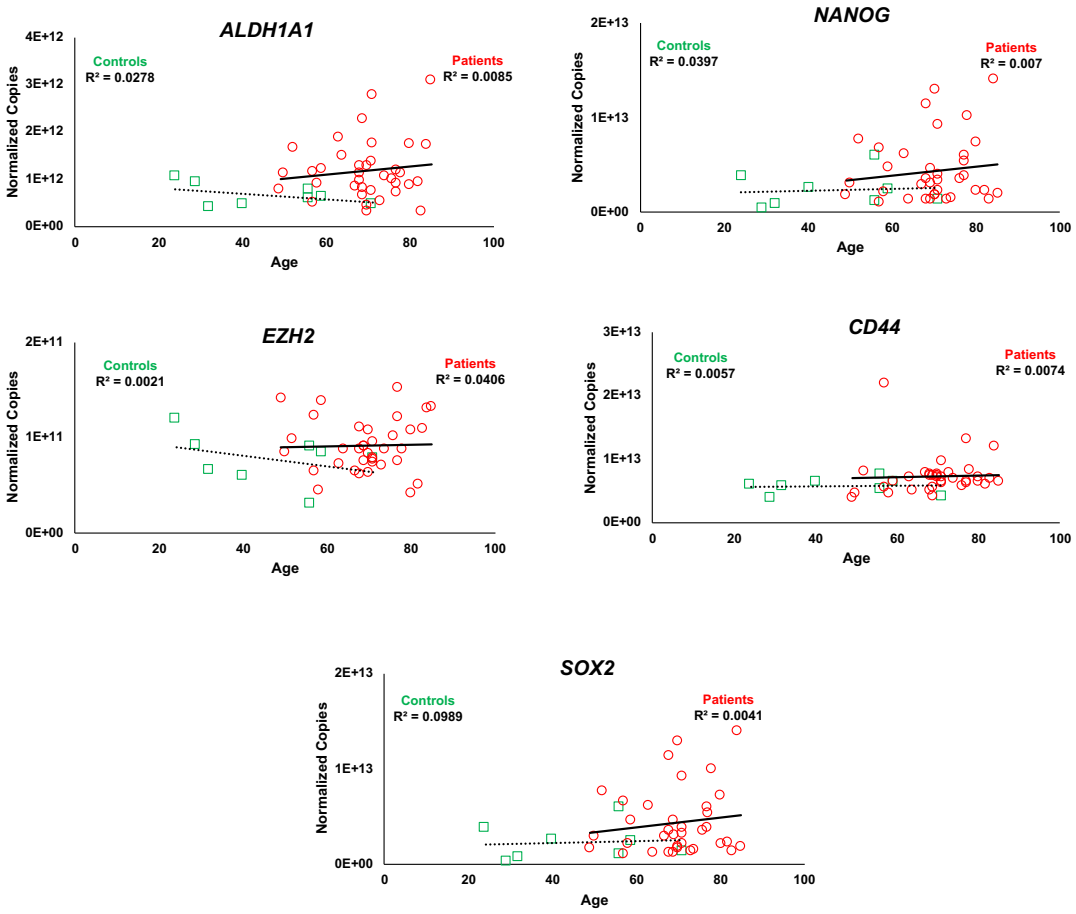


Table S1. Threshold determination for overexpression of prostate cell subtype genes in liquid biopsies.

Target Gene	Threshold at 99.5% in CN (FC)
ARV7	1.6E+10 (4.4)
ARFL	1.7E+10 (3.1)
KLK3	0
FOLH1	0
NSE	6.5E+11 (1.9)
SYP	6.9E+10 (2.2)
VEGFA	1.0E+11 (2.1)
NCAM1	7.4E+11 (2.9)
ALDH1A1	1.5E+12 (2.0)
POU5F1	1.2E+13 (2.4)
EZH2	3.1E+11(3.0)
NANOG	9.3E+12 (3.1)
CD44	1.0E+13(1.6)
SOX2	1.7E+12 (5.8)

Overexpression was defined as gene expression greater than the threshold, which was set for each gene marker at 2.58 standard deviations (SD) above the mean expression in blood RNA from 11 healthy controls at a 99.5 % confidence interval.

CN: Copy number, FC: Fold-change to the mean of controls.

Table S2. Patients' characteristics of the first series

	Patients	Controls
Number	15	11
Age, median (range), years	71 (52-82)	56 (24-71) p<0,005
PSA, median (IQR)	22,42 (1,25-144,3)	
Gleason at biopsy		
6	0 (0%)	
7	4 (26%)	
8	1 (7%)	
9	7 (47%)	
10	2 (13%)	
not reported	1 (7%)	
Initital treatment at diagnosis		
curative treatment	8 (53%)	
surgery	6 (40%)	
radiotherapy	2 (13%)	
initially metastatic disease	7 (47%)	
ADT	4 (27%)	
ADT + RT	0 (0%)	
ADT + CT	3 (20%)	
Time, median, IQR (month)		
Time between diagnostic and CRPC	46 (15-113)	
Time between CRPC and inclusion	24,5 (14-35,5)	
Metastatic sites		
None	0 (0%)	
Bone	14 (93%)	
Visceral	3 (20%)	
LN alone	1 (0,07)	
Current line of therapy		
2	5 (0,33%)	
3	10 (67%)	
Current treatment		
ADT only	5 (33%)	
AR-inhibitor	10 (67%)	
Abiraterone	8 (53%)	
Enzalutamide	2 (13%)	
Chemotherapy	6 (40%)	
Docetaxel	4 (27%)	
Cabazitaxel	2 (13%)	
Progressive disease	9 (60%)	

ADT: Androgen-Deprivation Therapy; AR: Androgen Receptor; CRPC: Castrate-Resistant Prostate Cancer; IQR: Inter Quartile Range (Q25-Q75); PSA: Prostate Specific Antigen

Table S3. Clinical relevance of circulating genes overexpressed in the series of 15 patients with advanced prostate cancer

		Luminal	Neuroendocrine	Stem
Age[1]		1,19	1,05	2,8
		(0.14-9.67)	(0.13-8.68)	(0.14-55.99)
PSA[1]		1,88	1,05	0,7
		(0.24-14.65)	(0.13-8.67)	(0.04-12.65)
Bone metastases		0,17	**0,04**	**0,01**
		(0.00-6.80)	**(0.00-0.45)**	**(0.00-0.51)**
Visceral metastases		0,39	NA	NA
		(0.01-10.50)		
Line of therapy		0,78	0,45	0,16
		(0.09-7.04)	(0.12-1.73)	(0.01-2.96)
Progressive disease		1,79	0,35	0,18
		(0.22-14.43)	(0.04-3.06)	(0.01-3.24)
Current mCRPC therapy				
	None	1,43	10,19	44,94
		(0.03-79.60)	(0.41-254.41)	(0.57-3554.92)
	Abiraterone	1,28	3,54	6,3
		(0.14-11.56)	(0.46-27.28)	(0.34-117.37)
	Any taxane	0,72	**0,13**	**0,05**
		(0.08-6.13)	**(0.019-0.88)**	**(0.00-0.59)**
	Docetaxel	0,24	0,13	0,05
		(0.03-2.23)	(0.01-1.59)	(0.00-1.47)
	Cabazitaxel	4,15	0,43	0,25
		(0.37-46.50)	(0.02-7.61)	(0.01-12.38)
Past mCRPC therapy				
	Any AR-inhibitor	0,67	0,1	0,11
		(0.08-5.73)	(0.01-1.08)	(0.01-1.78)
	Abiraterone	0,19	0,43	NA
		(0.01-3.83)	(0.02-7.61)	
	Enzalutamide	1,01	NA	0,3
		(0.10-9.77)		(0.01-6.31)
	Docetaxel	3,62	1,66	2,21
		(0.38-34.78)	(0.17-16.54)	(0.10-51.42)
Initial therapy				
	Surgery	0,08	0,52	0,54
		(0.01-1.13)	(0.05-5.00)	(0.02-11.79)
	Radiotherapy	2,83	2,4	2,79
		(0.22-35.98)	(0.24-24.18)	(0.09-82.88)
	Non-curative therapy	0,84	0,95	0,36
		(0.10-6.88)	(0.12-7.81)	(0.02-7.13)

Odds ratios and 99% confidence intervals identifying variables associated with luminal, neuroendocrine and stem cell markers. [1]:Patients were stratified below and above the median. Multi-level, mixed effects logistic regression models were used to calculate odds ratios and their respective 99% confidence intervals, in order to adjust for repeated measurements within individuals. Bold values are significant ($p<0.005$). NA: Non-Applicable.

Table S4. Clinical relevance of circulating genes overexpressed in the series of 15 patients with advanced prostate cancer.

Gene and cell subtype	Disease progression	Death
KLK3	1.44 (0.35-5.85)	2.42 (0.15-38.63)
ARFL	1.02 (0.12-8.49)	NA
ARV7	0.85 (0.17-4.24)	NA
FOLH1	0.56 (0.11-2.85)	NA
SYP	1.02 (0.12-8.49)	7.35 (0.45-119.17)
VEGFA	0.33 (0.04-2.67)	NA
NCAM1	NA	NA
ALDH1A1	0.92 (0.18-4.57)	NA
CD44	NA	NA
NANOG	NA	NA
POU5F1	NA	NA
SOX2	NA	NA
Any luminal marker	0.57 (0.15-2.16)	0.87 (0.05-13.95)
Any neuroendocrine marker	0.60 (0.12-2.98)	2.68 (0.16-45.34)
Any stem cell marker	0.60 (0.12-2.98)	NA
Any luminal + any neuroendocrine marker	0.53 (0.06-4.30)	NA
Any luminal + any stem cell marker	0.53 (0.06-4.30)	NA
Any neuroendocrine + any stem cell marker	NA	NA
Overexpression of at least one marker in all three groups	NA	NA
No marker	1.55 (0.38-6.27)	NA
Number of luminal markers	0.92 (0.49-1.82)	0.61 (0.11-3.31)
Number of neuroendocrine markers	0.55 (0.17-1.79)	1.23 (0.21-7.14)
Number of stem cell markers	0.58 (0.23-1.47)	NA
Number of markers	0.81 (0.56-1.18)	0.73 (0.25-2.16)

Hazards ratios and 99.5% confidence intervals for disease progression and death in patients overexpressing the selected circulating markers. Hazards ratios and respective 99.5% confidence intervals were calculated using Cox proportional hazard models. NA: Non-Applicable.

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Titre de la thèse : Suivi longitudinal de l'évolution dynamique de sous-types cellulaires de cancer de la prostate résistant à la castration à partir d'ARN de sang total

Thèse - Médecine - Lille 2021

Cadre de classement : Uro-Oncologie

DES + spécialité : DES Chirurgie Générale + Spécialité Urologique

Mots-clés : hétérogénéité tumorale ; biomarqueurs ; biopsies liquides ; cancer de prostate métastatique

Résumé :

Introduction : Le cancer de prostate (PCa) est une maladie hétérogène. Les décès en résultant sont surtout liés à la phase de résistance à la castration. L'antigène de prostate spécifique (PSA), marqueur utilisé aujourd'hui en routine du diagnostic jusqu'aux stades avancés présente des limites. Il ne reflète pas les modifications tumorales au cours de l'évolution de la maladie et l'impact des différentes lignées thérapeutiques. D'autres marqueurs, comme l'ARN de sang total, sont prometteurs pour répondre à ces limites. Nous avons étudié à partir de prélèvements sanguins l'évolution de sous-types de gènes de cellules épithéliales prostatiques (luminale, neuroendocrine et cellules souches) et leur dynamique au cours de la maladie.

Méthodes : Une étude prospective monocentrique a été réalisée à partir d'ARN de sang total de patients métastatiques et résistants à la castration (mCRPC) prélevé au cours de leur suivi en oncologie. 9 patients avec 2, 3 ou 4 visites de suivi ont été inclus, après une première série de 15 patients avec prélèvement unique, ainsi que 11 volontaires sains. 14 gènes ont été étudiés par qPCR en temps réel (TaqMan). Une régression logistique univariée a été réalisée pour relever la corrélation de ces résultats avec les données cliniques des patients.

Résultats : 7 de ces patients ont présenté une évolution de phénotype au cours de la progression de la maladie. Le phénotype le plus représenté en fin de trajectoire est le neuroendocrinien (55%). La progression sous chimiothérapie était associée négativement avec la surexpression de marqueurs indifférenciées (OR=0,12 p<0,005).

Conclusion : Le renouvellement dynamique de sous-ensemble de gènes épithéliaux de cellules prostatiques issus des tumeurs ou métastases est visualisé à partir de biopsies liquides. Leur utilisation pourrait aider au suivi de la progression de la maladie et au choix du traitement adapté au patient à chaque séquence de la maladie.

<u>Composition du Jury</u> :

Président :

Monsieur le Professeur Arnauld VILLERS

Assesseurs :

Monsieur le Professeur Xavier LEROY
Monsieur le Docteur David PASQUIER
Monsieur le Docteur Quentin VESVAL