

A systematic review of prostate cancer heterogeneity – understanding the clonal ancestry of multifocal disease

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Abstract

Context: Studies characterizing the genomic changes of prostate cancer (PCa) during natural progression have greatly increased our understanding of the disease. A better understanding of the evolutionary history of PCa would enable advancements in diagnostics, prognostication, and novel therapies that together will improve patient outcomes.

Objective: In this systematic review, we discuss molecular heterogeneity of PCa by assessing recent efforts to profile intratumoral heterogeneity and clonal evolution.

Evidence Acquisition: We screened a total of 1313 publication abstracts from PUBMED between 2009-2020, of which we reviewed 84 full-text articles. We excluded 49, resulting in a final 35 studies.

Evidence Synthesis: In studies of primary PCa (16 studies, 4793 specimens), there is a lack of consensus regarding the monoclonal or polyclonal origin of primary PCa. There is no consistent mutation giving rise to primary prostate cancer. Detailed clonal analysis of primary prostate cancer has been limited by current techniques. By contrast, clonal relationships between PCa metastases and a potentiating clone have been consistently identified (19 studies, 732 specimens). Metastatic specimens demonstrate consistent truncal genomic aberrations that suggest monoclonal metastatic progenitors.

Conclusions: The relationship between clonal dynamics of PCa and clinical outcomes needs further investigation. It is likely that this will provide the biological rationale for whether radical treatment of the primary prostate benefits patients with oligometastatic PCa. Future work studying the mutational burden in primary disease at single cell resolution should permit the identification of clonal patterns underpinning the origin of lethal PCa.

Patient summary: Prostate cancers arise in different parts of the prostate due to DNA mutations that occur by chance at different times. These cancer cells and their origin can be tracked by DNA mapping.

37 In this review we summarize the 'state-of-the-art' and outline what further science is needed to provide
38 the missing answers.

1. Introduction

Prostate cancer (PCa) is a heterogeneous disease. A single biopsy of the prostate, even if it does manage to hit a focus of cancer, is often inadequate for a comprehensive assessment of the disease¹⁻³. This is true for assessment of somatic genetic changes just as it is for histological morphology^{4,5}.

The accessibility, cost, coverage and resolution of genomic assays has improved rapidly since the first sequencing of the human genome. Even a decade ago relatively little was known about the wider landscape of genomic alterations in PCa. As new technologies have permitted broader profiling of tumors, PCa investigators were unable to find high incidence mutations as detected in other cancers such as pancreatic cancer where 90% of cases are *KRAS* mutant⁶. Large scale efforts to sequence primary PCa from multiple patients have revealed extensive genomic heterogeneity, particularly copy-number changes, and relative lack of distinct driver mutations⁷⁻¹³. In contrast, efforts to sequence advanced disease have revealed more consistent aberrations in many genes such as *AR*, *PTEN*, *TP53*, *ETS* genes, *BRAF* and DNA-Damage Repair gene pathway members¹⁴⁻¹⁷. While these efforts have been instrumental in advancing knowledge about prostate cancer genomics, they have largely failed to characterize actionable changes in early prostate cancer and thus targeted therapies have not made it into routine clinical practice as with other tumour types. It is increasingly apparent that this is due to the extensive intra-tumoral heterogeneity in prostate cancer as revealed by other, more focused, contemporary sequencing studies, particularly in primary disease^{18,19}.

In this review, we aggregate knowledge regarding prostate cancer intra-tumoral heterogeneity and clonal evolution as determined by genomic assays, as applied to multiple tumor regions, to identify the gaps in technology and understanding that need to be filled in order for us to correctly interpret clinical findings in this multifaceted disease.

2. Evidence Acquisition

2.1 Search Strategy

We conducted a systematic literature search of PubMed for all articles up to December 2020. The search terms and strategy are recorded in Supplementary Material 1. We limited our searches to publications from the past 10 years.

2.2 Inclusion and exclusion criteria

We followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) criteria (Figure 1) for evaluating records during the literature search. The lead author (AE) inspected all studies against the inclusion criteria, with two further authors reviewing all included and excluded studies to provide oversight (ADL & IGM). Specifically, we included original research articles, from DNA or RNA-based assays capable of resolving intra-tumoral heterogeneity and clonal evolution, from two or more readouts from human patient specimens. We excluded studies that reported results from model systems alone or non-human studies (eg., cell lines or mice), review articles, and reports from single readouts from a single cancer specimen.

3. Evidence Synthesis

3.1 Methods for Determining Heterogeneity

There are many methods for determining heterogeneity and the methods chosen by investigators vary in scope, size and in the biological entities targeted by the assay. It is therefore important to consider these technical aspects at the outset (Table 1, Figure 2A).

3.2.1 Intra-Prostatic Heterogeneity

Descriptions of heterogeneity may differ so we begin with some definitions. We consider 'intra-prostatic' to refer to localized disease and including local invasion (for example, spread to the seminal vesicles). We define 'Intra-tumoral heterogeneity' as observed phenotypic or genotypic differences

between cancer cells. Furthermore, it is important to distinguish between foci of disease that are unrelated -- termed 'polytumors' or 'polyclonal', from those that share a common ancestor 'monotumors', 'monoclonal', or subclonal(Figure 2A&B). Within the past ten years, increasing efforts to study these phenomena have used an assortment of methods to broadly characterize genomic intra-prostatic heterogeneity by assaying and analyzing multiple, separately extracted, regions of PCa lesions as well as adjacent non-cancerous regions.

3.2.2 Field Effect

Field cancerization, or field effect, was described by Slaughter and colleagues in 1953 as morphological changes occurring in adjacent, non-cancerous cells near tumour lesions²⁰, and this can include immune or inflammatory field effects. Despite evidence from an increasing number of different field effect markers²¹, the landscape of specific carcinogenic field effect changes in PCa is still relatively unknown. Genomic characterization of prostatic lesions and adjacent tissue regions has provided insights into the molecular origins and drivers of PCa development and progression.

Recent studies have reported results from the comparison of normal-adjacent prostate tissue and cancerous regions. The first study to use WGS found that "normal" tissues harbored distinct mutations not shared by blood or cancer samples taken from the same patients¹⁸. Separate efforts to characterize cancer adjacent stroma found that the stroma does not share the same genomic profile as adjacent cancerous regions, further suggesting that stromal cells are not most recent common ancestors of cancerous regions²². However, given that cross-talk between stromal cells, for example cancer associated fibroblasts, and neighboring PCa epithelial cells can occur²³ and evidence from other cancers has shown cancer associated fibroblasts influence cancer stemness²⁴, further work is needed to further elucidate how stromal cells may influence PCa clonal dynamics.

Additional efforts have focused on potential clonal relationship of prostatic intra-epithelial neoplasia (PIN) and adjacent cancer regions as assessed by genome sequencing. In one case, authors assessed multiple PIN and PCa regions of a single patient, and found a shared mtDNA mutation harbored by all PIN regions and at least one PCa clone²⁵. Similarly, Gerrin and colleagues re-analyzed multiple high-grade PIN (HGPIN) and PCa regions, previously characterized by WES, and found that HGPIN harbored a distinct *OR2AP1* mutation that was present in both the Gleason 3 and Gleason 4 tumor foci²⁶. Additionally, a separate study reported conserved copy-number variation in all regions from cases having both HGPIN and adjacent cancer regions²⁷. Finally, a recent report used single-cell WGS of individual cells “washed” from diagnostic prostate biopsies in addition to frozen punch biopsies from radical prostatectomy specimens²⁸. While these studies were able to establish clonal relationships, they also found that in some regions the cancers featured no copy number alterations, whereas other regions from the same patient, without any cancer had copy number alterations. Taken together, although not conclusive, the results suggest that PIN regions may be clonal ancestors of PCa, but there is no evidence, as yet, to link PIN with lethal clones.

3.2.3 Clonal Origin of Primary Prostate Cancer – Mono or Polyclonal?

Primary PCa is often multifocal²⁹. Uncertainty remains regarding whether common ancestral clones, also termed as a “tumor initiating cells”, can be identified from primary PCs. Monoclonal tumors are defined as arising from a single common ancestor, whereas polyclonal tumors are tumors with no shared common ancestry.

Analyses of several primary PCa collections have concluded that a monoclonal origin of PCa cannot be consistently detected from genomic sequencing (Table 2, [Supplementary Table 1](#))^{18,19,30–36}. An initial report could not identify a shared common ancestry in a majority of cases profiled by multi-region WES³⁵. These results were consistent with later findings of multi-region sequencing by WGS, which

129 additionally found multiple clones even within individually profiled foci, as well as distinct mutational
130 profiles of adjacent normal prostate ¹⁸. In addition, another report found that in at least one patient
131 whose tumor regions were assessed by WGS, the profiled tumors contained at least 2 subclones with no
132 common identifiable genomic alterations¹⁹. Furthermore, a larger study performed WES of multiple
133 regions from a cohort of patients and found that 76% of individual patients' tumour specimens shared
134 no copy-number variation or single-nucleotide variants ³⁷. Finally, a recent study of multiple mpMRI-
135 visible and invisible lesions in primary PCa reported results that suggest either a monoclonal or
136 polyclonal origin of PCa as assessed by WGS³⁶.

137 In contrast, a number of other studies identified individual cases where a monoclonal origin to tumors
138 was detected ³⁸⁻⁴⁰. An effort using WES on regions of both Gleason 3 and Gleason 4 disease was able to
139 identify shared common genomic alterations in all profiled regions ³⁹. A separate study using a similar
140 approach was also able to identify shared common genomic ancestry in all profiled regions ⁴⁰, although
141 the authors acknowledged from their data that genomic alterations from an individual profiled sub-
142 region could represent as few as 20% of all alterations in the patient's overall cancer burden. A more
143 recent study profiled individual single-cells from a radical prostatectomy specimen using WGS and this
144 approach identified features of monoclonality in all cells from at least one patient ³⁸.

145 Taken together, these reports show that there is no clear consensus on whether clinically presenting
146 primary PCa can be consistently assumed to be of either monoclonal or polyclonal origin. This has clear
147 clinical implications for both molecular diagnostics and focal therapy ⁴. A recent study has suggested
148 that clonality can be computationally deconvoluted from bulk-sequencing of single malignant foci, and
149 clonal status is prognostic for clinical outcome ⁴¹. It is also likely that sampling and detection bias could
150 also play a role, given that different molecular assays have different levels of coverage. Similarly, the
151 assays used in these studies are only applied to a fraction of the cells in the entire prostate gland or
152 specimen. To date, there has been no single comprehensive study utilizing the same molecular assay

and computational pipeline to characterize the prevalence of both monoclonal and polyclonal origin of tumor-initiation within PCa patients.

3.3.1 Metastatic Heterogeneity

Studies assessing extra-prostatic cancer heterogeneity have largely focused on decoding the heterogeneity within distantly disseminated metastatic PCa (Table 3, [Supplementary Table 1](#)). It is important to recognize that in this setting, these cellular relationships are usually inferred mutational aberrations identified at a single collection timepoint as there are almost no clinical indications for serial resection or biopsying of metastatic lesions. Much of the evidence presented is derived from “rapid autopsy” studies from metastatic sites, along with samples from the prostate gland and, in some cases, with control samples, or from research studies permitting the biopsying of metastatic lesions. A nascent but growing body of work has added the ability to interrogate circulating tumor cells allowing comparisons to molecular profiles from metastatic lesions.

3.3.2 Clonality and Origin of Metastatic Prostate Cancer

A number of studies have sought to profile biological features from multiple metastatic lesions to uncover the landscape of genomic alterations in advanced PCa. The first comprehensive report of the pattern of metastatic spread of PCa from autopsies proposed a monoclonal origin of PCa⁴². Another study assayed metastatic specimens and circulating tumor cells taken at the time of autopsy, and found their genomic profiles to be 90-99% concordant⁴³. Robbins et al. used a combinatorial array comparative genomic hybridization and targeted next-generation sequencing profiling approach, and validated previous findings identifying a genomic profile consistent with an individual metastatic progenitor⁴⁴. Similarly, a recent report by Rodrigues et al used WGS to profile 3 metastatic lesions from 1 patient, which identified a distinct RB1 deletion that was shared between three metastases in one patient, suggesting a monoclonal origin of disease⁴⁵.

176 Additionally studies have used WES⁴⁶ and WGS⁴⁷ to profile multiple metastatic lesions. These analyses
177 found evidence for the sequential accumulation of mutations in metastases⁴⁶ and observed complex
178 patterns of metastatic spread, including metastasis-to-metastasis seeding but also the potential for
179 multiple primary PCa clones to give rise to metastases⁴⁷. This finding of multiple mutational clones
180 existing in the metastatic space is important as, if corroborated, it implies that sampling a single site of
181 metastatic disease may be inadequate as a companion diagnostic tool to determine preferential
182 targeted treatment.

183 Taken together, these studies present our current state of knowledge of the pattern of spread of
184 disseminated metastatic PCa. In contrast to the challenges highlighted in identification of tumour-
185 initiating clones of origin in the prostate gland, these studies suggest that it is comparatively straight-
186 forward to identify clonal relationships between PCa metastases. Collections of PCa metastases
187 commonly arise from a common clonal progenitor within the prostate gland, but evidence also suggests
188 the likelihood that additional subclones arise in the metastatic space and that these may preferentially
189 give rise to further metastases.

190 **3.3.3 Temporal Heterogeneity**

191 Several studies have assessed temporal heterogeneity by observing true differences in states over time.
192 Under selective pressure of therapies, treatment resistance can emerge as a result of the expansion of
193 pre-existing subclonal populations. Studies assessing temporal heterogeneity typically use liquid
194 biopsies, however, a small subset also include PCa tissues (Figure 2B). A recent case report analyzed
195 genomic alterations through WGS in metastatic tissue and CTCs from multiple time points during disease
196 progression in a single patient⁴⁸. The authors found comprehensive loss of early clones by the last
197 observed timepoint, suggesting extensive changes to clonal profiles due to the selection pressures
198 induced by treatment. These findings are in concordance with a separate study that analyzed clonality

through copy number profiling and targeted deep-sequencing of multiple metastatic PCa specimens during the course of disease progression during treatment ⁴⁹. Treatment-related temporal heterogeneity has been implicated in the development of neuroendocrine PCa (NEPC). In a subset of patients with specimens taken at multiple time points, Beltran et al found genomic profiles to be shared between precursor adenocarcinoma and NEPC specimens suggesting that the NEPC phenotype had not arisen independently but was subclonally derived from the original acinar clone ⁵⁰. In a separate case report analyzing primary prostate material (cancerous and benign), as well as metastatic samples, Jiang et al found no copy-number variation in the primary tumor, but were able to identify a subset of single-nucleotide variants shared between both metastatic tissue samples and all CTCs, suggesting a common clonal progenitor cell had escaped the primary tumour ⁵¹. While these studies reported changes in clonal dynamics over time, nearly all were able to identify shared genomic alterations present in an individual putatively parental subclone.

3.4.1 Intra-Prostatic Heterogeneity and Metastatic Clonal Evolution

Clonal evolution describes the phylogenetic relationship between largely homogenous clusters of cancer cells and relates to temporal mutations which could arise from ‘cell extrinsic’ processes, such as exposures to environmental and treatment stress as well ‘cell intrinsic’ processes, such as sustained changes in signaling linked to DNA damage.

While there is great interest in the efficient identification of specific clonal regions of primary PCa tumours that may give rise to metastases, there is comparatively little experimental evidence to describe this phenomenon. We now describe the small number of studies that address together both intra-prostatic and metastatic clonal evolution by profiling both the primary prostate malignancy and matched metastatic lesions from the same patient (Table 4, [Supplementary Table 1](#)).

221 Haffner and colleagues analyzed the radical prostatectomy specimen, a regional lymph node
222 metastasis, metastatic biopsies, and multiple rapid autopsy metastatic lesions from a single patient⁵².
223 While the authors employed WGS on the metastatic biopsies, they were only able to perform
224 molecular assays of limited targets across the breadth of all available material, given that the primary
225 specimen had been in a pathology archive for 20 years and they were unable to attain quality DNA.
226 Interestingly, they were able to identify a region of Gleason pattern 3 disease with a PTEN deletion,
227 while the remainder of the prostatic tumor foci had intact PTEN, including certain regions of Gleason
228 pattern 4 disease. In the subsequent metastatic samples that lead to the patient's death, the authors
229 were able to identify the same PTEN deletion, suggesting that the PTEN negative Gleason Pattern 3
230 focus gave rise to the metastatic lesions. To identify the region of interest in the primary malignancy
231 that presumptively gave rise to the metastases, the authors assayed all primary tumor blocks with
232 PTEN IHC. Despite this, the authors made a concerted effort to validate specific tissue regions,
233 undertaking LCM of the PTEN deleted region and performing genomic analysis of the PTEN gene,
234 which confirmed a genomic alteration shared with the metastases. Another noteworthy observation
235 was that a local lymph node metastasis resected at the time of surgery harbored a separate clonal
236 ancestry than the other disseminated metastases. The authors conclude that it may be possible for a
237 spatially distinct Gleason pattern 3 focus of PCa to metastasize, but acknowledge the necessarily
238 limited genomic analysis undertaken and, of course, it possible that the metastatic foci arose from a
239 Gleason pattern 4 region that underwent temporally distinct PTEN deletion after seeding.

240 VanderWeele and colleagues assessed a subset of primary PCa tumour regions, along with normal
241 prostate, and lymph node metastases. The authors performed LCM to sub-analyze different Gleason
242 pattern regions of malignancy, and employed whole-exome sequencing on all samples. In two (of a
243 total of four) cases, the authors found shared mutations between low grade and high-grade disease,
244 supporting evidence for a common ancestor for both. Unlike Haffner et al, the authors found no

evidence in any cases observed that low-grade disease contributed directly to metastatic progression, and the majority of identified mutations were shared between high-grade disease and metastases corroborating previous wisdom that Gleason pattern 3 disease does not develop into higher grade disease, but that higher grade disease arises from *de novo* mutations⁵³. These findings support the notion of active surveillance in men with low-risk disease.

In contrast to Vanderweele et al., Lindberg and colleagues analyzed two lymph node metastases and one region of primary PCa using WES as a follow-on study⁵⁴ to their original report⁵⁵. In their first report, the authors could not find common ancestry between the lymph node metastases and primary tumour samples when molecularly characterizing these tumors using bulk tissue WES. In their follow-up study, Lindberg and colleagues re-analyzed patient' specimens, from 25 distinct morphological tissue regions isolated by LCM. They instead performed a "wider but shallower" analysis, using low coverage whole-genome sequencing, and this approach identified break point regions from copy-number analysis. Analysis of break point regions in primary tumour regions, as a proportion of those identified in the lymph node metastases, identified a region of Gleason pattern 4 disease in common ancestry with regions of intra-ductal carcinoma (an aggressive subform of acinar prostate cancer⁵⁶) that then shared the most genomic overlap with the lymph node metastases⁵⁴.

Chen et al. analyzed PCa subregions from a single patient who, rather unusually, had received neoadjuvant abiraterone followed by radical prostatectomy. The authors identified residual tumour in two tissue blocks, from which they isolated tumour regions and normal prostate separately using LCM, and performed whole-exome sequencing. While the authors were able to identify separate sets of unique mutations for both tumour foci, they shared a number of discrete mutations, strongly suggesting a common ancestral origin⁵⁷.

Hong and colleagues analyzed multiple regions, from both primary and metastatic tumour sites from four patients. All patients had WGS performed on primary and metastatic samples, and each patient

had a very different temporal and clinical profile. These differences were borne out in biological complexity. The authors were able to identify common clones of origin for all metastases. The authors found a number of interesting clinical and biological findings. For example, they found evidence to suggest that lateral spatial spread of primary tumour is associated with late-arising clones; that distant metastasis can re-seed the surgical bed, that therapy can lead to changes in the subclonal proportions of different subclones, and that the primary tumour can seed multiple waves of metastases. The authors conclude that their work demonstrates significant heterogeneity in the pattern of metastatic spread, but that we need more detailed analysis of clonal relationships in the primary prostate and in larger patient populations⁵⁸.

Gillard et al. assessed WES in two primary foci (one ductal and one acinar PCa), as well as a synchronous LN metastasis as part of a larger comparative study of acinar and ductal PCa. Mutational profiling suggests that all specimens shared common ancestry, with the ductal PCa foci diverging from acinar PCa, and the LNMet diverging from the ductal. These results suggest a monoclonal origin of PCa and metastases⁵⁹. Similarly, Ritch et al., used WES to compare multiple tumor foci from RP specimens and cfDNA in 2 patients as part of a larger study assessing cfDNA from patients with metastatic PCa. Despite observing a lack of concordance in mutational profiles between specimens in primary PCa and cfDNA, the authors found strong evidence monoclonal ancestry through phylogenetic analyses of PCa and metastatic progression⁶⁰. Most recently, Woodcock⁶¹ analysed multiple non-adjacent formalin-fixed, paraffin embedded samples from within the prostate and local expansion in the same 10 patients studied in Gundem et al⁴⁷. They created a bespoke target DNA-sequencing panel to capture single-nucleotide variants identified in the previously-studied metastatic and intra-prostatic samples, and used this to perform deep-sequencing on LCM regions in the new samples. With these, they were able to delineate the intra- and extra-prostatic phases of clonal evolution in unprecedented detail. They found that most clonal evolution occurred within the prostate in a single lineage, after which branching into

multiple distinct lineages occurred that often coexisted in a spatially intermixed state. In all ten men, they observed that one lineage was represented in all of the metastases, indicating that this so-called dominant lineage drove metastatic proliferation. Almost all other lineages were confined to the prostate at the time of death of the patients, although some were also observed in a subset of metastatic sites as a result of multiple seeding events from the prostate. The authors compared the genomic alterations found in the metastatic lineage to those that did not escape the prostate and found significant heterogeneity both between and within patients. Indeed, in one patient, the intra-prostatic lineage experienced at least 5 known driver alterations that were not observed in the lineage that metastasized. The extra-prostatic phase of evolution proceeded in a pattern that indicated that cells at metastatic sites were branches from a continually evolving lineage. In two cases, they could identify that this occurred from within the prostate, but this behavior was also observed in patients who had undergone radical prostatectomy.

Historically, intra-tumoral heterogeneity and clonal evolution typically have been studied as distinct phenomena, but these studies demonstrate that these concepts are intertwined. Broadly speaking, these reports demonstrate the difficulty in determining clonal dynamics between distinct regions of primary PCa, but that it may be more straightforward to find the 'seeds' for clonal metastatic mutations. While the detailed analyses discussed here has led to an improved understanding of PCa, it is difficult to know if the results can be generalizable to the majority of patients with prostate cancer, as they have not been broadly examined in larger patient cohorts.

3.5. Implications for the Clinical Management of Prostate Cancer

Given the limited number of patients with detailed molecular information from multiple sites of primary PCa, further work is required to conclusively link clonal intratumoral heterogeneity in primary PCa with clinical outcomes. The recent Eur Urol San Francisco Consensus statement⁶² provides a standard for

assessing the clinical impact of a prognostic biomarker. PSA is the original such biomarker in prostate cancer (Figure 2C) and many others have been proposed both in blood (for example, PCA3) and tissue (for example Prolaris, Oncotype DX and Decipher)^{12,63–65}. However, recent results highlighting transcriptomic heterogeneity in multiple primary PCa foci⁶⁶ call into question the efficacy of single-site assays to prognosticate patient outcomes. Espiritu et al. found similar results, in showing that patients with multiclonal PCas are more likely to experience poor clinical outcome⁴¹. Future biomarker work could seek to address this through integrated analysis of clonal status and multi-site assaying to improve prognostication.

Precedent exists for the impact of intertumoral heterogeneity on clinical outcome in other solid tumors. Famously, work in the 1980's identified a Her2 amplification in a subset of primary breast cancers⁶⁷. Later trials in patients with this specific alteration demonstrated increased overall survival with Her-2 directed treatment, now widely used as standard of care in breast cancer (for example, Herceptin)^{68,69}. However, to date PCas have not been found to harbor a high-incidence of mutations allowing for such specific targeting. In renal cancers, Gerlinger and colleagues⁷⁰ highlighted heterogeneous tumor evolution and the hindrance to developing precision medicine to either improve genetic diagnostics, or develop target-specific precision medicine therapies.

Studies of clonal evolution in metastatic PCa seem consistently to identify a monoclonal metastatic-potentiating origin which resides, unobserved, within primary PCa. This suggests that radical treatment of primary PCa in the context of metastatic disease could reduce further metastatic seeding events, reducing risk of a poor outcome. This is supported by recent evidence from the STAMPEDE trial found improved survival in oligometastatic PCa patients whose primary prostate was radically treated⁷⁰. However, further work is required to enable identification of such lethal clones during the primary diagnostic process.

3.6 Future Directions

Taken together, we identify from this review an unmet need to interrogate the clonal element of primary PCa intra-tumoral heterogeneity. There remains significant disagreement about whether primary PCa is of monoclonal or polyclonal origin, and whether a tumor-initiating clonal population can be identified. So far, studies have relied heavily on LCM, however newer methodologies to comprehensively profile tissues in a spatial manner are emerging to reduce selection bias and allow larger regions of tissue to be comprehensively profiled at a cellular level⁷¹.

Questions remain about how we might develop methods to consistently profile clonal ancestry, particularly in primary PCa. Additional insight into the clonal dynamics of PCa may be gained through further application of integrated genomics, which seeks to integrate the various molecular ‘-omics’. . Two of the studies discussed above assessed methylation status in combination with genomic profiling to analyze primary ⁷² and metastatic PCa samples ⁷³. The authors found that clonal methylation profiles were not only concordant, but when combined together with genomic information, were superior in analyzing clonal origins of disease than either assay alone. Future efforts to derive clonal dynamics could build on previous integrated approaches such as combined DNA and RNA signatures ^{9,10} which could be assessed in relation to long term patient outcomes. Combinatorial approaches incorporating morphology, transcriptomics, DNA and methylation are likely to deliver synergy in determining clonal ancestry.

The "width" and "depth" of any assay is important. For example, how many cells or regions are being assayed in a given method? Are the areas of prostatic cells, which have been selected by macro/microdissection, actually representative of the relevant underlying clonal relationships? LCM techniques, as used by many studies here, allow for meticulous excision and profiling of regions or cells,

but these techniques are extremely labour intense and are “low-throughput” and still dependent on bulk sequencing, limiting their utility in clinical decision-making.

The future will be increasingly dependent on new “spatial” single cell approaches, including spatial transcriptomics and other slide-based sequencing technologies. These technologies seek to combine genomics analyses with spatial tissue architecture, providing a fine-resolution spatial genomic atlas. Analyzing clonal dynamics through these technologies will allow comparison of multiple foci at unprecedented resolution, and has the potential to decode intratumoral heterogeneity and clonal evolution in PCa.

4. Conclusions

There is a need to identify the clonal source of metastatic disease in primary PCa. Without such knowledge, we cannot truly determine the course and optimal treatment paradigms from initial diagnosis. A number of factors contribute to huge complexity in carrying out these studies, including sample availability, resources, time, but also limited technology to spatially map genomics *in-situ*. Macrodissection and laser-capture microdissection combined with metastatic biopsies and rapid autopsies have raised important questions in our understanding of the relationship between the primary focus of PCa and lethal metastases. Recent advancements in technologies to map spatial biology will undoubtedly start to deliver the answers to these important questions, thereby leading to improved and personalized treatments for men with this common and ubiquitous malignancy.

References

1. Stewart, C. S., Leibovich, B. C., Weaver, A. L. & Lieber, M. M. Prostate cancer diagnosis using a saturation needle biopsy technique after previous negative sextant biopsies. *J. Urol.* **166**, 86–92 (2001).
2. AA, R. Adequate Tissue Sampling of Prostate Core Needle Biopsies. *Am. J. Clin. Pathol.* **107**, (1997).
3. Murphy, W. M., Dean, P. J., Brasfield, J. A. & Tatum, L. Incidental carcinoma of the prostate. How much sampling is adequate? *Am. J. Surg. Pathol.* **10**, 170–174 (1986).
4. Lamb, A. D., Zargar, H., Murphy, D. G., Corcoran, N. M. & Hovens, C. M. Disrupting the Status Quo in Prostate Cancer Diagnosis. *European Urology* **71**, 193–194 (2017).
5. Wei, L. *et al.* Intratumoral and Intertumoral Genomic Heterogeneity of Multifocal Localized Prostate Cancer Impacts Molecular Classifications and Genomic Prognosticators. *Eur. Urol.* **71**, 183–192 (2017).
6. Kanda, M. *et al.* Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. *Gastroenterology* **142**, (2012).
7. Mao, X. *et al.* Distinct genomic alterations in prostate cancers in Chinese and Western populations suggest alternative pathways of prostate carcinogenesis. *Cancer Res.* **70**, 5207–12 (2010).
8. Fraser, M. *et al.* Genomic hallmarks of localized, non-indolent prostate cancer. *Nature* **541**, 359–364 (2017).
9. Ross-Adams, H. *et al.* Integration of copy number and transcriptomics provides risk stratification

- 400 in prostate cancer: A discovery and validation cohort study. *EBioMedicine* **2**, 1133–1144 (2015).
- 401 10. Lalonde, E. *et al.* Tumour genomic and microenvironmental heterogeneity for integrated
 402 prediction of 5-year biochemical recurrence of prostate cancer: A retrospective cohort study.
 403 *Lancet Oncol.* **15**, 1521–1532 (2014).
- 404 11. Taylor, B. S. *et al.* Integrative Genomic Profiling of Human Prostate Cancer. *Cancer Cell* **18**, 11–22
 405 (2010).
- 406 12. Ross-Adams, H. & Lamb, A. D. The genetic classification of prostate cancer: what's on the
 407 horizon? *Futur. Oncol.* **12**, 729–733 (2016).
- 408 13. Dunning, M. J. *et al.* Mining Human Prostate Cancer Datasets: The “camcAPP” Shiny App.
 409 *EBioMedicine* **17**, 5–6 (2017).
- 410 14. Grasso, C. S. *et al.* The mutational landscape of lethal castration-resistant prostate cancer. *Nature*
 411 **487**, 239–243 (2012).
- 412 15. Robinson, D. *et al.* Integrative clinical genomics of advanced prostate cancer. *Cell* **161**, 1215–1228
 413 (2015).
- 414 16. Varambally, S. *et al.* Integrative genomic and proteomic analysis of prostate cancer reveals
 415 signatures of metastatic progression. *Cancer Cell* **8**, 393–406 (2005).
- 416 17. Baca, S. C. *et al.* Punctuated evolution of prostate cancer genomes. *Cell* **153**, 666–677 (2013).
- 417 18. Cooper, C. S. *et al.* Analysis of the genetic phylogeny of multifocal prostate cancer identifies
 418 multiple independent clonal expansions in neoplastic and morphologically normal prostate
 419 tissue. *Nat. Genet.* **47**, 367–372 (2015).
- 420 19. Boutros, P. C. *et al.* Spatial genomic heterogeneity within localized, multifocal prostate cancer.

- 421 *Nat. Genet.* **47**, 736–45 (2015).
- 422 20. Slaughter, D. P., Southwick, H. W. & Smejkal, W. “Field cancerization” in oral stratified squamous
423 epithelium. Clinical implications of multicentric origin. *Cancer* **6**, 963–968 (1953).
- 424 21. Nonn, L., Ananthanarayanan, V. & Gann, P. H. Evidence for field cancerization of the prostate.
425 *Prostate* **69**, 1470–1479 (2009).
- 426 22. Bianchi-Frias, D. *et al.* Cells Comprising the Prostate Cancer Microenvironment Lack Recurrent
427 Clonal Somatic Genomic Aberrations. *Mol. Cancer Res.* **14**, 374–84 (2016).
- 428 23. Nash, C. *et al.* Genome-wide analysis of AR binding and comparison with transcript expression in
429 primary human fetal prostate fibroblasts and cancer associated fibroblasts. *Mol. Cell. Endocrinol.*
430 **471**, 1–14 (2018).
- 431 24. Chen, W. J. *et al.* Cancer-associated fibroblasts regulate the plasticity of lung cancer stemness via
432 paracrine signalling. *Nat. Commun.* **5**, 1–17 (2014).
- 433 25. Gaisa, N. T. *et al.* Clonal architecture of human prostatic epithelium in benign and malignant
434 conditions. *J. Pathol.* **225**, 172–80 (2011).
- 435 26. Gerrin, S. J., Sowalsky, A. G., Balk, S. P. & Ye, H. Mutation Profiling Indicates High Grade Prostatic
436 Intraepithelial Neoplasia as Distant Precursors of Adjacent Invasive Prostatic Adenocarcinoma.
437 *Prostate* **76**, 1227–36 (2016).
- 438 27. Boyd, L. K. *et al.* High-resolution genome-wide copy-number analysis suggests a monoclonal
439 origin of multifocal prostate cancer. *Genes. Chromosomes Cancer* **51**, 579–89 (2012).
- 440 28. Alexander, J. *et al.* Utility of Single-Cell Genomics in Diagnostic Evaluation of Prostate Cancer.
441 *Cancer Res.* **78**, 348–358 (2018).

- 442 29. Andreoiu, M. & Cheng, L. Multifocal prostate cancer: biologic, prognostic, and therapeutic
443 implications. *Human Pathology* **41**, 781–793 (2010).
- 444 30. Guinney, J. *et al.* Prediction of overall survival for patients with metastatic castration-resistant
445 prostate cancer: development of a prognostic model through a crowdsourced challenge with
446 open clinical trial data. *www.thelancet.com/oncology* **18**, (2017).
- 447 31. Stürenberg, C. *et al.* Abstract 1396: Detection and local histological staging of prostate cancer foci
448 in H&E whole slide images using convolutional neural networks. in *Cancer Research* **79**, 1396–
449 1396 (American Association for Cancer Research (AACR), 2019).
- 450 32. Albertsen, P. C. *et al.* 20-Year Outcomes Following Conservative Management of Clinically
451 Localized Prostate Cancer. *JAMA* **293**, 2095 (2005).
- 452 33. Lokman, U., Erickson, A. M., Vasarainen, H., Rannikko, A. S. & Mirtti, T. PTEN Loss but Not ERG
453 Expression in Diagnostic Biopsies Is Associated with Increased Risk of Progression and Adverse
454 Surgical Findings in Men with Prostate Cancer on Active Surveillance. *Eur. Urol. Focus* **4**, 867–873
455 (2018).
- 456 34. Barentsz, J. O. *et al.* ESUR prostate MR guidelines 2012. *Eur. Radiol.* **22**, 746–757 (2012).
- 457 35. Lindberg, J. *et al.* Exome sequencing of prostate cancer supports the hypothesis of independent
458 tumour origins. *Eur. Urol.* **63**, 347–53 (2013).
- 459 36. Parry, M. A. *et al.* Genomic Evaluation of Multiparametric Magnetic Resonance Imaging-visible
460 and -nonvisible Lesions in Clinically Localised Prostate Cancer. *Eur. Urol. Oncol.* **2**, 1–11 (2019).
- 461 37. Løvf, M. *et al.* Multifocal Primary Prostate Cancer Exhibits High Degree of Genomic
462 Heterogeneity. *Eur. Urol.* **75**, 498–505 (2019).

- 463 38. Su, F. *et al.* Spatial Intratumor Genomic Heterogeneity within Localized Prostate Cancer Revealed
464 by Single-nucleus Sequencing. *Eur. Urol.* **74**, 551–559 (2018).
- 465 39. Sowalsky, A. G. *et al.* Gleason Score 7 Prostate Cancers Emerge through Branched Evolution of
466 Clonal Gleason Pattern 3 and 4. *Clin. Cancer Res.* **23**, 3823–3833 (2017).
- 467 40. Kim, T.-M. *et al.* Regional biases in mutation screening due to intratumoural heterogeneity of
468 prostate cancer. *J. Pathol.* **233**, 425–35 (2014).
- 469 41. Espiritu, S. M. G. *et al.* The Evolutionary Landscape of Localized Prostate Cancers Drives Clinical
470 Aggression. *Cell* **173**, 1003-1013.e15 (2018).
- 471 42. Liu, W. *et al.* Copy number analysis indicates monoclonal origin of lethal metastatic prostate
472 cancer. *Nat. Med.* **15**, 559–65 (2009).
- 473 43. Wu, Y. *et al.* High-Resolution Genomic Profiling of Disseminated Tumor Cells in Prostate Cancer. *J.*
474 *Mol. Diagn.* **18**, 131–43 (2016).
- 475 44. Robbins, C. M. *et al.* Copy number and targeted mutational analysis reveals novel somatic events
476 in metastatic prostate tumors. *Genome Res.* **21**, 47–55 (2011).
- 477 45. Rodrigues, D. N. *et al.* Rb1 heterogeneity in advanced metastatic castration-resistant prostate
478 cancer. *Clin. Cancer Res.* **25**, 687–697 (2019).
- 479 46. Nickerson, M. L. *et al.* Somatic alterations contributing to metastasis of a castration-resistant
480 prostate cancer. *Hum. Mutat.* **34**, 1231–41 (2013).
- 481 47. Gudem, G. *et al.* The evolutionary history of lethal metastatic prostate cancer. *Nature* **520**, 353–
482 357 (2015).
- 483 48. Dago, A. E. *et al.* Rapid phenotypic and genomic change in response to therapeutic pressure in

- 484 prostate cancer inferred by high content analysis of single circulating tumor cells. *PLoS One* **9**,
 485 e101777 (2014).
- 486 49. Carreira, S. *et al.* Tumor clone dynamics in lethal prostate cancer. *Sci. Transl. Med.* **6**, 254ra125
 487 (2014).
- 488 50. Beltran, H. *et al.* Divergent clonal evolution of castration-resistant neuroendocrine prostate
 489 cancer. *Nat. Med.* **22**, 298–305 (2016).
- 490 51. Jiang, R. *et al.* A comparison of isolated circulating tumor cells and tissue biopsies using whole-
 491 genome sequencing in prostate cancer. *Oncotarget* **6**, 44781–93 (2015).
- 492 52. Haffner, M. C. *et al.* Tracking the clonal origin of lethal prostate cancer. *J. Clin. Invest.* **123**, 4918–
 493 22 (2013).
- 494 53. VanderWeele, D. J. *et al.* Low-grade prostate cancer diverges early from high grade and
 495 metastatic disease. *Cancer Sci.* **105**, 1079–85 (2014).
- 496 54. Lindberg, J., Kristiansen, A., Wiklund, P., Grönberg, H. & Egevad, L. Tracking the origin of
 497 metastatic prostate cancer. *Eur. Urol.* **67**, 819–22 (2015).
- 498 55. Labbé, D. P. *et al.* High-fat diet fuels prostate cancer progression by rewiring the metabolome
 499 and amplifying the MYC program. *Nat. Commun.* **10**, 4358–4358 (2019).
- 500 56. Lawrence, M. G. *et al.* Knowing what’s growing: Why ductal and intraductal prostate cancer
 501 matter. *Science Translational Medicine* **12**, (2020).
- 502 57. Chen, E. J. *et al.* Abiraterone treatment in castration-resistant prostate cancer selects for
 503 progesterone responsive mutant androgen receptors. *Clin. Cancer Res.* **21**, 1273–80 (2015).
- 504 58. Hong, M. K. H. *et al.* Tracking the origins and drivers of subclonal metastatic expansion in

- 505 prostate cancer. *Nat. Commun.* **6**, 6605 (2015).
- 506 59. Gillard, M. *et al.* Integrative Genomic Analysis of Coincident Cancer Foci Implicates CTNNB1 and
507 PTEN Alterations in Ductal Prostate Cancer. *Eur. Urol. Focus* **5**, 433–442 (2019).
- 508 60. Ritch, E. *et al.* Identification of hypermutation and defective mismatch repair in ctDNA from
509 metastatic prostate cancer. *Clin. Cancer Res.* **26**, 1114–1125 (2020).
- 510 61. Woodcock, D. J. *et al.* Prostate cancer evolution from multilineage primary to single lineage
511 metastases with implications for liquid biopsy. *Nat. Commun.* **11**, 5070 (2020).
- 512 62. Cooperberg, M. R. *et al.* The State of the Science on Prostate Cancer Biomarkers: The San
513 Francisco Consensus Statement. *European Urology* **76**, 268–272 (2019).
- 514 63. Marrone, M., Potosky, A. L., Penson, D. & Freedman, A. N. A 22 gene-expression assay, decipher®
515 (GenomeDx biosciences) to predict five-year risk of metastatic prostate cancer in men treated
516 with radical prostatectomy. *PLoS Curr.* **7**, (2015).
- 517 64. Knezevic, D. *et al.* Analytical validation of the Oncotype DX prostate cancer assay - a clinical RT-
518 PCR assay optimized for prostate needle biopsies. *BMC Genomics* **14**, 690 (2013).
- 519 65. Cuzick, J. *et al.* Prognostic value of an RNA expression signature derived from cell cycle
520 proliferation genes in patients with prostate cancer: A retrospective study. *Lancet Oncol.* **12**,
521 245–255 (2011).
- 522 66. Salami, S. S. *et al.* Transcriptomic heterogeneity in multifocal prostate cancer. *JCI insight* **3**,
523 (2018).
- 524 67. Slamon, D. J. *et al.* Human breast cancer: Correlation of relapse and survival with amplification of
525 the HER-2/neu oncogene. *Science (80-.)*. **235**, 182–191 (1987).

68. Vogel, C. L. *et al.* Efficacy and Safety of Trastuzumab as a Single Agent in First-Line Treatment of HER2 -Overexpressing Metastatic Breast Cancer . *J. Clin. Oncol.* **20**, 719–726 (2002).
69. Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A. & Greene, M. I. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell* **41**, 695–706 (1985).
70. Gerlinger, M. *et al.* Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. *N. Engl. J. Med.* **366**, 883–892 (2012).
71. Ståhl, P. L. *et al.* Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. *Science* **353**, 78–82 (2016).
72. Brocks, D. *et al.* Intratumor DNA methylation heterogeneity reflects clonal evolution in aggressive prostate cancer. *Cell Rep.* **8**, 798–806 (2014).
73. Aryee, M. J. *et al.* DNA methylation alterations exhibit intraindividual stability and interindividual heterogeneity in prostate cancer metastases. *Sci. Transl. Med.* **5**, 169ra10 (2013).

Key points:**• What do we know:**

- Primary PCa is genetically heterogeneous
- There is no consistent mutation giving rise to primary prostate cancer
- Detailed clonal analysis of primary prostate cancer has been limited by current techniques
- Metastatic specimens demonstrate consistent truncal genomic aberrations that suggest monoclonal metastatic progenitors

• What are the key questions:

- Where are the metastatic progenitor populations in primary PCa?
- Can spatial genomic techniques be used to define clonal relationships in primary prostate cancer?
- Given the low mutational burden in primary prostate cancer, could alternative levels of biological information (eg RNA/proteins/methylation), help identify clonal populations?

565 **Glossary:**

566 PCa: Prostate Cancer

567 AR: Androgen Receptor

568 SNV: Single-Nucleotide Variants

569 ITH: Intra-tumoral Heterogeneity

570 CE: Clonal Evolution

571 CGH: comparative genomic hybridization

572 FISH: Fluorescent In-Situ Hybridization

573 IHC: Immunohistochemistry

574 HGPIN: High Grade Prostate Intraepithelial Neoplasia

575 PTEN: Phosphatase and tensin homolog

576 TP53: Tumor protein 53

577 ETS: Erythroblast transformation-specific

FIG 1

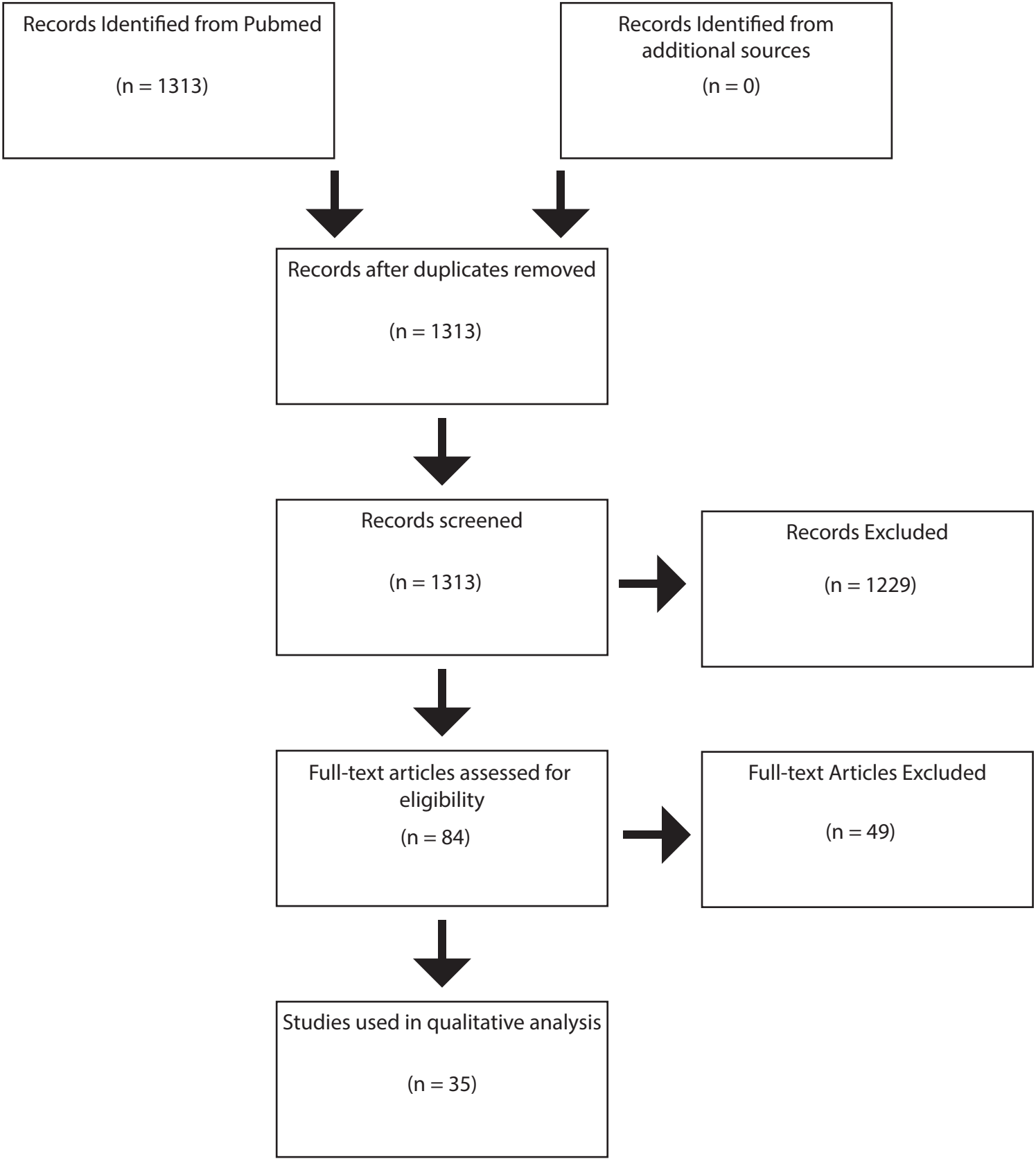
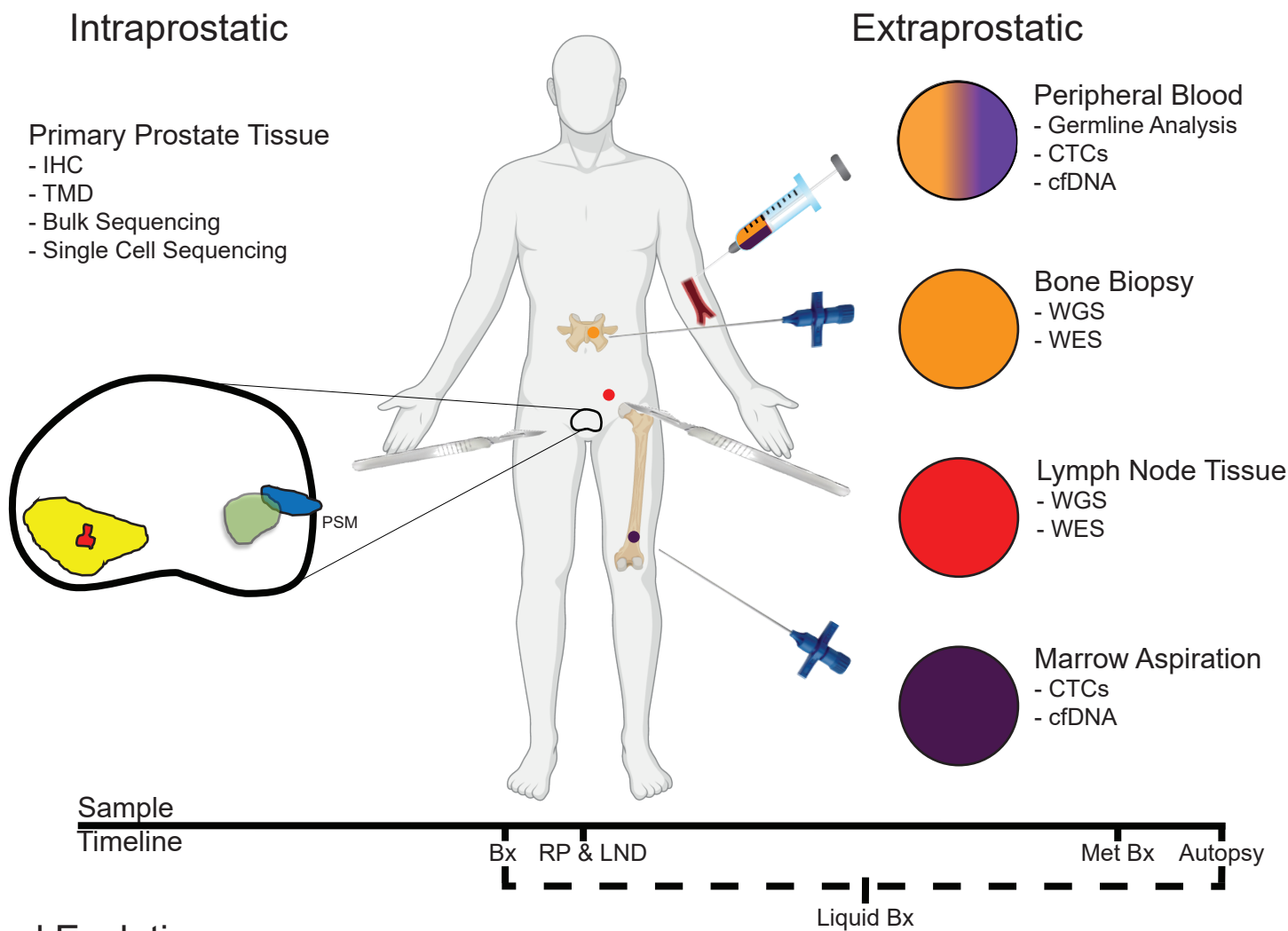
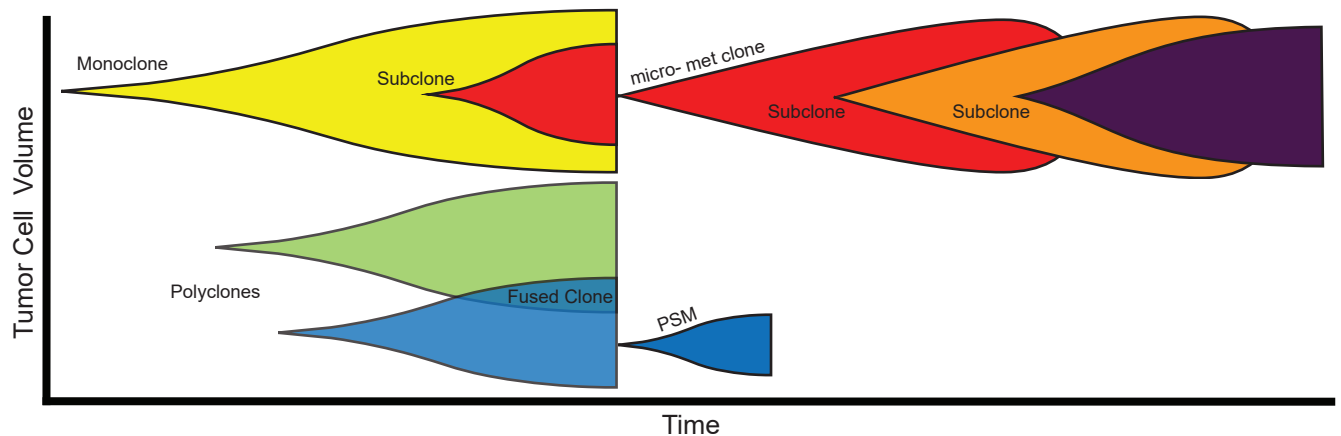


Fig. 1. Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) flowchart.

A. Tumour Heterogeneity



B. Clonal Evolution



C. Tumour Burden

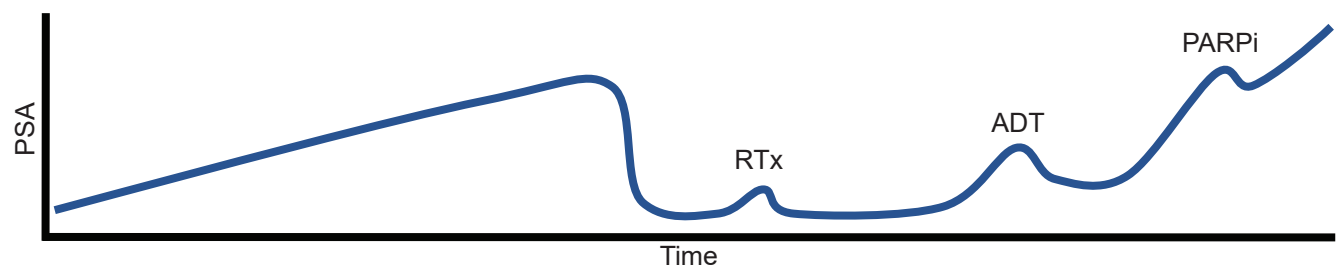


Figure 2. A) Methodologies and potential sample sites for determination of intraprostatic and extraprostatic heterogeneity. The prostate can be sampled by image-guided biopsy or at the time of radical prostatectomy. Multifocal heterogeneity may be evident at biopsy but is certainly seen at prostatectomy. Lymph node dissection at the time of initial surgery also enables sampling of presumed monoclonal cellular expansions of metastasised cells. Once the primary gland is removed, further options for sampling include selective sampling of sites of metastasis in bone or lymph nodes (or, occasionally, other soft tissue sites) or aspiration of tumour cell-bearing fluid from the blood or bone marrow. Circulating tumour cells (CTCs) or cell-free DNA (cfDNA) can be extracted from this fluid. B) Tumour clones arise from metachronous mutations in a polyclonal or subclonal fashion. Spatially proximate clones can overlap ("fused clone"). Clones naturally expand over time unless treated (by surgery or therapy). Clones retain the ability to undergo subclonal mutation under environmental pressure such as with systemic therapy from androgen targeted therapy or small molecule treatment. C) Tumour burden is most commonly assessed by prostate specific antigen (PSA; KLK3) levels. PSA usually falls to un-recordable levels after radical local treatment but residual tumour clones at the site of a positive surgical margin (PSM) or in the lymphatic system/circulation can gradually expand to release detectable levels of PSA. Subsequent systemic treatments limit such cellular expansions but genotypic versatility enables tumours clones to avoid extinction but continued expansion despite the altered environment. IHC = Immunohistochemistry, TMD = Tissue Microdissection, CTCs = circulating tumour cells, WGS = Whole Genome Sequencing, WES = Whole Exome Sequencing, cfDNA = cell-free DNA, Bx = Biopsy, RP = Radical Prostatectomy, LND = Lymph Node Dissection, MetBx = Metastatic Biopsy, PSM = Positive Surgical Margin, RTx = Radiotherapy, ADT = Androgen Deprivation Therapy, PARPi = PARP Inhibition.

Table 1 - Overview of the molecular assays used in the reviewed studies

Methodology	Abbreviation	Molecular State Assayed
Whole-Genome Sequencing	WGS	DNA
Whole-Exome Sequencing	WES	DNA
Array Comparative Genomic Hybridization	Array CGH	DNA
Methylation Array	Methylation Array	Methylation
Mitochondrial DNA	mtDNA	Mitochondrial DNA
Targeted Panel		DNA
Single-Nucleotide Polymorphism Array	SNP Array	DNA
Immunohistochemistry	IHC	Protein
Fluorescent In-situ Hybridization	FISH	DNA/RNA

Coverage and Resolution

Genome (3 billion individual base pairs) at 1bp Resolution

Exome (30 million individual base pairs) at 1bp Resolution

Genome (3 billion individual base pairs) at 5-10 megabase resolution

Genome wide at Distinct Methylation Sites (15000-450000 total)

Mitochondrial Genome (16,000 bp) at 1 bp Resolution

Dependent on Design, 1bp resolution

Genome wide, between 500,000-2million SNPs at 5-25 kilobase resolution

Single Protein

Single Gene/Transcript

Table 2. Overview of reviewed studies assessing clonal origin of primary prostate cancer.

Citation	Author	Date	Journal	Specimens	Specimens	Patients	Assays
²⁵	Gaisa et al.	2011	J. Pathol.	Multiple Primary PCa regions	1	1	mtDNA seq, IHC
²⁷	Boyd et al.	2012	Genes. Chromosomes Cancer	Primary PCa, Normal, HGPIN	48	18	SNP arrays
³⁵	Lindberg et al.	2013	Eur. Urol.	Primary PCa	10	4	WES, Array CGH
⁴⁰	Kim et al.	2014	J. Pathol.	Primary PCa Foci	12	3	WES, SNP & RNA arrays
¹⁹	Boutros et al.	2015	Nat. Genet.	Regions from Primary PCa	23	5	WGS, WES
¹⁸	Cooper et al.	2015	Nat. Genet.	Primary PCa, Benign, Matched blood	18	3	WGS
⁷²	Brocks et al.	2014	Cell Rep.	Regions from Primary PCa	72	5	Methylation Array
²⁶	Gerrin et al.	2016	Prostate	HGPIN regions and Primary PCa	6	1	WES, Targeted Deep Seq
²²	Bianchi-Frias et al.	2016	Mol. Cancer Res.	Primary PCa and Adjacent Stroma	40	20	Array CGH, mtDNA
²⁸	Alexander et al.	2018	Cancer Res.	Single cells from Primary PCa	4021	11	scWGS
³⁹	Sowalsky et al.	2017	Clin. Cancer Res.	Primary PCa regions	6	2	WES, Gene Expression
⁵	Wei et al.	2017	Eur. Urol.	Primary PCa cores	26	4	WES, SNP array, RNAseq
³⁷	Løvf et al.	2019	Eur. Urol.	Distinct Primary PCa regions	153	41	WES
³⁸	Su et al.	2018	Eur. Urol.	Single cells from Primary PCa + Benign	21	2	scWGS
⁴¹	Espiritu et al.	2018	Cell	Primary PCa samples	293	293	WGS
³⁶	Parry et al.	2019	Eur. Urol. Oncol.	Multiple Primary PCa regions	43	6	Low-Pass WGS, WES

PCa = Prostate cancer, mtDNA = mitochondrial DNA, IHC = Immunohistochemistry, PIN = prostatic intra-epithelial neoplasia, SNP = single-nucleotide polymorphism, HGPIN = high-grade prostatic intra-epithelial neoplasia, WES = Whole Exome Sequencing, CGH = comparative genomic hybridization, WGS = whole genome sequencing, scWGS = single-cell whole genome sequencing,

Table 3. Overview of reviewed studies assessing metastatic heterogeneity in prostate cancer.

Citation	Author	Date	Journal	Specimens	Specimens	Patients	Assays
⁴²	Liu et al.	2009	Nat. Med.	R. Autopsy - Distant Mets and Normal	94	30	Array CGH, SNP array
⁴⁴	Robbins et al.	2011	Genome Res.	R. Autopsy - Distant Mets	8	6	Array CGH
⁷³	Aryee et al.	2013	Sci. Transl. Med.	R. Autopsy - Distant Mets and Normal	95	13	Methylation Array
⁴⁶	Nickerson et al.	2013	Hum. Mutat.	R. Autopsy - Distant Mets and Normal Kidney	6	1	WES
⁴⁷	Gundem et al.	2015	Nature	Rapid Autopsy - Distant Mets*	51	10	WGS
⁴⁹	Carriera et al.	2014	Sci. Transl. Med.	CRPC Bx and plasma, Pre-Castrate samples	102	16	targeted DNA panel
⁴⁸	Dago et al.	2014	PLoS One	CTCs + Bone Met Bx	41 cells	1	WGS
⁵⁰	Beltran et al.	2016	Nat. Med.	NEPC and Adenocarcinoma Bx	50	17	WES, RNA seq, Methylation
⁵¹	Jiang et al.	2015	Oncotarget	Primary PCa + Normal, CTCs, Met Bx	14	1	WGS, WES, Array cCGH
⁴³	Wu et al.	2016	J. Mol. Diagn.	Autopsy CTCs + Metastatic PCa tissue	Unclear	2	SNP Array
⁴⁵	Rodrigues	2019	Clin. Can. Res.	Met Bx	3	1	WGS

CGH = comparative genomic hybridization, SNP = single-nucleotide polymorphism, WES = whole exome sequencing, WGS = whole genome sequencing, CRPC = castration resistant prostate cancer, CTCs = circulating tumour cells, met = metastases

Table 4. Overview of reviewed studies assessing intra-prostatic heterogeneity and metastatic clonal evolution

Citation	Author	Date	Journal	Specimens	Specimens	Patients	Assays
⁵²	Haffner et al.	2013	J. Clin. Invest	Primary RP, LN Mets, Distant Mets	18*	1	WGS (mets), IHC & FISH
⁵³	VanderWeele et al.	2014	Cancer Sci.	Primary RP, LN Metastases	10	4	WES
⁵⁴	Lindberg et al.	2015	Eur. Urol.	Primary RP, LN Metastases	4	1	WES
⁵⁷	Chen et al.	2015	Clin. Cancer Res.	Primary RP	26	4	WES
⁵⁸	Hong et al.	2015	Nat. Commun.	Primary and Distant Mets*	45	1	WGS, RNA seq, SNP arrays
⁵⁹	Gillard et al.	2019	Eur. Urol. Foc.	Primary RP, LN Metastasis	3	1	WES
⁶⁰	Ritch et al.	2019	Clin. Cancer Res.	Primary RP and cfDNA	12	2	WES
⁶¹	Woodcock et al.	2020	Nat. Commun.	Primary RP, Rapid Autopsy	150	10	WGS

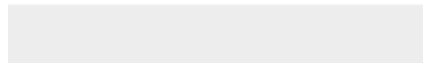
PCa = prostate cancer, RP = radical prostatectomy, LN = lymph node, Mets = metastases, WGS = whole genome sequencing, IHC = immunohistochemistry, FISH = fluorescent in-situ hybridization, WES = whole exome sequencing, cfDNA = cell-free DNA



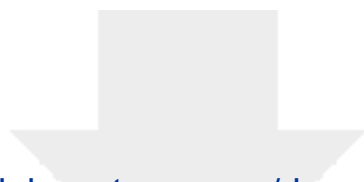
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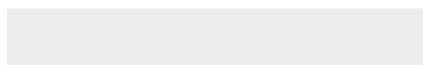
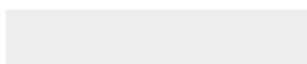




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Studies characterizing genomic changes of prostate cancer increased our understanding of the disease, however, its multifocal nature has hindered clinical translation and few reports have profiled multiple regions. We discuss recent efforts to profile prostatic intratumoral heterogeneity and clonal evolution.

A systematic review of prostate cancer heterogeneity – understanding the clonal ancestry of multifocal disease

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Abstract

Context: Studies characterizing the genomic changes of prostate cancer (PCa) during natural progression have greatly increased our understanding of the disease. A better understanding of the evolutionary history of PCa would enable advancements in diagnostics, prognostication, and novel therapies that together will improve patient outcomes.

Objective: In this systematic review, we discuss molecular heterogeneity of PCa by assessing recent efforts to profile intratumoral heterogeneity and clonal evolution.

Evidence Acquisition: We screened a total of 1313 publication abstracts from PUBMED between 2009-2020, of which we reviewed 84 full-text articles. We excluded 49, resulting in a final 35 studies.

Evidence Synthesis: In studies of primary PCa (16 studies, 4793 specimens), there is a lack of consensus regarding the monoclonal or polyclonal origin of primary PCa. There is no consistent mutation giving rise to primary prostate cancer. Detailed clonal analysis of primary prostate cancer has been limited by current techniques. By contrast, clonal relationships between PCa metastases and a potentiating clone have been consistently identified (19 studies, 732 specimens). Metastatic specimens demonstrate consistent truncal genomic aberrations that suggest monoclonal metastatic progenitors.

Conclusions: The relationship between clonal dynamics of PCa and clinical outcomes needs further investigation. It is likely that this will provide the biological rationale for whether radical treatment of the primary prostate benefits patients with oligometastatic PCa. Future work studying the mutational burden in primary disease at single cell resolution should permit the identification of clonal patterns underpinning the origin of lethal PCa.

Patient summary: Prostate cancers arise in different parts of the prostate due to DNA mutations that occur by chance at different times. These cancer cells and their origin can be tracked by DNA mapping.

37 In this review we summarize the 'state-of-the-art' and outline what further science is needed to provide
38 the missing answers.

1. Introduction

Prostate cancer (PCa) is a heterogeneous disease. A single biopsy of the prostate, even if it does manage to hit a focus of cancer, is often inadequate for a comprehensive assessment of the disease¹⁻³. This is true for assessment of somatic genetic changes just as it is for histological morphology^{4,5}.

The accessibility, cost, coverage and resolution of genomic assays has improved rapidly since the first sequencing of the human genome. Even a decade ago relatively little was known about the wider landscape of genomic alterations in PCa. As new technologies have permitted broader profiling of tumors, PCa investigators were unable to find high incidence mutations as detected in other cancers such as pancreatic cancer where 90% of cases are *KRAS* mutant⁶. Large scale efforts to sequence primary PCa from multiple patients have revealed extensive genomic heterogeneity, particularly copy-number changes, and relative lack of distinct driver mutations⁷⁻¹³. In contrast, efforts to sequence advanced disease have revealed more consistent aberrations in many genes such as *AR*, *PTEN*, *TP53*, *ETS* genes, *BRAF* and DNA-Damage Repair gene pathway members¹⁴⁻¹⁷. While these efforts have been instrumental in advancing knowledge about prostate cancer genomics, they have largely failed to characterize actionable changes in early prostate cancer and thus targeted therapies have not made it into routine clinical practice as with other tumour types. It is increasingly apparent that this is due to the extensive intra-tumoral heterogeneity in prostate cancer as revealed by other, more focused, contemporary sequencing studies, particularly in primary disease^{18,19}.

In this review, we aggregate knowledge regarding prostate cancer intra-tumoral heterogeneity and clonal evolution as determined by genomic assays, as applied to multiple tumor regions, to identify the gaps in technology and understanding that need to be filled in order for us to correctly interpret clinical findings in this multifaceted disease.

2. Evidence Acquisition

2.1 Search Strategy

We conducted a systematic literature search of PubMed for all articles up to December 2020. The search terms and strategy are recorded in Supplementary Material 1. We limited our searches to publications from the past 10 years.

2.2 Inclusion and exclusion criteria

We followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) criteria (Figure 1) for evaluating records during the literature search. The lead author (AE) inspected all studies against the inclusion criteria, with two further authors reviewing all included and excluded studies to provide oversight (ADL & IGM). Specifically, we included original research articles, from DNA or RNA-based assays capable of resolving intra-tumoral heterogeneity and clonal evolution, from two or more readouts from human patient specimens. We excluded studies that reported results from model systems alone or non-human studies (eg., cell lines or mice), review articles, and reports from single readouts from a single cancer specimen.

3. Evidence Synthesis

3.1 Methods for Determining Heterogeneity

There are many methods for determining heterogeneity and the methods chosen by investigators vary in scope, size and in the biological entities targeted by the assay. It is therefore important to consider these technical aspects at the outset (Table 1, Figure 2A).

3.2.1 Intra-Prostatic Heterogeneity

Descriptions of heterogeneity may differ so we begin with some definitions. We consider 'intra-prostatic' to refer to localized disease and including local invasion (for example, spread to the seminal vesicles). We define 'Intra-tumoral heterogeneity' as observed phenotypic or genotypic differences

between cancer cells. Furthermore, it is important to distinguish between foci of disease that are unrelated -- termed 'polytumors' or 'polyclonal', from those that share a common ancestor 'monotumors', 'monoclonal', or subclonal(Figure 2A&B). Within the past ten years, increasing efforts to study these phenomena have used an assortment of methods to broadly characterize genomic intra-prostatic heterogeneity by assaying and analyzing multiple, separately extracted, regions of PCa lesions as well as adjacent non-cancerous regions.

3.2.2 Field Effect

Field cancerization, or field effect, was described by Slaughter and colleagues in 1953 as morphological changes occurring in adjacent, non-cancerous cells near tumour lesions²⁰, and this can include immune or inflammatory field effects. Despite evidence from an increasing number of different field effect markers²¹, the landscape of specific carcinogenic field effect changes in PCa is still relatively unknown. Genomic characterization of prostatic lesions and adjacent tissue regions has provided insights into the molecular origins and drivers of PCa development and progression.

Recent studies have reported results from the comparison of normal-adjacent prostate tissue and cancerous regions. The first study to use WGS found that "normal" tissues harbored distinct mutations not shared by blood or cancer samples taken from the same patients¹⁸. Separate efforts to characterize cancer adjacent stroma found that the stroma does not share the same genomic profile as adjacent cancerous regions, further suggesting that stromal cells are not most recent common ancestors of cancerous regions²². However, given that cross-talk between stromal cells, for example cancer associated fibroblasts, and neighboring PCa epithelial cells can occur²³ and evidence from other cancers has shown cancer associated fibroblasts influence cancer stemness²⁴, further work is needed to further elucidate how stromal cells may influence PCa clonal dynamics.

Additional efforts have focused on potential clonal relationship of prostatic intra-epithelial neoplasia (PIN) and adjacent cancer regions as assessed by genome sequencing. In one case, authors assessed multiple PIN and PCa regions of a single patient, and found a shared mtDNA mutation harbored by all PIN regions and at least one PCa clone²⁵. Similarly, Gerrin and colleagues re-analyzed multiple high-grade PIN (HGPIN) and PCa regions, previously characterized by WES, and found that HGPIN harbored a distinct *OR2AP1* mutation that was present in both the Gleason 3 and Gleason 4 tumor foci²⁶. Additionally, a separate study reported conserved copy-number variation in all regions from cases having both HGPIN and adjacent cancer regions²⁷. Finally, a recent report used single-cell WGS of individual cells “washed” from diagnostic prostate biopsies in addition to frozen punch biopsies from radical prostatectomy specimens²⁸. While these studies were able to establish clonal relationships, they also found that in some regions the cancers featured no copy number alterations, whereas other regions from the same patient, without any cancer had copy number alterations. Taken together, although not conclusive, the results suggest that PIN regions may be clonal ancestors of PCa, but there is no evidence, as yet, to link PIN with lethal clones.

3.2.3 Clonal Origin of Primary Prostate Cancer – Mono or Polyclonal?

Primary PCa is often multifocal²⁹. Uncertainty remains regarding whether common ancestral clones, also termed as a “tumor initiating cells”, can be identified from primary PCs. Monoclonal tumors are defined as arising from a single common ancestor, whereas polyclonal tumors are tumors with no shared common ancestry.

Analyses of several primary PCa collections have concluded that a monoclonal origin of PCa cannot be consistently detected from genomic sequencing (Table 2, Supplementary Table 1)^{18,19,30–36}. An initial report could not identify a shared common ancestry in a majority of cases profiled by multi-region WES³⁵. These results were consistent with later findings of multi-region sequencing by WGS, which

129 additionally found multiple clones even within individually profiled foci, as well as distinct mutational
130 profiles of adjacent normal prostate¹⁸. In addition, another report found that in at least one patient
131 whose tumor regions were assessed by WGS, the profiled tumors contained at least 2 subclones with no
132 common identifiable genomic alterations¹⁹. Furthermore, a larger study performed WES of multiple
133 regions from a cohort of patients and found that 76% of individual patients' tumour specimens shared
134 no copy-number variation or single-nucleotide variants³⁷. Finally, a recent study of multiple mpMRI-
135 visible and invisible lesions in primary PCa reported results that suggest either a monoclonal or
136 polyclonal origin of PCa as assessed by WGS³⁶.

137 In contrast, a number of other studies identified individual cases where a monoclonal origin to tumors
138 was detected³⁸⁻⁴⁰. An effort using WES on regions of both Gleason 3 and Gleason 4 disease was able to
139 identify shared common genomic alterations in all profiled regions³⁹. A separate study using a similar
140 approach was also able to identify shared common genomic ancestry in all profiled regions⁴⁰, although
141 the authors acknowledged from their data that genomic alterations from an individual profiled sub-
142 region could represent as few as 20% of all alterations in the patient's overall cancer burden. A more
143 recent study profiled individual single-cells from a radical prostatectomy specimen using WGS and this
144 approach identified features of monoclonality in all cells from at least one patient³⁸.

145 Taken together, these reports show that there is no clear consensus on whether clinically presenting
146 primary PCa can be consistently assumed to be of either monoclonal or polyclonal origin. This has clear
147 clinical implications for both molecular diagnostics and focal therapy⁴. A recent study has suggested
148 that clonality can be computationally deconvoluted from bulk-sequencing of single malignant foci, and
149 clonal status is prognostic for clinical outcome⁴¹. It is also likely that sampling and detection bias could
150 also play a role, given that different molecular assays have different levels of coverage. Similarly, the
151 assays used in these studies are only applied to a fraction of the cells in the entire prostate gland or
152 specimen. To date, there has been no single comprehensive study utilizing the same molecular assay

and computational pipeline to characterize the prevalence of both monoclonal and polyclonal origin of tumor-initiation within PCa patients.

3.3.1 Metastatic Heterogeneity

Studies assessing extra-prostatic cancer heterogeneity have largely focused on decoding the heterogeneity within distantly disseminated metastatic PCa (Table 3, Supplementary Table 1). It is important to recognize that in this setting, these cellular relationships are usually inferred mutational aberrations identified at a single collection timepoint as there are almost no clinical indications for serial resection or biopsying of metastatic lesions. Much of the evidence presented is derived from “rapid autopsy” studies from metastatic sites, along with samples from the prostate gland and, in some cases, with control samples, or from research studies permitting the biopsying of metastatic lesions.

A nascent but growing body of work has added the ability to interrogate circulating tumor cells allowing comparisons to molecular profiles from metastatic lesions.

3.3.2 Clonality and Origin of Metastatic Prostate Cancer

A number of studies have sought to profile biological features from multiple metastatic lesions to uncover the landscape of genomic alterations in advanced PCa. The first comprehensive report of the pattern of metastatic spread of PCa from autopsies proposed a monoclonal origin of PCa⁴². Another study assayed metastatic specimens and circulating tumor cells taken at the time of autopsy, and found their genomic profiles to be 90-99% concordant⁴³. Robbins et al. used a combinatorial array comparative genomic hybridization and targeted next-generation sequencing profiling approach, and validated previous findings identifying a genomic profile consistent with an individual metastatic progenitor⁴⁴. Similarly, a recent report by Rodrigues et al used WGS to profile 3 metastatic lesions from 1 patient, which identified a distinct RB1 deletion that was shared between three metastases in one patient, suggesting a monoclonal origin of disease⁴⁵.

176 Additionally studies have used WES⁴⁶ and WGS⁴⁷ to profile multiple metastatic lesions. These analyses
177 found evidence for the sequential accumulation of mutations in metastases⁴⁶ and observed complex
178 patterns of metastatic spread, including metastasis-to-metastasis seeding but also the potential for
179 multiple primary PCa clones to give rise to metastases⁴⁷. This finding of multiple mutational clones
180 existing in the metastatic space is important as, if corroborated, it implies that sampling a single site of
181 metastatic disease may be inadequate as a companion diagnostic tool to determine preferential
182 targeted treatment.

183 Taken together, these studies present our current state of knowledge of the pattern of spread of
184 disseminated metastatic PCa. In contrast to the challenges highlighted in identification of tumour-
185 initiating clones of origin in the prostate gland, these studies suggest that it is comparatively straight-
186 forward to identify clonal relationships between PCa metastases. Collections of PCa metastases
187 commonly arise from a common clonal progenitor within the prostate gland, but evidence also suggests
188 the likelihood that additional subclones arise in the metastatic space and that these may preferentially
189 give rise to further metastases.

190 **3.3.3 Temporal Heterogeneity**

191 Several studies have assessed temporal heterogeneity by observing true differences in states over time.
192 Under selective pressure of therapies, treatment resistance can emerge as a result of the expansion of
193 pre-existing subclonal populations. Studies assessing temporal heterogeneity typically use liquid
194 biopsies, however, a small subset also include PCa tissues (Figure 2B). A recent case report analyzed
195 genomic alterations through WGS in metastatic tissue and CTCs from multiple time points during disease
196 progression in a single patient⁴⁸. The authors found comprehensive loss of early clones by the last
197 observed timepoint, suggesting extensive changes to clonal profiles due to the selection pressures
198 induced by treatment. These findings are in concordance with a separate study that analyzed clonality

through copy number profiling and targeted deep-sequencing of multiple metastatic PCa specimens during the course of disease progression during treatment ⁴⁹. Treatment-related temporal heterogeneity has been implicated in the development of neuroendocrine PCa (NEPC). In a subset of patients with specimens taken at multiple time points, Beltran et al found genomic profiles to be shared between precursor adenocarcinoma and NEPC specimens suggesting that the NEPC phenotype had not arisen independently but was subclonally derived from the original acinar clone ⁵⁰. In a separate case report analyzing primary prostate material (cancerous and benign), as well as metastatic samples, Jiang et al found no copy-number variation in the primary tumor, but were able to identify a subset of single-nucleotide variants shared between both metastatic tissue samples and all CTCs, suggesting a common clonal progenitor cell had escaped the primary tumour ⁵¹. While these studies reported changes in clonal dynamics over time, nearly all were able to identify shared genomic alterations present in an individual putatively parental subclone.

3.4.1 Intra-Prostatic Heterogeneity and Metastatic Clonal Evolution

Clonal evolution describes the phylogenetic relationship between largely homogenous clusters of cancer cells and relates to temporal mutations which could arise from ‘cell extrinsic’ processes, such as exposures to environmental and treatment stress as well ‘cell intrinsic’ processes, such as sustained changes in signaling linked to DNA damage.

While there is great interest in the efficient identification of specific clonal regions of primary PCa tumours that may give rise to metastases, there is comparatively little experimental evidence to describe this phenomenon. We now describe the small number of studies that address together both intra-prostatic and metastatic clonal evolution by profiling both the primary prostate malignancy and matched metastatic lesions from the same patient (Table 4, Supplementary Table 1).

221 Haffner and colleagues analyzed the radical prostatectomy specimen, a regional lymph node
222 metastasis, metastatic biopsies, and multiple rapid autopsy metastatic lesions from a single patient⁵².
223 While the authors employed WGS on the metastatic biopsies, they were only able to perform
224 molecular assays of limited targets across the breadth of all available material, given that the primary
225 specimen had been in a pathology archive for 20 years and they were unable to attain quality DNA.
226 Interestingly, they were able to identify a region of Gleason pattern 3 disease with a PTEN deletion,
227 while the remainder of the prostatic tumor foci had intact PTEN, including certain regions of Gleason
228 pattern 4 disease. In the subsequent metastatic samples that lead to the patient's death, the authors
229 were able to identify the same PTEN deletion, suggesting that the PTEN negative Gleason Pattern 3
230 focus gave rise to the metastatic lesions. To identify the region of interest in the primary malignancy
231 that presumptively gave rise to the metastases, the authors assayed all primary tumor blocks with
232 PTEN IHC. Despite this, the authors made a concerted effort to validate specific tissue regions,
233 undertaking LCM of the PTEN deleted region and performing genomic analysis of the PTEN gene,
234 which confirmed a genomic alteration shared with the metastases. Another noteworthy observation
235 was that a local lymph node metastasis resected at the time of surgery harbored a separate clonal
236 ancestry than the other disseminated metastases. The authors conclude that it may be possible for a
237 spatially distinct Gleason pattern 3 focus of PCa to metastasize, but acknowledge the necessarily
238 limited genomic analysis undertaken and, of course, it possible that the metastatic foci arose from a
239 Gleason pattern 4 region that underwent temporally distinct PTEN deletion after seeding.

240 VanderWeele and colleagues assessed a subset of primary PCa tumour regions, along with normal
241 prostate, and lymph node metastases. The authors performed LCM to sub-analyze different Gleason
242 pattern regions of malignancy, and employed whole-exome sequencing on all samples. In two (of a
243 total of four) cases, the authors found shared mutations between low grade and high-grade disease,
244 supporting evidence for a common ancestor for both. Unlike Haffner et al, the authors found no

evidence in any cases observed that low-grade disease contributed directly to metastatic progression, and the majority of identified mutations were shared between high-grade disease and metastases corroborating previous wisdom that Gleason pattern 3 disease does not develop into higher grade disease, but that higher grade disease arises from *de novo* mutations⁵³. These findings support the notion of active surveillance in men with low-risk disease.

In contrast to Vanderweele et al., Lindberg and colleagues analyzed two lymph node metastases and one region of primary PCa using WES as a follow-on study⁵⁴ to their original report⁵⁵. In their first report, the authors could not find common ancestry between the lymph node metastases and primary tumour samples when molecularly characterizing these tumors using bulk tissue WES. In their follow-up study, Lindberg and colleagues re-analyzed patient' specimens, from 25 distinct morphological tissue regions isolated by LCM. They instead performed a "wider but shallower" analysis, using low coverage whole-genome sequencing, and this approach identified break point regions from copy-number analysis. Analysis of break point regions in primary tumour regions, as a proportion of those identified in the lymph node metastases, identified a region of Gleason pattern 4 disease in common ancestry with regions of intra-ductal carcinoma (an aggressive subform of acinar prostate cancer⁵⁶) that then shared the most genomic overlap with the lymph node metastases⁵⁴.

Chen et al. analyzed PCa subregions from a single patient who, rather unusually, had received neoadjuvant abiraterone followed by radical prostatectomy. The authors identified residual tumour in two tissue blocks, from which they isolated tumour regions and normal prostate separately using LCM, and performed whole-exome sequencing. While the authors were able to identify separate sets of unique mutations for both tumour foci, they shared a number of discrete mutations, strongly suggesting a common ancestral origin⁵⁷.

Hong and colleagues analyzed multiple regions, from both primary and metastatic tumour sites from four patients. All patients had WGS performed on primary and metastatic samples, and each patient

had a very different temporal and clinical profile. These differences were borne out in biological complexity. The authors were able to identify common clones of origin for all metastases. The authors found a number of interesting clinical and biological findings. For example, they found evidence to suggest that lateral spatial spread of primary tumour is associated with late-arising clones; that distant metastasis can re-seed the surgical bed, that therapy can lead to changes in the subclonal proportions of different subclones, and that the primary tumour can seed multiple waves of metastases. The authors conclude that their work demonstrates significant heterogeneity in the pattern of metastatic spread, but that we need more detailed analysis of clonal relationships in the primary prostate and in larger patient populations⁵⁸.

Gillard et al. assessed WES in two primary foci (one ductal and one acinar PCa), as well as a synchronous LN metastasis as part of a larger comparative study of acinar and ductal PCa. Mutational profiling suggests that all specimens shared common ancestry, with the ductal PCa foci diverging from acinar PCa, and the LNMet diverging from the ductal. These results suggest a monoclonal origin of PCa and metastases⁵⁹. Similarly, Ritch et al., used WES to compare multiple tumor foci from RP specimens and cfDNA in 2 patients as part of a larger study assessing cfDNA from patients with metastatic PCa. Despite observing a lack of concordance in mutational profiles between specimens in primary PCa and cfDNA, the authors found strong evidence monoclonal ancestry through phylogenetic analyses of PCa and metastatic progression⁶⁰. Most recently, Woodcock⁶¹ analysed multiple non-adjacent formalin-fixed, paraffin embedded samples from within the prostate and local expansion in the same 10 patients studied in Gundem et al⁴⁷. They created a bespoke target DNA-sequencing panel to capture single-nucleotide variants identified in the previously-studied metastatic and intra-prostatic samples, and used this to perform deep-sequencing on LCM regions in the new samples. With these, they were able to delineate the intra- and extra-prostatic phases of clonal evolution in unprecedented detail. They found that most clonal evolution occurred within the prostate in a single lineage, after which branching into

multiple distinct lineages occurred that often coexisted in a spatially intermixed state. In all ten men, they observed that one lineage was represented in all of the metastases, indicating that this so-called dominant lineage drove metastatic proliferation. Almost all other lineages were confined to the prostate at the time of death of the patients, although some were also observed in a subset of metastatic sites as a result of multiple seeding events from the prostate. The authors compared the genomic alterations found in the metastatic lineage to those that did not escape the prostate and found significant heterogeneity both between and within patients. Indeed, in one patient, the intra-prostatic lineage experienced at least 5 known driver alterations that were not observed in the lineage that metastasized. The extra-prostatic phase of evolution proceeded in a pattern that indicated that cells at metastatic sites were branches from a continually evolving lineage. In two cases, they could identify that this occurred from within the prostate, but this behavior was also observed in patients who had undergone radical prostatectomy.

Historically, intra-tumoral heterogeneity and clonal evolution typically have been studied as distinct phenomena, but these studies demonstrate that these concepts are intertwined. Broadly speaking, these reports demonstrate the difficulty in determining clonal dynamics between distinct regions of primary PCa, but that it may be more straightforward to find the 'seeds' for clonal metastatic mutations. While the detailed analyses discussed here has led to an improved understanding of PCa, it is difficult to know if the results can be generalizable to the majority of patients with prostate cancer, as they have not been broadly examined in larger patient cohorts.

3.5. Implications for the Clinical Management of Prostate Cancer

Given the limited number of patients with detailed molecular information from multiple sites of primary PCa, further work is required to conclusively link clonal intratumoral heterogeneity in primary PCa with clinical outcomes. The recent Eur Urol San Francisco Consensus statement⁶² provides a standard for

assessing the clinical impact of a prognostic biomarker. PSA is the original such biomarker in prostate cancer (Figure 2C) and many others have been proposed both in blood (for example, PCA3) and tissue (for example Prolaris, Oncotype DX and Decipher)^{12,63–65}. However, recent results highlighting transcriptomic heterogeneity in multiple primary PCa foci⁶⁶ call into question the efficacy of single-site assays to prognosticate patient outcomes. Espiritu et al. found similar results, in showing that patients with multiclonal PCas are more likely to experience poor clinical outcome⁴¹. Future biomarker work could seek to address this through integrated analysis of clonal status and multi-site assaying to improve prognostication.

Precedent exists for the impact of intertumoral heterogeneity on clinical outcome in other solid tumors. Famously, work in the 1980's identified a Her2 amplification in a subset of primary breast cancers⁶⁷. Later trials in patients with this specific alteration demonstrated increased overall survival with Her-2 directed treatment, now widely used as standard of care in breast cancer (for example, Herceptin)^{68,69}. However, to date PCas have not been found to harbor a high-incidence of mutations allowing for such specific targeting. In renal cancers, Gerlinger and colleagues⁷⁰ highlighted heterogeneous tumor evolution and the hindrance to developing precision medicine to either improve genetic diagnostics, or develop target-specific precision medicine therapies.

Studies of clonal evolution in metastatic PCa seem consistently to identify a monoclonal metastatic-potentiating origin which resides, unobserved, within primary PCa. This suggests that radical treatment of primary PCa in the context of metastatic disease could reduce further metastatic seeding events, reducing risk of a poor outcome. This is supported by recent evidence from the STAMPEDE trial found improved survival in oligometastatic PCa patients whose primary prostate was radically treated⁷⁰. However, further work is required to enable identification of such lethal clones during the primary diagnostic process.

3.6 Future Directions

Taken together, we identify from this review an unmet need to interrogate the clonal element of primary PCa intra-tumoral heterogeneity. There remains significant disagreement about whether primary PCa is of monoclonal or polyclonal origin, and whether a tumor-initiating clonal population can be identified. So far, studies have relied heavily on LCM, however newer methodologies to comprehensively profile tissues in a spatial manner are emerging to reduce selection bias and allow larger regions of tissue to be comprehensively profiled at a cellular level⁷¹.

Questions remain about how we might develop methods to consistently profile clonal ancestry, particularly in primary PCa. Additional insight into the clonal dynamics of PCa may be gained through further application of integrated genomics, which seeks to integrate the various molecular ‘-omics’. . Two of the studies discussed above assessed methylation status in combination with genomic profiling to analyze primary ⁷² and metastatic PCa samples ⁷³. The authors found that clonal methylation profiles were not only concordant, but when combined together with genomic information, were superior in analyzing clonal origins of disease than either assay alone. Future efforts to derive clonal dynamics could build on previous integrated approaches such as combined DNA and RNA signatures ^{9,10} which could be assessed in relation to long term patient outcomes. Combinatorial approaches incorporating morphology, transcriptomics, DNA and methylation are likely to deliver synergy in determining clonal ancestry.

The "width" and "depth" of any assay is important. For example, how many cells or regions are being assayed in a given method? Are the areas of prostatic cells, which have been selected by macro/microdissection, actually representative of the relevant underlying clonal relationships? LCM techniques, as used by many studies here, allow for meticulous excision and profiling of regions or cells,

but these techniques are extremely labour intense and are “low-throughput” and still dependent on bulk sequencing, limiting their utility in clinical decision-making.

The future will be increasingly dependent on new “spatial” single cell approaches, including spatial transcriptomics and other slide-based sequencing technologies. These technologies seek to combine genomics analyses with spatial tissue architecture, providing a fine-resolution spatial genomic atlas. Analyzing clonal dynamics through these technologies will allow comparison of multiple foci at unprecedented resolution, and has the potential to decode intratumoral heterogeneity and clonal evolution in PCa.

4. Conclusions

There is a need to identify the clonal source of metastatic disease in primary PCa. Without such knowledge, we cannot truly determine the course and optimal treatment paradigms from initial diagnosis. A number of factors contribute to huge complexity in carrying out these studies, including sample availability, resources, time, but also limited technology to spatially map genomics *in-situ*. Macrodissection and laser-capture microdissection combined with metastatic biopsies and rapid autopsies have raised important questions in our understanding of the relationship between the primary focus of PCa and lethal metastases. Recent advancements in technologies to map spatial biology will undoubtedly start to deliver the answers to these important questions, thereby leading to improved and personalized treatments for men with this common and ubiquitous malignancy.

References

1. Stewart, C. S., Leibovich, B. C., Weaver, A. L. & Lieber, M. M. Prostate cancer diagnosis using a saturation needle biopsy technique after previous negative sextant biopsies. *J. Urol.* **166**, 86–92 (2001).
2. AA, R. Adequate Tissue Sampling of Prostate Core Needle Biopsies. *Am. J. Clin. Pathol.* **107**, (1997).
3. Murphy, W. M., Dean, P. J., Brasfield, J. A. & Tatum, L. Incidental carcinoma of the prostate. How much sampling is adequate? *Am. J. Surg. Pathol.* **10**, 170–174 (1986).
4. Lamb, A. D., Zargar, H., Murphy, D. G., Corcoran, N. M. & Hovens, C. M. Disrupting the Status Quo in Prostate Cancer Diagnosis. *European Urology* **71**, 193–194 (2017).
5. Wei, L. *et al.* Intratumoral and Intertumoral Genomic Heterogeneity of Multifocal Localized Prostate Cancer Impacts Molecular Classifications and Genomic Prognosticators. *Eur. Urol.* **71**, 183–192 (2017).
6. Kanda, M. *et al.* Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. *Gastroenterology* **142**, (2012).
7. Mao, X. *et al.* Distinct genomic alterations in prostate cancers in Chinese and Western populations suggest alternative pathways of prostate carcinogenesis. *Cancer Res.* **70**, 5207–12 (2010).
8. Fraser, M. *et al.* Genomic hallmarks of localized, non-indolent prostate cancer. *Nature* **541**, 359–364 (2017).
9. Ross-Adams, H. *et al.* Integration of copy number and transcriptomics provides risk stratification

- 400 in prostate cancer: A discovery and validation cohort study. *EBioMedicine* **2**, 1133–1144 (2015).
- 401 10. Lalonde, E. *et al.* Tumour genomic and microenvironmental heterogeneity for integrated
 402 prediction of 5-year biochemical recurrence of prostate cancer: A retrospective cohort study.
 403 *Lancet Oncol.* **15**, 1521–1532 (2014).
- 404 11. Taylor, B. S. *et al.* Integrative Genomic Profiling of Human Prostate Cancer. *Cancer Cell* **18**, 11–22
 405 (2010).
- 406 12. Ross-Adams, H. & Lamb, A. D. The genetic classification of prostate cancer: what's on the
 407 horizon? *Futur. Oncol.* **12**, 729–733 (2016).
- 408 13. Dunning, M. J. *et al.* Mining Human Prostate Cancer Datasets: The “camcAPP” Shiny App.
 409 *EBioMedicine* **17**, 5–6 (2017).
- 410 14. Grasso, C. S. *et al.* The mutational landscape of lethal castration-resistant prostate cancer. *Nature*
 411 **487**, 239–243 (2012).
- 412 15. Robinson, D. *et al.* Integrative clinical genomics of advanced prostate cancer. *Cell* **161**, 1215–1228
 413 (2015).
- 414 16. Varambally, S. *et al.* Integrative genomic and proteomic analysis of prostate cancer reveals
 415 signatures of metastatic progression. *Cancer Cell* **8**, 393–406 (2005).
- 416 17. Baca, S. C. *et al.* Punctuated evolution of prostate cancer genomes. *Cell* **153**, 666–677 (2013).
- 417 18. Cooper, C. S. *et al.* Analysis of the genetic phylogeny of multifocal prostate cancer identifies
 418 multiple independent clonal expansions in neoplastic and morphologically normal prostate
 419 tissue. *Nat. Genet.* **47**, 367–372 (2015).
- 420 19. Boutros, P. C. *et al.* Spatial genomic heterogeneity within localized, multifocal prostate cancer.

- 421 *Nat. Genet.* **47**, 736–45 (2015).
- 422 20. Slaughter, D. P., Southwick, H. W. & Smejkal, W. “Field cancerization” in oral stratified squamous
423 epithelium. Clinical implications of multicentric origin. *Cancer* **6**, 963–968 (1953).
- 424 21. Nonn, L., Ananthanarayanan, V. & Gann, P. H. Evidence for field cancerization of the prostate.
425 *Prostate* **69**, 1470–1479 (2009).
- 426 22. Bianchi-Frias, D. *et al.* Cells Comprising the Prostate Cancer Microenvironment Lack Recurrent
427 Clonal Somatic Genomic Aberrations. *Mol. Cancer Res.* **14**, 374–84 (2016).
- 428 23. Nash, C. *et al.* Genome-wide analysis of AR binding and comparison with transcript expression in
429 primary human fetal prostate fibroblasts and cancer associated fibroblasts. *Mol. Cell. Endocrinol.*
430 **471**, 1–14 (2018).
- 431 24. Chen, W. J. *et al.* Cancer-associated fibroblasts regulate the plasticity of lung cancer stemness via
432 paracrine signalling. *Nat. Commun.* **5**, 1–17 (2014).
- 433 25. Gaisa, N. T. *et al.* Clonal architecture of human prostatic epithelium in benign and malignant
434 conditions. *J. Pathol.* **225**, 172–80 (2011).
- 435 26. Gerrin, S. J., Sowalsky, A. G., Balk, S. P. & Ye, H. Mutation Profiling Indicates High Grade Prostatic
436 Intraepithelial Neoplasia as Distant Precursors of Adjacent Invasive Prostatic Adenocarcinoma.
437 *Prostate* **76**, 1227–36 (2016).
- 438 27. Boyd, L. K. *et al.* High-resolution genome-wide copy-number analysis suggests a monoclonal
439 origin of multifocal prostate cancer. *Genes. Chromosomes Cancer* **51**, 579–89 (2012).
- 440 28. Alexander, J. *et al.* Utility of Single-Cell Genomics in Diagnostic Evaluation of Prostate Cancer.
441 *Cancer Res.* **78**, 348–358 (2018).

- 442 29. Andreoiu, M. & Cheng, L. Multifocal prostate cancer: biologic, prognostic, and therapeutic
443 implications. *Human Pathology* **41**, 781–793 (2010).
- 444 30. Guinney, J. *et al.* Prediction of overall survival for patients with metastatic castration-resistant
445 prostate cancer: development of a prognostic model through a crowdsourced challenge with
446 open clinical trial data. *www.thelancet.com/oncology* **18**, (2017).
- 447 31. Stürenberg, C. *et al.* Abstract 1396: Detection and local histological staging of prostate cancer foci
448 in H&E whole slide images using convolutional neural networks. in *Cancer Research* **79**, 1396–
449 1396 (American Association for Cancer Research (AACR), 2019).
- 450 32. Albertsen, P. C. *et al.* 20-Year Outcomes Following Conservative Management of Clinically
451 Localized Prostate Cancer. *JAMA* **293**, 2095 (2005).
- 452 33. Lokman, U., Erickson, A. M., Vasarainen, H., Rannikko, A. S. & Mirtti, T. PTEN Loss but Not ERG
453 Expression in Diagnostic Biopsies Is Associated with Increased Risk of Progression and Adverse
454 Surgical Findings in Men with Prostate Cancer on Active Surveillance. *Eur. Urol. Focus* **4**, 867–873
455 (2018).
- 456 34. Barentsz, J. O. *et al.* ESUR prostate MR guidelines 2012. *Eur. Radiol.* **22**, 746–757 (2012).
- 457 35. Lindberg, J. *et al.* Exome sequencing of prostate cancer supports the hypothesis of independent
458 tumour origins. *Eur. Urol.* **63**, 347–53 (2013).
- 459 36. Parry, M. A. *et al.* Genomic Evaluation of Multiparametric Magnetic Resonance Imaging-visible
460 and -nonvisible Lesions in Clinically Localised Prostate Cancer. *Eur. Urol. Oncol.* **2**, 1–11 (2019).
- 461 37. Løvf, M. *et al.* Multifocal Primary Prostate Cancer Exhibits High Degree of Genomic
462 Heterogeneity. *Eur. Urol.* **75**, 498–505 (2019).

- 463 38. Su, F. *et al.* Spatial Intratumor Genomic Heterogeneity within Localized Prostate Cancer Revealed
464 by Single-nucleus Sequencing. *Eur. Urol.* **74**, 551–559 (2018).
- 465 39. Sowalsky, A. G. *et al.* Gleason Score 7 Prostate Cancers Emerge through Branched Evolution of
466 Clonal Gleason Pattern 3 and 4. *Clin. Cancer Res.* **23**, 3823–3833 (2017).
- 467 40. Kim, T.-M. *et al.* Regional biases in mutation screening due to intratumoural heterogeneity of
468 prostate cancer. *J. Pathol.* **233**, 425–35 (2014).
- 469 41. Espiritu, S. M. G. *et al.* The Evolutionary Landscape of Localized Prostate Cancers Drives Clinical
470 Aggression. *Cell* **173**, 1003-1013.e15 (2018).
- 471 42. Liu, W. *et al.* Copy number analysis indicates monoclonal origin of lethal metastatic prostate
472 cancer. *Nat. Med.* **15**, 559–65 (2009).
- 473 43. Wu, Y. *et al.* High-Resolution Genomic Profiling of Disseminated Tumor Cells in Prostate Cancer. *J.*
474 *Mol. Diagn.* **18**, 131–43 (2016).
- 475 44. Robbins, C. M. *et al.* Copy number and targeted mutational analysis reveals novel somatic events
476 in metastatic prostate tumors. *Genome Res.* **21**, 47–55 (2011).
- 477 45. Rodrigues, D. N. *et al.* Rb1 heterogeneity in advanced metastatic castration-resistant prostate
478 cancer. *Clin. Cancer Res.* **25**, 687–697 (2019).
- 479 46. Nickerson, M. L. *et al.* Somatic alterations contributing to metastasis of a castration-resistant
480 prostate cancer. *Hum. Mutat.* **34**, 1231–41 (2013).
- 481 47. Gudem, G. *et al.* The evolutionary history of lethal metastatic prostate cancer. *Nature* **520**, 353–
482 357 (2015).
- 483 48. Dago, A. E. *et al.* Rapid phenotypic and genomic change in response to therapeutic pressure in

- 484 prostate cancer inferred by high content analysis of single circulating tumor cells. *PLoS One* **9**,
 485 e101777 (2014).
- 486 49. Carreira, S. *et al.* Tumor clone dynamics in lethal prostate cancer. *Sci. Transl. Med.* **6**, 254ra125
 487 (2014).
- 488 50. Beltran, H. *et al.* Divergent clonal evolution of castration-resistant neuroendocrine prostate
 489 cancer. *Nat. Med.* **22**, 298–305 (2016).
- 490 51. Jiang, R. *et al.* A comparison of isolated circulating tumor cells and tissue biopsies using whole-
 491 genome sequencing in prostate cancer. *Oncotarget* **6**, 44781–93 (2015).
- 492 52. Haffner, M. C. *et al.* Tracking the clonal origin of lethal prostate cancer. *J. Clin. Invest.* **123**, 4918–
 493 22 (2013).
- 494 53. VanderWeele, D. J. *et al.* Low-grade prostate cancer diverges early from high grade and
 495 metastatic disease. *Cancer Sci.* **105**, 1079–85 (2014).
- 496 54. Lindberg, J., Kristiansen, A., Wiklund, P., Grönberg, H. & Egevad, L. Tracking the origin of
 497 metastatic prostate cancer. *Eur. Urol.* **67**, 819–22 (2015).
- 498 55. Labbé, D. P. *et al.* High-fat diet fuels prostate cancer progression by rewiring the metabolome
 499 and amplifying the MYC program. *Nat. Commun.* **10**, 4358–4358 (2019).
- 500 56. Lawrence, M. G. *et al.* Knowing what’s growing: Why ductal and intraductal prostate cancer
 501 matter. *Science Translational Medicine* **12**, (2020).
- 502 57. Chen, E. J. *et al.* Abiraterone treatment in castration-resistant prostate cancer selects for
 503 progesterone responsive mutant androgen receptors. *Clin. Cancer Res.* **21**, 1273–80 (2015).
- 504 58. Hong, M. K. H. *et al.* Tracking the origins and drivers of subclonal metastatic expansion in

- 505 prostate cancer. *Nat. Commun.* **6**, 6605 (2015).
- 506 59. Gillard, M. *et al.* Integrative Genomic Analysis of Coincident Cancer Foci Implicates CTNNB1 and
507 PTEN Alterations in Ductal Prostate Cancer. *Eur. Urol. Focus* **5**, 433–442 (2019).
- 508 60. Ritch, E. *et al.* Identification of hypermutation and defective mismatch repair in ctDNA from
509 metastatic prostate cancer. *Clin. Cancer Res.* **26**, 1114–1125 (2020).
- 510 61. Woodcock, D. J. *et al.* Prostate cancer evolution from multilineage primary to single lineage
511 metastases with implications for liquid biopsy. *Nat. Commun.* **11**, 5070 (2020).
- 512 62. Cooperberg, M. R. *et al.* The State of the Science on Prostate Cancer Biomarkers: The San
513 Francisco Consensus Statement. *European Urology* **76**, 268–272 (2019).
- 514 63. Marrone, M., Potosky, A. L., Penson, D. & Freedman, A. N. A 22 gene-expression assay, decipher®
515 (GenomeDx biosciences) to predict five-year risk of metastatic prostate cancer in men treated
516 with radical prostatectomy. *PLoS Curr.* **7**, (2015).
- 517 64. Knezevic, D. *et al.* Analytical validation of the Oncotype DX prostate cancer assay - a clinical RT-
518 PCR assay optimized for prostate needle biopsies. *BMC Genomics* **14**, 690 (2013).
- 519 65. Cuzick, J. *et al.* Prognostic value of an RNA expression signature derived from cell cycle
520 proliferation genes in patients with prostate cancer: A retrospective study. *Lancet Oncol.* **12**,
521 245–255 (2011).
- 522 66. Salami, S. S. *et al.* Transcriptomic heterogeneity in multifocal prostate cancer. *JCI insight* **3**,
523 (2018).
- 524 67. Slamon, D. J. *et al.* Human breast cancer: Correlation of relapse and survival with amplification of
525 the HER-2/neu oncogene. *Science (80-.).* **235**, 182–191 (1987).

68. Vogel, C. L. *et al.* Efficacy and Safety of Trastuzumab as a Single Agent in First-Line Treatment of HER2 -Overexpressing Metastatic Breast Cancer . *J. Clin. Oncol.* **20**, 719–726 (2002).
69. Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A. & Greene, M. I. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell* **41**, 695–706 (1985).
70. Gerlinger, M. *et al.* Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. *N. Engl. J. Med.* **366**, 883–892 (2012).
71. Ståhl, P. L. *et al.* Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. *Science* **353**, 78–82 (2016).
72. Brocks, D. *et al.* Intratumor DNA methylation heterogeneity reflects clonal evolution in aggressive prostate cancer. *Cell Rep.* **8**, 798–806 (2014).
73. Aryee, M. J. *et al.* DNA methylation alterations exhibit intraindividual stability and interindividual heterogeneity in prostate cancer metastases. *Sci. Transl. Med.* **5**, 169ra10 (2013).

546 **Glossary:**

547 PCa: Prostate Cancer

548 AR: Androgen Receptor

549 SNV: Single-Nucleotide Variants

550 ITH: Intra-tumoral Heterogeneity

551 CE: Clonal Evolution

552 CGH: comparative genomic hybridization

553 FISH: Fluorescent In-Situ Hybridization

554 IHC: Immunohistochemistry

555 HGPIN: High Grade Prostate Intraepithelial Neoplasia

556 PTEN: Phosphatase and tensin homolog

557 TP53: Tumor protein 53

558 ETS: Erythroblast transformation-specific