Tailoring the tools to study prostate cancer metastasis

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Chapter 1

Introduction

Prostate anatomy, function and physiology

Anatomy and physiology

The human prostate is a walnut-size, exocrine, male sex accessory gland. It is positioned within the pelvis and below the bladder, supporting the internal urethral sphincter and providing the anatomical connection between the bladder urethra and the ejaculatory ducts (**Fig. 1**). Its main function is to produce and secrete the prostatic fluid, which accounts for around the 30% of the ejaculate and contains citrate, high concentrations of zinc and various enzymes (1,2). Albeit the specific roles of the prostatic fluid within the semen are still largely unknown, prostatic fluid is required to fully support spermatozoa during the process of fertilization (3). One of the secreted enzymes is the prostate specific antigen (PSA), whose function contributes to the regulated release of sperms after ejaculation in the acidic environment of the vagina, and that greatly enhances male fertility (1).

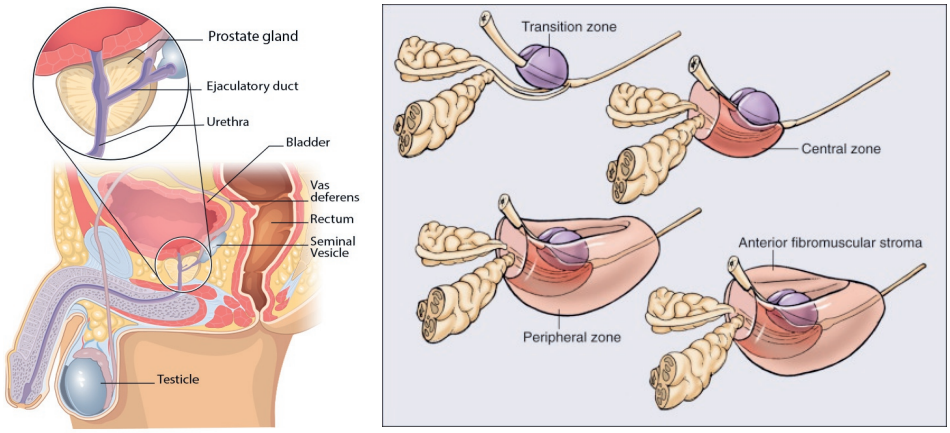

Figure 1. Anatomy of the prostate.
Illustration of the prostate anatomical location (left panel) and zonal description (right).

The prostate is unilobular and can be divided in three main zones (**Fig. 1**). The transition zone (TZ) surrounds the proximal urethra as it enters the prostate, accounting for about 10% of the volume of the organ, although it keeps growing throughout life. The central zone (CZ) surrounds the ejaculatory ducts and the portion of the urethra at the insertion site of the ejaculatory ducts and accounts for about 20% of the volume of the organ. The peripheral zone (PZ), which accounts for 70% of the organ, comprises most of the glandular tissue, surrounds the distal urethra and is the site where prostate malignancies most frequently develop (2,4).

Cellular composition

The mature prostate epithelium consists of different cell types that can be identified by morphohistological characteristics as wells as by the expression of different molecular markers. Luminal cells line the innermost layer of the prostate ducts and form a tall columnar pseudostratified epithelium (**Fig. 2A**). They are the main secretory cells of the prostate, secreting PSA and other proteolytic enzymes, prostatic acid phosphatase, high concentrations of zinc and citrate. The luminal cell layer lies on top of a layer of non-secretory cells termed basal cells, which contacts the basal membrane and forms a simple, continuous, squamous epithelium (1,5–7). Neuroendocrine cells are rare, terminally differentiated secretory cells, histologically identified by specific stainings (8). They are localized in the basal layer, from which they're supposed to derive, and may project dendritic-like processes through the luminal layer. Neuroendocrine cells secrete neuropeptides (serotonin, chromogranin A, synaptophysin), hormones and growth factors (including VEGF), exerting a

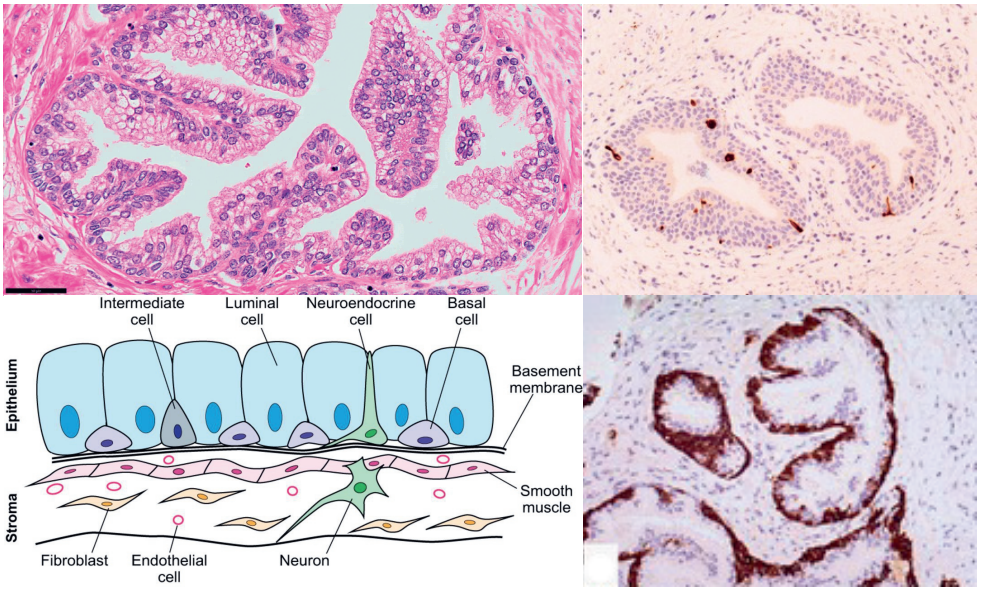

Figure 2 - Prostate histology and cellular components

A. *Prostate glands show secretory and basal cells surrounded by fibromuscular stroma.* ***B-C***. *Neuroendocrine cells (B) and basal cells (C) within prostate glands, as evidenced by chromogranin A staining and cytokeratins 5,14 staining respectively (200x). A-C from Lopez-Beltran, A., Cheng, L., Montironi, R., & Raspollini, M. (2017). Basic Anatomy and Histology of the Prostate. In Pathology of the Prostate: An Algorithmic Approach. Cambridge: Cambridge University Press.* ***D***. *Schematic of cell types in the adult prostate. From Prostate organogenesis: tissue induction, hormonal regulation and cell type specification. Roxanne Toivanen, Michael M. Shen. Development 2017 144: 1382-1398*

tissue homeostatic function; however, their integrated role within prostate physiology has not been completely cleared yet (9).
Basal, luminal and neuroendocrine cells are characterized by the expression of different molecular markers. Luminal cells express cytokeratins (CK) 8 and 18, Nkx3.1, AR and PSA. Basal cells can be identified by the expression of CK 5 and 14 and of p63 while neuroendocrine cells express chromogranin A, synaptophysin, enolase 2 and CD56 (**Fig. 2B-C**) (1,9).
The stromal compartment of the prostate includes smooth muscle cells, which surround the epithelium forming a thick fibromuscular layer whose contractions favour the expulsion of the prostatic fluid into the semen. Additional stromal components include tissue fibroblasts, blood and lymphatic vessels, nerves and cells of the immune system (**Fig. 2D**).
The prostate response to sex hormones is mediated mainly by the androgen receptor (AR), a nuclear receptor that consists of a transactivation domain, a DNA-binding domain and a ligand-binding domain (10). In the prostate epithelium, testosterone is reduced intracellularly to its more potent form dihydrotestosterone (DHT) by the enzyme 5α-reductase. Compared to testosterone, DHT has a higher affinity for the AR, which upon binding to its ligand enters the nucleus, binds to androgen-responsive elements (AREs) and regulates gene transcription (11). In addition to its transcription factor function, AR can activate extra-nuclear signal transduction pathways that include PI3K/Akt activation, Src, Ras and MAPK activation and stimulation of focal adhesion kinases (10,12–14).

Organ development and homeostasis

Prostate embryological development

The prostate is an organ of endodermic origin and its development is androgen regulated, with testosterone being the main sex hormone involved. Testosterone production starts in males at about week 9 of gestation, by Leydig cells in the testis, and will proceed in waves according to male sex development, reaching its peak at puberty. At this time the prostate reaches its definitive size and its functional maturity. At 10 weeks of gestation the urogenital sinus (UGS) epithelium buds into the surrounding UGS mesenchyme, initiating androgen receptor (AR)-driven ducts formation composed of solid epithelial cords. Subsequently, the ductal outgrowth mediated by branching morphogenesis process leads to the formation of the previously described mature zones. Finally, the glandular epithelium develops into its final form, with the canalization of the solid epithelial cords to form the

ductal lumen. This process is concomitant with cytological differentiation of the cells within the prostate tissue (1,15).

Developmental studies performed in rodents have shown that the prostate is one of the most posterior organs in the male, *Hox* genes regulating its development are in the most 5', posterior cluster, *Hox13* paralogs (16). In a study by Huang *et al*., *Hox13* co-expression with additional, anterior *Hox* genes in the different lobes of the developing rat prostate, was associated with the different branching patterns of the various rat prostate lobes (17). The study revealed also that testosterone upregulates the expression of posterior *Hox* genes, including the *Hox13* cluster, *in vitro* in cultures of rat prostate tissue. In contrast with prostate development in rodents, the process of prostate ductal branching in humans is complete at the time of birth. Despite the lack of information on human prostate development, *HOXB13* expression was consistently detected in adult human prostate tissue, as well as in prostate cancer samples (18). Multiple studies performed on prostate cancer found the recurrent dysregulated expression of genes in this cluster, suggesting a role for this *HOX* family in human prostate homeostasis (18–21).

Additional key transcription factor for prostate identity is the *NK* superfamily member *Nkx3.1*. This gene is expressed in the mouse UGS before the emergence of the prostatic epithelial buds, suggesting its early role in fate determination (22,23), a role that was ultimately associated to the prostate luminal stem cells compartment in a study by Kruithof-de Julio *et al.* in 2013 (24). *Nkx3.1* is induced by androgens in the developing lobes of the prostate and is in direct and co-dependent relation with sonic hedgehog (Shh) and canonical Wnt pathway expression during prostate formation (24,25). Expression of *Nkx3.1* is maintained in prostate epithelium throughout life, with tumor-suppressing and epithelial homeostasis functions (26–29).

Following prostate fate specification, epithelium cueing is supported by *FoxA1*, a member of the forkhead box (Fox) gene family, widely expressed in tissues of endodermal origin. *FoxA1* plays a determining role in ductal morphogenesis and epithelial cell maturation of the developing prostate (30). *FoxA1* is required for the expression of the PSA-encoding gene (*KLK3* in humans, *Klk1* family in mice, by binding *FoxA* cis-regulatory elements and *AR* on gene promoters (31).

Prostate homeostasis

Most adult tissues in the body contain a population of local stem cells, which replenish the pool of tissue-specific cells lost during physiological turnover. Despite the multiple definition of stemness that can be provided, stem cells

are essentially characterized by their capacity to self-renew, either by symmetric or by asymmetric division, respectively generating two stem daughter cells or one stem and one committed daughter cells. Most frequently, stem cells do not immediately generate terminally differentiated cells but progress towards their final fate through multiple cell divisions, generating a population of transient cells with reduced stemness potential. The adult prostate exhibits a slow cellular turnover becoming a relatively quiescent after morphogenesis. However, following rounds of androgens deprivation and replacement, the prostate epithelium shows alternating cycles of regression and regeneration, indicating the existence of stem cells in the adult prostate capable of self-renewing and of multi-lineage differentiation.

Different studies suggest the existence of multiple prostate stem/progenitor cells compartments, with distinct functions in organogenesis and regeneration. Initial hypothesis of prostate stem cells focused on the basal layer, as these cells are essentially androgen-independent and showed multiple stem cell features (32,33). p63 expression was found as a necessary requirement for the generation of prostate basal cells, identifying p63-expressing cells as a stem/progenitor pool of basal cells (34). This study was further confirmed by the additional finding that the UGS from $p63^{null}$ mice, grafted under the renal capsule of a host mouse, generated prostate tissue without basal cells (35). Additional markers identifying prostate basal stem cells included Trop2, CD49f, EpCAM and CD44 (36–39). During tissue regeneration following damage, both basal progenitors and luminal cells contribute to the regeneration of the luminal cell compartment, highlighting the bipotency of the basal progenitor as well as the existence of a luminal stem cells population (40,41) (42,43). The hypothesis of a luminal progenitor was supported by the identification of castration-resistant *Nkx3.1*-expressing (CARN) cells, a subpopulation of exclusively luminal cells expressing *Nkx3.1*, responsible for prostate budding and luminal epithelial cells differentiation (24).

While most luminal epithelial cells undergo apoptosis during androgen deprivation, rare CARN cells were found in the regressed prostates of castrated mice and, upon androgen replacement, could act as tissue stem cells to regenerate the luminal and basal epithelial compartment (44). In addition, the administration of rounds of androgen deprivation and supplementation were paralleled by $p63^{null}$ prostate tissue regression and regeneration, supporting the hypothesis of fully luminal stem/progenitor cells within the prostate (35).

Multiple models have been formulated on the ontogeny of the prostate epithelium. In a widely acknowledged model, prostate stem cells reside in the basal compartment, exhibiting a basal-to-luminal differentiation direction.

This model acknowledges the existence of transitioning progenitors, characterized by their proliferative capacity as well as by defined differentiation potential and termed "transit-amplifying" cells. By means of asymmetric divisions, the transit-amplifying cells directly replenish the basal cells pool and generate a luminal-committed progeny of cells. These latter cells, identified as "intermediate" cells, show an inherently proliferative capacity and exhibit an intermediate phenotype characterized by both basal and luminal markers, terminally differentiating into secretory luminal cells (45). An alternative model describes the basal, luminal and neuroendocrine cells as originating from separate epithelial lineages. This model was supported by the identification of luminal stem cells as well as by the lack of evidence of intermediate cells within the developing human prostate (46). According to this hypothesis, basal, luminal and neuroendocrine cells have separate progenitor cells originating the differentiated epithelial cell progeny. Both models however lack a robust explanation for the origin of neuroendocrine cells, that could originate from stem cells within the basal compartment, belong to an independent lineage or even being replenished by a separate stem cell population (47).

Prostate metabolism

Prostatic fluid production, which takes place primarily in the peripheral zone (PZ), is one of the main functions of the prostate. Prostatic fluid contains exceptionally high concentrations of citrate and zinc, as well as proteolytic enzymes like PSA and prostatic acid phosphatase (3,48). While the main function of the prostatic enzymes is ascribed to increased or facilitated sperm motility, the high concentrations of citrate and zinc have been implicated in different roles, including energetic reserve for sperm cells, increased anti-oxidative power and preservation of the acrosomal functions of the sperm cells. In the PZ of a normal prostate, both citrate and zinc are found to a ~100x higher concentration than other soft tissues and, compared to blood, both these elements can be found in semen at a 500-1000x higher concentrations (3). Such a high concentration of secreted zinc and citrate is the result of a highly specialized metabolic pathway specific to the luminal prostate epithelium. Luminal epithelial cells of the prostate upregulate *ZIP1*, a cell membrane zinc transporter that increases intracellular zinc concentrations, that is further transported into the mitochondria. Here, the high Zn^{2+} concentration inhibits the function of the mitochondrial aconitase, truncating the Krebs cycle and preventing further metabolization of the mitochondrial citrate. The accumulating citrate is then exported from the mitochondria via

the CTP transporter and secreted into the prostatic fluid as "net citrate production" (49). To sustain citrate secretion, prostate luminal cells need to undergo substantial metabolic adaptations to provide the anabolic blocks for citrate production and to compensate the energetic loss from the truncated Krebs cycle. These adaptations include the androgen-induced upregulation of different metabolic enzymes pyruvate dehydrogenase E1a (*PDH*), to supplement acetyl-coenzyme A (AcCoA) from the glycolytic branch, the aspartate transporter *EAAC1* and the mitochondria aspartate aminotransferase (*mAAT*) to provide increased oxaloacetate substrate for citrate conversion. The missed citrate oxidation via the Krebs cycle directly translates to a net reduction of ATP yield, that drops from 38 ATP/glucose mol to 24 ATP/glucose mol in the luminal cells. To compensate for this bioenergetic cost, prostate luminal cells exhibit increased aerobic glycolysis, a characteristic otherwise found in cancer cells (50).

Diseases of the prostate (non-cancerous)

Diseases of the prostate are commonly detectable by the appearance of lower urinary tract symptoms (LUTS) like slow or irregular urine stream, increased frequency and urgency to urinate, feeling of incomplete voiding, nocturia or incontinence (51). Commonly, some of these symptoms can be ascribed to benign prostatic enlargement (BPE), a condition primarily caused by benign prostatic hyperplasia (BPH), a normal process of aging in men whose incidence increases linearly with age (52). In most cases, it affects the central or the transition zone of the prostate and consists in an increase of both the stromal and the epithelial fractions of the prostate, leading eventually to an obstruction of the prostatic urethra and the development of LUTS. Development of BPH is associated to a chronic inflammatory state of the prostate epithelium. This is sustained by macrophage, T and B lymphocytes infiltration, that cause an inflammatory damage-induced remodelling of the prostate architecture, leading to structural changes associated to the benign disease (53,54).

LUTS can develop also as a consequence of prostatitis, an inflammation of the prostate with multiple possible aetiological agents. The National Institute of Health (NIH) classified prostatitis in four groups, basing on the symptoms and microbiological investigations (55,56), reported in Table 1.

About 2-16% of men experience prostatitis at one point of their life and about 90% of them will be diagnosed with chronic prostatitis/chronic pelvic pain syndrome (CPPS) (57). Etiology of CPPS is poorly understood, it affects men of all ages and its therapy is focused primarily on symptomatic relief. Bacterial

Table 1. Classification of prostatitis

Group	NIH classification	Characteristics	Incidence
I	Acute bacterial prostatitis	Acute infection of the prostate gland	2-5%
II	Chronic bacterial prostatitis	Persistent or recurrent bacterial infection of the prostate	2-5%
IIIa **IIIb**	Non-bacterial chronic prostatitis/Chronic pelvic pain syndrome (CPPS)	Non-bacterial inflammation of the prostate, causing pelvic pain, LUTS and sexual dysfunctions	90-95%
IV	Asymptomatic inflammatory prostatitis	Asymptomatic patients with inflammatory infiltrate in the prostate tissue or fluids.	Incidental diagnosis

prostatitis is mainly caused by bacteria of the *Enterobacteriaceae* family, primarily uropathogenic *E. Coli* (UPEC). Chronic bacterial prostatitis typically affects men aged 35-50 years, whereas the acute form shows a bimodal prevalence in men aged 20-40 and >60 years (58).

Primary prostate cancer: natural history and clinical problems

Statistics and definitions

Prostate cancer is the second most commonly diagnosed malignancy and the fifth most common cancer-related cause of death in men worldwide, with an estimated 1.6 million cases and over 350.000 deaths annually (59–61). Its incidence and mortality are closely linked to geographical variation, with a higher incidence and a lower mortality in more developed countries and an opposite trend in less developed ones. The average age of diagnosis is 66 years, with rare familiar forms being detected in men younger than 40 years (62). The introduction of PSA screening as a diagnostic tool can be identified as the most relevant prostate cancer-related epidemiologic event so far and its introduction lead to a "cohort effect" that translated into an apparent increased incidence of prostate cancer. Due to its increased sensitivity, PSA screening allowed for the detection of more prostate cancer cases at an earlier stage of the disease, shifting in fact the stage at which prostate cancer was

diagnosed. With more men diagnosed a less advanced disease, better therapeutic options opened and contributed significantly to lower prostate cancer mortality in states where PSA screening was introduced (63).

Cancer as a disease

The term cancer refers to diseases caused by an uncontrolled division of abnormal cells or, as Hanahan and Weinberg defined it, to "complex tissues composed of multiple distinct cell types that participate in heterotypic interactions with one another"(64). This specific definition highlights the multifactoriality and the tissue interdependence that concur to generate the disease. The same authors provided, in a previous milestone work, a synthesis of traits or hallmarks that identify a cancer cell and the process of malignant transformation (65). These identified hallmarks represent the dysregulation of underlying biological pillars that are being actively and tightly overseen by multicellular organisms and are reported in **Fig. 3**. It is relevant to note that

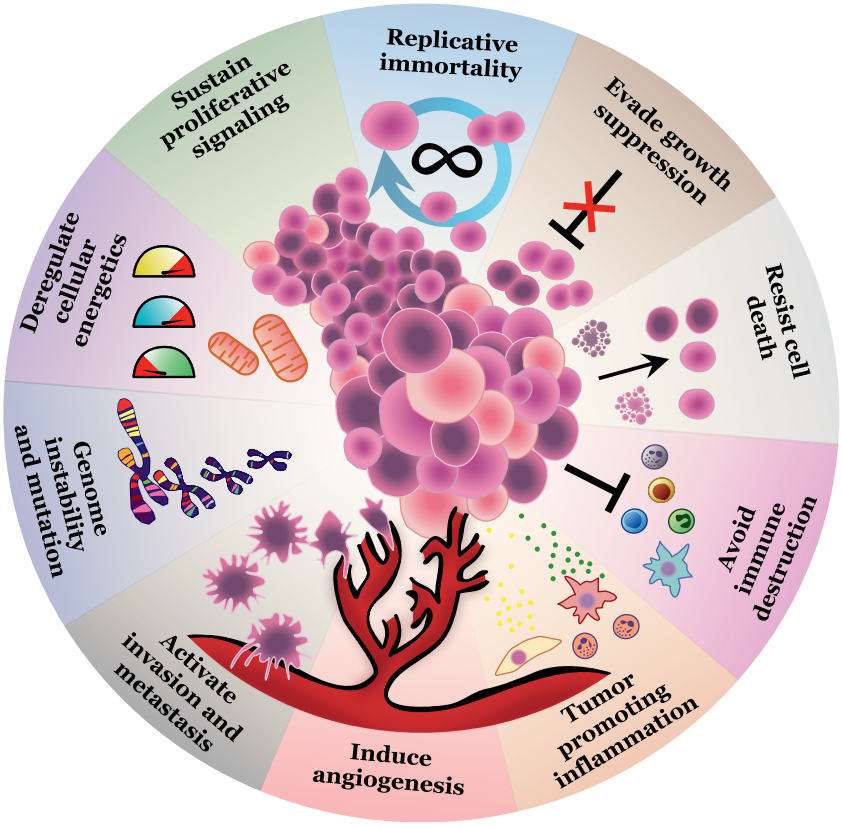

Figure 3 – The hallmarks of cancer, as reported by Hanahan and Weinberg.

rather than discussing individual pathways or elements contributing to the development of cancer, a topic- or function-oriented approach can better highlight what bottleneck features are required for the development of cancer. It became evident in time that all the elements involved in cancer development are intertwined and a single dysregulated pathway may promote distinct features at once.

Models of carcinogenesis and tumor progression

The transformation of normal cells into cancerous ones requires the co-occurrence of two key factors. On the one hand, genetic mutations have to take place and to become fixed in portions of the genome actively used by the cells. On the other hand, a pro-tumorigenic microenvironment is required to support the survival and the proliferation of the transformed cells. As these two aspects are required to co-occur, it is mainly possible to talk about risk factors and protective factors, that can increase or decrease respectively the likelihood of developing the cancer. All living cells have evolved mechanisms to contrast the accumulation of mutations in the DNA: the redundancy of the genetic code, the evolution of multiple DNA damage-scavenging and repair mechanisms, the functionalization of the genome into introns and exons, the adjustment of ploidy, etc. DNA mutation is the result of unrepaired or wrongly repaired DNA damage arising from the exposure of an organism to physical, chemical or biological carcinogens. Mutations can have a broad range of effects on a DNA sequence, from altering a single nucleotide (point mutation) to changing the order in which a DNA sequence is arranged, to whole chromosomal rearrangements, an event that affects the position or even the presence of whole segments of DNA in a cell. Mutations that open up the oncogenic process are identified as "driver" mutations and cause the weakening of cellular genome control mechanisms. Other mutations that facilitate the oncogenic transformation but that are not sufficient alone to cause cancer are referred as "passenger" mutations (66,67). Driver mutations, while still requiring a pro-oncogenic microenvironment to result in cancer formation, are always found in clinically detectable cancers and can be associated to varying degrees of passenger mutations depending on the background and risk factors of the patient (68,69). Moreover, mutations can either increase the function of a gene (gain of function mutations) or reduce it, down to its complete inactivation (loss of function mutations). Genes that promote cancer hallmarks are identified as oncogenes or proto-oncogenes, while genes involved in the control machinery or that inhibit the function of oncogenes are termed oncosuppressor genes. On average, oncogenes

harbour gain of function mutations, while oncosuppressor genes are hit by inactivating ones (70), however, as the hypothesis of a coherent and univocal "cancer genome" shared by all cancers is not supported by scientific evidence, oncogenes and oncosuppressor genes have different relevance depending on the natural history of each cancer.
Nevertheless, despite the discrimination of genes into oncogenes and oncosuppressors is widely accepted and benefits of solid experimental evidence, different theories have been postulated to explain the link between genomic instability and cancer development (see **Box 1**), including some recurrent cancer traits like heterogeneity and drug resistance (79–83).

Cancer stem cell

In healthy tissues, true stem cells can often be identified from transient or differentiated cells by the expression of a defined set of markers, as well as by distinctive properties (i.e. multilineage differentiation potential, *in vivo* or long-

BOX 1 – The biosemiotic theory in cancer genomes evolution
Comparative, multi-disciplinary approaches have generated hypotheses and theories useful to explain some empirically relevant cancer features. In the "biosemiotic theory", the genome is seen as a coded sign system (semiotic) that is being interpreted by the transcriptional and translational machinery in order to result in phenotypic outputs (71). DNA mutations would increase biosemiotics entropy, resulting in a deviation from a healthy, homeostatic state. The DNA encodes the information to generate each of the key semiotic elements: the "sign" (the DNA itself), the "semantic" (the translational machinery) and the "object" (the translated proteins). As many large-scale studies and models report (68,72,73), cancer does not proceed by steady, gradual accumulation of random mutations: mutational hotspots accumulate in oncogenes/tumor suppressor genes. According to the biosemiotic theory, genes directly involved in the semantic (tRNA, ribosomal RNA, etc) are identified as critical and mutations affecting their function would decrease cellular fitness too drastically, resulting in a quick removal by purifying selection (74). Ribosomal RNA genes (rDNA) and transfer RNA genes (tDNA) are in fact highly redundant in eukaryotic organisms, a feature that contributes to lower the phenotypic impact of mutations and to maintain genomic stability (75). At support of this notion, epigenetic dysregulation rather than core mutation of these genes has been correlated with cancer (76–78). Commonly mutated oncogenes/oncosuppressors instead are frequently part of the regulatory machinery within the biosemiotic process and their mutation leads to a more gradual increase in biosemiotic entropy. The higher the biosemiotic entropy, the noisier is the biosemiotic process itself and the further the cell moves from the evolutionary optimum, resulting in a decrease of cellular fitness seen in cancer-related processes like cancer cells growth or metastatic dissemination.

term tissue repopulation capacity, etc). Cancerous tissues may also harbour stem cells (or cancer stem cells, CSC), an hypothesis that while being commonly acknowledged, has raised an intense debate owing both to its underlying biology and its clinical translation (84–87). Despite CSCs were identified in many different cancers including prostate cancer, research has struggled to associate specific markers or phenotypic traits with cancer stemness. In addition, when a specific CSC population could be identified, its selective suppression failed to result in cancer eradication, owing to a high plasticity of the cells within the residual disease. Multiple mechanisms are at the base of this plasticity and differ among the different cancer types. Genetic hits on normal tissue stem cell may originate CSC, but also genetic deregulation of committed, transit-amplifying cells may result in CSC generation, dedifferentiation and lineage infidelity (88–90). The lack of a defined association between molecular markers and CSC potency hampered the development of CSC-targeted therapies and contributed to the concept that stemness in cancer could not be linked to a discrete population, but rather to a dynamic state (88,91). In addition to genetic and epigenetic factors, clues from the tumor microenvironment including crosstalk with the stromal and immune compartments, the overall local inflammatory state and the effects of therapeutic insults all shape the dynamic CSC equilibrium. This intrinsic and equilibrium-induced plasticity of CSCs ultimately contributes to the genetic and clonal heterogeneity of many cancers, rendering the CSC functional units of selection of cancer evolution (92,93).

Prostate cancer formation

Prostate cancer presents in its most common form as an adenocarcinoma, for which a generally agreed natural evolution is shown in **Fig 4**. Malignant transformation of the prostate tissue is generally a gradual process that can originate from cells of both luminal (94,95) and basal origin (37,94,96,97). However, the clinical relevance of distinguishing prostate cancer cell of origin remains unclear: unlike breast or bladder cancers, the extreme rarity of basal-like prostate cancers does not support a molecular classification based on the luminal/basal differentiation status (98); to this end, larger prospective studies need to be carried out to draw more solid conclusions.

Initiation of cellular transformation leads to the development of prostate intraepithelial neoplastic (PIN) lesions, that can further progress to localized prostate cancer and to advanced, locally invasive adenocarcinoma, progressing eventually to metastatic prostate cancer. PIN lesions, characterized by the localized hyperplasia of prostate epithelial cells within

their physiological location, can be in turn classified as low and high grade, with the former showing a higher level of conservation of the prostate glandular pattern compared to the latter. The multifocal nature of prostate cancer is already evident in PIN lesions, as patients frequently develop multiple, synchronous lesions in different areas of the prostate (99,100). Progression from PIN lesions to the more advanced stages of the disease is often associated with a loss of differentiation of epithelial cells, causing in turn the disruption of the functional tissue architecture.

This histological property holds the foundation for the main diagnostic tool used to stage the disease, the Gleason grading system, introduced by Donald Gleason in 1974 and used to define prostate cancer aggressiveness based on its histopathological features (101). The score is composed of a primary value reflecting the pattern most represented within the tissue and a second value grading the most dysplastic pattern. The score ranges from 1, indicating a well-differentiated tissue to 5, indicating only occasional gland formation and is usually reported in the format of score 1 + score 2 (i.e. 3+3, 3+4, 4+3, etc.) (102).

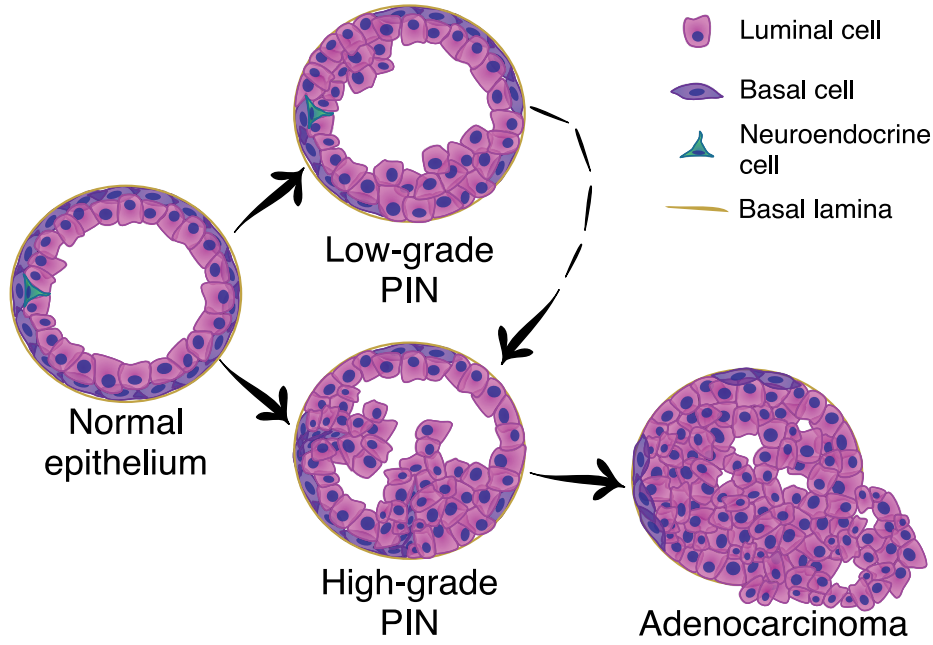

Figure 4 - Prostate cancer formation

Initial formation of adenocarcinomatous prostate cancer. Low-grade prostate intraepithelial neoplastic lesions (PIN) are often benign but can progress to high-grade PIN, robustly associated with the development of prostate adenocarcinoma. With progression, tissue architecture becomes less recognizable and epithelial cells can proliferate beyond the basal lamina.

Further accumulation of genetic hits within a PIN lesion, as well as a progressive dysregulation of tissue homeostasis and of cellular biochemical pathways can lead to progression to prostate adenocarcinoma. The natural history of prostate cancer at this stage can be variable, with some (usually lower-grade) lesions showing a more indolent progression and other (usually higher-grade) ones having a more rapid malignant course.

Hormone responsiveness and androgen-deprivation therapy (ADT)

A key parameter in the definition of prostate cancer evolution, that proved a relevant prognostic value, is prostate cancer hormone responsiveness, initially observed by Huggins and Hodges in 1941 (103). Androgen dependence has been exploited clinically with the development of androgen-deprivation therapies (ADT), that aim at reducing testosterone availability to cancer cells resulting in tumor shrinkage and clinical regression. Initially ADT was realized by bilateral orchiectomy or oral treatment with the estrogen diethylstilbestrol (DES). Treatment with DES suppressed the production of the luteinizing hormone-releasing hormone (LHRH), required to release LH and, in turn, to produce testosterone in the testis. Currently, pharmacological castration is achieved by two main strategies: LHRH agonists and LHRH antagonists. The first generate an initial flare of testosterone due to the increase release of LH. However, after a few days the cells of the pituitary gland become exhausted and stop releasing LH. Treatment with LHRH antagonists instead directly block LHRH action, resulting in the same testosterone suppressing effect (104,105). ADT has relevant side effects: immediate, acute and chronic. Immediate effects are linked to the flare of LHRH agonists and linked to the sudden rise of testosterone. Acute effects include sexual dysfunctions and hot flashes. Chronic effects include cardiovascular, hematological and musculoskeletal events. Osteoporosis is related to the concomitant reduction of estrogens, which production is linked to testosterone levels, and can be attenuated by estrogen administration (106,107).

Spatial and clonal tumor heterogeneity

Evidence from multiple tumor types has confirmed that cancer cells can undergo a process of Darwinian evolution, with the co-existence of multiple, heterogeneous cancer cells subpopulations (80,108,109). Cancer clones bearing tumor-promoting traits are under positive selection pressure, a process that can lead to the generation of intra-tumor clonal heterogeneity, ultimately resulting in spatially and genetically distinct tumor foci. The formation of multifocal lesions may occur both by intrinsic cancer cells

population dynamics and by external perturbations, like anti-cancer treatments, that can increase the selective pressure. The increased pressure further leads to the two complementary processes of genomic instability and clonal plasticity, contributing to additional evolutive waves promoting tumor aggressiveness and progression (80,110).

Prostate cancer commonly presents as a multifocal disease already at diagnosis, with a high degree of spatial heterogeneity (111–116). Findings from multiple studies have highlighted that the different foci may harbour different genetic mutations, conferring to cancer clones different degrees of aggressiveness within the same prostate (87,111–113,117). Albeit their common multifocality, low-grade prostate cancers are consistently more homogeneous than more aggressive ones, while the lethal metastatic disease shows clear subclonal features (98,118). Phylogenetic analysis of the different clones has increased our understanding of the traits that can result most beneficiary to cancer development, ultimately directing our therapeutic efforts to the key oncogenic hubs.

Despite the improvements in detecting and overcoming spatial heterogeneity of prostate cancer, patients relapsing after radical prostatectomy indicate that also temporal heterogeneity plays a major role in determining recurrence risk (118). Clinically relevant lesions are therefore not only those with the highest histological grade in their spatial surroundings, but also those with the potential of causing a relapse of the disease, in time. Targeting the potentially clinically relevant lesions is therefore not only a matter of diagnosis, but also a molecular question.

Molecular causes of prostate cancer

Evolutionary molecular mechanisms

The classification in molecular subtypes has changed clinical practice for many tumor types, associating patients bearing tumors with specific genomic alterations to clinically actionable targets (119–122).

The intrinsic heterogeneity of most primary prostate cancers hampered this process, requiring the analysis of large cohorts of patients to identify significant oncogenic drivers and validate molecular subtypes (98,123,124). Moreover, the genetic alterations driving primary prostate cancer differ from those most relevant for progression of advanced or castration-resistant prostate cancers so that a distinction between an early and a late stage of the disease needs to be made (125).

The development of new bioinformatics tools allowed not only to detect oncogenic events relevant for prostate cancer, but also to associate them to

the various landscapes of prostate cancer including the early-onset prostate cancer. Of note, some of these tools can help to infer genomic alteration dependencies, identifying which mutation or mutations are likely to occur before the appearance of a given one (126–128).

Compared to tumors where genomic alterations are accumulated through point mutations and small insertions or deletions (*indels*), prostate cancer shows a prevalence of chromosomal rearrangements and extensive copy number alterations (CNAs) as primary tumorigenic drivers (129–131).

There are few key chromosomal gains or losses in prostate cancer and the overall mutation rate is generally low, on average about 0.9 mutations/Mb in the primary lesion (123), a value that increases to about 4.4 mutations/Mb in the metastatic disease (129). A few possible mechanisms have been proposed and are linked to prostate-specific epigenetic regulation as well as to a few consistent molecular subtypes.

All actively transcribed DNA is subjected to increased mutational stress, or transcription-associated mutagenesis (TAM). This is particularly true in fast-cycling cells, at locations of intensely transcribed genes, where the transcription machinery and the replication fork have the highest chance of clashing, an event that can likely generate single- or double-strand DNA breaks (SSB and DSB) (132). This process has been documented also in PCa, where the distribution of DSB was associated to hotspots of AR-regulated genes (133). In particular, neoplastic and pre-neoplastic cells might associate an intense AR-driven transcriptional activity to an increased proliferation rate, overriding the suppression of proliferation that AR expression imposes to normal cells (33). AR-TAM contributes to explain the frequent detection of aberrations in proximity of AR-transcribed genes, as well as supporting the prostate-specificity of many reported DNA anomalies (134,135).

Clonal and subclonal tumorigenic events

Clonal genetic events associate to the initial steps of tumorigenesis, marking the aberrations that will be shared by most cancer cells within the focal lesion. Early events in prostate cancer development are therefore frequently linked to the decrease of epithelial differentiation, to an increased proliferative potential and, most relevantly, to the further loosening of genomic stability. Transcription-associated DSB, in the context of pre-neoplastic lesions has a high chance of generating chromoplexy, a pattern of complex inter-chromosomal rearrangements (136). Chromoplexy is frequently copy-number neutral, resulting undetectable to commonly used techniques like array comparative genome hybridization (aCGH). It is estimated to have a

prevalence of about 90% in PCa and therefore it is expected to be a very early event in PCa evolution (137). Somatic CNAs are an independent indicator of prostate cancer progression, with losses marking an earlier stage and gains defining a more advances stage. Losses are also about five time more common than gains and frequently involve a few recurrent loci, like 8p (32%), 13q (32%), 6q (22%), 16q (19%), 18q (19%) and 9p (16%) (138).
Subclonal events are genetic aberrations that are frequently detected in only a fraction of cells within each tumor focus. These later mutations are frequently associated to the acquisition of further malignant traits, like increased metastatic spread and survival (139,140) and can be tracked to metastatic lesions (118,140).
However, it is not possible to draw one-for-all boundaries between early and late tumorigenic events as the incidence of mutations is the outcome of different variables, including microenvironment, treatments and lifestyle. Nonetheless, some lesions have been shown to be tumor-initiating and consistently associated to the onset of the lesions, while others have been more frequently found in later stages of the disease (123).

Clonal events

ETS gene rearrangements

Chromosomal rearrangements between genes of the E26 transformation specific (ETS) family and the transmembrane protease serine 2 (*TMPRSS2*) gene are very common genetic aberrations in prostate cancer, being detected in 40-60% of patients (123). The detection of a genomic ETS fusion event within prostate cancer foci involves most commonly *ERG*, *ETV1/4* or *FLI1*, and classifies prostate tumors into ETS-positive or ETS-negative. TMPRSS2-ETS fusion is typically an early, highly clonal event, being detected already in PIN lesions (141). The most common fusion product consists in the interstitial deletion of about 3Mb at 21q22.2-3. The most frequent fusion product consists of the AR-regulated 5'-UTR of *TMPRSS2* fused with *ERG* at exon 4. The resulting product leads to the androgen-induced overexpression of an N-truncated isoform of *ERG*, an oncogene and key regulator of cell proliferation, differentiation and survival (124). The TMPRSS2-ERG fusion is the single most prevalent genetic lesion in prostate cancer (142).

SPOP, CHD1, FoxA1 and SPINK1 mutations

Speckle-type POZ protein (*SPOP*) is a substrate-binding component of an E3 ubiquitin ligase. Its mutations are the most common non-synonymous mutations in prostate cancer and can be detected in 6-15% primary prostate cancers (113,143–145). *SPOP* mutations are also an early occurring event in prostate tumorigenesis, as evidenced by their high clonality (130,144).

Dependencies analysis found no other mutations were required upstream of *SPOP*, in *SPOP*-mutated prostate cancers (144). *SPOP*-mutant tumors display an increased genomic instability that proceeds in patterns markedly different from that of non-*SPOP* mutant ones. While genomic instability of most prostate cancers is associated to interchromosomal rearrangements (i.e. chromoplexy, chromothripsis) that of *SPOP*-mutant ones is significantly higher than average and is associated to phenomena of kataegis (intrachromosomal rearrangements) (146). *SPOP* mutations occur solely as heterozygous, missense mutations, inducing loss-of-function in the remaining wild-type allele (136,147,148). Moreover, *SPOP* mutations inversely correlated with mutations in other key tumor suppressor genes as *TP53* and *PTEN* (130,145).
Forkhead box A1 (*FoxA1*) is a chromatin modulator that de-compacts condensed chromatin, making it accessible to other transcription factors, including the AR. Aberrations in the *FoxA1* locus have been found in about 3-13% of primary prostate cancers (130,149,150). A recent work has demonstrated that aberration in the *FoxA1* locus can be clustered in 3 different classes, named by the authors "FAST" (class 1), "FURIOUS" (class 2) and "LOUD" (class 3) (151). The FAST class of mutations affects the C-terminus of the Forkhead domain, increasing the genome-scanning efficiency of FoxA1 and, in turn, AR transcriptional activity. This class of mutations could be detected in about 8-9% of primary prostate cancers, where it was mutually exclusive of other clonal mutations (like *ETS* rearrangements or *SPOP* mutations). The LOUD class involve aberrations of the *FoxA1* locus, which bears regulatory element capable of the outlier *cis*-expression of oncogenes (123,151). Additionally, the *FoxA1* locus is a frequent target site of *ETV1* translocations, an event mechanistically supported by the hypermutation of *FoxA1* 3'-UTR, detected in about 12% of prostate cancer patients (123,135).
SPOP mutations associate with deletions at the 5q21 and 6q21 loci, harbouring *CHD1* and *FoxA1* respectively (130). Chromodomain helicase DNA-binding protein 1 (*CHD1*) is a chromatin remodeler that facilitates transcription and maintains chromatin architecture and genome stability (152,153). Loss of *CHD1* is an event uniquely restricted to prostate cancer and is detected in about 5-15% of primary prostate cancer cases. *CHD1* loss results in the nuclear redistribution of AR to ARE sites made accessible by other transcription factors, including FoxA1 and HOXB13. Most importantly, loss of *CHD1* was sufficient to initiate tumorigenesis in experimental models (154,155). The increased intrachromosomal rearrangements and overall mutagenic profiles associated to *SPOP*-mutated prostate cancers are seen also in *CHD1*- and *FOXA1*-mutated ones, identifying a specific prostate cancer cluster, distinct from other *ETS*-negative prostate cancers.

Serine protease inhibitor Kazal type 1 (*SPINK1*) is a secreted protein whose role in prostate cancer has not yet been completely defined; it was however shown to activate EGFR downstream pathway (156). It is highly overexpressed in 5-10% of prostate tumors and is almost exclusively found in *ETS*-negative, *SPOP*^{mut}, Ras/Raf-wild type tumors. *SPINK1* mutations have been linked to more aggressive cancers and higher risk of biochemical recurrence (157–159).

NKX3.1 mutations

The *NKX3.1* is a fundamental regulator of differentiation of the prostate epithelium and localized on chromosome 8p21. Its dysregulation can be detected in as many as 60-80% of prostate cancers, at a highly clonal level, evidencing an early occurrence and a positive selection of this mutation (23,136,160). Inactivating mutations of *NKX3.1* manifest as loss of heterozygosity, supporting the notion of haploinsufficiency of this tumor suppressor gene (161).

Subclonal oncogenic events

TP53

The role of *TP53* in integrating multiple tumor-suppressing functions makes this gene a frequent target of oncogenic mutations. About 25-30% of localized prostate cancers harbor alterations in 17p31.1, the *TP53* locus, either as deletions or as loss-of-function point mutations, suggesting an involvement of *TP53* in the early stages of the disease (136,149,159). However, in addition to a lesion-initiating role, multiple studies are linking *TP53* alterations with metastatic spread (118,140). *TP53* mutations are more frequently detected in *ETS*-positive prostate cancers and positively associate with *PTEN* mutations in primary prostate cancers.

PTEN

Loss of phosphatase and tensin homolog (*PTEN*), at locus 10q23.31, is a landmark mutation of prostate cancer, being one of the molecular events that identify progression to advanced prostate cancer. PTEN is part of the PI3K/Akt/mTOR pathway and contributes to its downregulation by inactivating PI3K. Loss of *PTEN* is most frequently seen as focal deletion in the *PTEN* locus (about 40%-60% of cases), but *PTEN* inactivating mutations are also relatively frequent events (5-10% of cases). *PTEN* alterations consistently associate with poorer prognosis, correlating positively with higher Gleason score, higher risk of metastasis and recurrence after therapy (130,149,150,159,162–167).

Together with loss of *TP53*, loss of *PTEN* is among the most common genomic events of prostate cancers and occur in both *ETS*-negative and *ETS*-positive

cancers, with a higher frequency for the latter. Deletion of the 3p13-14 region, spanning *FoxP1* is detectable in about 12% of primary prostate cancers and was recently associated with *PTEN* loss (168).

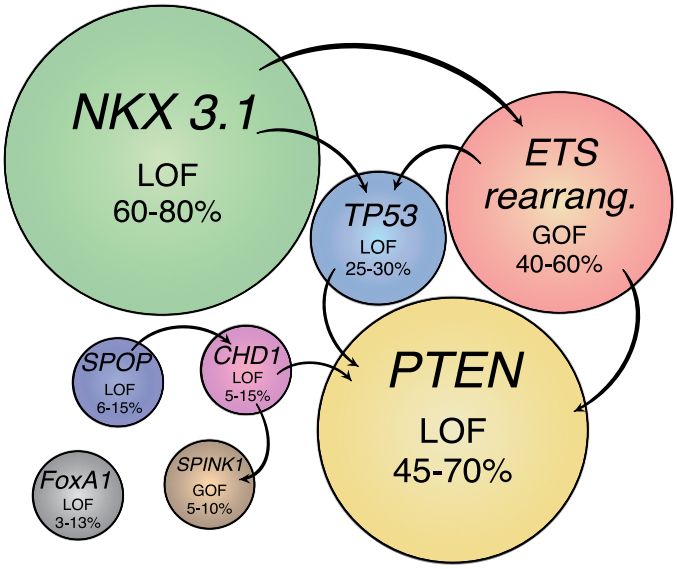

Figure 5 – The genomic landscape of primary PCa
Graphic summary of the most common genomic alterations found in primary prostate cancer, including their functional effect on the mutated protein product (LOF/GOF) and the average frequency of detection. The arrows indicate the average directionality of the mutational event.
LOF, loss of function.
GOF, gain of function.

Prostate cancer subtypes

The two main molecular event that contribute to classify prostate cancer based on their molecular evolution are *ETS* rearrangements and *SPOP* mutations (130,144,169). The Cancer Genome Atlas (TCGA) consortium provided a 7-cluster classification for primary prostate cancer, based on the detection of gene fusions involving *ETS* family members (*ERG* being the most prominent representative), *ETV1*, *ETV4* or *FLI1*, or of mutations in *SPOP*, *FOXA1* or *IDH1* (123). The consortium further supported the analysis of CNA as an independent parameter correlated to worse prognosis.

At the aetiological level, prostate cancer can be divided in sporadic and familiar depending on the genetic background of the patient. Familiar prostate cancer tends to be more aggressive and to have a worse outcome than the sporadic type (170). Although the precise mechanisms have still to be fully elucidated, high-risk mutations do predispose to familiar prostate cancers by jump-starting the oncogenic transformation. Mutations in Lynch syndrome-associated genes and, more generally, in genes involved in DNA stability and mismatch repair pathways increase genomic instability, a feature shared among all cancers but particularly relevant for prostate cancers (98,171).

BRCA2 and, although more controversial, *BRCA1* mutations correlate with a higher risk of prostate cancer and contribute to the young onset of prostate cancer (<65 years). The two largest clinical studies investigating the role of *BRCA1/2* mutations and prostate cancers are the EMBRACE and the IMPACT studies, currently still ongoing (172,173). Men carrying *BRCA2* germline mutations have an over 8-fold increased risk of being diagnosed for prostate cancer and show overall worse clinical parameters than non-carriers (174). Germline *BRCA2* loss predisposes to global genomic instability and localized prostate tumors from *BRCA2* carriers show features commonly detected in mCRPC (173,175–178).

De novo or primary neuroendocrine prostate cancer (NEPC), that is a NEPC diagnosis that does not follow nor that can be traced back to adenocarcinomatous prostate cancer (Adeno-PC), is an extremely rare type of cancer, with an incidence of about 1 case every 1'000'000 persons/year (8,179–181). Small-cell carcinoma (SCC) accounts for about 60% of the cases, the remaining being non-small cell carcinoma NEPC; in both cases the overall survival is about 10-12 months, with the SCC subtype showing the least favourable outcome. About 64% of *de novo* NEPC patients have metastasis at diagnosis and for patients without metastasis, radical prostatectomy confers the highest protective effect.

Diagnostic approach in primary PCa

Risk factors

Prostate cancer is a heterogeneous disease both at the molecular and at the clinical level. This intrinsic heterogeneity imposes the diversification between general risk factors for prostate cancer and risk factors associated for advanced, lethal prostate cancer. General risk factors for prostate cancer are older age, African descent and a positive family history of prostate cancer (60). Analysis of risk factors for lethal prostate cancer led to the identification of obesity, smoking and a taller height as positive risk factors and of physical activity as a negative one.

The identification of ethnicity and positive family history among prostate cancer risk factors strongly supported the genetic component as a susceptibility risk factor. To date, reliable markers for predicting susceptibility to prostate cancer have not yet entered clinical practice, although this field has been a target of intense investigation and newer, genome-wide association studies (GWAS) are emerging with promising results (182–184).

In these studies, a significant association was found between a missense single-nucleotide polymorphism (SNP) in the *ATM* (rs1800057, G>C,

p.Pro1054Arg) and in the *CDKN1B* (rs2066827, T>G, p.Val109Gly) genes. Panels of potentially clinically-relevant SNP are being developed, with the aim of identifying patients at higher risk of developing prostate cancer (183–185). Rare germline mutations in the *BRCA2* (175,186–188) and in the *BRCA1* (189) genes were also found associated with a higher risk of developing prostate cancer.

Among non-genetic risk factors, chronically elevated oestrogen levels as well as endocrine disruptors with estrogenic activity correlate an increased prostate cancer risk (190). Despite the lack of correlation between total serum oestradiol and prostate cancer risk, there is evidence that prostate cancer risk increases with age. This is marked in men by a decreasing testosterone-to-oestradiol ratio, suggesting that the relative ratio of these two sex hormones could have an impact, rather than their absolute levels (12).

Biomarkers for risk assessment

PSA as diagnostic tool

Serum PSA values are normally barely detectable (as a reference value, healthy men commonly show serum PSA values below 4 ng/ml) (191) and could be transiently increased by activities like practicing sport, ejaculation, receptive anal intercourse, after a digital rectal exam (DRE) or surgical procedures involving the prostate (192). Elevated serum PSA levels therefore do not necessarily imply a prostate cancer diagnosis: pathological conditions other than cancer, like prostatitis, lower urinary tract infections or benign prostatic hyperplasia (BPH) can also lead to increased serum PSA levels.

A sustained serum PSA increase however, is a common symptom of prostate cancer. Nevertheless, its multifactorial dependency limits the generation of a consensus threshold of serum PSA for a sure diagnosis of prostate cancer.

Higher grade lesions (Gleason score ≥ 8) are frequently associated with increased PSA but the strength of the association decreases for lower grade ones, lowering its decisional value for less advanced lesions (193,194). On the other hand, a non-irrelevant fraction of prostate cancers fails to increase serum PSA levels above the agreed threshold of 4 ng/ml (195).

Additional PSA derivatives have been introduced into clinical practice in the effort to improve specificity. Such derivatives include the measurement of free PSA (fPSA), % fPSA, complexed PSA (cPSA) and the specific quantification of PSA isoforms (196,197).

Other molecular markers of prostate cancer

The need of increasing specificity and sensitivity of diagnosis, as well as of differentiating a cancer diagnosis from other non-cancerous conditions of the

prostate, fostered the research of additional, clinically relevant prostate cancer-associated markers. Among them, the non-coding RNA prostate cancer antigen 3 (*PCA3*) and fusion transcripts of *TMPRSS2* with *ETS* gene family members showed a significant association with prostate cancer and their combined used enhanced the sensitivity of prostate cancer diagnosis (198–200). An advantage of these two markers is their detection in urine, especially after a DRE. This characteristic prompted the development of a non-invasive platform that integrates the detection of *PCA3* and *TMPRSS2-ERG* in the urine with PSA levels detected in serum: the Mi-Prostate Score (MiPS), a parameter that showed significantly greater AUC compared to serum PSA measurement alone or in combination with *PCA3*, in diagnosing prostate cancer (201).

Diagnostic imaging tools and histopathological evaluation

The current golden standard for prostate cancer diagnosis is the histopathologic evaluation of prostate biopsies. According to the European Society for Medical Oncology (ESMO) guidelines, in the event of a suspicious digital rectal examination (DRE) of the prostate and after confirming elevated blood PSA levels by two independent PSA tests, patients can be advised to proceed to sampling of the prostate (202). The most used techniques for making prostate cancer multifocal biopsy are the transrectal ultrasound-guided prostate biopsy (TRUS), the multiparametric magnetic prostate resonance imaging-guided biopsy (mpMRI) and the integration of the two, the fusion-mpMRI (146,203–205). In particular two recent clinical trials, PROMIS and PRECISION, supported the use of mpMRI to improve the detection of clinically significant prostate cancer (204,206).

The ESMO guidelines advise to proceed to prostate biopsy after integrating the PSA screening results with the other relevant risk factors of the patient, as discussed in the previous paragraphs. Conventionally, prostate biopsy is performed by sampling 10 to 12 prostate tissue cores in a grid-like pattern. The different cores will be microscopically analysed by a pathologist whom, in case of positivity to cancer, will issue a Gleason score for each lesion, with a higher score representing a less differentiated tumor.

After obtaining a Gleason score, patients may undergo an MRI exam of the prostate to assess whether the disease is localized or if it had invaded neighbouring or distant tissues. The system used to stage prostate cancer is the TNM system (207), widely used in many solid cancers and accepted by Union for International Cancer Control (UICC) and the American Joint

Committee on Cancer (AJCC), see **Box 2** for further details on the TNM system (208).

In men undergoing radical prostatectomy, the post-operatory histopathological assessment of the prostate will provide a pT score, which may revise the initial cT score determined pre-operatively. While understaging is generally the most common event, overstaging occurs in a non-negligible fraction of prostate cancers (209).

Prostate cancer risk determination

Among the main clinical challenges of primary prostate cancer is the discrimination of patients' risk class.

Assessment of the TNM stage, together with the other clinical information allows the stratification of patients into three main classes of risk: low, intermediate or high risk (202), which constitutes the most used risk group classification system to date. Recently, a 5-tier system adding a sub-classification of low- and high-risk prostate cancer has been proposed and validated by the National Comprehensive Cancer Network (210). This new tool, while not revolutionizing the existing risk categories, increases coping of the heterogeneity within each group.

While patients belonging to a low- or high-risk group have more defined therapeutic options, patients with an intermediate risk are the most exposed receive insufficient or over-treatment. Up to one-third of patients classified as intermediate-risk relapse, developing a more aggressive disease that could have benefited from a harsher therapeutic approach (211,212). On the other hand, many intermediate-risk patients potentially eligible for watchful waiting or other organ-sparing approaches receive unnecessary surgical or radiation therapy, with relevant consequences on their quality of life. Many clinical studies have focused on the subset of patients bearing these types of intermediate risk prostate cancer, in the attempt to determine a clearer patient stratification and to provide more effective therapeutic options (112,136,146,149,214).

Markers to help intermediate risk patient stratification have been difficult to find and to validate and are only recently starting to prove some clinical value, compared to the available options, as illustrated in the previous paragraphs (146,214,215).

The complex nature of prostate cancer does not allow the identification of a patient risk group solely by interrogating a few variables: in the last 20 years, numerous algorithms and nomograms have been developed to aid the urologists in decision-making at the different stages of prostate cancer

diagnosis and treatment. These tools have a relatively broad range of complexity, integrating as little as a few patient-related variables up to software-bound complex algorithms (216–218). While the integration of more variables does not necessarily correlate with an increased accuracy of prediction, it does reduce usability and availability of the tool to the final user, either the clinician or the patient (217). Many developed nomograms have been validated and proved useful to improve patients' risk stratification, both before and after definitive treatment. Among the developed tools, the pre-operative CAncer of the Prostate Risk Assessment (CAPRA) and the post-operative CAPRA (CAPRA-S) scores have met a widespread due to their powerful integration of multivariable risk stratification with ease of use (219–222).

Box 2 – The TNM system in cancer diagnosis

The TNM system informs on the status of the primary cancer, of the adjacent lymph nodes and on the presence of metastases. The primary cancer is identified as "T" followed by a number ranging 1-4 and, depending on the stage and on the disease, by a letter from "a" to "c". Higher numbers and letters indicate a more advanced disease. Lymph nodes involvement is coded with the letter "N", followed either by a "0" or a "1", indicating negative or positive adjacent lymph nodes invasion, respectively. In case of impossibility to assess either the primary tumor or the adjacent lymph nodes involvement, the respective letter code is followed by an "X". Metastatic spread is indicated by the letter "M", followed by a "0" for absence of detectable metastasis, whereas detection of metastasis is assigned the code "1" followed by a letter depending on the localization of the detected cancer cells: "a" for non-adjacent lymph nodes, "b" for the bones and "c" for other places.

Additionally, there are two types of "T" category, clinical and pathological T stages, "cT" and "pT" respectively. The former is inferred by data gathered from clinical tests like PSA tests, histopathological evaluation of prostate biopsies and DRE or other diagnostic imaging techniques and represents the best estimate of tumor stage evaluation. As the cT is an indirect measure of the extent of the disease, it has an inherent risk of lacking accuracy; however the cT will be the main parameter to delineate the therapeutic options and treatment strategy (213).

Clinical options for primary prostate cancer.

Risk assessment determines the ground on which clinical decisions will be taken. In addition to the three main risk groups, low, intermediate and high, primary prostate cancer can be localized, locally advanced or advanced, discussed in the next paragraph. Therapeutic options for localized prostate cancer are different for low- and intermediate-risk patients.

Low-risk and intermediate-risk prostate cancer

Patients with low-risk prostate cancer (T1-T2a) can be advised with the broadest range of therapeutic options, as these types of cancers are usually slow-growing, have a low risk of spreading and in many cases do not need treatment. Intermediate-risk patients (T2b) are generally advised to take additional diagnostic exams, to refine the therapeutic options most suitable for their specific situation.

Watchful waiting is the most conservative option and consists of a monitoring plan for prostate cancer without any additional treatment, until symptoms appear. This choice is particularly suitable for men with other health problems that would be unfit for other treatments and for prostate cancers that are unlike to impact on a patient life span.

Like watchful waiting, active surveillance is also a monitoring plan but consists of more, and more in-depth, regular tests. It aims to avoid or delay unnecessary treatments in low-risk prostate cancers, sparing the patients from side effects that might reduce their quality of life. Patients can opt for active surveillance until their cancer shows signs of progression, in which case they can be offered further treatments.

The surgical removal of the entire prostate, or radical prostatectomy, is one of the most common treatment options for prostate cancer, albeit patients in the low-risk group are those most exposed to overtreatment with this option. Another common treatment, usually alternative to the surgical approach, is radiotherapy, either as external beam radiotherapy or as internal radiotherapy of brachytherapy. Additional treatments are available, like high-intensity focused ultrasounds (HIFU) or cryotherapy but are less common. Radical radiotherapy efficacy and safety in the treatment of localized prostate cancer was assessed in numerous randomized, phase-III clinical trials, focussing especially on the intermediate- and high-risk disease. This cohort of non-metastatic patients, without lymph nodes involvement, benefited more from the combination of ADT and radiotherapy compared to ADT alone (223–225). The PEACE-2 trial (NCT01952223) is currently investigating the benefit of radiotherapy in the same cohort of patients also in terms of progression-free survival.

High-risk and locally advanced prostate cancer

Patients with high-risk (T2c-T4) or locally advanced (T3-T4) prostate cancers will be advised with more stringent options compared to the low- and intermediate-risk groups. Prostate cancer in these patients is likely to spread outside the prostate and has shown sign of aggressiveness. Prostate cancer is

staged as locally advanced when it has already spread outside the prostate capsule (into the seminal vesicles, bladder or pelvic wall, rectum or draining lymph nodes), but it has no detectable signs of spreading to distant organs.
Therapeutic options for patients with high-risk or locally advanced prostate cancer include radiotherapy, with extensive hormonal therapy, or radical prostatectomy, eventually in association with radiotherapy. It is currently controversial whether the concomitant treatment with chemotherapy (docetaxel) is beneficial for the treatment of high-risk prostate cancer patients: while multiple studies and their meta-analyses have confirmed that adding docetaxel can delay progression, overall survival was unchanged (226–230).

Progression to Advanced prostate cancer

Advanced prostate cancer refers to a stage where cancer cells have spread to distant organs. The metastatic disease has overall a worse outcome, with a 5-year survival rate of 30% if diagnosed at presentation. As a comparison, 5-year survival rate is nearly 100% for the localized disease, eroded to 98% when considering the 10-year rate (62,231). Nevertheless, owing to better diagnostic techniques and to the implementation of PSA screening, currently 77% of men are diagnosed with local disease, 16% of men with locally advanced prostate cancer and only 6% of patients are initially diagnosed with distant metastasis (231). The first sites of metastasis are often the adjacent or regional lymph nodes that, while predicting a worse prognosis, rarely are a leading cause of prostate cancer mortality (232). The most frequent site of metastasis in men diagnosed with metastatic CRPC (mCRPC) are the bones, and about 4 out of 5 patients show evidence of bone metastases in their clinical history (233). About 90% of patients that die of prostate cancer have metastatic disease to the bone, marking bone colonization as the most lethal event in prostate cancer progression (234,235). Other relevant sites of metastatic colonization are the liver, the lungs and the brain.

The metastatic spread

The leading cause of mortality in many cancer types, including prostate cancer, is the formation of distant metastasis. Different models to explain the generation and the temporal development of metastasis will be treated in deeper detail in **Chapter 2** of this work. At one point of tumor evolution, some cancer subclones may acquire the ability to disseminate from the primary site into the blood or lymphatic stream. This key feature marks one of the first steps of dissemination, that is the generation of circulating cancer cells (CTCs):

the vast majority of CTCs however will not survive in in the blood and will be progressively cleared from circulation. CTCs however might be clinically detectable, both by their presence in the blood (CTC count per ml of blood or per million blood cells) and by their by-products, mainly cell-free DNA (cfDNA) or circulating DNA (cDNA). A minor fraction of CTCs might carry or evolve the ability to extravasate the circulation and engraft distant tissue, generating disseminated tumor cells (DTCs). In prostate cancer, the most common sites of dissemination are the bones, with about 90% of patients showing bone involvement in their clinical history, the lungs, liver or brain; lymph nodes are frequently an intermediate harbour, before further colonization (236,237). Engrafted DTCs may enter an undetermined latency phase or dormancy that can last for years, characterized by a stop or a great reduction in proliferation rate, by a metabolic adaptation to and acquisition of markers specific of the engrafted tissue (tissue mimicry) and by the resistance to therapies, mostly owing to the reduced metabolism and proliferation rates. This latency phase is generally difficult to detect as it is normally asymptomatic and as the dormant cancer cells are often too few to be detected by current diagnostic tools.

Genetic studies on tumor evolution have increased our knowledge on the metastatic process, allowing to trace patient-specific phylogenetic tree of cancer evolution. This in turn has allowed to assess common genetic traits associated to specific aspects of the metastatic process (extravasation, survival, metabolic adaptation, etc), to defined tropism of metastasis for determined organs or for increased therapy resistance (118,136,238–243). Moreover, tracing the evolutionary trajectories have allowed to gain more insights on the process of cancer dissemination, including clonal dynamics, that informed us on the risk of further metastasis-derived dissemination. This in turn can indicate patient groups that can benefit most from specific treatments, refining and tailoring the therapeutic approach.

The appearance of clinically relevant metastases

An important difference is whether metastases are diagnosed together with the primary prostate cancer at presentation (synchronous metastasis) or if they are detected as a progression event of an already diagnosed and treated primary prostate cancer (metachronous metastasis) (244). About 35% of patients that were treated for the localized disease experience a biochemical relapse (BCR), that is a consistent increase of serum PSA after prostatectomy or radiotherapy. This increase could be due to persistent local disease (local recurrence), pre-existing metastasis or residual benign prostate tissue (245).

However only about 30% of these patients (that is, about 11% of patients with BCR) will experience a clinical recurrence (246–248).
A very relevant "grey area" between the localized disease and the advanced disease that has already spread to multiple distant sites is the "oligometastatic" disease. Oligometastatic patients show a limited number of clinically detectable metastasis ((249) and more recently revised by the same authors (250)). The relevance of the oligometastatic disease is that cancers that have shown only a limited spread could benefit from localized forms of treatment, potentially achieving curative intents. In contrast, in patients showing extensive metastatic colonization, cancer treatments are mostly aimed at containing rather than curing the disease (250). The underlying hypothesis is that, as metastatic spread is an acquired trait that must be evolved by cancer cells, the eradication of a metastatic lesion in its early onset is likely more effective than at a later timepoint of metastatic evolution. Currently however, no clear definitions of "limited spread" and of "oligometastatic" can be provided to clearly separate patients more likely to benefit from localized treatments from those in a later stage of the disease (251).

Monitoring and staging of advanced prostate cancer

The removal or disruption the prostate by an ablative treatment requires the use of other markers and tools to monitor disease progression. One of these tools is PSA, that might still be produced by prostate cancer metastatic cells, depending on the evolution stage of the cancer. PSA monitoring can offer a sensitive way to detect prostate cancer events, like effectiveness of therapy or progression of the disease. However, also in this case absolute numbers are difficult to draw as serum PSA threshold levels depend on the PSA nadir of the patient (the lowest detected PSA level after radical treatment) as well as on the overall kinetic of PSA modulation. Patients experiencing BCR may be addressed to undergo further exams, in order to detect the site of relapse. Bone scans and pelvic-abdominal CT scans are routinely performed, despite their low sensitivity, especially at low PSA levels. New PET-based imaging techniques have shown increased sensitivity compared to bone scintigraphy and CT scans, especially when performed with radiolabelled choline or prostate-specific membrane antigen (PSMA)-targeting tracers (252,253). The advancements of imaging techniques as well as the lowering of PSA thresholds of detection improved the sensitivity of metastasis detection, increasing the fraction of "oligometastatic" patients. Treatment of metastasis could be an indication for oligometastatic patients, as well as for patients developing

symptoms at the sites of metastasis. However, PSA could show an increase post-nadir also in absence of detectable metastatic lesions, a situation identified as "M0" or "non-metastatic" advanced prostate cancer. In particular, PSA doubling time (PSADT), a parameter that indicates the slope of PSA increase over time, is proving particularly useful to stratify risk in patients with advanced prostate cancer. Shorter PSADT were strongly associated with reduced time to metastasis and overall survival in M0 patients, identifying low PSADT patients as a high-risk group. Studies are ongoing to assess the PSADT cut-off thresholds that best predict high-risk patients (254,255). The most relevant parameter for the classification of advanced prostate cancer is the assessment of its responsiveness to hormonal therapy. Hormone responsiveness is not linked to the appearance of clinically detectable metastasis and consists of three stages:

Hormone-naïve/sensitive prostate cancer (HSPC). At this stage, the relapsed disease could still be controlled by castration, either surgical or, most frequently, pharmacological with androgen deprivation therapy (ADT).

Castration-resistant prostate cancer (CRPC). The progression of the disease in spite of ADT characterizes this stage of advanced prostate cancer. Patients can receive additional lines of treatment to control the disease.

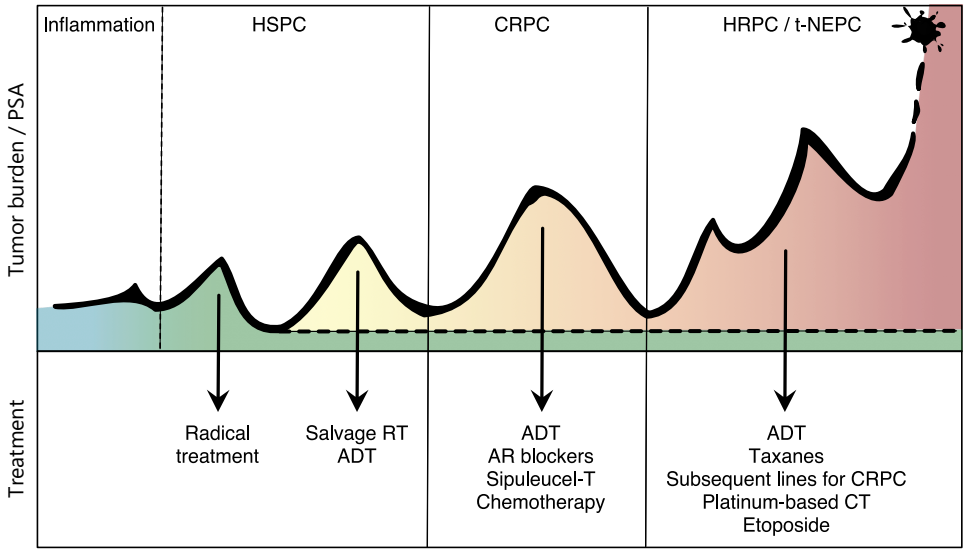

Figure 6 - Prostate cancer natural history

Progression of prostate cancer (PCa), as evidenced by tumor burden and PSA levels. In 3 out of 4 patients, the disease will be eradicated after initial diagnosis (green layer). If not eradicated, prostate cancer will progress and could reach androgen resistance (CRPC) or insensitiveness (HRPC or t-NEPC).

RT, radiotherapy; ADT, androgen deprivation therapy; HSPC, hormone-sensitive PCa; CRPC, castration-resistant PCa; HRPC, hormone-refractory PCa; t-NEPC, treatment-induced neuroendocrine PCa

Hormone-refractory prostate cancer (HRPC). At this last stage of prostate cancer, the disease does not respond anymore to ADT nor to AR-inhibitors, marking the start of further treatment lines (**Fig. 6**).

Hormone-sensitive metastatic prostate cancer (HSPC)

While 70-75% of patients initially diagnosed with prostate cancer will eradicate the disease, about 20-25% will experience relapse, requiring further treatment to control the disease and the symptoms that derive from it (248). As prostate cells need androgen for survival and proliferation, one of the first and more common treatment strategies for symptomatic advanced prostate cancer is the suppression of androgen production in the body. Albeit bilateral orchiectomy can achieve effective testosterone suppression, it is a definitive treatment and usually has a low compliance. The most frequently used treatment is ADT, that can pharmacologically induce castrate levels of testosterone without the need of surgery.

ADT in relapsing prostate cancer

Depending on the risk group and on the treatment choices performed for managing their primary prostate cancer, patients might have already been exposed to ADT in their clinical history. Progression and treatment of prostate cancer differs, both biologically and clinically, whether it happens during or shortly after ADT, long after ADT or in patients not previously exposed to ADT. ADT however is also rising questions that lack a shared consensus: when to start ADT and how long to keep patients on treatment is an intensely debated question. In order to attenuate the side effects and increase patients' compliance, many patients and urologists are opting for intermittent ADT. However, the consequences of this schedule on clinical outcomes of patients are not clear. Another open question is the maximum androgen blocking to reach, that is the optimal level of androgen blockade to maximize anti-cancer effects (244,252,256).

While ADT can block the LH-dependent production of testosterone, about 5% of this hormone is produced by the adrenal glands, in a LH-independent mechanism (256). In order to reach a stronger androgen blockade, androgens synthesis inhibitors have been developed. 17-α-hydroxylase/C17,20-lyase (CYP17), an enzyme produced by the testes, adrenal glands, as well as prostate cancer cells, required for the biosynthesis of androgens. After the discovery of its inhibitory action on CYP17, ketoconazole was used for over 30 years as an androgen synthesis inhibitor, increasing the time to progression by about 3-10 months (257). However, the low specificity of ketoconazole for CYP17

inhibition led to the development of abiraterone, a selective inhibitor of CYP17A1 (258). Compared to ketoconazole, abiraterone is more potent, more selective and has a better toxicity profile, making this drug a standard of care for patients failing ADT (259).

Treatment options for HSPC

According to the latest guidelines of the European society for Medical Oncology (ESMO), patients relapsing after a radical treatment with no evidence of metastasis (M0) can be recommended with specific treatments, depending on which radical treatment they underwent for treating the primary cancer. The aim is to target early, pelvis-localized metastatic foci or remaining cancer cells that might have escaped the radical treatment. Relapsing patients that underwent radiotherapy as a radical treatment might benefit from intermittent ADT, whereas patients who underwent radical prostatectomy may benefit from a "salvage" radiotherapy to the prostate bed or pelvis early after detection of biochemical relapse (202,260).

During the last years, different clinical trials explored the efficacy and safety of adding concomitant treatments to ADT, with the general aim of increasing overall survival (OS) and progression-free survival (PFS) in patients with metastatic HSPC. Docetaxel, a chemotherapeutic drug that targets the mitotic spindle of actively proliferating cells, improved patients' OS and PFS across multiple studies. A limitation of this treatment was the requirement of adequate patient clinical fitness, as the drug induced relevant side effects. Another option that was recently introduced in clinical practice, basing on the results of a few phase-III, randomized clinical trials, is the administration of abiraterone, enzalutamide or apalutamide in association to ADT. Patients receiving one of these agents in association to ADT showed in general an increased OS and PFS compared to patients receiving ADT alone (227–229,261–266). HSPC is a research hotspot for a growing range of clinical trials, aimed at improving current therapeutic settings as well as sparing treatments with little or no proven benefit for the patients. Among the many, a question of particular interest is the benefit of adjuvant radiotherapy compared to salvage radiotherapy, that is local radiotherapy administered shortly after radical prostatectomy or at biochemical relapse: the RAVES (NCT00860652), RADICALS-RT (NCT00541047) and GETUG-AFU-17 (NCT00667069) trials are currently investigating this setup.

Mechanisms of progression to castration-resistant prostate cancer (CRPC)

While ADT can control disease progression for some years, virtually all patients that experiences a relapse will progress to CRPC, with about 10-20% of prostate cancer patients developing CRPC by 5 years (233). In fact, over 80% of patients diagnosed with CRPC present clinically detectable metastases (233,267). The most recent criteria to determine progression to CRPC are testosterone levels below 1.7 nmol/l (castrate level) and either BCR with a kinetic of increasing PSA over 3 weeks or radiologic relapse as per RECIST criteria (268,269).

The natural history of CRPC patients can vary significantly depending on the presence of metastasis at the time of CRPC diagnosis. Recent clinical trials have shown that nonmetastatic (M0) CRPC patients that received a new generation AR blocker in association to continued ADT had prolonged metastasis-free interval (270–272). Another factor greatly influencing patient's outcome is the AR dependency status of the progressing prostate cancer. About 60% of CRPC are AR-dependent, showing various alterations in the AR pathway or in AR-linked pathway. However, in the remaining 40% of cases the tumor is "AR-indifferent", having developed alternative mechanisms to progress: in 10-20% of the cases the tumor acquires features of neuroendocrine prostate cancer (NEPC) and in about 30%, with some degree of overlap, the lesions bear alterations in genes involved in the DNA damage response or in the DNA mismatch repair pathway.

Genetic aberrations in CRPC

Compared to cancer cells in the primary tumor, metastatic cancer cells frequently show a higher degree of genome plasticity, as reflected by an overall higher rate of mutations accumulation (129,239). Moreover, the genomics commonly observed in adenomatous CRPC (Adeno-CRPC) are associated with treatment resistance to AR-directed therapies (129,149,273).

Key mutations at this stage of the disease involve mechanisms to resist androgen deprivation and drug treatments (129,135,240). Overall aneuploidy and copy number alterations were also shown to correlate with progression and with a more aggressive disease; however, no correlation has emerged so far between specific subsets of clonal or subclonal genomic alterations and metastasis tropism or resistance to chemotherapy (146,238,274–276). Recurrent genomic lesions have been found at the metastatic sites, supporting the notion of metastasis-enabling genetic hits. Common clonal, metastasis-enabling genomic lesions include mutations in the *AR* as well as in the *TP53*,

PTEN, *RB1*, *MYC* and *FOXA1* loci (150,239,240,273,277). As genetic dysregulations in CRPC occur on a background of an already genomically destabilized cell, frequent CRPC genetic alterations will be discussed within their main biochemical pathways.

Alterations in the AR pathway

The AR is the most commonly affected pathway in mCRPC, with over 70% of patients bearing genetic aberrations in the *AR* or in key elements of its pathway (278). Metastatic cells commonly show convergent evolution to overcome the AR inhibition induced by ADT; AR independency moreover increases the likelihood of survival of disseminated metastatic cells (240,279). The fraction of patients with *AR* activating mutations increases from about 15-20% to over 50% following treatment with anti-androgens like abiraterone or nonsteroidal antiandrogens (123,129,136,149,150,280,281). Androgen deprivation can be overcome in cancer cells by multiple mechanisms, the most common being focal amplifications of the *AR* locus (Xq12) and/or of *AR* gene enhancers, detected in about 50% of mCRPC patients. The amplification leads to an increase of *AR* transcription, a condition that is sufficient to drive the development of a castration-resistant phenotype (282,283).

Alterations of the ligand-binding domain (LBD) of the AR are also a major source of resistance to androgen deprivation and anti-androgens. Point mutations in the LBD of the AR can increase the avidity of the binding pocket to testosterone derivatives, decrease its specificity to allow the activation of the AR also by less potent androgens or even shape it to turn AR antagonists into AR activating ligands (284–286). Alternatively, truncated isoforms or splice variants of the AR (*AR-V*) partially or completely lacking the LBD can emerge. These isoforms, despite missing the LBD, retain the ability to bind to DNA and to activate transcription, uncoupling the transcriptional activity of the AR from ligand-induced activation. Among the different truncated isoforms, AR-V7 shows the highest correlation with increased resistance to therapy and reduced overall survival. Moreover, its detection in prostate cancer patients' circulating tumor cells (CTC) could be a prognostic marker for therapy resistance (287,288). However, large-cohort studies could not confirm a causative role of *AR-V7* or other *AR-Vs* in CRPC progression (123,129).

The increased expression of AR coactivators like FOXA1, NCOA1/2, EP300, TNK2 and SOX9 or the decreased expression of repressors like NCOR1/2 and NRIP1 have also found in a significant fraction of mCRPC genomes. Altering the balance between nuclear activators and repressors results in an increased AR activity and in a higher androgen sensitivity (129,149).

Additional resistance mechanisms override the AR-ligand interaction requirement, inducing AR transcriptional activity by increasing its

phosphorylation level, a mechanism occurring at physiological level also in the normal prostate. Overexpression of cyclin D1 (*CCND1*), was shown to increase CDK4/6-mediated AR phosphorylation, inducing resistance to AR antagonists. *CCND1* amplifications were detected in about 9% of mCRPC cases and less common mutational events were detected in CCND1-related pathway (129). Activation of HER2 and other growth factor receptors like EGFR, IL-6R, IGF-1R also increase AR activity, mediating its phosphorylation via the RAS/MAPK, SRC, JAK/STAT and other ACK1-mediated pathways, including Akt (289–292). Prostate cells dependency on the AR implies proapoptotic regulatory loops that are activated in absence of AR engagement. AR can regulate the expression of anti-apoptotic proteins like BCL-2 and BCL-XL, and this regulation is mediated by RB1 (293,294). Loss-of-function mutations in the *RB1* locus were detectable in about 21% of mCRPC patients and mutations in the *BCL-2* locus, albeit less frequent, were linked to progression to mCRPC (129,295).

Alterations in the PI3K/Akt/mTOR pathway

About 50% of metastatic prostate cancers shows a dysregulation of the PI3K pathway, rendering it the second most commonly dysregulated pathway in prostate cancer. The PI3K pathway coordinates key cellular functions like proliferation and survival, metabolism adaptation, immune system and angiogenesis regulation. Alterations in this pathway tend to be hyperactivating and occur most commonly with inactivating mutations in the *PTEN* locus or by amplification and other activating mutations in the *PIK3CA\B* and *AKT1* loci (129,149,296,297). *PTEN*-inactivating mutations can already be traced back in primary lesions, where they mark the onset of aggressive clones (136); however their high prevalence (about 40%) in metastatic samples supports a correlation between PI3K pathway dysregulation and metastatic potential. When co-occurring with MYC overexpression, PTEN loss or AKT activation were sufficient to drive tumorigenesis in luminal prostate cells (298,299), and have been associated to poorer clinical outcome, biochemical relapse after radical treatment and resistance to treatments (300).

Alterations in DNA repair and cell cycle pathways

Mutations in genes involved in DNA repair, either caretakers or gatekeepers, have a destabilizing effect on the genome. *TP53* encodes for a crucial tumor suppressor gene and therefore, as for other cancer types, the p53 is one of the most frequently affected pathways in CRPC, with >50% of patients bearing *TP53* deletions or inactivating mutations (129,301). Early inactivation of *TP53* in prostate cancer evolution marks frequently aggressive tumors, with a higher tendency to develop neuroendocrine-like features (273,302). However, *TP53*-inactivating mutations are much more frequent in CRPC, supporting a driver

function for *TP53* loss during progression (123,138,277). Other genes involved in DNA repair pathway frequently mutated during progression are *BRCA2* (10%), *ATM* (11%), *CDK12* (11%) and different mismatch repair genes like *MLH1* and *MSH2/6* (129,140,239,277). Mutations in *BRCA2* and *ATM* could be of clinical relevance, as tumors with this genomic asset have been shown to respond to poly ADP-ribose polymerase (PARP) inhibitors (303).

The MYC oncogene family comprise three members, *C-MYC*, *MYCN* and *MYCL*. All MYC proteins are multi-functional transcription factors involved in many different cellular functions, including cell cycle progression as well as in metabolism regulation and signal transduction (304). C-MYC is the most pleiotropic member, whereas N-MYC shows higher tissue restriction, being expressed preferentially in cells of the nervous and neuroendocrine system. The role of L-MYC is less well understood and while some reports have identified an association of *MYCL* aberrations with prostate cancer (112), others could not confirm them (123). Both N-MYC and C-MYC have been attributed causative role in many different cancers, including prostate cancer (305,306). In particular, while about 2-20% of primary prostate cancers bear MYC amplification, the fraction of cancer showing this aberration increases with progression, with frequent hotspots of amplification in the 8q24 chromosomal region, which includes MYC. However, many different studies could not link the frequent amplification of this region during progression to a specific role of MYC, as this and neighbouring regions contain MYC-related enhancers and other non-MYC related potential oncogenes, like *NCOA2* (129,149,150,238,273,276,297,307). Other aberrations in cell cycle-related genes include the amplification of cyclin-dependent kinase 4 (*CDK4*) and cyclin D1 (*CCND1*), this latter frequently co-occurring with *CDK12*-inactivating mutations (129,296).

Mutations in the *RB1* locus, encoding for RB1, a key checkpoint controlling S-phase entry, can be found in about 20-30% of CRPC cases. *RB1* mutations strongly correlate with neuroendocrine differentiation of CRPC and will be discussed more in detail in the paragraph about neuroendocrine progression.

Alterations in genes involved in chromatin remodeling

A locus commonly mutated in mCRPC is *FOXA1*, whose aberrations were already introduced in the previous paragraphs. About 34% of mCRPC patients show alterations in this locus, with a pattern of mutations that differs between primary and advanced prostate cancer (129,150,151,297). While the "FAST" and the "LOUD" classes of *FOXA1* mutations are highly prevalent in primary tumors, the "FURIOUS" class has been found to be mostly enriched in mCRPC samples, only rarely appearing in primary tumors and generally as a subclonal event. This class covers all mutations with truncating effect on the C-terminus

regulatory domain of FOXA1. This mutation class increases FOXA1 binding to DNA, with dominant effects over wild-type FOXA1, and disrupts FOXA1 recruitment and activation of the Wnt repressor TLE3. This altered interaction increases cell migration and invasion, ultimately promoting Wnt-driven metastasis formation.

Alterations in the Wnt pathway

APC, a locus mutated in different epithelial cancers including colon, colorectal and lung cancers, is a frequent target of inactivating mutation in prostate cancer as well. APC main function is within the Wnt pathway, where it acts as a repressor by sequestrating cytosolic beta-catenin (*CTNNB1*), thus preventing signal transduction. Mutations in other nodes of the Wnt pathways, including *CTNNB1*, have been detected as well, albeit as a lower frequency compared to *APC*. *APC* and *CTNNB1* mutations tend to occur more frequently in AR-dependent tumors, as it was shown that beta-catenin associated with the AR to drive gene transcription (297,308).

Therapeutic approach in CRPC

Clinical trials have progressively expanded the therapeutic options for patients progressing to CRPC. The patients will be advised on the treatments most suitable for their specific case basing on factors like previous treatments, PSA doubling time (PSADT), genetic or histological profile of sampled lesions (if available), degree of fitness and life expectancy of the patient and, most importantly, presence of symptoms. The discovery of the effectiveness of a more extensive AR blockade led to a new paradigm in prostate cancer: while only a few years ago CRPC and HRPC were frequently used as synonyms, nowadays tumors progressing on ADT but responding to AR-blockade are commonly indicated as CRPC. Only "true" AR-insensitive tumors are now being indicated as HRPC, a stage of the disease with different treatment options compared to CRPC. At the same time, progresses in knowledge of the biology of prostate cancer progression lead to the development of two currently ongoing clinical phase-III trials, CTC-STOP (NCT03327662) and ProBio (NCT03903835), assessing the benefit of a biomarker-driver assignation of therapy. In these mentioned trials, tumor cytogenetic characteristics will be analysed by means of CTC of circulating free DNA in patients' blood.

AR inhibitors and androgen synthesis blockers

The COU-AA-301 and COU-AA-302 clinical trials have shown a survival benefit for patients treated with the CYP17A inhibitor abiraterone that have progressed ADT and, for the latter trial, also chemotherapy with docetaxel.

This result demonstrated the effectiveness of extended AR signaling inhibition and included abiraterone in the standard-of-care- choices available for CRPC patients (309,310).

An effective strategy to control AR-dependent tumors is represented by AR-inhibitors, directly targeting and blocking AR signaling. First-generation AR-inhibitors, mostly represented by flutamide, nilutamide and bicalutamide, have been used for long time for the treatment of CRPC. While their use is now restricted to bicalutamide for its better efficacy and toxic profile, they have been mostly replaced by second-generation AR-inhibitors, mainly represented by enzalutamide, darolutamide and apalutamide (311). These newer drugs have a better toxicity profile and addressed mechanistic issues showed by first-generation drugs, like production of AR-agonist by some of their metabolized byproducts. Second-generation AR-inhibitors not only block AR signaling by directly competing with testosterone for AR binding but also prevent AR translocation into the nucleus, inhibiting AR binding to chromosomal DNA and ultimately blocking AR-controlled genes. Of note, the different drugs in this category may also target some AR mutants (312–314). Treatment with a second-generation anti-androgen has become a very common choice for CRPC, as this class of drugs demonstrated a clear survival benefit in many milestone, phase-III, randomized clinical trials (270–272,315,316). This evidence led to the approval of second-generation anti-androgens as independent first- or second-line therapy for CRPC (244).

The beneficial effects of both abiraterone and of second-generation AR inhibitors lead to the investigation of their effects at earlier stages of the disease. Results from very recent phase-III, randomized clinical trials ARAMIS, SPARTAN and PROSPER have demonstrated a benefit in terms of metastasis-free survival for M0 CRPC patients receiving either apalutamide, enzalutamide or darolutamide in association with ADT, compared to ADT alone (270–272). These results support the use of second-generation antiandrogens already at biochemical progression, an event frequently preceding radiological progression. The LATITUDE, ENZAMET, TITAN and STAMPEDE trials moreover have shown a significant survival advantage for patients with metastatic HSPC receiving ADT in combination with abiraterone or a new-generation AR-inhibitors, further expanding the therapeutic spectrum of these drugs (ARASENS, the trial for darolutamide in currently still ongoing, NCT02799602) (227,264–266). Ongoing trials are currently investigating the impact of these drugs in an earlier or later frameworks of prostate cancer natural history or in combination with other treatments commonly used, like radiotherapy (ATLAS NCT02531516; DASL-HiCaP NCT04136353; PEACE-1 NCT01957436; OSTRICh NCT03295565; PRESIDE NCT02288247).

Immunotherapies

Immune-based therapies for prostate cancer have been controversial in the past 10 years, owed partially to the overall unclear survival benefit granted and partially for the cost-benefit ratio of these treatments. The first immune-based treatment introduced for metastatic CRPC was Sipuleucel-T, a dendritic cells-based vaccine prepared with autologous cells from the patients pulsed with a recombinant protein composed of prostatic acid phosphatase (PAP) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Treatment with Sipuleucel-T, currently available only in the United States, showed a survival benefit for CRPC patients, both previously treated with docetaxel and chemotherapy-naïve, but could not significantly delay the time to disease progression. However, the increased survival of patients treated with this therapy lead to its approval for metastatic CRPC patients in 2010 (317,318). GVAX is a tumor cell-based vaccine, prepared by genetically modifying either patient's autologous cancer cells or allogeneic prostate cancer cells to express immunostimulatory molecules, increasing prostate cancer visibility to the immune system. However, VITAL-1 and VITAL-2, the two phase-III clinical trials of GVAX for advanced prostate cancer, had to be terminated due to lack of efficacy and to increased toxicity of the GVAX and docetaxel combination in one of the arms (319,320). Another phase-III clinical trial tested the efficacy and safety of PROSTVAC, a vaccine based on recombinant poxvirus genetically modified to express PSA and immune costimulatory molecules. Unfortunately, the results of the trial concluded that there was no benefit for PROSTVAC treatment in CRPC patients (321). A currently ongoing clinical trial is assessing the effects of DCVAC on chemotherapy naïve, metastatic CRPC patients (VIABLE trial, NCT02111577). DCVAC is a dendritic cells-based vaccine that uses patient's autologous cells pulsed with PSMA.

In more recent years, the interest in immunotherapy was reignited by the discovery of immune checkpoint (IC) inhibitors and their role in the immunologic escape of different cancers. The two main pillars on which IC therapy is based are 1. that the immune system has negative feedback mechanisms that prevent prolonged activation and 2. that tumors, including prostate cancer, may evolve mechanism to directly induce immune tolerance. Ipilimumab, an antibody directed against the IC CTLA-4 on lymphocytes approved for the treatment of melanoma, was the first IC inhibitor tested in a phase III trial for prostate cancer. Despite a successful phase I/II trial, treatment with ipilimumab as monotherapy did not show a survival benefit for metastatic CRPC patients (322,323). It is however currently being tested in combination

with other therapies, including other IC inhibitors (PROSTRATEGY trial, NCT03879122). Other IC inhibitors directed against PD-1 (pembrolizumab, nivolumab) or PD-L1 (atezolizumab) are currently being explored in different phase-III trials on metastatic CRPC patients (CheckMate 7DX NCT04100018; KEYNOTE-641 NCT03834493; KEYNOTE-921 NCT03834506; KEYLYNK-010 NCT03834519; IMbassador 150 NCT03016312).

Chemotherapy

Until the last 15 years, the standard-of-care first-line chemotherapy for advanced prostate cancer patients was represented by mitoxantrone, a DNA topoisomerase-II inhibitor, and by ketoconazole, an antifungal with sterol synthesis-blocking effects at high doses. Both drugs had relevant side effects, particularly mitoxantrone, complicating the management of patients with advanced prostate cancer. Treatment was substantially improved by the introduction into clinical practice of taxanes, inhibitors of microtubules polymerization whose cytotoxic effect is mainly due to the stabilization of the mitotic spindle in dividing cells. Docetaxel was the first taxane approved as first-line treatment for CRPC, in the metastatic setting (324,325). Different clinical trials, including the multi-arm multi-stage STAMPEDE trial, have investigated the use of docetaxel also in a non-metastatic, high-risk setting, with results that varied among the cohorts examined and length of follow-up. The benefit of adding docetaxel to standard-of-care in non-metastatic, high-risk prostate cancer patients was less pronounced and more controversial, compared to that achieved on metastatic patients (227,229,230,326). Trial outcomes largely depended on the cohort examined, as well as on the length of follow up and patient inclusion criteria.

Cabazitaxel, a newer and less toxic taxane, received approval as a second-line therapy for CRPC patients and, more recently, challenged docetaxel-based therapy as first-line treatment in the metastatic CRPC setting (247,327). Results proved both docetaxel and cabazitaxel were equally effective in controlling metastatic CRPC, however the fewer or less severe side effects of cabazitaxel increased the compliance to therapy – a result very recently confirmed by another study directly comparing docetaxel and cabazitaxel (328). In a recent dose-reduction study moreover, the lower 20 mg/m^2 dose of cabazitaxel was better tolerated and proved non-inferior to the standard 25 mg/m^2 dose (329).

Platinum-based chemotherapy, albeit common for other malignant disease, did not receive positive recommendation for use in prostate cancer (330,331). However, patients failing first- and second-line treatments and progressing to HRPC can opt for a platinum-based therapy.

Other investigational drugs and therapeutic strategies

Radiotherapy is normally regarded as a therapeutic option for primary prostate cancer or in case of relapse after radical prostatectomy (salvage radiotherapy). The VISION and the PCS IX trials are exploring the effect of radiotherapy on metastatic CRPC patients, the former via systemic administration of a PSMA-directed antibody radiolabeled with ^{117}Lu, the latter by administration of stereotactic body radiotherapy in association with ADT and enzalutamide. Additional trials are currently investigating the effect of radiotherapy in combination with standards of care therapies in earlier setups, like metastatic HSPC patients (PEACE-1, NCT01957436; PRESTO, NCT04115007).

Additional pharmacological approaches have been investigated in a number of phase-III clinical trials, assessing their impact on survival or disease progression mostly on metastatic CRPC patients. Drugs directed against the tumor microenvironment, like the endothelin receptor inhibitors atrasentan and zibozentan (NCT00036556 and refs. (332–335)), the VEGF-A inhibitor bevacizumab (336) or the immunomodulatory, anti-angiogenic drugs lenalidomide and tasquinimod have been assessed (337,338). Other drugs with a more direct anti-tumoral action have also been tested, like the receptor tyrosin kinase (RTK) inhibitors suramin, dasatinib and cabozantinib (339–342) or the antisense-oligonucleotide against the anti-apoptotic chaperone protein clusterin custirsen (343). Drug analogues of abiraterone with a CYP17A inhibitory action have also been tested and include galeterone and orteronel (344–347). Unfortunately, all these approaches have failed to increase survival in CRPC patients, and some combinations even resulted in increased toxicity and worse outcome.

As a result of the more advanced genomic knowledge on advanced prostate cancer, a number of phase-III clinical trials are now focusing on drugs against specific pathways dysregulated in CRPC. These include the PI3K/AKT/mTOR pathway, targeted by ipatasertib (NCT03072238) and everolimus (NCT03580239) or the (ADP)-ribose polymerase (PARP) inhibitors olaparib, rucaparib and talazoparib in patients with DNA damage repair defects (NCT03732820; NCT02987543; NCT02975934; NCT03395197).

Progression to hormone-refractory and neuroendocrine prostate cancer (HRPC and NEPC)

The distinction between CRPC and "true" HRPC became more consistent in the last 5-10 years, paralleling the development of more effective first- and

second-line treatments to control the disease after ADT. A more effective targeting of the AR pathway as well as the emergence of additional options to control CRPC progression drastically stretched the timeframe that CRPC patients could experience with a high or relatively high level of quality of life. Despite the therapeutic advances and the increased knowledge of the biology of this type of cancer however, resistance almost inevitably develops, redirecting patients to more aggressive forms of therapies and to palliative care. Challenges imposed by HRPC can be linked to two main areas: development of prostate cancer with neuroendocrine features and, mostly, progression of the bone metastatic disease.

Development of neuroendocrine prostate cancer (NEPC)

Given the rarity of *de novo* NEPC, most NEPC cases are detected in patients previously diagnosed with Adeno-PC frequently when diagnosing progression to CRPC. Recent analyses confirmed that NEPC differentiation of prostate cancer correlates with disease progression and revealed its link to exposure to AR-targeting therapies (348–350) and as such, this type of NEPC is frequently referred to as treatment-induced NEPC or t-NEPC. Up to about 20% of patients with CRPC receive a diagnosis of NEPC at one point of their clinical history, however this amount is likely to increase in the next years, according to the more extensive use of ADT and of anti-androgens (296,351–354). These tumors are characterized in most cases by the loss of prostate luminal markers (including PSA) and of AR expression, as well as by the acquisition of a typical neuroendocrine signature (302). Loss or reduction of AR expression in this context is associated with a more aggressive form of the disease, with very little sensitivity to androgen deprivation, reflecting an increased epithelial plasticity in response to therapy (302,351). While some of these AR-independent PCa do not express neuroendocrine markers (354), a relevant fraction displays NEPC features in association to AR-independence. Evidence suggests that the most likely evolution of CRPC to NEPC occurs by divergent evolution from a more adenocarcinoma-like CRPC lesion, that acquire new genomic and epigenomic drivers associated with increased epithelial plasticity and decreased AR signalling (273,302,349).

Genetic signature of NEPC

NEPC display recurrent genetic features that distinguish this form from Adeno-CRPC; the degree of difference however depends not only on the biology of this cancers but also on the time of appearance of clones with NEPC features in CRPC evolution. Different models have been formulated to explain NEPC

lineage evolution, that include linear evolution from primary to CRPC to NEPC, independent or parallel models in which Adeno-CRPC and NEPC clones evolve independently and divergent NEPC evolution from an Adeno-CRPC precursor. This latter model is the most supported by genomic (273,355,356). The spectrum of genomic alterations is similar between Adeno-CRPC and NEPC, with over 30% of the genome displaying aberrations and a higher mutation rate compared to localized prostate cancer. A most typical difference between NEPC and Adeno-CRPC involves the AR locus and AR signalling, that is much less affected in NEPC compared to Adeno-CRPC. While in some NEPC patient AR-V7 can be detected, in support of a late clonal divergence and resistance adaptation of the tumor to ADT and anti-androgens, in most NEPC cases no focal amplification of the AR locus nor gain-of-function point mutations are detected, and overall expression of the AR is generally much lower compared to Adeno-CRPC.

Genomic alterations directly associated to NEPC are *RB1* loss and *NMYC* amplification. *RB1* loss is highly enriched in NEPC, being present in about 70-90% of NEPC compared to about 15-30% of Adeno-CRPC, and it is frequently associated to *TP53* loss or loss-of-function mutations (*RB1* and *TP53* loss is detected in about 53% of NEPC (273,357)). Loss of *TP53* and, mostly, of *RB1* can shift the tumor from AR-dependent to AR-indifferent, contribute to the loss of epithelial markers like PSA and NKX3.1 and increase the expression of NMYC (358,359).

NMYC is not normally expressed in the prostate epithelium, but its overexpression is detectable in most NEPC cases as well as in about 20% of Adeno-CRPC. NMYC cooperates with enhancer of Zeste homolog 2 (EZH2), an epigenetic silencer and component of the polycomb repressive complex 2 (PRC2) to repress AR signalling and drive the NEPC program (355,359). *EZH2* transcription level is twice as high in NEPC compared to Adeno-CRPC and its expression was correlated with the enhanced expression of the neuroendocrine marker CD56 and of NMYC, in a positive feedback loop (273). In about 70% of NEPC, *NMYC* amplification is associated to amplification of the Aurora kinase A (*AURKA*), that stabilizes NMYC inhibiting its degradation (273,355,357,360,361).

Bone involvement in prostate cancer progression

Development of bone metastasis is the most severe and invalidating feature of advanced prostate cancer, and about 90% of metastatic CRPC patients show bone involvement (362). Once prostate cancer spreads to the bone, it is no longer possible to eradicate it, but multiple options became available in time

to contain the bone-metastatic disease. Prostate cancer bone metastases often appear as mainly osteoblastic lesions, frequently with osteolytic features, and the main symptom is severe pain that requires adequate management. Additionally, bone metastasis can cause skeletal-related events (SREs), like spinal cord compression or bone fractures, pathological side effects originated by the bone metastasis and related to a functional degeneration of the bone tissue (236,363).

Bone structure and physiology

The bone tissue, despite its static function supporting the muscular and articular system, is a highly dynamic tissue. Two main histological components can be identified and undergo specific regulation: the compact bone, involved in the mechanical support of muscles and in morphological identity of the organism, and the trabecular bone, hosting fat reserves and most importantly supporting long-term hematopoiesis. Within bone trabeculae a specialized circulatory system is generated, allowing a highly regulated and intense cellular trafficking from systemic circulation to the marrow space. At the cellular level the bone tissue consists of osteocytes, terminally differentiated cells embedded in a hard matrix of calcium hydroxyapatite, constantly in biochemical communication with the osseous microenvironment. Breakage of this communication is a key signal that triggers bone resorption, a process performed by osteoclasts, highly specialized, giant, multinucleated cells that dissolve the mineral together with the organic matrix deposited by the osteocytes (364,365). In physiological conditions, the exposure of digested matrix together with the release of matrix-embedded growth factors stimulates the proliferation of osteoprogenitor cells at the surface of the bone. At the molecular level, the main biochemical axis responsible for coupling bone resorption and deposition is the osteoprotegerin (OPG) - receptor activator of nuclear receptor kB ligand (RANKL) axis, together with other cytokines and growth factors like ephrins and semaphorins and the gp130 signaling system (366,367). RANKL, normally secreted at endosteal surfaces, interacts with RANK on the surface of osteoclasts, recruiting them at the site of bone resorption. In homeostatic conditions, this interaction is dampened by OPG, a decoy receptor for RANKL that sustains osteoblasts differentiation while preventing osteoclast recruitment and terminal maturation. Additional molecules are involved in the recruitment of osteoprogenitor cells at the remodelling site, like TGF-β1, and the chemokine CXCL12, both strong homing signals for osteoprogenitor cells (368,369). A major regulator of bone

metabolism is the Wnt pathway, whose activation via canonical β-catenin promotes bone formation and inhibits bone resorption (370).

Altered bone function in the metastatic disease

Although the mechanisms have not been fully elucidated yet, the appearance of bone metastasis is an event that can dysregulate both mechanical and hemopoietic bone functions. Osteoblastic bone metastases originate from a non-physiological exacerbation of bone formation that is uncoupled from resorption and from mechanical clues. Bone-engrafted cancer cells can stimulate osteoblasts to induce new bone formation both by direct and indirect mechanism. Direct mechanisms involve the secretion of inflammatory cytokines like IL-6, IL-1β, TNFα, M-CSF or PGE_2 that recruit and activate both osteoprogenitor cells and osteoclast precursors. In a second phase, the bone matrix resorbed by osteoclasts will release additional trophic growth factors and matrix remodelling factors like IGF-I, TGF-β, matrix metalloproteins and PDGF, which can both fuel bone formation and directly contribute to sustain metastatic tumor growth. Cancer cells may also directly alter the RANKL\OPG balance by secreting these factors in the metastatic niche (371). The net result will be a constant remodelling phase, in which functional bone tissue will be progressively replaced by disorganized bone deposition, further recruiting osteoclasts and releasing growth factors, in a pathologic feedback loop that many authors have identified as "vicious cycle" of bone metastasis (235,363,372–374). The process of bone metastasis formation will be presented in more detail in **Chapter 2** of this work.

Treatment of the bone metastatic disease

Despite the detailed mechanisms leading to bone pain are not completely understood, there are currently a few treatments available that can alleviate and control bone pain in bone metastatic patients. External beam radiotherapy (EBRT) is the most common form of palliative radiotherapeutic treatment for symptomatic bone metastasis, either in single or in multiple fractions. Other used approaches include surgical removal (particularly for spinal cord compression) or thermal ablation. The limiting factor of the former strategies is that it is not feasible to treat multiple metastases, leaving these options open mainly for oligometastatic patients or for specific lesions.

Radiotherapy

An effective strategy to target multiple bone lesions involves the administration of radioactive nuclides that are either calcium mimetics or phosphonates with high affinity for calcium, to target active bone remodelling

sites. Among the various radioisotopes, ^{223}Ra is the only one that showed an increased survival benefit, in addition to pain relief and delayed onset of SREs in bone metastatic CRPC patients (362). The same treatment however, when investigated in a similar setup on early-stage metastatic CRPC patients had to be terminated, due to increased toxicity in the treatment arm. This result recapitulates those of an earlier trial on ^{89}Sr, a radionuclide with similar mechanism of action to ^{223}Ra, that showed increased toxicity in the treatment arm (375,376). Overall, these results highlight the complexity of the bone metastatic disease, warning of the need for more reliable models to better stratify patients and disease stages. In this direction, a few ongoing clinical trials are exploring the impact of ^{223}Ra on more specific subsets of bone metastatic CRPC patients (ESCALATE, NCT04237584; NCT03574571; PEACE-3, NCT02194842).

Targeted therapy

In time, additional treatments have emerged, taking advantage of the increase understanding of the biology and dynamics of bone metastasis. Among the most used bon-targeted therapies are bisphosphonates. The mechanism of action of bisphosphonates is still not completely clear, however their bone effect is mainly based on their osteoclast-directed toxicity (see **Box 3**). Given their affinity for the bone tissue, bisphosphonates accumulate in bone, transferring to bone-resorbing osteoclasts where they inhibit key biochemical pathways required for osteoclasts function and survival. The use of bisphosphonates, despite not providing a survival benefit for bone metastatic CRPC patients, can significantly delay the onset of SREs and reduce bone pain, increasing their quality of life (377,378).

The benefits brought by bisphosphonates treatment were further improved by the introduction of denosumab, a recombinant antibody against RANKL. Denosumab effectively delayed the onset of SREs in CRPC patients, both with and without overt bone metastasis, and proved superior to zoledronic acid in a phase-III clinical trial (379,380). Moreover, a low dose denosumab treatment in HSPC patients contributed to an increase bone mineral density and reduced fracture risk in patients receiving ADT, a treatment directly associated with a reduction in bone health (381). As for bisphosphonates, denosumab reduces bone turnover by targeting osteoclasts. However, rather than by cytotoxic effects, it inhibits their formation by inactivation of RANKL, an essential growth factor for osteoclasts differentiation and survival (382).

A relevant side effect of both classes of drugs is reduced bone turnover, which leaves unresorbed progressively more damaged bone, ultimately resulting in bone symptoms like fractures and osteonecrosis (383,384). The symptoms are more severe in areas of more intense bone wear, like the jaw, where bone is subject to intense mechanical stress.

Box 3 – Work exposure and drug discovery
Bisphosphonates have a long history of interaction with the bone. Already in the XIX century, workers in friction matches factories around Europe were developing a work-associated condition that had a characteristic appearance and involved bone-related symptoms like sequestration of alveolar crest bone, gingivitis and osteonecrosis of the jaw (385). Later it became clear that this condition was triggered by the phosphorous fumes that factory workers were constantly exposed to and that upon contact with mucosal surfaces generated bisphosphonates compounds. These chemical species contain two reactive phosphonate groups ($PO(OH)_2$), leading to the identification of the condition as "phossy jaw".

Research tool for the investigation of prostate cancer

The multifaceted nature of prostate cancer has prompted the development of many different research tools. While the most classical tools are best fitted for investigating core aspects of the disease, like biochemical alterations or the genetic lesions most relevant to prostate cancer development and progression, other are more bound to patient-specific characteristics. These latter can be implemented in therapy-oriented assays and can assist in clinical decision-making.

Classical research tools for the investigation of PCa

Immortalized cell lines

The derivation of cell lines from human tumors greatly impacted cancer research as it provided a cost-effective, accessible and virtually endless source of material for research studies, with ample associated genomic data (386,387). Still today, cell lines represent a widely exploited tool for molecular and genetic studies, facilitating the understanding of relevant research areas like drug mechanism of action and resistance. The phenotypic consistency and relative stability *in vitro* of cell lines that promote their use however represent also their major drawback (388). Being immortalized and adapted to

laboratory culture conditions, all cell lines present defects in their DNA repair machinery, accumulating mutations and chromosomal aberrations proportionally to passages. This translates directly into drifts, both epigenetic (389) and in biologically relevant functions like their drug resistance profile (81,390). The different cell lines commonly available for the study of prostate cancer were frequently derived from metastatic lesions, either to the bone (PC-3 (391), VCaP (392)), to the lymph nodes (LNCaP (393)) or to the brain (Du145, (394)) and have been thoroughly reviewed by Sobel and Sadar in 2005 (395). This latter aspect directly highlights a limitation of this model: early prostate cancer lesions are not adequately represented by cell line models. This could be explained most likely by the extent of genetic plasticity required to adapt to prolonged *in vitro* culture.
Despite being a flexible and highly accessible tool, cell lines frequently fail to recapitulate many fundamental stages of tumorigenesis, mostly owing to the lack of interactions with the tumor environment and the extensive adaptations to *in vitro* culturing. The selective pressure of *in vitro* culture, moreover, results in the enrichment of the cancerous cells most fit to culture conditions, losing most if not all of the clonal heterogeneity generated during tumor development.

Animal models

Animal models, particularly mice and rats, have provided not only fundamental insights into the process of prostate cancer formation and progression, but have also allowed the generation of patient-derived xenografts (PDX), greatly supporting research on those aspects most challenging to recapitulate *in vitro*. A variety of transgenic and syngeneic mouse models have been developed and explored, providing the advantage of a fully in-mouse system. In genetically engineered mouse models (GEMM), the expression of oncogenic elements (like transforming viral proteins or proto-oncogenes) is commonly induced in the prostatic epithelium to generate a tumor. Tissue specificity is achieved by associating the expression of the engineered targets with genetic promoters specific for the prostate epithelium like *ARR2PB,* or by inserting the targets under the control of prostate-specific loci like *Nkx3.1, Klk1, Tmprss2* or, more widely, *Hoxb13* (396,397). The expression of known oncogenes like *Myc*, *Ras* or members of the ERG family can be associated with the inactivation of oncosuppressor genes, like *Trp53, Nkx3.1* or *Pten*, resulting in accelerated progression and increased invasiveness or penetrance (396,398).
The development of inducible systems, based on the Cre/*loxP* or on the CRISPR/Cas9 genome editing technologies, allowed the expression of the

engineered targets not only in a specific tissue, but also at a defined time (399). This latter aspect is particularly relevant for the study of organs like breast and prostate that have separated developmental and maturation stages, regulated by sex hormones waves during fetal development and puberty (15). Delaying tumor initiation significantly increased the power and the translational value of GEMMs, allowing the neoplastic lesions to develop in an environment and epigenetic context more closely resembling that of human prostate cancers. A relevant characteristic of GEMM and syngeneic animal models is that tumor-stroma interactions can develop according to the natural pathophysiology of the lesion and that this process happens within the same species. While this could reduce the translational value of these models, it allows to track the onset of a pathological tumor microenvironment, without the adaptation step required in PDX and owed to the human-derived tumor tissue cross-talking with mouse-derived stroma. It is noteworthy to highlight the presence of a functional immune system in syngeneic and GEMM animal models, which renders these models most suited for studying tumor immunology, including anti-cancer vaccines and immune checkpoint inhibitors (396,399). This characteristic needs to be accurately addressed when designing models based, for instance, on the highly immunogenic Cas9, whose expression could trigger the premature clearance of Cas9-expressing cells (400). The development and validation of GEMM however can be extremely time consuming, labor-intensive and expensive, limiting the usability of these models and further reducing their translational implications. Nevertheless, newer research approaches are once more shifting the attention on GEMMs. Patient-specific missense mutations in tumor suppressor genes have a different impact on tumor development compared to *null* alleles found in knock-out GEMMs. The possibility to model key prostate cancer missense (point) mutations, like $SPOP^{F133V}$ (169), can better mimic the biochemical milieu found in prostate cancer patients.

The early mechanisms of metastasis formation, like dissemination and extravasation, and of initial metastatic quiescence of disseminated tumor cells (DTC) are most commonly investigated using metastatic mouse models, inoculated with prostate cancer cells (401). Lateral tail vein injection of prostate cancer cells in immunodeficient mice was among the first widely used techniques to induce metastasis formation. In order to form bone metastases with a significant success rate, the injected cells had to be serially selected for bone tropism or to be genetically engineered: this effect was most likely owed to clearance of the injected cells in the lung capillary bed. The intracardiac injection of prostate cancer cells in the left ventricle of immunocompromised rodents overcame this latter limitation, bypassing the lung passage by

targeting the arterial bloodstream. The cells injected with this technique had a prompter access to visceral districts and to the bone marrow cavity, increasing the rate of bone metastasis formation (388).

In this scenario, another *in vivo* tool is increasing in popularity in cancer research due to its versatility, resources effectiveness and robustness: the zebrafish. Intensively studied in developmental biology research, this model organism is proving very useful to study the mechanisms of cancer biology as well (402–404). The zebrafish is a vertebrate system with an *ex-utero* development, making embryonic manipulation (including genome editing) much easier compared to mammal species. While genetic and biologic divergence from humans has to be accounted for, about 70% of human genes have at least one orthologue in the zebrafish (405). Many aspects of cancer biology can be effectively modeled in the zebrafish, including tropism to the bone marrow, represented in this model by the caudal hematopoietic tissue during embryonal development. During the embryonal stage moreover, the immune system is not fully developed, allowing the engraftment of xenogeneic cells and microtissues. This feature is of particular interest for cancer research, as human cancer cells can be successfully introduced in zebrafish embryos to study cancer features like metastatic dissemination, neoangiogenesis and organ tropism as well as cancer cells extravasation, proliferation and invasion (406,407). Its transparent body allows the use of high-resolution imaging techniques, enabling the direct visualization of cancer cells interactions with their microenvironment (408,409). In addition to the in-depth research that can be conducted with this model, its high fecundity and ease of manipulation facilitate its use for high-throughput screens. In cancer research, both chemical and drug screens are of relevance and the water environment of this model can facilitate the delivery of both, bypassing the administration step required for other laboratory animals. However, care must be taken when translating dose-related responses of zebrafish to humans as pharmacokinetics of various compounds can vary between the two species (410,411).

A third array of *in vivo* tools is represented by bone implants, in which natural or synthetic bone, as well as scaffolds of various materials are seeded with cells and then implanted, generally subcutaneously, in immunocompromised mice (412–414). Prostate cancer cells could be pre-loaded on the implant or could be delivered to the animal at a later stage, assessing cancer cells homing to or colonization of the implant, as well as microenvironment interactions (415–417). Compared to other techniques, like intraosseous inoculation of cancer cells, this approach allows a higher control of the experimental variables by means of implant functionalization and pre-loading with active compounds or

stromal cells, including human cells. While all these models contributed to the understanding of the pathophysiology of bone metastasis formation, they have little direct translational implications, mainly owing to the time required for their establishment, the high technical skills required and their often-low successful uptake rate. Cost, labor intensiveness and ethical implications are further reasons that limit the use of this model for translational assays.

Translational tools for PCa research

Near-patient *in vitro* systems: early cell lines and *ex vivo* tissue cultures

Near-patient tools conjugate the possibility of laboratory manipulations with a high degree of fidelity to the original tissue, resulting from a relatively preserved biology of the patient-derived sample. Organoids and patient-derived xenografts represent attractive and intensely investigated tool and will be discussed in further details in the following paragraphs.

Patient-derived cell lines (PDCL) differ from established cell lines for the higher degree of genetic similarity they retain, compared to the original tissue. A relevant aspect is that the near-patient origin of PDCL could help to obtain models of early prostate cancer. As reported in the previous paragraph, most cell lines available are representative of advanced prostate cancer lesions. *In vitro* models of cells with fewer genetic hits and with a molecular asset still relatable to the original tissue could improve our understanding of the biochemical dynamics of earlier prostate cancer lesions. Although there are no clear or widely agreed definitions of PDCL, it was shown that a cell line can take on average about 5-10 passages before genetically stabilizing to the *in vitro* culture conditions (81). In addition, PDCL could be derived in culture media with specific formulation (i.e. serum-free, addition of culture supplements of growth factors) to support the growth of the desired cellular population (418). The increased awareness on the biology of these models raised the attention on tracking culture conditions and passage number, as well as highlighting the need to perform genomic and transcriptomic controls to track genetic drift (419).

The lack of biochemical gradients and of tissue architecture of cell lines can be overcome by *ex vivo* cultures of tissue slices. This model is a relevant approach for applications demanding the analysis of tissue architecture, like drug screenings or immune assays, and can support the use of more resource-intensive approaches, including co-clinical trials (420,421). A noteworthy aspect is that the reaction of cells to a treatment greatly depends on their microenvironment, which includes stromal or immune cells as well as its spatial localization and adhesion substrate. It however does not typically support

prolonged culture and requires specific optimizations and culture strategies to yield reliable results, with different adaptations required by different tissues. Despite the differences in protocols, it generally consists of the *in vitro* culture of thin tissue slices, in a controlled atmosphere and on a semi-permeable membrane (422).

Patient-derived xenografts (PDX)

Patient-derived xenografts are powerful tools to study complex aspects of cancer biology like metastasis formation, microenvironment interactions or drug response. In established PDX, human cancer tissue is engrafted into an immunocompromised rodent, usually mice. The cancer tissue can be implanted orthotopically or, most commonly, subcutaneously in form of small pieces of tissue. The use of gel plugs or other constructs loaded with a cell suspension of the original tumor, or the direct injection of cancer cells in circulation is an alternative approach, adopted for specific types of tumors (i.e. leukemias and blood-born cancers) or for specific research needs.

The use of immunodeficient hosts greatly facilitates engraftment success, as it very unlikely to establish xenografts in immune competent ones. The clear drawbacks of this approach are the lack of a functional immune system, hence dampening studies on cancer immune editing, and the cleanliness standards needed for the husbandry and maintenance of these immunodeficient strains. Newer strategies to preserve the immune system functionality without jeopardizing xenograft uptake are gaining momentum and imply either a transient suppression of the host's native immune system (by pharmacological treatment or radiation, for instance), or the development of "humanized mice", that is mice engrafted with a human immune system (423,424). However, these strategies are significantly more resource-intensive and are generally used to address specific research needs. There is currently a wide range of choice of immunocompromised mouse strains that can allow the engraftment of xenotransplanted human cancers, a brief overview is shown in Tables 2 and 3 (425).

The advantage of PDXs is the stabilization and preservation of many physiopathological characteristics of the original tumor, including its histopathology and genomic aberrations (436–438). However, histological modifications as well as additional genomic alterations tend to accumulate during PDX passaging, requiring regular screening of the PDX to confirm the preservation of its key characteristics (419).

Table 2 *– Most common mouse strains with genetic immunodeficiency*

Common name	Genetic strain	Adaptive immune cells functionality		Innate immune system functionality			Refs
		T cells	B cells	NK cells	Comp	APC Neutr Granul	
Athymic nude	Foxn1^{nu} (1)	Abs	Funct	Funct	Funct	Funct	(426,427)
SCID	Prkdc^{scid} (2)	Abs	Abs	Funct	Funct	Funct	(428,429)
Beige	Lyst^{bg-J} (3)	Def	Def	Def	Funct	Def	(430)
Rag-deficient	Rag1^{tm1Mom} or Rag2$^{tm1.1Cgn}$ (4)	Abs	Abs	Funct	Funct	Funct	(431–433)
Non-obese diabetic (NOD)	See (5)	Funct	Funct	Def	Abs	Def	(434,435)

Comp, complement; *APC,* antigen presenting cell*; Neutr*, neutrophils; *Granul*, granulocytes. *Abs*, absent; *Def*, defective; *Funct*, functional.

[1]*Foxn1* is required for both hair follicle and thymic development

[2]*Prkdc* is required for DNA repair after V(D)J recombination of T cell receptor (TCR) and immunoglobulin (Ig) genes of maturing T and B cells

[3]*Lyst* is a lysosomal trafficking regulator required for the cytotoxic functions of different components of the immune system

[4]*Rag1* and *Rag2* genes are both required for the somatic recombination of the TCR and Ig genes in T and B cells.

[5]polygenic. Multiple mutations resulting in generalized immune system defects

Compared to other research resources, PDX can reflect more closely and more accurately the biology of many different cancers, including prostate cancer (439,440). Moreover, as shown in the work included in this thesis and in other scientific works, the transcriptomic profile and cellular heterogeneity of the original tumor positively correlate with those found in their PDXs (142,441).
This latter is one of the most relevant features of PDX, as other *in vivo* models like GEMMs are often unable to reach the molecular and clonal diversity typically seen in human cancers. Given their substantial contribution to research, many efforts have been dedicated to the generation of PCa PDX. In recent years, multiple PDX cohorts have been generated and characterized, from their *in vivo* growth kinetics to molecular and histological analyses, to pharmacological responses (437,442,443). Of note, the derivation of new PDX lines is generally more successful using more advanced lesions, due to their increased plasticity (444,445).
This reduced the availability of PDX suitable for modeling the earlier and most critical events for the pathophysiology of the disease, like the initial androgenic switch of CRPC or early metastatic dissemination. Therefore, although the current PDXs are best suited at modeling the most lethal disease, including therapy-driven NEPC, models of lower-stage, more commonly diagnosed prostate cancers are largely needed.
The translational value of PDX is further supported by the observation that PDXs drug response profiles can recapitulate the clinical responses of their matched human cancers, (446–448). This not only motivates their use in pharmacological research but lead to the development of the so-called "co-clinical" trials, a precision medicine approach in which PDXs from patients included in clinical trials receive the same (and possibly additional) therapies to follow clinical responses. This approach is particularly relevant to study new drug combinations (423,449,450). To this end, the "Co-clinical Trial Project", started in 2011 and currently ongoing, associates PDX of patients enrolled in different clinical trials with GEMMs of clinically relevant genetic lesions, aiming at tailoring effective anti-cancer therapies for advanced prostate and lung cancers (451,452).
Despite their relevant advantages, research involving PDX also has major limitations. Tumor engraftment in a murine host can be low, despite their immune permissiveness and uptake rates varying greatly among tumor types, with prostate cancer being among those not readily engrafting (440). Moreover, PDX rarely generate spontaneous bone metastasis, and those that do require either extensive manipulation or a direct intraosseous inoculation, blunting the key initial steps of bone metastasis formation (453).

An additional common limitation of PDX is their lack of a functional immune system, jeopardizing their implementation in the development of immunological treatments of prostate cancer, including immune checkpoint inhibitors or anti-cancer vaccines. This last disadvantage however could be neutralized with the development of PDX in humanized mouse models, as reported earlier in this paragraph (423).

Table 3 *– Multigenic immunodeficient mouse strains commonly used in PDX research*

Common name	Genetic strain	Adaptive immune cells functionality		Innate immune system functionality		
		T cells	B cells	NK cells	Comp	APC Neutr Granul
SCID-Beige	$Prkdc^{scid}$ $Lyst^{bg-J}$	Abs	Abs	Def	Funct	Def
NOD SCID	$Prkdc^{scid}$ in NOD background	Abs	Abs	Def	Abs	Def
NOD-$Rag1^{null}$ $IL2rg^{null}$ (NRG)	$Rag1^{tm1Mom}$ $IL2rg^{tm1Wjl}$ in NOD background	Abs	Abs	Abs	Abs	Def
NOD-scid $IL2Rg^{null}$ (NSG)	$Prkdc^{scid}$ $IL2rg^{tm1Wjl}$ in NOD background	Abs	Abs	Abs	Abs	Def

Comp, complement; *APC,* antigen presenting cells; *Neutr*, neutrophils; *Granul*, granulocytes. *Abs*, absent; *Def*, defective; *Funct*, functional.

Organoids

The use of organoids as a culture technique is proving a valuable tool to preserve and enhance the translational value of patient-derived samples. Organoids are self-organizing 3D structures, generated either by the

asymmetric division of tissue-resident stem cells or by aggregation of a heterogeneous population of cells from different lineages and at different stages of differentiation (454). Their fundamental characteristics lie in the generation of functional 3D structure, resembling the architecture of the tissue they derive from, as well as in the inclusion of more than one cell type within their structure (455,456). While the use of 3D cultures systems was well known to researchers and promoted the study of numerous key aspects of cell biology, the main breakthroughs introduced by the organoid technique were the use of a fully defined medium and the possibility to culture normal epithelial cells in 3D, stably and for long-term. Culturing in a defined medium opened the possibility for the standardization of culture technique, paving the way for the clinical use of organoids by means of Good Manufacturing Practices (GMP) and Good Laboratory Practices (GLP), strict requirements for a full translational use of research products. It also allowed to control and finely tune the biochemical microenvironment that cells are exposed to, greatly enhancing our knowledge of the biology behind tissue and organ regulation. This in turn lead to the development of *ad hoc* media and culture conditions, promoting the generation and maintenance of tissue-like 3D structures of cells from different epithelia, including intestinal, gastric, oesophageal, pancreatic, pulmonary, kidney, prostatic, mammary and others, including brain-like structures (455,457). Furthermore and in contrast to other research models like cell lines and animal models, the organoids represent a flexible and scalable system that can be adapted to studying elusive aspects like cellular biophysics, the interaction of tissues with the resident microbiome and its cross-talk with the immune system, this latter a player notoriously difficult to include in preclinical models (458–460).

Despite lineage-tracing experiments individuated distinct mouse prostate stem cells, allowing to draw functional parallels with the human prostate tissue (461,462), the factors regulating human prostate epithelium have not yet been fully elucidated. Different labs established protocol variations to generate prostate organoids, mostly relying on components like foetal bovine serum or Matrigel®, whose composition cannot be exactly determined and protocols relying on a fully-defined medium have been challenging to develop and replicate (463–465). Another limitation of patient-derived organoid assays regards the number of analyses that can be performed on each sample. This is especially true both for low-stage prostate cancers, as clinical advances allowed the sampling of smaller lesions and with less invasive techniques, and for metastatic lesions, where extensive sampling could be difficult to perform or might raise safety concerns for the patients.

Adequate research tools need to be developed to support a precision medicine approach, preserving at best the value of clinical samples while allowing for their controlled manipulation. The following chapters of this thesis work will present the results of research studies conducted using this precision medicine approach.

Outline and general aims of the study

The studies presented in the following chapters of this thesis work are focused on the development of near-patient, clinically usable tools for the implementation of a precision medicine approach to advanced prostate cancer patients. For this group of patients, multiple treatment options are available including pharmacological approaches that are amenable to *in vitro* testing. The ultimate goal of this work is to support the feasibility and clinical relevance of an organoid-based *in vitro* drug screening, using anti-cancer drugs already in clinical use, on patient-derived material. In order to do so, the different chapters will focus on specific sub-aims, covering different aspects of this research project. In particular, **Chapter 1** focused on the general aspects of prostate physiology and pathology, specifically illustrating the natural history, the challenges and the current treatment options for prostate cancer. An entire section was dedicated to the current tools available for the study of prostate cancer, highlighting advantages and disadvantages of each, including their clinical usability.

Chapter 2 provides an overview of the current knowledge on metastatic prostate cancer with a specific focus on bone metastatic prostate cancer, the leading cause of prostate cancer-related deaths. In **Chapter 3**, molecular analyses of bone metastatic prostate cancer are further developed using PDX models *in vitro*, *ex vivo* and *in vivo* tools. A case-study drug screening assay, using patient-derived organoids from bone metastatic prostate cancer, is provided as an initial proof of feasibility of the assay. In **Chapter 4** the investigation is further developed and scaled up. A novel PDX model is presented, including its extensive characterization, and implemented alongside with two others additional PDX models in a mid-throughput drug screening, aimed at repurposing drugs already in use for other malignancies. In this chapter, additional assays using patient-derived organoids are included and further characterized, validating not only the feasibility of this assay but also the relevance of this precision medicine approach. **Chapter 5** streamlines a strategy for data mining and literature searching, of utmost relevance for obtaining updated knowledge on investigational drugs, (pre)clinical trials and emerging markers. The proposed strategy was applied to Cripto, a protein with oncogenic function in multiple cancers including prostate cancer and with a currently investigated role as a biomarker for cancer. Lastly, **Chapter 6** discusses the results of all the studies presented, applying a patient-oriented perspective and consolidating the findings in the wider landscape of current prostate cancer research.

Figure references

Figure 1

Left panel, from https://www.cdc.gov/cancer/prostate/basic_info/what-is-prostate-cancer.htm

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Figure 2

A-C. licensed from Lopez-Beltran, A., Cheng, L., Montironi, R., & Raspollini, M. (2017). Basic Anatomy and Histology of the Prostate. In Pathology of the Prostate: An Algorithmic Approach. Cambridge: Cambridge University Press. doi:10.1017/9781108695947.002 (license # 45731) **D**. licensed from Roxanne Toivanen, Michael M. Shen. "Prostate organogenesis: tissue induction, hormonal regulation and cell type specification". Development 2017 144: 1382-1398 (license # 1096650-1)

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