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HUMORAL RESPONSE AGAINST HERV-K AND HERV-H EPITOPES, AND GENETIC VARIABILITY WITHIN THE MAJOR HISTOCOMPATIBILITY COMPLEX AND A MULTIGENE PANEL THROUGH NGS IN PROSTATE CANCER PATIENTS

A Thesis Submitted for The Degree of Doctor of Philosophy

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PhD Thesis

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Foreword

This thesis is based on a manuscript published during my PhD and on unpublished data.

The work and results presented in this thesis has been carried out during my enrolment as PhD student at the Department of Biomedical Sciences, University of Sassari, Italy, in the period from 1st November 2018 to 31st January 2022.

The samples used in the studies derived from subjects enrolled by the Urology Department of the University Hospital of Sassari and from the Transfusion Center of Sassari.

Preface

The thesis submission is in line with the requirements for the PhD degree in Life Sciences and Biotechnologies and the work was carried out at Department of Biomedical Sciences, Viale San Pietro 43b, University of Sassari, Italy.

Abstract

Prostate cancer (PCa) is the main cause of death in the male population. Incidence rates seem to vary by ethnicity, geography, and race, yet additionally by aging, obesity, diet and family history. Both genitourinary infections and chronic inflammation can contribute to the development of prostate cancer and tumor progression. PCa is a heterogeneous disease and one of the challenges is that current diagnostic tests such as protease-specific antigen (PSA) screening and histopathological examinations can not discriminate between indolent and invasive tumors. Nearly 8% of the human genome consists of sequences derived from endogenous retroviruses (ERVs). Human endogenous retroviruses (HERVs) are not infective and the pathogenicity has been weakened by the accumulation of genetic and epigenetic modifications responsible for the diminished HERV expression. Evidence indicates a possible involvement of HERV envelope proteins in several types of cancer, particularly the association between PCa and HERV-K. Furthermore, the application of next-generation sequencing (NGS) techniques to cancer research paved the way to new routes of investigation concerning the genetic background of individuals affected by PCa as well the potential impact of gene variants on cancer progression, metastases, and the response to cancer therapy. MHC class I and II play a critical role in mediating the immune responses against cancer cells. In PCa, loss of HLA class I expression has been observed in the primary tumor and lymph node metastases. The first part of this project focuses on the evaluation of the antibody response against highly immunogenic peptides obtained by the envelope protein of HERV-H, HERV-K, and HERV-W in plasma of patients with PCa, BPH, a borderline group of individuals with atypical small acinar proliferation (ASAP) and prostatic intraepithelial neoplasia (PIN), and a population of healthy controls (HCs). The second part focuses on the application of a NGS-based approach to characterize the variability within the Major Histocompatibility Complex (MHC) class I and II, one of the most variable region of human genome, and to analyze a panel of genes in a cohort of PCa patients and HCs.

The multigene panel analyzed was composed of 35 genes involved in innate and adaptative immune response, cancer, autophagy, and previously associated-PCa genes. Data presented in this thesis provides further evidence for the association between PCa and HERV-K, and new proof for HERV-H possible involvement in PCa pathogenesis emphasizing its potential to be used as a disease biomarker. Additionally, genotyping analysis of the MHC class I and II provides new data about the association of HLA-A and HLA-B and PCa risk. The analysis of the multigene panel detected novel genetic modifications in a cohort of individuals with PCa and one of healthy individuals. These findings shed light on novel SNPs and their potential impact on PCa risk, prognosis, survival and the patient response to cancer therapy.

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List of Abbreviations

AR	Androgen Receptor
ASAP	Atypical small acinar proliferation
BPH	Benign prostate hyperplasia
CK	Cytokeratins
CRPC	Castration Resistant Prostate Cancer
CT	Computer tomography
CZ	Central Zone
DDR	DNA damage repair
DHT	5α-dihydrotestosterone
ELISA	Enzyme-linked immunosorbent assay
ERG1	ETS-related gene
ETV1	ETS translocation variant
FDA	Food and Drug Administration
GS	Gleason grading system
GWAS	Genome Wide Association Studies
HERV	Human endogenous retrovirus
HERV-H	Human endogenous retrovirus-H
HERV-K	Human endogenous retrovirus-K
HERV-W	Human endogenous retrovirus-W
HGPIN	High grade prostatic intraepithelial neoplasia
HLA	Human leukocyte antigen
HPCa	Hereditary Prostate Cancer
hSGT	Human small glutamine rich tetratricopeptide
HWE	Hardy-Weinberg equilibrium
IEBD	Immune epitope database

IN	Integrase
LH	Luteinizing hormone
LNX	Numb protein X
LTR	Long terminal repeat
LUTS	Lower urinary tract symptoms
MHC	Major histocompatibility complex
MMR	DNA mismatch repair genes
MRI	Multiparametric resonance imaging
NGS	Next generation sequencing
OD	Optical density
PARP	Poly-adenosine diphosphate-ribose polymerase
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline and Tween-20
PCa	Prostate Cancer
PCR	Polymerase chain reaction
PDE-5	Phosphodiesterase 5
PIA	Proliferative Inflammatory Atrophy
PIN	Prostatic intraepithelial neoplasia
PLZF	Promyelocytic Leukemia Zinc-Finger Protein
PPT	Polypurine tract
PR	Protease
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PZ	Peripheral Zone
RT	Reverse transcriptase

SU	Surface
TM	Transmembrane
TMPRSS2	Transmembrane serine protease 2
TURP	Transurethral resection of the prostate
TZ	Transition Zone
TZFP	Testicular zinc-finger protein

Chapter 1 - Overview

Chapter 1 - Overview

1.1 Prostate Cancer Epidemiology and Pathology

1.1.1 Prostate Cancer Epidemiology

Prostate cancer (PCa) is the second most diffused cancer in men, after lung cancer. PCa is mainly a disease of the elderly, and the diagnosis tends to increase after the age of 50 years. The incidence and mortality rates vary considerably by population, economic status, and lifestyle [1]. In 2020, PCa has been the most diagnosed form of cancer in men in Italy with more than 39,000 cases [2] and more than 200,000 new cases have been diagnosed in the USA [3]. Globally, the odds to develop PCa are higher in high developed countries compared to low developed ones (Figure 1) [4]. The higher incidence rates may well reflect economical settings, lifestyles, and especially an easier access to medical care, including screening campaigns and early diagnosis [5]. The introduction of prostate-specific antigen (PSA) screening can be considered responsible for the increases in prostate cancer diagnosis and incidence rates, even though the mortality rates underwent a decline in Western and some European countries, included Italy [3]. In contrast, African American men have higher mortality rate than that of US men, included Hispanics, Whites and Asiatic men. Albeit the reason behind this decline are not clear, early detection and improved treatment solutions may be considered responsible. Several epidemiological studies show as prostate cancer risk, aggressiveness and prognosis are influenced by ethnicity, race, and geography [1,3,6].

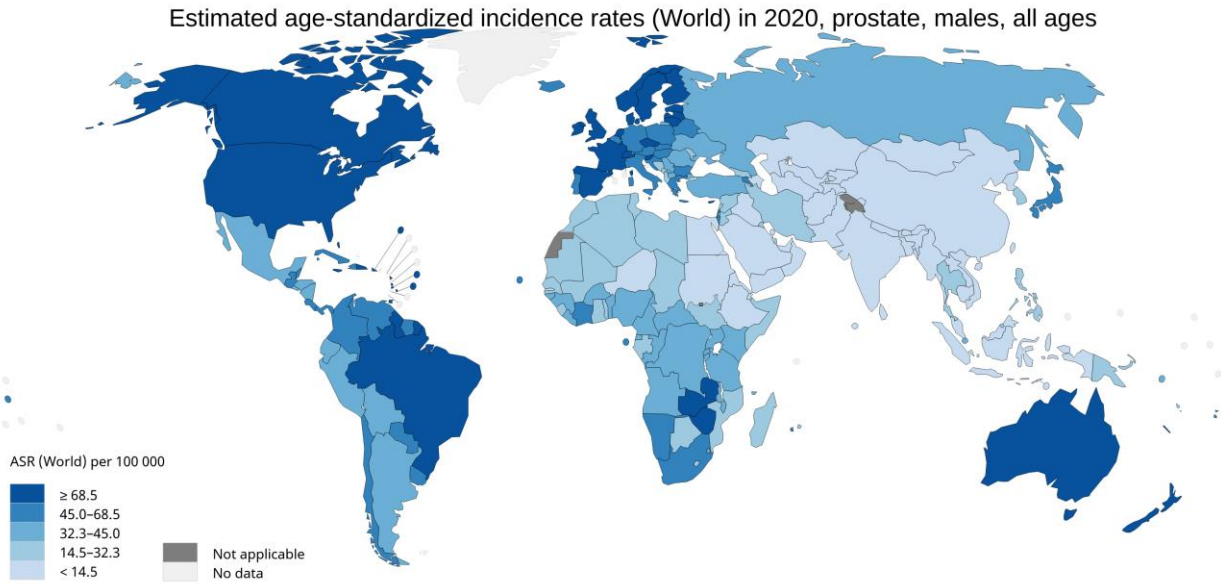

Figure 1. Age-standardized incidence rates of Prostate Cancer in 2020 [3].

1.1.2 Prostate Cancer Risk Factors

Given the high incidence of prostate cancer among men population, different risk factors, associated to lifestyle, have been identified in large, prospective cohort studies. Some of the risk factors identified seem to be associated to a higher risk of advanced prostate cancer, especially smoking and obesity [7]. Diet and the intake of specific food seem to have a relevant impact on prostate cancer risk [8]. Some studies have instead found an association between dairy intake and overall prostate cancer risk and an inverse association between lycopene intake, contained in cooked tomato, and risk of advanced disease [9,10]. Diet and smoke are the only modifiable risk factors identified and their role in prostate cancer pathogenesis is yet poorly understood. However, PCa shows the highest hereditability compared to other major cancers.

1.1.2.1 Genetic Factors

Cancer possesses a genetic nature since different mutations are necessary to induce the cancerogenic process. Mainly, there are two classes of significant mutations: activating mutations in oncogenes and inactivating mutations in oncosuppressor genes. Loss of gene function comes from genomic rearrangements involving loss of genomic DNA resulting in deletions or fusion with other genes. To date, several mutations have been associated to an increased risk of PCa. BRCA1 and BRCA2 are two suppressor genes implicated in the mechanisms of repair of damaged DNA and transcriptional regulation in response to DNA damage [11,12]. Both BRCA1 and BRCA2 have been associated with an increased risk of ovarian and breast cancer [13]; additionally, BRCA2 and BRCA1 are mutated in 5.3% and 0.3% of patients with metastatic PCa, respectively [14]. Recently, the US Food and Drug Administration (FDA) authorized the use of two poly-adenosine diphosphate-ribose polymerase (PARP) inhibitors for the treatment of biomarker-positive metastatic castrate resistant prostate cancer [15]. Other mutations have been reported in ATM, CHEK2, HOXB13, NBS1 genes [14,16,17]. More than 200 loci associated with early onset PCa, aggressive PCa, or gene × gene interactions have been identified through family-based linkage and genome wide association (GWAS) studies [18]. Eighty six novel risk variants that affects PCa susceptibility have been identified through a multiancestry GWAS meta-analysis by Conti et al. along with new candidate genes potentially implicated in the development of PCa [19].

Androgen receptor (AR) plays a pivotal role in the physiology of prostate gland, the first evidence of mutations in its ligand-binding domain was observed in an androgen responsive cell line LNCaP. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens [20].

AR mutations have been found in 50% of patients with castrate-resistant prostate cancer (CRPC), demonstrating that AR gene mutations underlie the mechanisms of AR resistance in AR targeted therapy [21]. With the advance of tissue technology, AR was found amplified in 23% of 47 patients with CRPC, whether in studies with hormone-naïve PCa cases no AR mutations were found. AR amplification and the splicing variant v7 are the main mechanism to drive AR resistance. In 1990, a loss of genetic portion in the region 10q was discovered in nearly 30% of localized PCa [22]. Later, the region of 10q was reported to encode a tumor suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome ten) involved in the PI3K-AKT pathway [23]. The amplification of the transcription factor c-Myc was reported along with the activation of the PI3K pathway. Somatic alterations, such as gene fusions, play a role in PCa etiology and involve the family of ETS transcription factors [24]. The most common fusion gene found in PCa patients is the TMPRSS2:ERG fusion whose prevalence can vary from 35% to 70% depending on the population in study, while ERG rearrangements with SLC45A3 and NDRG1 result less frequent [24–26]. Other members of the ETS family, such as ETV1, ETV4 and ETV5, were as well found rearranged in PCa cases. Given the heterogenous nature of PCa, patients can present an aggressive disease, characterized by a rapid progression and metastasis, or a slow disease with low tendency to progress. Generally, individuals with a hereditary form show an early onset, an aggressive disease, and an advanced stage.

1.1.2.2 Hereditary Prostate Cancer

PCa has one of the highest hereditability rates among cancers. Individuals with a family history of PCa have a two- to four-fold greater probability to develop cancer. Hereditary PCa (HPCa) accounts for 5-15% of PCa cases and is characterized by an early onset and an aggressive progression [27]. Over 170 susceptibility loci have been associated to HPCa and mutations in the DNA damage repair genes (DDR) (BRCA1, BRCA2) and in the DNA mismatch repair genes (MMR) (MLH1, MSH2, MSH6) are used as HPCa biomarkers [28].

1.1.2.3 Aging, Race and Ethnicity

Advanced age can most certainly be considered a risk factor associated to prostate cancer development since most men who receive a diagnosis of prostate cancer are over 70 years old [3]. Epidemiological studies show as the incidence as well the mortality of prostate cancer seem to be higher in specific ethnic group, such as African and Afro-American men. It would probably be helpful to detect specific African-alleles and clarify PCa etiology in these populations. Intriguingly, some of the loci associated to PCa in Asian or European men are not present in African men [29].

1.1.3 Prostate Anatomy and Histology

The prostate is a tubule-alveolar exocrine gland that forms part of the male reproductive system and contributes secretions to the ejaculate [30]. It sits adjacent to the bladder and in front of the rectum, and it is surrounded by a thin capsule consisting of collagen, elastin, and smooth muscle. Conventionally portrayed as "walnut-shaped", it surrounds the proximal urethra as it exits from the bladder [31]. The prostate parenchyma has been divided into three main zones: a central zone (CZ), encompassing the ejaculatory ducts and accounting for 25% of the prostate; a transition zone (TZ) which represents 10% of prostate tissue and close to the urethra; and a peripheral zone (PZ), surrounding the posterolateral region of the prostate (Figure 2) [32–34]**.** These zones have different embryologic origins and differ for their histology, anatomic characteristics, biologic functions, and their proneness to pathologic disorders. The majority of all PCa, nearly 70%, arise from the PZ [35,36], along with Proliferative Inflammatory Atrophy (PIA) and Prostatic Intraepithelial Neoplasia (PIN) [37–39]. Conversely, CZ has a very low incidence of PCa, whist TZ, despite its similar embryologic origin to PZ, has a lower cancer incidence rate. The stroma of TZ is fibromuscular and it also is the zone where Benign Prostate Hyperplasia (BPH) mainly occurs [37]. The prostate epithelium consists of different cell types that vary in their morphology and function. The luminal cells are columnar epithelial cells involved in the synthesis of cytokeratins (CK) as well as secretory proteins, such as PSA [40]. Below there is a layer of non-secretory basal cells that line the basement membrane and express several CK (CK5, CK14 and p63), they also express very low levels of AR compared to luminal cells [41,42]. Lastly, neuroendocrine cells, which are basally localized and present a dendritic-like processes, secrete neuropeptides, serotonin and other peptide hormones [43].

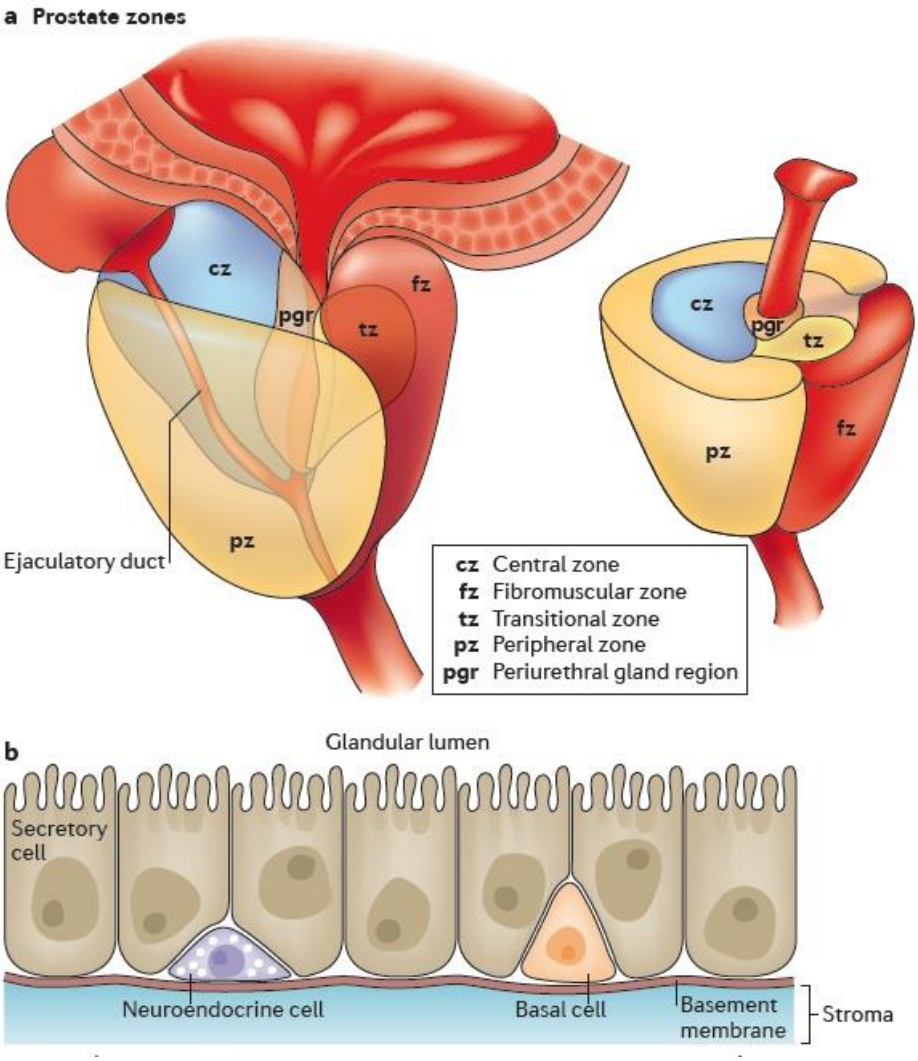

Figure 2. Macroscopic and microscopic anatomy of the prostate gland. a|Human prostate is characterized by three main zones: the peripheral zone, where ~70% of prostate cancers arise [35,36]; the central zone; and (3) the transition zone [32,34]. b| Microscopic structure of the prostate. The glandular epithelium of the prostate is encased in a fibromuscular stroma. The secretory luminal layer of tall columnar cells consists of cells that produce prostatic acid phosphatase, PSA and human kallikrein 2, all of which are secreted as part of the seminal fluid [44].

The normal growth and development of the prostate is regulated by androgenic hormones, particularly testosterone, produced by Leydig cells in the testes under stimulation with luteinizing hormone (LH) and then converted by 5α-reductase (nicotinamide-adenine dinucleotide phosphate-dependent δ4-3-ketosteroid 5α-oxidoreductase) to 5α-dihydrotestosterone (DHT) [45,46]. The latter binds to intracellular AR which results in a conformational change and AR dimerization, followed by its transport in the nucleus where it activates the expression of target genes. Androgens and ARs play a pivotal role in PCa initiation and progression [46]. Moreover, androgen deprivation by orchiectomy determined the regression of cancer, even though patients tend to later develop CRPC, a lethal form of cancer unresponsive to traditional androgen deprivation therapy and therefore patients are treated with chemotherapy which confers a survival advantage.

1.1.4 Prostate Pathology

1.1.4.1 Benign Prostatic Hyperplasia

Benign Prostatic Hyperplasia (BPH) is a non-malignant enlargement of the prostate gland defined as a benign proliferation in the number of stromal and glandular epithelial cells within the TZ (**Fig. 3**). BPH patients usually present lower urinary tract symptoms (LUTS) which comprise urinary frequency, urgency, nocturia, incomplete bladder emptying, and hesitancy [31,47]. Such symptoms can negatively affect the quality of life of the patient, and in most severe cases, patients sustain urinary retention [48]. The prevalence of BPH increases with age [48], though the pathophysiology is incompletely understood. Multiple pre-clinical and clinical studies have pinpointed numerous risk factors for BPH [49].

Age-related metabolic aberrations, such as type 2 diabetes, obesity, and dyslipidemia, have been related to the development and progression of BPH/LUTS [50] alongside inflammation during which the secretion of cytokines, chemokines and growth factors will determine the growth of epithelial and stromal prostatic cells [51]. Treatment of BPH includes lifestyle changes where applicable, and pharmacological management and surgery. Numerous drugs are currently used in the treatment of BPH, such as α-blockers, 5α-reductase inhibitors and phosphodiesterase 5 (PDE-5) inhibitors [52].

Despite the new technologies, Transurethral Resection of the prostate (TURP) remains the cornerstone of surgical treatment for BPH [52].

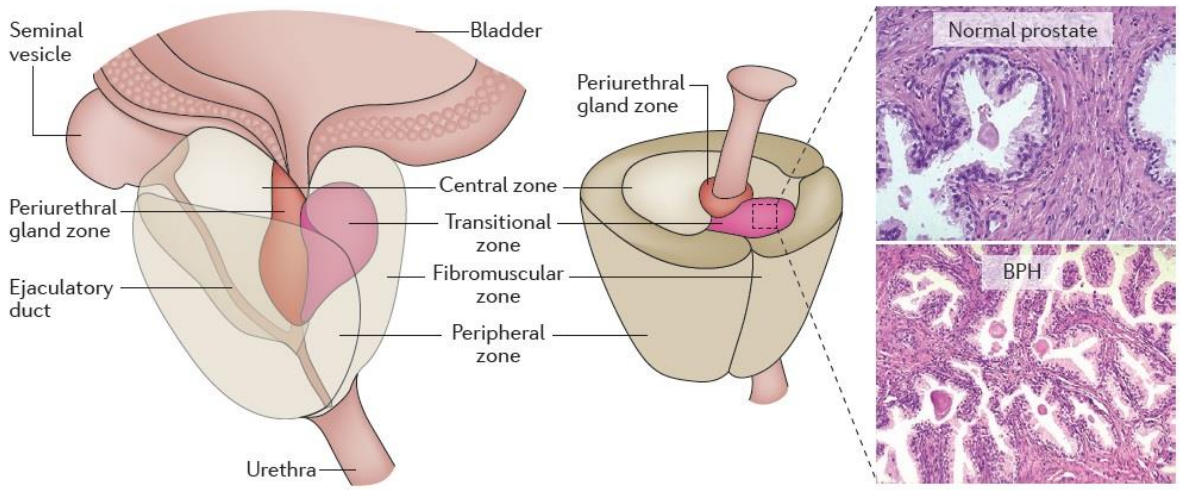

Figure 3. Graphical representation of human prostate gland and histological slides of both a normal prostate and BPH tissue. Pleomorphism with regard to stromal/epithelia ratio is typically observed in BPH; the BPH slide below shows proliferation of the glandular epithelial cells [49].

1.1.4.2 Atypical Small Acinar Proliferation and High-Grade Prostatic Intraepithelial Neoplasia

High-Grade prostatic intraepithelial neoplasia (HGPIN) and atypical small acinar proliferation (ASAP) are considered a possible precursor and a primary premalignant lesion of PCa along with [38]. Microscopically, HGPIN is described as an atypical, proliferative change in the physiological architecture of prostatic acini and ducts characterized by the presence of prominent nucleoli (Figure 4a). [53]. Whereas ASAP is identified by small foci of atypical glands with some feature of adenocarcinoma (Figure 4b). The percentage of patients to develop PCa after an initial diagnosis of HGPIN or ASAP is around 22-23% and 21%-51%, respectively [54,55].

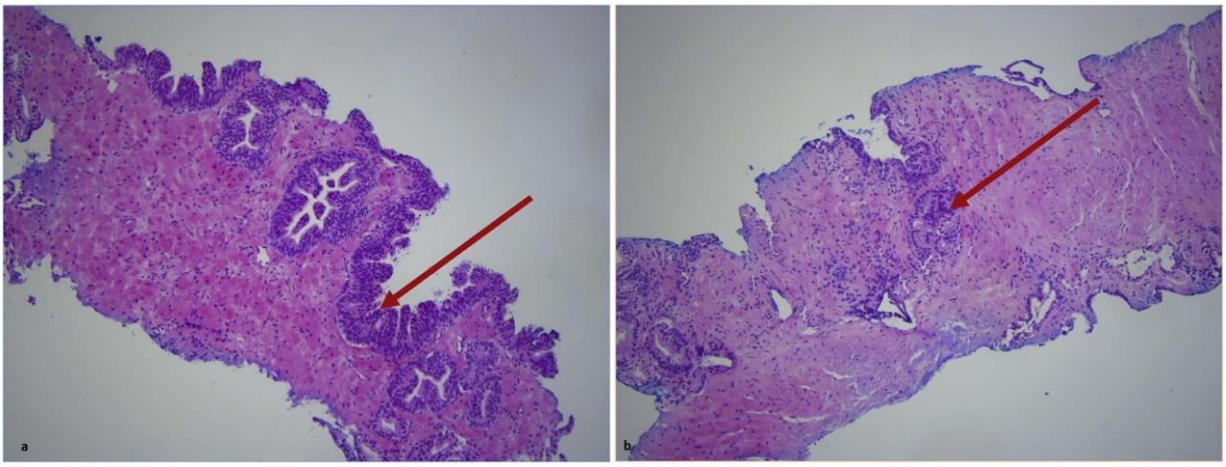

Figure 4. Hematoxylin and eosin staining of high-grade prostatic intraepithelial neoplasia (a) and atypical small acinar proliferation (b) [56].

1.1.4.3 Prostate Cancer

PCa exhibits a noteworthy heterogeneity in neoplastic regions, also multifocal. Histopathological examination of the cancer tissue reveals benign glandular tissue, PIN tissue and adenocarcinomas areas characterized by distinct levels of differentiation and severity. In 1992, Gleason puts in place a system to help evaluate the degree of severity [57]. A score is attributed summing up the two most prevalent neoplastic lesions in the biopsy sample (e.g., 3+3; 3+4), a higher score denotes a more advanced adenocarcinoma (Fig. 5). Acinar adenocarcinoma is the most diffused among all primary prostate carcinomas, and as previously described, it arises mostly in the peripheral zone [58]. Ductal adenocarcinoma is rarer and occurs along the cells lining the ducts of the prostate gland and, unlike acinar adenocarcinoma, it tends to grow faster and spread to testis, penis, and lung [59]. A tall columnar, pseudostratified epithelium with papillary, cribriform, glandular, or solid structure characterize ductal adenocarcinoma, whilst, acinar adenocarcinoma portrays cuboidal cells arranged in acini [59]. Non-acinar prostate adenocarcinomas account for nearly 5-10% of PCa and include ductal and intraductal adenocarcinoma, squamous neoplasms, urothelial adenocarcinoma, basal cell carcinoma, and neuroendocrine tumors. Intraductal adenocarcinoma

has been introduced in the 2016 World Health Organization classification, it describes intraductal spread or in situ progress of acinar or ductal adenocarcinoma of the prostate gland as well an intraductal proliferation of urothelial carcinoma [60]. Squamous and basal cell carcinoma are extremely rare, the first is characterized by the presence of intercellular bridges or keratinization, the latter shows cystic/cribriform patterns and metastasis has been reported in nearly 15% of cases [61]. Neuroendocrine prostate cancer is a lethal form of cancer, most patients die within 1 to 2 years of diagnosis [62].

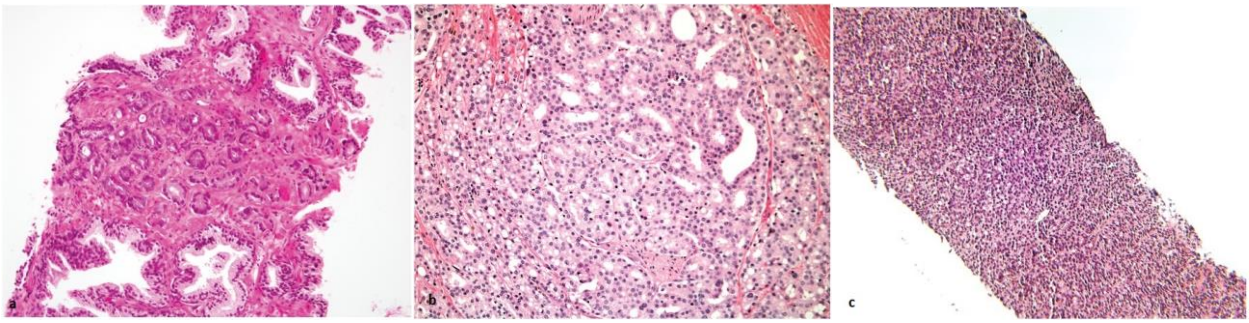

Figure 5. Hematoxylin and eosin staining of the adenocarcinoma of the prostate showing different PCa stages with different Gleason grades. A| Needle core biopsy with Gleason grade 3+3 (score of 6) with single, separate glands. B| Needle core biopsy with Gleason grade 4+4 (score of 8) with cribriform and fused glands. C| Needle core biopsy with Gleason grade 5+5 (score of 10) with a solid pattern, the tissue may lack gland formation or present necrosis [61].

1.2 Management of Prostate Cancer

1.2.1 Prostate Cancer Diagnosis

Needle biopsy remains the gold standard procedure when a case of PCa is suspected. A prostate biopsy is performed using transrectal ultrasound to collect 10 to 12 tissue samples. The sample inspection is executed by a pathologist who issues the predominant histological pattern and, accordingly, assigns a primary Gleason score for the predominant histological pattern and a secondary grade for the highest pattern, both on a scale of 1 to 5 based on the sample microscopic architecture [63,64]. To help assess the severity of the type of cancer and the prognosis, physicians stratify patients in low, intermediate and high risk by exploiting Gleason score patterns, PSA serum levels, and the clinical stage[63]. Guidelines have been understandably outlined in order to standardize the procedures related to the number, location and collection of the prostate cores, assignation of Gleason score and grade group [64,65]. The accuracy of the diagnosis is highly dependent on the precision with which the biopsy has been performed, especially because this technique misses 21% to 28% of PCa cases [66]. In the last years, a great number of new FDA-approved biomarkers have been introduced in the clinical practice, such as prostate cancer antigen 3 test, Prostate Health Index, to help lower the rate of false negative individuals [67,68]. Multiparametric MRI, bone scan and computer tomography (CT) are employed to research for metastasis and assess their clinical stage [5].

1.2.2 Management and Therapies

Prostate adenocarcinoma is unfortunately the most diffused cancer in the male Western population. Androgen receptor (AR) plays a crucial role in PCa since its development and progression depends on androgenic stimulation [45,46]. Thereby, androgens deprivation represents one of the therapies undertaken to hinder the progression of the disease. Radical prostatectomy and/or radiotherapy constitutes the definitive treatment for localized PCa.

1.3 Human Endogenous Retroviruses

Human genome is composed for about 50% of repeat sequences, described as "junk", though the advance in genome sequencing revealed how wrong this predicament was [69]. Especially since nearly 8% of these repeats are remnants of exogenous retrovirus, called human endogenous retroviruses (HERVs). Millions of years ago, the integration of such exogenous retroviruses in germ line cells has guaranteed the vertical transmission of ERV sequences to the host's offspring [70]. HERVs have been found in all vertebrates [71,72], they share the same mechanism used by exogenous retroviruses to integrate in the genome. Once in the cytoplasm, the retroviral genome is reverse transcribed into a double-strand DNA by the viral reverse transcriptase [73]. Through the interaction of the viral integrase and cellular cofactors, the proviral genome is then integrate in the host genome. Once integrated, the retroviral sequences can express mRNA encoding several proteins. HERVs share the same proviral structure of exogenous retroviruses which consists of two long terminal repeats (LTRs) flanking the viral genes *gag*, *pro-pol* and *env* (Figure 6).

The LTRs play a regulatory function in HERV expression, acting as promoters, enhancer, and polyadenylation signals, while *gag*, *pro-pol* and *env* encode for the structural components. Summarily, *gag* encode the structural components of matrix, capsid and nucleocapsid; *pro-pol* expresses the proteases (PR), reverse transcriptase (RT) and integrase (IN); whilst *env* encode an envelope protein with two distinct subunits, surface (SU) and transmembrane (TM).

The HERV provirus also contains a primer binding site (PBS) flanked by 5'-LTR and *gag*, and a polypurine tract (PPT) located between *env* and 3'-LTR; the PBS serves as a binding site for the cellular tRNA priming the (-) strand DNA synthesis, whereas PPT acts as primer for the (+) strand DNA synthesis. HERVs are classified in three main classes: class I (*Gammaretrovirus*- and *Epsilonretrovirus*-like), class II (*Betaretrovirus*-like) and class III (*Spumaretrovirus*-like). Many HERVs have been named according to the cellular tRNA bound by their PBS, such as HERV-K for lysine or HERV-H for histidine; others have been nominated after the name of a neighbor gene, such as HERV-FRD.

Figure 6. Complete sequence of a genomic HERV RNA sequence comprising *gag*, *pro*, *pol*, *env* flanked by 5' and 3' LTR. (MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; RH, RNase H; IN, integrase; SU, surface; TM, transmembrane).

1.3.1 Molecular Mechanisms by Which HERVs May Induce Cancer

HERVs proteins perform a variety of functions and appear to affect the course of several disease. Several mechanisms have been proposed about the role of HERVs in cancer: insertional mutagenesis, chromosomal re-arrangements, HERV-K Rec and Np9 acting as oncogenes, immunomodulation, cell-fusion and aberrant signaling (Figure 7).

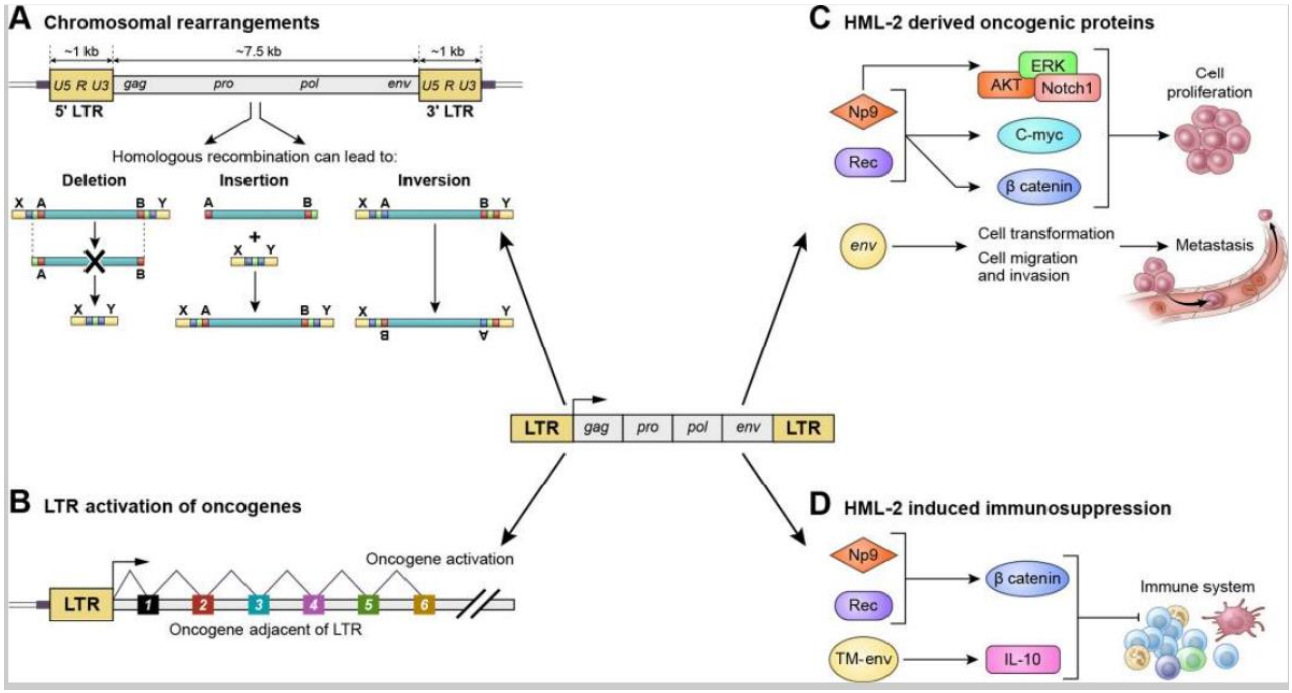

Figure 7. Potential mechanism for HERV-K(HML2) induced carcinogenesis. A| Chromosomal rearrangements: HML2-LTRs activity may contribute to carcinogenesis causing deletions, insertions, and inversions. B| LTR-induced activation of oncogenes: HML2-LTR may act as alternative promoter of adjacent oncogenes and recruit transcription factors, thus affecting cell proliferation gene expression. C| HERK-K(HML2) derived oncoproteins: Np9 and Rec activate ERK, AKT, Notch, c-myc and beta-catenin. HERV-K env appears to induce cell transformation, migration and invasion. D| Env-derived Immunosuppression: Np9 and Rec can enhance beta-catenin expression which leads to immune tolerance to tumors, whilst Env presents an immunosuppressive domain in the TM domain able to upregulate IL-10 [74].

Non-allelic recombination of HERV sequences can result in deletions, duplications, and chromosomal re-arrangements which may alter or inhibit the expression of other host genes. Some patients with PCa have been described with HERV-K_22q11.23 5'-LTR-UTR translocations upstream of the transcription factor ETS translocation variant 1 (ETV1), which leads to an enhanced expression of the ETV1 oncogene promoting cancerogenesis [75]. Alternatively, LTRs can function as alternate promoters and dysregulate nearby proto-oncogenes or growth-promoting genes. Particularly, In human prostate cancer, oncogenic gene fusions involving the ETS (E26 transformation-specific) gene family occur as a result of recurrent chromosomal translocation, one of the 5' fusion partners identified for such translocations was a HERV-K provirus [75]. Vogt et al. demonstrated how the loss of the mismatch repair endonuclease *PMS2* gene was correlated to a chromosomal rearrangement involving the HERV provirus; the inactivation of PMS2 therefore prompted mismatch repair cancer syndrome and colorectal cancer [76]. HERV Env protein expression may prove to be deleterious because of its ability to induce cell-cell fusion and assist in cancer development [77,78] along with the ability to evade immune surveillance of the tumor microenvironment thanks to the immunosuppressive properties of the TM domain [79–81]. The immunosuppressive properties of the TM domains have been described for syncytin-2 [82] as well as the Env proteins of HERV-K [83], HERV-H [79] and HERV-E [84]. The expression of HERV-K(HML-2) proteins Rec and Np9 has been linked to tumorigenesis despite *Rec* and *Np9* transcripts have been found in healthy human tissues [85]. Rec and Np9 are both known to interact with the cellular promyelocytic leukemia zinc-finger protein (PLZF) [86], a transcriptional repressor of the c-Myc proto-oncogene. Rec also binds to the testicular zinc-finger protein (TZFP) and the human small glutamine rich tetratricopeptide repeat (hSGT), which are both involved in repressing

androgen receptors [87]. Whether, Np9 interacts with Numb protein X (LNX), hence the Numb/Notch signaling pathway [88] whose dysregulation has been reported in several cancer [89–91]. In leukemia stem/progenitor cells, Np9 regulates cell growth by activating and upregulating ERK, AKT, Notch and beta-catenin pathways [92]. Cell fusion occurs when the cellular membrane of one or more cell merge together, generating multinucleated cell. Cell fusion occurs not only in cancer [93], but it is an important process during placenta development. Furthermore, in mammals, syncytiotrophoblast formation is mediated by two fusogens deriving by HERV-W and HERV-FDR env genes (syncytin 1 and syncytin 2, respectively) [94,95]. Both syncytins have been also found expressed in cancer tissue [78,96–98] in which cancer cells seem to be able to merge with other non-cancer cells, such as bone marrow derived cells [99], leading to metastasis and heightened malignancy [100].

1.3.2 Human Endogenous Retroviruses and Prostate Cancer

HERV families possess distinct expression patterns and can be found in cancerous and normal tissues [101]. Both HERV-H and HERV-K have been found expressed in testicular cancer as well normal tissues. HERV-H is prominent in cancerous tissues, such as colon, stomach and prostate [101]. HERV-K, along with HERV-H and HERV-W, retains at least one full-length open reading frame. The expression of HERV-K mRNA and proteins is noticeable in prostate tissue of patients with a diagnosis of cancer. HERV-K (HML2) Gag protein was indeed isolated in serum of PCa patients by serological recombinant cDNA technology [102]. In PCa cell lines, HERV-K was detected in both full-length and spliced forms [103]. While, increased HERV-K Gag mRNA expression was found in PBMCs of older men and smokers with PCa compared to healthy controls [104].

Another study confirmed HERV-K involvement in PCa founding an increased expression of HERV-K Gag in prostate tissue and detected a correlation between high autoantibody levels against HERV-K Gag and PCa progression [105]. Recently, Rezaei et al. reported an increase in HERV-K Gag mRNA and protein levels in malignant regions of PCa patients as opposed to non-malignant tissue; HERV-K Gag protein was additionally found in 66.7% of malignant sections and only in 5.6% of benign tissue [106].

Another aspect to keep in consideration is gene fusion of androgen-regulated promoter with member of the ETS family of transcription factors. Particularly, the fusion of the transcription factors ETS translocation variant (ETV1) or ETS-related gene (ERG1) to the transmembrane protease serine 2 (TMPRSS2) is the most common gene fusion identified, the TMPRSS2-ERG fusion is found in between 40 and 50% of PCa patients [24,107]. Other two studies revealed HERV-K17 [108] and HERV-K_22q11.23 [75] as novel ETV1 fusion partners, prostate-specific and androgen-regulated like TMPRSS2.

Several studies pinpoint a general increase in HERV-W expression in tumor samples compare to healthy tissue [109]. Cancer cells sustain abnormal hypomethylation of CpG nucleotides which may be partly responsible for HERV reactivation in tumor tissues, whereas normal cells silence HERV expression by epigenetic mechanism. Unfortunately, few studies addressed the methylation status of HERV-W sequence in tumor tissue. In ovarian carcinomas though, an upregulation of HERV-W sequences due to a reduction in promoter methylation has been reported [110]. However, some of HERV-W sequences appear to maintain a hypermethylated state in malignant tissue. Similarly, HERV-W upregulation was also reported in neuroblastoma cancer cells and colon carcinoma cells [111,112], but in these conditions HERV-W increased expression may be a consequences of the cancer cell line environment instead of a specific cancer trait. Upregulation of HERV-H env-related transcripts have been correlated with colon cancer *in vitro* and in colon tumor tissue compared to adjacent normal tissue [113].

Furthermore, HERV-H transcript increased in metastatic tumor cells undergoing epithelial-to-mesenchymal transition (EMT) and in advanced colon cancer sample, besides HERV-H mediated the recruitment of immunoregulatory cells playing a critical role in tumor metastasis and immune escape [81].

1.4 Next-Generation Sequencing and Advances in Prostate Cancer

The advance of the Next-Generation Sequencing (NGS) has undoubtedly revolutionized cancer research in the last decade. NGS allowed to simultaneously sequence millions of DNA fragments differing from traditional sequencing methods with which only few selected fragments were sequenced because of high costs and intensive work. In cancer research, NGS provided the means to deepen and widen the current knowledge of tumor biology by providing new insights into tumor heterogeneity, genetic variability of the disease, genes involved in the mechanisms of resistance to drugs and the roles of the immune system. In other words, NGS helped to move cancer research toward "personalized medicine" which refers to the development of targeted therapies able to affect the expression of critical cancer genes, identified by cancer genome profiling, and directly attack cancer cells [114]. Finally, the advances of NGS technologies helped to classify this diversified disease into subgroups taking advantage of the different genomic, transcriptomic, and epigenetic features.

1.4.1 The Human Major Histocompatibility Complex

The major histocompatibility complex (MHC) is the most polymorphic region in vertebrates [115]. MHC is a 4 Mb region, located on chromosome 6, and contains the human leukocyte antigen (HLA) genes. The HLA system encode cell-surface proteins involved in the regulation of adaptative immune responses. MHC initiates the adaptative immune response by presenting peptides to T lymphocytes in several context, such as cancer, autoimmunity, and infections. The two major classes of MHC molecules have different functions. Class I molecules (encoded by HLA-A, HLA-B and HLA-C) present intracellular molecules to $CD8^+$ T cells, whereas class II molecules (encoded by HLA-DR, HLA-DQ and HLA-DP) exhibits exogenous antigens to $CD4^+$ T cells [116]. HLA class I molecules are prevalently expressed on nucleated cell in the body, while HLA class II molecules are found in B cells, dendritic cells, macrophages [117,118]. The analysis of HLA variants is routinely used for tissue matching in allogenic organ transplants [119].

However, recent evidence revealed how alterations or loss of the expression patterns of HLA genes constitute a critical immune escape mechanism that allows tumor cells to escape immunosurveillance [120,121]. Currently, the knowledge about HLA class I and II alterations and underlying molecular mechanisms in PCa is limited and controversial. Nonetheless, both MHC class I and class II appear to be expressed at different stages of mouse and human prostate tumor cells [122].

1.4.2 Gene-panel analysis

Genome profiling of cancer using NGS technologies is earning popularity in the clinical setting. By sequencing the tumor genome, researchers and clinicians have access to new information about genomic aberrations upon which to develop new therapeutic strategies. Multigene panel testing aims to reduce the likelihood of a false negative assessment of genetic risk and to allow the identification of risk alleles for a particular disease in a cost-effective way [123]. Multigene panel testing allows to obtain genetic risk information in a more time-efficient way. NGS is a cost-efficient technique which is usually employed to sequence the entire coding region of the genes of interest and part of the intronic sequences, or to analyze specific genetic variants [124].

1.5 Aims

The aims of this thesis can be summarized in the following point:

I. To assess the presence and specificity of the humoral response against highly immunogenic peptides derived from the envelope protein of HERV-K, HERV-H and HERV-W in PCa patients compared to HC, BPH, and borderline cohorts.

II. To identify new MHC class I and class II loci associated with PCa risk by Next-generation Sequencing.

III. To investigate novel relevant genetic variants in a population of PCa patients utilizing a NGS custom-made multigene panel approach.

Chapter 2

Chapter 2

2. HERV-K and HERV-H Env proteins Induce a Humoral Response in Prostate Cancer Patients

2.1 Materials and Methods

2.1.1 Study Population

This study was approved by the Ethical Committee of AOU Sassari, and all patients signed an informed consent form. A cohort consisting of 105 patients with PCa, 74 patients with BPH, a borderline group of 31 patients diagnosed with ASAP and PIN, and 105 HCs matched by age. PCa, BPH and borderline cohorts were recruited at the Urology Department of University Hospital, Sassari, while HCs were recruited at the Transfusion Center, AOU, Sassari. Peripheral venous blood was collected in K+ -EDTA test tubes for both patients and HCs at the time of the subject recruitment. Whole blood was separated by standard Ficoll–Histopaque (Sigma-Aldrich, St. Louis, MO, USA) gradient centrifugation and newly separated plasma was stored at -20 °C. Thereafter, plasma samples were employed in indirect ELISA experiments to test the presence of autoantibodies against HERV env-derived peptides. Below, table 1 shows the clinical information related to patients and HCs.

Table 1. Demographic and clinical information of patient groups and healthy controls.

	PCa (n=105)	**HCs** (n=104)	**BPH** (n = 74)	**Borderline** (n = 31)
Age (mean±SD)	71.3 ± 8.2	60.1 ± 5.8	66.3 ± 7.6	68.3 ± 7.4
Serum PSA (ng/ml, mean±SD)	33.6±107.9		7.1 ± 6.9	6.0 ± 4.2
≤ 4 ng/ml	16		24	8
> 4 ng/ml	86		48	21
Unknown	1		1	1
Gleason Score (GS)				
GS=6	45			
GS=7	30			
GS≥8	26			
Unknown	4			

Abbreviations: SD, standard deviation; PSA, prostate-specific antigen; PCa, prostate cancer; HCs, healthy controls, BPH, benign prostatic hyperplasia.

2.1.2 Highly Immunogenic Peptides

The Immune Epitope Database (IEBD) is the online tool used to design and synthetize the peptides (HERV-K env-su$_{19–37}$, HERV-K env-su$_{109–126}$, HERV-W env-su$_{93–108}$, HERV-W env-su$_{248–262}$, HERV-H env-su$_{229–241}$, HERV-H env$_{387–399}$) at >95% purity (LifeTein, South Plainfield, NJ, USA). The peptides have been resuspended in DMSO and stored in single-use aliquots at -80°C (Table 2).

Table 2. Aminoacidic sequences of peptides used as antigens in the indirect ELISA assay.

	Epitope Position	**Epitope Sequence**	**UniProtKb Accession Number**
HERV-K env-su$_{19–37}$	19–37	VWVPGPTDDRCPAKPEEEG	O42043
HERV-K env-su$_{109–126}$	109–126	RPKGKTCPKEIPKGSKNT	
HERV-W env-su$_{93–108}$	93–108	NPSCPGGLGVTVCWTY	Q9UQF0
HERV-W env-su$_{248–262}$	248–262	NSQCIRWVTPPTQIV	Q9UQF0
HERV-H env-su$_{229–241}$	229–241	LGRHLPCISLHPW	Q9N2J8
HERV-H env$_{387–399}$	387–399	RVIPLIPLMVGLG	Q9N2J8

2.1.3 Indirect Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was performed to detect specific antibodies against the following peptides: HERV-K env-su$_{19-37}$, HERV-K env-su$_{109–126}$, HERV-W env-su$_{93–108}$, HERV-W env-su$_{248–262}$, HERV-H env-su$_{229–241}$ and HERV-H env$_{387–399}$. Ninety-six-wells Nunc immune-plates were incubated overnight at 4°C with a solution composed of carbonate-bicarbonate 0.05 M, pH 9.5 (Sigma-Aldrich, St. Louis, MO, USA) and the respective peptides at 10 μg/mL.

A one-hour step was carried out in a blocking solution with 5% non-fat dried milk at room temperature (Sigma-Aldrich, St. Louis, MO, USA) and phosphate-buffered saline (PBS).

The plates were washed twice with a solution of PBS and 0.05% Tween-20 (PBS-T) and plasma samples were added at 1:100 concentration and incubated for two hours at room temperature. Next, each plate was washed five times in PBS-T and incubated one hour at room temperature with an alkaline phosphate-conjugated anti-human IgG polyclonal antibody (1:1000, Sigma-Aldrich, St. Louis, MI, USA) and PBS. After another washing step in PBS-T, plates were incubated in a dark environment at room temperature with milli-Q water and p-nytrophenyl phosphate (Sigma-Aldrich, St. Louis, MI, USA). For each plate, absorbance was read at 405 nm using SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). All samples were analyzed in duplicate and positive controls were used for each peptide. A highly positive control serum was used to normalize the absorbance values.

2.1.4 Statistical Analysis

D'Agostino–Pearson omnibus normality test and the Shapiro–Wilk normality test were used to analyze the normality and distribution of the data. Mann–Whitney U test and Kruskal–Wallis test with Dunn's multiple comparisons test were used to check the presence of differences between two or more groups, respectively. A cut-off value was chosen using the receiver-operating characteristic (ROC) and the sample positivity was verified using Fisher's exact test. Specificity and sensitivity were properly selected for all the samples analyzed. The correlation between the antibody responses against HERV-env peptides and PSA levels was calculated by Spearman's correlation test. The level of statistical significance was set up as $p < 0.05$. GraphPad Prism 8.2.0 software (GraphPad Software, San Diego, CA, USA) was used to carry out statistical analysis.

2.2 Results

2.2.1 Analysis of the Humoral Response Against HERV Env-derived Specific Peptides

The results presented in this chapter have been published as "**Manca M.A.**, Solinas T., Simula E.R., Noli M., Ruberto S., Madonia M., Sechi L.A. HERV-K and HERV-H Env Proteins Induce a Humoral Response in Prostate Cancer Patients. Pathogens. 2022 Jan 14;11(1):95. doi: 10.3390/pathogens11010095. PMID: 35056043; PMCID: PMC8778306" [125].

The peptides used in the indirect ELISA experiments derive from the most immunogenic portions of the envelope protein of HERV-H, HERV-K and HERV-W and were employed in the detection of specific antibodies within the PCa, HCs, BPH and the borderline populations. The figure 8D shows that 15 out of 105 (14%) individuals with PCa ($p = 0.004$), 6 out of 104 (6%) of the HCs, 6 out of 74 (8%) individuals with PBH, and 2 out of 31 (6%) individuals of the borderline cohort showed a positive response against HERV-K env-su$_{109–126}$. Whereas antibodies against HERV-H env-su$_{229–241}$ (Figure 8C) were found in 28 out of 105 (26%) individuals with PCa ($p < 0.0001$), 15 out of 104 (14%) of HCs, 16 out of 74 (15%) individuals with PBH ($p = 0.008$), 8 out of 31 (25%) individuals of the borderline cohort. About the other peptides used in the study, no seroreactivity was detected against HERV-K env-su$_{19–37}$ (Figure 8A), HERV-W env-su$_{93–108}$ (Figure 8B), HERV-W env-su$_{248–262}$ (Figure 8E) and HERV-H env$_{387–399}$ (Figure 8F) between PCa and HCs, BPH or borderline groups.

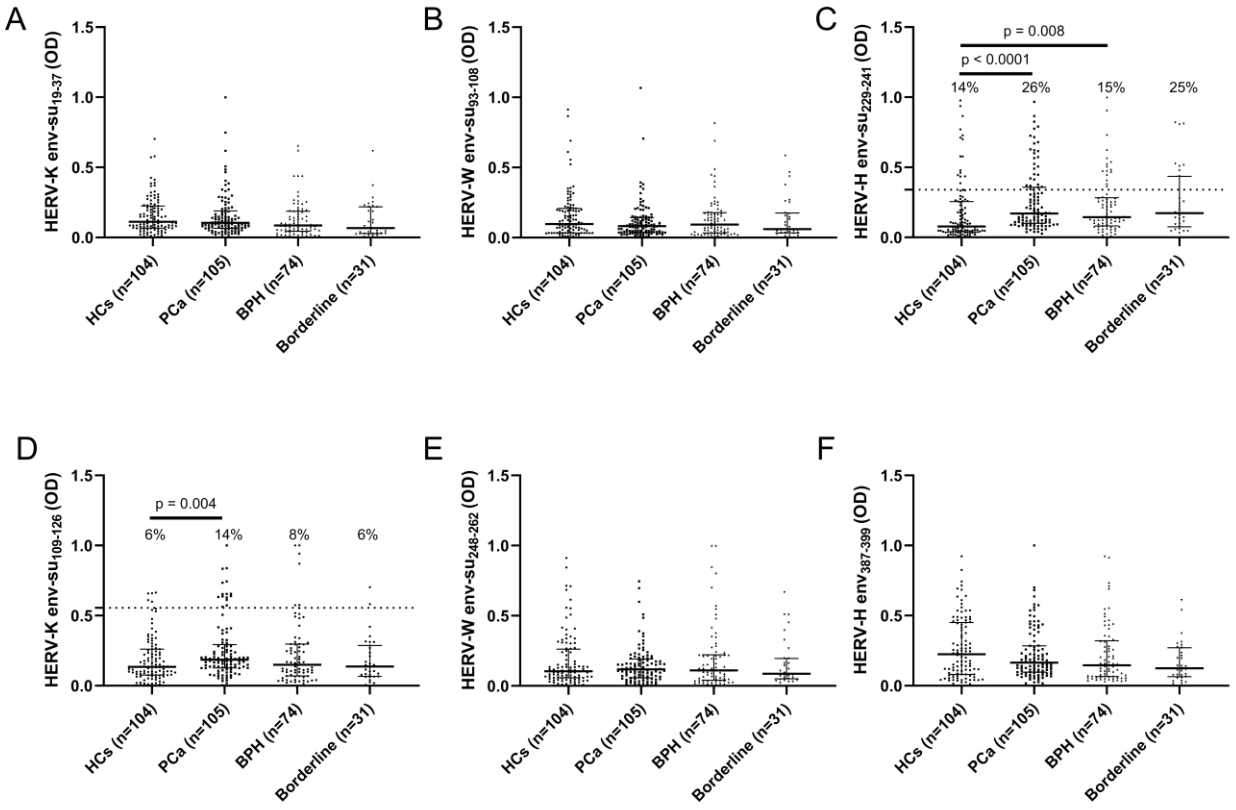

Figure 8. ELISA-based analysis of the antibody reactivity against HERV-env derived peptides. Plasma samples from HCs, PCa, BPH, and borderline cohorts were tested against HERV-K env-su$_{19–37}$ (A), HERV-K env-su$_{109–126}$ (D), HERV-W env-su$_{93–108}$ (B), HERV-W env-su$_{248–262}$(E), HERV-H env-su$_{229–241}$ (C), HERV-H env$_{387–399}$ (F). Black bars correspond median plus interquartile range and the dotted lines stand for the cut-off values attained after ROC analysis to assess sample seropositivity.

2.2.2 Stratification of PCa patients by Gleason Score to assess seroreactivity against HERV env-derived peptides

The stratification of PCa patients was performed in accordance with the Gleason grading system (GS) in order to evaluate if the antibody response directed to HERV env-derived peptides was affected by the patient grading system and the level of progression of PCa. A statistically significant response against HERV-K env-su$_{109–126}$ was found between GS ≥ 8 group and HCs ($p = 0.03$) (Figure 9D). Similarly, the analysis of HERV-H env-su$_{229–241}$ antibody levels revealed a significant difference between HCs and patients with GS = 6 ($p = 0.001$), GS = 7 ($p = 0.03$) and GS ≥ 8 ($p = 0.01$) (Figure 9C). Furthermore, antibody levels against HERV-H env-su$_{229–241}$ were similar across the various GS classes suggesting that the Gleason grading and the level of differentiation of prostate tissue did not affect the humoral response directed against the HERV-H env-su$_{229–241}$ epitope of the envelope protein. This further analysis confirmed the lack of noticeable humoral response against the other HERV env-derived epitopes used in this study: HERV-K env-su$_{19–37}$ (Figure 9A), HERV-W env-su$_{93–108}$ (Figure 9B), HERV-W env-su$_{248–262}$ (Figure 9E) and HERV-H env$_{387–399}$ (Figure 9F).

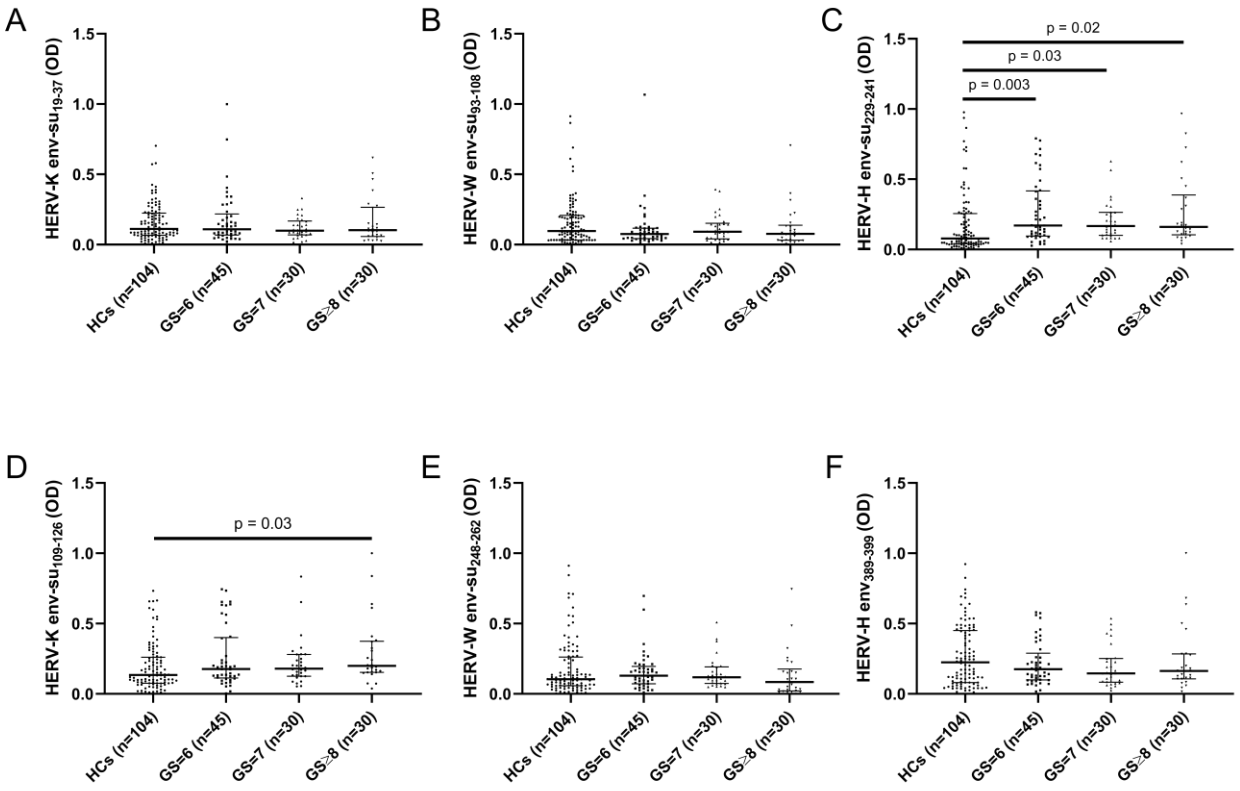

Figure 9. ELISA-based analysis of antibody response against HERV-K env-su$_{19–37}$ (A), HERV-W env-su$_{93–108}$ (B), HERV-H env-su$_{229–241}$ (C), HERV-K env-su$_{109–126}$ (D), HERV-W env-su$_{248–262}$(E) and HERV-H env$_{387–399}$ (F) in PCa population stratified according to Gleason grading system. Black bars correspond to median plus interquartile range.

2.3 Spearman's Correlation Analysis between PSA Plasmatic Levels and Antibody Levels Against HERV env-derived Epitopes

Since prostate-specific antigen (PSA), a kallikrein-serine protease produced by the epithelial cells of the prostate gland, is presently used as PCa biomarker due to the increased circulating levels during cancer [126], correlation analysis was carried out to evaluate the presence of an association between PSA plasmatic levels and the antibody response against HERV env-derived peptides. As shown in Figure 10, the analysis indicated that PSA plasmatic levels and the humoral response against HERV env-derived peptides are not correlated in patients with PCa (Figure 10A) as well as in patients with PBH (Figure 10B), and in patients of the borderline group (Figure 10C). Moreover the following correlations were detected in PCa patients (Figure 10A) between HERV-K env-su$_{19–37}$ and HERV-K env-su$_{109–126}$ ($r = 0.34$, $p = 0.0004$), HERV-W env-su$_{93–108}$ ($r = 0.31$, $p = 0.001$), HERV-W env-su$_{248–262}$ ($r = 0.34$, $p = 0.0004$), HERV-H env-su$_{229–241}$ ($r = 0.33$, $p = 0.0005$) and HERV-H env$_{387–399}$ ($r = 0.23$, $p = 0.01$); low correlations were also detected between HERV-K env-su$_{109–126}$ and HERV-W env-su$_{93–108}$ ($r = 0.07$, p = ns), HERV-W env-su$_{248-262}$ ($r = 0.24$, $p = 0.01$), HERV-H env-su$_{229-241}$ ($r = 0.16$, p = ns) and HERV-H env$_{387-399}$ ($r = -0.003$, p = ns). Further, HERV-W env-su$_{93-108}$ exhibited a low correlation with HERV-W env-su$_{248-262}$ ($r = 0.39$, $p < 0.0001$), HERV-H env-su$_{229-241}$ ($r = 0.41$, $p < 0.0001$), HERV-H env$_{387-399}$ ($r = 0.46$, $p < 0.0001$), and a low correlation was also found between HERV-W env-su$_{248-262}$ and HERV-H env-su$_{229-241}$ ($r = 0.37$, $p = 0.0001$), HERV-H env$_{387-399}$ ($r = 0.21$, $p = 0.02$). A moderate correlation was noticed between HERV-H env-su$_{229-241}$ and HERV-H env$_{387-399}$ ($p < 0.0001$).

The correlation analysis performed using the antibody levels of HERV env-derived peptides in BPH patients (Figure 10B) exhibited a moderate correlation between HERV-K env-su$_{19–37}$ and HERV-K env-su$_{109–126}$ ($r = 0.50$, $p < 0.0001$), HERV-W env-su$_{93–108}$ ($r = 0.54$, $p < 0.0001$), HERV-H env-su$_{229–241}$ ($r = 0.49$, $p < 0.0001$) and a low correlation between HERV-K env-su$_{19–37}$ and HERV-W env-su$_{248–262}$ ($r = 0.35$, $p = 0.002$), and HERV-H env$_{387–399}$ ($r = 0.28$, $p = 0.01$). HERV-K env-su$_{109–126}$ showed a low correlation with HERV-W env-su$_{93–108}$ ($r = 0.37$, $p = 0.001$), HERV-W env-su$_{248–262}$ ($r = 0.28$, $p = 0.01$), and HERV-H env-su$_{229–241}$ ($r = 0.26$, $p = 0.02$); while HERV-K env-su$_{109–126}$ exhibited no significant correlation with HERV-H env$_{387–399}$ ($r = 0.14$, $p =$ ns).

Moderate correlations were found between HERV-W env-su$_{93–108}$ and HERV-W env-su$_{248–262}$ ($r = 0.45$, $p < 0.0001$), HERV-H env-su$_{229–241}$ ($r = 0.67$, $p < 0.0001$), and HERV-H env$_{387–399}$ ($r = 0.57$, $p < 0.0001$). In regard to HERV-W env-su$_{248–262}$, the following correlations were found with: HERV-H env-su$_{229–241}$ ($r = 0.40$, $p < 0.0001$), HERV-H env$_{387–399}$ ($r = 0.22$, $p =$ ns); whilst, a moderate correlation was found between HERV-H env-su$_{229–241}$ and HERV-H env$_{387–399}$ ($r = 0.59$, $p < 0.0001$). As for the Borderline group (Figure 10C), a strong correlation was observed between HERV-K env-su$_{19–37}$ and HERV-K env-su$_{109–126}$ ($r = 0.80$, $p < 0.0001$), HERV-W env-su$_{248–262}$ ($r = 0.73$, $p < 0.0001$), HERV-H env-su$_{229–241}$ ($r = 0.76$, $p < 0.0001$), and between HERV-W env-su$_{93–108}$ and HERV-W env-su$_{248–262}$ ($r = 0.77$, $p < 0.0001$), HERV-H env-su$_{229–241}$ ($r = 0.70$, $p < 0.0001$). A strong correlation was also observed between HERV-H env-su$_{229–241}$ and HERV-W env-su$_{248–262}$ ($r = 0.72$, $p < 0.0001$). A moderate correlation was noticed between HERV-K env-su$_{109–126}$ and HERV-W env-su$_{93–108}$ ($r = 0.47$, $p = 0.007$), HERV-W env-su$_{248–262}$ ($r = 0.50$, $p = 0.004$), HERV-H env$_{387–399}$ ($r = 0.39$, $p = 0.02$), as well between HERV-K env-su$_{19–37}$ and HERV-W env-su$_{93–108}$ ($r = 0.65$, $p = 0.0001$); alongside the moderate correlation found between HERV-H env$_{387–399}$ and HERV-W env-su$_{248–262}$ ($r = 0.43$, $p = 0.01$), HERV-K env-su$_{19–37}$ ($r = 0.49$, $p = 0.005$), HERV-W env-su$_{93–108}$ ($r = 0.56$, $p = 0.001$), HERV-H env-su$_{229–241}$ ($r = 0.58$, $p = 0.007$).

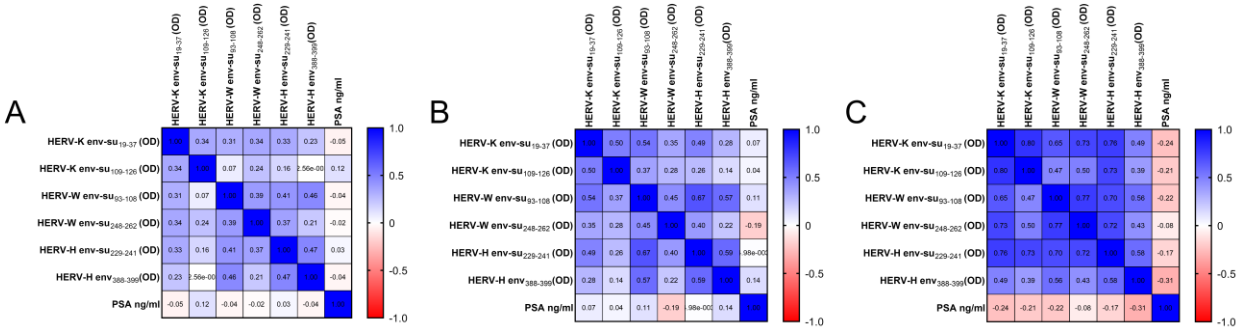

Figure 10. Heatmap of the Spearman's correlation coefficients obtained among HERV-env derived peptides and PSA plasmatic levels in PCa (A), PBH (B), and borderline (C) cohorts.

2.4 Discussion

The association between HERVs and several diseases, such as amyotrophic lateral sclerosis [127,128], multiple sclerosis [129], autoimmune diseases [130,131] and several types of cancer [102,132–135], has been widely investigated over the years. The envelope (env) protein of HERVs family has immunosuppressive properties and it is involved in the modulation of transcription factors of cancer-related pathways [92,136]. Cancer management requires screening methods for cancer diagnosis and prognosis, and currently healthcare exploits both prostate biopsy and PSA screening for diagnosing PCa. To investigate how HERVs contribute to cancer pathogenesis, several studies used different approaches. In a recent study by Razei et al., higher levels of HERV-K gag RNA and protein were observed in PCa malignant regions as opposed to matched benign regions [106]. Other studies have previously shown that serum antibody against HERV-K gag is present in PCa patients [105] and HERV-K gag expression is elevated in the PBMCs of PCa patients, with smoking status and age being significant determinants [137]. According to Chie et al., HERV-H enhances epithelial-to-mesenchymal transition and plays a role in cancer immune evasion [81]._According to this latter research, an HERV-H peptide was able to boost the production of CCL19 which in turn led to the recruitment of immunosuppressive immune cells into HERV-H^+ $CCL19^+$ colon cancer tissues. The work by Pérot et al. suggests HERV-H plays a prominent role in colorectal cancer by demonstrating the association between HERV-H reactivation and clinical parameters, like lymph node metastasis [138]. The purpose of this study was to investigate whether specific humoral responses to HERVs, in particular to epitopes derived from the most immunogenic portions of the env proteins of HERV-H, HERV-K, and HERV-W, could be an appropriate disease biomarker for PCa. Plasma samples were chosen because these body fluids are readily accessible and favorable for analyzing HERV-specific immune responses.

Furthermore, the presence of HERV env antibodies was also studied in two distinct cohorts consisting of patients affected by BPH and a borderline populations to further assess the specificity of the association found between PCa, HERV-K and HERV-H. In the PCa population, 14% of the subjects analyzed were found seropositive against HERV-K env-su$_{109–126}$, while the humoral response against peptides derived from the env protein of HERV-W (HERV-W en-su$_{93–108}$ and HERV-W env-su$_{248–262}$) did not differ among groups.

Additionally, our investigation revealed for the first time that 26% of PCa patients had strongly positive antibody response against HERV-H env-su$_{229–241}$ in comparison to HCs, BPH, and the borderline cohorts. In the BPH group, 14% of patients were found seropositive to HERV-H env-su$_{229–241}$ as well, although T-Fisher exact test did not confirm this result. HERV-env-specific humoral response was further explored by stratifying the PCa population according to the Gleason Score system, a strong predictor of PCa prognosis. Our findings revealed that tumor progression and Gleason grading system were not correlated with the antibody levels against HERV-H env-su$_{229–241}$, in fact no difference was observed among the GS-based groups, whereas patients with a GS ≥ 8 displayed a stronger antibody response against HERV-K env-su$_{109–126}$.

These findings support the hypothesis that HERV-K env and HERV-H env autoantibodies may play a role as prostate cancer biomarkers; it may be useful to inquire into the expression levels of the env protein of HERV-K and HERV-H in prostate tissue samples as well as PBMCs to help shed light on the diagnostic and prognostic potential of HERV serum antibodies.

Chapter 3

Chapter 3

3. Characterization of MHC class I and class II loci by Next-Generation Sequencing in Prostate Cancer Patients (Unpublished Data)

3.1 Material and methods

3.1.1 Sample collection and PBMCs isolation

The study was approved by the Ethical Committee of AOU, Sassari. A cohort of 50 patients with PCa and 50 HCs matched by age were used in this work (Table 3). PCa patients were enrolled at the Urology Department of University Hospital of Sassari and the HCs were enrolled at the Transfusion Center of AOU, Sassari. Peripheral whole blood samples were collected in K^+ -EDTA test tubes for both PCa and HCs individuals. Peripheral blood mononuclear cells (PBMCs) were separated from the other blood components by Ficoll-Histopaque gradient centrifugation (Sigma-Aldrich, St. Louis, MO, USA). PBMCs were then stored at -80°C in Fetal Bovine Serum and dimethyl-sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) until further use.

Table 3. Demographic and clinical information about PCa patients and HCs.

	PCa (n=50)	**HCs (n=50)**
Age (mean±SD)	70.7±8.1	58.4±7
Serum PSA (ng/ml, mean±SD)		
≤ 4 ng/ml	7	
> 4 ng/ml	43	
Gleason Score (GS)		
GS=6	23	
GS=7	16	
GS≥8	9	
Unknown	2	

3.1.2 Genomic DNA extraction

Genomic DNA was extracted by PBMCs using DNeasy Blood and Tissue kit (Qiagen, CA, USA). Cells were washed twice in Phosphate Buffer Saline 1X (PBS) and resuspended in 200 µl PBS 1X. Genomic DNAs were extracted according to the manufacturer's instructions. The final DNA concentration of each sample was measured using Nanodrop One (Thermo Scientific) and DNA quality was evaluated by calculating the following absorbance ratios: A260/A280 and A260/A230.

3.1.3 Library Preparation and HLA Genotyping

Each individual of both populations was genotyped for HLA-A, HLA-B, HLA-C, HLA-DQB1, HLA-DRB1, HLA-DPB1 using a CRS4-NGSC in-house protocol. Briefly, the entire gene region from 5'UTR to 3'UTR of Class I HLA (HLA-A, HLA-B and HLA-C loci) and of Class II (HLA-DRB1, -DPB1, -DQB1 loci) were amplified by long range PCR using a modified protocol from Hosomichi et al. (BMC Genomics 2013, 14:355). Primers for HLA-A and HLA-DRB1 were redesigned, and the amplifications were carried out in duplex PCR (two loci for each reaction). PCR products were quantified by Qubit fluorimeter and pooled in equimolar quantities. Library were obtained using NexteraDNA Flex with 100ng of DNA and indexed with IDT for Illumina Nextera DNA UD Indexes Primer Set (Illumina, San Diego, CA, USA). After purification of PCR products with 1X AMPure XP beads (Beckman Coulter), the libraries were quantified by Qubit fluorimeter. A loading pool consisting of 96 samples was diluted to 9 pM before sequencing on a MiSeq Reagent Kit v3 600-cycle (Illumina).

3.1.4 Bioinformatic and Statistical analysis

Demultiplexing and fastq file generation was conducted on BaseSpace Sequence Hub (Illumina). HLA typing data were analyzed using NGSengine software (GenDX) and manual data review. The allele frequencies for each HLA locus were calculated using a direct counting method and the differences between PCa patients and HCs were calculated by two-sided T-Fisher Exact test. P-values < 0.05 were considered statistically significant. GraphPad Prism 8.2.0 software (GraphPad Software, San Diego, CA, USA) was used to carry out statistical analysis.

3.2 Results

3.2.1 Allele frequencies of MCH class I in PCa patients and HCs

Table 4 below summarizes the allele frequencies and the differences found for MHC class I loci (HLA-A, HLA-B, HLA-C) in a cohort of individuals with PCa and one of HCs. The number of HLA-A, HLA-B and HLA-c alleles identified in this study were 22, 35 and 23, respectively. The most frequent alleles found in locus A in patients with PCa were A*02:01 (16.66%), A*02:05 (13.54%), A*30:02 (12.5%), A*24:02 (9.38%), A*32:01 (9.38%), A*03:01 (6.25%) and A*26:01 (6.25%). On the locus B, B*18:01 (18.8%), B*35:01 (9.57%), B*58:01 (9.57%), B*07:02 (5.32%), B*14:02 (5.32%), B*38:01 (5.32%) were the most frequent alleles in the PCa cohort. Instead in the HCs group, the most frequent alleles were the following: A*24:01 (20.21%), A*02:01 (15.96%), A*11:01 (14.89%), A*30:02 (12.76%), A*32:01 (9.32%), B*12:01 (30.61%), B*35:01 (9.18%), B*58:01 (8.16%), C*05:01 (16.67%), C*04:01 (13.33%), C*07:01 (16.67%), C*07:18 (13.33%), C*12:03 (8.33%).

The analysis to verify the differences in HLA allele frequencies between the two populations identified two HLA-A alleles more commonly present in the HCs than PCa patients. HLA-A*11:01 was found in 5.21% of PCa patients and 14.89% of HCs ($p = 0.0305$, OR: 0.314), whilst A*24:02 occurred in 9.38% of PCa and 20.21% of HCs ($p = 0.0414$, OR: 0.408). On the HLA-B locus, B*18:01 was the only one to show a significant difference between PCa and HCs, 18.8% and 30.61% ($p = 0.046$, OR: 0.500), respectively. On the locus HLA-C, C*03:03 was exclusively observed in PCa patients with a 5% frequency.

Table 4. Allele frequencies of MHC class I (HLA-A, HLA-B, HLA-C) and frequency differences between PCa patients and HCs analyzed using T-Fisher exact test. Statistically significant alleles have been marked in bold.

Allele Frequencies of MHC class I				
	PCa (2n=100)	**HCs (2n=100)**	**OR**	**p-value**
A*01:01	3 (3.1%)	3 (3.2%)	0.978	ns
A*01:02	/	1 (1.06%)	0.000	ns
A*02:01	16 (16.66%)	15 (15.96%)	1.053	ns
A*02:05	13 (13.54%)	8 (8.51%)	1.684	ns
A*02:06	1 (1.04%)	/	0.000	ns
A*02:09	1 (1.04%)	/	0.000	ns
A*03:01	6 (6.25%)	2 (2.13%)	3.067	ns
A*11:01	**5 (5.21%)**	**14 (14.89%)**	**0.314**	**0.0305**
A*23:01	1 (1.04%)	2 (2.12%)	0.484	ns
A*24:02	**9 (9.38%)**	**19 (20.21%)**	**0.408**	**0.0414**
A*24:03	/	1 (1.06%)	0.000	ns
A*25:01	/	1 (1.06%)	0.000	ns
A*26:01	6 (6.25%)	3 (3.2%)	2.022	ns
A*29:01	1 (1.04%)	2 (2.13%)	0.484	ns
A*29:02	2 (2.08%)	1 (1.06%)	1.979	ns
A*30:01	3 (3.12%)	3 (3.2%)	0.978	ns
A*30:02	12 (12.5%)	12 (12.76%)	0.976	ns
A*30:04	1 (1.04%)	/	0.000	ns
A*31:01	1 (1.04%)	/	0.000	ns
A*32:01	9 (9.38%)	5 (5.32%)	1.841	ns
A*33:01	5 (5.21%)	2 (2.13%)	2.527	ns

Table 4 (continued).

A*68:01	1 (1.04%)	/	0.000	ns
A* NA	4	6		
B*07:02	5 (5.32%)	2 (2.04%)	2.697	ns
B*07:05	2 (2.13%)	2 (2.04%)	1.043	ns
B*08:01	2 (2.13%)	2 (2.04%)	1.043	ns
B*13:02	3 (3.19%)	3 (3.06%)	1.044	ns
B*14:02	5 (5.32%)	4 (4.08%)	1.320	ns
B*15:01	4 (4.25%)	1 (1.02%)	4.311	ns
B*15:17	1 (1.06%)	2 (2.04%)	0.516	ns
B*15:18	1 (1.06%)	1 (1.02%)	1.043	ns
B*18:01	**17 (18.08%)**	**30 (30.61%)**	**0.500**	**0.046**
B*18:03	/	1 (1.02%)	0.000	ns
B*27:05	/	1 (1.02%)	0.000	ns
B*35:01	9 (9.57%)	9 (9.18%)	1.047	ns
B*35:02	3 (3.19%)	4 (4.08%)	0.775	ns
B*35:03	/	2 (2.04%)	0.000	ns
B*35:08	1 (1.06%)	/	0.000	ns
B*38:01	5 (5.32%)	3 (3.06%)	1.779	ns
B*39:06	1 (1.06%)	/	0.000	ns
B*40:02	1 (1.06%)	/	0.000	ns
B*40:06	/	1 (1.02%)	0.000	ns
B*44:02	2 (2.13%)	2 (2.04%)	1.043	ns
B*44:03	2 (2.13%)	1 (1.02%)	2.132	ns
B*44:05	2 (2.13%)	/	0.000	ns
B*45:01	1 (1.06%)	1 (1.02%)	1.043	ns

Table 4 (continued).

B*47:01	1 (1.06%)	1 (1.02%)	1.043	ns
B*49:01	4 (4.25%)	4 (4.08%)	1.044	ns
B*51:01	3 (3.19%)	4 (4.08%)	0.775	ns
B*51:08	/	1	0.000	/
B*52:01	3 (3.19%)	3 (3.06%)	1.044	ns
B*53:01	1 (1.06%)	1 (1.02%)	1.043	ns
B*55:01	3 (3.19%)	2 (2.04%)	1.582	ns
B*56:01	1 (1.06%)	/	0.000	/
B*57:01	1 (1.06%)	/	0.000	/
B*58:01	9 (9.57%)	8 (8.16%)	1.191	ns
B*58:22	/	1 (1.02%)	0.000	ns
B*73:01	1 (1.06%)	1 (1.02%)	1.043	ns
B* NA	6	2		
C*01:02	1 (1.25%)	3 (5.0%)	0.240	ns
C*02:02	4 (5.0%)	1 (1.67%)	3.105	ns
C*03:02	1 (1.25%)	/	0.000	/
C*03:03	4 (5.0%)	/	0.000	/
C*04:01	13 (16.25%)	8 (13.33%)	1.261	ns
C*05:01	10 (12.50%)	10 (16.67%)	0.714	ns
C*06:02	5 (6.25%)	3 (5.0%)	1.267	ns
C*07:01	8 (10.0%)	10 (16.67%)	0.555	ns
C*07:02	5 (6.25%)	2 (3.33%)	1.933	ns
C*07:04	/	1	0.000	/
C*07:18	8 (10.0%)	8 (13.33%)	0.722	ns
C*07:682	1 (1.25%)	/	0.000	/

Table 4 (continued).

C*08:02	4 (5.0%)	1 (1.67%)	3.105	ns
C*12:02	2 (2.50%)	2 (3.33%)	0.744	ns
C*12:03	9 (11.25%)	5 (8.33%)	1.394	ns
C*15:02	2 (2.5 %)	/	0.000	ns
C*15:04	/	1	0.000	ns
C*15:05	2 (2.50%)	1 (1.67%)	1.513	ns
C*15:22	/	1	0.000	/
C*16:01	/	1		/
C*16:02	/	1		/
C*16:04	1 (1.25%)	1 (1.67%)	0.747	ns
C* NA	20	40		

Abbreviations: OR, odd ratio; ns = not significant; NA: Not available.

3.2.2 Allele frequencies of MCH class II in PCa patients and HCs

Table 5 below summarizes allele frequencies of MHC class II calculated in both PCa patients and HCs. No significant difference was observed between PCa and HCs, though numerous alleles were found most represented in both groups. In the PCa cohort, the following HLA-DRB1 alleles DRB1*16:01, DRB1* 03:01, DRB1*11:04, DRB1*13:01, DRB1*15:02, DRB1*01:01 accounted for 14.29%, 12.86%, 10%, 7.14%5.71% and 5.71%, respectively. On the locus DQB1, the most frequent alleles were DQB1*03:01 (19.10%), DQB1*05:02 (14.61%), DQB1*02:01 (13.48%), DQB1*03:02 (7.87%), DQB1*06:03 (5.62%). As for HLA-DBP1, the most common alleles were DPB1*02:01 (28.75%), DPB1*04:01 (20%), DPB1*04:02 (15%), DPB1*03:01 (10%). Additionally, the most observed HLA-DRB1 alleles in the cohort of HCs were DRB1*03:01 (19.32%), DRB1*11:04 (14.77%), DRB1*16:01 (13.64%), DRB1*01:01 (6.82%), DRB1*11:01 (6.82%).

On the loci HLA-DQB1 and HLA-DRB1, the most frequent alleles were the following: DQB1*03:01 (18.99%), DQB1*02:01 (13.92%), DQB1*05:02 (13.92%), DRB1*04:01 (28.13%), DRB1*04:02 (21.88%), DRB1*02:01 (20.83%), DRB1*10:01 (5.21%). A novel allele (DRB1*16:64) belonging to HLA-DRB1 locus, not reported in any of the online database (e. g. the IPD-IMG/HLA Database and the Allele Frequency Database), was identified in the genotyping of both populations.

Table 5. Allele frequencies of MHC class II (HLA-DRB1, HLA-DQB1, HLA-DPB1) and frequency differences between PCa patients and HCs analyzed using T-Fisher exact test.

Allele Frequencies of MHC class II				
	PCa (2n=100)	**HCs (2n=100)**	**OR**	**p-value**
DRB1*01:01	4 (5.71%)	6 (6.82%)	0.828	ns
DRB1*01:02	3 (4.29%)	2 (2.27%)	1.925	ns
DRB1*01:03	/	3	0.000	ns
DRB1*03:01	9 (12.86%)	17 (19.32%)	0.616	ns
DRB1*04:01	3 (4.29%)	1 (1.14%)	3.896	ns
DRB1*04:02	3 (4.29%)	2 (2.27%)	1.925	ns
DRB1*04:03	1 (1.43%)	1 (1.14%)	1.261	ns
DRB1*04:04	1 (1.43%)	3 (3.41%)	0.410	ns
DRB1*04:05	3 (4.29%)	6 (6.82%)	0.612	ns
DRB1*07:01	3 (4.29%)	5 (5.68%)	0.743	ns
DRB1*08:01	1	/	0.000	ns

Table 5 (continued).

DRB1*08:04	1	/	0.000	ns
DRB1*10:01	1 (1.43%)	1 (1.14%)	1.261	ns
DRB1*11:01	3 (4.29%)	6 (6.82%)	0.612	ns
DRB1*11:02	1 (1.43%)	1 (1.14%)	1.261	ns
DRB1*11:03	1	/	0.000	ns
DRB1*11:04	7 (10.0%)	13 (14.77%)	0.472	ns
DRB1*13:02	/	1	0.000	ns
DRB1*12:01	1 (1.43%)	1 (1.14%)	1.261	ns
DRB1*13:01	5 (7.14%)	1 (1.14%)	6.692	ns
DRB1*13:02	/	4	0.000	ns
DRB1*13:03	2	/	0.000	ns
DRB1*14:04	/	1	0.000	ns
DRB1*14:54	2 (2.86%)	1 (1.14%)	2.559	ns
DRB1*15:01	1 (1.43%)	3 (3.41%)	0.410	ns
DRB1*15:02	4 (5.71%)	2 (2.27%)	2.606	ns
DRB1*16:01	10 (14.29%)	12 (13.64%)	1.056	ns
DRB1*16:64	/	1	0.000	ns
DRB1* NA	30	12		
DQB1*02:01	12 (13.48%)	11 (13.92%)	0.963	ns
DQB1*02:02	3 (3.37%)	3 (3.80%)	0.884	ns
DQB1*03:01	17 (19.10%)	15 (18.99%)	1.007	ns
DQB1*03:02	7 (7.87%)	3 (3.80%)	2.163	ns
DQB1*03:03	/	1 (1.27%)	0.000	ns
DQB1*03:04	1 (1.12%)	1 (1.27%)	0.886	ns
DQB1*03:05	1 (1.12%)	/	0.000	ns

Table 5 (continued).

DQB1*03:19	1 (1.12%)	/	0.000	ns
DQB1*04:02	2 (2.24%)	/	0.000	ns
DQB1*05:01	7 (7.87)	5 (6.33%)	1.263	ns
DQB1*05:02	13 (14.61%)	11 (13.92%)	1.057	ns
DQB1*05:03	1 (1.12%)	1 (1.27%)	0.886	ns
DQB1*06:01	3 (3.37%)	2 (2.53%)	1.343	ns
DQB1*06:02	2 (2.25%)	1 (1.27%)	1.793	ns
DQB1*06:03	5 (5.62%)	1 (1.27%)	4.643	ns
DQB1*06:04	/	2 (2.53%)	0.000	ns
DQB1* NA	11	21		
DPB1*01:01	1 (1.25%)	1 (1.04%)	1.203	ns
DPB1*02:01	23 (28.75%)	20 (20.83%)	1.533	ns
DPB1*03:01	8 (10.0%)	6 (6.25%)	1.667	ns
DPB1*04:01	16 (20.0%)	27 (28.13%)	0.639	ns
DPB1*04:02	12 (15.0%)	21 (21.88%)	0.630	ns
DPB1*05:01	1 (1.25%)	1 (1.04%)	1.203	ns
DPB1*09:01	1 (1.25%)	/	0.000	ns
DPB1*10:01	2 (2.50%)	5 (5.21%)	0.467	ns
DPB1*13:01	/	2 (2.08%)	0.000	ns
DPB1*14:01	4 (5.0%)	2 (2.08%)	2.474	ns
DPB1*16:01	/	2 (2.08%)	0.000	ns
DPB1*17:01	2 (2.50%)	4 (4.17%)	0.589	ns
DPB1*19:01	1 (1.25%)	/	0.000	ns
DPB1*20:01	/	1 (1.04%)	0.000	ns
DPB1*23:01	2	/	0.000	ns

Table 5 (continued).

DPB1*104:01	1 (1.25%)	3 (3.13%)	0.392	ns
DPB1*124:01	1 (1.25%)	1 (1.04%)	1.203	ns
DPB1*126:01	1 (1.25%)	/	0.000	ns
DPB1*665:01	1 (1.25%)	/	0.000	ns
DPB1*905:01	1 (1.25%)	/	0.000	ns
DPB1*NA	20	4		

Abbreviations: OR, odd ratio; ns = not significant; NA: Not available.

3.3 Discussion

Immunocompromised individuals have an increased risk to develop cancer, hence the immune system is capable to recognize and dispose of malignant cells before they start to become clinically noticeable [139,140]. Several evidences outlined the importance of the immune system involvement in the treatment of cancer, pointing out how cancer tissues infiltrated by activated T cells have a better prognosis compared to those with low T cell infiltration [141–144]. Antibodies able to inhibit negative regulatory molecules (PD-1/PD-L1 or CTLA4) are administered in checkpoint blockade therapy since they are known to curtail T cell responses. Unfortunately, as the cancer becomes clinically evident, continuing to grow and progress untreated, the probability to fail in responding to the current immunotherapies increase as well. Understanding the mechanisms by which cancer cells appear to be able to evade the immune system alongside the immunogenetic traits for each cancer type are of utmost importance to improve the efficacy of immunotherapy. Certainly, both MHC class I and II play critical roles in mediating the attack of CD8+ and CD4+ cells against the neoantigens present on the surface of the cancer cells. However, the MHC expression can be downregulated by epigenetic mechanisms [145–147] and the loss of MHC class I has been reported for several cancer hampering each attempt to develop effective immunotherapies. In PCa, loss of HLA class I expression has been observed in 34% of primary PCa and 80 % of lymph node metastases [148]. Altered expression patterns and loss of expression were also detected in PCa patient biopsies, but not in BPH specimen in which a strong and homogenous expression was found [149]. Furthermore, IFN-γ is critical in eliciting a tumor-specific T cell-mediated memory immune response against TRAMP-C2 tumor by enhancing MHC class I-dependent antigen presentation of tumor-derived immunogenic peptides and antagonizing the antiinflammatory effects of tumor-derived-TGF-β [150].

The aim of this study was to genotype both MHC class I and II in PCa patients employing NGS technologies, despite the low sample size and the missing genotyping data of some HLA loci, we provide new insight into the genetic diversity of HLA class I and II in PCa.

Genotyping analysis of MHC class I and class II led us to identify three new alleles associated to PCa, two belonging to HLA-A locus and one to HLA-B locus. From the analysis of the allele frequencies between HCs and PCa patients, it emerged that A*24:02 and A*11:01 had a lower allele frequency in PCa patients compared to the HCs. A*24:02 has been previously linked to PCa, a study from Stokidis S. et al. reported that A*24:02 positive patients presented a more favorable clinical outcome and a slower cancer progression [151]. Another work demonstrates the correlation between immune parameters and clinical outcome in PCa patients vaccinated with a HER-2/neu hybrid polypeptide vaccine highlighting how A*24 positive patients presented increased overall survival and stronger immune responses [152]. This result corroborates the hypothesis of a protective role for A*24:02 in PCa, considering that the patients used in this study presented lower levels of A*24:02 than HCs, however further studies are needed to evaluate A*24:02 expression levels in prostate tissue as well to assess whether A*24:02 presence might affect the overall survival of the patients, the progression rate as well as metastasis occurrence. The presence of A*24:02 may clearly increase the immunogenicity of cancer cells in addition to enhance the immunosurveillance of the tumor area by presenting tumor-derived antigens and elicit cytotoxic T lymphocytes responses. A*11:01 allele frequency was higher in HCs appeared less frequent in PCa patients than HCs as well, suggesting a protective function of this allele in PCa. A protective effect of A*11:01 was observed in skin cancer where none of the patients enrolled in the study were positive for A*11:01 [153]. Conversely, A*11:01 frequency was found higher in patients with stable and progressive lung cancer than in the control group [154]. Given the contradictory results, further analysis ought to be considered in order to thoroughly explore the role of this allele in the biology of PCa.

Lastly, B*18:01 has been linked to PCa the first time, it was found mostly present in HCs than patients with PCa suggesting a possible protective role in PCa as well. In conclusion, our study is the first to investigate MHC genetic diversity in PCa by NGS paving the way to the analysis of the newly associated allele identified. Further investigation is required to improve the knowledge about the role of A*24:02, A*11:01 and B*18:01 in PCa onset and progression.

Chapter 4

Chapter 4

4. Multigene Panel Testing and Prostate Cancer Risk (Unpublished Data)

4.1 Materials and methods

4.1.1 Study population

The study was approved by the Ethical Committee of AOU, Sassari. A cohort of 47 patients with PCa and 43 HCs were used in this work, the table below summarizes demographic and clinical information of each cohort (Table 6). Patients diagnosed with PCa were recruited by the Urology Department of University Hospital of Sassari, whether healthy subject were recruited by the Transfusion Center of AOU, Sassari. K^+-EDTA test tubes were used to collect peripheral whole blood from each PCa patients and HCs.

Table 6. Demographic and clinical information about PCa patients and HCs.

	PCa (n=47)	HCs (n=43)
Age (mean±SD)	70.6±8.0	49.7±14.9
Serum PSA (ng/ml, mean±SD)		
≤ 4 ng/ml	7	
> 4 ng/ml	40	
Gleason Score (GS)		
GS=6	20	
GS=7	16	
GS≥8	8	

Abbreviations: PSA, prostate-specific antigen; SD, standard deviation.

4.1.2 Peripheral blood mononuclear cell isolation and Genomic DNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Histopaque gradient centrifugation (Sigma-Aldrich, St. Louis, MO, USA). PBMCs were stored at -80°C in Fetal Bovine Serum and dimethyl-sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) until further use. Genomic DNA was extracted by PBMCs using DNeasy Blood and Tissue kit (Qiagen, CA, USA). Cells were washed twice in Phosphate Buffer Saline 1X (PBS) and resuspended in 200 μl PBS 1X. Genomic DNAs were extracted according to the manufacturer's instructions. The final DNA concentration of each extracted sample was measured using Nanodrop One (Thermo Scientific), DNA quality was evaluated by calculating the raios of absorbance at A260/A280 and A260/A230.

4.1.3 Library preparation and genomic sequencing

Thirty-four genes involved in immune response and linked to PCa were sequenced. Gene panel analysis was performed using two AmplySeq primer pools designed and purchased from Illumina. To amplify the entire coding region of 34 genes, PCR protocol and reagents were adapted from QIAGEN Application Note (Qiagen 1104745 10/2016). PCR products were quantified by Qubit and equimolarly pooled. Gene-panel PCR pool was grouped to achieve the appropriated sequencing coverage. Library were obtained using NexteraDNA Flex with 100ng of DNA and indexed with IDT for Illumina Nextera DNA UD Indexes Primer Set (Illumina). PCR products were purified with 1X AMPure XP beads (Beckman Coulter) and the libraries were quantified by Qubit. A loading pool consisting of 96 samples was diluted to 9pM before sequencing on a MiSeq Reagent Kit v3 600-cycle (Illumina).

Demultiplexing and fastq file generation was conducted on BaseSpace Sequence Hub (Illumina) and for each sample two distinct analysis were carried out. Gene panel sequences were analyzed using an in-house pipeline.

4.1.4 Statistical analysis

The allele and genotype frequency estimates and deviation from Hardy-Weinberg equilibrium (HWE) were carried out (http://ihg2.helmh-oltzmuenchen.de/cgi-bin/hw/hwa1.pl). T-Fisher exact test, Chi square test and allelic odds ratios (ORs) were used to compare genetic variants between PCa and HC cohorts. To test the relationships between the illness and the nucleotide substitution for a given locus, a multivariate regression analysis was carried out by applying a backward selection in which non-significant covariates were removed step by step, until the final model was obtained. A Generalized Linear Mixed Models (GLMM), implemented in the R-package lme4 [155] in R environment version 4.1.1 (R Core Team available at https://www.r-project.org/), was used to perform the analysis. Initial dataset was composed by 1247 loci in 35 genes sequenced from 90 individuals, 47 of which affected by PCa and 43 HCs. After the first quality check 1238 loci remained. The first GLMMs runs were applied individually on each single gene in order to find loci statistically associated with the occurrence of the illness. In this step, statistical significance was set at $P < 0.05$ with few exceptions for borderline values which were maintained in order to avoid premature selections in the intermediate models. A new dataset was built with the obtained statistical significance variables that represent potential predictors and GLMMs runs were applied.

Again, the statistical significance was set at $P < 0.05$ and the GLMMs were also used to find a final model capable of discerning useful covariate predictors for the estimation of illness odds in patient with the given mutation. Indeed, the final model was used to calculate the odds ratio with 97.5% CI by using the R-package "oddsratio" (available at https://cran.r-project.org/web/packages/oddsratio/).

4.2 Results

4.2.1 Significant allele and genotype frequencies detected in PCa patients and HCs.

A total of 47 PCa patients and 43 HCs underwent NGS and comprehensive gene profiling was conducted in 35 genes. Table 7 below summarizes the genes examined in this work.

Table 7. Summary of the genes examined by NGS.

Genes characterized by NGS			
TNFSF1B	*IL2*	*IL4R*	*PACRG*
TNFRS	*IL7R*	*IL18*	*TRAF6*
PTPN22	*IL5*	*IL12RB1*	*LAG3*
CD28	*IL13*	*IL12RB2*	*VDR*
IFNG	*IL4*	*IL18R1*	*TMPRSS*
IFNGR1	*IL12A*	*MAPT*	*2*
IFNGR2	*IL12B*	*ACE*	*ATG7*
IRF5	*IL17A*	*PARK2*	*NR1I2*
IL2RA	*IL6*	*SCLC11A1*	*ACE2*

Both genotype and allele frequencies of the analyzed genes were estimated in both populations and HWE was assessed for each variant. Chi-square test and T-Fisher's exact test were used to estimate the presence of significant differences in the genotype and allele frequencies, respectively, in the two groups. The distribution of significant allele and genotype frequency is summarized in Table 8. In the *IL4R*, the rs2301807 (C) was most frequently present in controls compare to PCa patients (OR: 0.007, $p < 0.0001$). (CC) genotype was found in 32 controls and 20 PCa patients (OR: 0.014, CI: 0.001-0.244, $p < 0.0001$).

In the 3' UTR of the *IL12RB1* gene, (AA) genotype was only found in 4 controls (OR: 0.062, CI: 0.003-1.243, p = 0.013). In the *IL12RB2* gene, rs2307145 (C) allele frequency, higher in the controls, was found to decrease (0.194 times) the risk to develop PCa in the study population (OR: 0.194, CI: 0.039-0.971, p = 0.038).

An intronic variant (rs2069832) was identified in the *IL6* gene, (G) allele frequency was higher in PCa patients than HCs. The analysis results indicate an increased risk to develop PCa by 2.5 times (OR: 2.583, CI: 1.092-6.114, p = 0.028). Two intronic mutations were also identified within the *TMPRSS2* gene, rs140141551 and rs11701576. rs140141551 (A) allele was correlated with a higher PCa risk (OR: 7.535, CI: 0.922-61.586, p = 0.0382). Additionally, rs140141551 (CA) genotype was also correlated to increased PCa risk (OR: 8.205, CI: 0.922-61.586, p = 0.024). Conversely, rs11701576 (G) allele frequency was associated with a lower PCa risk (OR: 0.309, CI: 0.097-0.977, p = 0.037) along with (AG) genotype (OR: 0.263, CI: 0.077-0.899, p = 0.027). A synonymous mutation was identified within the *ACE2* gene, rs35803318 (T) allele was found associated to a higher PCa risk by more than 11 times (OR: 11.743, CI: 0.659-209.216, p = 0.022).

Table 8. Distribution of Statistically significant allele and genotype frequencies in PCa patients and HCs. Values in bold represent significant results.

Gene	SNP	Gene feature	Gentoypes	PCa N (F)	HCs N (F)	OR	95% CI	p-value
IL4R	rs2301807	intronic	AA	22 (0.512)	0 (0.000)	Ref		
			AC	1 (0.023)	0 (0.000)	0.067	0.001-4.693	ns
			CC	20 (0.465)	32 (1.000)	0.014	0.001-0.244	**<0.0001**
			AC+CC	21 (0.466)	32 (1.000)	0.015	0.001-0.255	**<0.0001**
			A	45 (0.523)	0 (0.000)	Ref		
			C	41 (0.477)	64 (1.000)	0.007	0.000-0.118	**<0.0001**
IL12RB1	rs3746190	3′ UTR	GG	22 (0.500)	12 (0.353)	Ref		
			GA	22 (0.500)	18 (0.529)	0.667	0.261-1.706	ns
			AA	0 (0.000)	4 (0.118)	0.062	0.003-1.243	**0.013**
			GA+AA	22 (0.500)	22 (0.647)	0.545	0.218-1.367	ns
			G	66	42	Ref		
			A	22	26	0.538	0.271-1.070	ns
IL12RB2	rs2307145	missense	GG	38 (0.950)	24 (0.800)	Ref		
			GC	2 (0.050)	5 (0.167)	2.857	0.045-1.407	ns
			CC	0 (0.000)	1 (0.033)	0.625	0.008-5.419	ns
			GC+CC	2 (0.050)	6 (0.200)	1.477	0.039-1.129	ns
			G	78 (0.975)	53 (0.883)	Ref		
			C	2 (0.025)	7 (0.117)	0.194	0.039-0.971	**0.038**
IL6	rs2069832	intronic	AA	1 (0.083)	2 (0.027)	Ref		
			AG	10 (0.500)	12 (0.270)	1.667	0.131-21.195	ns
			GG	26 (0.417)	10 (0.703)	5.200	0.423-63.909	ns
			AG+GG	36 (0.917)	22 (0.973)	3.273	0.280-38.244	ns
			A	12 (0.162)	16 (0.333)	Ref		
			G	62 (0.838)	32 (0.667)	2.583	1.092-6.114	**0.028**
TMPRSS2	rs140141551	intronic	CC	39 (0.830)	40 (0.976)	Ref		
			CA	8 (0.170)	1 (0.024)	8.205	0.980-68.710	**0.024**
			AA	0 (0.000)	0 (0.000)	1.025	0.020-52.950	ns
			CA+AA	8 (0.170)	1 (0.024)	8.205	0.980-68.710	**0.024**
			C	86 (0.915)	81 (0.988)	Ref		
			A	8 (0.150)	1 (0.012)	7.535	0.922-61.586	**0.0382**

Table 8. (continued)

Gene	SNP	Gene feature	Gentoypes	PCa N (F)	HCs N (F)	OR	95% CI	p-value
TMPRSS2	rs11701576	intronic	AA	38 (0.884)	18 (0.667)	Ref		
			AG	5 (0.116)	9 (0.333)	0.263	0.077-0.899	**0.027**
			GG	0 (0.000)	0 (0.000)	0.481	0.009-25.810	ns
			AG+GG	5 (0.116)	9 (0.333)	0.263	0.077-0.899	**0.027**
			A	81 (0.942)	45 (0.833)	Ref		
			G	5 (0.058)	9 (0.167)	0.309	0.097-0.977	**0.037**
ACE2	rs35803318	synonymous	CC	43 (0.915)	34 (1.000)	Ref		
			CT	1 (0.021)	0 (0.000)	2.379	0.094-60.247	ns
			TT	3 (0.064)	0 (0.000)	5.552	0.277-131.141	ns
			CT+TT	4 (0.085)	0 (0.000)	7.128	0.371-137.158	ns
			C	87 (0.925)	68 (1.000)	Ref		
			T	7 (0.075)	0 (0.000)	11.743	0.659-209.216	**0.022**

Abbreviations: ns, not significant; Ref, reference allele; N, number of individuals; F, frequency; OR, odds ratio; CI, confidence interval.

4.2.2 Evaluation of the relationship between PCa and nucleotide substitution for each locus examined using a Generalized Linear Mixed Model

To test the relationships between nucleotide substitution for a given locus and PCa, a multivariate regression analysis was performed through a backward selection procedure in which non-significant covariates were deleted step by step, until the final model was attained. Analyses were performed by means of a Generalized Linear Mixed Models (GLMM). The first GLMMs runs were applied individually on each single gene to find loci statistically associated with the occurrence of PCa. In this step, statistical significance was set at $p < 0.05$ with few exceptions for borderline values, which were maintained to avoid premature selections in the intermediate models. A new dataset was built with the obtained statistically significant variables that represent potential predictors and GLMMs runs were applied (Table 9). Multivariate analysis pointed out 4 SNPs within *TNFRSF1B*, 3 SNPs within *PTPN22*, 3 SNPs within *IL18R1*, 3 SNPs within *CD28*, 2 within *SLC11A1*, 1 SNP within *ATG7*, 1 SNP *NR1I2*, 1 SNP *IL7R*, 1 SNP *IL5*, 1 SNP *IL13*, 1 SNP *IL4*, 1 SNP *IL12B*, 7 SNPs within *PARK2*, 1 SNP within *IL6*, 1 SNP within *IL2RA*, 1 SNP within *TRAF6*, 1 SNP within *IL18*, 1 SNP within *LAG3*, 1 SNP within *VDR*, 3 SNPs within *IFNG*, 3 SNPs within *IL4R*, 2 within *MAPT*, 3 SNPs within *IL12RB1*, 1 SNP within *IFNGR2*.

Table 9. Results of multivariate analysis on each single gene. Table shows the 48 potential predictors with statistical significance with the occurrence of the cancer and the summary of found coefficients. NM indicates the number of intermediate models used to obtain the final model.

Gene	Position	Estimate	Std. Error	t value	Pr(>|t|)	NM
TNFRSF1B	12251317	-0.41505	0.09149	-4.537	1.89e-05 ***	3
	12252522	0.71043	0.17963	3.955	0.000159 ***	
	12253351	-0.19031	0.08854	-2.149	0.034484 *	
	12253888	-0.32971	0.13489	-2.444	0.016604 *	
	12254590	0.70924	0.14309	4.956	3.68e-06 ***	
PTPN22	114380534	5.710e-01	2.379e-01	2.400	0.01856 *	6
	114380640	-5.130e-01	2.424e-01	-2.116	0.03720 *	
	114401592	5.550e-01	1.796e-01	3.091	0.00269 **	
IL18R1	103001294	-1.07744	0.35991	-2.994	0.00360 **	4
	103001313	1.11261	0.36755	3.027	0.00326 **	
	103013432	0.50393	0.14815	3.401	0.00102 **	
CD28	204571470	0.3274	0.1293	2.532	0.01314 *	2
	204591736	0.4273	0.1524	2.803	0.00625 **	
	204591851	-0.4086	0.1487	-2.748	0.00731 **	
SLC11A1	219248015	-0.21680	0.09983	-2.172	0.0326 *	3
	219258795	0.62760	0.13311	4.715	9.13e-06 ***	
ATG7	11399746	0.5644	0.1471	3.836	0.000235 ***	2
NR1I2	119526373	0.22078	0.10666	2.07	0.0414 *	3
IL7R	35861076	5.732e-01	1.768e-01	3.241	0.00168 **	3

Table 9 (continued).

IL5	131877942	0.2080	0.1090	1.908	0.0597 .	2
IL13	131993850	0.42803	0.11143	3.841	0.00023 ***	2
IL4	132010107	0.26173	0.11047	2.369	0.020014 *	2
IL12B	158749524	5.802e-01	1.664e-01	3.488	0.000763 ***	2
PARK2	161781314	0.37435	0.18751	1.996	0.04921 *	3
	161969941	-0.25665	0.12434	-2.064	0.04217 *	
	161990286	0.36843	0.18294	2.014	0.04730 *	
	161990483	-0.12856	0.05244	-2.452	0.01634 *	
	161990516	0.10818	0.04805	2.251	0.02704 *	
	162394341	0.48702	0.18368	2.651	0.00962 **	
	162683759	-0.40913	0.19278	-2.122	0.03683 *	
IL6	22766834	0.4306	0.1443	2.983	0.00369 **	3
IL2RA	6061246	0.58333	0.11770	4.956	3.46e-06 ***	3
TRAF6	36511631	0.4143	0.1202	3.446	0.000874 ***	2
IL18	112020852	0.35577	0.11119	3.200	0.00191 **	2
LAG3	6883743	0.40986	0.10905	3.758	0.000307 ***	3
VDR	48251239	5.732e-01	1.768e-01	3.241	0.00168 **	3
IFNG	68549177	0.2377	0.1279	1.858	0.0666 .	3
	68549274	0.7348	0.3005	2.445	0.0165 *	
	68549283	-0.6343	0.3050	-2.080	0.0405 *	

Table 9 (continued).

IL4R	27358098	-0.15236	0.02543	-5.991	4.72e-08 ***	5
	27358103	0.60576	0.17933	3.378	0.00110 **	
	27370257	0.53428	0.18529	2.884	0.00497 **	
MAPT	44060859	0.16713	0.08505	1.965	0.05260 .	3
	44068964	0.41742	0.14425	2.894	0.00481 **	
IL12RB1	18170384	-0.09311	0.05099	-1.826	0.071317 .	2
	18188452	0.40721	0.10672	3.816	0.000255 ***	
	18191621	0.24199	0.12999	1.862	0.066080 .	
IFNGR2	34787312	0.30995	0.07351	4.217	5.99e-05 ***	2

Significance codes: 0<'***'<0.001<'**'<0.01< '*'<0.05< '.'<0.1

Statistical significance was set at $p < 0.05$ and the GLMMs were also used to reach a final model capable of detecting useful covariate predictors for the estimation of PCa odds in patient with the given mutation. The covariate predictors identified in the analysis and summarized in Table 10, account for a synonymous mutation in the *TNFRSB1* gene (position: 12252522), two intronic mutations (positions: 12253351 and 12254590) in the *TNFRSB1* gene, an intronic SNP in the *SLC11A* gene (position: 219248015), two intronic SNP located in the *PARK2* gene (rs3765474 and rs3765475) and two intronic SNPs located in the IL4R gene (positions: 27358098 and 27358103).

Table 10. Results of multivariate analysis applied on the subset including all the 48 variables with statistical significance previously reported. Listed statistically significant variables below represent useful predictors. NM indicates the number of intermediate models used to obtain the final model.

Significance codes: 0<'***'<0.001<'**'<0.01< '*'<0.05< '.'<0.1

Gene	Position	Estimate	Std. Error	t value	Pr(>|t|)	NM
TNFRSF1B	12252522	0.47001	0.18140	2.591	0.011346 *	9
	12253351	-0.16534	0.07680	-2.153	0.034301 *	
	12254590	0.42147	0.12463	3.382	0.001111 **	
SLC11A1	219248015	-0.25240	0.08653	-2.917	0.004571 **	
PARK2	161990483	-0.09529	0.03710	-2.569	0.012042 *	
	161990516	0.09292	0.03328	2.792	0.006529 **	
IL4R	27358098	-0.13954	0.02246	-6.214	2.12e-08 ***	
	27358103	0.57510	0.16789	3.425	0.000966 ***	

To measure the association between PCa and the covariate predictors detected in the final model, the odds ratio were estimated with 97.5% confidence interval (C.I.). Table 11 reports the results obtained after multivariate analysis was performed on each individual gene. Unfortunately, the odds ratios calculation was not achievable for some loci. However, any of the 3 SNPs detected within the *TNFRS1B* gene (rs636964) were associated with PCa. Within *PARK2* gene, the rs3765474 intronic variant was found associated with PCa risk, in fact individuals harboring such variant have a lower risk to develop PCa (OR: 0.87; CI_low: 0.80; CI_high: 0.96).

On the contrary, the rs3765475 intronic variant also identified within the *PARK2* gene appears to have an opposite effect compared to rs3765474 increasing the risk to develop PCa (OR: 1.18; CI_low: 1.07; CI_high: 1.30). Furthermore, rs2301807 intronic variant within *IL4R* gene was found to be associated with a lower risk, 0.9 times, of developing PCa.

Table 11. Results of odds ratios calculation on multivariate analysis applied on the statistically significant variables representing useful predictors.

Gene	SNP ID	Position	OR	CI_low (2.5%)	CI_high (97.5%)
TNFRSF1B	NA	12252522	NA	-	-
	rs636964	12253351	0.99	0.98	0.99
	NA	12254590	NA	-	-
SLC11A1	NA	219248015	NA	-	-
PARK2	rs3765474	161990483	0.87	0.80	0.96
	rs3765475	161990516	1.18	1.07	1.30
IL4R	rs2301807	27358098	0.9	0.86	0.93
	NA	27358103	NA	-	-

Abbreviations: OR, Odds Ratio; CI, confidence interval; NA, not available.

4.3 Discussion

We examined thirty-five genes involved in innate and adaptative immune responses, cancer, autophagy as well as previously associated with PCa (Table 7). Initially, we evaluated the distribution of allele and genotypes frequencies between PCa patients and HCs. rs2301807 *IL4R*, rs3746190 *IL12RB1*, rs2307145 *IL12RB2*, rs11701576 *TMPRSS2*, rs636964 *TNFRS1B*, rs3765474 *PARK2* were found inversely associated to PCa, whether rs2069832 *IL6*, rs140141551 *TMPRSS2* and rs35803318 *ACE2*, rs2301807 *PARK2* were positively associated to a higher PCa risk. The genetic variant CC and C of *IL4R* rs2301807 were significantly associated with a lower PCa risk. IL-4, a T-helper 2 (Th2) cytokine, is known to foster pro-metastatic phenotypes and enhance metastatic tumor burden through IL-4 receptor (IL-4R) in epithelial cancers [156]. IL-4R upregulation in the tumor microenvironment has been reported in several types of cancer, as breast, colon, pancreatic and prostate cancer. Accordingly, IL4R appears a plausible candidate to attenuate metastatic tumor growth. IL-4 neutralization by monoclonal antibody was able to lessen metastatic lung tumor burden *in vivo* [157] and the loss of IL-4Rα in tumor cells enabled the reduction of metastatic tumor growth [158]. Furthermore, IL-4 is known to activate androgen receptor in conditions of low androgen levels [159] as well to boost the growth of androgen-sensitive LNCaP cells in androgen-deprived conditions [160].

The genetic variant AA of rs3746190 *IL12RB1* and the alternative allele A of rs2307145 *IL12RB2* were found associated with a lower PCa risk and thus, they appear to have a protective effect toward PCa. IL-12, along with IL-6, IL-23 and IL-27, belong to the class I hematopoietin family of cytokines and mediate signaling through the JAK (Janus kinase)-STAT (signal transducer and activator of transcription protein) pathways [161]. IL-12 plays a critical role in stimulating natural killer (NK)-cell cytotoxicity, IFN-γ production during infection and inflammation and in the generation of T-helper type I cells [161].

The action of IL-12 is mediated by IL-12 receptor, a heterodimeric complex composed of IL-12Rβ1 and IL-12Rβ2. Serum levels of IL-12 p40 monomer are higher in PCa patients compared to HCs. IL-12 p40 neutralization stimulates apoptosis in prostate cancer cells since p40 helps cancer cell survival by internalizing of IL12RB1 [162].

The association between innate immunity, inflammation and advanced PCa was investigated in a case-control study in which inflammatory and innate immunity pathways played a modest role in the etiology of advanced PCa. However, no association was found between PCa and IL-12 receptor (IL-12Rβ1 and IL-12Rβ2) [163]. Furthermore, prostate tumor cells specifically stimulated the up-regulation of activation markers IL-12R, CD25 and PD-1 on invariant natural killer T (iNKT) cells [164].

Our data shows that the SNP located within *IL6* gene (rs2069832) is associated with a 2.5 fold increased risk of PCa than HCs. IL-6 is a pleiotropic cytokine implicated in the regulation of several cellular functions, such as proliferation, differentiation, apoptosis, angiogenesis and immune response [165]. Elevated serum IL-6 levels have been associated with an unfavorable prognosis in patients with different types of cancer, such as ovarian cancer, multiple myeloma, breast cancer, lung cancer and PCa [166]. Particularly, several studies confirmed an increase in IL-6 levels in patients with CRPC and metastatic PCa compared to individuals with earlier stages of the disease and healthy controls [167–169]. IL-6 genomic variants have been previously correlated with PCa risk, the A C haplotype (rs2069845 and rs1800795) was associated with PCa and BPH risk whilst the A G haplotype (rs2069845 and rs1800795) resulted to be protective against PCa and PBH risk [170]. The presence of rs2069832 *IL6* GG genotype and exposure to passive smoke have been associated to a four-fold increased risk to develop breast cancer [171]. In contrast, several studies failed to observe any association between *IL6* genetic variants and PCa risk [172–174]. IL-6 rs2069832 is an intronic SNP which may alter the expression of *IL6* gene.

TMPRSS2 gene encodes for a transmembrane serine protease 2 expressed by prostate epithelial cells. TMPRSS2 is upregulated in response to androgens in PCa cells, even though TMPRSS2 physiologic function remains unclear [175]. The most common chromosomal aberration is the fusion of the 5'UTR of *TMPRSS2* and *ERG*, an oncogenic transcription factor, which leads to the silencing of the promoterless copy of *TMPRSS2*. The contribution of TMPRSS2 to prostate carcinogenesis may also be associated to aberrant localization in subcellular compartment due to the loss of polarity in transformed cells. In these conditions, TMPRSS2 may gain access and/or activate some cancer-promoting substrates, whereas TMPRSS2 physiological subcellular location would preclude it [175]. Both rs140141551 and rs11701576 identified within *TMPRSS2* are intronic mutations; strikingly, rs11701576 falls within the recombination subregion of *TMPRSS2* that recombines with ERG recombination subregion. The alternative G allele of rs11701576 was found associated with a lower PCa risk, suggesting a possible protective effect of this SNP in PCa.

The *ACE2* gene encodes for the angiotensin-converting enzyme 2 (ACE2) which results overexpressed in several types of cancer, including PCa [176]. ACE2 expression appears to be protective against tumor progression in cancer tissue and is associated with favorable prognosis[177]. The rs35803318 identified within *ACE2* is a synonymous variant which, according to our analysis, appears to be positively associated to a higher PCa risk. A recent study pinpointed though an increased presence of rs35803318 in Italian, European, and American cohorts compared to the very low frequency detected in Asian and African populations [178]. The rs35803318 is a synonymous variant and, it does not determine any amino acid change in the protein sequence nor in the protein function. New evidence suggests a critical role for synonymous mutations in human disease, cancer, clinical outcome and therapy response.

Furthermore, synonymous mutations can alter mRNA stability, miRNA binding sites, protein levels or conformation by modifying splicing regulatory sites [179]. Oncogenes exhibits high levels of synonymous substitutions even though no evidence is found in tumor suppressor genes [180,181].

The *PARK2* gene encodes for an E3 ubiquitin ligase (*Parkin*) and plays a pivotal role in protein ubiquitination and degradation. *Parkin* protein was first identified as a gene implicated in autosomal recessive juvenile Parkinsonism [182]. Several studies attributed a tumor suppressive function to *PARK2*. Particularly, inactivating somatic mutations and intragenic deletions seem to abrogate *PARK2*'s E3 ligase activity and growth-suppressive effect by undermining *Parkin* ability to ubiquitinate cyclin E [183]. Additionally, *parkin* -/- mice lacking exon 3 had enhanced hepatocyte proliferation and develop macroscopic tumors with the traits of hepatocellular carcinoma [184]. The presence of somatic mutations in solid cancers is not common and mutational events may be even rare in colorectal, gastric, breast, lung, and prostate cancer[185].

In the present study, the multivariate analysis performed on the significant predictors pointed out two intronic mutations within the *PARK2* gene to be associated with PCa risk: rs3765474 and rs3765475. The first was found correlated with a lower (PCa) risk (OR: 0.87), whilst the latter was associated with a higher PCa risk (OR: 1.18). This result is difficult to interpret given the lack of studies regarding *PARK2* polymorphisms in PCa as well the function of the E3 ubiquitin ligase in prostate carcinogenesis. This study presents some limitations. First, the sample size of our cohort is small and this affects the reliability of the results. Second, few studies are available for some of the genes analyzed, therefore further studies are necessary to evaluate the prevalence, penetrance and functional impact of the variants in PCa pathogenesis.

Overall, we used a gene panel approach to characterize genetic modifications in a cohort of individuals with PCa compared to a cohort of healthy individuals. These findings shed light on novel SNPs and their possible impact not only on PCa risk, but also prognosis, survival and the patient response to cancer therapy.

Chapter 5 - References

1. Taitt, H.E. Global Trends and Prostate Cancer: A Review of Incidence, Detection, and Mortality as Influenced by Race, Ethnicity, and Geographic Location. *Am. J. Mens. Health* **2018**, *12*, 1807–1823, doi:10.1177/1557988318798279.

2. Spandonaro, F.; D'angela, D.; Polistena, B.; Bruzzi, P.; Iacovelli, R.; Luccarini, I.; Stagni, P.; Brigido, A. Prevalence of prostate cancer at different clinical stages in italy: Estimated burden of disease based on a modelling study. *Biology (Basel).* **2021**, *10*, 1–7, doi:10.3390/biology10030210.

3. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J. Clin.* **2021**, *71*, 209–249, doi:10.3322/caac.21660.

4. Fitzmaurice, C.; Abate, D.; Abbasi, N.; Abbastabar, H.; Abd-Allah, F.; Abdel-Rahman, O.; Abdelalim, A.; Abdoli, A.; Abdollahpour, I.; Abdulle, A.S.M.; et al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-Adjusted life-years for 29 cancer groups, 1990 to 2017: A systematic analysis for the global burden of disease study. *JAMA Oncol.* **2019**, *5*, 1749–1768, doi:10.1001/jamaoncol.2019.2996.

5. Barsouk, A.; Padala, S.A.; Vakiti, A.; Mohammed, A.; Saginala, K.; Thandra, K.C.; Rawla, P.; Barsouk, A. Epidemiology, Staging and Management of Prostate Cancer. *Med. Sci.* **2020**, *8*, 28, doi:10.3390/medsci8030028.

6. Rebbeck, T.R.; Devesa, S.S.; Chang, B.-L.; Bunker, C.H.; Cheng, I.; Cooney, K.; Eeles, R.; Fernandez, P.; Giri, V.N.; Gueye, S.M.; et al. Global Patterns of Prostate Cancer Incidence, Aggressiveness, and Mortality in Men of African Descent. *Prostate Cancer*

2013, *2013*, 1–12, doi:10.1155/2013/560857.

7. Allott, E.H.; Masko, E.M.; Freedland, S.J. Obesity and prostate cancer: Weighing the evidence. *Eur. Urol.* **2013**, *63*, 800–809, doi:10.1016/j.eururo.2012.11.013.

8. Zhu, Y.; Li, Q.; Xu, X. Dietary inflammatory index and the risk of prostate cancer: a dose-response meta-analysis. *Eur. J. Clin. Nutr.* **2020**, *74*, 1001–1008, doi:10.1038/s41430-019-0500-3.

9. Preble, I.; Zhang, Z.; Kopp, R.; Garzotto, M.; Bobe, G.; Shannon, J.; Takata, Y. Dairy product consumption and prostate cancer risk in the United States. *Nutrients* **2019**, *11*, 1–12, doi:10.3390/nu11071615.

10. Matsushita, M.; Fujita, K. Influence of Diet and Nutrition on Prostate Cancer. **2020**, 1–18.

11. Yoshida, K.; Miki, Y. Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci.* **2004**, *95*, 866–871, doi:10.1111/j.1349-7006.2004.tb02195.x.

12. Zhao, W.; Wiese, C.; Kwon, Y.; Hromas, R.; Sung, P. The BRCA tumor suppressor network in chromosome damage repair by homologous recombination. *Annu. Rev. Biochem.* **2019**, *88*, 221–245, doi:10.1146/annurev-biochem-013118-111058.

13. Paul, A.; Paul, S. The breast cancer susceptibility genes (BRCA) in breast and ovarian cancers. *Front. Biosci. - Landmark* **2014**, *19*, 605–618, doi:10.2741/4230.

14. Pritchard, C.C.; Mateo, J.; Walsh, M.F.; De Sarkar, N.; Abida, W.; Beltran, H.; Garofalo, A.; Gulati, R.; Carreira, S.; Eeles, R.; et al. Inherited DNA-Repair Gene Mutations in Men with Metastatic Prostate Cancer. *N. Engl. J. Med.* **2016**, *375*, 443–453, doi:10.1056/nejmoa1603144.

15. Risdon, E.N.; Chau, C.H.; Price, D.K.; Sartor, O.; Figg, W.D. PARP Inhibitors and Prostate Cancer: To Infinity and Beyond BRCA . *Oncologist* **2021**, *26*, e115–e129,

doi:10.1634/theoncologist.2020-0697.

16. Xu, J.; Lange, E.M.; Lu, L.; Zheng, S.L.; Wang, Z.; Thibodeau, S.N.; Cannon-Albright, L.A.; Teerlink, C.C.; Camp, N.J.; Johnson, A.M.; et al. HOXB13 is a susceptibility gene for prostate cancer: Results from the International Consortium for Prostate Cancer Genetics (ICPCG). *Hum. Genet.* **2013**, *132*, 5–14, doi:10.1007/s00439-012-1229-4.

17. Leongamornlert, D.; Mahmud, N.; Tymrakiewicz, M.; Saunders, E.; Dadaev, T.; Castro, E.; Goh, C.; Govindasami, K.; Guy, M.; O'Brien, L.; et al. Germline BRCA1 mutations increase prostate cancer risk. *Br. J. Cancer* **2012**, *106*, 1697–1701, doi:10.1038/bjc.2012.146.

18. Rebbeck, T.R. Prostate Cancer Genetics: Variation by Race, Ethnicity, and Geography. *Semin. Radiat. Oncol.* **2017**, *27*, doi:10.1016/j.semradonc.2016.08.002.

19. Conti, D. V.; Darst, B.F.; Moss, L.C.; Saunders, E.J.; Sheng, X.; Chou, A.; Schumacher, F.R.; Olama, A.A. Al; Benlloch, S.; Dadaev, T.; et al. Trans-ancestry genome-wide association meta-analysis of prostate cancer identifies new susceptibility loci and informs genetic risk prediction. *Nat. Genet.* **2021**, *53*, 65–75, doi:10.1038/s41588-020-00748-0.

20. J.Veldscholte, C.Ris-Stalpers, G.G.J.M.Kuiper, G.Jenster, C.Berrevoets, E.Claassen, H.C.J.van Rooij, J.Trapman, A.O.B.; E.Mulder1 A mutation in the ligand binding domain of the androgen receptor of human L NCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem. Biophys. Res. Commun.* **1990**, *173*, 534–540.

21. M. E. Taplin, G. J. Bubley, T. D. Shuster, M. E. Frantz, A. E. Spooner, G. K. Ogata, H. N. Keer, S.P.; Balk Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N. Engl. J. Med.* **1995**, *332*, 1393–8.

22. Carter, B.S.; Ewing, C.M.; Ward, W.S.; Treiger, B.F.; Aalders, T.W.; Schalken, J.A.;

Epstein, J.I.; Isaacs, W.B. Allelic loss of chromosomes 16q and 10q in human prostate cancer. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87*, 8751–8755, doi:10.1073/pnas.87.22.8751.

23. Wise, H.M.; Hermida, M.A.; Leslie, N.R. Prostate cancer, PI3K, PTEN and prognosis. *Clin. Sci.* **2017**, *131*, 197–210, doi:10.1042/CS20160026.

24. Tomlins, S.A.; Bjartell, A.; Chinnaiyan, A.M.; Jenster, G.; Nam, R.K.; Rubin, M.A.; Schalken, J.A. ETS Gene Fusions in Prostate Cancer: From Discovery to Daily Clinical Practice. *Eur. Urol.* **2009**, *56*, 275–286, doi:10.1016/j.eururo.2009.04.036.

25. Han, B.; Mehra, R.; Dhanasekaran, S.M.; Yu, J.; Menon, A.; Lonigro, R.J.; Wang, X.; Gong, Y.; Wang, L.; Shankar, S.; et al. A fluorescence in situ hybridization screen for E26 transformation-specific aberrations: Identification of DDX5-ETV4 fusion protein in prostate cancer. *Cancer Res.* **2008**, *68*, 7629–7637, doi:10.1158/0008-5472.CAN-08-2014.

26. Esgueva, R.; Perner, S.; Lafargue, C.; Scheble, V.; Lein, M.; Fritzsche, F.R.; Dietel, M.; Kristiansen, G.; Rubin, M.A. Prevalence of TMPRSS2-ERG and SLC45A3-ERG gene fusions in a large prostatectomy cohort. *Mod. Pathol.* **2010**, *23*, 539–546, doi:10.1038/modpathol.2009.193.Prevalence.

27. Vietri, M.T.; D'elia, G.; Caliendo, G.; Resse, M.; Casamassimi, A.; Passariello, L.; Albanese, L.; Cioffi, M.; Molinari, A.M. Hereditary prostate cancer: Genes related, target therapy and prevention. *Int. J. Mol. Sci.* **2021**, *22*, doi:10.3390/ijms22073753.

28. Zhen, J.T.; Syed, J.; Nguyen, K.A.; Leapman, M.S.; Agarwal, N.; Brierley, K.; Llor, X.; Hofstatter, E.; Shuch, B. Genetic testing for hereditary prostate cancer: Current status and limitations. *Cancer* **2018**, *124*, 3105–3117, doi:10.1002/cncr.31316.

29. Rebbeck, T.R. Prostate Cancer Genetics: Variation by Race, Ethnicity, and Geography. *Semin. Radiat. Oncol.* **2017**, *27*, 3–10, doi:10.1016/j.semradonc.2016.08.002.

30. JOHN E. NIEDERHUBER, JAMES O. ARMITAGE, JAMES H. DOROSHOW, MICHAEL B. KASTAN, J.E.T. *Abeloff's Clinical Oncology*; 2020; ISBN 9781119130536.

31. Omar Franco, M.D., P.. et al. Review of Prostate Anatomy and Embryology and the Etiology of BPH. *Urol Clin North Am* **2016**, *43*, 279–288, doi:10.1016/j.ucl.2016.04.012.Review.

32. McNeal, J.E. Origin and development of carcinoma in the prostate. *Cancer* **1969**, *23*, 24–34, doi:10.1002/1097-0142(196901)23:1<24::AID-CNCR2820230103>3.0.CO;2-1.

33. McNeal, J.E. The zonal anatomy of the prostate. *Prostate* **1981**, *2*, 35–49, doi:10.1002/pros.2990020105.

34. McNeal Normal Histology of the Prostate.pdf. *Am. J. Surg. Pathol.* 1999, *12*.

35. McNeal J.E., Redwine E.A., Freiha F.S., S.T.A. Zonal distribution of prostatic adenocarcinoma.

36. Haffner, J.; Potiron, E.; Bouyé, S.; Puech, P.; Leroy, X.; Lemaitre, L.; Villers, A. Peripheral zone prostate cancers: Location and intraprostatic patterns of spread at histopathology. *Prostate* **2009**, *69*, 276–282, doi:10.1002/pros.20881.

37. Lee, C.H.; Akin-Olugbade, O.; Kirschenbaum, A. Overview of Prostate Anatomy, Histology, and Pathology. *Endocrinol. Metab. Clin. North Am.* **2011**, *40*, 565–575, doi:10.1016/j.ecl.2011.05.012.

38. Bostwick, D.G.; Qian, J. High-grade prostatic intraepithelial neoplasia. *Mod. Pathol.* **2004**, *17*, 360–379, doi:10.1038/modpathol.3800053.

39. Wang, Y.; Hayward, S.W.; Cao, M.; Thayer, K.A.; Cunha, G.R. Cell differentiation lineage in the prostate. *Differentiation* **2001**, *68*, 270–279, doi:10.1046/j.1432-0436.2001.680414.x.

40. Ramaekers, F.C.S.; Aalders, T.W.; Debruyne, F.M.J.; Schalken, J.A. Colocalization of Basal and Luminal Cell-type Cytokeratins in Human Prostate Cancer. *Cancer Res.* **1992**, *52*, 6182–6187.

41. Mirosevich, J.; Bentel, J.M.; Zeps, N.; Redmond, S.L.; D'Antuono, M.F.; Dawkins,

H.J.S. Androgen receptor expression of proliferating basal and luminal cells in adult murine ventral prostate. *J. Endocrinol.* **1999**, *162*, 341–350, doi:10.1677/joe.0.1620341.

42. El-Alfy, M.; Luu-The, V.; Huang, X.F.; Berger, L.; Labrie, F.; Pelletier, G. Localization of type 5 17β-hydroxysteroid dehydrogenase, 3β- hydroxysteroid dehydrogenase, and androgen receptor in the human prostate by in situ hybridization and immunocytochemistry. *Endocrinology* **1999**, *140*, 1481–1491, doi:10.1210/endo.140.3.6585.

43. Abrahamsson, P.A. Neuroendocrine cells in tumour growth of the prostate. *Endocr. Relat. Cancer* **1999**, *6*, 503–519, doi:10.1677/erc.0.0060503.

44. Verze, P.; Cai, T.; Lorenzetti, S. The role of the prostate in male fertility, health and disease. *Nat. Rev. Urol.* **2016**, *13*, 379–386, doi:10.1038/nrurol.2016.89.

45. Dai, C.; Heemers, H.; Sharifi, N. Androgen signaling in prostate cancer. *Cold Spring Harb. Perspect. Med.* **2017**, *7*, doi:10.1158/1078-0432.CCR-17-0528.

46. Tan, M.E.; Li, J.; Xu, H.E.; Melcher, K.; Yong, E.L. Androgen receptor: Structure, role in prostate cancer and drug discovery. *Acta Pharmacol. Sin.* **2015**, *36*, 3–23, doi:10.1038/aps.2014.18.

47. Dornbier, R.; Pahouja, G.; Branch, J.; McVary, K.T. The New American Urological Association Benign Prostatic Hyperplasia Clinical Guidelines: 2019 Update. *Curr. Urol. Rep.* **2020**, *21*, doi:10.1007/s11934-020-00985-0.

48. Wei, J.T.; Calhoun, E.; Jacobsen, S.J. Urologic Diseases in America Project: Benign Prostatic Hyperplasia. *J. Urol.* **2008**, *179*, 1256–1261, doi:10.1016/j.juro.2008.03.141.

49. Chughtai, B.; Forde, J.C.; Thomas, D.D.M.; Laor, L.; Hossack, T.; Woo, H.H.; Te, A.E.; Kaplan, S.A. Benign prostatic hyperplasia. *Nat. Rev. Dis. Prim.* **2016**, *2*, 1–15, doi:10.1038/nrdp.2016.31.

50. Ming Jianga,Douglas W. Stranda, Omar E. Francoa, Peter E. Clarka, and S.W.H.

PPARγ: A Molecular Link between systemic metabolic disease and benign prostate hyperplasia. *Differentiation* **2011**, *82*, 220–236, doi:10.1016/j.diff.2011.05.008.PPAR.

51. Robert, G.; Descazeaud, A.; Nicolaïew, N.; Terry, S.; Sirab, N.; Vacherot, F.; Maillé, P.; Allory, Y.; De La Taille, A. Inflammationin benign prostatic hyperplasia: A 282 patients' immunohistochemical analysis. *Prostate* **2009**, *69*, 1774–1780, doi:10.1002/pros.21027.

52. Gravas, S.; Cornu, J.; Gacci, M.; Gratzke, C.; Herrmann, T.; Mamoulakis, C.; Rieken, M.; Speakman, M.; Tikkinen, K. EAU Guidelines on Non-Neurogenic Male LUTS incl. BPO 2020. **2020**.

53. Wiener, S.; Haddock, P.; Cusano, J.; Staff, I.; McLaughlin, T.; Wagner, J. Incidence of Clinically Significant Prostate Cancer After a Diagnosis of Atypical Small Acinar Proliferation, High-grade Prostatic Intraepithelial Neoplasia, or Benign Tissue. *Urology* **2017**, *110*, 161–165, doi:10.1016/j.urology.2017.08.040.

54. Ploussard, G.; Plennevaux, G.; Allory, Y.; Salomon, L.; Azoulay, S.; Vordos, D.; Hoznek, A.; Abbou, C.C.; la Taille, A. High-grade prostatic intraepithelial neoplasia and atypical small acinar proliferation on initial 21-core extended biopsy scheme: Incidence and implications for patient care and surveillance. *World J. Urol.* **2009**, *27*, 587–592, doi:10.1007/s00345-009-0413-1.

55. López, J.I. Prostate adenocarcinoma detected after high-grade prostatic intraepithelial neoplasia or atypical small acinar proliferation. *BJU Int.* **2007**, *100*, 1272–1276, doi:10.1111/j.1464-410X.2007.07122.x.

56. Manning, T.G.; Cheung, E.; Perera, M.; Christidis, D.; O'Brien, J.S.; Mitchell, C.; Bolton, D.M.; Lawrentschuk, N. Atypical Small Acinar Proliferation and High-grade Prostatic Intraepithelial Neoplasia in the Era of Multiparametric Magnetic Resonance Imaging: A Contemporary Review. *Urology* **2017**, *107*, 5–10, doi:10.1016/j.urology.2017.04.021.

57. Gleason, D.F. Histologic grading of prostate cancer: A perspective. *Hum. Pathol.* **1992**, *23*, 273–279, doi:10.1016/0046-8177(92)90108-F.

58. Li, J.; Wang, Z. The pathology of unusual subtypes of prostate cancer. *Chinese J. Cancer Res.* **2016**, *28*, 130–143, doi:10.3978/j.issn.1000-9604.2016.01.06.

59. Ranasinha, N.; Omer, A.; Philippou, Y.; Harriss, E.; Davies, L.; Chow, K.; Chetta, P.M.; Erickson, A.; Rajakumar, T.; Mills, I.G.; et al. Ductal adenocarcinoma of the prostate: A systematic review and meta-analysis of incidence, presentation, prognosis, and management. *BJUI Compass* **2021**, *2*, 13–23, doi:10.1002/bco2.60.

60. Moch, H.; Cubilla, A.L.; Humphrey, P.A.; Reuter, V.E.; Ulbright, T.M. The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs—Part A: Renal, Penile, and Testicular Tumours. *Eur. Urol.* **2016**, *70*, 93–105, doi:10.1016/j.eururo.2016.02.029.

61. Humphrey, P.A. Histopathology of prostate cancer. *Cold Spring Harb. Perspect. Med.* **2017**, *7*, 1–21, doi:10.1101/cshperspect.a030411.

62. Aggarwal, R.; Zhang, T.; Small, E.J.; Armstrong, A.J. 719 Neuroendocrine Prostate Cancer: Subtypes, Biology, and Clinical Outcomes NCCN: Continuing Education Accreditation Statement. *J. Natl. Compr. Cancer Netw.* | **2014**, *12*, 719–726.

63. Litwin, M.S.; Tan, H.J. The diagnosis and treatment of prostate cancer: A review. *JAMA - J. Am. Med. Assoc.* **2017**, *317*, 2532–2542, doi:10.1001/jama.2017.7248.

64. Epstein, J.I.; Egevad, L.; Amin, M.B.; Delahunt, B.; Srigley, J.R.; Humphrey, P.A. The 2014 international society of urological pathology (ISUP) consensus conference on gleason grading of prostatic carcinoma definition of grading patterns and proposal for a new grading system. *Am. J. Surg. Pathol.* **2016**, *40*, 244–252, doi:10.1097/PAS.0000000000000530.

65. Magi-galluzzi, C.; Vickers, A.J.; Parwani, A. V; Reuter, V.E. Epstein 2016 A contemporary prostate cancer grading system.pdf. *Eur Urol* **2015**, *69*, 428–435,

doi:10.1016/j.eururo.2015.06.046.A.

66. Bjurlin, M.A.; Carter, H.B.; Schellhammer, P.; Cookson, M.S.; Gomella, L.G.; Troyer, D.; Wheeler, T.M.; Schlossberg, S.; Penson, D.F.; Taneja, S.S. Optimization of initial prostate biopsy in clinical practice: Sampling, labeling and specimen processing. *J. Urol.* **2013**, *189*, 2039–2046, doi:10.1016/j.juro.2013.02.072.

67. Uhr, A.; Glick, L.; Gomella, L.G. An overview of biomarkers in the diagnosis and management of prostate cancer. *Can. J. Urol.* **2020**, *27*, 24–27.

68. Saini, S. PSA and beyond: alternative prostate cancer biomarkers. *Cell. Oncol.* **2016**, *39*, 97–106, doi:10.1007/s13402-016-0268-6.

69. Lander, E.S.; Linton, L.M.; Birren, B.; Nusbaum, C.; Zody, M.C.; Baldwin, J.; Devon, K.; Dewar, K.; Doyle, M.; Fitzhugh, W.; et al. Erratum: Initial sequencing and analysis of the human genome: International Human Genome Sequencing Consortium (Nature (2001) 409 (860-921)). *Nature* **2001**, *412*, 565–566, doi:10.1038/35087627.

70. Vargiu, L.; Rodriguez-Tomé, P.; Sperber, G.O.; Cadeddu, M.; Grandi, N.; Blikstad, V.; Tramontano, E.; Blomberg, J. Classification and characterization of human endogenous retroviruses mosaic forms are common. *Retrovirology* **2016**, *13*, 1–29, doi:10.1186/s12977-015-0232-y.

71. Hayward, A.; Grabherr, M.; Jern, P. Broad-scale phylogenomics provides insights into retrovirus - Host evolution. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 20146–20151, doi:10.1073/pnas.1315419110.

72. Hayward, A.; Cornwallis, C.K.; Jern, P. Pan-vertebrate comparative genomics unmasks retrovirus macroevolution. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 464–469, doi:10.1073/pnas.1414980112.

73. Poeran Reverse Transcription of Retroviruses and LTR Retrotransposons STEPHEN. *Physiol. Behav.* **2015**, *3*, 3–27, doi:10.1128/microbiolspec.MDNA3-0027-2014.Reverse.

74. Garcia-Montojo, M.; Doucet-O'Hare, T.; Henderson, L.; Nath, A. Human endogenous retrovirus-K (HML-2): a comprehensive review. *Crit. Rev. Microbiol.* 2018, *44*, 715–738.

75. Tomlins, S.A.; Laxman, B.; Dhanasekaran, S.M.; Helgeson, B.E.; Cao, X.; Morris, D.S.; Menon, A.; Jing, X.; Cao, Q.; Han, B.; et al. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature* **2007**, *448*, 595–599, doi:10.1038/nature06024.

76. Vogt, J.; Wernstedt, A.; Ripperger, T.; Pabst, B.; Zschocke, J.; Kratz, C.; Wimmer, K. PMS2 inactivation by a complex rearrangement involving an HERV retroelement and the inverted 100-kb duplicon on 7p22.1. *Eur. J. Hum. Genet.* **2016**, *24*, 1598–1604, doi:10.1038/ejhg.2016.75.

77. Duelli, D.; Lazebnik, Y. Cell fusion: A hidden enemy? *Cancer Cell* **2003**, *3*, 445–448, doi:10.1016/S1535-6108(03)00114-4.

78. Bjerregaard, B.; Holck, S.; Christensen, I.J.; Larsson, L.I. Syncytin is involved in breast cancer-endothelial cell fusions. *Cell. Mol. Life Sci.* **2006**, *63*, 1906–1911, doi:10.1007/s00018-006-6201-9.

79. Mangeney, M.; De Parseval, N.; Thomas, G.; Heidmann, T. The full-length envelope of an HERV-H human endogenous retrovirus has immunosuppressive properties. *J. Gen. Virol.* **2001**, *82*, 2515–2518, doi:10.1099/0022-1317-82-10-2515.

80. Mangeney, M.; Pothlichet, J.; Renard, M.; Ducos, B.; Heidmann, T. Endogenous retrovirus expression is required for murine melanoma tumor growth in vivo. *Cancer Res.* **2005**, *65*, 2588–2591, doi:10.1158/0008-5472.CAN-04-4231.

81. Kudo-Saito, C.; Yura, M.; Yamamoto, R.; Kawakami, Y. Induction of immunoregulatory CD271+ cells by metastatic tumor cells that express human endogenous retrovirus H. *Cancer Res.* **2014**, *74*, 1361–1370, doi:10.1158/0008-5472.CAN-13-1349.

82. Lavialle, C.; Cornelis, G.; Dupressoir, A.; Esnault, C.; Heidmann, O.; Vernochet, C.; Heidmann, T. Paleovirology of "syncytins", retroviral env genes exapted for a role in placentation. *Philos. Trans. R. Soc. B Biol. Sci.* **2013**, *368*, doi:10.1098/rstb.2012.0507.

83. Morozov, V.A.; Dao Thi, V.L.; Denner, J. The Transmembrane Protein of the Human Endogenous Retrovirus - K (HERV-K) Modulates Cytokine Release and Gene Expression. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0070399.

84. Le Dantec, C.; Vallet, S.; Brooks, W.H.; Renaudineau, Y. Human endogenous retrovirus group E and its involvement in diseases. *Viruses* **2015**, *7*, 1238–1257, doi:10.3390/v7031238.

85. Schmitt, K.; Heyne, K.; Roemer, K.; Meese, E.; Mayer, J. HERV-K(HML-2) Rec and Np9 transcripts not restricted to disease but present in many normal human tissues. *Mob. DNA* **2015**, *6*, doi:10.1186/s13100-015-0035-7.

86. Kaufmann, S.; Sauter, M.; Schmitt, M.; Baumert, B.; Best, B.; Boese, A.; Roemer, K.; Mueller-Lantzsch, N. Human endogenous retrovirus protein Rec interacts with the testicular zinc-finger protein and androgen receptor. *J. Gen. Virol.* **2010**, *91*, 1494–1502, doi:10.1099/vir.0.014241-0.

87. Hanke, K.; Chudak, C.; Kurth, R.; Bannert, N. The Rec protein of HERV-K(HML-2) upregulates androgen receptor activity by binding to the human small glutamine-rich tetratricopeptide repeat protein (hSGT). *Int. J. Cancer* **2013**, *132*, 556–567, doi:10.1002/ijc.27693.

88. Armbruester, V.; Sauter, M.; Roemer, K.; Best, B.; Hahn, S.; Nty, A.; Schmid, A.; Philipp, S.; Mueller, A.; Mueller-Lantzsch, N. Np9 Protein of Human Endogenous Retrovirus K Interacts with Ligand of Numb Protein X. *J. Virol.* **2004**, *78*, 10310–10319, doi:10.1128/jvi.78.19.10310-10319.2004.

89. Downey, R.F.; Sullivan, F.J.; Wang-Johanning, F.; Ambs, S.; Giles, F.J.; Glynn, S.A. Human endogenous retrovirus K and cancer: Innocent bystander or tumorigenic

accomplice? *Int. J. Cancer* **2015**, *137*, 1249–1257, doi:10.1002/ijc.29003.

90. Roy, M.; Pear, W.S.; Aster, J.C. The multifaceted role of Notch in cancer. *Curr. Opin. Genet. Dev.* **2007**, *17*, 52–59, doi:10.1016/j.gde.2006.12.001.

91. Rolle, K.; Rivero-müller, A.; Nees, M. Progression and Metastasis. **2021**.

92. Huang, W.; Chen, T.; Meng, Z.; Gan, Y.; Wang, X.; Xu, F.; Gu, Y.; Xu, X.; Tang, J.; Zhou, H.; et al. The viral oncogene Np9 acts as a critical molecular switch for co-activating β-catenin, ERK, Akt and Notch1 and promoting the growth of human leukemia stem/progenitor cells. *Leukemia* **2013**, *27*, 1469–1478, doi:10.1038/leu.2013.8.

93. Zhang, J.; Lou, X.; Zellmer, L.; Liu, S.; Xu, N.; Liao, D.J. Just like the rest of evolution in Mother Nature, the evolution of cancers may be driven by natural selection, and not by haphazard mutations. *Oncoscience* **2014**, *1*, 580–590, doi:10.18632/oncoscience.83.

94. Nevedomskaya, E.; Baumgart, S.J.; Haendler, B. Recent advances in prostate cancer treatment and drug discovery. *Int. J. Mol. Sci.* **2018**, *19*, doi:10.3390/ijms19051359.

95. Pérot, P.; Cheynet, V.; Decaussin-Petrucci, M.; Oriol, G.; Mugnier, N.; Rodriguez-Lafrasse, C.; Ruffion, A.; Mallet, F. Microarray-based identification of individual HERV loci expression: application to biomarker discovery in prostate cancer. *J. Vis. Exp.* **2013**, 1–13, doi:10.3791/50713.

96. Yi, J.M.; Kim, H.M.; Kim, H.S. Expression of human endogenous retrovirus HERV-W family in various human tissues and cancer cells. *J. Gen. Virol.* **2004**, *85*, 1203–1210, doi:10.1099/vir.0.79791-0.

97. Larsen, J.M.; Christensen, I.J.; Nielsen, H.J.; Hansen, U.; Bjerregaard, B.; Talts, J.F.; Larsson, L.I. Syncytin immunoreactivity in colorectal cancer: Potential prognostic impact. *Cancer Lett.* **2009**, *280*, 44–49, doi:10.1016/j.canlet.2009.02.008.

98. Strissel, P.L.; Ruebner, M.; Thiel, F.; Wachter, D.; Ekici, A.B.; Wolf, F.; Thieme, F.;

Ruprecht, K.; Beckmann, M.W.; Strick, R. Reactivation of codogenic endogenous retroviral (ERV) envelope genes in human endometrial carcinoma and prestages: Emergence of new molecular targets. *Oncotarget* **2012**, *3*, 1204–1219, doi:10.18632/oncotarget.679.

99. Pawelek, J.M.; Chakraborty, A.K. Fusion of tumour cells with bone marrow-derived cells: a unifying explanation for metastasis John. *Nat. Rev. Cancer* **2008**, *8*, 377–386.

100. Their, J.P. Epithelial-mesenchymal transitions in tumor progression. *Nat. Rev. Cancer* **2002**, *2*, 442–454, doi:10.1038/nrc822.

101. Stauffer Y., Theiler G., Sperisen P., Lebedev Y., and J.C. V. Digital expression profiles of human endogenous retroviral families in normal and cancerous tissues. *Cancer Immun.* **2004**, *4*.

102. Ishida, T.; Obata, Y.; Ohara, N.; Matsushita, H.; Sato, S.; Uenaka, A.; Saika, T.; Miyamura, T.; Chayama, K.; Nakamura, Y.; et al. Identification of the HERV-K gag antigen in prostate cancer by SEREX using autologous patient serum and its immunogenicity. *Cancer Immun.* **2008**, *8*, 1–10.

103. Agoni, L.; Guha, C.; Lenz, J. Detection of human endogenous retrovirus K (HERV-K) transcripts in human prostate cancer cell lines. *Front. Oncol.* **2013**, *3 JUL*, 1–12, doi:10.3389/fonc.2013.00180.

104. Wallace, T.A.; Downey, R.F.; Seufert, C.J.; Schetter, A.; Dorsey, T.H.; Johnson, C.A.; Goldman, R.; Loffredo, C.A.; Yan, P.; Sullivan, F.J.; et al. Elevated HERV-K mRNA expression in PBMC is associated with a prostate cancer diagnosis particularly in older men and smokers. *Carcinogenesis* **2014**, *35*, 2074–2083, doi:10.1093/carcin/bgu114.

105. Reis, B.S.; Jungbluth, A.A.; Frosina, D.; Holz, M.; Ritter, E.; Nakayama, E.; Ishida, T.; Obata, Y.; Carver, B.; Scher, H.; et al. Prostate cancer progression correlates with increased humoral immune response to a human endogenous retrovirus GAG

protein. *Clin. Cancer Res.* **2013**, *19*, 6112–6125, doi:10.1158/1078-0432.CCR-12-3580.

106. Rezaei, S. D.; Hayward, J. A.; Norden, S.; Pedersen, J.; Millis, J.; Hearps, A. C.; Tachedjian, G.. HERV-K Gag RNA and Protein Levels Are Elevated Prostate Cancer. **2021**, *13*, 1–12, doi:doi.org/10.3390/v13030449.

107. Abeshouse, A.; Ahn, J.; Akbani, R.; Ally, A.; Amin, S.; Andry, C.D.; Annala, M.; Aprikian, A.; Armenia, J.; Arora, A.; et al. The Molecular Taxonomy of Primary Prostate Cancer. *Cell* **2015**, *163*, 1011–1025, doi:10.1016/j.cell.2015.10.025.

108. Hermans, K.G.; Van Der Korput, H.A.; Van Marion, R.; Van De Wijngaart, D.J.; Van Der Made, A.Z.; Dits, N.F.; Boormans, J.L.; Van Der Kwast, T.H.; Van Dekken, H.; Bangma, C.H.; et al. Truncated ETV1, fused to novel tissue-specific genes, and full-length ETV1 in prostate cancer. *Cancer Res.* **2008**, *68*, 7541–7549, doi:10.1158/0008-5472.CAN-07-5930.

109. Grandi, N.; Tramontano, E. Type W human endogenous retrovirus (HERV-W) integrations and their mobilization by L1 machinery: Contribution to the human transcriptome and impact on the host physiopathology. *Viruses* **2017**, *9*, 1–37, doi:10.3390/v9070162.

110. Menendez, L.; Benigno, B.B.; Mcdonald, J.F. L1 and HERV-W retrotransposons are hypomethylated in human ovarian carcinomas. **2004**, *5*, 1–5.

111. Díaz-Carballo, D.; Acikelli, A.H.; Klein, J.; Jastrow, H.; Dammann, P.; Wyganowski, T.; Guemues, C.; Gustmann, S.; Bardenheuer, W.; Malak, S.; et al. Therapeutic potential of antiviral drugs targeting chemorefractory colorectal adenocarcinoma cells overexpressing endogenous retroviral elements. *J. Exp. Clin. Cancer Res.* **2015**, *34*, 1–13, doi:10.1186/s13046-015-0199-5.

112. Hu, L.; Uzhameckis, D.; Hedborg, F.; Blomberg, J. Dynamic and selective HERV RNA expression in neuroblastoma cells subjected to variation in oxygen tension and demethylation. *Apmis* **2016**, *124*, 140–149, doi:10.1111/apm.12494.

113. Liang, Q.; Xu, Z.; Xu, R.; Wu, L.; Zheng, S. Expression patterns of non-coding spliced transcripts from human endogenous retrovirus HERV-H elements in colon cancer. *PLoS One* **2012**, *7*, doi:10.1371/journal.pone.0029950.

114. Apostolia M. Tsimberidou, Elena Fountzilas, M.N.R.K. Review of Precision Cancer Medicine: Evolution of the Treatment Paradigm. *Cancer Treat Rev* **2020**, *86*, doi:10.1016/j.ctrv.2020.102019.Review.

115. Bodmer, W.F. Evolutionary significance of the HL-A system. *Nature* **1972**, *237*, 139–183, doi:10.1038/237139a0.

116. Jatin M. Vyas, Annemartha G. Van der Veen, H.L.P. The known unknowns of antigen processing and presentation. *Nat. Rev. Immunol.* **2008**, *8*, 607–618, doi:10.1038/nri2368.The.

117. Fu, C.; Jiang, A. Dendritic Cells and CD8 T Cell Immunity in Tumor Microenvironment. *Front. Immunol.* **2018**, *9*, 1–11, doi:10.3389/fimmu.2018.03059.

118. Hillman, G.G.; Kallinteris, N.L.; Lu, X.; Wang, Y.; Wright, J.L.; Li, Y.; Wu, S.; Forman, J.D.; Gulfo, J. V.; Humphreys, R.E.; et al. Turning tumor cells in situ into T-helper cell-stimulating, MHC class II tumor epitope-presenters: Immuno-curing and immuno-consolidation. *Cancer Treat. Rev.* **2004**, *30*, 281–290, doi:10.1016/j.ctrv.2003.08.002.

119. Mahdil, B. mutar A glow of HLA typing in organ transplantation. *Clin. Transl. Sci.* **2013**, *2*, 1, doi:10.1186/2001-1326-2-6.

120. Hazini, A.; Fisher, K.; Seymour, L. Deregulation of HLA-I in cancer and its central importance for immunotherapy. *J. Immunother. Cancer* **2021**, *9*, 1–17, doi:10.1136/jitc-2021-002899.

121. Dhatchinamoorthy, K.; Colbert, J.D.; Rock, K.L. Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation. *Front. Immunol.* **2021**, *12*, doi:10.3389/fimmu.2021.636568.

122. Nanda, N.K.; , Lynn Birch, Norman M. Greenberg, and G.S.P. MHC Class I and Class II Molecules Are Expressed in Both Human and mouse prostate tumor microenvironment. *Prostate* **2006**, *66*, 1257–1284.

123. Collins, S.C. Precision reproductive medicine: multigene panel testing for infertility risk assessment. *J. Assist. Reprod. Genet.* **2017**, *34*, 967–973, doi:10.1007/s10815-017-0938-y.

124. Cragun, D.; Radford, C.; Dolinsky, J.S.; Caldwell, M.; Chao, E.; Pal, T. Panel-based testing for inherited colorectal cancer: A descriptive study of clinical testing performed by a US laboratory. *Clin. Genet.* **2014**, *86*, 510–520, doi:10.1111/cge.12359.

125. Manca, M.A.; Solinas, T.; Simula, E.R.; Noli, M.; Ruberto, S.; Madonia, M.; Sechi, L.A. HERV-K and HERV-H Env Proteins Induce a Humoral Response in Prostate Cancer Patients. *Pathogens* **2022**, *11*, 1–9, doi:10.3390/pathogens11010095.

126. Romero Otero, J.; Garcia Gomez, B.; Campos Juanatey, F.; Touijer, K.A. Prostate cancer biomarkers: An update. *Urol. Oncol. Semin. Orig. Investig.* **2014**, *32*, 252–260, doi:10.1016/j.urolonc.2013.09.017.

127. Douville, R.; Liu, J.; Rothstein, J.; Nath, A. Identification of active loci of a human endogenous retrovirus in neurons of patients with amyotrophic lateral sclerosis. *Ann. Neurol.* **2011**, *69*, 141–151, doi:10.1002/ana.22149.

128. Arru, G.; Mameli, G.; Deiana, G.A.; Rassu, A.L.; Piredda, R.; Sechi, E.; Caggiu, E.; Bo, M.; Nako, E.; Urso, D.; et al. Humoral immunity response to human endogenous retroviruses K/W differentiates between amyotrophic lateral sclerosis and other neurological diseases. *Eur. J. Neurol.* **2018**, *25*, 1076-e84, doi:10.1111/ene.13648.

129. Küry, P.; Nath, A.; Créange, A.; Dolei, A.; Marche, P.; Gold, J.; Giovannoni, G.; Hartung, H.P.; Perron, H. Human Endogenous Retroviruses in Neurological Diseases. *Trends Mol. Med.* **2018**, *24*, 379–394, doi:10.1016/j.molmed.2018.02.007.

130. Mameli, G.; Erre, G.L.; Caggiu, E.; Mura, S.; Cossu, D.; Bo, M.; Cadoni, M.L.; Piras,

A.; Mundula, N.; Colombo, E.; et al. Identification of a HERV-K env surface peptide highly recognized in Rheumatoid Arthritis (RA) patients: a cross-sectional case–control study. *Clin. Exp. Immunol.* **2017**, *189*, 127–131, doi:10.1111/cei.12964.

131. Grandi, N.; Tramontano, E. HERV envelope proteins: Physiological role and pathogenic potential in cancer and autoimmunity. *Front. Microbiol.* 2018, *9*.

132. Kewitz, S.; Staege, M.S. Expression and regulation of the endogenous retrovirus 3 in Hodgkin's lymphoma cells. *Front. Oncol.* **2013**, *3 JUL*, doi:10.3389/fonc.2013.00179.

133. Benešová, M.; Trejbalová, K.; Kovářová, D.; Vernerová, Z.; Hron, T.; Kučerová, D.; Hejnar, J. DNA hypomethylation and aberrant expression of the human endogenous retrovirus ERVWE1/syncytin-1 in seminomas. *Retrovirology* **2017**, *14*, 1–17, doi:10.1186/s12977-017-0342-9.

134. Barth, M.; Gröger, V.; Cynis, H.; Staege, M.S. Identification of human endogenous retrovirus transcripts in Hodgkin Lymphoma cells. *Mol. Biol. Rep.* **2019**, *46*, 1885–1893, doi:10.1007/s11033-019-04640-x.

135. Johanning, G.L.; Malouf, G.G.; Zheng, X.; Esteva, F.J.; Weinstein, J.N.; Wang-Johanning, F.; Su, X. Expression of human endogenous retrovirus-K is strongly associated with the basal-like breast cancer phenotype. *Sci. Rep.* **2017**, *7*, doi:10.1038/srep41960.

136. Lemaître, C.; Tsang, J.; Bireau, C.; Heidmann, T.; Dewannieux, M. A human endogenous retrovirus-derived gene that can contribute to oncogenesis by activating the ERK pathway and inducing migration and invasion. *PLoS Pathog.* **2017**, *13*, doi:10.1371/journal.ppat.1006451.

137. Wallace, T.A.; Downey, R.F.; Seufert, C.J.; Schetter, A.; Dorsey, T.H.; Johnson, C.A.; Goldman, R.; Loffredo, C.A.; Yan, P.; Sullivan, F.J.; et al. Elevated HERV-K mRNA expression in PBMC is associated with a prostate cancer diagnosis particularly in older men and smokers. *Carcinogenesis* **2014**, *35*, 2074–2083,

doi:10.1093/carcin/bgu114.

138. Pérot, P.; Mullins, C.S.; Naville, M.; Bressan, C.; Hühns, M.; Gock, M.; Kühn, F.; Volff, J.N.; Trillet-Lenoir, V.; Linnebacher, M.; et al. Expression of young HERV-H loci in the course of colorectal carcinoma and correlation with molecular subtypes. *Oncotarget* **2015**, *6*, 40095–40111, doi:10.18632/oncotarget.5539.

139. Mortaz, E.; Tabarsi, P.; Mansouri, D.; Khosravi, A.; Garssen, J.; Velayati, A.; Adcock, I.M. Cancers related to immunodeficiencies: Update and perspectives. *Front. Immunol.* **2016**, *7*, 1–13, doi:10.3389/fimmu.2016.00365.

140. Mayor, P.C.; Eng, K.H.; Singel, K.L.; Abrams, S.I.; Odunsi, K.; Moysich, K.B.; Fuleihan, R.; Garabedian, E.; Lugar, P.; Ochs, H.D.; et al. Cancer in primary immunodeficiency diseases: Cancer incidence in the United States Immune Deficiency Network Registry. *J. Allergy Clin. Immunol.* **2018**, *141*, 1028–1035, doi:10.1016/j.jaci.2017.05.024.

141. Pautu, J.L.; Kumar, L. Intratumoral T cells and survival in epithelial ovarian cancer. *Natl. Med. J. India* **2003**, *16*, 150–151.

142. Sato, E.; Olson, S.H.; Ahn, J.; Bundy, B.; Nishikawa, H.; Qian, F.; Jungbluth, A.A.; Frosina, D.; Gnjatic, S.; Ambrosone, C.; et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 18538–18543, doi:10.1073/pnas.0509182102.

143. Ryschich, E.; Nötzel, T.; Hinz, U.; Autschbach, F.; Ferguson, J.; Simon, I.; Weitz, J.; Fröhlich, B.; Klar, E.; Büchler, M.W.; et al. Control of T-Cell-Mediated Immune Response by HLA Class I in Human Pancreatic Carcinoma. *Clin. Cancer Res.* **2005**, *11*, 498–504.

144. Matsumoto, H.; Thike, A.A.; Li, H.; Yeong, J.; Koo, S.L.; Dent, R.A.; Tan, P.H.; Iqbal, J. Increased CD4 and CD8-positive T cell infiltrate signifies good prognosis in a

subset of triple-negative breast cancer. *Breast Cancer Res. Treat.* **2016**, *156*, 237–247, doi:10.1007/s10549-016-3743-x.

145. Serrano, A.; Tanzarella, S.; Lionello, I.; Mendez, R.; Traversari, C.; Ruiz-Cabello, F.; Garrido, F. Expression of HLA class I antigens and restoration of antigen-specific CTL response in melanoma cells following 5-aza-2′-deoxycytidine treatment. *Int. J. Cancer* **2001**, *94*, 243–251, doi:10.1002/ijc.1452.

146. Chiappinelli, K.B.; Strissel, P.L.; Desrichard, A.; Li, H.; Henke, C.; Akman, B.; Hein, A.; Rote, N.S.; Cope, L.M.; Snyder, A.; et al. Inhibiting DNA Methylation Causes an Interferon Response in Cancer via dsRNA Including Endogenous Retroviruses. *Cell* **2015**, *162*, 974–986, doi:10.1016/j.cell.2015.07.011.

147. Stone, M.L.; Chiappinelli, K.B.; Li, H.; Murphy, L.M.; Travers, M.E.; Topper, M.J.; Mathios, D.; Lim, M.; Shih, I.M.; Wang, T.L.; et al. Epigenetic therapy activates type I interferon signaling in murine ovarian cancer to reduce immunosuppression and tumor burden. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, E10981–E10990, doi:10.1073/pnas.1712514114.

148. Blades, R.A.; Keating, P.J.; McWilliam, L.J.; George, N.J.R.; Stern, P.L. Loss of HLA class I expression in prostate cancer: Implications for immunotherapy. *Urology* **1995**, *46*, 681–687, doi:10.1016/S0090-4295(99)80301-X.

149. Bander, N.H.; Yao, D.; Liu, H.; Chen, Y.T.; Steiner, M.; Zuccaro, W.; Moy, P. MHC class I and II expression in prostate carcinoma and modulation by interferon-alpha and -gamma. *Prostate* **1997**, *33*, 233–239, doi:10.1002/(SICI)1097-0045(19971201)33:4<233::AID-PROS2>3.0.CO;2-I.

150. Martini, M.; Testi, M.G.; Pasetto, M.; Picchio, M.C.; Innamorati, G.; Mazzocco, M.; Ugel, S.; Cingarlini, S.; Bronte, V.; Zanovello, P.; et al. IFN-γ-mediated upmodulation of MHC class I expression activates tumor-specific immune response in a mouse model of prostate cancer. *Vaccine* **2010**, *28*, 3548–3557,

doi:10.1016/j.vaccine.2010.03.007.

151. Novo, D.; Prostate, M.; Stokidis, S.; Fortis, S.P.; Kogionou, P.; Anagnostou, T. HLA Class I Allele Expression and Clinical Outcome in De Novo Metastatic Prostate Cancer. **2020**, 1–14.

152. Anastasopoulou, E.A.; Voutsas, I.F.; Keramitsoglou, T.; Gouttefangeas, C.; Kalbacher, H.; Thanos, A. A pilot study in prostate cancer patients treated with the AE37 Ii - key - HER - 2 / neu polypeptide vaccine suggests that HLA - A * 24 and HLA - DRB1 * 11 alleles may be prognostic and predictive biomarkers for clinical benefit. *Cancer Immunol. Immunother.* **2015**, *64*, 1123–1136, doi:10.1007/s00262-015-1717-1.

153. On a possible protective effect of HLA-A11 against skin cancer and keratotic skin lesions in renal transplant recipients.pdf.

154. Araz, O.; Ucar, E.Y.; Meral, M.; Yalcin, A.; Acemoglu, H.; Dogan, H.; Karaman, A.; Aydin, Y.; Gorguner, M.; Akgun, M. Frequency of Class I and II HLA alleles in patients with lung cancer according to chemotherapy response and 5-year survival. *Clin. Respir. J.* **2015**, *9*, 297–304, doi:10.1111/crj.12143.

155. Bates, D.; Mächler, M.; Bolker, B.M.; Walker, S.C. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **2015**, *67*, doi:10.18637/jss.v067.i01.

156. Bankaitis, K.V.; Fingleton, B. Targeting IL4/IL4R for the treatment of epithelial cancer metastais. *Clin Exp Metastasis* **2016**, *32*, 847–856, doi:10.1007/s10585-015-9747-9.Targeting.

157. DeNardo, D.G.; Barreto, J.B.; Andreu, P.; Vasquez, L.; Tawfik, D.; Kolhatkar, N.; Coussens, L.M. CD4+ T Cells Regulate Pulmonary Metastasis of Mammary Carcinomas by Enhancing Protumor Properties of Macrophages. *Cancer Cell* **2009**, *16*, 91–102, doi:10.1016/j.ccr.2009.06.018.

158. Venmar, K.T.; Carter, K.J.; Hwang, D.G.; Dozier, E.A.; Fingleton, B. IL4 receptor

ILR4α regulates metastatic colonization by mammary tumors through multiple signaling pathways. *Cancer Res.* **2014**, *74*, 4329–4340, doi:10.1158/0008-5472.CAN-14-0093.

159. Lee, S.O.; Lou, W.; Hou, M.; Onate, S.A.; Gao, A.C. Interleukin-4 enhances prostate-specific antigen expression by activation of the androgen receptor and Akt pathway. *Oncogene* **2003**, *22*, 7981–7988, doi:10.1038/sj.onc.1206735.

160. Soo Ok Lee, Elaine Pinder, Jae Yeon Chun, Wei Lou, Meng Sun, A.C.G. Interleukin-4 Stimulates Androgen-Independent Growth in LNCaP Human Prostate Cancer Cells. *Prostate* **2008**, *68*, 85–91, doi:10.1002/pros.

161. Tait Wojno, E.D.; Hunter, C.A.; Stumhofer, J.S. The Immunobiology of the Interleukin-12 Family: Room for Discovery. *Immunity* **2019**, *50*, 851–870, doi:10.1016/j.immuni.2019.03.011.

162. Kundu, M.; Roy, A.; Pahan, K. Selective neutralization of IL-12 p40 monomer induces death in prostate cancer cells via IL-12 – IFN- γ. **2017**, *114*, doi:10.1073/pnas.1705536114.

163. Mefford, J.A.; Cheng, I.; Plummer, S.J.; Levin, A.M.; Rybicki, B.A.; Casey, G.; Witte, J.S. Association of the Innate Immunity and Inflammation Pathway with Advanced Prostate Cancer Risk. **2012**, *7*, 1–8, doi:10.1371/journal.pone.0051680.

164. Nowak, M.; Arredouani, M.S.; Tun-Kyi, A.; Schmidt-Wolf, I.; Sanda, M.G.; Balk, S.P.; Exley, M.A. Defective NKT cell activation by CD1d+ TRAMP prostate tumor cells is corrected by interleukin-12 with alpha-galactosylceramide. *PLoS One* **2010**, *5*, doi:10.1371/journal.pone.0011311.

165. Azevedo, A. IL-6/IL-6R as a potential key signaling pathway in prostate cancer development. *World J. Clin. Oncol.* **2011**, *2*, 384, doi:10.5306/wjco.v2.i12.384.

166. Twillie, D.A.; Eisenberger, M.A.; Carducci, M.A.; Hseih, W.S.; Kim, W.Y.; Simons, J.W. Interleukin-6: A candidate mediator of human prostate cancer morbidity.

Urology **1995**, *45*, 542–549, doi:10.1016/S0090-4295(99)80034-X.

167. Daniel J. George, Susan Halabi, Timothy F. Shepard, Ben Sanford, Nicholas J. Vogelzang, Eric J. Small, A.; Kantoff, P.W. The Prognostic Significance of Plasma Interleukin-6 Levels in Patients with Metastatic Hormone-Refractory Prostate Cancer: Results from Cancer and Leukemia Group B 9480. *Clin. Cancer Res.* **2005**, *11*, 1815–1820, doi:10.1158/1078-0432.CCR-04-1560.

168. Akimoto, S.; Okumura, A.; Fuse, H. Relationship between serum levels of interleukin-6, tumor necrosis factor-α and bone turnover markers in prostate cancer patients. *Endocr. J.* **1998**, *45*, 183–189, doi:10.1507/endocrj.45.183.

169. Shariat, S.F.; Andrews, B.; Kattan, M.W.; Kim, J.H.; Wheeler, T.M.; Slawin, K.M. Plasma levels of interleukin-6 and its soluble receptor are associated with prostate cancer progression and metastasis. *Urology* **2001**, *58*, 1008–1015, doi:10.1016/S0090-4295(01)01405-4.

170. Mohammad Taheri, Rezvan Noroozi, Azadeh Rakhshan, Molud Ghanbari, Mir Davood Omrani, S.G.-F. IL-6 Genomic Variants and Risk of Prostate Cancer. *Urol. J.* **2019**, *16*, 463–468.

171. Slattery, M.L.; Curtin, K.; Giuliano, A.R.; Sweeney, C.; Baumgartner, R.; Edwards, S.; Wolff, R.K.; Baumgartner, K.B.; Byers, T. Active and passive smoking, IL6, ESR1, and breast cancer risk. *Breast Cancer Res. Treat.* **2008**, *109*, 101–111, doi:10.1007/s10549-007-9629-1.

172. Michaud, D.S.; Daugherty, S.E.; Berndt, S.I.; Platz, E.A.; Yeager, M.; Crawford, E.D.; Hsing, A.; Huang, W.Y.; Hayes, R.B. Genetic polymorphisms of interleukin-1B (IL-1B), IL-6, IL-8, and IL-10 and risk of prostate cancer. *Cancer Res.* **2006**, *66*, 4525–4530, doi:10.1158/0008-5472.CAN-05-3987.

173. Sun, J.; Hedelin, M.; Zheng, S.L.; Adami, H.O.; Bensen, J.; Augustsson-Bälter, K.; Chang, B.; Adolfsson, J.; Adams, T.; Turner, A.; et al. Interleukin-6 sequence variants

are not associated with prostate cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **2004**, *13*, 1677–1679.

174. Winchester, D.A.; Gurel, B.; Till, C.; Goodman, P.J.; Tangen, C.M.; Santella, R.M.; Johnson-pais, T.L.; Leach, R.J.; Thompson, I.M.; Xu, J.; et al. Key Genes Involved in the Immune Response Are Generally Not Associated With Intraprostatic In fl ammation in Men Without a Prostate Cancer Diagnosis : Results From the Prostate Cancer Prevention Trial. **2016**, *574*, 565–574, doi:10.1002/pros.23147.

175. Thunders, M.; Delahunt, B. Gene of the month: TMPRSS2 (transmembrane serine protease 2). *J. Clin. Pathol.* **2020**, *73*, 773–776, doi:10.1136/jclinpath-2020-206987.

176. Shafiee, S.; Cegolon, L.; Khafaei, M.; Gholami, N.; Zhao, S.; Khalesi, N.; Moosavian, H.; Fathi, S.; Izadi, M.; Ghadian, A.; et al. Gastrointestinal cancers, ACE-2/TMPRSS2 expression and susceptibility to COVID-19. *Cancer Cell Int.* **2021**, *21*, 1–10, doi:10.1186/s12935-021-02129-x.

177. Wang, X.S. Angiotensin-converting enzyme 2 connects COVID-19 with cancer and cancer immunotherapy. *World J. Gastrointest. Oncol.* **2021**, *13*, 157–160, doi:10.4251/wjgo.v13.i3.157.

178. Strafella, C.; Caputo, V.; Termine, A.; Barati, S.; Gambardella, S.; Borgiani, P.; Caltagirone, C.; Novelli, G.; Giardina, E.; Cascella, R. Analysis of ace2 genetic variability among populations highlights a possible link with covid-19-related neurological complications. *Genes (Basel).* **2020**, *11*, 1–10, doi:10.3390/genes11070741.

179. Sauna, Z.E.; Kimchi-Sarfaty, C. Understanding the contribution of synonymous mutations to human disease. *Nat. Rev. Genet.* **2011**, *12*, 683–691, doi:10.1038/nrg3051.

180. Sharma, Y.; Miladi, M.; Dukare, S.; Boulay, K.; Caudron-Herger, M.; Groß, M.; Backofen, R.; Diederichs, S. A pan-cancer analysis of synonymous mutations. *Nat. Commun.* **2019**, *10*, doi:10.1038/s41467-019-10489-2.

181. Supek, F.; Miñana, B.; Valcárcel, J.; Gabaldón, T.; Lehner, B. Synonymous mutations

frequently act as driver mutations in human cancers. *Cell* **2014**, *156*, 1324–1335, doi:10.1016/j.cell.2014.01.051.

182. Farrer, M.J. Genetics of Parkinson disease: Paradigm shifts and future prospects. *Nat. Rev. Genet.* **2006**, *7*, 306–318, doi:10.1038/nrg1831.

183. Veeriah, S.; Taylor, B.S.; Meng, S.; Fang, F.; Yilmaz, E.; Vivanco, I.; Janakiraman, M.; Schultz, N.; Hanrahan, A.J.; Pao, W.; et al. Somatic mutations of the Parkinson's disease-associated gene PARK2 in glioblastoma and other human malignancies. *Nat. Genet.* **2010**, *42*, 77–82, doi:10.1038/ng.491.

184. Fujiwara, M.; Marusawa, H.; Wang, H.Q.; Iwai, A.; Ikeuchi, K.; Imai, Y.; Kataoka, A.; Nukina, N.; Takahashi, R.; Chiba, T. Parkin as a tumor suppressor gene for hepatocellular carcinoma. *Oncogene* **2008**, *27*, 6002–6011, doi:10.1038/onc.2008.199.

185. Je, E.M.; Yoo, N.J.; Lee, S.H. Somatic mutation of PARK2 tumor suppressor gene is not common in common solid cancers. *Pathol. Oncol. Res.* **2013**, *19*, 393–395, doi:10.1007/s12253-012-9591-z.

Pubblications

1. Under review: Noli M., Meloni G., Simula E.R., **Manca M.A.**, Jasemi S., Ruberto S., Cossu D., Palermo M., Sechi LA. Autoantibodies against proinsulin, human endogenous retrovirus W (HERV-W) and Mycobacterium avium subspecies paratuberculosis (MAP) slowly decrease after years since T1D diagnosis. Zoonotic Disease.

2. **Manca M.A.**, Solinas T., Simula E.R., Noli M., Ruberto S., Madonia M., Sechi L.A. HERV-K and HERV-H Env Proteins Induce a Humoral Response in Prostate Cancer Patients. Pathogens. 2022 Jan 14;11(1):95. doi: 10.3390/pathogens11010095. PMID: 35056043; PMCID: PMC8778306.

3. Arru G., Galleri G., Deiana G.A., Zarbo I.R., Sechi E., Bo M., Cadoni M.P.L., Corda D.G., Frau C., Simula E.R., **Manca M.A.**, Galistu F., Solla P., Manetti R., Sechi G.P., Sechi L.A. HERV-K Modulates the Immune Response in ALS Patients. Microorganisms. 2021 Aug 23;9(8):1784. doi: 10.3390/microorganisms9081784. PMID: 34442863; PMCID: PMC8399181.

4. Simula E.R., **Manca M.A.**, Jasemi S., Uzzau S., Rubino S., Manchia P., Bitti A., Palermo M., Sechi L.A. HCoV-NL63 and SARS-CoV-2 Share Recognized Epitopes by the Humoral Response in Sera of People Collected Pre- and during CoV-2 Pandemic. Microorganisms. 2020 Dec 14;8(12):1993. doi: 10.3390/microorganisms8121993. PMID: 33327507; PMCID: PMC7764996.

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