A chi mi ha sopportata e supportata,

riuscendo sempre a farmi vedere la prospettiva migliore delle cose…

Ai miei genitori.

UNIVERSITÀ DEGLI STUDI DI UDINE

PHD COURSE IN BIOMEDICAL AND BIOTECHNOLOGICAL SCIENCES

XXIX CYCLE

EPIDEMIOLOGIC AND EPIGENETIC INSTRUMENTS TO STUDY MECHANISMS INVOLVED IN PROSTATE CANCER RELAPSE

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LIST OF ABBREVIATIONS

PC	Prostate Cancer
SEER	Surveillance, Epidemiology and End Results Program
IARC	International Agency for Research on Cancer
PSA	Prostate Specific Antigen
RP	Radical Prostatectomy
RT	Radiotherapy
ADT	Androgen Deprivation Therapy
CAB	Combined Androgen Blockade
AR	Androgen Receptor
CRPC	Castrate Resistant Prostate Cancer
GSTP1	Glutathione S-Transferase Pi 1
ncRNAs	Non-coding RNAs
FVG	Friuli Venezia Giulia
US	United States
AK	Alaska
AIRTUM	Associazione Italiana dei Registri Tumori
ISS	Istituto Superiore di Sanità
E	Estrogens
AAG	Antiandrogens (group G)
BIP	Drugs used in benign prostatic hypertrophy
HP	Systemic hormonal preparations (excl. Sex hormones and insulins)
C	Antineoplastic agents
AGnRH	Gonadotropin releasing hormone analogues
AE	Antiestrogens
AAL	Antiandrogens (group L)
AAG	Antiandrogens (group G)
AAO	Other hormone antagonists and related agents
IA	Intestinal Antiinfectives
FD	Drugs used in diabetes
LM	Lipid modifying agents
AA	Antiinflammatory and antirheumatic agents, non steroids.
R1	Relapse according to medical doctors (explicitly defined).
R2	Relapse according to the guidelines (increasing OPSA levels over 0.2 ng/ml).

BCR	Biochemical recurrence
SD	Standard deviation
IQR	Interquartile Range
CpG island	Sites of DNA rich of cytosines and Guanines
OR	Odds Ratio
HR	Hazard Ratio
95 % CI	95% Confidence Interval

ABSTRACT

Prostate Cancer (PC) is the most common cancer in elderly males (>70 years) in Europe, but also American and African populations are characterized by high incidence and mortality related to this malignancy. Incidence rates of PC became higher after the introduction of the Prostate Specific Antigen (PSA) screening. Actually, there isn't a unique method to treat this pathology, so several therapeutic procedures could be used. The treatments frequently used are watchful waiting, prostatectomy and radiotherapy however often a pharmacological treatment is also used (in particular the anti-hormonal medications); sometimes two different options are used.

The main aims of this study are the following:

1) Study the use of medications in patients with a diagnosis of PC in Friuli Venezia Giulia considering the period between 1998 and 2014.
2) Study the methylation status of a part of the Glutathione S-transferase Pi 1 (GSTP1) promoter in patients who underwent Radical Prostatectomy (RP) and try to understand if methylation of this promoter could influence the risk of relapse.
3) Study the features of patients who underwent Radical Prostatectomy, comparing those characterized by a relapse to those who are not characterized by a relapse.

The first step has been the identification of subjects with a diagnosis of PC in Friuli Venezia Giulia (15079 cases) from Tumor's register (data available from 1995 to 2009). In a second phase, subjects with only one tumor (PC) were selected (11521 people).

Later, data on subjects, were crossed with data about medications (especially those related to prostatic problems) which were taken by these 2915 subjects. From data obtained, were selected those subjects underwent to Radical Prostatectomy (RP)

(530 people) to whom was possible extract clinical information more detailed (TNM, Gleason score, PSA etc., obtaining 149 people). Finally, the last selection was based to the availability of biological samples (paraffin-embedded prostates specimens) on pathological anatomy, which were available for 122 patients.

People selected were divided into people characterized by a disease relapse (PC) and subjects without a relapse (for whom no relapse was documented). The purpose of the two groups identified above was to understand if there were some differences in terms of methylation (related to the promoter of GSTP1), hence to determine whether this elements can influence the risk of relapse.

During the studied period (from 1998 to 2014) in Friuli Venezia Giulia, the class of medications most used from patients with PC is that including Hormonal Preparations (HP), which shows a continuous increase, whereas the use of antiandrogens medications (AA) shows a decrease over time with the exception of new antiandrogens medications (belonging to the AAO class).

The results show that people with higher levels of methylation have a higher risk of relapse, compared to those subjects with lower levels of methylation. Additionally, a higher Gleason score increases the risk of relapse, as shown in many papers in literature. The results obtained from Cox models shows that bivariate models (adjusted by age) are characterized by a trend of relapse's risk, which increases with levels of methylation. This trend is not maintained in multiple models.

In conclusion, the methylation of GSTP1 promoter might be useful to identify patients with higher risk of biochemical recurrence, however further studies based on an epidemiologic epigenetic approach are needed.

1 INTRODUCTION

1.1 Prostate Cancer (PC)

1.1.1 Definition

Prostate Cancer (PC) begins when cells in the prostate gland start to grow uncontrollably. The prostate is a gland found in the male reproductive system. It makes some of the fluid that is part of semen. The prostate is localized below the bladder and in front of the rectum. The size of the prostate changes with age, being smaller in younger men, and much larger in older men. Behind the prostate there are glands called seminal vesicles that make most of the fluid for semen. The urethra, which is the tube that carries urine and semen out of the body through the penis, goes through the center of the prostate[1] (*Figure 1.1.1.1*).

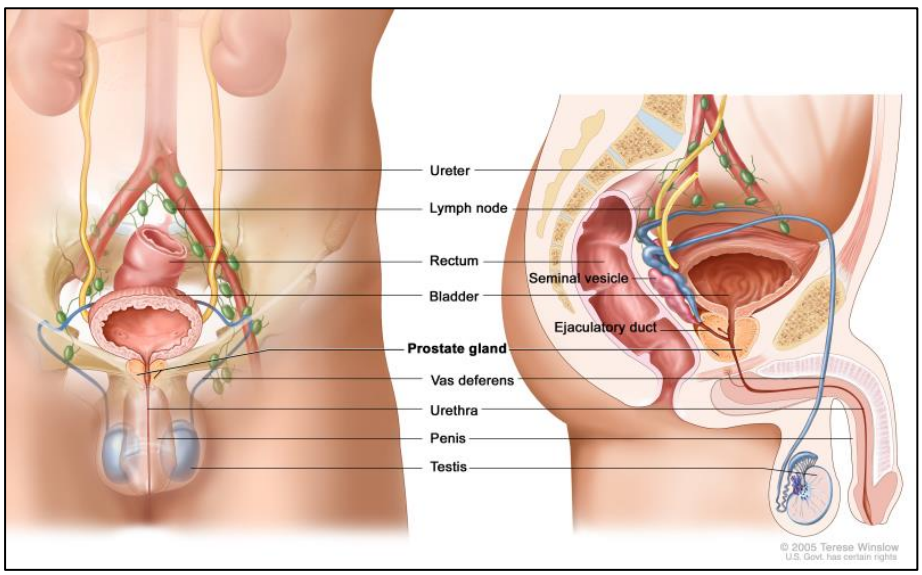

Figure 1.1.1.1

Position of prostate gland in the human body[1a].

1.1.2 Incidence

According to the European Association of Urology[2], PC is the most common cancer in elderly males (>70 years) in Europe but several articles show different data about the incidence of PC (for example articles define PC as the third most common cancer in men in Europe, North America and in many states of Africa[3]). Nevertheless, in all articles it represents one of the majors health problems, especially in developed countries with their greater proportion of elderly men in the general population. Based on Surveillance, Epidemiology and End Results Program (SEER) data, the number of new cases of prostate cancer in the American population is 129.4 per 100,000 men per year[4]. Based on European data, Northern end Western Europe are characterized by highest incidence compared to Eastern and Southern Europe, and have showed a continuous increase[2]. In Italian guidelines on Prostate carcinoma, the PC is described as the most common malignancy among men (20% of all diagnosed tumors) from 50 years of age. In the European Community, age-standardized incidence rates show 86.7 cases for 100.000 people. It is possible to identify a gradient from Northern Italian regions to Southern ones; in particular, among residents in Northern Italy, there are 109.5 new cases/year, the central regions reveal a -22% (85.3/100000) and the Southern Italy a -44% (61.4/100000). These differences can be caused by a lot of factors and the most important is the difference in the distribution of PSA screening[5]. The *Figure 1.1.2.1* was obtained by AIURTUM databases and confirmed what described before, based on the available standardized registers in terms of incidence and mortality rates[1b].

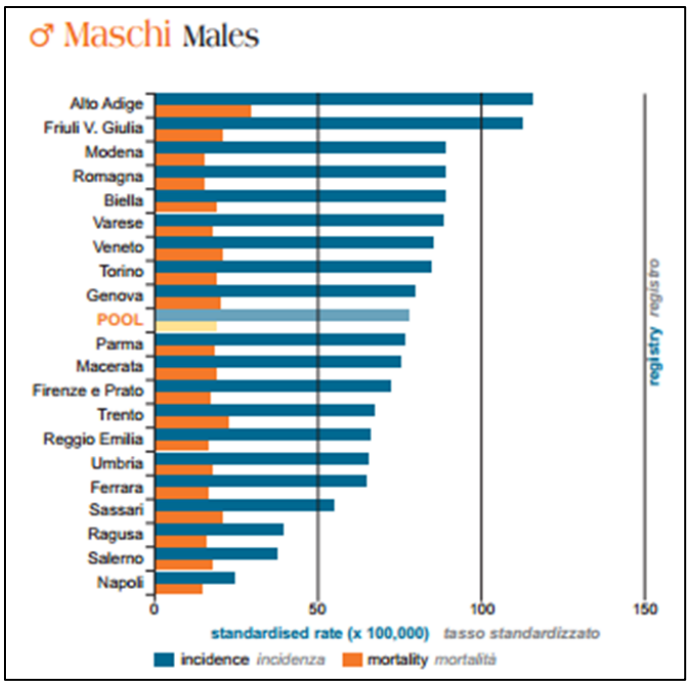

Figure 1.1.2.1

Incidence and mortality rates of PC related to the pool of registers obtained from AIRTUM databases[1b].

1.1.3 Mortality

In American population, the number of deaths was 20.7 per 100.000 men per year. These rates are age-adjusted and based on 2009-2013 cases and deaths[4].

PC is the most common malignancy in Italy but represented the third place in mortality, and in the majority of cases is related to men older than 70 years. The PSA screening and the subsequent early diagnosis is often related to an over-diagnosis in Northern Italy, but does not show differences in mortality among several regions in Italy set at 17-18 cases every 100.000 people/year[5].

1.1.4 Etiology, Risk Factors and Protective factors

The risks' factors of developing PC are not well known[5], but many works describe PC as a disease caused by a multifactorial etiology driven by an interaction of genetic factors and environmental factors[5].

- ***Risk factors***
  - Age
  - Race
  - Hormonal factors
  - Familiar history of PC
  - Genetic factors
  - Lifestyle
- ***Protective factors***
  - Vitamins (A, D)
  - Oligoelements
  - Antioxidants

The increasing age is one of the most important factors related to Prostate Cancer, while the second most important element is related to the levels of biologically active androgens. Also, race is a feature that plays a role in PC; for example, the Afro-American population is characterized by an higher risk of developing PC compared to non-Hispanic[6], Caucasian, Asiatic or Latino-american[7] men. In the United States, Afro-American population shows higher rates of disease and an advanced disease with higher risk of metastasis at diagnosis. Although skin color is not associated with mortality or progression of the disease, lower education of Afro-American people is positively associated to the worst outcome[7].

1.1.5 Diagnostic evaluation

There are a lot a of ways to evaluate prostate cancer, such as screening (to an early detection) [9], digital rectal examination, use of prostate specific antigen (PSA) levels [10, 11], prostate biopsy[12], use of imaging, histopathology of prostate specimens and clinical staging (TNM staging (T=tumor, N=number of lymph nodes, M=metastases), Gleason score) [13].

1.2 Therapy and prostate cancer

There are several approaches to manage and treat PC, the available possibilities are:

a) Deferred treatment (active surveillance/watchful waiting).[14,15,16]
b) Radical prostatectomy.[17]
c) Radiotherapy.[18]
d) Brachytherapy.[19]
e) Cryosurgery.[20]
f) Hormonal therapy.[21]
g) Combination therapies.[22]

a) Deferred treatment

These types of treatments are often used when PC progresses slowly and especially in older men with a higher incidence of comorbidity and different risks of mortality due to other causes. [14-16, 23]

b) Radical prostatectomy (RP)

RP is a surgical treatment of PC that will be treated in detail in the paragraph 1.2.1.[24]

c) Radiotherapy

Radiotherapy (RT) is a therapy that concerns the use of intensity-modulated radiations and that can be image-guided. Often this type of therapy completes the treatment of RP in patients with positive margins derived from surgical treatment.[18] The radiation therapy may not decrease the PSA to an undetectable level, and there is a debate regarding the timing and level of PSA into the serum that indicates treatment failure following radiation therapy.[24-26]

d) Brachytherapy

Low dose rate (LDR) brachytherapy is a safe and effective technique that needs some eligible criteria as very early stage (T1-T2, N0; M0), a Gleason score ≤6, an initial level of PSA ≤10 ng/ml, ≤50% of biopsies cores involved with cancer, etc.
For these reasons, often it is not possible to use this technique.[19]

e) Cryosurgery

Cryosurgery is a technique that uses freezing to induce cell death by dehydration, resulting in protein denaturation, rupture of cell membranes and vascular stasis and microthrombi resulting in ischaemic apoptosis. The potential candidates for this therapy are those who have organ-confined PC and those with a minimal extension of tumor beyond the prostate. [20]

f) Hormonal therapy

There are several types of Androgen Deprivation Therapy (ADT), and the medications can be divided into those that suppress the secretion of testicular androgens and those which inhibit the actions of androgens with their receptor (ex. antiandrogens); these two types of drugs can be combined in the Combine Androgen Blockade (CAB). Paragraph 1.2.2 outlines this in more detaile.[21]

g) Combination therapies

CAB is an example of combination therapies. Some reviews show that CAB appears to provide more small survival advantages than monotherapy (surgical castration or LH-RH agonists) beyond 5 years, but this type of therapies are more expensive and present more side effects compared to monotherapy. Currently, there are heterogeneous results on whether it is better to use of CAB or monotherapy.[2, 22]

1.2.1 Radical Prostatectomy (RP)

RP is a surgical treatment of PC, consisting in the removal of the entire prostate gland and the resection of both seminal vesicles, along with sufficient surrounding tissue to obtain a negative margin for PC. The goal of this treatment should be the eradication of the disease. At the moment, the RP is the only treatment for localized PC that shows benefits related to overall survival and cancer-specific survival.[17, 24]

1.2.1.1 Definition of Biochemical Recurrence (BCR) after radical prostatectomy

There are many definitions of biochemical recurrence (BCR), and a review, published in 2007, analyzed the various definitions, (including 145 articles contained 53 different definitions of BCR for patients treated with radical

prostatectomy (RP)). It found that the most common definition was a Prostate Specific Antigen (PSA) of > 0.2 ng/ml or a slight variation thereof. The authors of this study concluded that BCR can be identified as an initial serum PSA of ≥0.2 ng/ml, with a second confirmatory level of PSA > 0.2 ng/ml.[27, 28]
This work will be consider only this type of recurrence therefore the introduction only describes this type.

1.2.2 Medications used in cases of PC

Hormone therapy is a cancer treatment that removes hormones or blocks their action and stops cancer cells from growing. This therapy may include:

- ***Luteinizing hormone-releasing hormone agonists or antagonists*** (LH-RH agonists/ LH-RH antagonists) can stop testicles from making testosterone.
  - ***a) LH-RH agonists***: are synthetic analogs of LH-RH and are delivered using injections, stimulate pituitary injections and so a rise in LH and FSH that induce a surge of testosterone. These medications have a long term action.
  - ***b) LH-RH antagonists:*** binds LH-RH receptors in the pituitary gland, the effect is a rapid decrease of LH, FSH and testosterone levels, but there are no formulations with a long action related to these compounds. Abarelix and Degarelix belong to this class.[2]
- ***Estrogens***: can stop testicles from making testosterone and are not associated to bone loss. A used estrogen is Diethylstilboestrol.[29]
- ***Antiandrogens***: can block the action of androgens as testosterone, can be divided in steroidal and non steroidal, both compete with androgens at the receptor level.
  - ***a) Steroidal:*** are synthetic derivatives of hydroxyprogesterone and they are cyproterone acetate, megestrol acetate and medroxyprogesterone acetate.

b) Non steroidal: the monotherapy with these compounds (nilutamide, flutamide and bicalutamide) seem to improve the quality of life and the compliance of patients compared to castration.[30]

- ***New compounds (for castrate resistant status):*** including abiraterone acetate (an inhibitor of CYP17 that suppress the synthesis of testosterone at adrenal level) and enzalutamide an antiandrogen with a higher affinity to the Androgen Receptor (AR) compared to bicalutamide. These compounds should be used after docetaxel in cases of Castrate Resistant Prostate Cancer (CRPC).[31, 32]
- ***Chemotherapy (CHT)***: drugs used to stop the growth of cancer cells by killing the cells or by stopping them from dividing; the most used is docetaxel combined with prednisone (to a palliative intent).[33]

1.3 Epigenetics

The term "epigenetics" indicates a branch of genetics that studies the heritable modifications which alter genetic expressions without modifying the DNA sequence: it is a regulatory process that controls the transcription of information encoded in the DNA sequence into RNA before their translation into proteins. Epigenetic factors are responsible for the dynamic remodeling chromatin processes that are related to the expression (or not expression) of genes. The most important ways through which epigenetics acts are related to DNA methylation, histone modifications and the action of small non-coding RNAs (ncRNAs): these systems interact to ensure epigenetic control.[41] Unlike DNA sequence, epigenetic patterns are different among tissues, shows changes related to advancing age, and are sensitive to environmental exposures.[34]

Figure 1.3.1 shows how epigenetics acts and the factors which may influence these processes related to remodeling processes.

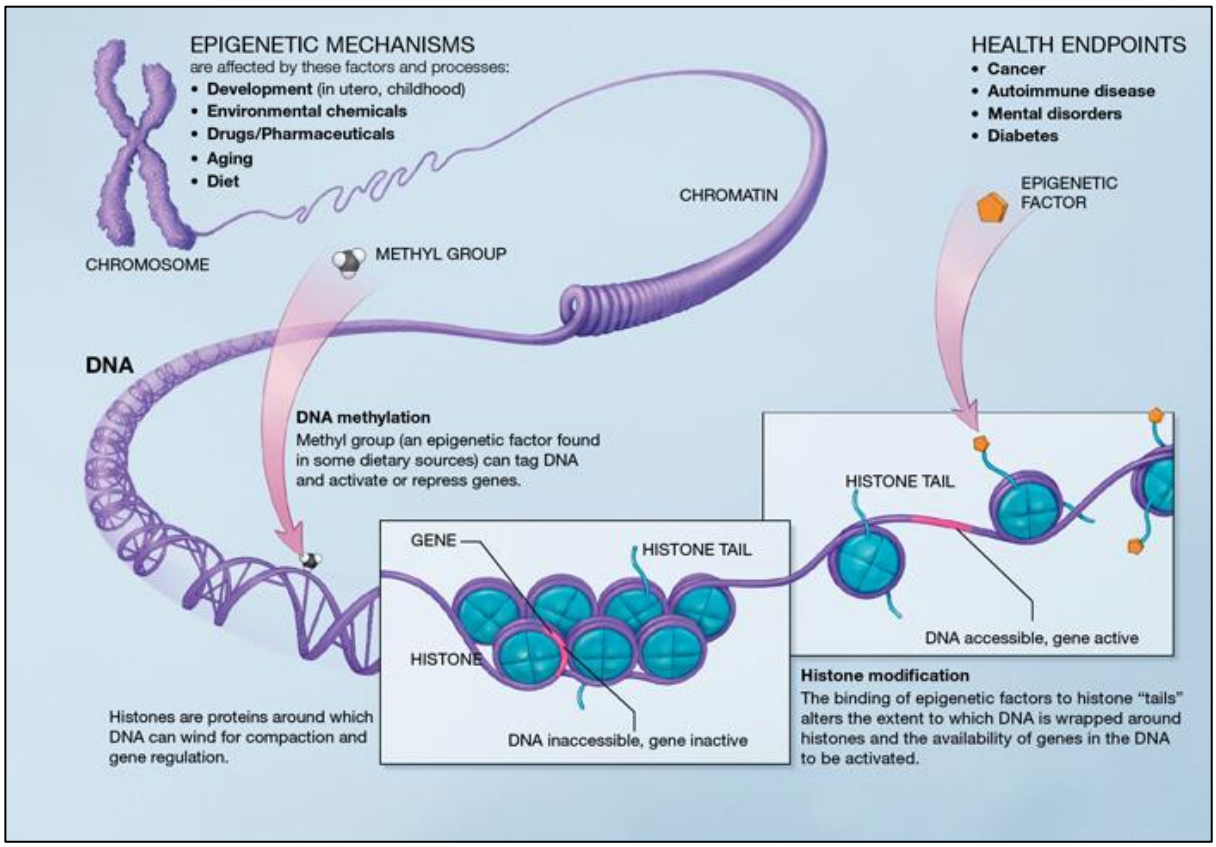

Figure 1.3.1

A summary figure of epigenetic mechanisms[1c].

Some examples of epigenetic processes are:

- Inactivation of X chromosome.
- Developmental biology.
- Tissue differentiation.

Moreover, the epigenetics is important to:

- Understand the mechanisms of some diseases.
- Identify biomarkers useful to predict information about the onset of some disease or their prognosis.
- Use epigenetics to find new therapies.

There are many factors which may influence the epigenome such as diet (ex. folate assumption, (folates are the major source of methyl groups)), lifestyle (ex. smoke or alcohol habits), hormonal factors (ex. during pregnancy) and air pollution.

1.3.1 Epigenetic Epidemiology

Epigenetic epidemiology is a branch of science which links two "epi" sciences; this new science is defined as the study of the associations between epigenetic variation and the risk of human diseases. Epigenetic epidemiology studies the role of epigenetic modifications in human disease etiology, the possible role of epigenetics between environmental exposures and disease outcomes, and, finally can be useful to discover new disease biomarkers.

The objective of studies in epigenetic epidemiology is try to identify the implications of epigenetics in the outcomes of the disease of interest, and this goal can be explored used the framework of epidemiologic studies; the identification of an appropriate design is related to the research question.

The choice of tissue to be analyzed is very important. Epigenetics patterns are different among tissues, and it is necessary to consider if the tissue to analyze is the target tissue or a surrogate tissue; the use of target tissue is better than the use of a surrogate because in the latter it is possible that some elements (ex. methylation levels) could be different from the target.[41]

1.3.2 DNA methylation

DNA methylation, more specifically hypomethylation and hypermethyation[36], are the most investigated epigenetic alterations in many diseases and also in PC.[35,44] This type of epigenetic mechanism consists in the addition of a methyl group (-CH_3) on an azotate base.

The most studied epigenetic modification related to methylation is the methylations of cytosines (C) located in "CpG islands", which are small regions of DNA that occur in the promoters of a substantial portion of human genes. Usually, these CpG islands are not methylated in normal tissues and this is associated with transcriptional competency, indeed methylations status of cytosines are involved in normal gene control. The abnormal methylation can be related to cancer or other diseases when the methylation of generally unmethylated CpG islands causes the silencing of a gene. The abnormal methylation of CpG islands, and so the silencing

of a gene, can derive from copying errors related to diet, carcinogens exposures, aging, etc. On the other hand, there are some elements such as repetitive elements or promoters of onco-genes that tend to be methylated and consequently to abnormal demethylation, presenting a situation that is exactly the opposite of the scenario described above.

DNA methylation is an interesting process for epidemiologic studies because the patterns of methylation can be measured using quantitative methods and, as described above, are known to influence gene expression when located in regulatory regions of genes and are subject to alterations associated with aging and exposure to some elements. The methylation of cytosines in regulatory regions of genes became important because it can directly contribute to the carcinogenic process by inducing the aberrant expression of some genes, or decrease the expression of others; moreover these processes can increase the frequency of mutations.[41]

1.3.2.1 Methods to study levels of methylation

There are several laboratory methods used in epigenetic epidemiology to analyze the biospecimens; the choice depends on:

- type of samples (fresh samples, formalin fixed paraffin embedded tissues);
- derived products to be analyzed (cells, DNA, RNA);
- the epigenetic elements of interest to the study (Histone modifications, mapping of DNA target for transcription factors, DNA methylation, transcription levels, allelic specific expression).

Two different approaches can be used to study methylation levels, depending on the type of analyses of interest. In particular, it can be loci-specific analyses or a global estimation.

In relation to analyses related to a specific locus, three several methods can be used:

1) ***Sensitive methods***, which include Bisulfite sequencing (Bs-Sequencing) and Methyl Specific PCR (MSP).
2) ***Semi-quantitative methods*** as Methylight (a real-time MSP) and Methylation Specific High Resolution Melting curve analyses (MS-HRM).

3) ***Quantitative-methods*** including COmBined Restriction enzyme Analyses (COBRA), Bisulfite PCR followed by MALDI-TOF (Bs-PCR w/MALDI-TOF), Bisulfite-Pyrosequencing (Bs-Pyrosequencing).

Figure 1.3.2.1.1 briefly present the characteristics of these methods.

To study globally methylation of a genome the following tests can be used: CpG methylase assay, ELISA-type methods, Cytosine extension assay or analysis of repetitive elements.[41]

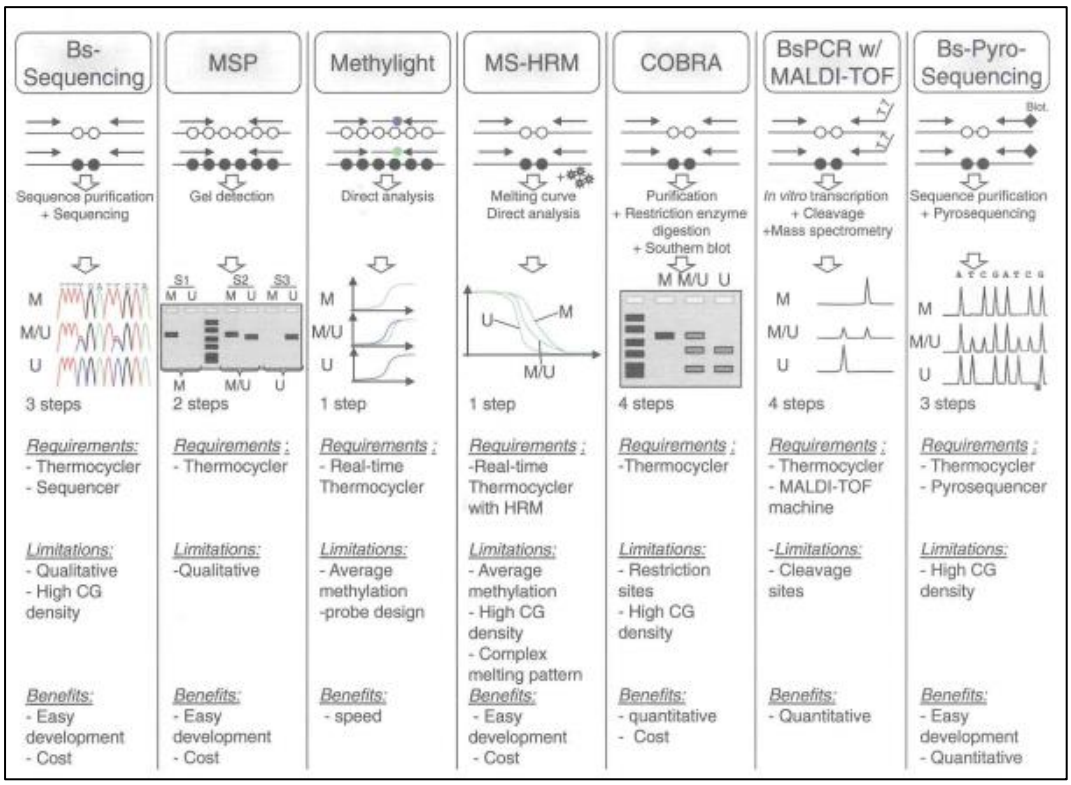

Figure 1.3.2.1.1

A schematic representation of the methods to perform methylation analyses locus-specific[1d].

1.4 Genes associated to PC

Recently many scientists focused their attention on the role of methylation in relation to the onset of many tumors[37-40].

Genomic studies, have often allowed the identification of mutations in many genes, in several tumors and also in Prostate Cancer (PC).

PC is characterized by a heterogeneity found in the cells of PC, indeed it is possible to find out clones with several genomic origins; some cells present a delection, other fusions, other mutations. Some of these occur in the early stages, others in later stage, but all published works declare that origins of PC are not driven by definitive genomic events. This is confirmed by the existence of convergent events which occur before the point mutations or other genomic changes.[42] Specific mutations are not found in all PC cases, on the contrary, some mutations related to the activation of the oncogene Ras or to the inactivation of TP53, were found only in a small number of patients; whereas other mutations (as those connected to the inactivation of PTEN) were found only at the later stages of the illness. The identification of epigenetic alterations seems to be more frequent than DNA modifications; moreover the timing of epigenetic changes appears in early stages compared to the mutations of some genes (as PTEN).[43] In particular, among epigenetic events, DNA methylation is the epigenetic change most studied[42] and it is relatively stable through subclonal evolution.[36]

The methylation of many genes was studied in relation to Prostate Cancer to analyze either the diagnostic or the prognostic potential of these genes. Some works focus their attention on the frequency of hypermethylation at several cancer-related genes in prostate tissues in different types of tissues (non malignant tissues, high-grade prostatic intraepithelial neoplasia, prostate adenocarcinoma (PCa)). The reviews written by Carmen Jeronimo at al. (2011) presented the percentages of hypermethylation of several genes such as: APC, AR, CD44, CDH1, CDH13, CRBP1, Cyclin D2, DAPK, EDNRB, ER (α-A, α-C, β), FHIT, GSTP1, HIC-1, MGMT, p16, PR (A, B), RARβ2, RASSF1A, TIMP-3.[43, 64]

Other reviews studied the prognostic markers of methylation for PC, presenting also other genes interesting for PC. According to the authors, one of the best candidates as prognostic marker is: PITX2, which seems to be an upstream regulator of both insulin-like growth factor 1 receptor and of the Androgen Receptor (AR); moreover it appears to be linked to initiation and progression of PC.[44] This

gene was also involved the regulation of cell-type specific proliferation, and when it is hypermethylated it seems to correlate with PC.[65]

One of the most studied genes is APC (adenomatous polyposis coli). Some studies found a hypermethylation[45] of both the APC's promoter and GSTP1's promoter in normal prostatic tissue adjacent to tumoral tissue in patients with a worst prognosis. The protein plays the role of tumor suppressor regulating the Wnt pathway that is important for tumorigenesis.[46]

PTGS2 (Prostaglandin-endoperoxide synthase 2) is a promising gene because their hypermethylation was found also in body fluids of patients with PC.[66]

RARB encodes for β Receptor of Retinoic acid and promotes the growth and survival of tumoral cells and neoangiogenesis. This gene is frequently hypermethylated and silenced in PC.[67]

RASSF1A encodes for a protein, which shows lower levels or altered levels in several tumors.

Another studied gene is EPHX3 (Epoxide hydrolase 3). The data on methylation of this gene, used in univariate analyses of a study, show a link between the hypermethylation and the progression of PC, however the multivariate analyses do not show the same results.[47]

The protein encoding from HOXD3 gene regulates the adhesion among cells, but its function is not so clear. There are three studies focused on the protein/gene mentioned above, but the results of these different studies are contradictory. [68-70]

Chr3-EST when hypermethylated seems to be a borderline predictor of relapse, both in univariate and multivariate analysis.[48]

Hypermethylation of GPR7 (that is a protein characterized by a neuroendocrine factor, but the function related to prostate tissue, at the moment, is not yet very clear) in univariate analysis appears to be a predictor of local relapse but in multivariate analysis, the results are not the same.[71] Also, the expression and methylation data related to CD44 was investigated in PC; hypermethylation of its promoter, and so the loss of the expression of the protein (which is involved in the cell-cell interaction) has been associated with the aggressiveness of the PC.[72] Data concerning the methylation of this gene are contradictory, but in the study which

analyzed more samples the hypothesis of the relation between the methylation of the promoter and the prediction of biochemical recurrence was not confirmed[73].
More promising results related to the prediction of recurrence of PC have been found in studies focused on the downregulation derived from hypermethylation. For example the aberrant addiction of methyl groups on the miR-205 promoter, both in univariate and multivariate analysis, seems to predict the progression of the disease.[76]
In another study, instead, the hypomethylation of KLK10 has given data on the potential prediction of biochemical recurrence of the illness therefore on the potential prognostic value of this gene as a biological useful marker.[77]

1.4.1 GSTP1

Glutathione S-Transferase pi 1, also named GSTP1, is a member of the Glutathione S-Transferase family (GST), which is a group of enzymes categorized in four classes (alpha (α), mu (μ), pi (π), and theta (θ)) in relation to the biochemical, immunologic and structural features. This family is involved in detoxification processes by catalyzing the conjugation of hydrophobic and electrophilic compounds with reduced glutathione. GSTP1 is a polymorphic gene located on the chromosome 11q13.2, the gene encoding for several variants of active and functionally proteins, which seems to have a role in xenobiotic metabolism and play a role in susceptibility to cancer and other diseases[49].
Some authors suggest that GSTP1 may act as a tumor suppressor gene and have a role in the JNK signaling. The inactivation of the gene seems related to tumor growth.[50] As far as its role in detoxification is concerned, the inactivation of the gene GSTP1 could result in the accumulation of somatic genome alterations that can promote tumor growth.[41, 51-54, 74]
DNA CpG islands hypermethylation causes gene silencing and it is a common event in carcinogenesis and progression; a lot of studies focus their attention on the gene GSTP1[50,75]. Methylation of the GSTP1 gene promoter region is one of the most common epigenetic changes in PC.[63] This gene was often analyzed as a diagnostic factor. In some cases it was described also as a possible prognostic factor and was

found also a correlation between the hypermetilation of GSTP1 and tumors characterized by high Gleason scores.[55] Data about diagnostic[56] and prognostic significance of GSTP1 are controversial and for this reason it is an interesting target. Moreover many works based on methylation of GSTP1 were made using Quantitative methylation specific PCR (MSP) instead of analyses using pyrosequencing.[55]

2 AIMS OF THE PROJECT

The first aim of this project was to learn to use the systems and software to obtain epidemiologic information and to manage the data (ex. Surveillance Epidemiology and End Results (SEER) and SAS statistical package). The second aim of the project was to study therapies prescribed to patients with PC (and related to the disease).

Another aim of this work was to identify a possible association between the methylation status of the GSTP1 promoter and the relapse of the above-mentioned disease.

Finally, the most important aim of my PhD work was to test myself to work independently, from the study design to the achievement of the results, encountering a lot of problems during my work to realize my purposes.

3 RESULTS

The following paragraphs set out the results obtained during the PhD. It is possible to group the results in three parts.

a) The first section presents data about incidence and mortality rates related to PC on American, Italian and FVG populations (paragraph 5.1).
b) The second part analyzes incidence data of 15.079 patients in FVG and medications used on 2915 patients in FVG (paragraph 5.2).
c) Lastly, the attention focuses on a selected population of 122 patients resident in FVG, with PC, who underwent a radical prostatectomy for whom biological samples were available to be used for methylation analyses (paragraph 5.3).

3.1 Analyses of different populations

The first aim of this thesis was to learn to use databases and to manipulate and use skills to analyze data in order to obtain information about people affected by PC. In particular were analyzed data related to three different populations (and databases), which are:

- American population;
- Italian population;
- The Population of Friuli Venezia Giulia (FVG), that is a region located in the Northern – East part of Italy.

About these three populations, in particular, were used data from different databases to study incidence rates, mortality rates, trends over the time and by age. With respect to the American population, moreover it was possible to perform a comparison of available data on various races (this was not possible for the Italian and FVG populations because these type of data are not available). Finally, a

comparison among the different databases used was performed as part of this study.

3.1.1 SEER data

The first part of results are related to Surveillance, Epidemiology, and End Results Program (SEER) data and are grouped by ethnicity and as whole as follows: White people (represented in pink in the figures), Black people (represented in green), Other people (characterized by yellow) and All of the above (in blue).

3.1.1.1 Trends of INCIDENCE rates for PC in American Population

F*igure 3.1.1.1.1* shows the trend of incidence rates for prostate cancer for a number of several ethnicities of the American population from 1973 to 2011.

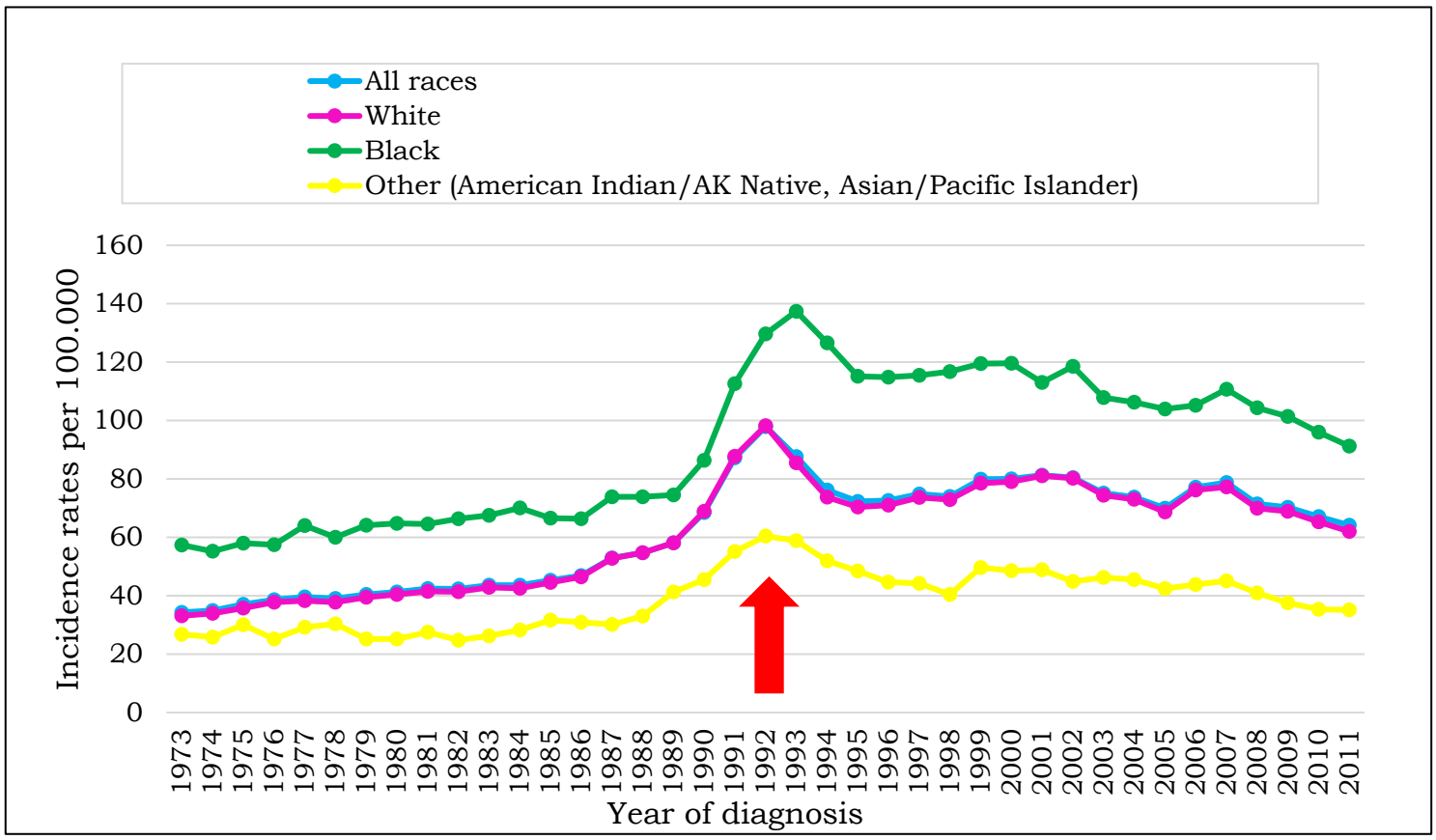

Figure 3.1.1.1.1

Trends of INCIDENCE rates for prostate cancer from 1973 to 2011.

Different populations show similar trends, and it is important to note the peak in the early nineties (related to the introduction of PSA screening).

The incidence data, extracted from SEER databases, were also analyzed using age classes and the results are presented in *Figure 3.1.1.1.2.* It is interesting to see the peaks of incidences, which show a higher incidence in the age range of 70 – 74 for black population and for the age bracket 75 – 79 when related to White, Other and also to the group named "All races".

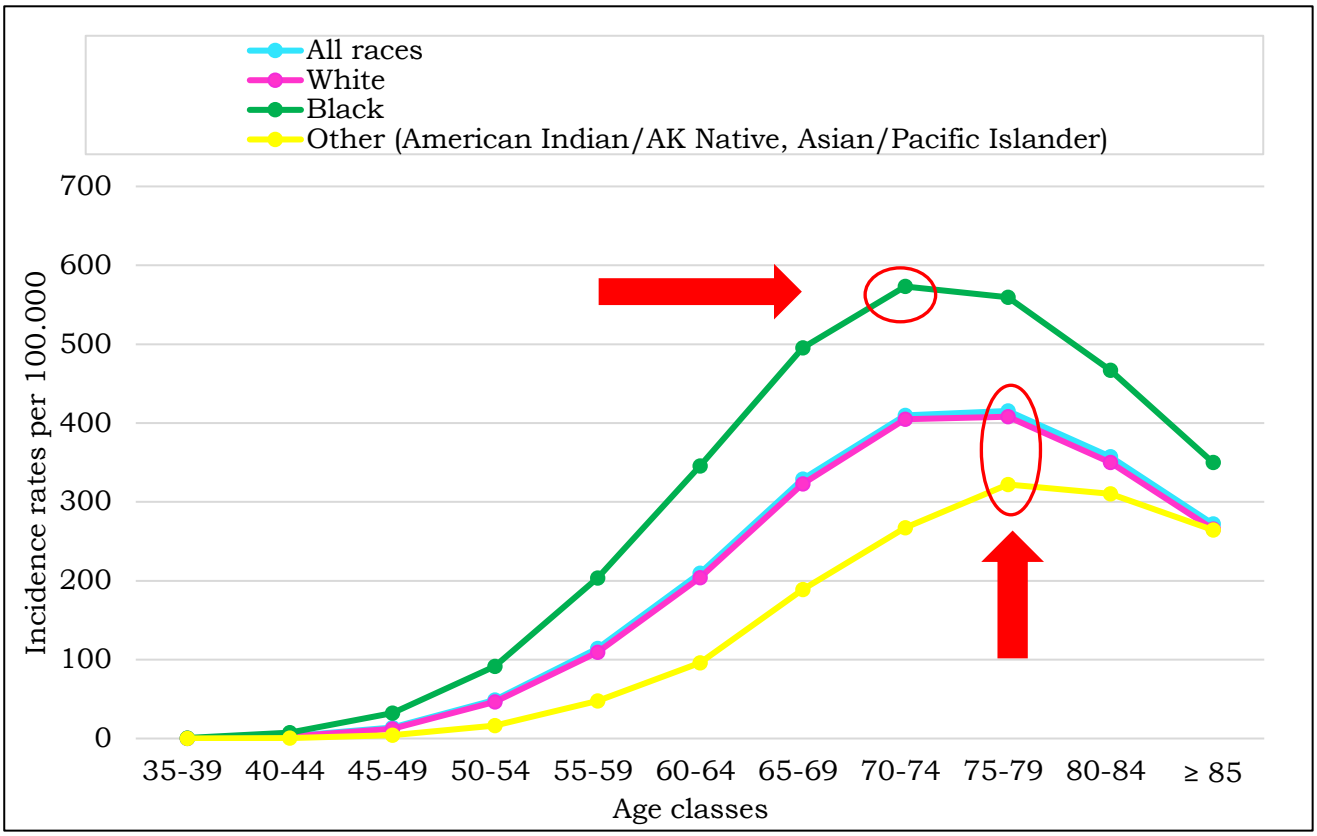

Figure 3.1.1.1.2

Trends of INCIDENCE rates for prostate cancer related to classes of age, between 1973 and 2011.

3.1.1.2 Trends of MORTALITY rates for PC in American Population

When analyzing the American population, the trends considered related to mortality rates concern the years between 1969 and 2011. It is very interesting to note the difference between the trend of Black people as it is higher than the other three populations (White, Other and All people together). All the four groups of

people considered show the same trend, with an increase in correspondence to the early years of the nineties but a decrease of mortality in all the populations analyzed in the last years considered. All these results are shown below in F*igure 3.1.1.2.1*.

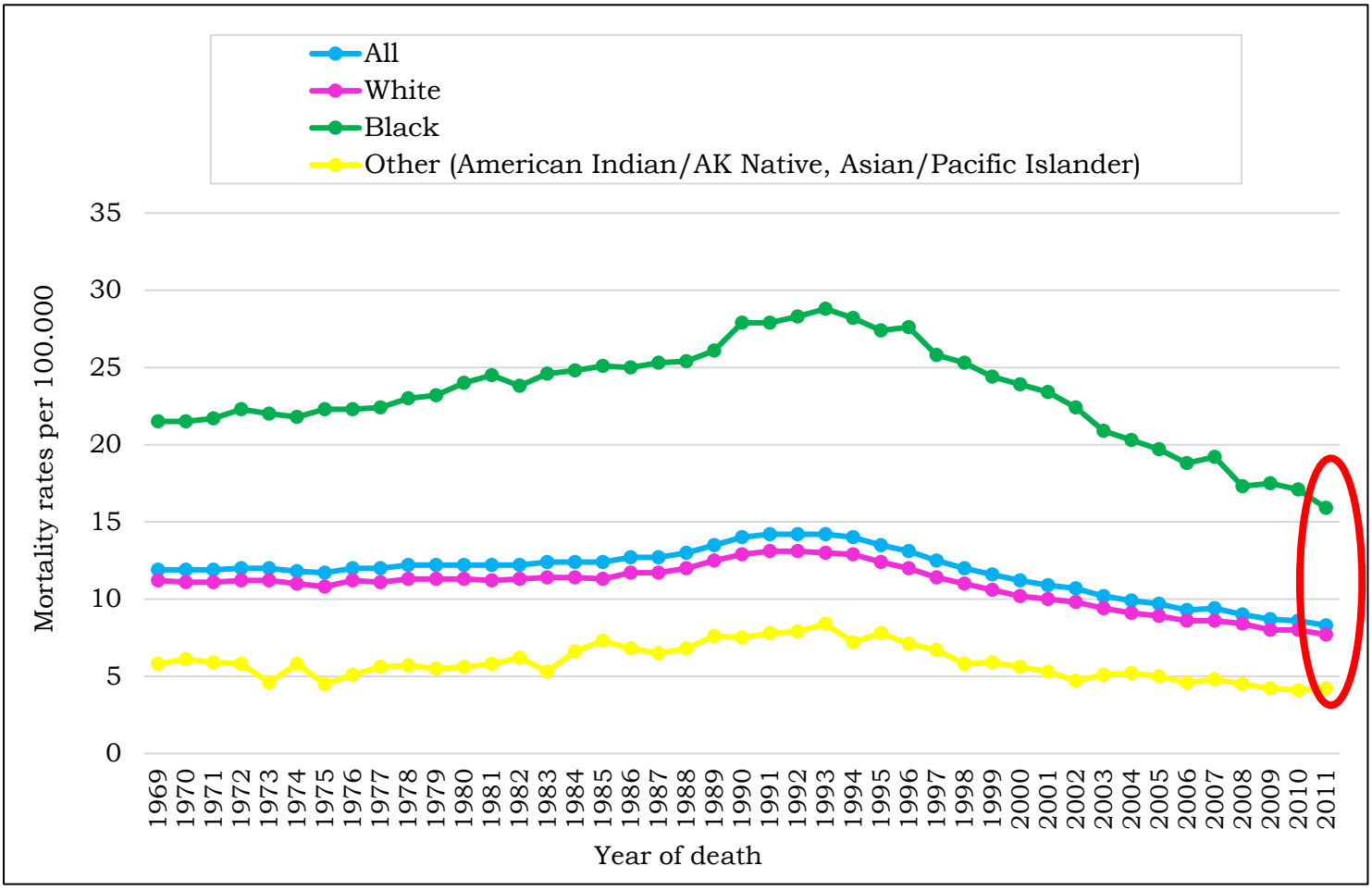

Figure 3.1.1.2.1

Trends of MORTALITY rates for prostate cancer from 1969 to 2011.

Following the study of trends over time, trends in relation to classes of age for all the four populations were analyzed (as shown in *Figure 3.1.1.2.2*). The mortality increases with age, and this is reflected in the higher-age classes showing a higher mortality rate. There is also a difference between Black people and the other populations analyzed with Black people; indeed data on Black people show higher mortality rates.

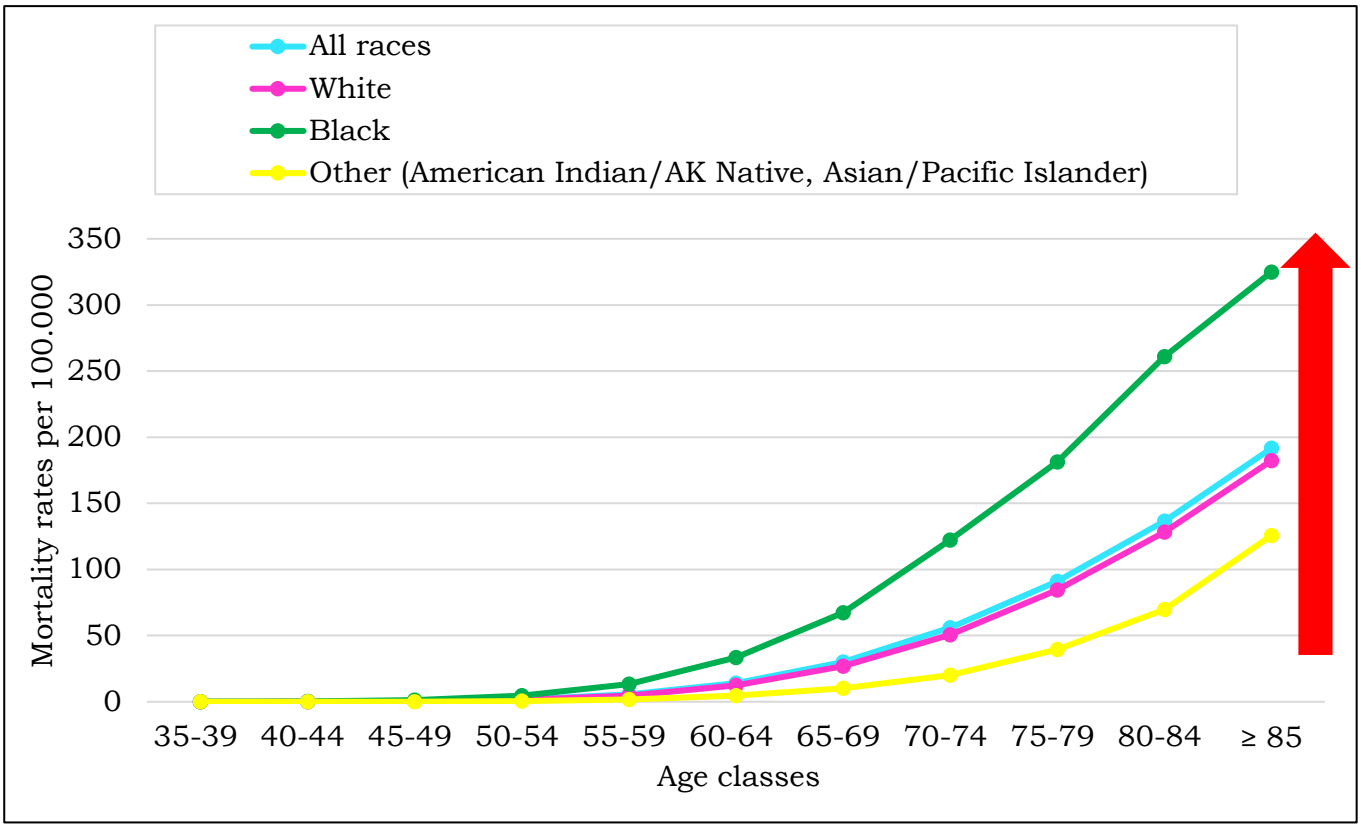

Figure 3.1.1.2.2

Trends of MORTALITY rates for prostate cancer related to classes of age, between 1969 and 2011.

3.1.1.3 Summary of results related to SEER data

The first important result is that Black people is the race, referred to the American population, characterized by higher incidence and mortality compared to the other races (White, Other and All races together). Another important element to consider, is the introduction of PSA screening, indeed the introduction of the use of that screening determined an increase in incidence of prostate cancer, as is possible to appreciate from the figures presented above.

Despite the increase of incidence of prostate cancer, the American population show a decrease in mortality from the late Nineties to 2011; moreover, this epidemiologic measure (mortality) is directly associated with the age in all races.

It is interesting to note that Black men are characterized by higher incidence and mortality rates compared to the other populations considered. The elements that could be related to the higher incidence of Black people are both socioeconomic

and biological. Specifically, lower levels of education and disadvantaged socioeconomic status are related to the first aspect; whereas biological elements are related to higher testosterone levels than other races and higher genetic predisposition to mutations and polymorphisms.
Higher risk of metastases at diagnoses (related to a poor outcome) and advanced disease at diagnosis are important factors to consider in relation to the higher mortality of the Black race. Biologically, this race shows differences in the Androgen Receptor (AR), for example the length of CAG repeats (shorter) in the gene coding for AR or a higher expression of the same receptor, but some studies described also a stronger bond between AR and their ligands in Black people.

3.1.2 AIRTUM data

This section presents the incidence data extracted from databases of Associazione Italiana dei Registri Tumori (AIRTUM). Specifically, the data analyzed relates to the years between 2006 and 2009 for data associated to Italian population. On the other hand, for the population of Friuli Venezia Giulia the data relates to the years between 2004 and 2007. For each population the chosen data refers to the most recent years available. In both the Italian and FVG populations, the trends of incidence rates related to classes of age were studied.
For the Italian population: the pink line represents data for the year 2006, blue line is related to 2007, the green line represents the year 2008, and the yellow line refers to 2009 data.
For the FVG population: the pink line represents 2004 data, blue line is related to 2005, green line 2006 data, and the yellow line data concerning 2007.

3.1.2.1 Trends of INCIDENCE rates for PC in Italian Population

Figure 3.1.2.1.1 shows data on the incidence rates in relation to classes of age for the Italian population from 2006 and 2009. Each color represents a different year (as described above); it is interesting to note that the trend of all lines is very similar as the all show a peak in correspondence of age class 70 – 74, therefore this age bracket is characterized by higher incidence rates. The trends of all lines show an

increase of incidence rates directly associated with age until class between 70 and 74 years, whereas older people (between 75 and more than 85 years) show a decrease in incidence rates.

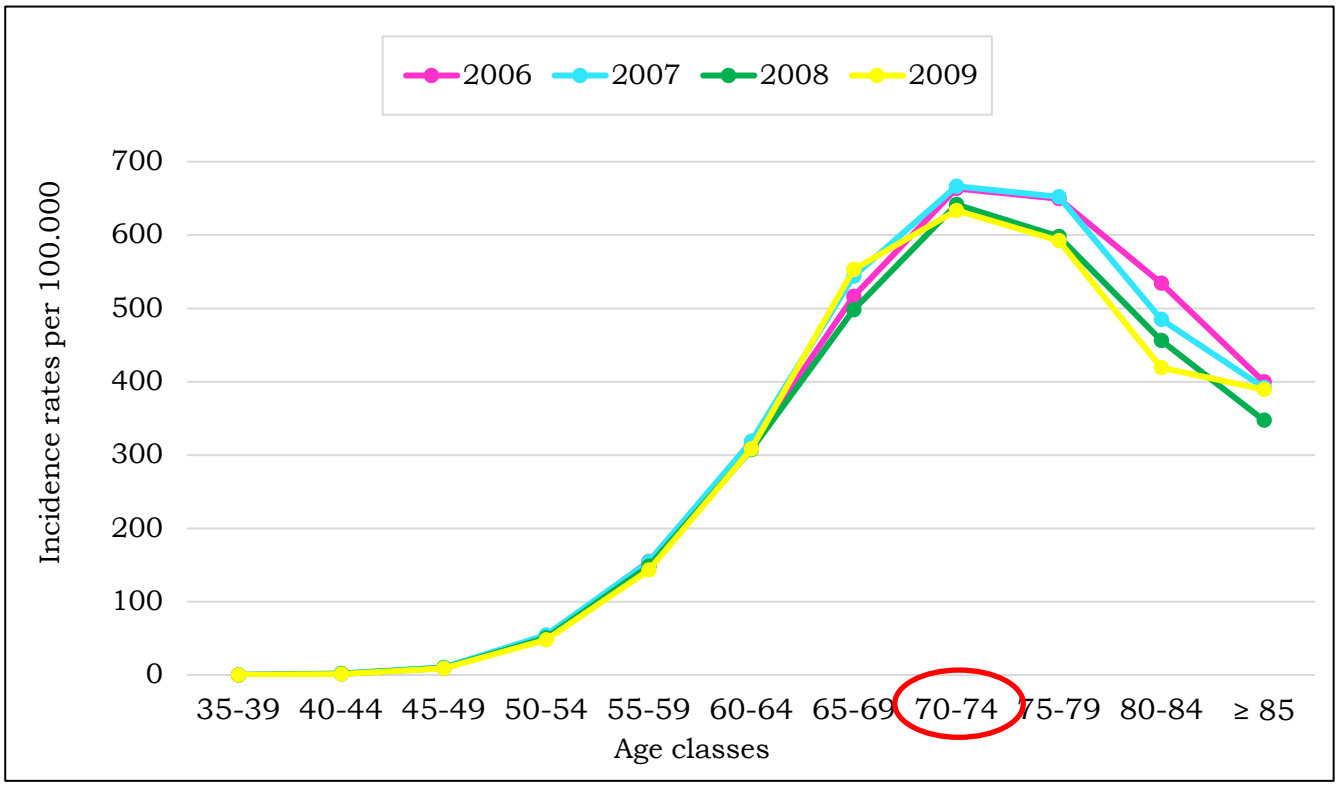

Figure 3.1.2.1.1

Trends of INCIDENCE for prostate cancer in Italy between 2006 and 2009.

3.1.2.2 Trends of INCIDENCE rates for PC in the population of Friuli Venezia Giulia

F*igure 3.1.2.2.1* shows FVG incidence rates by year and related to classes of age. Trends of all considered years shown are similar and it is possible to see an increase of incidence rates, also in this population, related to age. Similarly to the other populations, for which the trends of incidence in relation to age have been studied, older people show a decrease, of incidence rates. In particular, for the years 2004 and 2007, the higher incidence rates correspond to the age class 75 – 79, whereas the peak in 2005, corresponds to the age class 70 – 74 and, lastly, in 2006 the higher incidence was documented in the age class 80 – 84. These results are summarized in *Figure 3.1.2.2.1.*

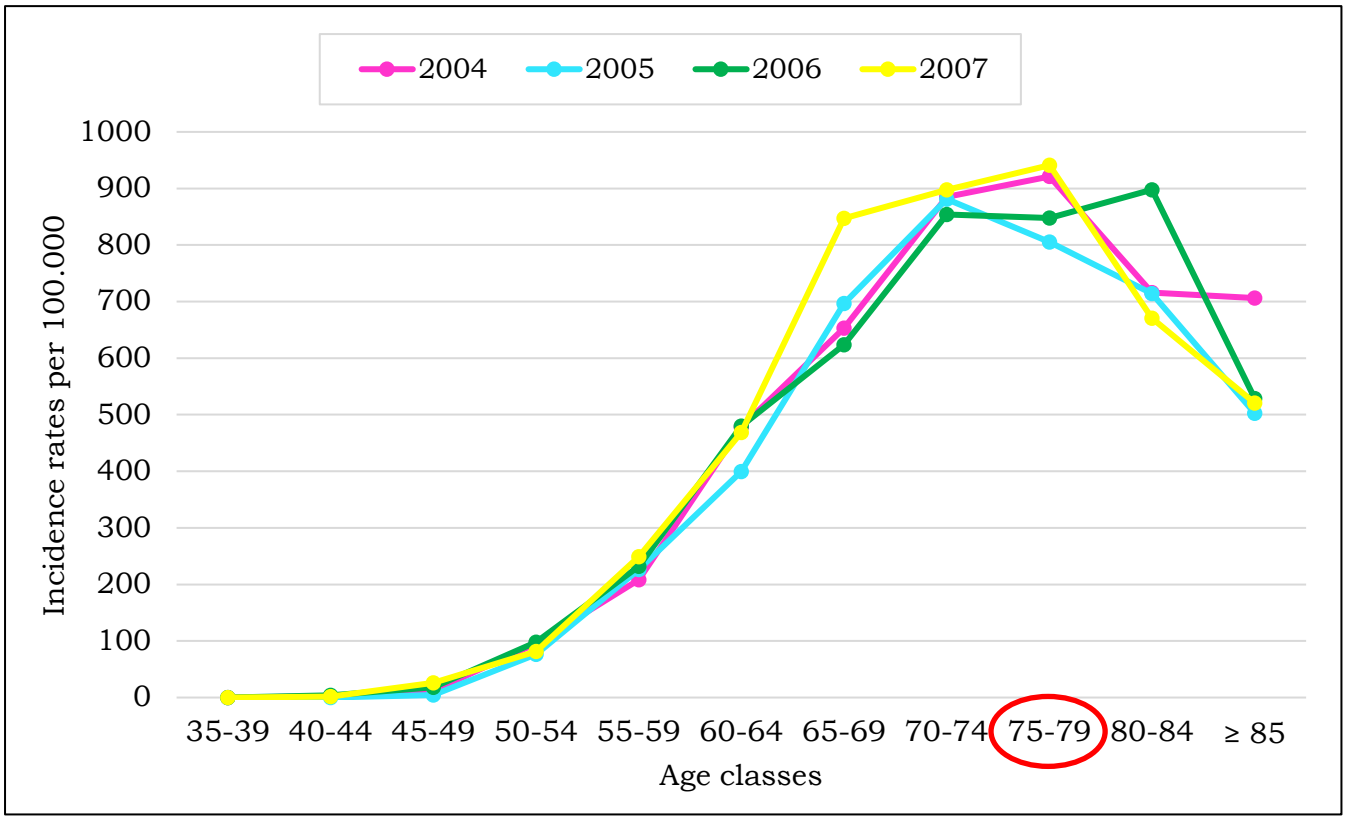

Figure 3.1.2.2.1

Trends of INCIDENCE for prostate cancer in FVG from 2004 to 2007.

3.1.2.3 Summary of results related to AIRTUM data

To summarize, Italian and FVG people show different peaks of incidence rates by age class. In fact Italian population is characterized by higher incidence rates corresponding to the class of age 70 – 75, whereas FVG residents, in three of the years studied, show higher rates in relation to older people (classes 75 – 79 and 80 - 84).

3.1.3 A comparison of trends of incidence rates

Figure 3.1.3.1 has been included to show a comparison in incidence rates among the American population (in yellow), Italian population (in blue) and FVG population (in pink). The year 2007 was chosen as it is the most recent year for which there are available data on all three populations considered. American and Italian populations show a similar trend of incidence rates and both are lower than

the FVG incidence rates. Moreover, the peak corresponds to the class of age 70- 74 relatively to the American and Italian populations whilst for the FVG population, the peak is related to the age class 80 – 84.

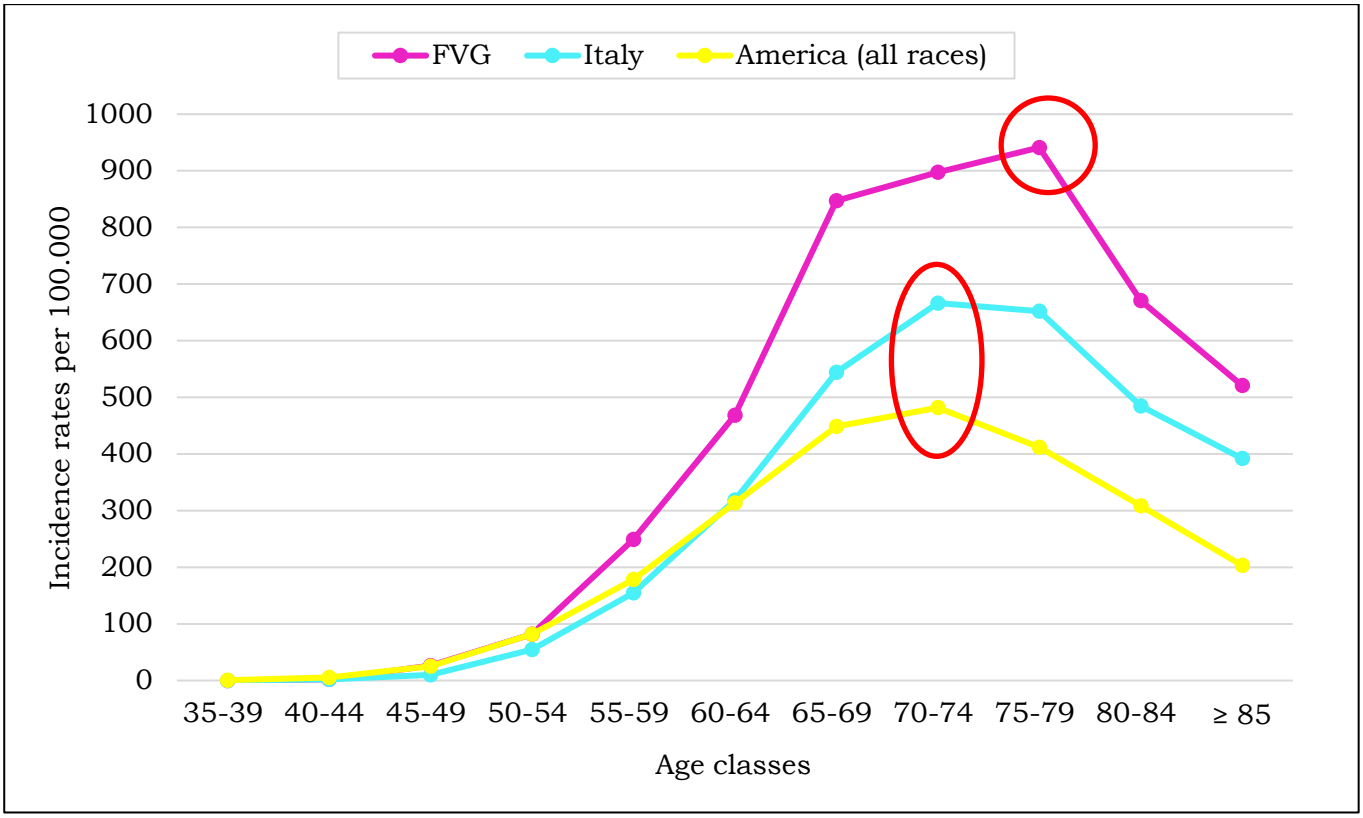

Figure 3.1.3.1

Trends of INCIDENCE rates for prostate cancer in 2007 for America, Italy and FVG.

3.1.4 ISS Data

Italian and FVG mortality's data were extracted from registers of Istituto Superiore di Sanità (ISS). In particular, for both these populations the mortality rates were analyzed every five years from 1990 to 2010, data related to 2004 and 2005 are not available, for this reason mortality rates concerning 2006 were used instead of those of the 2005. 1990 is represented by the pink line, 1995 is indicated by the blue line, 2000 is represented by the green line, 2006 is showed in red and 2010 in yellow. These colors were maintained both, in figures related to Italian

population and in those which represents FVG people. Trends of mortality rates were studied by class of age for Italian and FVG populations.

3.1.4.1 Trends of MORTALITY rates in Italy

Trends of mortality rates in Italy show an increase which is directly associated with age. Another interesting point to consider is the decrease of mortality rates in the last years; indeed F*igure 3.1.4.1.1* underlines this trend especially for years 2006 and 2010 (the red and yellow lines are lower than the other lines demonstrating a reduction in mortality rates for all classes of age considered; the red arrow aims to emphasize this concept).

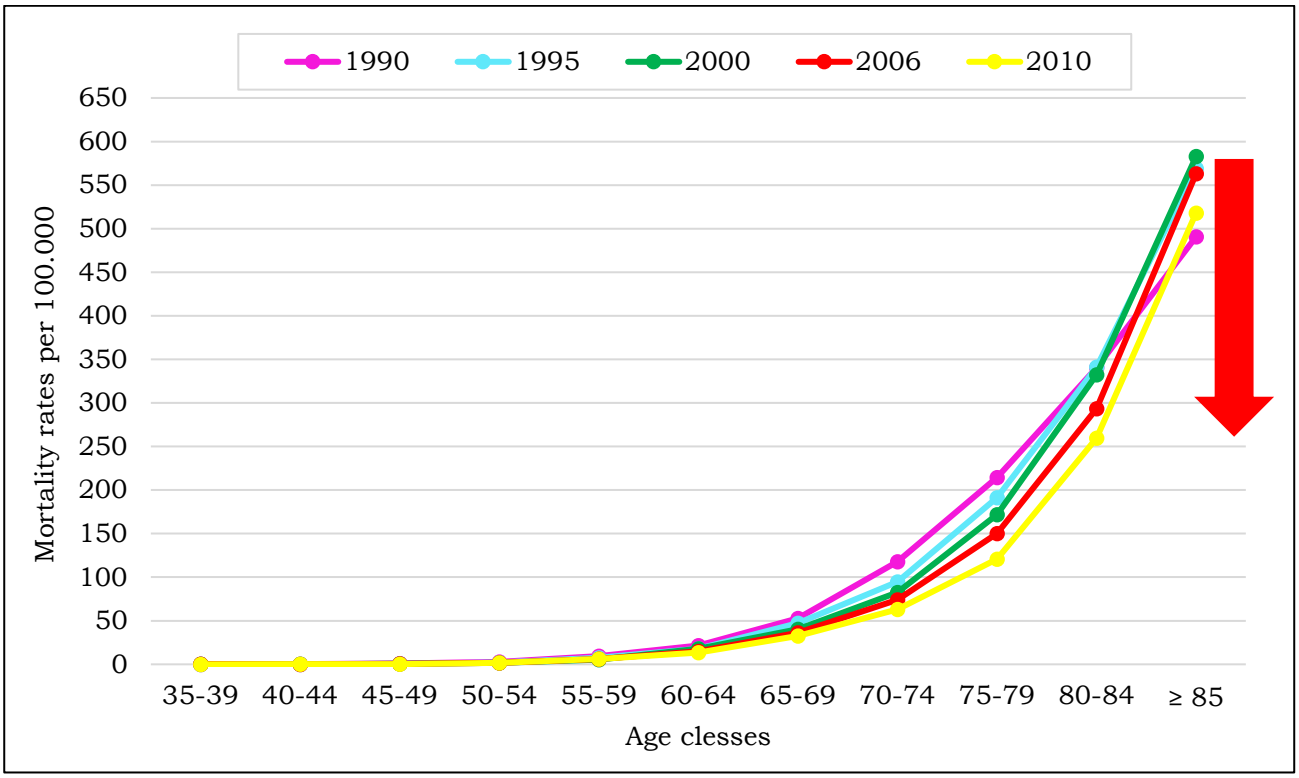

Figure 3.1.4.1.1

Trends of MORTALITY rates in Italy.

Figure 3.1.4.2.1 shows the mortality rates of Italian population in relation to the five years considered above (1990, 1995, 2000, 2006, 2010). Some of the data used, were also used to obtain *Figure 3.1.4.1.1*, however, *Figure 3.1.4.2.2* represents the

trends related to the classes of age 55 - 59, 60 - 64, 65 - 69, 70 - 74, 80 - 84 and ≥85, where the ages between 35 and 54 were excluded because the mortality rates are very low. This figure represents the trends of mortality rates in Italy in a different way, showing a decrease in mortality rates from 2000 to 2010 for all considered classes of age.

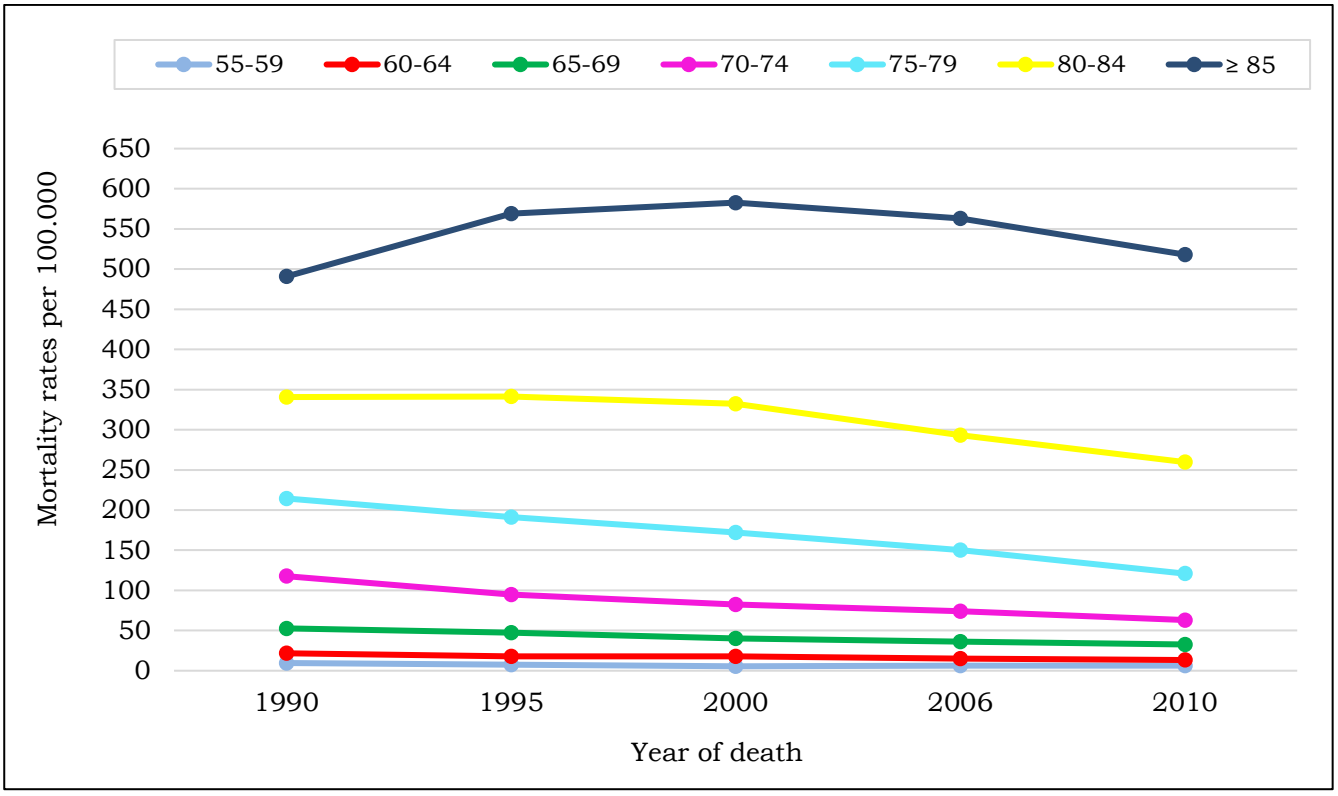

Figure 3.1.4.1.2

Trends of MORTALITY rates in Italy.

3.1.4.2 Trends of MORTALITY rates in Friuli Venezia Giulia

Mirroring the trends concerning the Italian population, also in FVG there is a reduction in mortality rates over the time; indeed the lines in F*igure 3.1.4.2.1* highlight the decrease of mortality from 1990 to 2010. Also in this population there is a direct association between mortality rates and age.

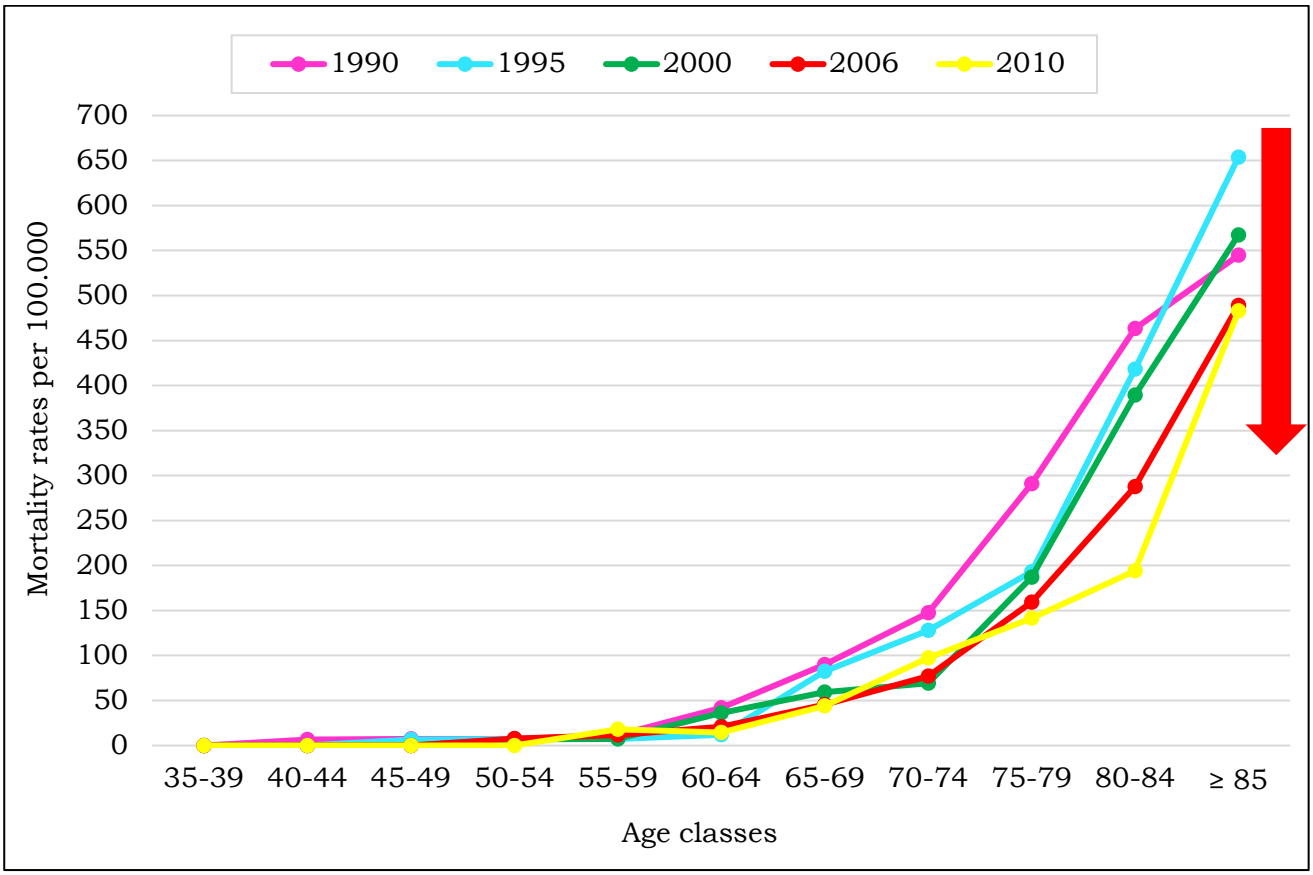

Figure 3.1.4.2.1

Trends of MORTALITY rates in FVG.

Figure 3.1.4.2.2 shows trends of mortality rates in FVG related to the years 1990, 1995, 2000, 2006, 2010 (which are the same years used for Italian population figure). The classes of age considered in this figure are: 55 – 59, 60 – 64, 65 -69, 70-74, 75 – 79, 80 – 84, ≥85. Trends show a decrease in mortality rates from 1995 to 2010 for the classes of age 65 – 69, 75 – 79, 80 – 84 and ≥85, whereas a different trend characterizes the class of age including people from 70 to 74 showing an increase of mortality trend from 2000 to 2010.

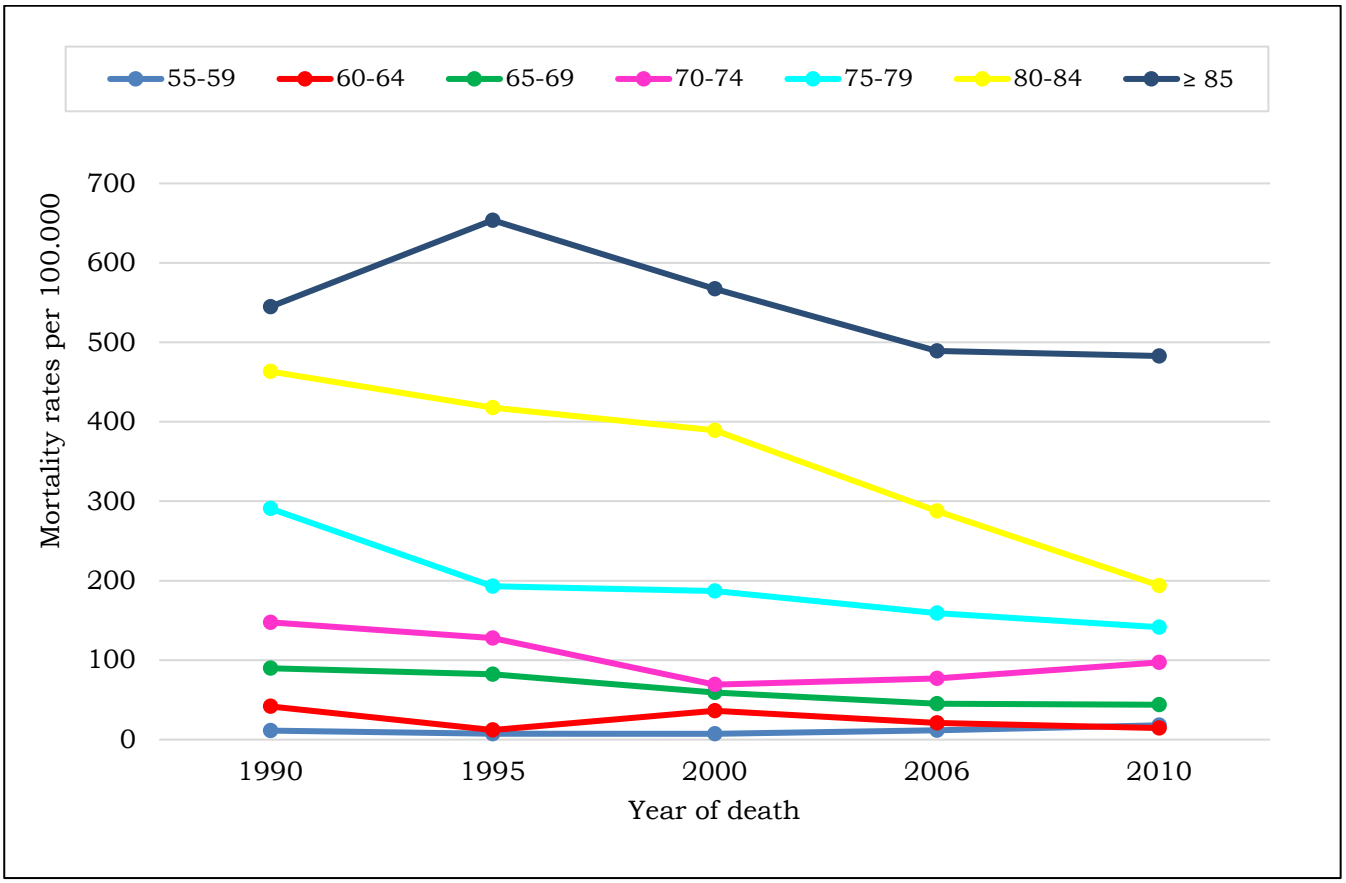

Figure 3.1.4.2.2

Trends of MORTALITY rates in FVG.

3.1.4.3 Summary of results related to ISS data

Both Italian and FVG populations show a decrease in mortality rates for prostate cancer from 1990 to 2010; in F*igures 3.1.4.1.1 and 3.1.4.2.1,* these trends are represented by red arrows. The mortality, in both populations, is directly related to age; indeed, despite an overall decrease in mortality rates over the time, there is an increase of mortality within age classes.

3.1.5 A comparison among trends of mortality rates

The comparison between ISS and SEER data underlines a similar trend for the Italian and American populations but a different trend for the FVG data, which is less linear and consistent. *Figure 3.1.5.1,* was made only to have an idea of the mortality trends between 1980 and 2011, show a decrease in mortality over the time for the three populations. Data about 2004 and 2005 were not included because they are not available for FVG and Italian populations. For the American population, the data considered included All races. The years (of death) are represented in abscissa, whereas the ordinate reports mortality rates. In order to collect more information, *Figure* (3*.1.5.2*), representing trends of mortality in relation to age for the three populations considered, in the year 2010, was also prepared. Also in this case the trends confirmed an increase of mortality rates in relation to the increase in age.

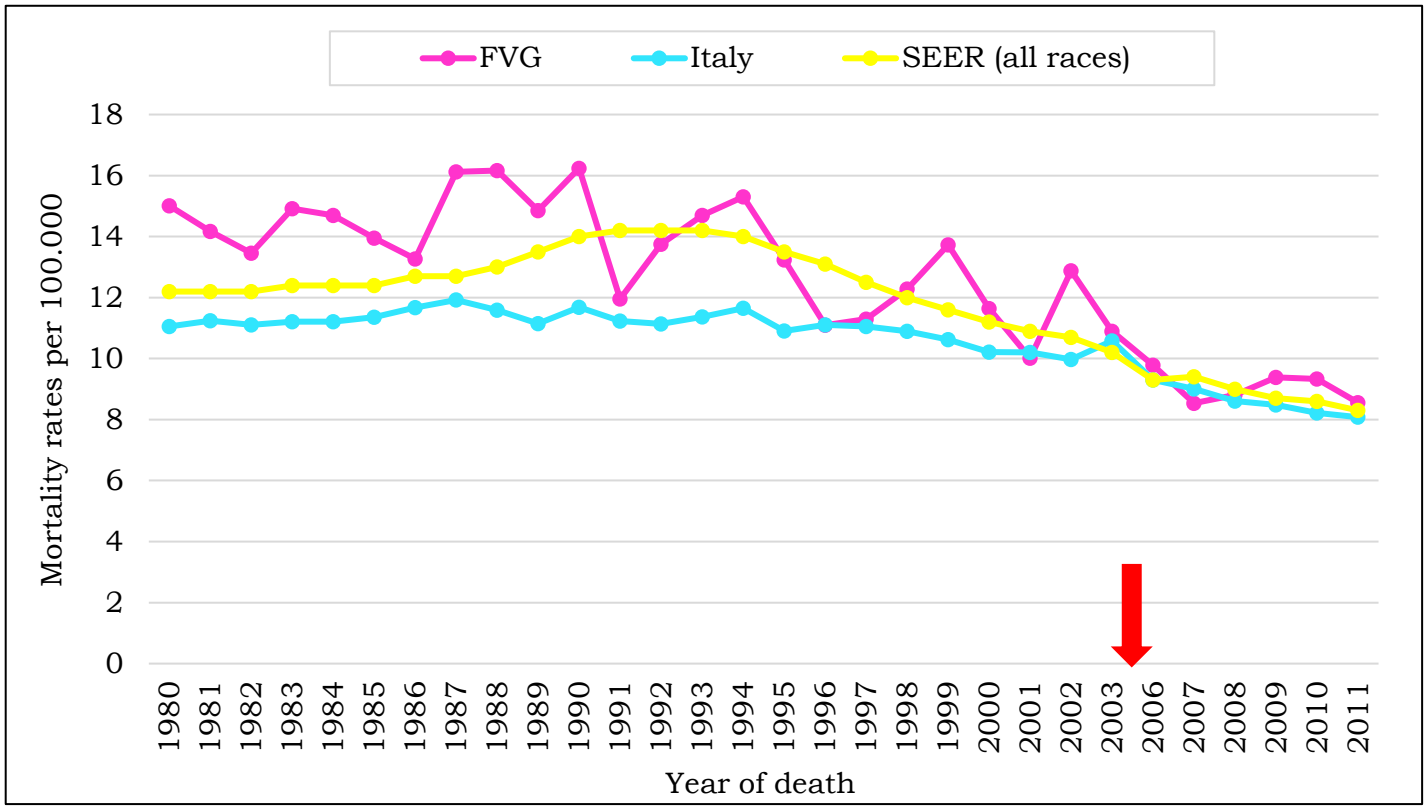

Figure 3.1.5.1

Trends of MORTALITY rates for prostate cancer from 1980 to 2011 in FVG, Italy and America.

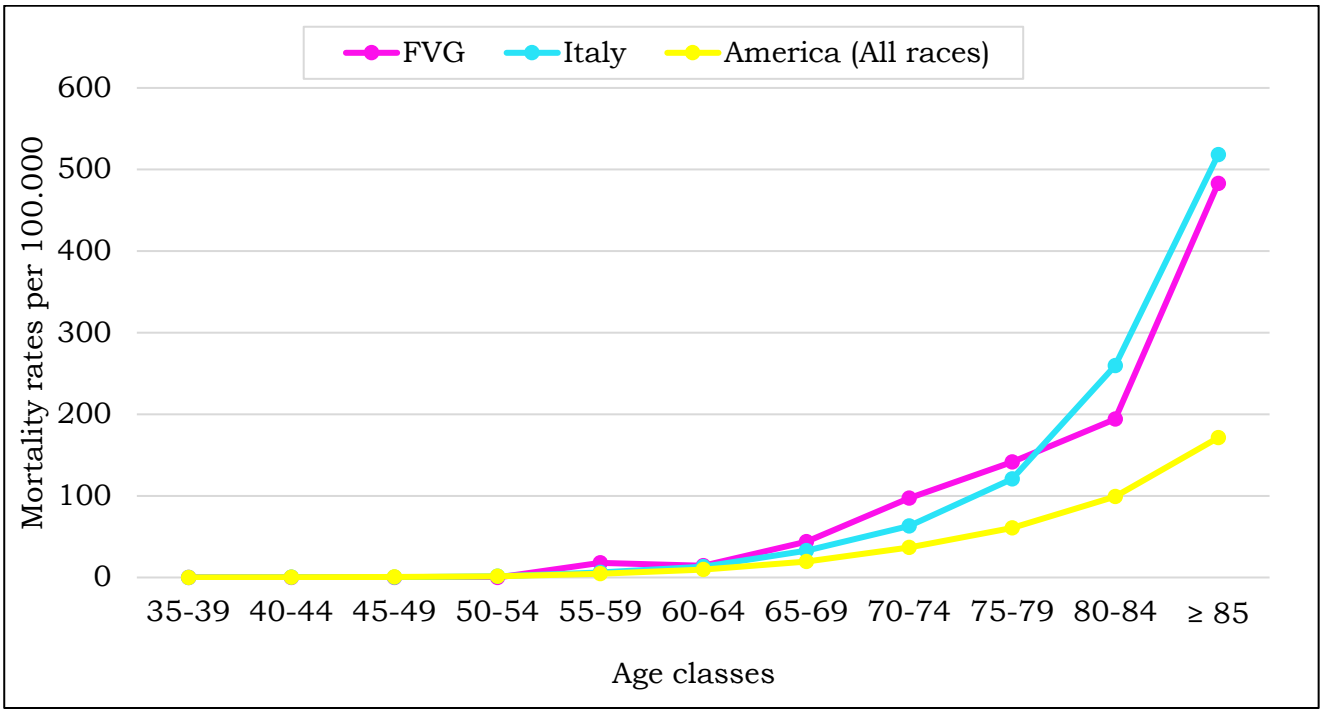

Figure 3.1.5.2

Trends of MORTALITY rates in 2010 in FVG, Italy and America.

3.2 Focus on data obtained from Regional Repository of microdata about FVG population

Another aim of this work was to try to understand what are the key points of PC and the data contained in the Tumor's register and in the regional repository of microdata, as prostate cancer cases were used to obtain these results. From the database mentioned above, data were extracted, referring to FVG population in the period between 1995 and 2009. From Tumor register the data obtained were in particular about the new diagnosis. Subsequently to the linking of the data of the Tumor's register and data of the regional repository of microdata, it was possible to obtain also information on deaths, medications and some other information about people affecting by prostate cancer.

3.2.1 New cases of PC in Friuli Venezia Giulia

From the link between Tumor register and regional repository of microdata it was possible to identify 15.079 people with a new diagnosis of PC in FVG. The data were analyzed considering age at diagnosis and year of diagnosis; for both these information frequencies and percentages were obtained (presented in *Tables 3.2.1.1 and 3.2.1.2*).

More than twenty-one percent (21.88%, 3300 subjects) of all considered people had a diagnosis of PC between 70 and 74 years of age; this class is followed by the class constituted by people with between 65 and 69 years of age (20.18%); also the class 75 – 79 is well represented (17.61%).

Data presented in more detail in the *Table 3.2.1.1*.

Age at diagnosis	Frequency (N)	Percentage (%)
30-34	1	0.01
40-44	12	0.08
45-49	72	0.48
50-54	373	2.47
55-59	1011	6.70
60-64	2062	13.67
65-69	3043	20.18
70-74	3300	21.88
75-79	2655	17.61
80-84	1603	10.63
≥ 85	947	6.28

Table 3.2.1.1

Data of new diagnosis of PC in FVG in relation to age at diagnosis.

When analyses were performed using the year of diagnosis as reference, it was possible to see an increase in the number of diagnosis in the early 2000s,

confirming that the introduction of PSA screening, had an effect on the number of diagnoses, however the peak of new diagnoses occurred in 2007 with (1259 new cases of PC). These data are presented in *Table 3.2.1.2*.

Year of diagnosis	Frequency (N)	Percentage (%)
1995	634	4.20
1996	735	4.87
1997	842	5.58
1998	918	6.09
1999	931	6.17
2000	967	6.41
2001	998	6.62
2002	1057	7.01
2003	1101	7.30
2004	1145	7.59
2005	1085	7.20
2006	1165	7.73
2007	1259	8.35
2008	1125	7.46
2009	1117	7.41

Table 3.2.1.2

Description of new cases of PC in FVG, based on years of diagnosis.

Table 3.2.1.3 summarizes the information about the country of birth of 15.079 new cases of PC. In most cases (96.46%) the country of birth is Italy, in 156 cases (1.03%) this information was not available, whereas other States are negligible.

Country of birth	Frequency (N)	Percentage (%)
Italy	14545	96.46
Missing	156	1.03

Ex Yugoslavia	105	0.70
France	80	0.53
Austria	15	0.10
Libya	14	0.09
Romania	13	0.09
Argentina	11	0.07
Belgium	11	0.07
Switzerland	11	0.07
Great Britain and Northern Ireland	10	0.07
Egypt	9	0.06
Germany	8	0.05
Slovenia	8	0.05
USA	8	0.05
Albania	7	0.05
Croatia	7	0.05
Ethiopia	4	0.03
Ghana	4	0.03
Turkey	4	0.03
Ex U.R.S.S	4	0.03
Bosnia- Herzegovina	3	0.02
Federal Germany	3	0.02
Netherlands	3	0.02
Serbia	3	0.02
Tunisia	3	0.02
Hungary	3	0.02
Bulgaria	2	0.01
Congo (Democratic republic)	2	0.01
Eritrea	2	0.01
Morocco	2	0.01
Poland	2	0.01
Russia	2	0.01
Venezuela	2	0.01
Australia	1	0.01
Brazil	1	0.01
Czechoslovakia	1	0.01
Chile	1	0.01
Philippines	1	0.01
Greece	1	0.01
India	1	0.01
Luxembourg	1	0.01
Macedonia	1	0.01
Norway	1	0.01
Czech Republic	1	0.01

Senegal	1	0.01
Spain	1	0.01

Table 3.2.1.3

Distribution of Country of birth of new cases of PC in FVG from 1995 to 2005.

3.2.2 Deaths caused by PC in Friuli Venezia Giulia

From regional repository of microdata, data of deaths caused by PC were also extracted. These data are described in *Table 3.2.2.1,* and are available up to 2014. Until 2014, 2513 deaths caused by PC were identify. In line with data collected on new diagnoses of PC, also in these case descriptive analyses were performed considering age at death and year of death (*Tables 3.2.2.1 and 3.2.2.2*).

More than one quarter (26.34 %) of deaths due to PC occurs in people with almost an age of almost 85 years; *Table 3.2.2.1* shows that there is an increase of the number of deaths for PC directly linked with age.

Age at death	Frequency (N)	Percentage (%)
40-44	1	0.04
45-49	5	0.20
50-54	13	0.52
55-59	58	2.31
60-64	128	5.09
65-69	229	9.11
70-74	385	15.32
75-79	473	18.82
80-84	559	22.24
≥ 85	662	26.34

Table 3.2.2.1

Deaths due to PC in relation to age at death.

To collect more complete descriptive information, analyses on subjects dead due to PC were also performed considering the year of death (from 1995 to 2014). In this case, there is a quite similar distribution over the time, as it is possible to see in *Table 3.2.2.2*.

Year of death	Frequency (N)	Percentage (%)
1995	118	1.55
1996	159	2.09
1997	215	2.83
1998	245	3.22
1999	295	3.88
2000	317	4.17
2001	361	4.74
2002	427	5.61
2003	424	5.57
2004	455	5.98
2005	464	6.10
2006	484	6.36
2007	470	6.18
2008	504	6.62
2009	569	7.48
2010	496	6.52
2011	476	6.25
2012	469	6.16
2013	430	5.65
2014	232	3.05

Table 3.2.2.2

Distribution of deaths due to PC from 1995 to 2014.

The nation of birth was also analyzed in relation to the data on death. Of 14.545 subjects affected from PC, 2401 (16.51%) died of this disease, whereas of 378

people born in other states, 68 (17.51 %) died for the same cause as presented in *Table 3.2.2.3*.

Nation of birth	Death for PC
Italy 14545 (97.47 %)	2401 (16.51 %)
Other states 378 (2.53 %)	68 (17.99 %)
Total 14.923	2469

Table 3.2.2.3.

Data linking state of birth and death for PC.

Lastly, the last table of this section provides information about the survival after the diagnosis for people who died of PC (T*able 3.2.2.4*). In particular, to obtain these data was necessary to use only the data of 2513 people with a date of death. More than one 79% survived 1 year post-diagnosis, more than 50% of patients were alive 3 years post-diagnosis. For 1.67% (42 people) of patients, PC diagnosis was done after their death.

Survival after diagnosis related to patients died to PC	Frequency (N)	Percentage (%)
Survived 1 year post-diagnosis	1996	79.43
Survived 2 years post-diagnosis	1599	63.63
Survived 3 years post-diagnosis	1294	51.49
Survived 4 years post-diagnosis	1033	41.11
Survived 5 years post-diagnosis	835	33.23
Survived 6 years post-diagnosis	660	26.26
Diagnosis after death	42	1.67

Table 3.2.2.4

Data on survival after a PC's diagnosis, of 2513 subjects died due to PC.

3.3 Analyses of medications used by subjects with PC

Whilst researching and collecting data, two different research approaches were applied about medications:

a) A priori: a review of literature and guidelines allowed the identification of all drugs used in the therapy of PC; in particular, 50 different medications (which have been validated by an urologist and an oncologist) had been found, and they are summarized in *Supplementary table 1.*

b) From databases obtained from the regional repository of microdata: all prescribed drugs were identified (starting from diagnosis); this allowed to identify 86 different ATC codes (belonging to the 13 groups chosen to be analyzed), thought attention was focused on nine of these groups, in particular on:
   - Estrogens (E),
   - antiandrogens (group G (AAG) and group L (AAL)),
   - drugs used in benign prostatic hypertrophy (BIP),
   - systemic hormonal preparations (HP),
   - antineoplastic agents (C),
   - gonadotropin releasing hormone analogs (AGnRH),
   - antiestrogens (AE),
   - other hormone antagonists and related agents (AAO).

   Table 3.3.1 shows the medications (included in the 9 classes considered) identified from the repository.

Class of medications	Medication	ATC code	Users
HP	Tetracosactide	H01AA02	1
HP	Desmopressin	H01BA02	5
HP	Octreotide	H01CB02	4
HP	Lanreotide	H01CB03	4
HP	Betametasone	H02AB01	304
HP	Dexamethasone	H02AB02	209

HP	Methylprednisolone	H02AB04	265
HP	Prednisone	H02AB07	1325
HP	Triamcinolone	H02AB08	62
HP	Idrocortisone	H02AB09	1
HP	Cortisone	H02AB10	23
HP	Levothyroxine sodium	H03AA01	183
HP	Lithyronine sodium	H03AA02	5
HP	Thiamazole	H03BB02	66
HP	Potassium perchlorate	H03BC01	5
HP	Glucagon	H04AA01	5
HP	Calcitonin	H05BA01	1
HP	Cinacalcet	H05BX01	4
HP	Paracalcitol	H05BX02	6
C	Corticotropin	L01AA01	16
C	Etoposide	L01CB01	6
C	Estramustine	L01XX11	191
AGnRH	Buserelin	L02AE01	131
AGnRH	Leuprorelin	L02AE02	715
AGnRH	Goserelin	L02AE03	145
AGnRH	Triptorelin	L02AE04	671
AE	Tamoxifen	L02BA01	32
AAL	Flutamide	L02BB01	248
AAL	Bicalutamide	L02BB03	1254
AAO	Degarelix	L02BX02	24
E	Ethinylestradiol	G03CA01	2
AAG	Cyproterone	G03HA01	459
BIP	Finasteride	G04CB01	204
BIP	Dutasteride	G04CB02	99

Table 3.3.1

Medications (belonging to the nine classes of medications of interest) found from the regional repository of microdata, assumed by people with PC.

3.3.1 Descriptive analyses on users and prescriptions from 1995 to 2014

Table 3.3.1.1 summarizes the numbers of users and prescriptions belonging to 9 groups of interest between 1995 and 2014, in subjects with an admission in one of the healthcare facilities in the city of Udine.

Considering the classes of medications, the most used are HP (1802 users, followed by users of AGnRH (1463) and of users of AAL (1373)). The AAG and the AAO users were less than the AAL users. People who used medications included in AE group are 32 whereas users of estrogens are 2.
With reference to prescriptions data, the results present some differences, indeed the most prescribed medications are drugs included in the group of AAL, followed by AGnRH and HP. The classes characterized by fewer prescriptions are AE (168), AAO (162) and E (only 19 prescriptions). Both users and prescriptions of a class of medications can be included also in another group of medications, indeed the several classes of medications are not mutually exclusives, so the same person could use several classes and could have more prescriptions for the same medication.

Classes of medications	***Prescriptions***	***Users***
AAG	5336	459
AAL	16708	1373
AE	168	32
AGnRH	16178	1463
C	1557	201
BIP	3621	284
HP	15028	1802
E	19	2
AAO	162	24

Table 3.3.1.1

Number of prescriptions and users of the 9 groups of medications of interest from 1995 to 2014, used (after diagnosis) by people with PC.

Data extracted from the regional repository of microdata, in particular about prescriptions and users, were also analyzed to study the trend over the time.
The trend of prescription's use is illustrated in F*igure 3.3.1.1* (Number of prescriptions in the Y-axis, and years in the X-axis). From 1998 to 2014, it is interesting to note the increasing use of medications included in HP group. Class

of AGnRH shows an opposite trend with a peak in 2000 and another peak in 2013. The use of antiandrogens shows two different trends; when AAL and AAG are considered the trends indicate a first increase from 1998/1999 to 2004/2005 followed by a decrease of prescriptions for these groups of medications, whereas the class of AAO shows an increase since its first use in 2011. The data about prescriptions and users started from 1998.

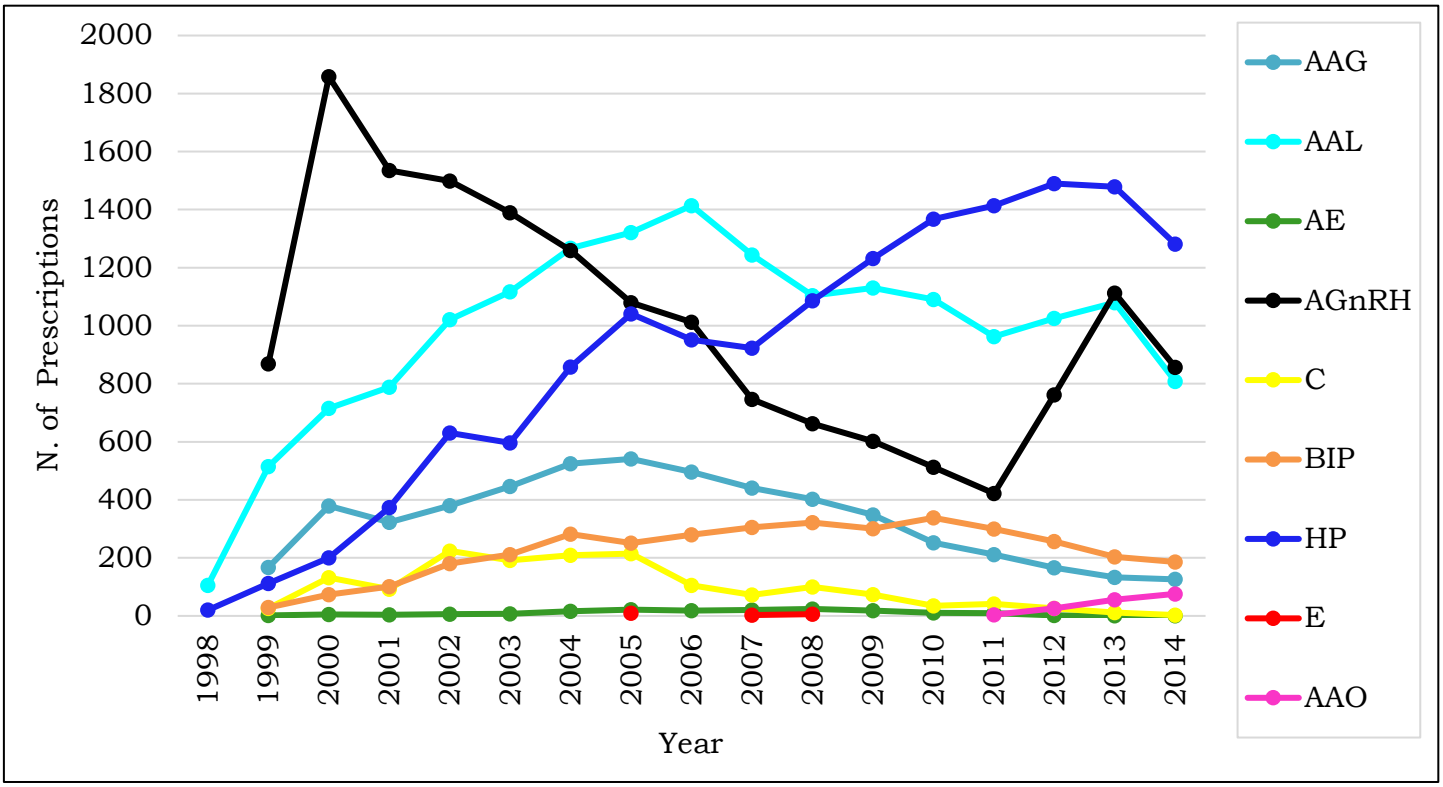

Figure 3.3.1.1

Trends of prescription' use of the nine classes of medications of interest from 1995 to 2014.

Figure 3.3.1.2 shows the trends of users for each class of medications of interest for the years between 1998 and 2014. Users of classes AAG and AAL show an increase until the years 2005/2006 followed by a decrease of users, whereas the users of medications included in HP show a continuous increase, together with the users of AAO's class. Users of BIP medications are quite unvaried. Lastly, use of the AGnRH's class decreased until 2011 and then showed an increment.

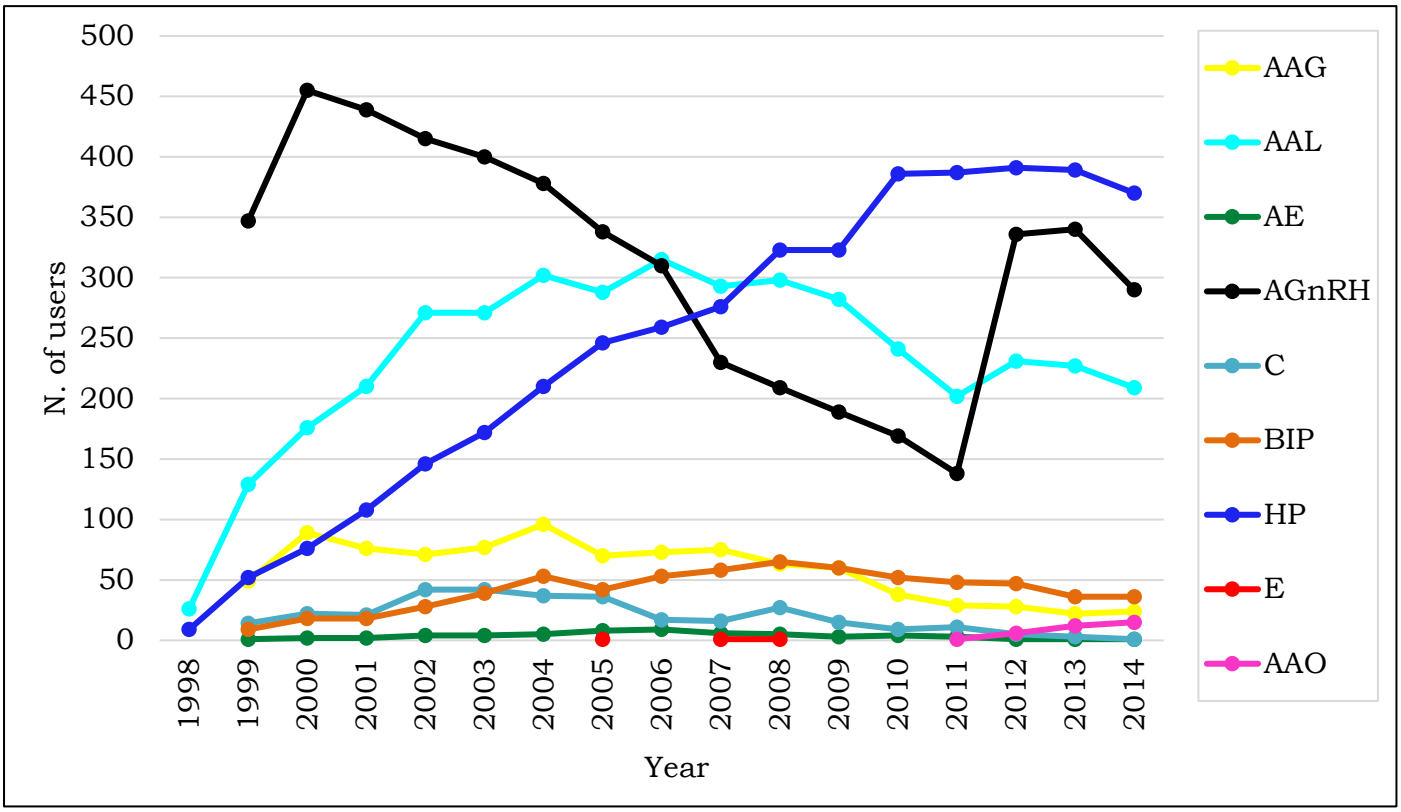

Figure 3.3.1.2

Trends of users of the nine classes of medications of interest from 1995 to 2014.

3.3.2 Combinations of medications used

Combinations of medications were studied to understand what classes are used alone or together and how frequently. *Table 3.3.2.1* lists the combinations used (in which the order of intake is important). Also in this case, medications as active substances were not used, instead only the groups they belong to were taken into consideration. This choice was made to reduce the number of combinations in order to limit the number of combinations represented by with very little numbers/percentages. *Table 3.3.2.1* lists the combinations (from the population of 2915 patients) used by almost 10 patients. More than seventy-five percent (76.58%) is the sum of combinations represented more than 1%; but most combinations were used in less than 1% of patients. The most used combination of medications is the class of HP (26.11%), followed by people taking only a drug of AGnRH's class

(7.72%). The third highest percentage is characterized by patients that take medications of both group of AAL and of AGnRH's group.

Additional information about the use of medications is presented in *Table 3.3.2.2*, where the patients are divided on the basis of the sum of the different groups used. More than 44 % used only one group of medications, and 29% of patients use two several groups but there is also a small number of people characterized by an intake of 5 or 6 different classes of medications.

Combinations of medications	***Frequency (N)***	***Percentage (%)***
HP	761	26.11
AGnRH	225	7.72
AAL + AGnRH	208	7.14
AAL	170	5.83
AAL + AGnRH + HP	145	4.97
AAL + HP	130	4.46
AGnRH + HP	94	3.22
AGnRH + AAL	92	3.16
BIP	69	2.37
AAG	53	1.82
AGnRH + AAL + HP	53	1.82
HP + AGnRH	49	1.68
AAG + AGnRH	47	1.61
HP + AAL	39	1.34
BIP + HP	34	1.17
AAG + AGnRH + AAL	32	1.10
AAG + AGnRH + HP	31	1.06
HP + AAL + AGnRH	28	0.96
AAG + HP	27	0.93
AAL + HP + AGnRH	27	0.93
HP + BIP	24	0.82
AGnRH + HP + AAL	20	0.69
AAL + AGnRH + C + HP	18	0.62
AAL + AGnRH + AAG	16	0.55
AAL + AAG	14	0.48
AAL + AGnRH + C	14	0.48
AAL + AGnRH + HP + C	13	0.45
AGnRH + AAL + C + HP	12	0.41
BIP + AAL + AGnRH	12	0.41
AAG + AGnRH + AAL + HP	10	0.34
AGnRH + BIP	10	0.34

HP + AAG	10	0.34

Table 3.3.2.1

Most used combinations of medications in patients with PC in FVG.

Sum of classes used	Patients (N)	Percentage (%)
1	1285	44.08
2	847	29.06
3	534	18.32
4	193	6.62
5	49	1.68
6	7	0.24

Table 3.3.2.2

Distribution of patients with PC in FVG which take medications, based on the number (sum) of different groups taken.

3.3.3. Patient's features

When considering the age at diagnosis, of the 2915 subjects studied, the most represented class is between 70 and 74 years (21.92 %), followed by the class of people with an age of 65 – 69 years (20.34 %), whereas the less represented classes of people are younger than 54 and older than 85 (data presented in *Table 3.3.3.1*).

Age at diagnosis	Patients (N)	Percentage (%)
≤ 54	74	2.54
55-59	178	6.11
60-64	432	14.82
65-69	593	20.34
70-74	639	21.92
75-79	560	19.21
80-84	292	10.02
≥ 85	147	5.04

Table 3.3.3.1

Distribution of patients with PC which intake medications in FVG in relation to age at diagnosis.

Other information about the 2915 people analyzed in the paragraphs above, referred to incidence data (*Table 3.3.3.2 and Figure 3.3.3.1*), mortality data and finally surgical information.

a) Incidence data

Based on incidence data (available from 1995 to 2009), *Table 3.3.3.2* underlines that in 2001 there is a slight increase in incidence of PC, but are roughly the same numbers over the time; also in this instance, the numbers show an increase after the years of introduction of the screening of PSA.

Year of diagnosis	Patients (N)	Percentage (%)
1995	60	2.06
1996	98	3.36
1997	132	4.53
1998	177	6.07
1999	221	7.58
2000	226	7.75
2001	256	8.78
2002	232	7.96
2003	231	7.92
2004	253	8.68
2005	219	7.51
2006	201	6.90
2007	231	7.92
2008	213	7.31
2009	165	5.66

Table 3.3.3.2

Incidence of PC in people residents in Udine.

Figure 3.3.3.1 represents the trend of incidence of PC in subjects with minimum one admission in one of the healthcare facilities of Udine; also in this case the trend show an increase in incidence until the firsts years of 2000 and then a trend that remains quite constant.

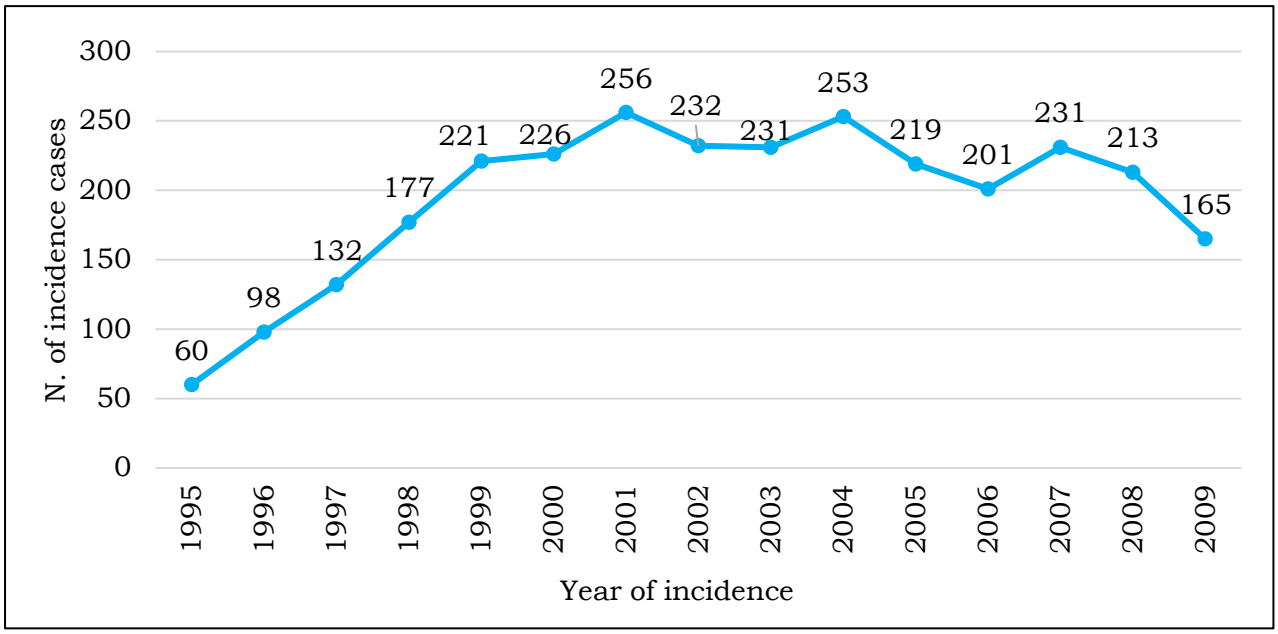

Figure 3.3.3.1

Trend of PC's incidence in patients resident in Udine.

b) Mortality data

Of the people considered (2915), 1402 patients are alive, whereas 1513 are dead.

c) Surgical information

Another feature considered (for further analyses) was the patients submission of a prostatectomy. *Figure 3.3.3.2* summarizes the types of prostatectomies which the patients underwent. More than six-houndred subjects (646) were underwent a prostatectomy. In 530 cases a Radical Prostatectomy (RP) was reported, in 90 cases a transurethral prostatectomy and 26 patients reported other prostatectomies.

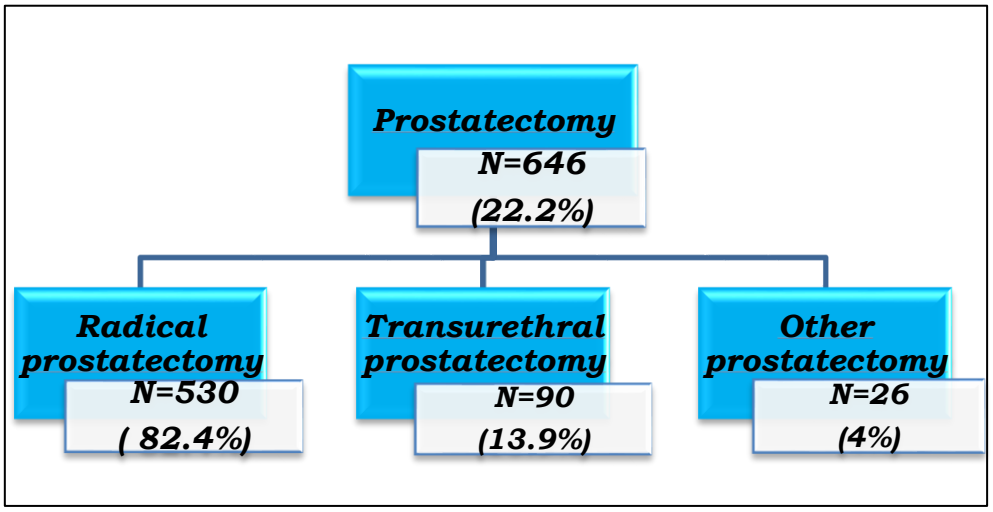

Figure 3.3.3.2

Types of prostatectomies to which some patients underwent.

3.4 A cohort study constituted by 122 people to study the features of patients with and without relapse of PC.

Stemming from the 2915 population studied in the previous paragraph, a relatively small study was conducted on 122 patients, who were divided into two groups: a group characterized by a relapse of PC and a group without a relapse of PC. It was possible to obtain more clinical and biological information about these subjects.

From the 2915 patients, the cohort selected represents those who underwent a radical prostatectomy and were monitored by University Hospital of Udine for whom clinical and biological data were available. (The selection is described more detailed in paragraph 6.2).

A description of the features related to the population considered can be found in *Table 3.4.1.* This table summarizes the variables considered for the analyses, which are presented below. The table shows a list of continuous and categorical variables. For the continuous variables, the table reports the number of subjects for which

the data are available (N), and the mean with the related values of standard deviation (SD); next to these values, median's values are shown in brackets.
Age at diagnosis, methylation levels of each site (CpG) analyzed (CpG1, CpG2, CpG3 and CpG4) individually and all together, PSA levels before and 30 days after RP and follow-up of patients are presented in the table as continuous variables, even if in some analyses they will be categorized. The age at diagnosis shows a mean of 66.51 years of age (median 68). On methylation levels of each CpG (expressed for each locus as a percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines), the mean values CpG1 shows the lower values with a mean of 34.88% (median 36%) followed by CpG3 with a mean of 36.59% (median 38.5%) and CpG2 characterized by a mean of 37.11% (median 39.5%) and the higher value is related to CpG4 that shows a mean of 41.21% (median 42%). When considering the mean of all the values of the four CpG sites, the mean obtained is 37.44% (median 39%). As far as PSA levels are concerned, the attention was focused in particular on levels before RP (data available only for 100 people), and on levels recorded 30 days after RP (data available for 118 patients). The mean related to levels before surgery is 14.54 ng/ml (median 7.21 ng/ml) whereas the mean concerning levels after surgery is 2.05 ng/ml (median 0.02 ng/ml). Finally, the last continuous variable considered is the follow-up of patients (from the data of RP to last medical examination for those patients without a relapse, and from the data of RP to the data of the relapse for the others): in this case the mean is 57.86 months and the median 71 months.
The categorical variables considered are: T(NM) stage, Gleason score, relapse/or not relapse on the first definition, and on the second definition (see paragraph 6.2.2), and data about mortality.
Data on TNM stage are often incomplete and defined in a lot of different ways (T2NxMx, T2N0Mx, T2NMetc…), for this reason, it was chosen to use only the data related to the T-stage (available for all the patients). One patient alone is characterized by a T-stage of 1, the major part of patients are characterized (60.66 %) by a T-stage 2, one third of the patients (31.97 %) by T-stage 3 and 8 patients of a T-stage 4.

Generally Gleason score is constituted by a Gleason total and a sum of a primary and secondary grade (for example 7 (3+4)). Also in this case it was chosen to use only the total grade because in some cases total grade was the only information available, moreover the number of several classes obtained would be too large. Gleason score under 6 is hardly represented, whereas people with a Gleason of 6 are 29, the most represented Gleason score is 7 (59 patients), and the higher scores 8 and 9 characterize 20 and 9 people respectively.

According to the first definition of relapse considered (where it is necessary that a medical doctor writes explicitly “Biochemical recurrence”), patients with a relapse are 40 (32.79%), whereas according to the second definition on biochemical recurrence (based on increasing levels of PSA) patients characterized by a relapse are 42 (34.43%). There are two patients who show a relapse according to the first definition of recurrence, but not according to the second definition. Of all patients, 108 (88.52%) are alive and 14 died.

Continuous variables	
Age at diagnosis (N=122), mean ± SD (median)	66.51 ± 5.41 (68.00)
Mehtylation levels CpG1 (N=122), mean ± SD (median)	34.88 ± 16.68 (36.00)
Mehtylation levels CpG2 (N=122), mean ± SD (median)	37.11 ± 16.91 (39.50)
Mehtylation levels CpG3 (N=122), mean ± SD (median)	36.59 ± 16.96 (38.50)
Mehtylation levels CpG4 (N=122), mean ± SD (median)	41.21 ± 17.53 (42.00)
Methylation levels (mean of 4 CpG) (N=122), mean ± SD (median)	37.44 ± 16.88 (39.00)
PSA levels before RP (N=100)	14.54 ± 19.40 (7.21)
PSA levels 30 days after RP (N=118)	2.05 ± 9.66 (0.02)
Follow-up (months) (N=122), mean ± SD (median)	57.86 ± 36.27 (71.00)
Categorical variables	
T-stage N (%):	***N = 122***
T1	1 (0.82)
T2	74 (60.66)
T3	39 (31.97)
T4	8 (6.56)
Gleason score N (%)	***N = 122***
3	1 (0.82)
4	1 (0.82)
5	3 (2.46)
6	29 (23.77)
7	59 (48.36)
8	20 (16.39)
9	9 (7.38)
Relapse R1 N (%)	***N = 122***
Yes	40 (32.79)
No	82 (67.21)
Relapse R2 N (%)	***N = 122***
Yes	42 (34.43)
No	80 (65.57)
Alive N (%)	***N = 122***
Yes	108 (88.52)
No	14 (11.48)

Table 3.4.1

Characteristics of the study population (N=122).

Tables 3.4.2 and 3.4.3 list all the combinations of medications taken by 122 patients from the date of diagnosis to the end of the follow-up. Medications until the end of the follow-up were considered for the people without a relapse, whereas the medication until the data of relapse were considered for those patients with a relapse. The tables differ only in the case of one of the two patients that had a relapse according to a definition, but not according to the other definition of relapse, indeed *Table 3.4.2* shows the combination of AGnRH and AAL, missing in *Table 3.4.3.* For the same reason in the *Table 3.4.2* there are 36 patients which not taken medications that become 37 in the *Table 3.4.3.*

When considering patients without consumption of medications (according to R1), of the 36 patients, eighteen are characterized by consumption of medications after relapse, and the same number of subjects (18) took medications only after the last medical examination (*Table 3.4.2).* When patients were considered according to R2, patients characterized by a consumption of medications only after the end of the follow-up (relapse or last medical examination) are 37 (*Table 3.4.3*).

Combinations of medications N (%) according to the relapse R1	***N = 122***
AAG and AGnRH	1 (0.82)
AAL	6 (4.92)
AAL and AAO	1 (0.82)
AAL and AGnRH	4 (3.28)
AAL and HP	1 (0.82)
AAO	1 (0.82)
AGnRH	6 (4.92)
AGnRH and AAL	1 (0.82)
BIP	9 (7.38)
BIP and AAL and AGnRH and HP	1 (0.82)
HP	55 (45.08)
No medications before relapse	36 (29.50)

Table 3.4.2

Combination of medications taken by patients according to the first definition of relapse (R1).

Combinations of medications N (%) according to the relapse R2	***N = 122***
AAG and AGnRH	1 (0.82)
AAL	6 (4.92)
AAL and AAO	1 (0.82)
AAL and AGnRH	4 (3.28)
AAL and HP	1 (0.82)
AAO	1 (0.82)
AGnRH	6 (4.92)
BIP	9 (7.38)
BIP and AAL and AGnRH and HP	1 (0.82)
HP	55 (45.08)
No medications before relapse	37 (30.33)

Table 3.4.3

Combination of medications taken by patients according to the second definition of relapse (R2).

In *Table 3.4.4* is possible to see the distribution of continuous variables; the table shows data on mean, median, minimum and maximum values related to all variables considered; moreover were reported also data on 20°, 40°, 60° and 80° percentiles. The age distribution ranges from 50 to 77 years.

Methylation levels show larger ranges; in particular, sites related on CpG1, CpG2 and CpG3 present a minimum value of 2% of methylated cytosines (in relation to methylated and unmethylated cytosines), and higher values are 80% of methylated cytosises for CpG1 and CpG2, and 79% for CpG3. CpG4 is characterized by a minimum of 5% and a maximum of 85%. The methylation level, obtained from the mean of values of all the four CpG sites considered, ranges from a minimum of 3 and a maximum of 81% methylated cytosines.

PSA levels before RP show a very large range of values, from 0.97 to 95 ng/ml. After 30 days of RP PSA ranges from 0 to 70 ng/ml.

The periods of follow-up show a minimum of 0 months and a maximum of 113 months.

Patients' features	N	Mean	SD[1]	Min[2]	20° pctl	40° pctl	50° pctl	60° pctl	80° pctl	Max[3]
Age at diagnosis	122	66.51	5.41	50.00	62.00	66.00	68.00	69.00	71.00	77.00
Methylation CpG1	122	34.88	16.68	2.00	20.00	30.00	36.00	41.00	48.00	80.00
Methylation CpG2	122	37.11	16.91	2.00	22.00	33.00	39.50	42.00	49.00	80.00
Methylation CpG3	122	36.59	16.96	2.00	21.00	33.00	38.50	42.00	49.00	79.00
Methylation CpG4	122	41.21	17.53	5.00	26.00	37.00	42.00	48.00	54.00	85.00
Methylation level (mean of CpG1, CpG2, CpG3 and CpG4)	122	37.44	16.88	3.00	22.00	34.00	39.00	44.00	50.00	81.00
PSA levels before RP	100	14.55	19.40	0.97	4.30	5.93	7.21	8.62	16.80	95.00
PSA levels 30 days after RP	118	2.05	9.66	0.00	0.01	0.02	0.02	0.03	0.15	70.00
Follow-up (months) according to R1	122	43.34	36.09	0.00	7.00	19.00	32.00	54.00	82.00	113.00
Follow-up (months) according to R2	122	43.05	35.95	0.00	7.00	19.00	32.00	52.00	82.00	113.00

[1] SD = Standard Deviation
[2] Min = Minimum
[3] Max=Maximum

Table 3.4.4

Distribution of continuous variables of the 122 patients with PC considered for the cohort study.

To perform statistical analyses, some variables were considered in two different ways, first as continuous variables and in a second time as categorical variables. The variables treated both as continuous and categorical are the variable related to methylation (in particular was considered the "mean" of the values related to the four CpG sites), age, and the variables related to PSA levels (both before and 30 days after RP). This choice was made because from the clinical point of view the categories done, it should be both correct and informative.

3.4.1 Features of patients in relation to the outcome

The features of people were analyzed in relation to the outcome of whether the relapse develops. As two different criteria were considered to identify the relapse, all the analyses of the variables related to the outcome, were done two times using first a definition (R1) and then the other (R2), hence also the summarizing tables (related to the outcome) will be presented likewise.

Tables 3.4.1.1 and 3.*4.1.2* summarize the features of the people, in particular in these cases the continuous variables were considered respectively, in relation to the outcome R1 and R2. These tables reported the statistics summaries including mean, median, SD and Interquartile Range (IQR), whilst the last column contains the P-values obtained from Wilcoxon-Mann-Whitney test. All the P-values (except that related to age) are significant (<0.05); on methylation data, cases (patients with a relapse) show means and medians characterized by higher values than the same statistical parameters referred to controls (patients without a relapse). Also data on PSA levels, both before and after 30 days of RP are higher in cases than in controls. These results are similar whether the outcome parameter considered be relapsed according to the first definition (R1) or the second definition (R2), respectively *Tables 3.4.1.1* and *3.4.1.2.*

Patients' features	***Relapse / Not Relapse***									***Wilcoxon - Mann - Whitney***
Continuous variables	***Subjects WITHOUT relapse 1 (R1)***					***Subjects WITH relapse 1 (R1)***				
	N	***Mean***	***SD[1]***	***Median***	***IQR[2]***	***N***	***Mean***	***SD1***	***Median***	***IQR[2]***	***P-value***
Age	82	66.34	5.84	68.00	9.00	40	66.85	4.46	67.50	7.00	0.88
Methylation (CpG1)	82	31.84	15.62	33.00	23.00	40	41.10	17.24	42.00	19.00	0.01
Methylation (CpG2)	82	34.13	16.06	36.00	24.00	40	43.20	17.18	43.00	21.00	0.02
Methylation (CpG3)	82	33.54	16.23	37.00	25.00	40	42.83	16.93	42.00	22.50	0.02
Methylation (CpG4)	82	37.83	16.97	39.50	24.00	40	48.15	16.78	48.50	22.50	0.01
Methylation (mean)	82	34.33	16.13	36.50	24.00	40	43.83	16.78	44.00	22.50	0.01
PSA before surgery	64	12.67	19.32	6.06	5.19	36	17.89	19.37	10.52	16.79	0.01
PSA 30 days after RP	80	0.32	1.93	0.02	0.03	38	5.69	16.34	0.10	1.59	<0.01
Follow-up (months)	82	51.72	38.88	64.00	75.00	40	26.21	21.21	20.00	21.00	0.01

[1] SD = Standard Deviation
[2] IQR = Interquartile range

Table 3.4.1.1

Features of continuous variables related to outcome (R1).

Patients' features	Relapse / Not Relapse										Wilcoxon - Mann - Whitney
Continuous variables	Subjects WITHOUT relapse 2 (R2)					Subjects WITH relapse 2 (R2)					
	N	Mean	SD[1]	Median	IQR[2]	N	Mean	SD[1]	Median	IQR[2]	P-value
Age	80	66.34	5.79	68.00	9.00	42	66.83	4.65	67.50	7.00	0.85
Methylation (CpG1)	80	31.48	15.63	33.00	23.00	42	41.36	16.82	42.00	19.00	<0.01
Methylation (CpG2)	80	33.86	16.16	36.00	24.00	42	43.29	16.77	43.00	17.00	0.01
Methylation (CpG3)	80	33.29	16.34	36.50	24.00	42	42.88	16.52	42.00	19.00	0.01
Methylation (CpG4)	80	37.54	17.08	39.00	24.50	42	48.21	16.37	48.50	21.00	<0.01
Methylation (mean)	80	34.04	16.23	36.00	23.50	42	43.93	16.38	44.00	21.00	<0.01
PSA before surgery	62	11.47	18.11	5.93	4.70	38	19.56	20.61	11.30	16.70	<0.01
PSA 30 days after RP	78	0.27	1.92	0.02	0.02	40	5.50	15.94	0.11	1.89	<0.01
Follow-up (months)	80	51.80	39.11	64.00	75.00	42	26.38	20.84	20.50	21.00	0.01

[1] SD = Standard Deviation
[2] IQR = Interquartile range

Table 3.4.1.2

Features of continuous variables related to outcome (R2).

The analyses were performed in relation to the outcome (relapse) also using the categorical variables. The results related to the outcome according to the first definition of relapse (R1), can be found in *Table 3.4.1.3*, whereas those related to the second definition of relapse in *Table 3.4.1.4.* The tables, mentioned in the previous lines, present in the last column the P-values obtained using the Chi-square test. In these tables, the significant P-values (<0.05) are related to: Gleason score and PSA levels 30 days after RP, considering the first definition of relapse (R1); whereas, when it is considered the second definition (R2), the significant P-values, can be found related to the same variables mentioned for the R1 but also for T-stage and PSA levels before RP.

Patients' features	Relapse / Not Relapse		Chi-square
Categorical variables	Subjects WITHOUT relapse 1 N (%)	Subjects WITH relapse 1 N (%)	P-value
Age at diagnosis	N = 82	N = 40	0.44
≤ 59	12 (14.63)	3 (7.50)	
60 - 64	16 (19.51)	10 (25.00)	
65 - 69	25 (30.49)	16 (40.00)	
≥ 70	29 (35.37)	11 (27.50)	
T (stage):	N = 82	N = 40	0.08
T1-T2	56 (58.29)	19 (47.50)	
T3	21 (25.61)	18 (45.00)	
T4	5 (6.10)	3 (7.50)	
Gleason score	N = 82	N = 40	0.01
≤ 6	29 (35.37)	5 (12.50)	
7	39 (47.56)	20 (50.00)	
8 - 9	14 (17.07)	15 (37.50)	
Pre-surgery PSA	N = 64	N = 36	0.06
< 4	13 (20.31)	4 (11.11)	
4 - 10 (10 excl)	36 (56.25)	14 (38.89)	
10 - 20	7 (10.94)	8 (22.22)	
> 20	8 (12.50)	10 (27.78)	
PSA after 30 days	N = 80	N = 38	<0.01
< 0.2	73 (91.25)	24 (63.16)	
≥ 0.2	7 (8.75)	14 (36.84)	
Methylation (mean/quintiles)	N =82	N = 40	0.27
≤ 22	21 (25.61)	5 (12.50)	
23 - 34	18 (21.95)	8 (20.00)	
35 - 44	19 (23.17)	8 (20.00)	
45 - 50	12 (14.63)	8 (20.00)	
≥ 51	12 (14.63)	11 (27.50)	

Table 3.4.1.3

Categorical variables related to the outcome (considering R1 as definition of relapse).

Patients' features	Relapse / Not Relapse		Chi-square
Categorical variables	**Subjects WITHOUT relapse 2 N (%)**	**Subjects WITH relapse 2 N (%)**	**P-value**
Age at diagnosis	**N = 80**	**N = 42**	**0.72**
≤ 59	11 (13.75)	4 (9.52)	
60 - 64	16 (20.00)	10 (23.81)	
65 - 69	25 (31.25)	16 (38.10)	
≥ 70	28 (35.00)	12 (28.57)	
T (stage):	**N = 80**	**N = 42**	**0.02**
T1-T2	56 (70.00)	19 (45.24)	
T3	21 (26.25)	18 (42.86)	
T4	3 (3.75)	5 (11.90)	
Gleason score	**N = 80**	**N = 42**	**<0.01**
≤ 6	29 (36.25)	5 (11.90)	
7	39 (48.75)	20 (47.62)	
8 - 9	12 (36.25)	17 (40.48)	
Pre-surgery PSA	**N = 62**	**N = 38**	**0.01**
< 4	13 (20.97)	4 (10.53)	
4 - 10 (10 excl)	36 (58.06)	14 (36.84)	
10 - 20	7 (11.29)	8 (21.05)	
> 20	6 (9.58)	12 (31.58)	
PSA after 30 days	**N = 78**	**N = 40**	**<0.01**
< 0.2	73 (93.59)	24 (60.00)	
≥ 0.2	5 (6.41)	16 (40.00)	
Methylation (mean/quintiles)	**N = 80**	**N = 42**	**0.23**
≤ 22	21 (26.25)	5 (11.90)	
23 - 34	18 (22.50)	8 (19.05)	
35 - 44	18 (22.50)	9 (21.43)	
45 - 50	11 (13.75)	9 (21.43)	
≥ 51	12 (15.00)	11 (26.19)	

Table 3.4.1.4

Categorical variables related to the outcome (considering R2 as definition of relapse).

a) Logistic regression analyses and Cox analyses using variables available for all 122 patients (Gleason Scores, age and methylation levels).

To obtain more information, logistic regressions and Cox analyses were performed. Tables containing results of logistic regressions analyses show only the values related to Odds Ratios (OR) and relative 95% Confidence Intervals (95% CI) for univariate analyses, whereas bivariate and multiple models show also P-values. Tables presenting data related to Cox analyses, show Hazard Ratios (HR) values and relative 95% CI for univariate analyses, these data was completed adding P-values on data concerning bivariate and multiple analyses.

Tables from *Table 3.4.1.5* to *Table 3.4.1.8,* show results obtained from logistic regression analyses in which were included variables whose values were available for all 122 patients studied. In particular, these analyses were performed using the following variables: Gleason scores, age, and methylation levels. Only T-stage variable was excluded, after a discussion with clinicians.

Results presented in *Tables 3.4.1.5 and 3.4.1.6* are similar, but *Table 3.4.1.5* concerns the data related to the first definition of relapse (R1) whereas *Table 3.4.1.6* is related to the second definition of relapse (R2).

When considering Gleason score, patients with higher values, show an increase of the risk of relapse. Univariate and bivariate analyses show an increase of ORs values related to the increase of methylation levels; this tendency seems to be maintained in multiple models, but these data are not significant.

Logistic regressions were performed using methylation both as a categorical variable (*Tables 3.4.1.5* and *3.4.1.6*, where methylation data were considered using quintiles, and *Supplementary Tables 7, 8, 9, 10* where methylation data were considered using tertiles and quartiles, paragraphs 7.6 and 7.7 respectively) and a continuous variable *(Tables 3.4.1.7 and 3.4.1.8*). *Tables 3.4.1.7 and 3.4.1.8* differ for the definition of relapse (R1 or R2) considered.

Cox analyses were performed to include also the time (from the date of RP until the relapse for those subjects that have a relapse and until the last medical examination for those who have not had a relapse).

Results related to Cox analyses in which the same variables mentioned above are used (Gleason scores, age and methylation levels) are shown in *Tables 3.4.1.9*,

3.4.1.10, 3.4.1.11 and 3.4.1.12. Methylation was considered using quintiles in *Tables 3.4.1.9 and 3.4.1.10*, whereas *Tables 3.4.1.11 and 3.4.1.12* use the same variables, but in these cases the methylation was considered as a continuous variable. Values of methylation considered in all the tables presented are those obtained from the mean of the values of all the four CpGs considered. Cox analyses were performed also using tertiles and quartiles (*Supplementary tables 11, 12, 13, 14)*.

In *Tables 3.4.1.9 and 3.4.1.10*, the significant results are related to those patients with a Gleason score of 8 or 9 that show a risk of relapse greater than 6 times comparing to those subjects with a Gleason score ≤6; this result is significant for both the definitions of relapse (according to R1 and R2).

Also in *Tables 3.4.1.11 and 3.4.1.12* significant data are related to higher Gleason scores but Gleason scores of 8 and 9 show risks that are little smaller than those presented in the *Tables 3.4.1.9 and 3.4.1.10* (Hazard Ratio (HR) of 4.93 in the Table 3.4.1.11 and HR of 6.03 in the *Table 3.4.1.9*).

A difference can be seen when methylation level is considered as a continuous variable, indeed the results of bivariate analyses show a significant P-value related to this variable, but the P-value doesn't maintain a significant value in the multiple analyses.

Logistic regression according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95 % CI**		**OR**	**95% CI**	**P-value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06		2.53	0.83 - 7.76	0.10
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01		5.38	1.51 - 19.17	0.01
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.87	0.52 - 6.73		1.84	0.51 - 6.70	0.35		1.64	0.83 - 22.29	0.47
35 - 44	1.77	0.49 - 6.35		1.77	0.49 - 6.36	0.38		1.23	0.32 - 4.74	0.76
45 - 50	2.80	0.75 - 10.52		2.73	0.71 - 10.58	0.15		1.85	0.45 - 7.60	0.40
≥ 51	3.85	1.08 - 13.75		3.82	1.07 - 13.69	0.04		2.30	0.59 - 8.87	0.23

[1] Adjusted by age

Table 3.4.1.5

Univariate, bivariate (age adjusted) and multiple logistic regressions, according to the first definition of relapse (R1), considering Gleason scores, age, and methylation levels (N=122).

Logistic regression according to the second definition of relapse (R2)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95% CI**		**OR**	**95% CI**	**P-value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 - 8.86		2.91	0.98 - 8.71	0.06		2.55	0.83 - 7.80	0.10
8 - 9	8.22	2.47 - 27.35		8.64	2.56 - 29.12	<0.01		7.16	2.00 - 25.62	<0.01
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.87	0.52 - 6.73		1.85	0.51 - 6.72	0.35		1.67	0.43 - 6.46	0.46
35 - 44	2.10	0.60 - 7.42		2.10	0.60 - 7.43	0.25		1.40	0.36 - 5.36	0.63
45 - 50	3.44	0.92 - 12.79		3.38	0.88 - 12.94	0.08		2.21	0.54 - 9.13	0.27
≥ 51	3.85	1.08 - 13.75		3.83	1.07 - 13.72	0.04		2.16	0.55 - 8.47	0.27

[1] Adjusted by age

Table 3.4.1.6

Univariate, bivariate (age adjusted) and multiple logistic regressions, according to the second definition of relapse (R2) considering Gleason scores, age, and methylation levels (N=122).

Logistic regression according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95% CI**		**OR**	**95% CI**	**P-Value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 – 8.86		2.92	0.98 – 8.72	0.05		2.45	0.80 - 7.49	0.12
8 - 9	6.21	1.88 – 20.56		6.47	1.94 – 21.63	<0.01		4.38	1.22 - 15.71	0.02
Methylation (mean/continuous variable)										
	1.04	1.01 - 1.06		1.04	1.01 - 1.06	<0.01		1.03	1.00 - 1.05	0.06
[1] Adjusted by age										

Table 3.4.1.7

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) considering Gleason scores, age, and methylation levels (N=122).

Logistic regression according to the second definition of relapse (R2)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95% CI**		**OR**	**95% CI**	**P-value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 – 8.86		2.91	0.98 – 8.71	0.06		2.45	0.80 - 7.49	0.12
8 - 9	8.22	2.47 – 27.35		8.64	2.56 – 29.12	<0.01		5.92	1.65 - 21.30	0.01
Methylation (mean/continuous variable)										
	1.04	1.01 - 1.07		1.04	1.01 - 1.07	<0.01		1.03	1.00 - 1.06	0.06
[1] Adjusted by age										

Table 3.4.1.8

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the second definition of relapse (R2) considering Gleason scores, age, and methylation levels (N=122).

Cox analyses according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**		**HR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08		2.41	0.87 - 6.63	0.09
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01		6.03	2.01 - 18.12	<0.01
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.30	0.42 - 3.97		1.32	0.42 - 4.15	0.63		1.18	0.36 - 3.80	0.79
35 - 44	1.26	0.41 - 3.87		1.27	0.41 - 3.88	0.68		0.81	0.25 - 2.58	0.72
45 - 50	1.53	0.50 - 4.71		1.57	0.49 - 5.03	0.45		0.98	0.29 - 3.32	0.98
≥ 51	1.86	0.65 - 5.37		1.87	0.65 - 5.40	0.25		0.96	0.31 - 2.94	0.96
[1] Adjusted by age										

Table 3.4.1.9

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) considering Gleason scores, age, and methylation levels (N=122).

Cox analyses according to the second definition of relapse (R2)									
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**			**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.40	0.90 - 6.40		2.40	0.90 - 6.41	0.08	2.44	0.89 - 6.69	0.08
8 - 9	6.60	2.42 - 18.00		6.60	2.42 - 18.00	<0.01	7.20	2.44 - 21.25	<0.01
Methylation (mean/quintiles)									
≤ 22	1	----		1	----		1	-----	
23 - 34	1.30	0.42 - 3.97		1.33	0.42 - 4.16	0.63	1.20	0.37 - 3.89	0.76
35 - 44	1.42	0.48 - 4.26		1.43	0.48 - 4.28	0.52	0.87	0.28 - 2.69	0.80
45 - 50	1.76	0.59 - 5.28		1.81	0.58 - 5.67	0.31	1.11	0.34 - 3.64	0.87
≥ 51	1.86	0.65 - 5.37		1.87	0.65 - 5.41	0.25	0.90	0.29 - 2.75	0.52
[1] Adjusted by age									

Table 3.4.1.10

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the second definition of relapse (R2) considering Gleason scores, age, and methylation levels (N=122).

Cox analyses according to the first definition of relapse (R1)									
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**			**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-Value**	**HR**	**95% CI**	**P-value**
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08	2.26	0.83 - 6.17	0.11
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01	4.93	1.64 - 14.87	<0.01
Methylation (mean/continuous variable)									
	1.02	1.00 - 1.04		1.02	1.00 - 1.04	0.04	1.01	0.99 - 1.03	0.55
[1] Adjusted by age									

Table 3.4.1.11

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) considering Gleason scores, age, and methylation levels (N=122).

Cox analyses according to the second definition of relapse (R2)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**		**HR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.40	0.90 - 6.40		2.40	0.90 - 6.41	0.08		2.29	0.84 - 6.23	0.11
8 - 9	6.60	2.42 - 18.00		6.60	2.42 - 18.00	<0.01		5.98	2.02 - 17.73	<0.01
Methylation (mean/continuous variable)										
	1.02	1.00 - 1.04		1.02	1.00 - 1.04	0.03		1.01	0.98 - 1.03	0.64
[1] Adjusted by age										

Table 3.4.1.12

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the second definition of relapse (R2) considering Gleason scores, age, and methylation levels (N=122).

b) *Logistic regression analyses and Cox analyses using Gleason scores, age, methylation levels and PSA values before RP (analyses performed on 100 patients).*

Logistic regression analyses and Cox analyses were also performed adding the variable related to PSA values before RP to the other variables considered above (Gleason score, age and methylation levels). In these analyses only data related to the first definition of relapse (R1) were considered. Analyses based on data concerning the second definition of relapse (R2) were not performed as the difference concerns only two subjects.

Data on PSA values before RP are available for 100 patients, hence also logistic regression and Cox analyses considered only those patients for whom all values are available (losing 22 patients when the analyses were performed including also the variable related to PSA before RP). In the Tables presented below variable related to PSA before surgery was considered both as categorical and continuous variable.

Logistic regression analyses including Gleason scores, age, methylation levels and PSA before RP were presented in *Tables 3.4.1.13, 3.4.1.14, 3.4.1.15 and 3.4.1.16*. *Tables 3.4.1.13 and 3.4.1.14* used methylation as a categorical variable, whereas *Tables 3.4.1.15 and 3.4.1.16* used methylation as a continuous variable.

In these analyses the most important data with a statistically significant value is related to the higher Gleason scores values (8 – 9), but only in the bivariate analyses, this result is maintained in the multiple analyses only when the variable related to PSA after RP is considered as a continuous variable (*Table 3.4.1.14*).

Cox analyses were also performed using the same variables described above (Gleason scores, age, methylation levels and PSA values before RP).

Tables 3.4.1.17 and 3.4.1.18 were obtained considering methylation as a categorical variable, whereas in the *Tables 3.4.1.19 and 3.4.1.20* methylation was considered as a continuous variable.

Cox analyses performed with the variables listed above show statistically significant data for higher values of Gleason scores, indeed people with higher Gleason sores shows a risk 3 times greater than people with Gleason scores ≤ 6.

Table 3.4.1.17 shows a statistically significant data for people characterized by higher Gleason scores (8 – 9) and higher levels of PSA before RP (>20 ng/ml) in relation to bivariate analyses, but only data concerning Gleason scores maintain the significance in the multiple model. People with higher Gleason scores have an higher risk to develop a relapse than those with a lower Gleason score, and people with higher levels of PSA (10 - 20 and <20 ng/ml) before RP have a risk of more than 3 times to develop a relapse than the subjects with a PSA < 4 ng/ml.
Table 3.4.1.19, where methylation was considered as a continuous variable, shows statistically significant HRs related to people with higher Gleason scores (8 – 9) whose and higher levels of PSA before RP (10 – 20 and >20 ng/ml) whose have an higher risk to develop a relapse than those belonging to the classes (Gleason ≤ 6, and PSA <4 respectively). These data are significant into the bivariate analyses but the significance was not maintained in the multiple analyses.
Logistic regressions and Cox analyses were also performed using tertiles instead of quintiles. These analyses consider only the first definition of relapse (R1). Data are shown in *Supplementary tables 15 and 16.*

Logistic regression according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95 % CI**		**OR**	**95% CI**	**P-value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06		2.12	0.62 - 7.30	0.23
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01		2.50	0.57 - 11.61	0.23
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.87	0.52 - 6.73		1.84	0.51 - 6.70	0.35		2.06	0.47 - 9.08	0.34
35 - 44	1.77	0.49 - 6.35		1.77	0.49 - 6.36	0.38		0.75	0.17 - 3.28	0.69
45 - 50	2.80	0.75 - 10.52		2.73	0.71 - 10.58	0.15		1.00	0.19 - 5.11	0.99
≥ 51	3.85	1.08 - 13.75		3.82	1.07 - 13.69	0.04		1.43	0.33 - 6.21	0.63
Pre-surgery PSA										
< 4	1	----		1	----			1	----	
4 - 10 (10 excl)	1.26	0.35 - 4.54		1.29	0.36 - 4.67	0.69		1.06	0.26 - 4.32	0.93
10 - 20	3.71	0.82 - 16.84		3.85	0.84 - 17.60	0.08		3.02	0.50 - 18.20	0.23
> 20	4.06	0.95 - 17.43		4.19	0.97 - 18.08	0.05		3.59	0.65 - 19.92	0.14
[1] Adjusted by age										

Table 3.4.1.13

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values before RP (N=100).

Logistic regression according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95 % CI**		**OR**	**95% CI**	**P-value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06		2.23	0.68 - 7.29	0.19
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01		3.96	1.02 - 15.34	0.05
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.87	0.52 - 6.73		1.84	0.51 - 6.70	0.35		1.85	0.43 - 7.99	0.41
35 - 44	1.77	0.49 - 6.35		1.77	0.49 - 6.36	0.38		0.84	0.20 - 3.48	0.81
45 - 50	2.80	0.75 - 10.52		2.73	0.71 - 10.58	0.15		1.32	0.28 - 6.16	0.72
≥ 51	3.85	1.08 - 13.75		3.82	1.07 - 13.69	0.04		1.56	0.38 - 6.38	0.53
Pre-surgery PSA (continuous variable)										
	1.01	0.99 - 1.04		1	0.99 - 1.04	0.19		1.01	0.99 - 1.03	0.40
[1] Adjusted by age										

Table 3.4.1.14

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values before RP (N=100).

Logistic regression according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95 % CI**		**OR**	**95% CI**	**P-value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06		2.05	0.61 - 6.92	0.95
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01		2.02	0.46 - 8.91	0.35
Methylation (mean/continuous variable)										
	1.04	1.01 - 1.06		1.04	1.01 - 1.06	0.01		1.02	0.99 - 1.05	0.25
Pre-surgery PSA										
< 4	1	----		1	----			1	----	
4 - 10 (10 excl)	1.26	0.35 - 4.54		1.29	0.36 - 4.67	0.69		0.85	0.21 - 3.39	0.82
10 - 20	3.71	0.82 - 16.84		3.85	0.84 - 17.60	0.08		2.10	0.38 - 11.66	0.39
> 20	4.06	0.95 - 17.43		4.19	0.97 - 18.08	0.05		2.35	0.46 - 12.12	0.31
[1] Adjusted by age										

Table 3.4.1.15

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values before RP (N=100).

Logistic regression according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95% CI**		**OR**	**95% CI**	**P-Value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 – 8.86		2.92	0.98 – 8.72	0.05		2.11	0.66 - 6.81	0.21
8 - 9	6.21	1.88 – 20.56		6.47	1.94 – 21.63	<0.01		3.05	0.80 - 11.62	0.10
Methylation (mean/continuous variable)										
	1.04	1.01 - 1.06		1.04	1.01 - 1.06	0.01		1.02	0.99 - 1.05	0.20
Pre-surgery PSA (continuous variable)										
	1.01	0.99 - 1.04		1	0.99 - 1.04	0.19		1.01	0.99 - 1.03	0.49
[1] Adjusted by age										

Table 3.4.1.16

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values before RP (N=100).

Cox analyses according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**		**HR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08		2.37	0.83 - 6.77	0.11
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01		3.48	1.03 - 11.74	0.04
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.30	0.42 - 3.97		1.32	0.42 - 4.15	0.63		1.00	0.30 - 3.30	0.99
35 - 44	1.26	0.41 - 3.87		1.27	0.41 - 3.88	0.68		0.52	0.16 - 1.75	0.29
45 - 50	1.53	0.50 - 4.71		1.57	0.49 - 5.03	0.45		0.43	0.12 - 1.63	0.22
≥ 51	1.86	0.65 - 5.37		1.87	0.65 - 5.40	0.25		0.57	0.18 - 1.84	0.57
Pre-surgery PSA										
< 4	1	----		1	----			1	----	
4 - 10 (10 excl)	1.62	0.53 - 4.91		1.62	0.53 - 4.93	0.40		1.56	0.49 - 4.95	0.45
10 - 20	3.40	1.02 - 11.34		3.40	1.02 - 11.33	0.05		3.19	0.80 - 12.78	0.10
> 20	3.86	1.21 - 12.35		3.87	1.21 - 12.38	0.03		3.59	0.90 - 14.38	0.07
[1] Adjusted by age										

Table 3.4.1.17

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values before RP (N=100).

Cox analyses according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**		**HR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08		2.51	0.88 - 7.14	0.08
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01		5.11	1.60 - 16.31	<0.01
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.30	0.42 - 3.97		1.32	0.42 - 4.15	0.63		0.94	0.28 - 3.13	0.92
35 - 44	1.26	0.41 - 3.87		1.27	0.41 - 3.88	0.68		0.59	0.18 - 1.94	0.38
45 - 50	1.53	0.50 - 4.71		1.57	0.49 - 5.03	0.45		0.65	0.18 - 2.36	0.52
≥ 51	1.86	0.65 - 5.37		1.87	0.65 - 5.40	0.25		0.67	0.21 - 2.14	0.50
Pre-surgery PSA (continuous variable)										
	1.01	1.00 - 1.02		1.01	1.00 - 1.02	0.14		1.01	0.99 - 1.02	0.52
[1] Adjusted by age										

Table 3.4.1.18

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values before RP (N=100).

Cox analyses according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-Value**		**HR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08		2.05	0.72 - 5.83	0.18
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01		2.83	0.83 - 9.65	0.10
Methylation (mean/continuous variable)										
	1.02	1.00 - 1.04		1.02	1.00 - 1.04	0.04		1.00	0.98 - 1.03	0.99
Pre-surgery PSA										
< 4	1	----		1	----			1	----	
4 - 10 (10 excl)	1.62	0.53 - 4.91		1.62	0.53 - 4.93	0.40		1.29	0.41 - 4.10	0.67
10 - 20	3.40	1.02 - 11.34		3.40	1.02 - 11.33	0.05		2.17	0.58 - 8.16	0.25
> 20	3.86	1.21 - 12.35		3.87	1.21 - 12.38	0.03		2.44	0.66 - 9.09	0.18
[1] Adjusted by age										

Table 3.4.1.19

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values before RP (N=100).

Cox analyses according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-Value**		**HR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08		2.22	0.79 - 6.21	0.13
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01		4.02	1.27 - 12.79	0.02
Methylation (mean/continuous variable)										
	1.02	1.00 - 1.04		1.02	1.00 - 1.04	0.04		1.00	0.98 - 1.08	0.90
Pre-surgery PSA (continuous variable)										
	1.01	1.00 - 1.02		1.01	1.00 - 1.02	0.14		1.00	0.99 - 1.02	0.60
[1] Adjusted by age										

Table 3.4.1.20

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values before RP (N=100).

c) *Logistic regression analyses and Cox analyses using Gleason scores, age, methylation levels and PSA values 30 days after RP (analyses involved 118 patients).*

Last models related to logistic regression and Cox analyses were built using the following variables: Gleason scores, age, methylation (also in this case both a categorical and a continuous variable) and PSA values 30 days after RP.
Data related to PSA 30 days after RP are available for 118 patients, therefore also the analyses were performed on the same number of subjects.
Logistic regression analyses show that people with PSA values ≥ 0.2 ng/ml (30 days after RP), have a risk to 6 times higher to develop a relapse than those with a PSA <0.2 ng/ml; this result is statistically significant both in bivariate and multiple analyses (*Tables 3.4.1.21 and 3.4.1.23*). Another significant result related to the multiple model concerns the increasing risk of relapse for those subjects with a higher Gleason scores (7 and 8-9) compared to those with a Gleason ≤ 6 (*Tables 3.4.1.22 and 3.4.1.24).*
The variable related to data of PSA 30 days after RP was considered also as a continuous variable (*Tables 3.4.1.22 and 3.4.1.24*). Tables mentioned above shows a risk of relapse (statistically significant in the multiple model) that increase with the increase of Gleason scores, indeed people with a Gleason of 7 have a risk four times higher compared to people with Gleason ≤ 6, and subjects with Gleason 8 – 9 have a risk five times higher compared people with Gleason to ≤ 6.
The variable related to PSA values 30 days after RP, was used also to perform Cox analyses. People with PSA values ≥ 0.2 ng/ml (30 days after RP), show an higher risk (4 times) to have a relapse compared to people with PSA values < 0.2 ng/ml, and this result is statistically significant both in the bivariate and multiple model (*Tables 3.4.1.25 and 3.4.1.27*). When the variable related to PSA values 30 days after RP was considered as a continuous variable, the statistically significance is maintained but the related HRs are lower (*Tables 3.4.1.26 and 3.4.1.28*).
Logistic regressions and Cox analyses were also performed using tertiles instead of quintiles. These analyses consider only the first definition of relapse (R1). Data are shown in *Supplementary tables 17 and 18.*

Logistic regression according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95 % CI**		**OR**	**95% CI**	**P-value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06		3.63	1.03 - 12.83	0.04
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01		3.27	0.74 - 14.46	0.12
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.87	0.52 - 6.73		1.84	0.51 - 6.70	0.35		0.96	0.22 - 4.16	0.96
35 - 44	1.77	0.49 - 6.35		1.77	0.49 - 6.36	0.38		0.97	0.24 - 4.00	0.97
45 - 50	2.80	0.75 - 10.52		2.73	0.71 - 10.58	0.15		1.40	0.32 - 6.20	0.66
≥ 51	3.85	1.08 - 13.75		3.82	1.07 - 13.69	0.04		2.03	0.50 - 8.29	0.32
PSA after 30 days										
< 0.2	1	----		1	----			1	----	
≥ 0.2	6.08	2.20 - 16.83		6.22	2.23 - 17.32	<0.01		5.08	1.58 - 16.28	<0.01
[1] Adjusted by age										

Table 3.4.1.21

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values 30 days RP (N=118).

Logistic regression according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95 % CI**		**OR**	**95% CI**	**P-value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06		4.29	1.13 - 16.24	0.03
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01		5.32	1.19 - 23.75	0.03
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.87	0.52 - 6.73		1.84	0.51 - 6.70	0.35		1.29	0.29 - 5.78	0.74
35 - 44	1.77	0.49 - 6.35		1.77	0.49 - 6.36	0.38		1.29	0.30 - 5.49	0.73
45 - 50	2.80	0.75 - 10.52		2.73	0.71 - 10.58	0.15		1.95	0.43 - 8.87	0.39
≥ 51	3.85	1.08 - 13.75		3.82	1.07 - 13.69	0.04		2.69	0.63 - 11.48	0.18
PSA after 30 days (continuous variable)										
	1.20	0.95 - 1.52		1.22	0.96 - 1.56	0.11		1.13	0.94 - 1.37	0.19
[1] Adjusted by age										

Table 3.4.1.22

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values 30 days RP (N=118).

Logistic regression according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95 % CI**		**OR**	**95% CI**	**P-value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06		3.28	0.95 - 11.33	0.06
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01		2.64	0.60 - 11.73	0.20
Methylation (mean/continuous variable)										
	1.04	1.01 - 1.06		1.04	1.01 - 1.06	0.01		1.03	1.00 - 1.06	0.06
PSA after 30 days										
< 0.2	1	----		1	----			1	----	
≥ 0.2	6.08	2.20 - 16.83		6.22	2.23 - 17.32	<0.01		4.82	1.49 - 15.65	<0.01
[1] Adjusted by age										

Table 3.4.1.23

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values 30 days RP (N=118).

Logistic regression according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	OR	**95% CI**		**OR**	**95% CI**	**P-Value**		**OR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 – 8.86		2.92	0.98 – 8.72	0.05		4.09	1.09 - 15.38	0.04
8 - 9	6.21	1.88 – 20.56		6.47	1.94 – 21.63	<0.01		4.49	1.01 - 20.04	0.05
Methylation (mean/continuous variable)										
	1.04	1.01 - 1.06		1.04	1.01 - 1.06	0.01		1.03	1.00 - 1.06	0.04
PSA after 30 days (continuous variable)										
	1.20	0.95 - 1.52		1.22	0.96 - 1.56	0.11		1.12	0.96 - 1.32	0.16
[1] Adjusted by age										

Table 3.4.1.24

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values 30 days RP (N=118).

Cox analyses according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**		**HR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08		2.82	0.92 - 8.66	0.07
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01		2.71	0.66 - 11.11	0.17
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.30	0.42 - 3.97		1.32	0.42 - 4.15	0.63		0.79	0.23 - 2.75	0.71
35 - 44	1.26	0.41 - 3.87		1.27	0.41 - 3.88	0.68		0.65	0.20 - 2.14	0.48
45 - 50	1.53	0.50 - 4.71		1.57	0.49 - 5.03	0.45		0.81	0.24 - 2.79	0.74
≥ 51	1.86	0.65 - 5.37		1.87	0.65 - 5.40	0.25		0.84	0.27 - 2.65	0.77
PSA after 30 days										
< 0.2	1	----		1	----			1	----	
≥ 0.2	4.50	2.32 - 8.74		4.80	2.42 - 9.52	<0.01		4.08	1.52 - 11.01	<0.01
[1] Adjusted by age										

Table 3.4.1.25

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values 30 days RP (N=118).

Cox analyses according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**		**HR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08		3.00	0.98 - 9.17	0.05
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01		5.50	1.60 - 18.84	<0.01
Methylation (mean/quintiles)										
≤ 22	1	----		1	----			1	----	
23 - 34	1.30	0.42 - 3.97		1.32	0.42 - 4.15	0.63		0.85	0.24 - 2.95	0.79
35 - 44	1.26	0.41 - 3.87		1.27	0.41 - 3.88	0.68		0.79	0.25 - 2.53	0.69
45 - 50	1.53	0.50 - 4.71		1.57	0.49 - 5.03	0.45		0.80	0.23 - 2.79	0.72
≥ 51	1.86	0.65 - 5.37		1.87	0.65 - 5.40	0.25		0.95	0.31 - 2.96	0.93
PSA after 30 days (continuous variable)										
	1.03	1.01 - 1.05		1.03	1.01 - 1.05	<0.01		1.02	1.00 - 1.04	0.05
[1] Adjusted by age										

Table 3.4.1.26

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values 30 days RP (N=118).

Cox analyses according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-Value**		**HR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08		2.40	0.80 - 7.24	0.12
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01		2.15	0.52 - 8.89	0.29
Methylation (mean/continuous variable)										
	1.02	1.00 - 1.04		1.02	1.00 - 1.04	0.04		1.01	0.99 - 1.04	0.41
PSA after 30 days										
< 0.2	1	----		1	----			1	----	
≥ 0.2	4.50	2.32 - 8.74		4.80	2.42 - 9.52	<0.01		3.88	1.49 - 10.12	<0.01
[1] Adjusted by age										

Table 3.4.1.27

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values 30 days RP (N=118).

Cox analyses according to the first definition of relapse (R1)										
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**				**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-Value**		**HR**	**95% CI**	**P-value**
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08		2.70	0.90 - 8.10	0.08
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01		4.41	1.28 - 15.28	0.02
Methylation (mean/continuous variable)										
	1.02	1.00 - 1.04		1.02	1.00 - 1.04	0.04		1.01	0.99 - 1.03	0.40
PSA after 30 days (continuous variable)										
	1.03	1.01 - 1.05		1.03	1.01 - 1.05	<0.01		1.02	1.00 - 1.04	0.05
[1] Adjusted by age										

Table 3.4.1.28

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) considering Gleason scores, age, methylation levels and PSA values 30 days RP (N=118).

d) Some distributions of people with PC (relapse vs not relapse), based on methylation levels and other features

There are also graphs (*Figures 3.4.1, 3.4.2, 3.4.3, 3.4.4*) in which the subjects without a relapse there are plotted in blue, and the subjects with a relapse are plotted in red (in these *Figures*, only the definition of relapse based on the first definition of relapse (R1) was considered because the two definition differs only for two subjects). *Figure 3.4.1* shows the distribution of patients with PC considering methylation levels (the mean of the four CpGs considered) on the Y-axis, and age (X-axis). *Figures 3.4.2 and 3.4.3* show the distribution of people considering methylation levels (Y-axis) and PSA values before RP. The difference between the *Figure 3.4.2 and 3.4.3* is the scale used on the X-axis: indeed in the first figure, the scale is included between 1 and 100 ng/ml, whereas in the second a logarithmic scale was used (base 10 (Log10)). This scale allows to have a better situation about those people with a PSA level until 20 ng/ml, which are too compact in *Figure 3.4.2*. *Figures 3.4.4 and 3.4.5* show the distribution of people based on methylation levels and PSA levels 30 days after RP; in particular the *Figure 3.4.4* shows PSA levels from 1 to 80 ng/ml, where all people are represented, whereas *Figure 3.4.5*, shows only those patients with PSA levels after RP from 0 to 0.5 ng/ml, excluding those patients with higher levels of PSA.

These information show an association between relapse and methylation for the higher values of methylation (in particular for the four upper quintiles); for this reason other analyses were performed to characterized those people belonging to the first quintile of methylation (26 people, of which five patients with relapse), data shown in Supplementary *Tables 3 and 4*, in paragraph 7.3). The dispersion graphs were made also considering methylation levels and Gleason score, and methylation levels and T-stage (data shown in the appendix (*Figures 7.4.1* and 7.4.2)).

Only 14 patients died so analyses on mortality were not performed, moreover the project was focused on relapse as primary endpoint.

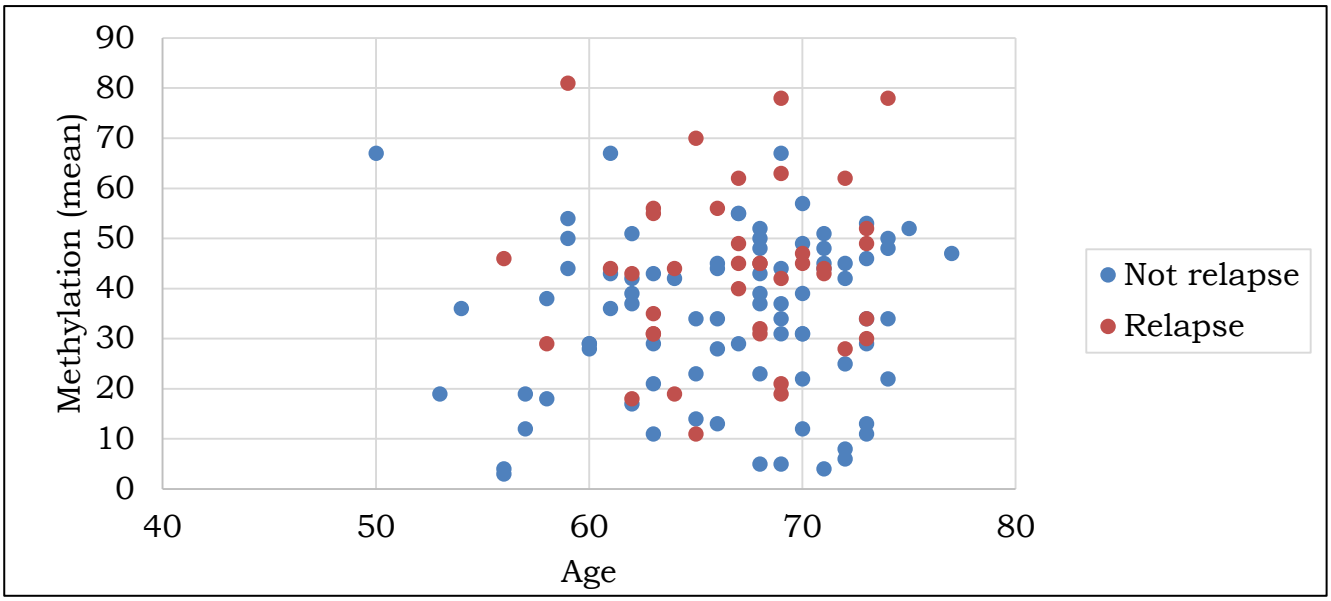

Figure 3.4.1

Distribution of people with PC (relapse vs not relapse), based on methylation levels and age.

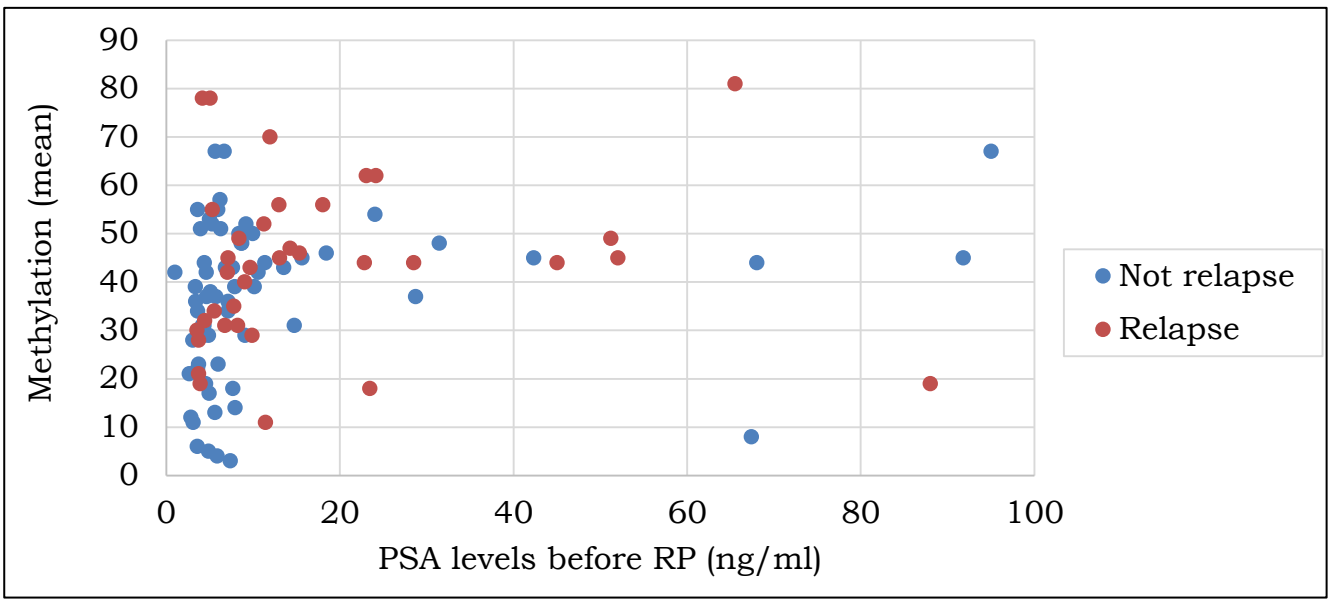

Figure 3.4.2

Distribution of people with PC (relapse vs not relapse), based on methylation levels and PSA levels before RP.

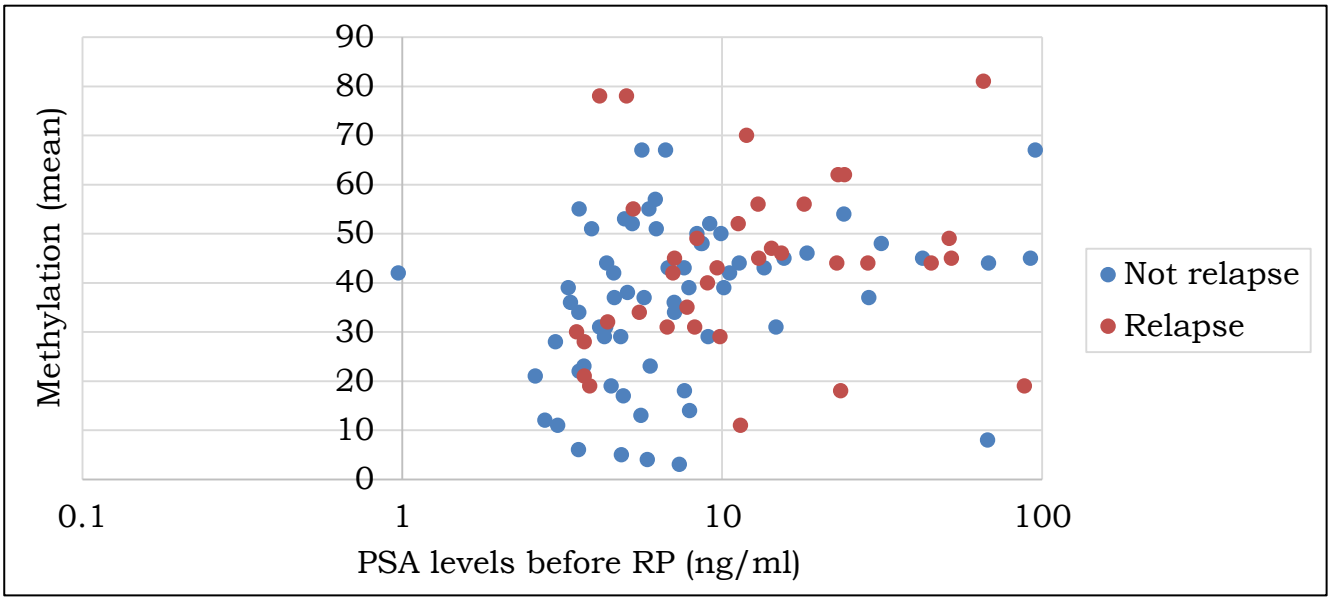

Figure 3.4.3

Distribution of people with PC (relapse vs not relapse), based on methylation levels and PSA levels before RP using a Log10 scale.

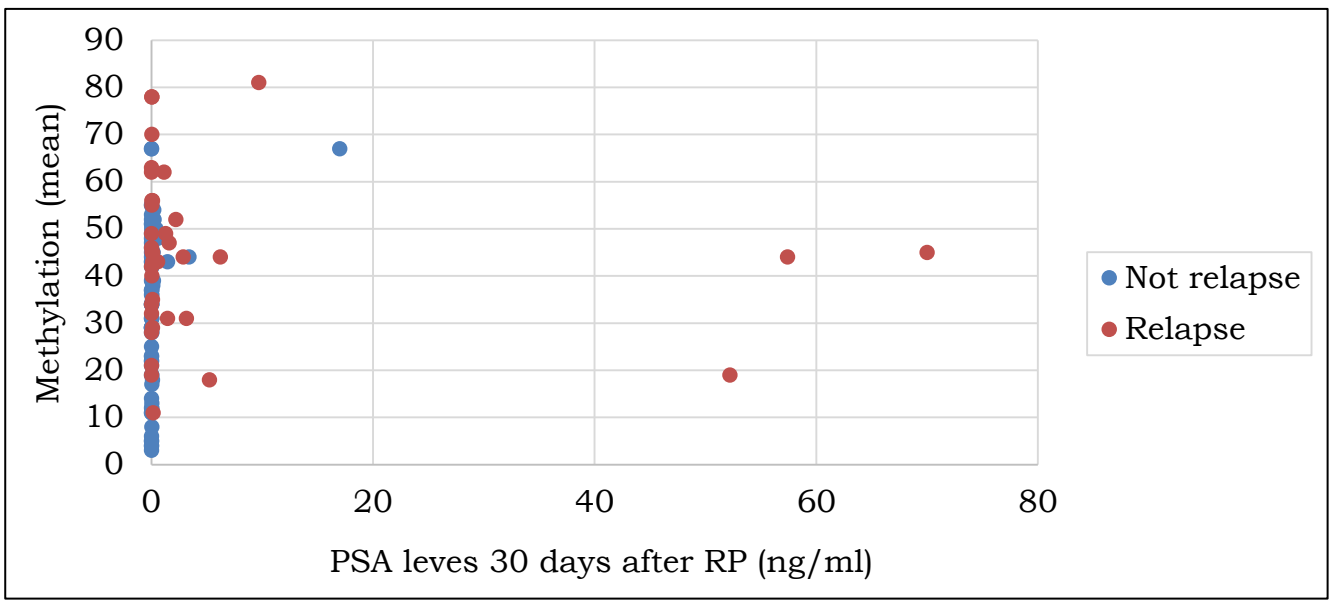

Figure 3.4.4

Distribution of people with PC (relapse vs not relapse), based on methylation levels and PSA levels 30 days after RP (using PSA levels between 0 and 80 ng/ml).

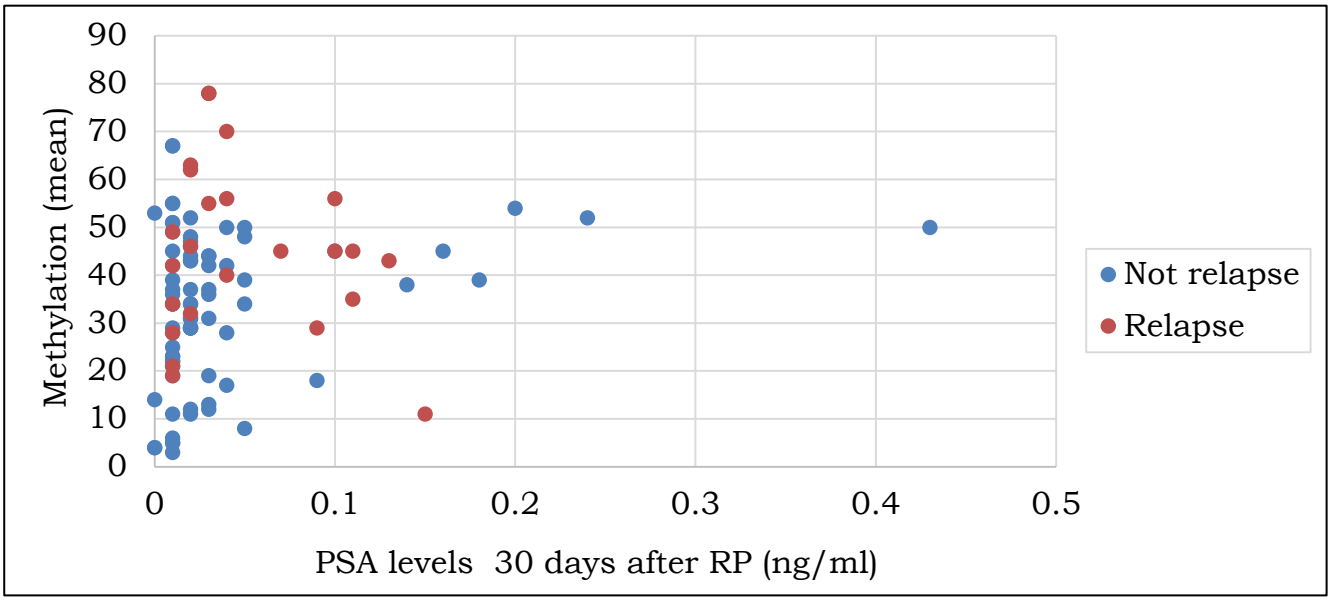

Figure 3.4.5

Distribution of people with PC (relapse vs not relapse), based on methylation levels and PSA levels 30 days after RP (using PSA levels between 0 and 0.5 ng/ml).

3.4.2 Descriptive analyses and comparisons between patients belonging to the first quintile of methylation and the others

Some descriptive analyses were performed in order to obtain more information. *Table 3.4.2.1* shows the distribution of patients when considering methylation levels and Gleason scores, but in this case the outcome (relapse/not relapse) was not considered. The cells contain row's percentages. Thirteen people with Gleason scores ≤ 6 belong to the lower class of methylation (≤ 22), whereas the same Gleason score was found in two subjects characterized by the higher level of methylation (≥ 51). Conversely 3 patients with a Gleason score of 8 – 9 belonging to the lower class of methylation (≤ 22) instead 9 patients characterized by the same Gleason score belonging to the higher class of methylation (≥ 51).

Methylation levels	Gleason Scores			Total
	≤ 6	7	8 - 9	
	N (%)	N (%)	N (%)	
≤ 22	13 (50.00)	10 (38.46)	3 (11.54)	26 (100)
23 - 34	8 (30.77)	16 (61.54)	2 (7.69)	26 (100)
35 - 44	7 (25.93)	11 (40.74)	9 (33.33)	27 (100)
45 - 50	4 (20.00)	10 (50.00)	3 (11.54)	20 (100)
≥ 51	2 (8.70)	12 (52.17)	9 (39.13)	23 (100)

Table 3.4.2.1

Distribution of people (N=122) based on methylation levels and Gleason scores.

Attention was also focused to study the features of people belonging to the lower class of methylation (≤ 22). To generate the tables presented below, patients were divided in two groups: a first group characterized by a level of methylation lower than 22, and a second group constituted by all the other patients (characterized by levels of methylation higher than 22). *Table 3.4.2.2* shows that 50% of people belonging to the first quintile of methylation are characterized by a Gleason ≤ 6, whereas people with higher levels of methylation are characterized by higher Gleason scores. *Table 3.4.2.3* presents data related to T-stage and methylation; also in this case, more than 96% of people with lower levels of methylation belong to the class T1-T2. *Table 3.4.2.4* concerns the relation between age and methylation. The distribution of people with lower methylation levels among classes of age does not show significant differences, whereas in the other group considered, classes of age most represented are those related to elderly people (65-69 and ≥ 70). *Table 3.4.2.5* shows information related to PSA before RP and methylation. More than 35% of people characterized by lower levels of methylation belong to the class of PSA < 4 ng/ml, whereas more than 50% of the people with higher levels of methylation are characterized by levels between 4 and 10 ng/ml. Finally, *Table 3.4.2.6* shows the relation with PSA values 30 days after RP. In this case, the majority of subjects of both groups of methylation were characterized by a level of PSA <0.2.

Subjects belonging to FIRST quintile (≤ 22) of methylation	Gleason Scores			Total
	≤ 6	7	8 - 9	
	N (%)	N (%)	N (%)	N (%)
Yes	13 (50.00)	10 (38.46)	3 (11.54)	26 (100)
No	21 (21.88)	49 (51.04)	26 (27.08)	96 (100)

Table 3.4.2.2

Distribution of Gleason scores based on methylation levels (N=122).

Subjects belonging to FIRST quintile (≤ 22) of methylation	T-stage			Total
	T1 - T2	T3	T4	
	N (%)	N (%)	N (%)	N (%)
Yes	25 (96.15)	0 (0.00)	1 (3.85)	26 (100)
No	50 (52.08)	39 (40.63)	7 (7.29)	96 (100)

Table 3.4.2.3

Distribution of T-stage based on methylation levels (N=122).

Subjects belonging to FIRST quintile (≤ 22) of methylation	Classes of age				Total
	≤ 59	60 - 64	65 - 69	≥ 70	
	N (%)	N (%)	N (%)	N (%)	N (%)
Yes	6 (23.08)	5 (19.23)	7 (26.92)	8 (30.77)	26 (100)
No	9 (9.38)	21 (21.88)	34 (35.42)	32 (33.33)	96 (100)

Table 3.4.2.4

Distribution of classes of age based on methylation levels (N=122).

Subjects belonging to FIRST quintile (≤ 22) of methylation	Pre-surgery PSA				Total
	< 4	4 - 10 (10 excl)	10 - 20	> 20	
	N (%)	N (%)	N (%)	N (%)	N (%)
Yes	7 (36.84)	8 (42.11)	1 (5.26)	3 (15.79)	19 (100)
No	10 (12.35)	42 (51.85)	14 (17.28)	15 (18.52)	81 (100)

Table 3.4.2.5

Distribution of pre-surgery PSA based on methylation levels (N=100)

Subjects belonging to FIRST quintile (≤ 22) of methylation	PSA after 30 days		Total
	< 0.2	≥ 0.2	
	N (%)	N (%)	N (%)
Yes	23 (92.00)	2 (8.00)	25 (100)
No	74 (79.57)	19 (20.43)	93 (100)

Table 3.4.2.6

Distribution of PSA 30 days after RP based on methylation levels (N=118).

Table 3.4.2.7 shows results obtained from univariate and bivariate logistic regression analyses where the outcome is methylation level, and the independent variables is Gleason score. The bivariate model was adjusted for age. The risk to have higher methylation levels is three times for those patients with a Gleason score of 7 compared to those characterized by a Gleason ≤ 6, and more than five times for patients with a Gleason of 8 – 9. The results are statistically significant.

Logistic regression according to the dichotomous classification of methylation						
Variables	Univariate			Bivariate[1] (Age-adjusted)		
	OR	95 % CI		OR	95% CI	P-value
Gleason						
≤ 6	1	----		1	----	
7	3.03	1.15 - 8.00		2.94	1.10 - 7.84	0.03
8 - 9	5.37	1.35 - 21.34		5.82	1.41 - 23.51	0.01
[1] Adjusted by age						

Table 3.4.2.7

Distribution of PSA 30 days after RP based on methylation levels (N=122).

4 DISCUSSION AND CONCLUSIONS

4.1 Trends of incidence and mortality rates in FVG, Italian and American populations

The first part of the work confirmed that the race in particular, Black ethnicity, is one of the risk factors for PC as presented in many papers as Prostate cancer and race[57]. This feature can be seen in the *Figures* obtained from SEER data. Unfortunately, the cohort did not shown people belonging to black race, so this risk factor cannot be included in the analyses on the people involved in the study. The first part of the study shows trends of incidence and mortality in three different populations (American, Italian and the population of FVG). The use of PSA screening caused an augment in incidence rates because bring to discover also PC that would remain asymptomatic. Another interesting risk factor is the age, indeed the incidence rates show an increase related to the age with a peak related to the class including 70-75 years (data confirmed also for the cohort analyzed in the second part of this work).

The mortality at the same time is directly related to the age, indeed all populations considered have a trend that increase with age; by analyzing data over time, it is possible to see a decrease in mortality rates from the last years of the past century and the most recent years.

4.1.1 A focus on data contained in the Regional Repository of microdata

From Tumor register and the regional repository of microdata, data were extracted about cases of PC and it was possible to describe the features of the population of interest by descriptive analyses. The analyses confirm, also in this case, that the peak of incidence of PC occurs in the class including an age between 70 and 74 years. Also when the year of diagnosis is considered, it is possible to see an increase of incidence after the introduction of the PSA screening (in the early 2000s). When considering the Country of birth of the patients analyzed, the majority are Italians, hence it was not possible to study the risk factor associated to the ethnicity. The deaths caused by PC also in this case confirmed the data found in literature showing an increase of deaths directly related to the age. More than one quarter of deaths for PC occurs 7 years after diagnosis.
The population considered was analyzed also to obtain information on the use of classes of medications. Medications that are often used for PC were chosen, but also those which could have a link to PC: in this case, being the prostate an organ relate to hormones, it is interesting also to analyze the class of human medications included in the class of HP.

4.2 Medications used by subjects with PC

The trends of users and prescriptions for people with PC in Udine, from 1998 to 2014, were analyzed. For example, the use of AGnRH shows a considerable decrease from 2000 to 2011, year which sees a reversion of the trend, as this class of medications shows a new increase. 2011 is also the year in which medical doctors started to use the medications included in the class of AAO.
The use of HP underlines a continuous increase over time (except in 2014, probably because it is the year in which data were extracted and so they were not complete). The analyses of combinations taken from people with PC confirm the last concept, as more than one quarter of people take a medication included in the class of HP.

The second type of therapy taken from these patients is the use of AGnRH that, as seen before, shows a decrease over the time but is still used a lot; a similar frequency was shown from the patients which take a combination including a medication of the class AAL, an another, belonging to the class of AGnRH. From these analyses it was possible to deduce that a lot of combinations are used by few people, confirming the guidelines and works in literature, which affirm that the therapy for PC is not unique for all patients characterized by this problem.
About half of the patients considered take only one medication, but a lot of them take two or three different drugs.
The last information about this cohort of patients is related to the types of prostatectomies to which these patients were submitted, underlining a preference for radical prostatectomy (more than 80%).

4.3 A cohort study of 122 people affected by PC with and without a relapse

The last part of the project was focused on a cohort of 122 people. The mean of age at diagnosis of the population studied in this part of the work shows an age at diagnosis that is lower than the peak related to incidence present in literature, as shown in many articles as the analyses of the European Cancer observatory[58] (66 years in the people studied compared to the peak of incidence from 70 to 79 years). T-stage more represented is the T2, and the total Gleason score with more subjects is 7. A relapse was found in one third of people studied. The values of methylation of all the four CpG sites, and also the mean of the four values (obtained using pyrosequencing), are higher for people that have had a relapse compared to the people not characterized by a relapse. PSA levels before and after RP shows values lower for those people without a relapse.
Multiple logistic analyses show that the risk of relapse increase for people with higher Gleason scores.
Methylation levels (considering only Gleason, age and methylation) show similar trends in univariate, bivariate and multiple logistic analyses (using quintiles

related to methylation data), indeed in univariate and bivariate analyses the methylation shows a trend in which the risk of relapse increases with the increase of methylation levels, this trend was also maintained in multiple logistic analyses (but data are not significant). The lack of meaningfulness could be explained by the small number of subjects considered. When it is considered also PSA before RP, the risk of relapse increases for those patients with higher levels of PSA, but also in this case multiple models (logistic and Cox regressions are not significant). Analyses considering PSA levels 30 days after RP show that people with a level >0.2 have an higher risk to develop a relapse compared to those subjects characterized by lower levels. Results related to PSA values 30 days after RP are statistically significant also in multiple logistic regressions and Cox analyses.

Results confirmed the importance of hypermethylation related to GSTP1 in carcinogenesis as described in the studies of Singal et al.[59] and Strand et al.[36], but also their prognostic value related to a possible relapse.

Many studies in literature confirmed the results obtained from our data, however there are also some differences in particular related to the type of patients, the methods used to analyze methylation and the primary outcomes considered.

Moritz R. et al.[55], analyzing methylation patterns related to GSTP1 only in patients with a Gleason score ≤7 and using quantitative methylation specific PCR to obtain methylation data. Data obtained by Moritz at al. show that DNA hypermethylation, could be used as a predictor of PSA recurrence. These authors found significant data related to RAR beta gene, but they considered hypermethylation also in other genes including GSTP1.

Zhou at al.[50] suggest that quantitative GSTP1 methylation may be considered a prognostic factor. These authors as Moritz at al., to evaluate the methylation not use pyrosequencing but quantitative fluorogenic real-time methylation specific polymerase chain reaction.

Concluding methylation of GSTP1 promoter might be useful to identify patients with higher risk of biochemical recurrence, however further studies based on an epidemiologic epigenetic approach, and with a greater number of subjects, are needed.

4.4 Limits

According to the first part of the work, the most important limit is the lack of analyses related to European and Asian data, as these data would allow to have a complete context to describe the situation of incidence and mortality related to PC. Another important limit is related to the groups containing the medications that, in particular in the class of HP, are very heterogeneous. Available data are often incomplete, and this does not allow to obtain information as doubling time of PSA, an interesting parameter to evaluate as demonstrated by Arlen et al.[60], Ramirez et al.[61], and Tracy et al.[62]; certainly the definition of relapse could be considered also using data related to doubling time PSA. The size of the sample is the most important limit, indeed the results, probably, are related to a small sample, thus it is not possible to generalize the results found. An interesting analysis could be study the effects of medications on methylation levels comparing to methylation levels (in the same patients) in the biopsies (used to have a diagnosis of PC) and the levels obtained by prostates from RP samples. Unfortunately, this type of analyses was not possible. When considering the use of medications, the data are very few and heterogeneous therefore it would be interesting to examine the effects of these medications on methylation levels and in relation to the risk of relapse.

5 MATERIAL AND METHODS

The work can be divided into three parts: the first part, in which the available data on incidence and mortality are used and elaborated to obtain trends of incidence and mortality in particular populations. In the second part a cohort of people affected by PC characterized by the use of some classes of medications was identified, and the attention was focused on some characteristics of the population and in particular on medications taken by these patients. Lastly, in the third part a small cohort studied was undertaken, starting from the identification within the cohort of people underwent a RP. This set was divided in two groups, a first group including the subjects characterized by a relapse of PC and a second group constituted by people without a relapse of the same illness.
The Material and Methods related to the first part are presented in the last part of this chapter, whereas the first paragraphs of this chapter describe the materials and methods related to the little cohort of 122 people that is the most innovative part of the work.

5.1 Studied Population

The population was studied in several moments, to obtain different information. In particular, the population was studied in three steps to obtain the information described below:

a) Brief descriptive analyses on the people resident in FVG affected by PC (incidence and mortality to obtain data related to age and year of incidence).
b) The use of selected types of medications (prescribed after the date of diagnosis) in people with PC (to understand what are the therapies used for

these kinds of patients (the analyses on these step were performed on a sample constituted by 2915 subjects).

c) The methylation status of the GSTP1 promoter in people with a PC (residing in FVG) and characterized by almost a hospitalization at the University Hospital of Udine (due to any cause).

Information on medications and on methylation status have also been used to perform some analyses on components, which could be related to biochemical recurrence PC.

The study started with the identification from Tumor register (considering the period between 1995 and 2009), of people with a diagnosis of PC (using the International Classification of Disease-9 (ICD-9) code "185"). Information were linked (using an anonymous unique personal identifier number named "key-anagrafe") with data included into the regional repository of microdata to select people with PC residents in Friuli Venezia Giulia (15.079 men). After this step, men with only one tumor (PC) were selected obtaining a reduction of the population of interest (11.521 men), also in this stage were considered data related to the period from 1995 to 2009. The next selection identified people who had minimum one hospitalization (due to any causes) in one of the healthcare facilities of Udine (all the structures of the province of Udine were included) between 1986 and 2014. This further phase generated 4182 eligible subjects. The database containing these subjects was linked with a database of all medications prescribed to the patients of FVG (including selected medications (the nine groups described in paragraph 5.3)) taken from patients with PC with minimum one admission in one of the healthcare facilities in Udine (and described later); from this link a population constituting of 2915 subjects was obtained. Data related to medications and operations (Radical Prostatectomies (RP)) were extracted from two databases including the years between 1986 to 2014, but information are most reliable from the year 2000. At this stage only patients underwent to a Radical Prostatectomy (RP) (from 2006 to 2014) were selected, because after this type of surgery it is simpler to identify a biochemical recurrence (530 men). In the next step, people monitored by the University Hospital of Udine (the bigger healthcare facility in Udine) were selected. From this selection, it was possible to obtain a database

constituting of 390 people. Only for 149 subjects out of 390, was possible to have clinical information (which are reliable from 2006) and the last bottleneck has been the availability of prostates samples at pathological anatomy (at University Hospital of Udine) in order to obtain biological samples thus the methylation data (paraffin-embedded prostates specimens) were available only for 122 patients). The most important steps, described above, are summarized in *Figure 5.1.1.*

Below a brief description of the populations considered on the two groups of analyses follows.

Figure 5.1.1

Steps to select eligible people for the study.

5.1.1 Analyses of medications in patients with PC with almost an admission in FVG. (A population constituted by 2.915 people).

The results of analyses, related to the population of 2915 people, are described on the paragraph 3.3. On this part of the study were considered data of people with a diagnosis of PC with minimum one admission at one of the healthcare facilities of Udine underwent to a pharmacological therapy and in particular with the classes of medications of interest (described above). For these patients, data about incidence, mortality, surgical procedures and especially about medications were analyzed.

5.1.2 Analyses of a cohort constituted by 122 patients.

The last part of the study focused on a small population constituted by 122 patients for whom it was possible to obtain more information especially on clinical data (as Gleason, TNM stage etc.) and biological data (availability of prostates samples) and therefore methylation data. To obtain the final cohort of 122 subjects from the 2915 patients used for previous analyses (point b)), it was necessary to selected patients underwent to a RP (starting from a database including data on the period between 1986 and 2014) and next chose only those monitored by the University Hospital of Udine (390). Then those patients underwent to RP from 2006 (data from which clinical data are reliable) were selected, obtaining 149 patients. Finally, of the 149 patients, it was possible to obtain biological samples for 122 of 149 patients. This small population was divided into people with or without a relapse of PC:

- ***PEOPLE WITH A RELAPSE***: Patients with PC with a relapse of the disease.
- ***PEOPLE WITHOUT A RELAPSE***: Patients with PC without a relapse of disease.

The relapse was considered using two several elements:

a) ***The first type of RELAPSE (R1)***: In the first case, relapse was considered that, when a medical doctor, in the medical report, talk about of biochemical recurrence (BCR), in an explicit way.
b) ***The second type of RELAPSE (R2)***: Instead in the second case, relapse was considered that when PSA levels showed an increase over the time (and were over 0.2 ng/ml.)

Some checks and comparisons were done between the two definitions, which correspond in all cases except for two patients, which show increasing levels of PSA but no medical doctor defines explicitly a relapse. For this reason, in the analyses of this population two variables were maintained for the relapse which differ for the two subjects described above; so these two subjects relapse according to the second definition but not for the first one.

The follow-up for patients without a relapse is the period from the date of RP to last medical examination, whereas the follow-up for patients with a relapse, is the time from the date of RP to the date of the relapse.

The results of analyses of these patients are presented in paragraph 3.4.

5.2 Selection of medications

From regional repository of microdata data about all prescriptions related to patients residents in FVG with PC were extracted (data referred to a period between 1995 and 2014). First, all prescriptions related to patients of FVG with PC (obtaining 463.903 total prescriptions) were identified. In a second phase, only prescriptions about the medications of interest, prescribed after the data of diagnosis for patients with minimum one admission at University Hospital of Udine were extracted and included in the 13 groups of potential interest. (The 13 groups include: estrogens, antiandrogens (group G), drugs used in benign prostatic hypertrophy, systemic hormonal preparations (excl. Sex hormones and insulins), antineoplastic agents, gonadotropin releasing hormone analogues, antiestrogens, antiandrogens (group L), other hormone antagonists and related agents, intestinal antiinfectives, drugs used in diabetes, lipid modifying agents, antiinflammatory and antirheumatic agents, (non steroids)). Later, the number of patients that intake

all drugs included in the 13 groups were identified; subsequently the patients selected were those who had intake drugs from the 9 groups chosen (estrogens, antiandrogens (group G and group L), drugs used in benign prostatic hypertrophy, systemic hormonal preparations (excl. Sex hormones and insulins), antineoplastic agents, gonadotropin releasing hormone analogues, antiestrogens, other hormone antagonists and related agents) to perform more specified analyses. At the end of this selection, 236 different combinations of drugs related to these 2915 patients were obtained. The term "combinations" means the type of drugs (presented as classes, included in the 9 selected groups of medications), taken in a particular order, where the order is important.

Steps of selection, described above, were summarized in *Figure 5.2.1.*

In *Table 5.2.1* are presented 13 groups of medications, which were selected in a first phase, as medications to study in patients with a diagnosis of PC in FVG, however after some considerations, it was decided to focus the attention only on 9 of 13 groups, obtaining only a few exploratory and descriptive data on the 4 groups excluded (see *Supplementary table 2*). In particular, the first column shows the group of medication analyzed, the second column represents the ATC code referred to the group of medications considered, the third column shows the abbreviation used on tables and results and the last column is necessary to understand what are the groups considered in this work.

Groups of medications	***ATC code***	***Abbreviation***	***Considered for analyses***
Estrogens	G03C	E	Yes
Antiandrogens (group G)	G03H	AAG	Yes
Drugs used in benign prostatic hypertrophy	G04C	BIP	Yes
Systemic hormonal preparations (excl. Sex hormones and insulins)	H	HP	Yes
Antineoplastic agents	L01	C	Yes
Gonadotropin releasing hormone analogs	L02AE	AGnRH	Yes
Antiestrogens	L02BA	AE	Yes
Antiandrogens (group L)	L02BB	AAL	Yes
Other hormone antagonists and related agents	L02BX	AAO	Yes

Intestinal Antiinfectives	A07A	IA	No
Drugs used in diabetes	A10	FD	No
Lipid modifying agents	C10	LM	No
Antiinflammatory and antirheumatic agents, non steroids	M01A	AA	No

Table 5.2.1

Groups of medications used from patients with PC and extracted from the regional repository of microdata.

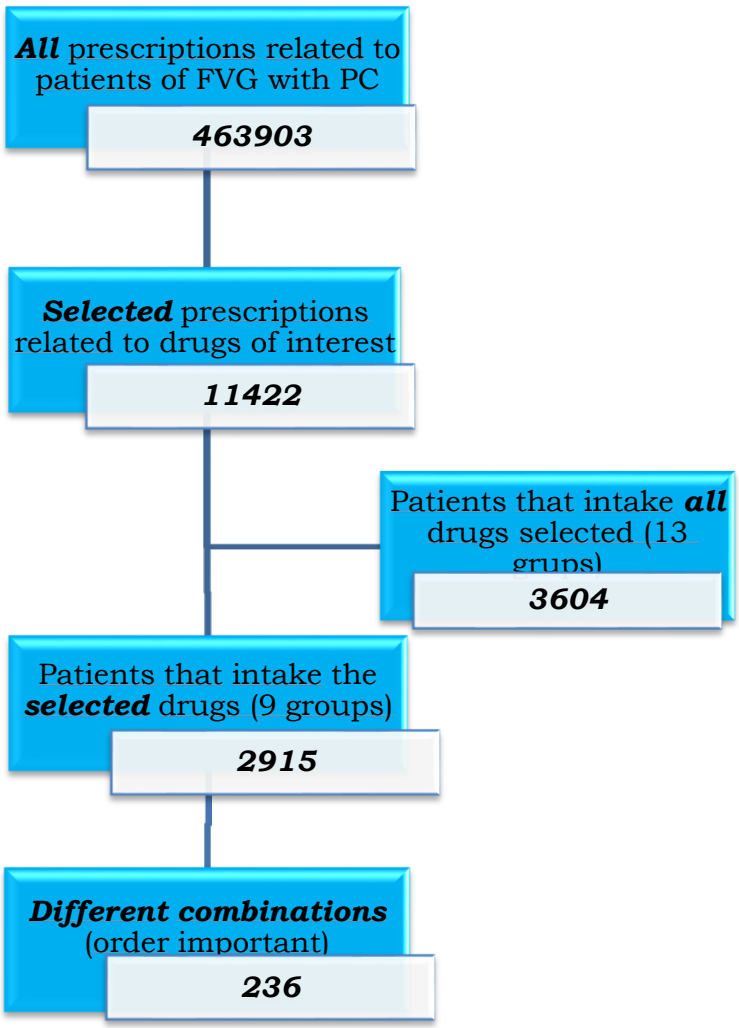

Figure 5.2.1

Steps of selection to identify the combinations of drugs of interest used in patients with PC in FVG.

5.3 Data sources

The study used several databases to obtain different information. Brief descriptions of databases used can be found in the paragraphs below.

5.3.1 Databases to study incidence and mortality rates of FVG, Italian and American population

5.3.1.1 Databases of Surveillance, Epidemiology, and End Results Program (SEER)

Data of SEER are referred to the American population, from these databases, were extracted several kinds of information, in particular, incidence data, these data are available to the period between 1973 and 2013, whereas mortality data are available to the period between 1969 and 2013. To the present study were used data until 2011. The data mentioned above, cover approximately 26 % of the population of United States (US). These incidence data are characterized by a coverage of many years, but geographically the features are not so better. The databases provide also rates for different races as: Black, White, Other races (which includes: American Indian/Alaska (Ak) native, Asian/ Pacific Islander), but provide also data of All races together.

5.3.1.2 AIRTUM data

From the registers of Associazione Italiana dei Registri Tumori (AIRTUM) incidence data related to Italian and FVG populations were extracted. Italian rates (obtained from a pool of 38 different tumor registers) are available from 2006 and 2009, whereas FVG data are available from 1995 to 2007.

5.3.1.3 ISS data

Data from Istituto Superiore di Sanità (ISS) were used to obtain information on mortality referred to Italian and FVG populations. Mortality rates are available from

1989 to 2011, but data concerning the years 2004 and 2005 are not available because ISTAT doesn't provide these data.

5.3.2 The Tumor's register

Tumor register is a register containing incidence data of several cancers from 1995 to 2009. In 2003 it was accredited by the International Agency Research on Cancer (IARC) and included on volume VIII of Cancer Incidence in Five Continents. This register was used to identify people with a PC (using ICD – 9 185 which identify Prostate Cancer).

5.3.3 Regional Repository of microdata

The study used data from the databases of the Friuli Venezia Giulia Regional Health Service. The databases register computerized information regarding the delivery of health care services on an individual basis. Individual records from the different databases can be linked by a unique personal identifier (key-anagrafe). The computerized structure is provided and maintained by INSIEL Spa, an information technology company partly owned by the FVG Region. The three databases used for the study are the Patient Identification Database, the Outpatient Prescription Database, and the Hospital Service Database.

The ***Patient Identification Database*** includes continuous and complete demographic and vital information of all residents in FVG since 1970. For each resident, the database contains the date of birth, the country of birth and the date of death.

The ***Outpatient Prescription Database*** includes data from prescription medications dispensed in the pharmacies of the region since 1995. Among the information recorded for each prescription, the drug name and date of prescription. Prescription medications are coded according to the Anatomical Therapeutic Chemical (ATC) classification. Only reimbursed medications, included in the Italian National Prescription Formulary, prescribed for approved indications, and written

by physicians on the official prescription pad, are registered in the prescription database.

The ***Hospital Service Database*** contains data on hospitalizations in all public and private hospitals of the region since 1986. The information includes dates of admission and discharge, up to six hospital discharge diagnoses, and up to six procedures. Discharge diagnoses include one primary diagnosis, listed in the first position, and up to five secondary diagnoses, listed from the second to the sixth position. Diagnoses and procedures are recorded using the Internal Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM).

5.4 Molecular Methods

5.4.1 DNA extraction

Genomic DNA was extracted from the FFPE blocks and purified using the commercially available QIAamp ® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) as described below.

Tissue slices cut from FFPE blocks were deparaffinizated with Deparaffinization Solution (Qiagen), incubated at 56°C for 3 min and then cool at room temperature. Samples were incubated with 180 µl of buffer ATL (tissue lysis buffer) and 20 µl of proteinase K for 1h at 56°C, and then for 1h at 90°C. To lyse prostate cells 200 µl of Buffer AL was added to the sample, and the obtained lysate was washed with 200 µl of absolute ethanol, and then transferred into a spin column containing a silica membrane of resin. The resin holds the DNA molecules by tying. The silica membrane was washed twice with washing buffers AW1 and AW2, then genomic DNA was eluted with a 50 µl of elution buffer (ATE). The extracted DNA was then stored at -80°C. The concentration of each DNA sample was assessed by UV-visible spectrophotometer (NanoDrop Technology).

5.4.2 Sodium bisulfite modification

The Epitect Bisulfite Kit (Qiagen, Hilden, Germany) was used to perform bisulphite modification of genomic DNA, along with positive controls for methylated [CpGenomeTM universal methylated DNA (Chemicon Co.)] and unmethylated status [CpGenomeTM universal unmethylated DNA (Chemicon Co.)].
1000 ng of DNA were modified in a volume of 40 μl, as recommended by the manufacturer's protocol. Genomic DNA was added to 85 μl of sodium bisulfite Mix, 15 μl or 35 μl of DNA Protect Buffer, and the final total volume of 140 μl was reached with distilled H_2O. Samples were placed in a thermal cycler for the 5 hours-long conversion reaction, with thermal profile as follows: 5' at 95°C, 25' at 60°C, 5' at 95°C, 85' at 60°C, 5' at 95°C, 175' at 60°C and final hold at 20°C.
Samples were then added to 310 μl of Buffer BL +10 μg of carrier RNA and washed 250 μl of absolute ethanol, after that were passed into a spin column and the washed with 500 μl of BW (wash buffer). Desulfonation was performed by adding 500 μl of BD (Desulfonation Buffer) to the column and incubating for 15' at room temperature. The spin column was washed twice with 500 μl of BW and allowed to dry. Bisulfite-converted DNA was eluted in 40 μl of EB (elution buffer) and stored at -80°C.

5.4.3 Quantification of GSTP1 methylation by Pyrosequencing

Analyses of GSTP1 (GenBank accession number M24485) promoter methylation status were performed on a PyroMark Q24 MDx (Qiagen, Hilden, Germany) using PyroMark Gold reagents (Qiagen). Primers, which amplify a 72bp-long fragment containing four CpG sites (positions 1038, 1040, 1043, 1049), were designed outside the CpG sites, according to PyroMark Assay Design software version 2.0 (Qiagen). PCR and sequencing primers are listed in *Table 5.4.3.1*. We performed PCR reaction using PyroMark PCR Kit (Qiagen) in a total volume of 35 μl. The PCR mix contained 1X PCR Master Mix, 1X Coral Load and 0.2μM of primer forward

and reverse and 2 μl of bisulfite-converted DNA with the following cycling profile: 95°C for 15 min followed by 45 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min. The PCR product (20 μl) was added to 18 μl of distilled water and incubated under shaking with 40 μl of binding buffer and 2 μl of streptavidin-coated beads. Pyrosequencing reaction was performed in a total volume of 25 μl, including 24.85 μl of 20 mM Tris-Acetate, 5 mM $MgAc_2$ and 0.3 μM sequencing primer in a PyroMark Q24 MD instrument. Pyrosequencing methylation assays were created according to the manufacturer's instruction and were set up using as the sequence to analyze and the dispensation order C/TGC/TGGC/TGATTTC/TGGGGA and GTCAGTCAGTCGTATTCGG, respectively. Methylation quantification was achieved using the provided software, and expressed for each DNA locus as the percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines. Positive controls for methylated [CpGenomeTM universal methylated DNA (Chemicon Co.)] and unmethylated status [CpGenomeTM universal unmethylated DNA (Chemicon Co.)] were included in each pyrosequencing run. Analyses on GSTP1 methylation were conducted ensuring that the case and control samples were analyzed within the same batch.

Primer	***GSTP1***
Forward	5'-GATTTGGGAAAGAGGGAAAGGT-3'
Reverse	5'-Biot-CAAAAAAACGCCCTAAAATCC- 3'
Sequencing	5'-GGTTTTTTYGGTTAGTTG-3'

Table 5.4.3.1

List of primers used in PCR and sequencing.

5.5 Statistical Analyses

Statistical analyses were performed on 122 people with a diagnosis of Prostate Cancer underwent to radical prostatectomy at University Hospital of Udine. The population described above, is also characterized by the use of particular classes of medications.

Descriptive analyses were used to describe the population's features. In particular, were considered the following variables: age at diagnosis, percentage of methylation of all the four CpG sites considered on the promoter of the GSTP1 gene and also the mean of these four values, PSA levels before surgery (RP), PSA levels after 30 days of RP, T-stage (related to TNM), and Gleason score (in particular Total Gleason score).

The continuous variables were described by the following indexes of position and dispersion: number, mean, standard deviation, Minimum, Maximum, 20° percentiles, 40° percentiles, median (50° percentiles), 60° percentiles and 80° percentiles.

Categorical variables were studied using a frequency of distribution and the respective percentage. The existence of statistically significant associations between the outcome variable (relapse/not relapse) with the covariates used, were analyzed using the Chi–Square test for categorical variables and the Wilcoxon–Mann–Whitney for continuous variables.

Univariate regression analyses were useful to study the associations between the relapse and the variables believed associated in the descriptive analyses (presented above). The first group of logistic regression analyses involved Gleason scores, age and methylation levels (considering methylation both as categorical and continuous variable).

Logistic regressions were performed in different ways, in particular using methylation as a categorical variable (considering the distribution using quintiles) but also as a continuous variable (data presented on paragraph 3.4.1). *Supplementary tables 7 and 8* show results of logistic regression analyses considering the distribution of methylation using tertiles, whereas *Supplementary*

tables 9 and 10 show the results of the same analyses using the distribution of methylation using quartiles.

The variable related to T-stage was excluded because associated with Gleason, the choice of use Gleason instead of T-stage was done (after a debate with a clinician), because this variable, it is more specific for PC than T of TNM value. PSA values were excluded from the first group of analyses because PSA values are missing for more than 20 patients and this would have caused a decrease in the number of people to include in the analyses, but this variable was considered in a second group of analyses which included fewer subjects (N=100) but consider a new information. The same decision was made in relation to the variable related to PSA values 30 after RP. Analyses considering PSA values both before and 30 days after RP, were performed using methylation as a continuous and a categorical variable (data related to quintiles are presented on paragraph 3.4.1, whereas data using tertiles can be found in *Supplementary tables* presented in paragraphs 7.10 and 7.11). Another choice was to adjust the variables using age as a continuous variable, but the preliminary analyses were perform also using classes (also grouped in several ways).

The last analyses performed were Cox analyses, in which there have been used the same variables, used in the multiple logistic regression, but in this case was considered also the time. For patients characterized by a relapse of PC, the time between the date of RP and the date of relapse was considered, for those subjects without a relapse the time between the date of RP and the last medical examination was used. Cox analyses were performed in different ways, in particular using methylation as a categorical variable considering the distribution using quintiles, but also as a continuous variable (data described in the paragraph 3.4.1). *Supplementary tables 11 and 12* show results of Cox analyses considering the distribution of methylation using tertiles, whereas *Supplementary tables 13 and 14* show the results of the same analyses using the distribution of methylation using quartiles. In these cases, the analyses were made using both the first and the second definition of relapse (according to R1 and R2).The first group of Cox analyses included Gleason, age, and methylation, whereas the second group considered also PSA values before RP, finally, the last group analyzed the same

variables but was considered PSA values 30 days after RP, instead of PSA before surgery. The analyses, in which variables related to PSA (before and after RP) were considered, were performed using only the data related to the first definition of relapse (R1).
Statistical analyses were performed using SAS © statistical package 9.3 (SAS Institute Inc., Cary, N.C., USA).

Ethics committee review

The study protocol was reviewed by the Ethics Committee at the University Hospital of Udine.

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6.1 References of figures

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1c. https://commonfund.nih.gov/sites/default/files/epigeneticmechanisms.pdf (*Figure 1.3.1*)

1d. Epigenetic Epidemiology; Springer Science+Business Media B.V. 2012; Michels Ed.; Karin B. Michels (page 41). (*Figure 1.3.2.1.1*)

7 APPENDIX

7.1 Supplementary table 1

Supplementary table 1 summarized all medications used in patients suffering of PC found from a literature review. This table shows a list of medications with their ATC codes. In some cases the ATC code was not found and in other cases the substances were not specified so well so, the same medication could correspond to many several ATC codes.

Medication	*ATC code*
Goserelin	L02AE03
Leuprorelin	L02AE02
Ciproterone acetate	G03HA01
Leuprolide	L02AE02
Triporelin	L02AE04
Abarelix	L02BX01
Degarelix	L02BX02
Tamoxifen	L02BA01
Prostvac	ATC not found
Docetaxel	L01CD02
Bicalutamide	L02BB03
Flutamide	L02BB01
Buserelin acetate	L02AE01
Finasteride	G04CB01
Cabazitaxel	L01CD04
Mitoxantrone	L01DB07
Azacitidine	L01BC07
Celecoxib	M01AH01
Nilutamide	L02BB02
Diethylstilbestrol (DES)	L02AA01
Nafarelina	H01CA02

Etinil estradiol	G03CA01
Abiraterone	L02BX03
Enzalutamide	L02BB
Estramustine	L01XX11
Prednisone	H02AB07
Prednisolone	H02AB06
Denosumab	M05BX04
Antracicline	L01DB
Zoledronic acid	M05BA08
Corticosteroidis (not better specified)	ATC not found
Estrogens	G03C
Progestogens	G03F
Inhibitors of adrenal steroidgenesis	Many ATC codes
Antiandrogens of several nature	Many ATC codes
Ciclofosfamide	L01AA01
Platinum derivatives	ATC not found
Etoposide	L01CB01
Vinca alkaloids	L01CA
Taxanes	L01CD
Denosumab	M05BX04
Decitabine (5-aza-2'-deoxycytidine)	L01BC08
Trichostatin A	ATC not found
Butyrate (not better specified what medication)	Many ATC codes
Hydrocortisone	Many ATC codes
Vitamin E, resveratrol, anti-inflammatory drugs, flufenamic acid, exisulind, selenium	Many ATC codes
Dutasteride/tamsulosin clorhydrate	G04CA52
Dutasteride	G04CB02

Supplementary table 1

List of medications used in patients with PC found from a literature review.

7.2 Supplementary table 2

Supplementary table 2 presents some descriptive data, related to groups of medications not considered for the main analyses done on the work presented; in particular these data include the four groups of medications included on *table 6.2.1.1* but not used to perform the analyses presented above.

The groups included are:

- Intestinal Antiinfectives (AI),
- drugs used in diabetes (FD),
- lipid modifying agents (ML),
- antiinflammatory and antirheumatic agents (non steroids) (AA).

The table summarized the number of users for each medication (described with the name of medication, the group which they referred to, and the ATC code).

Group	Medication	ATC code	Users
IA	Nystatin	A07AA02	257
FD	Insulin (human)	A10AB01	65
FD	Insulin lispro	A10AB04	67
FD	Insulin aspart	A10AB05	25
FD	Insulin glulisine	A10AB06	25
FD	Insulin (human)	A10AC01	58
FD	Insulin lispro	A10AC04	8
FD	Insulin (human)	A10AD01	39
FD	Insulin lispro	A10AD04	9
FD	Insulin aspart	A10AD05	10
FD	Insulin (human)	A10AE01	2
FD	Insulin glargine	A10AE04	93
FD	Insulin detemir	A10AE05	20
FD	Metformin	A10BA02	388
FD	Glibenclamide	A10BB01	46
FD	Chlorpropamide	A10BB02	1
FD	Glipizide	A10BB07	2
FD	Gliquidone	A10BB08	5
FD	Gliclazide	A10BB09	145
FD	Glimepiride	A10BB12	87
FD	Phenformin and sulfonamides	A10BD01	43
FD	Metformin and sulfonamides	A10BD02	301

FD	Metformin and rosiglitazone	A10BD03	3
FD	Metformin e pioglitazone	A10BD05	20
FD	Glimepiride and pioglitazone	A10BD06	3
FD	Metformin and sitagliptin	A10BD07	16
FD	Metformin and vildagliptin	A10BD08	4
FD	Metformin and linagliptin	A10BD11	1
FD	Acarbose	A10BF01	9
FD	Rosiglitazone	A10BG02	1
FD	Pioglitatone	A10BG03	21
FD	Sitagliptin	A10BH01	13
FD	Vildagliptin	A10BH02	2
FD	Linagliptin	A10BH05	2
FD	Repaglinide	A10BX02	129
FD	Exenatide	A10BX04	2
FD	Liraglutide	A10BX07	4
LM	Simvastatin	C10AA01	625
LM	Lovastatin	C10AA02	21
LM	Parvastatin	C10AA03	154
LM	Fluvastatin	C10AA04	62
LM	Atorvastatin	C10AA05	771
LM	Cerivastatin	C10AA06	19
LM	Rosuvastatin	C10AA07	346
LM	Bezafibrate	C10AB02	13
LM	Gemfibrozil	C10AB04	22
LM	Fenofibrate	C10AB05	51
LM	Colestyramine	C10AC01	16
LM	Omega3 Triglycerides	C10AX06	252
LM	Ezetimibe	C10AX09	23
LM	Simvastatina and ezetimibe	C10BA02	63
AA	Celecoxib	M01AH01	383

Supplementary table 2

Exploratory data based on users of the four groups (presented on table 6.2.1.1) not considered on analyses performed and presented in the main part of this work.

7.3 Supplementary tables 3 and 4

In *Supplementary table 3* and *Supplementary table 4* are summarized the features of people belonging to the first quintile in relation to the outcome considered, according to the first definition of relapse (R1) The analyses were performed only using the first definition of relapse, because the two subjects which differ from definitions according to R1 and R2, does not belong to the first quintile, and so the results would be the same. In *Supplementary table 3,* there are considered the continuous variables, whereas in *Supplementary table 4,* there are summarized results related to categorical variables.

Patients' features	***Relapse / Not Relapse***									***Wilcoxon - Mann - Whitney***
Continuous variables	***Subjects WITHOUT relapse 1 (R1)***					***Subjects WITH relapse 1 (R1)***				
	N	***Mean***	***SD[1]***	***Median***	***IQR[2]***	***N***	***Mean***	***SD1***	***Median***	***IQR[2]***	***P-value***
Age	21	65.14	6.78	66.00	13.00	5	65.80	3.11	65.00	5.00	0.53
Methylation (CpG1)	21	11.14	6.04	12.00	11.00	5	13.60	4.10	14.00	4.00	<0.01
Methylation (CpG2)	21	12.52	6.51	12.00	13.00	5	16.00	4.36	17.00	1.00	<0.01
Methylation (CpG3)	21	11.71	6.84	12.00	15.00	5	17.80	4.32	18.00	1.00	<0.01
Methylation (CpG4)	21	14.48	6.82	14.00	12.00	5	23.40	3.58	24.00	1.00	<0.01
Methylation (mean)	21	12.33	6.34	12.00	12.00	5	17.60	3.85	19.00	1.00	<0.01
PSA before surgery	14	9.38	16.78	4.87	3.78	5	26.07	35.54	11.40	19.57	0.98
PSA 30 days after RP	20	0.02	0.02	0.01	0.01	5	11.52	22.85	0.15	5.22	0.93

[1] SD = Standard Deviation
[2] IQR = Interquartile range

Supplementary table 3

Features (continuous variables) of patients with PC belonging to the first quintile (N=26).

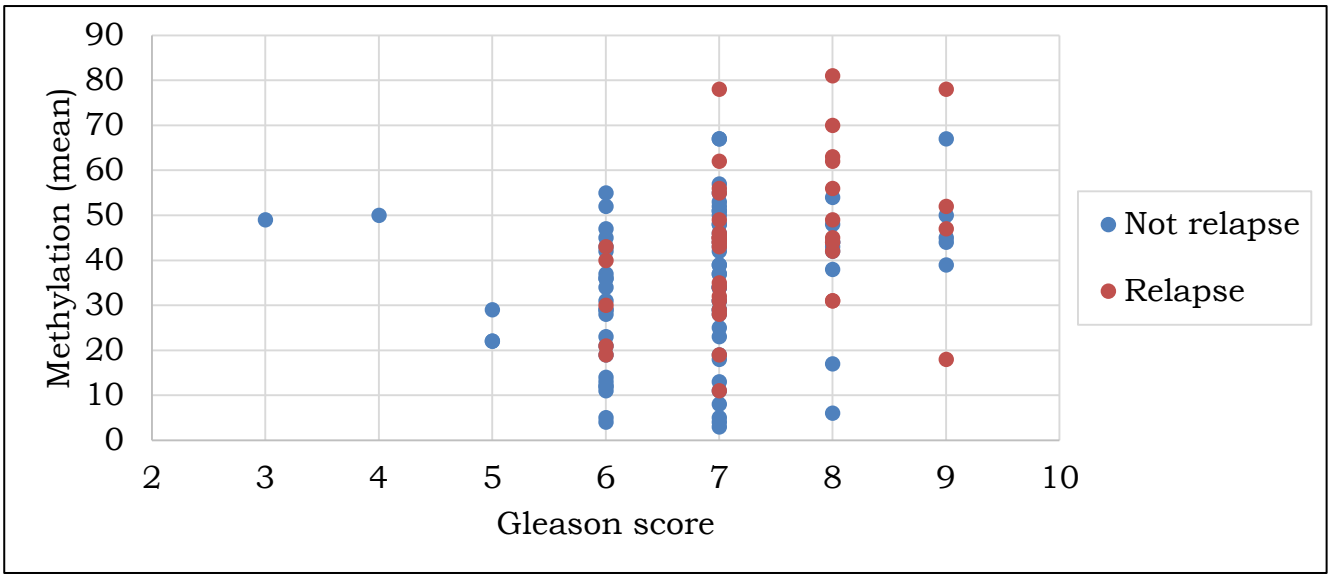
Patients' features	Relapse / Not Relapse		Chi-square
Categorical variables	Subjects WITHOUT relapse 1 N (%)	Subjects WITH relapse 1 N (%)	P-value
Age at diagnosis	**N = 21**	**N = 5**	**0.06**
≤ 59	6 (28.57)	0 (0.00)	
60 - 64	3 (14.29)	2 (40.00)	
65 - 69	4 (19.05)	3 (60.00)	
≥ 70	8 (38.10)	0 (0.00)	
T (stage):	**N = 21**	**N = 5**	**0.04**
T1-T2	21 (100.00)	4 (80.00)	
T3	0 (0.00)	0 (0.00)	
T4	0 (0.00)	1 (20.00)	
Gleason score	**N = 21**	**N = 5**	**0.77**
≤ 6	11 (52.38)	2 (40.00)	
7	8 (38.10)	2 (40.00)	
8 - 9	2 (9.52)	1 (20.00)	
Pre-surgery PSA	**N = 14**	**N = 5**	**0.04**
< 4	5 (35.71)	2 (40.00)	
4 - 10 (10 excl)	8 (57.14)	0 (0.00)	
10 - 20	0 (0.00)	1 (20.00)	
> 20	1 (7.14)	2 (40.00)	
PSA after 30 days	**N = 20**	**N = 5**	**<0.01**
< 0.2	20 (100.00)	3 (60.00)	
≥ 0.2	0 (0.00)	2 (40.00)	

Supplementary table 4

Features (categorical variables) of patients with PC belonging to the first quintile (N=26).

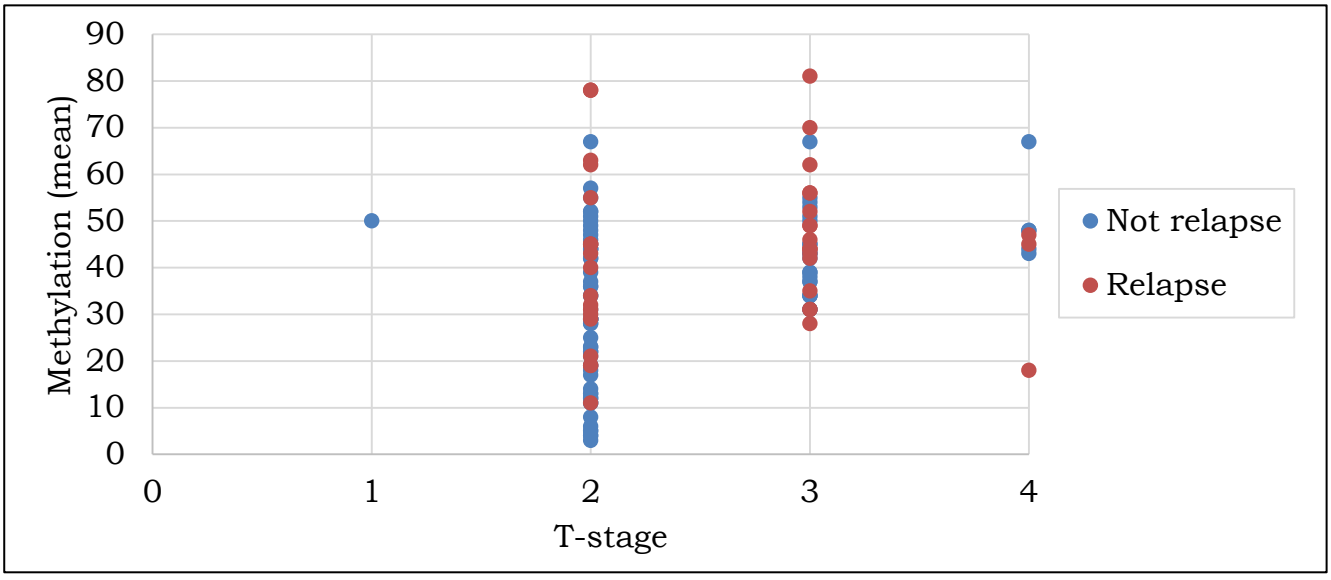
7.4 Supplementary figures

To have information about the distribution of patients in relation to some features (to complete the data presented on paragraph 3.4.1), there were made *Figures* that give the distribution of patients with and without relapse in relation to Methylation levels and Gleason score and methylation levels and T-stage.



Supplementary figure 7.3.1

Distribution of people in relation to Methylation levels and Gleason score (N=122).



Supplementary figure 7.3.2

Distribution of people in relation to Methylation levels and T-stage (N=122).

7.5 Supplementary tables 5 and 6

The distribution of subject according to the first definition of relapse (R1) based on the distribution of methylation using tertiles (*Supplementary table 5*) and quartiles (*Supplementary table 6*).

Distribution of subjects based on tertiles	Subjects WHITOUT relapse (R1)	Subjects WITH relapse (R1)	Total
	N (%)	N (%)	N (%)
≤ 31	34 (75.56)	11 (24.44)	45 (100)
32 - 45	27 (65.85)	14 (34.15)	41 (100)
>45	21 (58.33)	15 (41.67)	36 (100)

Supplementary table 5

Distribution of subjects using tertiles.

Distribution of subjects based on quartiles	Subjects WHITOUT relapse (R1)	Subjects WITH relapse (R1)	Total
	N (%)	N (%)	N (%)
≤ 28	26 (81.25)	6 (18.75)	32 (100)
29 - 39	22 (73.33)	8 (26.67)	30 (100)
40 - 48	18 (58.06)	13 (41.94)	31 (100)
> 48	16 (55.17)	13 (44.83)	29 (100)

Supplementary table 6

Distribution of subjects using quartiles.

7.6 Supplementary tables 7 and 8

Supplementary table 7 and *8* show results of logistic regression analyses considering the distribution of methylation using tertiles. Methylation's values are related to the mean of the four CpGs. *Supplementary table 7* presents results according the first definition of relapse (R1) whereas *Supplementary table 8* shows the results according the second definition of relapse (R2).

Logistic regression according to the first definition of relapse (R1)									
Variables	***Univariate***			***Bivariate[1] (Age-adjusted)***			***Multiple[1]***		
	OR	***95 % CI***		***OR***	***95% CI***	***P-value***	***OR***	***95% CI***	***P-value***
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06	2.76	0.91 -8.41	0.07
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01	5.80	1.64 – 20.49	0.01
Methylation (mean/tertiles)									
≤ 31	1	----		1	----		1	----	
32 - 45	1.60	0.63 - 4.09		1.59	0.62 - 4.07	0.33	1.15	0.42 – 3.11	0.78
≥ 45	2.21	0.85 - 5.70		2.17	0.83 - 5.65	0.11	1.38	0.49 – 3.85	0.54
[1] Adjusted by age									

Supplementary table 7

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) (N=122).

Logistic regression according to the second definition of relapse (R2)									
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**			**Multiple[1]**		
	OR	**95% CI**		**OR**	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.97	1.00 - 8.86		2.91	0.98 - 8.71	0.06	2.72	0.98 – 8.28	0.79
8 - 9	8.22	2.47 - 27.35		8.64	2.56 - 29.12	<0.01	7.56	2.13 – 26.87	<0.01
Methylation (mean/tertiles)									
≤ 31	1	----		1	----		1	----	
32 - 45	1.78	0.70 - 4.52		1.77	0.70 - 4.50	0.23	1.24	0.46 – 3.36	0.68
> 45	2.47	0.96 - 6.37		2.44	0.94 - 6.32	0.07	1.46	0.52 – 4.10	0.47
[1] Adjusted by age									

Supplementary table 8

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the second definition of relapse (R2) (N=122).

7.7 Supplementary tables 9 and 10

Supplementary tables 9 and 10 show results of logistic regression analyses where the distribution of methylation was considered using quartiles. Methylation's values are related to the mean of the four CpGs. *Supplementary table 9* presents data according the first definition of relapse (R1). *Supplementary table 10* shows results according the second definition of relapse (R2).

Logistic regression according to the first definition of relapse (R1)									
Variables	***Univariate***			***Bivariate[1] (Age-adjusted)***			***Multiple[1]***		
	OR	***95 % CI***		***OR***	***95% CI***	***P-value***	***OR***	***95% CI***	***P-value***
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06	2.60	0.85 – 7.91	0.09
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01	4.80	1.35 – 17.03	0.02
Methylation (mean/quartiles)									
≤ 28	1	----		1	----		1	----	
29 - 39	1.58	0.47 - 5.24		1.57	0.47 - 5.23	0.46	1.33	0.39 – 4.60	0.65
40 - 48	3.13	1.00 - 9.77		3.10	0.98 - 9.74	0.05	2.12	0.63 - 7.07	0.22
> 48	3.52	1.11 - 11.13		3.49	1.10 - 11.08	0.03	2.27	0.67 - 7.68	0.19
[1] Adjusted by age									

Supplementary table 9

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) (N=122).

Logistic regression according to the second definition of relapse (R2)									
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**			**Multiple[1]**		
	OR	**95% CI**		**OR**	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.97	1.00 - 8.86		2.91	0.98 - 8.71	0.06	2.60	0.85 – 7.97	0.09
8 - 9	8.22	2.47 - 27.35		8.64	2.56 - 29.12	<0.01	6.35	1.78 – 22.70	<0.01
Methylation (mean/quartiles)									
≤ 28	1	----		1	----		1	----	
29 - 39	1.58	0.47 - 5.24		1.57	0.47 - 5.23	0.46	1.32	0.38 – 4.60	0.66
40 - 48	4.06	1.31 - 12.62		4.04	1.29 - 12.63	0.02	2.63	0.79 – 8.80	0.12
> 48	3.52	1.11 - 11.13		3.5	1.11 - 11.11	0.03	2.12	0.62 – 7.23	0.23
[1] Adjusted by age									

Supplementary table 10

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the second definition of relapse (R2) (N=122).

7.8 Supplementary tables 11 and 12

Supplementary tables 11 and 12 show results related to Cox analyses analyses where the distribution of methylation was considered using tertiles. Methylation's values are related to the mean of the four CpGs. *Supplementary table 11* presents results according to the first definition of relapse (R1), whereas *Supplementary table 12* shows results according to the second definition of relapse (R2).

Cox analyses according to the first definition of relapse (R1)									
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**			**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08	2.51	0.92 – 6.87	0.07
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01	6.10	2.04 – 18.21	<0.01
Methylation (mean/tertiles)									
≤ 31	1	----		1	----		1	----	
32 - 45	1.25	0.57 - 2.76		1.25	0.57 - 2.77	0.58	0.81	0.35 – 1.87	0.62
> 45	1.50	0.69 - 3.27		1.51	0.69 - 3.29	0.30	0.87	0.38 - 2.02	0.75
[1] Adjusted by age									

Supplementary table 11

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) (N=122).

Cox analyses according to the second definition of relapse (R2)									
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**			**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.40	0.90 - 6.40		2.40	0.90 - 6.41	0.08	2.48	0.91 – 6.79	0.08
8 - 9	6.60	2.42 - 18.00		6.60	2.42 - 18.00	<0.01	7.08	2.41 – 20.85	<0.01
Methylation (mean/tertiles)									
≤ 31	1	----		1	----		1	-----	
32 - 45	1.34	0.62 - 2.93		1.35	0.62 - 2.94	0.46	0.82	0.36 – 1.89	0.64
> 45	1.62	0.75 - 3.50		1.63	0.75 - 3.51	0.22	0.90	0.39 - 2.06	0.80
[1] Adjusted by age									

Supplementary table 12

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the second definition of relapse (R2) (N=122).

7.9 Supplementary tables 13 and 14

Supplementary tables 13 and 14 show results related to Cox analyses analyses where the distribution of methylation was considered using quartiles. Methylation's values are related to the mean of the four CpGs. *Supplementary table 13* shows the results according the first definition of relapse (R1) whereas *Supplementary table 14* shows results according the second definition of relapse (R2).

Cox analyses according to the first definition of relapse (R1)									
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**			**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.4	0.90 - 6.39		2.4	0.90 - 6.42	0.08	2.29	0.84 – 6.25	0.11
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01	5.31	1.75 – 16.11	<0.01
Methylation (mean/quartiles)									
≤ 28	1	----		1	----		1	----	
29 - 39	1.40	0.48 - 4.02		1.41	0.49 - 4.06	0.53	1.23	0.42 – 3.62	0.70
40 - 48	1.92	0.73 - 5.05		1.94	0.73 - 5.16	0.19	1.10	0.39 – 3.14	0.86
> 48	2.23	0.85 - 5.87		2.24	0.85 - 5.89	0.10	1.31	0.47 – 3.66	0.60
[1] Adjusted by age									

Supplementary table 13

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) (N=122).

Cox analyses according to the second definition of relapse (R2)									
	Univariate			Bivariate[1] (Age-adjusted)			Multiple[1]		
	HR	95% CI		HR	95% CI	P-value	HR	95% CI	P-value
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.4	0.90 - 6.40		2.4	0.90 - 6.41	0.08	2.29	0.84 – 6.24	0.11
8 - 9	6.6	2.42 - 18.00		6.6	2.42 - 18.00	<0.01	6.14	2.06 – 18.34	<0.01
Methylation (mean/quartiles)									
≤ 28	1	----		1	----		1	-----	
29 - 39	1.40	0.49 - 4.03		1.41	0.49 - 4.08	0.53	1.25	0.43 – 3.67	0.68
40 - 48	2.25	0.97 - 5.81		2.28	0.88 - 5.94	0.09	1.23	0.44 – 3.43	0.70
> 48	2.23	0.85 - 5.87		2.24	0.85 - 5.90	0.10	1.24	0.45 – 3.47	0.23

[1] Adjusted by age

Supplementary table 14

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the second definition of relapse (R2) (N=122).

7.10 Supplementary tables 15 and 16

Supplementary tables 15 and 16 show results related to logistic regression analyses (*Supplementary table 15*) and Cox analyses (*Supplementary table 16*) where the distribution of methylation was considered using tertiles and are considered as variables: Gleason, age, methylation and PSA values before RP. Methylation's values are related to the mean of the four CpGs. *Tables* presented in this paragraph show the results according the first definition of relapse (R1).

Logistic regression according to the first definition of relapse (R1)										
Variables	***Univariate***			***Bivariate[1] (Age-adjusted)***				***Multiple[1]***		
	OR	***95 % CI***		***OR***	***95% CI***	***P-value***		***OR***	***95% CI***	***P-value***
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06		2.30	0.67 - 7.91	0.19
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01		2.53	0.58 - 11.04	0.22
Methylation (mean/tertiles)										
≤ 31	1	----		1	----			1	----	
32 - 45	1.60	0.63 - 4.09		1.59	0.62 - 4.07	0.33		0.72	0.23 - 2.27	0.58
≥ 45	2.21	0.85 - 5.70		2.17	0.83 - 5.65	0.11		0.96	0.30 - 3.10	0.94
Pre-surgery PSA										
< 4	1	----		1	----			1	----	
4 - 10 (10 excl)	1.26	0.35 - 4.54		1.29	0.36 - 4.67	0.69		1.04	0.26 - 4.12	0.96
10 - 20	3.71	0.82 - 16.84		3.85	0.84 - 17.60	0.08		2.69	0.49 - 15.10	0.26
> 20	4.06	0.95 - 17.43		4.19	0.97 - 18.08	0.05		3.08	0.59 - 16.03	0.18
[1] Adjusted by age										

Supplementary table 15

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) (N=100).

Cox analyses according to the first definition of relapse (R1)									
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**			**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08	2.50	0.86 - 7.22	0.09
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01	3.63	1.07 - 12.35	0.04
Methylation (mean/tertiles)									
≤ 31	1	----		1	----		1	----	
32 - 45	1.25	0.57 - 2.76		1.25	0.57 - 2.77	0.58	0.53	0.21 - 1.34	0.18
> 45	1.50	0.69 - 3.27		1.51	0.69 - 3.29	0.30	0.57	0.22 - 1.45	0.24
Pre-surgery PSA									
< 4	1	----		1	----		1	----	
4 - 10 (10 excl)	1.62	0.53 - 4.91		1.62	0.53 - 4.93	0.40	1.48	0.46 - 4.70	0.51
10 - 20	3.40	1.02 - 11.34		3.40	1.02 - 11.33	0.05	2.70	0.71 - 10.28	0.15
> 20	3.86	1.21 - 12.35		3.87	1.21 - 12.38	0.03	3.06	0.80 - 11.70	0.10
[1] Adjusted by age									

Supplementary table 16

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) (N=100).

7.11 Supplementary tables 17 and 18

Supplementary tables 17 and 18 show results related to logistic regression analyses (*Supplementary table 17*) and Cox analyses (*Supplementary table 18*) where the distribution of methylation was considered using tertiles and are considered as variables: Gleason, age, methylation and PSA values 30 days after RP. Methylation's values are related to the mean of the four CpGs. *Tables* presented in this paragraph show the results according the first definition of relapse (R1).

Logistic regression according to the first definition of relapse (R1)										
Variables	***Univariate***			***Bivariate[1] (Age-adjusted)***				***Multiple[1]***		
	OR	***95 % CI***		***OR***	***95% CI***	***P-value***		***OR***	***95% CI***	***P-value***
Gleason										
≤ 6	1	----		1	----			1	----	
7	2.97	1.00 - 8.86		2.92	0.98 - 8.72	0.06		3.63	1.06 - 12.44	0.04
8 - 9	6.21	1.88 - 20.56		6.47	1.94 - 21.63	<0.01		3.38	0.78 - 14.57	0.10
Methylation (mean/tertiles)										
≤ 31	1	----		1	----			1	----	
32 - 45	1.60	0.63 - 4.09		1.59	0.62 - 4.07	0.33		1.35	0.46 - 3.95	0.59
≥ 45	2.21	0.85 - 5.70		2.17	0.83 - 5.65	0.11		1.55	0.51 - 4.73	0.44
PSA after 30 days										
< 0.2	1	----		1	----			1	----	
≥ 0.2	6.08	2.20 - 16.83		6.22	2.23 - 17.32	<0.01		5.02	1.57 - 16.11	0.01
[1] Adjusted by age										

Supplementary table 17

Univariate, bivariate (age adjusted) and multiple logistic regression, according to the first definition of relapse (R1) (N=118).

Cox analyses according to the first definition of relapse (R1)									
Variables	**Univariate**			**Bivariate[1] (Age-adjusted)**			**Multiple[1]**		
	HR	**95% CI**		**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Gleason									
≤ 6	1	----		1	----		1	----	
7	2.40	0.90 - 6.39		2.40	0.90 - 6.42	0.08	2.73	0.90 - 8.23	0.08
8 - 9	5.61	2.03 - 15.52		5.61	2.03 - 15.52	<0.01	2.78	0.70 - 11.09	0.15
Methylation (mean/tertiles)									
≤ 31	1	----		1	----		1	----	
32 - 45	1.25	0.57 - 2.76		1.25	0.57 - 2.77	0.58	0.89	0.36 - 2.15	0.79
> 45	1.50	0.69 - 3.27		1.51	0.69 - 3.29	0.30	0.91	0.36 - 2.26	0.83
PSA after 30 days									
< 0.2	1	----		1	----		1	----	
≥ 0.2	4.50	2.32 - 8.74		4.80	2.42 - 9.52	<0.01	3.87	1.48 - 10.13	<0.01
[1] Adjusted by age									

Supplementary table 18

Univariate, bivariate (age adjusted) and multiple Cox analyses, according to the first definition of relapse (R1) (N=118).

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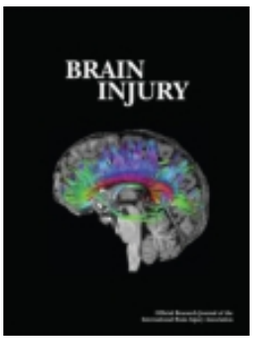
9 PUBLISHED ARTICLES

1. *Prescribing practice and off-label use of psychotropic medications in post-acute brain injury rehabilitation centres: a cross-sectional survey*. Pisa FE, ***Cosano G***, Giangreco M, Giorgini T, Biasutti E, Barbone F; Group for the Study of Medication Use in Centers for Post-acute Brain Injury Rehabilitation. Brain Inj. 2015; 29(4):508-16. doi: 10.3109/02699052.2014.992474. Epub 2014 Dec 30.

2. *Polypharmacy and the use of medications in inpatients with acquired brain injury during post-acute rehabilitation: A cross-sectional study*. ***Cosano G***, Giangreco M, Ussai S, Giorgini T, Biasutti E, Barbone F, Pisa FE; Group for the Study of Medication Use in Centres for Post-acute Brain Injury Rehabilitation. Brain Inj. 2016; 30(3):353-62.
   doi: 10.3109/02699052.2015.1118767. Epub 2016 Feb 18.



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ORIGINAL ARTICLE

Prescribing practice and off-label use of psychotropic medications in post-acute brain injury rehabilitation centres: A cross-sectional survey

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Abstract

Objective: Guidance on pharmacotherapy of neurobehavioural sequelae post-acquired brain injury (ABI) is limited. Clinicians face the choice of prescribing off-label. This survey assesses prescribing practice and off-label use of psychotropic medications in Italian brain injury rehabilitation centres and factors associated with atypical antipsychotics use.

Materials and methods: Centres were identified through the roster of the Italian Society for Rehabilitation Medicine. Information was collected through a structured questionnaire. This study calculated the prevalence of centres reporting to use off-label individual medications and unconditional logistic regression Odds Ratio (OR), with 95% confidence interval (95% CI) of atypical antipsychotics use.

Results: Psychotropic medications were commonly used. More than 50% of the 35 centres (participation ratio 87.5%) reported to use off-label selected antipsychotics, mostly for agitation (90.5%) and behavioural disturbances (19.0%), and antidepressants, mostly for insomnia (37.5%) and pain (25.0%). Atypical antipsychotic use was directly associated with age <40 years (OR = 2.68; 95% CI = 1.25–5.76), recent ABI (1.74; 0.74–4.09), not with reported off-label use (0.98; 0.44–2.18).

Conclusion: In clinical practice, the effectiveness and safety of medications, in particular off-label, should be systematically monitored. Studies are needed to improve the quality of evidence guiding pharmacotherapy and to evaluate effectiveness and safety of off-label prescribing.

Keywords

Acquired brain injuries, antidepressants, antiepileptic agents, antipsychotics, off-label, pharmacoepidemiology, prescribing, prevalence, psychotropic medications, survey

History

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Introduction

During post-acute rehabilitation, patients with acquired brain injury (ABI), traumatic and non-traumatic, may suffer neurobehavioural sequelae [1–4] requiring treatment with psychotropic medications. The quantity and quality of evidence guiding pharmacotherapy of neurobehavioural sequelae of ABI is limited. Recent systematic reviews reported supporting evidence of efficacy of methylphenidate, donepezil and dopamine enhancing agents to improve cognition [5, 6], of beta-blockers on aggression [7] and methylphenidate on behaviour (anger/aggression, psychosocial functioning) [5] after traumatic brain injury (TBI). Serotoninergic antidepressants and sertraline had the strongest evidence of efficacy on depression following TBI [8, 9] and a positive effect of methylphenidate has also been reported [10]. SSRIs proved to be effective on post-stroke depression [11]. Paroxetine and buspirone may be effective in reducing anxiety symptoms in stroke patients with co-morbid anxiety and depression [12]. For most treatments, however, the available evidence is insufficient to promulgate practice standards or guidelines and only treatment options (lowest level of recommendation) are currently available [5–9, 13–15].

Therefore, clinicians rely on clinical experience and on treatment options. Moreover, they often face the choice of prescribing off-label.

In several contexts of clinical practice, it is not uncommon to use a medication outside the indications, dose range and patient population approved by regulatory agencies after the scrutiny of pre-clinical and clinical data has confirmed its efficacy and safety [16]. Off-label use of medications raises complex issues [16–21]. Little is known about the prevalence of off-label prescribing in the post-acute rehabilitation of persons with ABI.

A survey was performed to assess the prescribing practice of psychotropic medications in Italian tertiary brain injury rehabilitation centres providing post-acute rehabilitation to inpatients who suffered a severe traumatic or non-traumatic

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brain injury. Objectives of this study were to assess (a) the prevalence of use of psychotropic medications; (b) the prevalence of the off-label use of psychotropic medications; and (c) the factors associated with the use of atypical antipsychotics.

Materials and methods

Study design

This study is a 1-day cross-sectional survey conducted in tertiary centres for post-acute brain injury rehabilitation in Italy. The survey had a centre-level and a patient-level part. The units under study were: (a) in the centre-level part, all the tertiary centres for post-acute brain injury rehabilitation in Italy; and (b) in the patient-level part, all the patients with ABI, following a traumatic or non-traumatic cause, receiving post-acute rehabilitation and hospitalized in the participating centres at the day of the survey. The centres were identified through the roster of the Italian Society for Physical and Rehabilitation Medicine (Società Italiana di Medicina Fisica e Riabilitazione, SIMFER).

Data collection

An invitation e-mail was sent to the medical director of each identified centre. A structured questionnaire and a guide with definitions and instructions were sent to participating centres. At each centre, a designated rehabilitation physician compiled the questionnaire, which encompassed a centre-level and a patient-level part. The centre-level part inquired on facility characteristics, including the number of beds and of inpatients at the time of the survey and prescribing practice. The questionnaire listed 108 psychotropic medications commonly used in the post-acute rehabilitation of persons with ABI and identified by means of a systematic review of the literature (list of medications displayed in Supplemental electronic Table e1). For each of the listed psychotropic medications, the following information was collected: use (yes/no), off-label prescription with reference to the Italian formulary (yes/no) and indication/s (a free text answer without limitations to the number of indications to be reported). In addition, the respondents could report up to 15 additional psychotropic medications, not included in the list. The survey questions are provided in Supplemental electronic Table e2.

The patient-level part of the questionnaire collected, for each included patient, demographic and clinical characteristics, such as gender and age, the Rancho Level of Cognitive Functioning Scale (LCF) score [http://www.rancho.org/Research_RanchoLevels.aspx, last accessed on July 1, 2012] at admission and time since the occurrence of ABI. For each patient a copy of the anonymized medication chart was obtained. The information on the medication used on one single day (the day before the survey) was extracted from the medication chart.

The survey was conducted on 15 September 2012. Centres with a high number of inpatients could collect data over 2 or more consecutive days. A form was sent to non-participating centres to collect information on reasons for not partaking in the survey and on facility characteristics.

Questionnaire delivery, quality controls and data input

Compiled questionnaires were delivered to the study co-ordination centre by mail or e-mail. Upon delivery, compiled questionnaires were inspected for the completeness and consistency of the recorded information. Any omission, error or inconsistent data was checked through immediate contact with the compiling physician and corrected as appropriate. The medications were then classified using the Anatomical Therapeutic Chemical (ATC) classification system codes. The data were recorded into an electronic database using an entry form created specifically for this study. For the input of selected variables, a range of allowable values was established. Further quality control of the data included the identification of missing and out-of-range values, tests for logical data relationships and generation of output for review. Any inconsistency was investigated by reviewing the recorded data, re-examining the questionnaire or through contact with the compiling physician and corrected as appropriate.

Statistical analysis

Descriptive statistics were calculated to characterize the centres. Continuous variables, such as number of beds and of inpatients, were categorized using the quartiles as cut-off values. For each medication, this study calculated the prevalence of centres (a) reporting its use, by dividing the number of centres reporting its use by the total number of participating centres; and (b) reporting its off-label use, by dividing the number of centres reporting its use off-label by the total number of centres reporting to use that medication. For each therapeutic class, the frequency of off-label indications was calculated by dividing the number of centres reporting the individual indication by the number of centres reporting to use off-label at least one medication in the same therapeutic class.

The Odds Ratio (OR), with 95% confidence interval (95%CI), of receiving an atypical antipsychotic agent was calculated by means of multivariate unconditional logistic regression. This analysis was performed on patient-level data and was, therefore, restricted to the 31 centres providing patient-level data. Before building the multivariate model, all variables (reported off-label use of atypical antipsychotics at the centre, yes/no; age; sex; number of weeks since ABI; LCF score; geographical area; number of beds) were evaluated by univariate logistic regression. The variables that explained the variability or modified the regression coefficient estimators were retained in the final model for other covariates.

Statistical analysis was performed using SAS© statistical package 9.2 (SAS Institute Inc., Cary, NC).

Ethics committee review

The study protocol was reviewed by the Ethics Committee at the University Hospital of Udine and at participating centres.

Results

A total of 40 centres were identified and 35 (87.5%) agreed to participate, 31 (77.5%) providing patient-level data (Figure 1). About two-thirds of the participating centres were located in northern Italy, half had 15 or more beds,

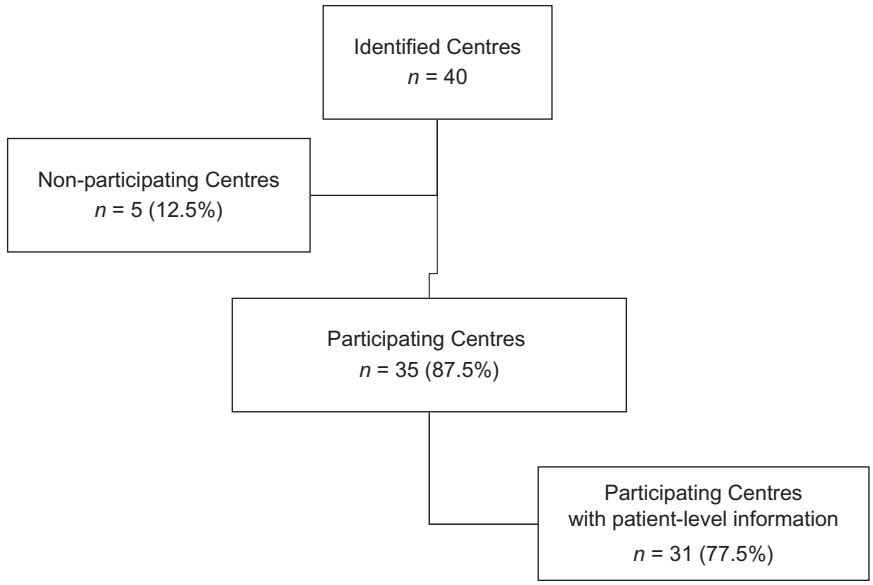

Figure 1. Flow diagram describing the inclusion of Centres for Post-acute Brain Injury Rehabilitation in the survey.

Table I. Characteristics of participating centres.

	n (*n* = 35)	%
Geographic area		
North East	13	37.1
North West	10	28.6
North – sub-total	23	65.7
Centre	6	17.1
South and Islands	6	17.1
Number of beds for patients with ABI		
<8	8	22.9
8–14	9	25.7
15–29	9	25.7
≥30	9	25.7
Minimum–maximum	4–67	
Number of inpatients with ABI		
<6	8	22.9
6–11	6	17.1
12–22	12	34.3
≥23	9	25.7
Minimum–maximum	2–65	

ranging from 4–67, and 60% had 12 or more inpatients at the time of the survey, ranging from 2–65 (Table I).

The prevalence of centres reporting to use and to use off-label individual medications is displayed in Table II. The most used analgesic was tramadol, by 88.6% of the centres, followed by codeine and oxycodone (57.1%). All the centres reported to use levetiracetam, but only one (2.9%) to use it off-label. The second most used antiepileptic was valproate (91.4%), followed by carbamazepine and benzodiazepines (82.9%).

The most used antipsychotics were the atypical agents quetiapine (97%) and olanzapine (83%). More than 50% of the centres reported to use off-label these medications and risperidone, while the prevalence was lower for typical antipsychotics, ranging from 9.5% ($n = 2$) for promazine to 42.9% ($n = 3$) for prometazine.

The most used antidepressants were citalopram (91.4%), sertraline (85.7%) and venlafaxine (74.3%). The antidepressants most used off-label were trazodone, by 57.9% of the centres, amitryptiline (54.5%) and mirtazapine (53.3%).

Bromocriptine was reported to be used off-label by 54.5% of the centres, amantadine by 41.9% and levodopa–benserazide by 35.7%. Table III displays the results for additional medications not included in the list. Of note, eight (22.9%) centres reported the use of beta blocking agents.

The indications for off-label use are displayed in Table IV. Antiepileptics were prescribed for a wide range of indications, including paroxysmal autonomic instability, depression, psychomotor agitation and disturbances such as myoclonus, muscle hypertonia and essential tremor. Three centres reported to use antiepileptics for depression. Psychomotor agitation (90.5%) was the main indication for antipsychotics, followed by behavioural disturbances (19.0%). Insomnia and sleep disturbances (37.5%, $n = 9$), were the main indications for antidepressants, followed by pain (25.0%, $n = 6$), sialorrhea (20.8%, $n = 5$) and psychomotor agitation or neurostimulation (16.7%). Neurostimulation was the main off-label indication for psychostimulants, dopaminergics and anti-dementia drugs.

Among the inpatients of the 31 centres providing patient-level data, the Odds Ratio (OR) of receiving an atypical antipsychotic agent was directly associated with age <40 years, recent (≤11 weeks) ABI and LCF score 4 (confused–agitated) and inversely associated with LCF score 1–2 (none or generalized response) (Table V). When the model was adjusted simultaneously for age, sex, LCF score, time since ABI and geographical location, inpatients of centres reporting atypical antipsychotics off-label use were not at increased risk of receiving these agents (OR = 0.98; 95% CI = 0.44–2.18) compared to inpatients of centres reporting no off-label use.

Discussion

This survey found that psychotropic medications were commonly used in Italian tertiary centres for post-acute brain injury rehabilitation. More than 90% of the centres reported to use levetiracetam, quetiapine, valproate and citalopram. More than 50% of centres reported to use off-label the atypical antipsychotics quetiapine, olanzapine and risperidone and the antidepressants trazodone and amitryptiline. Fewer centres

Table II. Prevalence of centres reporting the use and the off-label use of psychotropic medications[a] by therapeutic class.

		Centres reporting use		Centres reporting off-label use	
Therapeutic class (ATC)	Medication	*n*	%	*n*	%
Analgesics (N02)	tramadol	31	88.6	0	—
	oxycodone	20	57.1	1	5.0
	codeine	20	57.1	0	—
	morphine	14	40.0	1	7.1
	buprenorphine	13	37.1	1	7.7
	methadone	4	11.4	2	50.0
	paracetamol	35	100.0	0	—
Antiepileptics (N03)	levetiracetam	35	100.0	1	2.9
	valproate	32	91.4	3	9.4
	carbamazepine	29	82.9	3	10.3
	benzodiazepines	29	82.9	3	10.3
	oxcarbazepine	27	77.1	2	7.4
	phenytoin	26	74.3	0	—
	pregabalin	25	71.4	1	4.0
	phenobarbital	24	68.6	0	—
	gabapentin	17	48.6	2	11.8
	lamotrigine	16	45.7	0	—
	topiramate	14	40.0	0	—
	tizanidine	14	40.0	2	14.3
	dantrolene	8	22.9	0	—
	zonisamide	3	8.6	0	—
	thiocolchicoside	3	8.6	0	—
	vigabatrin	2	5.7	0	—
	ethosuximide	1	2.9	1	100.0
Antipsychotics (N05A)					
Typical	haloperidol	24	68.6	6	25.0
	promazine	21	60.0	2	9.5
	chlorpromazine	16	45.7	0	—
	promethazine	7	20.0	3	42.9
Atypical	quetiapine	34	97.1	18	52.9
	olanzapine	29	82.9	15	51.7
	risperidone	18	51.4	10	55.6
	clozapine	8	22.9	3	37.5
	sulpiride	6	17.1	3	50.0
	aripiprazole	4	11.4	2	50.0
Anxiolytics, hypnotics and sedatives (N05B, N05C)	lorazepam	29	82.9	1	3.5
	clonazepam	28	80.0	5	17.9
	zolpidem	27	77.1	6	22.2
	alprazolam	26	74.3	0	—
	diazepam	26	74.3	2	7.7
	bromazepam	20	57.1	0	—
	triazolam	17	48.6	1	5.9
	melatonin agonists	8	22.9	1	12.5
	lormetazepam	7	20.0	1	14.3
	flurazepam	4	11.4	0	—
	others[b]	5	14.5	0	—
Antidepressants (N06A)	citalopram	32	91.4	1	3.1
	sertraline	30	85.7	3	10.0
	venlafaxine	26	74.3	4	15.4
	paroxetine	24	68.6	1	4.2
	escitalopram	24	68.6	1	4.2
	duloxetine	23	65.7	1	4.3
	amitriptyline	22	62.9	12	54.5
	trazodone	19	54.3	11	57.9
	mirtazapine	15	42.9	8	53.3
	fluoxetine	15	42.9	2	13.3
	reboxetine	7	20.0	1	14.3
	clomipramine	4	11.4	1	25.0
	lithium	4	11.4	0	—
	fluvoxamine	3	8.6	1	33.3
	imipramine	1	2.9	0	—
Psychostimulants, dopaminergics and anti-dementia drugs (N06B, N06D, N04B)	amantadine	31	88.6	13	41.9
	L-dopa/benserazide	28	80.0	10	35.7
	L-dopa/carbidopa	26	74.3	7	26.9
	bromocriptine	11	31.4	6	54.5
	donepezil	5	14.3	1	20.0

(*continued*)

Table II. Continued

Therapeutic class (ATC)	Medication	Centres reporting use		Centres reporting off-label use	
		n	%	*n*	%
	memantine	4	11.4	0	—
	rivastigmine	4	11.4	1	25.0
	methyilphenidate	3	8.6	0	—
	modafinil	1	2.9	0	—
	galantamine	1	2.9	0	—

[a]Results for psychotropic medications included in the questionnaire list. The complete list of the 108 psychotropic medications assessed in the questionnaire is available in Supplemental electronic Table 1.
[b]This category includes temazepam, chlordiazepoxide, vigabatrin, buspirone and zaleplon, each used by 1 centre.

Table III. Prevalence of centres reporting the use of additional psychotropic medications.[a]

Medication	ATC	Centres reporting use	
		n	%
beta blocking agents[b]	C07A	8	22.9
clotiapine	N05AH06	3	8.6
L deprenyl	N04BD01	1	2.9
agomelatine	N06AX22	1	2.9
amisulpride	N05AL05	1	2.9
biperiden	N04	1	2.9
clonidine	C02AC01	1	2.9
lacosamide	N03AX18	1	2.9
levomepromazine	N05AA02	1	2.9
acetylcarnitine	N06BX12	1	2.9
oxycodone and naloxone	N02AA55	1	2.9
oxcarbazepine	N03AF02	1	2.9
piracetam	N06BX03	1	2.9
pramipexole	N04BC05	1	2.9
tetrabenazine	N07XX06	1	2.9
trihexyphenidyl	N04AA01	1	2.9
valpromide	N03AG02	1	2.9
zuclopenthixol	N05AF05	1	2.9

[a]Results for additional psychotropic medications not included in the questionnaire list and reported by respondents.
[b]atenolol, $n = 1$; propranolol, $n = 7$.

reported to use off-label typical antipsychotics than atypical ones. Off-label prescribing is not uncommon in several contexts of clinical practice and it raises complex issues [16–21]. The absence of approval for a specific indication or group of patients does not necessarily mean that the medication use is inappropriate for that indication or population. No request to expand the labelling may have been submitted to the regulatory agency, even in the presence of additional supporting evidence [21]. Off-label prescribing may provide a treatment for patients with an orphan disease, prompting access to therapeutic options based on new emerging evidence or new options when approved treatments have failed [22]. On the other hand, off-label prescribing involves the extrapolation of evidence on both effectiveness and safety to an indication not assessed in development trials or to an unstudied population [23]. Therefore, it raises concern, in particular when occurring without scientific evidence on efficacy and safety [17].

In this study, psychomotor agitation was the main off-label indication for atypical antipsychotic agents. Consistently, a previous survey in Italian rehabilitation centres [24] found that atypical antipsychotics were the most used medication for agitation, followed by antiepileptics, typical antipsychotics, benzodiazepines and antidepressants. A recent study found that lorazepam and methotrimeprazine were the most common agitation medications on an 'as needed' basis, while methotrimeprazine and quetiapine were the most used in long-standing treatments [25].

In this survey, the other agitation medications were antidepressants and antiepileptics. The questionnaire list did not include beta blocking agents, reported as additional medications by eight centres.

In clinical practice, antipsychotics and benzodiazepines are commonly used as first line treatment for acute agitation and aggressive behaviour [26]. Atypical antipsychotics are preferred to typical agents for their lower cognitive and motor adverse effects [8, 26]. The strongest evidence of efficacy, however, has been found for beta-blocking agents and methylphenidate [5, 7, 13]. The evidence of efficacy of atypical antipsychotic medications used off-label in other patient populations has been reviewed. This evidence is restricted to a few indications: aripiprazole, olanzapine and risperidone were associated with small but statistically significant benefits for the treatment of agitation and behavioural symptoms in dementia [27].

A retrospective audit conducted in a neurorehabilitation unit for patients with ABI found that the assessment for agitation was not consistently performed to support pharmacological treatment decisions, not to monitor the effectiveness of treatment. Moreover, the results of assessments did not correlate well with pharmacological management [25].

It was found that SSRIs and, in particular, sertraline were the most used antidepressants, consistently with the results of a survey on rehabilitation specialists in the Netherland and the UK [28]. SSRIs [9, 29] and sertraline [7, 9] have been recommended as first choice agents for treating depression in patients with brain injury. In this study, selected antidepressants, such as amitriptyline, trazodone and mirtazapine, were used off-label. The main non-licensed indications were insomnia and sleep disturbances, followed by pain and sialorrhea. Several agents and therapeutic classes were used for neurostimulation, including psychostimulants, anti-dementia medications, anxiolytics, hypnotics and sedatives, antiepileptics and antidepressants. No firm evidence exists to support any pharmacological intervention for neurofacilitation or neurostimulation [8, 30].

This study found that age <40 years, recent (≤11 weeks) ABI and LCF score 4 are associated with an increased probability of receiving atypical antipsychotics. Agitated and aggressive behaviour, the main off-label indication of antipsychotics in this study, may characterize patients with

Table IV. Number and distribution of reported off-label indications, by therapeutic class.

Therapeutic class	Indication	Centres reporting the off-label indication	
		n	%
Antiepileptics (N03)	paroxysmal autonomic instability	5	38.5
	depression	3	23.1
	psychomotor agitation	2	15.4
	pain	2	15.4
	myoclonus	2	15.4
	muscle hypertonia	2	15.4
	other[a]	9	69.2
	total[b]	13	100.0
Antipsychotics (N05A)	psychomotor agitation	19	90.5
	behavioural disturbance	4	19.0
	depression	2	9.5
	other[c]	6	28.6
	total[b]	21	100.0
Anxiolytics, hypnotics and sedatives (N05B, N05C)	neurostimulation	4	40.0
	muscle hypertonia	2	20.0
	other[d]	7	70.0
	total[b]	10	100.0
Antidepressants (N06A)	insomnia and sleep disturbances	9	37.5
	pain	6	25.0
	sialorrhoea	5	20.8
	psychomotor agitation	4	16.7
	neurostimulation	4	16.7
	inappetence	2	8.3
	dyskinesia	2	8.3
	other[e]	11	45.8
	total[b]	24	100.0
Psychostimulants, anti-dementia drugs (N06B, N06D, N04B)	neurostimulation	13	81.3
	paroxysmal autonomic instability	2	12.5
	other[f]	2	12.5
	total[b]	16	100.0

[a]Includes essential tremor, behavioural disturbance, migraine, infectious fever, seizures, anxiety, neurostimulation, paresthesia, hiccup.
[b]The totals are the number of centres reporting the use off-label of at least one medication of the individual therapeutic class. The totals do not sum up to the total number of indications because more than one indication could be written.
[c]Includes anxiety, inertia, insomnia, frontal lobe syndrome, vertiginous syndrome, hiccup.
[d]Includes pain, paroxysmal autonomic instability, inertia, insomnia and sleep disturbances, anxiety, psychomotor agitation, aggressive behaviour.
[e]Includes compulsive behaviour, emotional incontinence, inertia, anorexia, muscle hypertonia, paroxysmal autonomic instability, paresthesia, psychosis, anxiety, hiccup, tremor.
[f]Includes psychomotor agitation, central fever.

Table V. Odds ratio (OR) and 95% confidence interval (95% CI) of receiving an atypical antipsychotic medication.

	Non-users ($n=420$) *n* (%)	Users ($n=54$) *n* (%)	Univariate			Age-adjusted			Multivariate[c]		
			OR	95% CI		OR	95% CI		OR	95% CI	
Center[a] reported off-label prescribing											
No[b]	99 (23.6)	11 (20.4)	1.00	—	—	1.00	—	—	1.00	—	—
Yes	321 (76.4)	43 (79.6)	1.21	0.60	2.43	1.19	0.59	2.41	0.98	0.44	2.18
Age class (years)											
≤39[b]	104 (24.8)	23 (42.6)	2.43	1.20	4.95				2.68	1.25	5.76
40–59	162 (38.6)	17 (31.5)	1.15	0.55	2.42	—	—	—	1.31	0.61	2.85
≥60	154 (36.7)	14 (25.9)	1.00	—	—	—	—	—	1.00	—	—
Sex											
Men[b]	265 (63.1)	34 (63.0)	1.00	—	—	1.00	—	—	1.00	—	—
Women	155 (36.9)	20 (37.0)	1.01	0.60	1.81	1.08	0.59	1.95	1.17	0.62	2.20
LCF score											
1–2 No or generalized response	155 (36.9)	5 (9.3)	0.21	0.06	0.78	0.19	0.05	0.71	0.20	0.05	0.76
3 Localized	76 (18.1)	13 (24.1)	1.13	0.37	3.42	1.14	0.37	3.48	1.20	0.39	3.74
4 Confused–agitated	38 (9.1)	14 (25.9)	2.43	0.79	7.47	2.08	0.66	6.51	2.25	0.69	7.35
5 Confused, inappropriate, non-agitated	75 (17.9)	13 (24.1)	1.14	0.38	3.47	1.15	0.37	3.53	1.17	0.38	3.63

(*continued*)

Table V. Continued

	Non-users ($n = 420$) *n* (%)	Users ($n = 54$) *n* (%)	Univariate OR	95% CI		Age-adjusted OR	95% CI		Multivariate[c] OR	95% CI	
6 Confused–appropriate	43 (10.2)	4 (7.4)	0.61	0.15	2.47	0.55	0.14	2.26	0.60	0.14	2.47
7–8 Automatic/purposeful and Appropriate[b]	33 (7.9)	5 (9.3)	1.00	—	—	1.00	—	—	1.00	—	—
Time since ABI (weeks)											
≤11[b]	110 (26.2)	22 (40.7)	2.08	0.94	4.60	2.12	0.95	4.74	1.74	0.74	4.09
12–18.5	95 (22.6)	11 (20.4)	1.20	0.49	2.96	1.34	0.54	3.34	1.10	0.42	2.90
18.5–32	111 (26.4)	11 (20.4)	1.03	0.42	2.53	1.11	0.45	2.74	0.99	0.39	2.52
>32	104 (24.8)	10 (18.5)	1.00	—	—	1.00	—	—	1.00	—	—
Geographical area											
Centre[b]	95 (22.62)	14 (25.9)	1.00	—	—	1.00	—	—	1.00	—	—
North-East	113 (26.9)	12 (22.2)	0.72	0.32	1.63	0.68	0.30	1.55	0.67	0.27	1.67
North-West	99 (23.6)	12 (22.2)	0.82	0.36	1.87	0.90	0.39	2.06	0.96	0.40	2.29
South and Islands	113 (26.9)	16 (29.6)	0.96	0.45	2.07	1.00	0.46	2.16	1.17	0.51	2.68

[a]This analysis is restricted to the 31 centres providing patient-level data.
[b]Reference category.
[c]The unconditional logistic regression model included terms for: off-label prescribing of atypical antipsychotics at the centre, age class (≤39 years; 40–59 years; ≥60 years), sex, LCF score, number of weeks since ABI (<11; 11–<18.5; 18.5–32; >32), geographical area (North; Centre; South and Islands).

LCF score 4 and have been positively associated with younger age [31, 32]. The results suggest that these symptoms may be more frequent in the first 3 months after ABI, although the course and timing of recovery has a high inter-patient variability.

Limitations

This survey was conducted in Italian rehabilitation centres and the generalizability of results may be limited. Several factors, including national regulation and drug marketing strategies, affect prescribing practices.

The non-participating centres did not provide any information. One cannot, therefore, evaluate reasons for not participating nor the differences from the participating centres.

This study assessed prescribing practices of psychotropic medication through a physician-compiled questionnaire. Although the compiling physicians were rehabilitation specialist aware of their centre prescribing practice, errors in reporting cannot be ruled out.

The questionnaire sought information about additional psychotropic medications not included in the list. It is, therefore, likely that few, if any, psychotropic medications used in the current practice of the participating centres have been missed.

Conclusions

This survey showed that off-label use of psychotropic medications, in particular selected antipsychotics and antidepressants, is not uncommon in the current clinical practice of ABI rehabilitation centres. Competent, effective and safe off-label prescribing requires high awareness about its scientific validity and medical evidence. Careful and systematic monitoring of the effectiveness and safety of medications, in particular when prescribed off-label, is highly recommended in patients with ABI.

Well designed studies are needed to improve the quality of evidence guiding pharmacological treatments of neurobehavioural sequelae of ABI and to evaluate the risks and benefits of off-label prescribing.

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Declaration of interest

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Good Pharmacoepidemiology Practice: The study adhered to the Guidelines for Good Pharmacoepidemiology Practices (GPP) (International Society for Pharmacoepidemiology, 2008). STROBE (http://www.strobe-statement.org) was used as a guideline for the reporting of observational studies.

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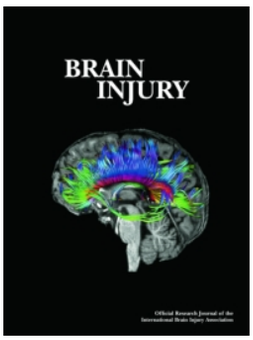

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ORIGINAL ARTCLE

Polypharmacy and the use of medications in inpatients with acquired brain injury during post-acute rehabilitation: A cross-sectional study

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Abstract

Background: This study assessed the use of medications during inpatient post-acute rehabilitation for acquired brain injury (ABI).
Materials and methods: All inpatients with ABI undergoing post-acute rehabilitation in centres identified through the roster of the Italian Society for Rehabilitation Medicine were included. A designated physician in each centre collected information through a structured questionnaire. This study calculated (a) prevalence of medication use, (b) logistic regression Odds Ratio (OR), with 95% confidence interval (95% CI), of polypharmacy (≥ 6 medications).
Results: A total of 484 patients (median age = 52 years, 63.4% men, median time from acute event = 18.5 weeks) were included; 33.8% had Rancho Los Amigos Levels of Cognitive Functioning Scale (RLAS) score 1–2, 8.1% had a score of 7–8, of whom 92.0% received medications, 51.8% had a score of 6–10, of whom 83.9% had at least one psychotropic medication and 66.9% had two or more; 51.8% received anti-epileptics, 32.1% anti-depressants, 14.5% anti-psychotics, peaking in RLAS 4 (37.3%) and decreasing in RLAS 7–8. Polypharmacy was directly associated with age (55–64 years, OR = 2.1; 95% CI = 1.1–4.1; ≥ 65 years, OR = 1.7; 95% CI = 0.9–3.3), inversely with RLAS score (1-2 vs 7–8, OR = 4.3; 95% CI = 1.9–9.8).
Conclusion: Polypharmacy and concurrent use of psychotropic medications was common, raising concern about drug–drug interactions. Safety and effectiveness of medications should be monitored, particularly when used concurrently.

Keywords

ABI, brain injury, polypharmacy, post-acute rehabilitation, psychotropic

History

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Introduction

Patients undergoing post-acute rehabilitation following acquired brain injury (ABI), traumatic and non-traumatic, may suffer multiple problems, both medical (such as gastrointestinal bleed, venous thromboembolism, infections, endocrine dysfunction) and neurobehavioural (such as disordered consciousness, delirium, agitation, seizures, depression, cognitive impairment, insomnia and sleep disturbances, chronic pain, fatigue) [1–17]. These sequelae, which impact negatively on patients morbidity and outcomes and interfere with the rehabilitation process [18], are often targeted with pharmacologic intervention [19–21].

Previous studies assessing the real-life prescribing practice of rehabilitation centres for ABI, in general, were focused on specific medications or clinical conditions, such as agitation [22], depression [20] and venous thromboembolism prophylaxis [23]. Other studies assessed the prescribing preferences of physiatrists for the treatment of neurobehavioural sequelae of traumatic brain injuries [24] or the prescribing practices of psychotropic medications in rehabilitation centres [25]. Recently, a study assessed the pattern of psychotropic medication administration during acute inpatient rehabilitation for traumatic brain injury [26].

Polypharmacy is common in patients with ABI undergoing post-acute rehabilitation [27,28]. Polypharmacy increases the potential for drug–drug and drug–disease interactions and, thus, the risk of adverse events or negative clinical consequences for patients with ABI during rehabilitation [29,30]. The extent of polypharmacy has been assessed in vulnerable patient populations, such as adults with multi-morbidity [31], the elderly [32] and nursing homes residents [33]. Few data are available on the prevalence of polypharmacy in patients with ABI during post-acute rehabilitation.

This study performed a survey in Italian tertiary centres for post-acute rehabilitation aimed to (a) describe the pattern of medications use in inpatients with ABI during post-acute rehabilitation and (b) assess the prevalence of polypharmacy and its association to patients characteristics.

Materials and methods

Study design

This study is a 1-day cross-sectional survey conducted in tertiary centres for post-acute Brain Injury Rehabilitation in Italy.

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Eligible centres provide rehabilitation to inpatients in the post-acute stage (≥ 4 weeks after injury) after a severe ABI following either a traumatic or non-traumatic cause. The centres were identified through the roster of the Italian Society for Physical and Rehabilitation Medicine (Società Italiana di Medicina Fisica e Riabilitazione, SIMFER). All the patients with ABI, following a traumatic or non-traumatic cause, undergoing post-acute rehabilitation and hospitalized in the participating centres at the day of the survey, were included.

Data collection

An invitation e-mail was sent to the medical director of each identified centre. A structured questionnaire and a guide with definitions and instructions were sent to participating centres. A designated physician in each centre completed the questionnaire, which encompassed (a) a centre-level part and (b) a patient-level part for each enrolled patient.

For each enrolled patient, the centre designated physician completed one patient-level questionnaire, which collected demographic and clinical characteristics, such as gender and age, the Rancho Level of Cognitive Functioning Scale (RLAS) score at admission [34] and time since the occurrence of ABI. For each patient, a copy of the anonymized medication chart for one single day (the day before the survey) was obtained and all medications prescribed and administered were abstracted.

The centre-level part of the study has been described in detail elsewhere [25]. The survey was conducted on 15 September 2012. Centres with a high number of inpatients could collect data over two or more consecutive days.

A form was sent to non-participating centres to collect information on reasons for not partaking in the survey and on facility characteristics.

Questionnaire delivery, quality controls and data input

Completed questionnaires were delivered to the study coordination centre by mail or e-mail. Upon delivery, compiled questionnaires were inspected for the completeness and consistency of the recorded information. Any omission, error or inconsistent data was checked through immediate contact with the compiling physician and corrected as appropriate. The name of medication and dosage were extracted from medication charts. The medications were then classified using the Anatomical Therapeutic Chemical (ATC) classification system codes, a standard tool for drug utilization research [35,36]. The ATC system classifies the active substances according to five hierarchical levels: a main anatomical group, two therapeutic sub-groups, a chemical-therapeutic sub-group and a chemical substance sub-group. It, therefore, simultaneously takes into account the therapeutic, pharmacological and chemical properties. The medications are divided into 14 main groups (1st level, anatomical main group), with therapeutic sub-groups (2nd level, therapeutic sub-group), pharmacological sub-groups (3rd level, pharmacological sub-group), chemical sub-groups (4th level, chemical sub-group) and the 5th level is the chemical substance (5th level, chemical substance). The data were recorded into an electronic database using an entry form created specifically for this study. For the input of selected variables, a range of allowable values was established. Further quality control of the data included the identification of missing and out-of-range values, tests for logical data relationships and generation of output for review. Any inconsistency was investigated by reviewing the recorded data, re-examining the questionnaire or through contact with the compiling physician and corrected as appropriate.

Statistical analysis

Descriptive statistics were calculated to characterize the patients. Continuous variables were categorized using as cut-off values the quartiles for the time (weeks) since the occurrence of ABI and 5-years intervals for age. The number of medications taken was calculated as the sum of medication listed in the chart. Polypharmacy was defined as the concurrent prescription of six or more medications. For each therapeutic class or active substance, the prevalence of use was calculated as the ratio between the number of patients taking it and the total number of enrolled patients.

The Odds Ratio (OR), with 95% confidence interval (95% CI), of polypharmacy was calculated by means of unconditional logistic regression uni- and multi-variate. Before building the multivariate model, terms for age, class, sex, RLAS score, number of weeks since ABI, geographical location of the centre and number of beds of the centre were evaluated by univariate logistic regression. The final model retained the variables that explained the variability or modified the regression coefficient estimators for other covariates. Sensitivity analyses were performed, defining polypharmacy as the daily concurrent use of: (a) ≥ 5 medications; (b) ≥ 7 medications. Statistical analysis was performed using SAS© statistical package 9.3 (SAS Institute Inc., Cary, NC).

Ethics committee review

The study protocol was reviewed by the Ethics Committee at the University Hospital of Udine and at participating Centres.

Results

A total of 40 centres were identified and 35 (87.5%) agreed to participate, 31 (77.5%) provided patient-level data for all inpatients at the day of the survey. The study included 484 patients (return rate = 100%), 55.6% aged 35–64 years and 63.4% men (Table I). About 29% of patients had RLAS score 2, 5% had RLAS score 1 and 8% score had RLAS score 7–8.

The most used classes of medications were medications for peptic ulcer and gastro-oesophageal disease (85.5%, $n = 414$), mostly PPIs (79.5%, $n = 385$) and antithrombotic agents (78.5%, $n = 380$), mostly heparins (70.9%, $n = 343$) (Table II). Beta-blocking agents were used by 39.5% ($n = 191$) of patients. Diuretics were used by 27.5% of patients, agents acting on Renin Angiotensin System by 25.4%, calcium channel blockers by 19.4% and anti-hypertensives by 14.9%.

Sixty patients (12.45%) were administered clonidine, a centrally acting antiadrenergic anti-hypertensive. Eleven per cent of patients ($n = 56$) were administered thyroid therapy, mostly levothyroxine (10.6%; $n = 51$). About 20% of patients received

Table I. Number and distribution of patients with ABI according to selected characteristics.

	n (*n* = 484)	%
Gender		
Women	177	36.6
Men	307	63.4
Age (years)		
median (25°–75° percentile)	52.0 (37.5–64.0)	
< 25	54	11.2
25–34	53	10.9
35–44	65	13.4
45–54	103	21.3
55–64	101	20.9
65–74	67	13.8
≥ 75	41	8.5
RLAS score[a]		
1. No response	23	4.8
2. Generalized response	140	28.9
3. Localized	92	19.0
4. Confused–agitated	52	10.7
5. Confused, inappropriate, not agitated	90	18.6
6. Confused–appropriate	48	9.9
7. Automatic/purposeful – 8. Appropriate	39	8.1
Time since ABI (weeks)		
median (25°–75° percentile)	18.5 (11.0–32.0)	
≤ 11	134	27.7
11–18.5	108	22.3
18.5–32	122	25.2
> 32	120	24.8

[a] Rancho Level of Cognitive Functioning Scale (RLAS) score (http://www.rancho.org/Research_RanchoLevels.aspx, last accessed on 1 July 2012 [34]).

muscle relaxants, mostly centrally acting agents. Antibacterials were used by 25.2% of patients. About 4.3% used systemic antimycotics, 1.0% (*n* = 5) antiprotozoals, 0.6% (*n* = 3) antimycobacterials and 0.4% (*n* = 2) systemic antivirals.

About one quarter of patients received medications for constipation (26.7%) and 18.0% medications for functional gastrointestinal disorders, mostly propulsives (15.7%). Cardiac therapy was used by 11.0% of patients, lipid modifying agents by 8.1% and vasoprotectives by four patients. About half of the patients received anti-epileptics (51.7%, *n* = 250). Levetiracetam was the most used anti-epileptic agent (51.2%, *n* = 128), followed by valproate (14.8%, *n* = 37) and phenobarbital (14.4%, *n* = 36) (Table III).

About one-third of patients received antidepressants and SSRIs were the most prescribed class (by 104 out of 155 users). Sertraline was the most used single agent (9.3%, *n* = 45). Less than one third of patients used analgesics (27.3%, *n* = 132). About 14.5% (*n* = 70) received anti-psychotics. A total of 55 (78.6%) patients received atypical agents, while only 24 (34.3%) received typical ones. Quetiapine was the most used anti-psychotic (*n* = 39, 8.1%) followed by promazine (*n* = 17, 3.5%).

Analgesics were used by 148 (30.6%) patients. Most used analgesics were non-opioid agents (132 patients; 27.3%), while opioid agents were used by 28 (5.8%) patients. Psychostimulants were used by 17 (3.5%) patients; the most commonly used psychostimulant was piracetam (*n* = 11; 2.3%), followed by acetylcarnitine (*n* = 5; 1.0%) and methylphenidate (*n* = 3; 0.6%).

Table II. Number of users by therapeutic class.

		Users (*n* = 483)[a]	
Therapeutic class (ATC code)	Active substance	*n*	%[b]
Medications for peptic ulcer (A02B)			
proton pump inhibitors	—	385	79.7
histamine-2 receptor antagonists	—	28	5.8
other medication for peptic ulcer	—	6	1.2
total users		414	85.7
Anti-thrombotic agents (B01A)			
heparin	—	343	71.0
platelet aggregation inhibitors[c]	—	50	10.4
other antithrombotic agents	—	13	2.7
vitamin k antagonists	—	12	2.5
total users		380	78.7
Beta blockers (C07A)	atenolol	33	6.8
	bisoprolol	56	11.6
	carvedilol	13	2.7
	metoprolol	33	6.8
	nebivolol	6	1.2
	propranolol	49	10.1
	sotalol	3	0.6
total users		191	39.5
Diuretics (C03)			
high-ceiling diuretics		114	23.6
potassium-sparing agents		28	5.8
low-ceiling diuretics		9	1.9
diuretics + potassium-sparing agents		6	1.2
total users		133	27.5
Medications for constipation (A06A)		129	26.7
Agents acting on RAS[d] (C09)			
ace inhibitors		99	20.5
angiotensin II antagonists		26	5.4
total users		123	25.5
Antibacterials for systemic use (J01)		122	25.3
Muscle relaxants (M03B)			
muscle relaxants, centrally acting		97	20.1
muscle relaxants, directly acting		6	1.2
total users		103	21.3
Systemic hormones[e] (H)			
thyroid therapy		56	11.6
	levothyroxine	51	10.6
	liothyronine	2	0.4
	hiamazole	6	1.2
corticosteroids, systemic		46	9.5
pituitary and hypothalamic hormones		3	0.6
	desmopressin	3	0.6
total users		96	19.9
Calcium channel blockers (C08C)			
selective, mainly vascular effects		90	18.6
selective, direct cardiac effects		4	0.8
total users		94	19.5

(*Continued*)

Table II. (Continued).

Therapeutic class (ATC code)	Active substance	Users (n = 483)[a] n	$\%^b$
Medications for functional gastrointestinal disorders (A03F)			
propulsives		76	15.7
other		16	3.3
total users		87	18.0
Anti-hypertensives (C02)			
anti-adrenergics, centrally acting (clonidine)		60	12.4
anti-adrenergics, peripherally acting (doxazosin)		22	4.6
total users		72	14.9
Medications for OAD[f] (R03B)		68	14.1
Vitamins (A11)		67	13.9
Mineral supplements (A12)		65	13.5
Anti-anaemic preparations (B03)			
iron preparations		32	6.6
vitamin b12 and folic acid		30	6.2
other anti-anaemic preparations		17	3.5
total users		64	13.3
Intestinal anti-inflammatory agents (A07)		59	12.2
Cardiac therapy (C01)			
anti-arrhythmics, class I and III		20	4.1
vasodilators		20	4.1
cardiac glycosides		10	2.1
cardiac stimulants excl. cardiac glycosides		4	0.8
other cardiac preparations		4	0.8
total users		53	11.0
Drugs used in diabetes (A10)			
insulins and analogues		44	9.1
blood glucose lowering drugs, excl. insulins		9	1.9
total users		50	10.4
Ophthalmologicals (S01)		40	8.3
Lipid modifying agents (C01)		39	8.1
Cough and cold preparations (R05)		39	8.1
NSAIDs (M01A)		38	7.9
Blood substitutes and perfusion solutions (B05)		35	7.3
Dermatologicals (D)		32	6.6
Bile and liver therapy (A05)		29	6.0
Genito urinary system and sex hormones (G)		25	5.2
Anti-mycotics for systemic use (J02)		21	4.4
Drugs for treatment of bone diseases (M05)		10	2.1
Anti-gout preparations (M04)		7	1.5
Anti-emetics and anti-nauseant (A04)		6	1.2
Anti-histamines for systemic use (R06)		5	1.0
Antiprotozoals (P01)		5	1.0
Vasoprotectives (C05)		4	0.8
Immunosuppressants (L04)		4	0.8
Other haematological agents (B06)		3	0.6
Anti-mycobacterials (J04)		3	0.6
Anti-virals for systemic use (J05)		2	0.4
Endocrine therapy (L02)		2	0.4
Nasal decongestants for topical use (R01A)		2	0.4
Anti-neoplastic agents (L01)		1	0.2
Other otologicals (S02D)		1	0.2

[a] One subject had missing information for the medication use.
[b] The number of total users per therapeutic group may not be the sum of users of active substances in that group, as patients may use more than one active substance per therapeutic group.
[c] Excludes heparins.
[d] RAS: renin-angiotensin system.
[e] Excludes sex hormones and insulin.
[f] OAD: obstructive airways diseases.

Figure 1 displays the prevalence of use of selected medications by RLAS score and time since ABI. A decreasing prevalence of use with increasing RLAS score was found for medications for peptic ulcer, from 82.6% in patients with RLAS score 1 to 69.2% in those with scores 7 and 8 and, for antithrombotic agents, from 73.9% to 61.5% Beta blocking agents, diuretics and medications for constipation showed a similar decreasing pattern. Increasing time since ABI did not affect the prevalence of use of medications for peptic ulcer (the number of users and the prevalence by RLAS class and by time since ABI is displayed in Supplemental Electronic Table e1).

Figure 2 displays the prevalence of use of selected psychotropic medications by RLAS score and time since ABI (the number of users and the prevalence by RLAS class is displayed in Supplemental Electronic Table e2). The use of anti-epileptics decreased by 15.5% from RLAS score 1 to 7–8, conversely antidepressants increased by 36.5%. Anti-Parkinson agents and analgesics showed a decreasing trend with increasing RLAS score. Anti-psychotics and, less markedly, anxiolytics had a peak in patients with RLAS score 4, characterized by a confused–agitated state. In these patients the prevalence of use was 37.3% and 21.6%, respectively. Patients who had ABI more than 32 weeks prior to the survey had the highest prevalence of use of anti-epileptic and anti-Parkinson agents, conversely those with the shortest interval, since ABI had the highest prevalence of use of anti-psychotic agents.

More than half of the patients (51.7%) used 6–10 medications and 28.3% used 11 or more (Table IV). The great majority of patients (n = 406, 83.9%) used at least one psychotropic medication, about two-thirds of them used two or more. Seventy-nine patients, 31.6% of anti-epileptics users, received two agents of this class. Eighteen (11.6% of users) had two anti-depressants and 11 (15.7% of users) had two

Table III. Psychotropic medications, number of users by therapeutic class.

Therapeutic class (ATC code)	Active substance	Users (n = 483)[a] n	$\%^b$
Anti-epileptics (N03A)			
	levetiracetam	128	26.5
	valproate	37	7.7
	phenobarbital	36	7.5
	clonazepam	31	6.4
	gabapentin	27	5.6
	oxcarbazepine	23	4.8
	pregabalin	22	4.6
	carbamazepine	19	3.9
	phenytoine	17	3.5
	lacosamide	4	0.8
	valpromide	3	0.6
	lamotrigine	3	0.6
	zonisamide	2	0.4
total users		250	51.8
Anti-depressants (N06A)			
SSRIs	sertraline	45	9.3
	citalopram	26	5.4
	escitalopram	21	4.3
	paroxetine	9	1.9
	fluoxetine	2	0.4
	fluvoxamine	1	0.2
sub-total SSRIs		104	21.5
other anti-depressants	trazodone cloridrato	24	5.0
	agomelatine	12	2.5
	duloxetine	9	1.9
	venlafaxine	8	1.7
	mirtazapine	5	1.0
	adenometionine	2	0.4
sub-total other anti-depressants		59	12.2
MRIs	amitriptyline	10	2.1
sub-total MRIs		10	2.1
total users		155	32.1
Analgesics (N02)			
analgesics opioids (N02A)		28	5.8
other analgesics (N02B)			
anilides		123	25.5
salicylic acid and derivatives		11	2.3
subtotal others		132	27.3
total users		148	30.6
Anti-Parkinson agents (N04B)			
dopaminergic agents	amantadine	39	8.1
	levodopa+benserazide	28	5.8
	levodopa+carbidopa	13	2.7
	pramipexole	4	0.8
	selegiline	3	0.6
	ropinirole	2	0.4
	levodopa	1	0.2
	melevodopa +carbidopa	1	0.2
sub-total dopaminergic agents		80	16.6
anti-cholinergic agents	biperiden	4	0.8
	trihexyphenidyl	4	0.8
sub-total anti-cholinergic agents		8	1.7
total users		86	17.8
anxiolytics (N05B)			
	lorazepam	24	5.0
	diazepam	21	4.3
	alprazolam	19	3.9
	delorazepam	6	1.2
	bromazepam	4	0.8
	clobazam	1	0.2
total users		71	14.7
Anti-psychotics (N05A)			
atypical	quetiapine	39	8.1
	olanzapine	12	2.5
	clotiapine	2	0.4
	amisulpride	2	0.4
	risperidon	1	0.2
	aripriprazole	1	0.2
typical	promazine	17	3.5
	chlorpromazine	3	0.6
	levomepromazine	1	0.2
	haloperidol	2	0.4
	periciazine	1	0.2
	tiapride	1	0.2
total users		70	14.5
Hypnotics and sedatives (N05C)		35	7.3
Psychostimulants, agents for ADHD, nootropics (N06B)			
	methylphenidate	3	0.6
	acetylcarnitine	5	1.0
	piracetam	11	2.3
Total users		17	3.5
Other nervous system medications (N07)		6	1.2
Anti-dementia medications (N06D)		4	0.8
Anaesthetics (N01)		1	0.2

[a] One subject had missing information for the medication use.
[b] The number of total users per therapeutic group may not be the sum of users of active substances in that group, as patients may use more than one active substance per therapeutic group.

anti-psychotics. Of patients taking SSRIs, 11 (10.6%) received concurrently an antipsycotic agent. Of patients taking quetiapine, 10 (25.6%) received concurrently an antidepressant (Supplemental Electronic Tables e5 and e6).

The Odds Ratio (OR), with 95% confidence interval (95% CI), of polypharmacy is displayed in Table V. When the model adjusted simultaneously for age, gender, RLAS score and time since ABI, the OR approximately doubled in patients aged 45–54 and 55–64 years and increased by 70% in those 65 years old or older compared to patients younger than 45 years. RLAS score had a strong inverse association with the OR: compared to patients with score 7–8, those with 1 or 2 had OR = 4.3 (95% CI = 1.9–9.8) and with 3 had OR= 3.0 (95% CI = 1.3–7.3).

Discussion

This survey found that the large majority of patients (92%) received at least one medication and that concurrent use of several medications is common, with about half of the patients receiving 6–10 different medications at the same time.

Psychotropic medications were largely used, 84.1% of patients received at least one medication of this group and 66.9% two or more. The most used psychotropic medications were anti-epileptic agents (administered to 51.8% of patients),

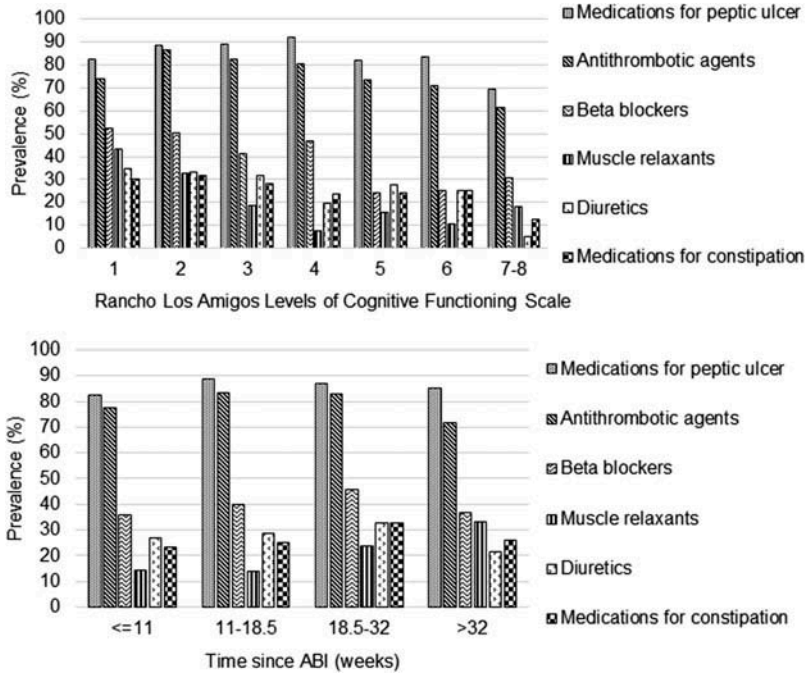

Figure 1. Prevalence of use of selected medications by RLAS score and by time since ABI.

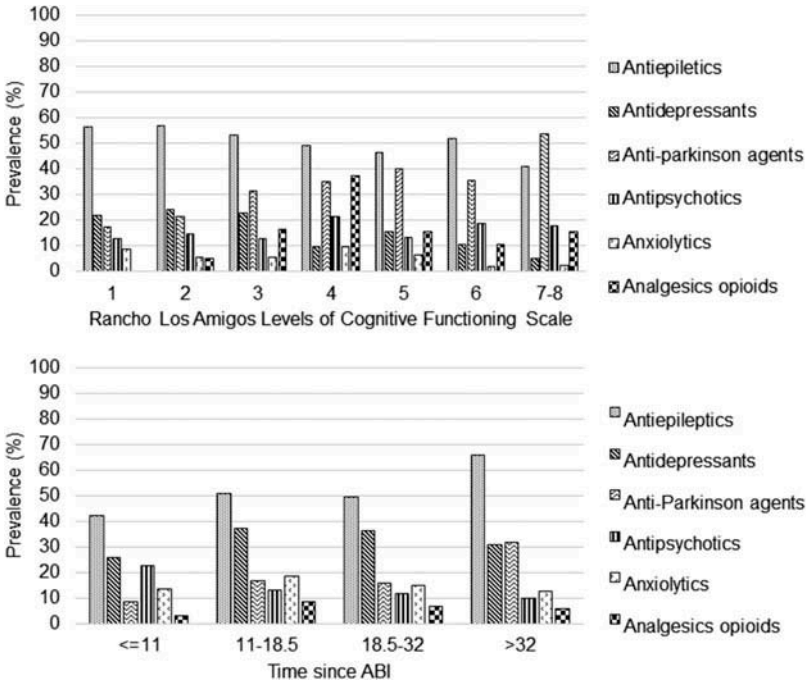

Figure 2. Prevalence of use of selected psychotropic medications by RLAS score and by time since ABI.

followed by anti-depressants (32.1%), analgesics (30.6%), anti-Parkinson medications (17.8%), anxiolytics (14.7%), anti-psychotics (14.5%), hypnotics and sedatives (7.3%) and psychostimulants (3.5%). Other psychotropic medications, such as anti-dementia agents and anaesthetics, were used by a small number of patients (< 1.5%).

A study conducted in the US and Canada on inpatients during acute rehabilitation for traumatic brain injury found that psychotropic medications were commonly used, 95% of patients received at least one at any time during rehabilitation and 31.8% six or more. The most used agents were narcotics analgesics (by 72% of patients), antidepressants (by two thirds of patients), anticonvulsants (47%), anxiolytics (33%), hypnotics (30%), stimulants (28%), anti-Parkinson medications (25%), anti-psychotics (25%) and miscellaneous (18%) [26]. Intra-Countries differences in prescribing practices and differences in the rehabilitation stage at which patients were assessed may explain some different findings. In this study, inpatients were in post-acute rehabilitation and were assessed at a median time

Table IV. Number and distribution of patients by number of medications and of psychotropic medications used.

	n (*n* = 483)[a]	%
Number of medications		
0	4	0.8
1–5	92	19.0
6–10	250	51.8
11–15	102	21.1
≥ 16	35	7.2
Number of psychotropic medications[b]		
1	160	33.1
2	116	24.0
3	74	15.3
4	29	6.0
5 and more	27	5.6
At least one	406	84.1

[a] One subject had missing information for the medication use.
[b] These include: N01, Anesthetics; N02, Analgesics; N03, Anti-epileptics; N04, Anti-Parkinson medications; N05, Psycholeptics; N06, Psychoanaleptics; N07, Other Nervous System medications.

since the acute event of 18.5 weeks (11.0–32.0). In the study by Hammond et al. [26], inpatients were in acute rehabilitation, with mean time from injury to rehabilitation admission of 29 days (SD = 34). In particular, analgesic opioids may be less used in this study, by 5.8% of patients, because a lower percentage of patients may be affected by severe pain. In this study the most used analgesics were non-opioid agents, used by 27.3% of patients.

Anti-epileptics were administered to about half of the patients, a finding similar to other studies [26]. The most used agent was levetiracetam.

The main indication was not reported in medication charts and, thus, this study lacks information on the main reason for medication prescribing. Therefore, this study could not disentangle anti-epileptic prescriptions intended as seizure prophylaxis from those for the treatment of post-injury epilepsy. Of note, 31.6% of anti-epileptic medications users and 16.3% of the total number of enrolled patients used concurrently two or more anti-epileptic medications. The concurrent use of more than one antiepileptic is explained mainly by cases in which monotherapy has not been effective in controlling the seizures or the type of anti-epileptic is being changed. Use of these medications off-label for pain management may also partly explain this finding.

Among anti-depressant agents, administered to one third of patients, SSRIs were the most used, in agreement with a previous survey in the Netherlands and the UK [20]. The evidence supports the efficacy of serotoninergic anti-depressants and sertraline in treating depression following both TBI [19,37,38] and stroke [39]. Supporting evidence of the use of SSRIs for chronic agitation and aggression was found [40]. Antidepressants may also reduce the frequency of emotionalism after stroke [41].

About 15% of patients were prescribed anti-psychotic agents. Quetiapine was the most used medication, consistently with other studies [26]. Patients with RLAS 4 had the highest frequency of use of anti-psychotics, while their use decreased with increasing time since ABI. RLAS score 4 is characterized by agitated and aggressive behaviour. Use of anti-psychotics in brain injury rehabilitation is controversial. Anti-psychotics are involved in clinically significant drug–drug interactions with several other medications, including anti-depressants and anti-epileptics [42], agents that from

Table V. Odds ratio (OR), with 95% confidence interval (95% CI), of polypharmacy.

	n of medications				Univariate		Multivariate[a]	
	< 6		6 or more					
	n	%	*n*	%	OR	95% CI	OR	95% CI
Gender								
Men[b]	66	68.7	240	62.0	1.0	—	1.0	—
Women	30	31.3	147	38.0	1.4	0.8–2.2	1.3	0.8–2.1
Age category (years)								
≤ 45[b]	45	46.9	126	32.6	1.0	—	1.0	—
45–54	18	18.7	85	22.0	1.7	0.9–3.1	1.9	1.0–3.5
55–64	15	15.6	86	22.2	2.1	1.1–3.9	2.1	1.1–4.1
≥ 65	18	18.7	90	23.3	1.8	1.0–3.3	1.7	0.9–3.3
RLAS score								
1 and 2	21	21.9	142	36.7	3.8	1.7–8.4	4.3	1.9–9.8
3	15	15.6	77	19.9	2.9	1.2–6.8	3.0	1.3–7.3
4	15	15.6	36	9.3	1.3	0.6–3.3	1.5	0.6–3.8
5	20	20.8	70	18.1	2.0	0.9–4.5	2.1	0.9–4.8
6	11	11.5	37	9.6	1.9	0.7–4.8	2.1	0.8–5.5
7 and 8[b]	14	14.6	25	6.5	1.0	—	1.0	—
Time since ABI (weeks)								
≤ 11[b]	34	35.4	99	25.6	1.0	—	1.0	—
11–18.5	15	15.6	93	24.0	2.1	1.1–4.2	2.0	1.0–3.9
18.5–32	21	21.9	101	26.1	1.7	0.9–3.0	1.5	0.8–2.8
> 32	26	27.1	94	24.3	1.2	0.7–2.2	1.1	0.6–2.1

[a] The model included terms for age, gender, RLAS score, time since ABI (weeks).
[b] Reference category.

these results are frequently concurrently administered with anti-psychotics.

In clinical practice, anti-psychotics and benzodiazepines are commonly used as first line treatment for acute agitation and aggressive behaviour [43]. Atypical anti-psychotics are preferred to typical agents for their lower cognitive and motor adverse effects [19,43]. Beta-blocking agents, used by ~40% of patients in this study, proved effective for treating aggression in persons with TBI [40]. However, the high doses needed to achieve a therapeutic effect may limit their clinical utility [19,43]. Methylphenidate, used by three patients, had also strong evidence of efficacy in the management of agitation [40,44,45].

A survey found that ~ 50% of patients receive medications for agitation [22]. Lacking information on the indication, this study could not assess the prevalence of patients treated for agitation. It is likely, however, that some prescriptions of agents such as SSRIs and anti-psychotics were intended to treat agitation.

In this survey, the majority of patients receive multiple medications per day, about half with 6–10 and about a third with 11 or more. About half of the patients received more than one psychotropic medication. This percentage was 71% in Veterans with mild TBI and Post Traumatic Stress Disorder [46].

Anti-ulcer medications, mostly PPIs, and anti-thrombotic agents, mostly heparins, were the most used medications in patients receiving post-acute rehabilitation in Italian referral centres for Brain Injury Rehabilitation. Pharmacological protection of gastrointestinal mucosae and venous thromboembolism prophylaxis seem, thus, to be common practice. Venous thromboembolism is a major cause of mortality and morbidity in patients with brain injury. There is no consensus regarding appropriate screening, prophylaxis or treatment of this condition during brain injury rehabilitation. In a recent study, prophylactic anti-coagulation during rehabilitation was safe, but did not conclusively reduce venous thromboembolism [21]. The prevalence of patients receiving anti-coagulation in this survey is higher than in other studies. About 50% of the patients with TBI had prophylactic anti-coagulation during rehabilitation [21]. Anti-coagulation prophylaxis was performed by 56% of rehabilitation centres for traumatic brain injury [23].

Polypharmacy, defined as the use of six or more medications per day, was directly associated with age and inversely with RLAS score.

Polypharmacy may be entirely appropriate and driven by multi-morbidity [47]. It has been argued that polypharmacy may not be always hazardous and represents inappropriate use of medications, depending on the clinical conditions for which those drugs are being prescribed [48]. In patients with multi-morbidity, as often are those with ABI, the use of multiple medications may be appropriate. However, accurate prescribing and surveillance of medication safety is highly advisable, due to the increased potential for drug–drug and drug–disease interactions and for adverse events. Moreover, the concurrent use of multiple medication in patients with traumatic brain injury has been associated with falls [30].

To define polypharmacy, this study used as a cut-off six medications taken concurrently. In published studies, the number of medications taken daily that define polypharmacy varies, from four or more [32], five or more [49] or six or more [50] medications. The clinically relevant cut-off may be relative to patients characteristics, such as age, morbidity and multi-morbidity.

Strengths

Information on the medications administered to patients was abstracted from medication charts. All medications administered to each patient were recorded, without any exclusion. This method of data collection allowed one to assess all the medications used in real-life clinical practice of acquired brain injury post-acute inpatient rehabilitation.

Limitations

Information on the cause of ABI, if traumatic or not traumatic, and on comorbidities was not available on the sources used for this study. Thus, one cannot assess if the pattern of medication use differs according to the cause of ABI. Moreover, one cannot disentangle medications taken for sequelae of ABI from those for comorbidities that may pre-exist.

In interpreting the results of this study, it has to be considered that medications are often used for purposes that are not clear from the drug classification and main indication. Some anti-depressants are used off-label to treat insomnia and pain, some anti-epileptics are used for pain management [25]. The indication was not reported on the medication chart and this information could not be abstracted. Thus, this study cannot report the frequency of use by indication and assess how frequently medications are prescribed to patients for indications other than those approved. Moreover, one cannot disentangle the frequency of use of anti-psychotics or anti-depressants for agitation and aggression, nor distinguish between the use of anti-convulsants for the treatment of seizures and for their prevention or report the frequency of use of medication for sleep disturbances.

The study is a 1-day survey. For each patient, there is information on the medications taken in one single day. Therefore, this study does not have information on the duration of treatment.

Conclusions

This survey showed that polypharmacy is common in patients undergoing post-acute rehabilitation following ABI. Patients often have multiple morbidities and polypharmacy may be appropriate. Future studies in patients with ABI should evaluate the role of comorbidities in polypharmacy.

Nevertheless, polypharmacy raises concern on drug–drug interactions. Careful and systematic monitoring of the effectiveness and safety of medications, in particular when used concurrently, is highly recommended in patients with ABI.

Declaration of Interest

The authors report no conflicts of interest. This study was funded by the non-profit organization Italian Association for

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