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GLOBAL DNA HYPOMETHYLATION AND GENE-SPECIFIC HYPERMETHYLATION AS POSSIBLE DIAGNOSTIC AND PROGNOSTIC BIOMARKERS FOR PROSTATE CANCER

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1. INTRODUCTION

1.1 EPIGENETICS

The genome contains information in two forms, genetic and epigenetic. The genetic information is stored in each cell in the form of DNA as genetic code, that provides the blueprint for the manufacture of all the proteins necessary to create a living thing, while the epigenetic information provides instructions on how, where, and when the genetic information should be used. Epigenetic mechanisms allow genetically identical cells to achieve diverse stable phenotypes by controlling the transcriptional availability of various parts of the genome through differential chromatin marking and packaging: ensuring that genes are turned on at the proper time is as important as ensuring that they are turned off when not needed [Shen and Laird, 2013].

The term epigenetics (literally 'over' or 'upon' genetics) was originally defined, in the early 1940s, to describe the events that could not be wholly explained by traditional genetics. Conrad Waddington (1905-1975) defined epigenetics as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being", and this concept was used to explain why genetic variations sometimes did not lead to phenotypic variations and how genes might interact with their environment to yield a phenotype [Waddington, 2012]. Waddington's definition initially referred to the role of epigenetics in embryonic development; however, the definition of epigenetics has evolved over time as it is implicated in a wide variety of biological processes. The current definition of epigenetics is "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence"; most of these heritable changes are established during differentiation and are stably maintained through multiple cycles of cell division. This heritability of gene expression patterns is mediated by epigenetic modifications, including methylation of the DNA bases, post-translational modifications of histone proteins as well as the positioning of nucleosomes along the DNA and the micro-RNAs. The complement of these modifications, collectively referred to as the epigenome, provides a mechanism for cellular diversity by regulating what genetic information can be accessed by the transcriptional machinery [Sharma et al., 2010].

1.2 DNA METHYLATION

The major form of epigenetic information in mammalian cells is DNA methylation. It consists in the reversible covalent addition of a methyl group ($-CH_3$) predominantly at the five position

of the cytosine pyrimidine ring, thereby creating 5-methylcytosine (5mC). 5mC accounts for the 3% of total cytosine bases in the DNA. In mammals, this reaction almost exclusively occurs in cytosines located 5 prime to the guanine base, commonly annotated as CpG dinucleotides, where the intervening 'p' represents the phosphodiester bond linking cytosine- and guanine-containing nucleotides [Rottach et al., 2009]. CG dinucleotides are infrequent in the genome, except at short DNA sequences termed "CpG islands", that often colocalize with gene promoters and regulatory regions. For this reason, DNA methylation has been functionally linked mainly to the control of gene expression [Li and Zhang; 2014].

1.3 ESTABLISHMENT OF THE DNA METHYLATION PATTERN

In the life cycle of an individual, the genome undergoes extensive reprogramming of methylation pattern during early development. In mammals, following fertilization, the levels of cytosine methylation globally decrease from the first cleavage stages up to the early blastocyst, before being reacquired during and after implantation. Immunofluorescence studies have shown that this global demethylation occurs asymmetrically on both parental genomes: the paternal DNA rapidly looses 5mC signal in the zygote before DNA replication, probably through a process of active demethylation, whereas the maternal DNA is thought to lose the methylation pattern over several cell divisions by a replication-dependent passive demethylation [Saitou et al., 2012]. This was confirmed by the observation that preimplantation blastocysts have a globally demethylated genome and one of the possible reasons is that it may facilitate the activation of the pluripotency program in the embryo. Exceptions are imprinting control regions (ICRs), that faithfully maintain allele-specific epigenetic pattern after fertilization and direct the monoallelic expression of clusters of imprinted genes in embryonic development. All ICRs identified so far are also known as differentially DNA methylated regions (DMRs) on the two parental chromosomes, mainly the maternal, thus indicating that the oocyte genome is more prone to transmit functional gene methylation states to the embryo than the paternal genome [Radford et al., 2011].

In postimplantation embryos, new patterns of DNA methylation are re-established. After implantation, the cells of the epiblast becomes the source of all embryonic lineages, including the primordial germ cells (PGCs). PGCs undergo a second wave of epigenetic reprogramming that is completed by embryonic day 13.5 and is characterized by exchange of histone variants, loss of histone modifications and global erasure of DNA methylation at all gene loci during their proliferation and migration. These kinetics of demethylation appear to be complex and

might reflect the use of a combination of passive and active processes to achieve full demethylation in PGCs. When epigenetic reprogramming is completed, PGCs enter meiotic prophase in females and mitotic arrest in males [Auclair et al., 2012].

1.4 DISTRIBUTION OF DNA METHYLATION SITES

The frequency of CpG dinucleotides in humans is 1%, only the 21% of the expected number based on the GC content in the human genome; this underrepresentation is due to the inherent mutability of methylated cytosine. The product of cytosine deamination, uracil, is readily recognized as aberrant by the base excision repair machinery and corrected: the enzymes TDG (thymine DNA glycosylase) and MBD4 (methyl-CpG domain protein 4) have been reported to selectively remove thymine from a T:G mismatch in the context of CpG dinucleotides. In contrast, the deamination product of methylated cytosine is thymine, leading to a potential C to T transition in the next round of replication. Consequently, methylated CpGs in the germ line are likely to be lost over time [Weber et al., 2007].

This low number of overall CpG dinucleotides is not uniformly distributed along the genome: as mentioned before, the human DNA is globally characterized by unusually dense clusters of CpG dinucleotides called “CpG islands” (CGIs). These regions are defined as usually of 200 bp-2 kb in length and characterized by higher density of G+C bases than the rest of the genome (at least 55%), a ratio observed/expected of CpG frequency of at least 0.6, and a consequent little CpG depletion [Illingworth et al., 2008]. There are about 29,000 CpG islands in the human genome, and the majority of gene promoters, roughly 60%, reside within CpG islands and are characterized by frequent absence of DNA methylation: in particular, the promoters for housekeeping genes are often embedded in CpG islands, as well as a proportion of tissue-specific genes, tumor suppressor and developmental regulator genes. In contrast, the 80% of CpG dinucleotides located outside of CpG islands of mammalians are heavily methylated and can be found in promoters on the inactive X chromosome, in one allele of imprinted genes and in gene bodies, as well as in and nonrepetitive intergenic sequences, in satellite DNA and within repetitive elements. This distribution pattern of methylated CpGs in human genome is not random: DNA methylation plays a crucial role in the regulation of gene expression, and, basically, its presence is associated with stable gene silencing [Meng et al., 2015].

1.5 IMPACT OF DNA METHYLATION ON GENE TRASCRIPTION

In general, gene expression and DNA methylation are inversely correlated. In the cell, DNA methylation exerts its biological function of transcription repressor in at least two ways.

Interference with Transcription Factor Binding. The presence of methyl groups bound to the cytosines can physically impede the binding of transcription factors to the gene promoter and, hence, directly interfere with gene activation. A number of transcription factors recognize GC-rich sequence motifs that can contain CpG sequences: several of these are unable to bind DNA when the CpG sequence is methylated [Li and Zhang, 2013]. A recent study revealed that DNA-binding factors can also shape DNA methylation patterns: whole genome bisulphite sequencing analysis of embryonic cells in mice revealed the existence of low-methylated regions (LMRs) at CpG-poor distal regulatory regions. LMRs are occupied by transcription factors and their binding is both necessary and sufficient to generate LMRs, indicating that transcription factors can vice-versa influence local DNA methylation [Stadler et al., 2011].

Recruitment of Methyl-CpG Binding Proteins and Repressor Complexes. The second mode of repression is opposite to the first as it involves proteins that are attracted to rather than repelled by methyl-CpG. These proteins, called methyl-CpG-binding domain proteins (MBDs) are characterized by a DNA-binding motifs able to specifically recognize and bind only methylated CpGs. In addition, the MBD proteins can in turns recruit co-repressor complexes such as histone deacetylases, histone lysine methyltransferase or other larger chromatin remodeling protein, leading to the formation of silenced states of chromatin that ensures a stable repression of gene transcription [Li and Zhang, 2013].

More recent researches have identified a new class of protein characterized by the CXXC domain, that can specifically recognize unmethylated CGs and can target other proteins or histone demethylases to unmethylated CpG islands. It has been suggested that this recruitment might help to maintain an unmethylated state similar to that seen with classic transcription factors, but evidences for this model are still missing [Shubeler, 2015].

1.6 FUNCTIONS IN NORMAL DEVELOPMENT

DNA methylation is essential for regulating several cellular processes during normal mammalian development, including the silencing of retrotransposon elements, the tissue-specific gene expression, the genomic imprinting, and the X chromosome inactivation.

Silencing of transposable elements. Mobile DNAs, also known as transposons or "jumping genes", are widespread in nature and comprise an estimated 45% of the human genome. These sequences consist in intragenomic repeated and endoparasitic elements dispersed throughout the genome and are divided in two different classes: DNA transposons are sequences which move by a "cut-and-paste" mechanism via an element-encoded transposase, while

retrotransposons are RNA transposons that use a “copy-and-paste” mode of moving in genomes through an RNA intermediate.

A substantial fraction of the human genome (> 30%), is derived directly or indirectly from LINE-1 retrotransposon activity. If expressed, these elements are potentially harmful as their replication and insertion can lead to chromosomal instability, translocations, gene disruption and DNA mutations. To prevent their movement around the genome these mobile elements need to be silenced completely and stably [Hancks and Kazazian, 2012]. The vast majority of these elements are inactivated by mutations acquired over time as the result of the deamination of 5mC, by rearrangements or heavily silenced by bulk hypermethylation. Despite the presence of more than 500,000 copies in the human genome, most of these elements are inactive, with only a subset, an estimated 80–100 elements, currently active. Full-length human L1 is ~6.0 kb, contains an internal promoter located in the 5’-untranslated region (UTR) and two non-overlapping open-reading frames (ORF1 and ORF2); both ORFs are required for L1 retrotransposition, since ORF1 encodes a 40 kDa protein that contains a coiled-coiled domain, a non-canonical RNA recognition motif domain and a basic C-terminal domain, while ORF2 encodes a 150 kDa protein that contains endonuclease and reverse transcriptase activity [Moore et al., 2013].

Apart from LINE-1, also SINE (short interspersed elements) elements have been discovered, of which the most abundant class is represented by the Alu insertional elements. Alu elements are dimeric sequences approximately 300 bp in length derived from the 7SL RNA gene. These sequences contain a bipartite RNA pol III promoter, a central poly A tract, a 3' poly A tail, numerous CpG islands and are bracketed by short direct repeats. An estimated 500,000-1,000,000 units are dispersed throughout the human haploid genome and their retroposition activity is determined by both internal and flanking regulatory elements as well as distant genes affecting transcription or transcript stability. As for LINE-1, Alu trasposable activity is turned-off by bulk methylation [Rodriguez et al., 2008].

Dosage compensation. In female mammalian development, X-chromosome inactivation (XCI) occurs during early embryogenesis to transcriptionally silence one of the pair of ~156-Mb X chromosomes, thereby achieving dosage compensation with males who have a single X chromosome. The X-inactivation process converts an X chromosome (X_i) from active euchromatin into transcriptionally silent and highly condensed heterochromatin through a series of events that include the coating of the X chromosome by *Xist* RNA, DNA methylation and histone modification. Several studies have shown that DNA methylation of the X_i plays a central role in the maintenance of its inactive state. While the active X (X_a) and X_i have very

similar global levels of methylation, CpG islands on the X_i have a tendency to be methylated, while those located on the X_a are unmethylated. In contrast, CpG dinucleotides of genes escaping from XCI often remain unmethylated on both the X_i and X_a [Cotton et al., 2015].

Genomic imprinting. Genomic imprinting is a process causing the mono-allelic expression of a specific subset of autosomic genes in a parental origin specific manner — *i.e.*, genes that are expressed either from the paternally inherited chromosome or from the maternally inherited chromosome (paternal allele and maternal allele henceforth) are imprinted. Today, over 100 imprinted genes have been identified, and are usually organized in clusters where they are controlled by a unique imprinted control region rich in CpGs, that are methylated in one of the two strands, thus leading to the transcriptional silencing of the methylated allele. The acquisition of methylation at ICRs occurs during the germ cell development through a *de novo* process with a small number of ICRs becoming methylated in intergenic regions of sperm DNA and the majority acquiring methylation at promoter sequences of oocyte genome. Several syndromes and complex deseases such as cancer are associated with deletions or aberrations in DNA methylation of ICRs [Adalsteinsson and Ferguson-Smith, 2014].

1.7 THE DNA METHYLATION MACHINERY

Epigenetic modifications concerning methylation are written, erased, read, and regulated by a set of specific proteins, that collectively form the DNA methylation machinery.

Writers of DNA methylation pattern. The reaction of addition of the methyl group to the cytosines is catalyzed by the enzymes belonging to the family of the DNA methyltransferases (DNMTs), that use the S-adenosyl-L-methionine (SAM, also known as AdoMet) as the methyl donor. The methyl group of AdoMet is bound to a sulfonium ion that thermodynamically destabilizes the molecule and makes the relatively inert methylthiol of the methionine part very reactive toward activated carbon atoms. This reaction involves the DNMT DNA binding domain, flipping the target cytosine out of the double helix, with the consequent formation of a transient covalent complex with the cytosine residue. The DNMT domain adds a cysteine thiolate to the 6-carbon of the substrate cytosine, followed by transfer of the methyl group to the 5-carbon [Daniel et al., 2011].

DNA methyltransferases are the family of enzymes responsible for methylation pattern acquisition during gametogenesis, embryogenesis, and somatic tissue development. To date, four active members of this family have been identified in mammals: DNMT1, DNMT3a, DNMT3b and DNMT3L. DNMT1 is often referred to as maintenance methyltransferase, because it is believed to be the primary enzyme responsible for the maintenance of methylation

during replication by copying the methylation pattern of the parent DNA strand onto the newly synthesized strand through its peculiar preference for hemimethylated templates. Consequently, it passes the epigenetic information over cell divisions. DNMT1 is particularly present at high concentrations in dividing cells, always located in replication foci; it also operates with its methylation co-factor UHRF1 in protein complexes that constitute an enzymatic platform and contains an N-terminal region that is associated with various chromatin-associated proteins, including the *de novo* methyltransferases, histone-modifying enzymes and MeCPs [Meng et al., 2015].

DNMT3a and DNMT3b are responsible for *de novo* DNA methylation, targeting cytosines of previously unmethylated CpG dinucleotides as well as working with DNMT1 to ensure propagation of methylation patterns during DNA replication. Particularly, these enzymes have an equal preference for hemimethylated and unmethylated DNA, essential for their roles in *de novo* methylation of the genome during development and for newly integrated retroviral sequences [Walton et al., 2011]. Following the first wave of genome-wide demethylation in the preimplantation embryo, DNMT3a and DNMT3b are highly expressed at implantation and re-establish a bimodal methylation pattern that effects more than 80% of the genome. Genetic and functional analyses carried on in mice indicate that Dnmt3a and Dnmt3b have non-overlapping functions during development: accordingly, Dnmt3a is necessary for maternal imprinting at differentially methylated regions, while Dnmt3b is required for methylation of pericentromeric repeats and CGIs on inactive X chromosome [Takeshima et al., 2006]. Of special interest is DNMT2 (also known as TRDMT1), which has reportedly only weak DNA methylation ability *in vitro* and appears to have the potential to methylate RNA instead of DNA. Finally, DNMT3L (DNMT3-like) has no catalytic activity itself: it stimulates the *de novo* methyltransferase activity of Dnmt3a or Dnmt3b through physical interaction by increasing their ability to bind to the methyl group donor, the *S*-adenosyl-*L*-methionine [Meng et al., 2015].

Strong and continual cooperation between different methyltransferase enzymes is needed for the maintenance of DNA methylation, especially in repeats and imprinted genes. Although this classical proposal based on a maintenance methyltransferase and on two *de novo* DNMTs has been supported for many years, more recent experimental observations that do not fit with this simple model have been accumulating. The observation that DNMT1 alone is incapable of perfect maintenance methylation and the localization of the DNMT3a and DNMT3b enzymes to specific chromatin regions containing methylated DNA has led to the proposal of a revised model. It predicts that Dnmt1 maintains the bulk of DNA methylation in dividing cells but it is

also able to perform error corrections, thus having a role in *de novo* methylation of genomic sequences. Dnmt3a and Dnmt3b, apart from their role in *de novo* methyltransferase activity, appear to be also involved in the maintenance of the DNA methylation pattern during each cellular replication [Jones and Liang, 2009; Walton et al., 2011].

Readers of the DNA methylation pattern. As mentioned before, the "written" methylation marks at CpG dinucleotides can be specifically recognised by various Methyl-CpG Binding Proteins (MBPs), which may specifically recognise and subsequently interpret the established methylated CpG marks by recruiting chromatin modifiers and remodeling complexes to establish a repressive pattern of gene expression. Almost all MBPs have been demonstrated to associate also with transcriptional repressors, implying an additional layer of regulation between DNA methylation and transcription. All the methyl-CpG binding proteins share binding specificity for symmetrical 5meCpG dinucleotides, although they retain differences in DNA binding specificity and unique roles [Zou et al., 2012].

The various MBPs could be divided in three structural families: the MBDs, the zinc finger and the SRA family. The MBD protein family consists of eleven known protein, including MeCP2, MBD1, MBD2, MBD4, SETDB1, SEDTB2, BAZ2A and BAZ2B, that specifically recognise 5-methyl-CpG dinucleotides via novel methyl-CpG-binding domain (MBD). The 70–85-amino acid MBD domain has the ability to bind single symmetrically methylated CpG dinucleotides. An exception is represented by MBD3, that is the only family member that does not bind specifically to methylated DNA but can bind to unmethylated DNA and has been reported to bind to 5-hydroxymethylated cytosines [Meng et al., 2015].

In addition to the MBD domain, MBD family members contain several differing domains that reflect their respective roles. Most family members contain a transcriptional repression domain (TRD) that mediates interactions with protein partners. Other family members possess unique domains such a glycosylase domain (MBD4) or unmethylated-CpG-binding zinc finger (CxxC) domain (MBD1) [Du et al., 2015]. The second KAISO family comprises structurally unrelated zinc-finger proteins, KAISO and its two close paralogs: ZBTB4 and ZBTB38. These proteins have been shown to bind to methylated DNA through zinc-finger motifs, but can also bind a non-methylated consensus. The third family includes two 5meCpG-binding ubiquitin-like proteins UHRF1 and UHRF2, which recognise methylated DNA via SET and RING finger-associated (SRA) domains. Specifically, UHRF1 is an essential protein that binds hemimethylated DNA and acts in conjunction with DNMT1 to facilitate maintenance DNA methylation; in the absence of UHRF1, there is a precipitous loss of DNA methylation [Buck-Koehntop and Defossez, 2013].

Erasers of DNA methylation pattern. Many developmental biologists have described the waves of genome-wide DNA demethylation occurring in the germline and in early embryogenesis, however, the process by which DNA methylation is erased has been elusive. Recent discoveries have established that the DNA demethylation process can be achieved through both a passive and an active mechanism: passive loss of DNA methylation refers to the failure of the maintenance of the methylation pattern during successive rounds of DNA replication either in the absence of DNMT1 or because of its inhibition, while active DNA demethylation refers to an enzymatic process that results in the removal of the methyl-group from 5mC [Wu and Zhang, 2014]. During preimplantation development the 5mC levels of the maternal genome went through a replication-dependent dilution process (i.e., passive demethylation); in contrast, the 5mC levels of the paternal genome dramatically decrease a few hours after. Given that no DNA replication occurs during this period, the loss of 5mC in the paternal genome is considered active. Another place where global loss of 5mC is observed is in the primordial germ cells (PGCs), specifically by the time they arrive at the genital ridge. Because PGCs have undergone several cell cycles in the presence of DNMT1 during this process, the loss of the epigenetic marks is likely to be active [Wu and Zhang, 2014]. Active DNA demethylation has also been reported in somatic cells in a locus-specific manner as a physiological process, and because no DNA replication takes place in the processes described above, active DNA demethylation is believed to be responsible for the loss of DNA methylation. The mechanism through which active DNA demethylation takes place has been elucidated after the identification of 5-hydroxymethylcytosine (5hmC) as a bona fide base of mammalian genomic DNA and the demonstration that the ten–eleven translocation (TET) protein family is responsible for the conversion of 5mC to 5hmC. It was experimentally shown both *in vitro* and *in vivo* that TET-mediated 5mC oxidation should be able to proceed further to generate 5-formylcytosine (5fC) and, more limited, 5-carboxylcytosine (5caC).

The TET enzyme family comprises three cytosine dioxygenases, TET1 and its two dioxygenase paralogues TET2 and TET3. C-terminal catalytic domains of TET proteins contain an indispensable cysteine-rich region adjacent to their DSBH domain, that is able to oxidise the 5-methyl group on thymine (T) to 5-hydroxymethyluracil (5hmU). In addition, TET1 and TET3 carry a cysteine–X–X–cysteine (CXXC) domain at the N-terminus that strongly binds to unmethylated DNA. The conversion of 5mC to 5hmC probably leads to loss of maintenance of existing DNA methylation patterns given that 5hmC is not recognized by DNMT1 during DNA replication leading to passive DNA demethylation during cell division. In addition, the 5mC oxidation products 5fC and 5caC may serve as intermediates for active

DNA demethylation as both can be cleaved by the thymine-DNA glycosylase (TDG). Tet-mediated 5mC oxidation followed by TDG-mediated excision of 5fC/5caC and base excision repair (BER) might be one of the pathways for active DNA demethylation [Pastor et al., 2013]. The substitution of 5mC residues with 5hmC may be translated in the inhibition of the recruitment of the classic 5mC interpreter MeCPs, thus weakening subsequent transcriptional repression activities. *in vitro* studies showed that MeCP2 has a markedly reduced binding affinity for 5hmC in contrast to its strong binding affinity for 5mC. The less bound MeCP2 may release the associated chromatin modifying enzymes, deregulating the repressive transcription environment [Meng et al., 2015].

1.8 ABERRANT DNA MEHYLATION AND CANCER

Gene function in cancer can be disrupted either through genetic alterations, which directly cause intragenic point mutations, allelic loss and deletions as well as amplifications and translocations, or epigenetic alterations, which alter the heritable state of gene expression. The epigenetic pattern present in normal cells undergoes extensive distortion in cancer: cancer epigenome is characterized by global changes in DNA methylation and histone modification patterns as well as altered expression profiles of chromatin-modifying enzymes. These epigenetic changes, namely epimutations, result in global dysregulation of gene expression profiles leading to the development and progression of disease states [Peltomaki, 2012]. Epimutations can induce inappropriate silencing of tumor suppressor genes and/or activation of oncogenes independently or in conjunction with deleterious genetic mutations or deletions: for this reason, they could represent the second hit required for cancer initiation as proposed by Knudson in the “two-hit” hypothesis [Knudson, 1971].

Above all the epigenetic abnormalities in the cancer cell, aberrant DNA methylation is the most common molecular lesion. Aberrant DNA methylation is deeply involved both in cancer development and progression because DNA methylation pattern is inherited with a high fidelity in somatic cells. Once aberrant DNA methylation is induced, it is accurately transmitted to daughter cells after cell division: therefore, they are selected in a rapidly duplicating cancer cell population, thus conferring a growth advantage to tumoral cells [Sharma et al., 2010].

Historically, the first discovery of epigenetic alterations goes back to 1983, when global hypomethylation of the cancer genome was described, and after a decade, site-specific CpG island hypermethylation within the promoter region of a tumor suppressor gene was demonstrated to cause its silencing. The CpG island methylator phenotype (CIMP), defined as

frequent methylation of multiple CGIs, was reported first in colorectal tumors in 1999 [Toyota et al., 1999] and thereafter extended in other types of cancers. More recently, mutations of epigenetic regulators, including those of DNMTs, TET and IDH have been revealed, and epigenetic drugs, such as DNA demethylating agents, have already become an option for cancer treatment [Hattori and Ushijima, 2014].

1.8.1 GENE-SPECIFIC HYPERMETHYLATION

Increased methylation level of the CpG islands in the promoter regions of tumor suppressor genes has been accepted as being a common feature of human cancer: in 1993, inactivation of the RB tumor-suppressor gene by DNA hypermethylation of CpG islands spanning its promoter region was reported as the first evidence in cases with retinoblastoma. Subsequently, aberrant DNA methylation of other tumor-suppressor genes, many of them being housekeeping genes, i.e. genes that need to be constitutively expressed and transcriptionally active throughout development and differentiation in all tissues, was also reported as a way of inactivation alternative to genetic alterations, thus contributing to tumor development and progression. Silencing of these genes by CpG promoter hypermethylation in cancer cells is known to affect a wide range of cellular processes and several cellular pathways: cell cycle (Rb, BRCA1, p16 INK4a, p15 INK4b), DNA repair (BRCA1, MGMT, MLH1), transcription factors (GATA-4, GATA-5, VHL), epigenetic enzymes (NSD1, RIZ1), receptors (CRBP1, ESR1, TSHR), signal transduction (APC, RASSF1A, WIF1), detoxification and carcinogen metabolism (GSTP), metastasis and cell invasion (CDH1, TIMP3), apoptosis (DAPK, TMS1, CASP8), and angiogenesis (THBS1). Furthermore, aberrant promoter hypermethylation can deregulate differentiation genes, i.e. genes that have to be expressed only in a time- and tissue-dependent manner [Berdasco and Esteller, 2010]. In addition to direct inactivation of tumor suppressor genes, DNA hypermethylation can also silence additional classes of genes indirectly: hypermethylation-mediated inactivation of the transcription factor RUNX3 (runt-related transcription factor 3) in esophageal and gastric cancer and of transcription factors GATA-4 and GATA-5 in colorectal and gastric cancers leads to inactivation of their downstream targets, as well as silencing of DNA repair genes (e.g. MLH1, BRCA1 etc.) enables cells to accumulate further genetic lesions leading to the rapid progression of cancer [Sharma et al., 2010].

Several reports have characterized a specific spectrum of gene hypermethylation for different tumor types, defining a “DNA hypermethylome” for each type of cancer, a sort of “map” that is even maintained in long-established human cancer cell lines. Some tumors have an higher

methylation level of the known CpG islands than others: for example, the most hypermethylated tumor types are those of the gastrointestinal tract, while significantly less hypermethylation has been reported in other types such as ovarian tumors and sarcomas [Esteller, 2007]. While the ability of DNA hypermethylation to silence tumor suppressor genes in cancer is well established, how genes are targeted for this aberrant DNA methylation is still unclear, as it still unknown why certain regions are more susceptible to hypermethylation, whereas others remain unmethylated in different cancer types. Recent epigenome-wide analyses revealed that in cancer cells a very high number (from several hundreds to one thousand) of genes with promoter CGIs is hypermethylated; however, since most of such genes are not expressed or expressed at very low levels in normal cells, they are considered not as "driver genes", which are causally involved in tumorigenesis, but as "passenger genes", that accompany the transformation process but have no effect per se on the process of carcinogenesis [Kalari and Pfeifer, 2010].

1.8.2 Global DNA hypomethylation

The hypothesis that the cancer cell genome undergoes a reduction of its 5mC content in comparison with the normal tissue has been firmly corroborated: genome-wide hypomethylation in general occurs at repetitive elements, retrotransposons, pericentromeric regions, introns and gene deserts.

Hypomethylation has been identified specifically in repeated elements and endogenous viruses but this is not surprising, since these DNA elements are highly abundant and comprise most of the CpG islands that are normally methylated in healthy somatic tissues. Currently, it is thought that the major contribution of transposons demethylation to tumor development is their activation and translocation to other genomic regions, leading to genomic and chromosomal integrity destabilization [Ehrlich, 2009]. It was hypothesized that DNA hypomethylation could lead to the activation and expression of classical oncogenes, but evidence suggests a greater involvement in the activation of developmentally critical genes or genes associated with tumor invasion or metastasis. However, activation of such genes by hypomethylation is still controversial because some of them do not have CpG islands in their promoters [Hattori and Ushijima, 2014].

1.8.3 Detection of aberrant DNA methylation: different approaches

Many different techniques for DNA methylation determination exist, and choosing the most appropriate one largely depends on the nature and number of the samples, information

required, and costs. The three main approaches are: methylation-specific restriction enzyme digestion, affinity purification of methylated DNA and DNA bisulfite conversion. The DNA obtained is further subjected to molecular-genetic approaches, which are for single locus analysis based on PCR, whereas in the case of genome-wide assay they are based either on microarray technology, mass spectroscopy or next generation sequencing analysis [Han and Garcia, 2013; Paska and Hudler, 2015].

Presently, bisulfite genomic sequencing is regarded as a gold-standard technology for detection of DNA methylation because it allows a qualitative, quantitative and efficient approach to identify 5mCs at single base-pair resolution, as well as the analysis of methylation in repeat sequences and rare methylation variants [Li and Tollefsbol, 2011]. This method is based on the finding that the amination reactions of cytosine and 5-methylcytosine proceed with very different consequences after the treatment of sodium bisulfite. In this regard, non methylated cytosines in single-stranded DNA will be converted into uracil residues and recognized as thymine in subsequent PCR amplification and sequencing. 5mCs are immune to this conversion and remain as cytosines, allowing 5mCs to be distinguished from unmethylated cytosines. The DNA obtained is further subjected to molecular-genetic approaches, which are for single locus analysis based on PCR, whereas in the case of genome-wide interrogation they are based either on microarray technology, mass spectroscopy or next generation sequencing analysis [Paska and Hudler, 2015].

1.8.4 How to measure global DNA hypomethylation

There are many approaches which can be applied to the analysis of global DNA methylation. Currently, the gold standard methods for measurement of global methylation are based on the high performance liquid chromatography (HPLC) approach and its variants, as well as the Liquid Chromatography-Mass Spectrometry (LC-MS), that provide an absolute measurement of 5mC in samples of DNA by enzymatic hydrolysis of genomic DNA.

As explained before, about 45% of the human genome is composed by repetitive regions, in which most of the CpG island methylation in the genome can be found. A substantial fraction of the human genome (> 30%), is derived directly or indirectly from LINE-1 retrotransposon, that is present in more than 500,000 copies. Also, Alu insertional elements have been discovered as highly abundant (500,000-1,000,000 units), dispersed in the genome and normally inactivated by heavy methylation [Hancks and Kazazian, 2012].

For these reasons, one of the currently most reliable methods to evaluate global DNA methylation level is based on the assessment of the hypomethylation of these repetitive

sequences, that can serve as a reliable surrogate marker for global genomic DNA methylation [Lisanti et al., 2013]. Results from a recent study of global DNA methylation in murine cells and tissues showed a good correlation between LINE-1 promoter methylation level and total 5-methylcytosine amount measured by LC-MS [Newman et al., 2012]. LINE-1 methylation levels are measurable through different technical approaches, each of them having specific advantages and disadvantages; however, due to its sensitivity, reproducibility, and the possibility to obtain quantitative methylation data, the bisulfite pyrosequencing in considered as one of the better choices for global DNA methylation analyses [Toraño et al., 2012].

1.8.5 Aberrant methylation as therapeutic target

Being a postsynthetic event, in proliferating cells DNA methylation is critically dependent on continued expression of DNA methyltransferases: this is an advantage for anticancer therapy since, unlike genetic changes, epigenetic changes may potentially be reverted by treatment with pharmacological agents. Inhibition of the expression of these enzymes would therefore result in progressive reduction in DNA methylation in newly divided cells, a phenomenon associated with reactivation of gene expression in hypomethylated cells [Issa and Kantarjian, 2009]. Basically, DNA methylation inhibitors can be classified as nucleoside inhibitors and are represented by the 2′-deoxycytidine and its derivatives. 5-azacytidine and 5-aza-2′-deoxycytidine are cytosine analogues that trap all DNA methyltransferases and target them for degradation. At low doses that do not inhibit proliferation, these drugs are effective hypomethylating agents and they have been proposed to have anti-tumour properties since they can inhibit tumour growth by specifically reversing the repression of tumour suppressor and cell cycle genes that are aberrantly methylated in tumour cells, and hence to have less side-effects than non-specific conventional chemotherapy. An important implication is that, unlike conventional cytotoxic agents, it may be best to use such drugs at concentrations lower than the maximum tolerated dose [Ghoshal and Bai, 2007]. In addition these demethylating agents can be indirectly implicated in the anticancer therapy due to their potential to restore sensitivity to a range of chemotherapeutic agents including cisplatin, epirubicin and temozolomide [Fojo and Bates, 2003].

1.9 DNA methylation as a potential biomarker in cancer

A biomarkers is any naturally-occurring characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, particular pathological processes or pharmacological response to a therapeutic intervention. An ideal biomarker must be able to

provide clinically-relevant information and be accurately measurable in multiple individuals, ideally across multiple populations. Biomarkers can be used at any stage of a disease and can be associated with its cause or latency (risk biomarkers), onset (diagnostic biomarkers), clinical course (prognostic biomarkers), response to treatment (predictive biomarkers), or can also be associated with specific environments (exposure biomarkers). As almost all complex human diseases are caused by a mixture of genetic and environmental variation, biomarkers, especially those antecedent to disease, can be influenced by either of these factors and can also reflect the mechanisms by which exposure and disease are related [Mayeux, 2004]. The process of development of a new biomarker to be used in the clinical setting is a very long way, that can be summarized as follows: single studies provide *potential biomarkers*, which could be *validated* using an independent technique and *replicated* in an independent study, also known as external validation. Following the systematic review and/or meta-analysis of a large number of independent studies, they become *candidate clinical biomarkers* that can enter clinical trials. Once approved, they become *proven clinical biomarkers*, used in clinical practice [Mikeska and Craig, 2014].

Throughout the process of carcinogenesis, there are many opportunities to identify cancer biomarkers, going from genetic to metabolic marks. The selected biomarkers must be specific, sensitive, and detectable in specimens obtained through minimally invasive procedures. Compared with molecular structures such as mRNA, miRNA and certain proteins, the use of DNA for the measurement of tumour markers can be more easily transferred from a research laboratory setting into routine diagnostics: it is a chemically stable molecule that can be readily amplifiable and easily detected; also, it can be stored and conserved for long periods of time [Mikeska and Craig, 2014].

Regarding DNA methylation, methyl groups on cytosines are part of the covalent structure of the DNA: once methylation is acquired, it is in most cases chemically and biologically stable over time and can survive in most sample storage conditions. Almost any biological tissue sample or bodily fluid can be used for DNA methylation analysis, archival formalin-fixed paraffin-embedded (FFPE) samples included [Thirlwell et al., 2010]. Currently, DNA methylation appears as one of the most promising epigenetic biomarkers in cancer, not only for their stability and the relative ease in their detection: aberrant DNA methylation has a great potential to be used for as an early indication of neoplastic transformation, as indicated by reports in which aberrantly hypermethylated sites were detected in seemingly normal epithelia from people years before the overt development of cancer. Several DNA methylation markers have been evaluated in a variety of clinical specimens for cancer diagnosis, prognosis and

predictive information on therapeutic responses; in addition, DNA methylation can potentially be used as a biomarker to detect a tumor in the minimal residual disease [Jankowska et al, 2015].

Apart from these advantages, DNA methylation-based biomarkers have some limitations, first the heterogeneity of clinical specimens, that may contain a mixture of components that bring different types of cells with its own methylation peculiarities. Even the adjacent sections of the same tumor may have slightly different composition and quantitatively different methylation patterns [Levenson, 2010]. Second, some DNA methylation changes are due to the exposure to environmental influences as well as to the aging, that should not to be misinterpreted as cancer predisposing alterations [Tost, 2009]. Third, the choice of region to be studied: the investigated locus should ideally be unmethylated in normal cases and methylated in cancer case, with methylation levels of a sample clearly classified as either normal or cancerous. In cancer, these ideal situations may be true for one region of a CpG island and not necessarily for another: multiple (epi)alleles are present, which differ in their pattern of methylated and unmethylated CpG positions [Mikeska et al., 2012].

1.10 PROSTATE CANCER

1.10.1 EPIDEMIOLOGY AND RISK FACTORS

Prostate cancer is the most common cancer in men in Europe, accounting for about 25% of all non-skin tumors diagnosed in men [Ferlay et al., 2013]. It is also the most common cancer diagnosed in males in the United States: in 2014, the American Cancer Society estimated that 233,000 patients would be diagnosed with prostate cancer and that prostate cancer alone would account for 27% of all new incident cases of cancer in men. Prostate cancer is also the second leading cause of cancer death in the US, accounting for 10% of all male cancer deaths [Siegel et al., 2014]. In Italy, 26% of all prevalent cases of male cancers (295,624) were patients with prostate cancer [Source: rapporto AIRTUM 2014]. The lifetime risk of being diagnosed with prostate cancer is approximately 16%, while the probability of dying is 3% [Source: SEER Cancer Statistics Reviews, 2010-2012 data].

Risk factors for prostate cancer include age, ethnicity, and family history: it is a disease that affects older men, with the median age at diagnosis of 67 years; furthermore, 59% of men older aged at least 80 years have histological evidences of latent disease [Bell et al., 2015]. Concerning family history, men with one or two first-degree relatives with this disease have a 2-fold and a 4-fold, respectively, increased lifetime risk of developing prostate cancer

[Tabayoyong and Abouassaly, 2015]. Prostate cancer has a strong ethnic propensity: high incidences are documented in descendents of the Northern Europeans and African Americans, while other groups, including native Africans and Asians, are much less susceptible to the disease. A sort of North-South gradient distribution of prostate cancer incidence can also be noted, especially in Europe [Gunderson et al., 2011].

1.10.2 ANATOMY AND GRADING

The prostate gland is divided in three glandular regions: the peripheral (located posteriorly), the central and the transition zone (in the front of the gland surrounding the urethra), which respectively comprise ~70%, 25% and 5% of the prostatic glandular tissue. The peripheral zone is the site of origin of most carcinomas (70-80%), but some (~15%) arise in the transitional zone and, rarely, in the central zone. Morphological differences between tumors in these zones [Greene et al., 1991], as well as markers of poor prognosis at higher rates among peripheral zone cancers have been observed, suggesting different aggressiveness [Erbersdobler et al., 2002]. While cancers of the peripheral zone are typically diagnosed after biopsy procedures or radical prostatectomies, those arising from transitional tissue are incidentally discovered in men undergoing transurethral resection of the prostate (TURP), a non-curative procedure intended to treat urinary symptoms [Sinnot et al., 2015].

The Gleason score for tumor-cell differentiation is the recommended methodology for prostate cancer grading. According to current international convention, the (modified) Gleason score of cancers detected in a prostate biopsy consists of the Gleason grade of the dominant (most extensive) carcinoma component plus the highest grade, regardless of its extent. To date, Gleason score represents the best-established prognostic indicator for prostate cancer progression [Heidenreich et al., 2014]. However, whether prostate cancers arise well-differentiated and then progress to less differentiated forms or if Gleason grade is an early and largely unchanging feature of the tumor still remains to be clarified [Penney et al., 2013; Hussein et al., 2015].

1.10.3 PSA-BASED SCREENING AND RELATED ISSUES

The introduction of PSA screening as an opportunistic screening for prostate cancer has had a profound impact on its incidence and mortality: in the US, a dramatic increase in prostate cancer incidence occurred with a peak in 1992, attributed to earlier detection of cases and to detection of cases that would never have been diagnosed or treated. After 1992 prostate cancer incidence declined until 1995, attributed to the cull effect resulting from the number of earlier

detected cases as a consequence of PSA screening. Moreover, prostate cancer mortality rates increased slowly between 1975 and the early 1990s with a peak in 1992, and has continued to decline steadily, such that today mortality rates are lower than that observed before the introduction of PSA screening [Tabayoyong and Abouassaly, 2015].

PSA is a serine protease whose transcription is driven by androgens. It is producted by the prostatic epithelium and released into the seminal fluid. PSA levels in the blood are typically low, but can be increased owing to disruption of normal prostatic architecture, which occurs with malignant processes such as cancer but also with benign processes [Lilja et al., 2008].

In 1991, Catalona and colleagues reported the first study using serum PSA as a screening test for prostate cancer. Using a PSA cutoff of 4 ng/mL as the trigger for initiating prostate biopsy, they demonstrated that the combination of PSA with digital rectal examination (DRE) was superior to DRE alone for the detection of prostate cancer [Catalona et al., 1991]. Shortly after, in the late 1980s and early 1990s, use of PSA for the screening of prostate cancer became widespread in the United States, albeit without the benefit of a large, prospective trial to guide optimal screening strategies.

After an elevated PSA dosage, the standard diagnostic technique for prostate cancer is the histopathological review of prostate tissue collected via needle biopsy. While this process is accurate for patients with extensive cancer, in many cases limited disease comprising small cancer foci, as the 80% of prostate cancer is, can be missed [Serenaite et al., 2015]. Prostate biopsy is associated with a false-negative rate of up to 30% due to sampling error and it is affected by the number of cores and pattern of sampling [Stewart et al., 2013]. Due to the substantial false-negative rate, needle biopsy of the prostate does not perform well at excluding a cancer diagnosis. Several nomograms containing different parameters have been proposed to aid the rebiopsy decision, but they are hampered by low accuracy [Chun et al., 2007; Benecchi et al., 2008]. Therefore, men with persistently elevated serum PSA values continue to undergo biopsy procedures, even though only 10-36% of second biopsies detect cancer; moreover, after each subsequent negative biopsy, cancer detection decreases [Trock et al., 2012]. Repeat biopsies in this group can affect patient care in two ways: it can delay cancer detection in patients with disease (and delay effective therapy) or subject cancer-free men to additional invasive biopsy procedures [Troyer et al., 2009].

Although use of PSA as a biomarker in screening for prostate cancer has been well-documented, its greatest limitation is that it is not specific for prostate cancer: serum PSA levels may rise not only during cancerogenesis, but also in benign processes such as benign prostatic hyperplasia, inflammation, infection, or trauma. Furthermore, the optimal upper limit

of the normal range for PSA is still unclear, and currently there is no consensus cutoff value for PSA that is specific for prostate cancer: PSA cutoff of 4 ng/ml has a calculated sensitivity of 78.7% and a specificity of 59.2%. PSA concentration is also a poor discriminator of low- and high-risk disease, and clinically insignificant prostate cancer is common. Prostate cancer of clinical irrelevance represents the vast majority of all cases, as confirmed by autopsy studies that have identified indolent prostate cancers in up to 75% of men 85 years or older who ultimately died of causes other than prostate cancer [Tabayoyong & Abouassaly, 2015]. As a result, PSA screening has led to a concern for overdiagnosis and overtreatment of clinically indolent prostate cancers that ultimately pose no harm to the patient, therefore subjecting men to the morbidity of prostate cancer treatment, increased side effects and long term complications and a decreased quality of life: indeed, a great increase in needle core biopsies and radical prostatectomies has paralleled the increased use of PSA testing [Liljia et al, 2008]. Lastly, this screening is associated to significant costs to the health care system, when the cost of the PSA test itself is considered in addition to the costs of diagnosis, staging, and treatment of screen-detected prostate cancers [Tawfik, 2015].

The effects of PSA screening on mortality from prostate cancer are not yet clear: a recent meta-analysis of five randomised controlled trials carried out in Europe and in US comparing the effect of PSA-screening vs. no screening on all-cause mortality and prostate cancer-specific mortality identified that screening does not significantly decrease prostate cancer-specific mortality and is associated with a high degree of overdiagnosis, treatment and screening-related harms [Ilic et al, 2013]. Furthermore, guidelines recently updated by the European Association of Urology do not recommend widespread population-based screening by PSA [Heidenreich et al., 2014].

The debate over the use of PSA testing for early detection of prostate cancer still continues, and its inability to distinguish between indolent and aggressive prostate cancers makes hard to establish which tumours may kill the patient if left untreated: therefore, there is a urgent need to investigate into new biomarkers and tools for both prostate cancer diagnosis and prognosis.

1.11 RATIONALE OF THE STUDY

Large amount of literature indicate that epigenetic alterations are frequent in prostate cancer and are thought to contribute both to the disease onset and progression. Although the exact mechanisms of how these epigenetic alterations arise in prostate cancer are not understood, the fact that they occur at a much higher frequency than mutations and are common in premalignant stages of the disease make them attractive biomarkers for diagnosis, prognosis

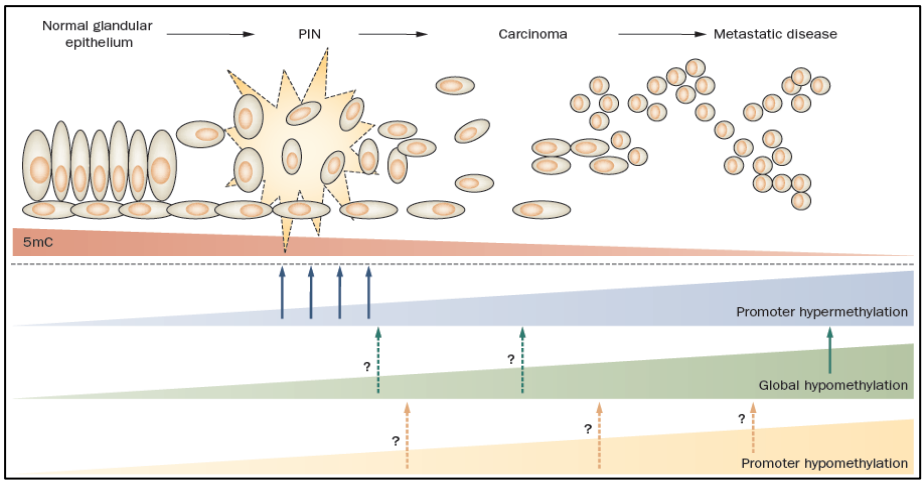
and treatment response [Chiam et al., 2014]. Aberrant DNA methylation is the best-characterized epigenetic alteration in prostate tumors: to date, almost 70 genes with promoter hypermethylation have been identified and reported as potential biomarkers of detection and progression. Notably, glutatione S-transferase pi 1 (GSTP1) gene is reported as hypermethylated in more of the 90% of prostate cancer and suggested as early detection biomarker [Valdes-Mora and Clark, 2015]. GSTP1 belongs to a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Aberrant GSTP1 methylation was detected in histologically negative biopsy samples, suggesting that epigenetic alterations in a first negative biopsy may be useful as potential markers of prostate cancer prediction on a repeat biopsy [Troyer et al., 2009; Trock et al., 2012; Stewart et al., 2013]. To improve the overall specificity and sensitivity, multiple studies have proposed that a panel of hypermethylated genes, including GSTP1, may be useful to discriminate prostate cancer from benign prostate hyperplasia [Bastian et al., 2005; Bastian et al., 2007; Ellinger et al., 2008; Baden et al., 2011; Yoon et al., 2012; Ashour et al., 2014]. Furthermore, promoter hypermethylation of GSTP1 and other candidate genes, measured both in tissues and in body fluids, has been found to be repeatedly associated with clinicopathologic indicators of poor prognosis: biochemical recurrence (PSA relapse) [Bastian et al., 2007; Kristensen et al., 2014; Ashour et al., 2014], Gleason score [Bastian et al., 2005; Liu et al., 2008; Baden et al., 2011; Yoon et al., 2012], and tumor stage [Bastian et al., 2005; Ellinger et al., 2008; Liu et al., 2008].

Global DNA hypomethylation, generally defined as loss of methylation of highly repetitive DNA sequences such as LINE-1, has been studied to a much lesser extent than gene-specific hypermethylation. It may occur in early stages of prostate cancer and may be involved in the development of prostate intraepithelial neoplasia lesions [Cho et al., 2009; Yang et al., 2013], but it was described also at later stages of prostate cancer such as in metastatic prostate cancer and associated with prognostic parameters [Florl et al., 2004; Cho et al., 2007; Yegnasubramanian et al., 2008; Delgado-Cruzata et al., 2012]. Evidences on the role of global DNA hypomethylation in prostate cancer development and progression have been summarized in a recent meta-analysis [Zelic et al., 2015a], that underlined the potential value of this epigenetic mechanism as diagnostic and prognostic marker, but also suggested the necessity to overcome problems about heterogeneity and small sample size of the studies through well-designed studies.

Reported data suggest that in prostate cancer hypermethylation and hypomethylation are not mutually-exclusive, but rather concomitant events in close relationship [Florl et al., 2004;

Yegnasubramanian et al., 2008; Cho et al., 2009]. Prostate cancer has been proposed as a model of "epigenetic catastrophe", particularly in relation to the widespread changes observed in DNA methylation patterns (Figure 1.1). An overall decrease in 5mC occurs since first stages; in addition, at specific gene loci, promoter hypermethylation is one of the earliest molecular aberrations and persists throughout disease progression. Global hypomethylation becomes more extensive as disease becomes metastatic [Perry et al., 2010].

Figure 1.1. Changes in methylation patterns during prostate cancer development, according to the model of the epigenetic catastrophe.



From Perry et al., Nat Rev Urol (2010)

Methylation status of selected genes has been explored also in non-neoplastic tissue adjacent to tumor, to assess if aberrant methylation already occurs in tissues without features of malignancy, to evaluate if it is comparable to the epigenetic events of primary tumor, according to the hypothesis of the "epigenetic field effect" in prostate cancer, and also if it is able to predict the risk of recurrence of malignant disease [Chai and Brown, 2009].

The concept of field effect (or field cancerization) was based on observations that cancer developed in multifocal areas and that abnormal tissue surrounded the tumor, suggesting that neoplastic or preneoplastic cells existed in a histologically normal field proximal to cancer tissue [Mehrotra et al., 2008]. Hanson and colleagues have reported hypermethylation of the promoter of two genes (GSTP1 and RARβ2) both in normal epithelium and stroma isolated from regions adjacent to the tumors [Hanson et al. 2006] and, more recently, promoter methylation of selected gene was found increased in non-neoplastic tissue adjacent to tumor [Mehrotra et al., 2008; Steiner et al., 2010; Jentzmic et al., 2012]. By contrast, to our knowledge, global DNA hypomethylation in non neoplastic tissue adjacent to prostate tumor has not been investigated yet.

DNA methylation, as discussed above, is mediated by enzymes belonging to the family of the DNA methyltransferases (DNMTs). Documented association between DNMT3b overexpression and increased methylation [Nosho et al., 2009], as well as between DNMT3b inhibition and global and gene-specific DNA hypomethylation [Biswal et al., 2012], indicate a direct relation between DNMT3b activity and DNA methylation status in the tumor. DNMT3b overexpression has been described in several types of cancers [Lin and Wang, 2014], including prostate [Kobayashi et al., 2011], and associated to disease progression [Hayette et al., 2012; Chen et al., 2014], although in prostate cancer this relationship has been observed only in *in vitro* assays [Gravina et al., 2013].

Single nucleotide polymorphisms (SNPs) in the DNMT3b gene have been reported to increase the transcriptional activity of the promoter of DNMT3b, thus producing an effect similar to enzyme overexpression. This effect lastly may play a role in aetiology and progression in a wide range of malignant solid tumors and hematologic neoplasms, prostate cancer included [Singal et al., 2005], as recently reviewed [Duan et al., 2015].

Regarding a possible effect of DNMT3b polymorphisms on global DNA hypomethylation in cancer cells, to our knowledge this has not been evaluated yet.

2. AIMS OF THE STUDIES

This research project includes three studies, conducted on three types of prostate tissue: non-neoplastic prostate tissue (STUDY 1), prostate tumor tissue (STUDY 2 and 3) and non-neoplastic tissue adjacent to tumor (STUDY 2). In general in these studies we explored the role of methylation status in the development and progression of prostate cancer, and the possible regulation of mechanisms leading to a given methylation status through the efficiency of DNMT3b enzyme. The relevance of the methylation pattern could suggest a possible role of global hypomethylation and gene-specific hypermethylation of selected genes as a diagnostic and a prognostic marker.

⇒ STUDY1

A case-control study conducted on an unselected series of men who received at least two prostate biopsies at the San Giovanni Battista Hospital, Turin, between 1993 and 2003 to evaluate if aberrant methylation detected on the first biopsy may serve as prediction marker of prostate cancer diagnosis in a subsequent biopsy;

⇒ STUDY2

A cohort study that involved consecutive prostate cancer patients diagnosed at the San Giovanni Battista Hospital between 1982 and 1996 aiming to understand if global DNA hypomethylation evaluated both in tumor and in non-neoplastic tissue adjacent to tumor may have prognostic value for prostate cancer;

⇒ STUDY 3

A cohort study performed on consecutive prostate cancer patients diagnosed at the San Giovanni Battista Hospital between 1982 and 1996, a part of them already involved in the STUDY 2, to evaluate if selected polymorphisms of DNMT3b gene could have an effect on aberrant methylation and may be implicated in prostate cancer prognosis.

3. STUDY 1

"LINE-1 HYPOMETHYLATION AND GSTP1 HYPERMETHYLATION ON INITIAL NEGATIVE PROSTATE BIOPSY AS MARKERS OF PROSTATE CANCER ON A REBIOPSY"

Overall aim of the study was to assess if aberrant methylation markers measured in a first negative prostate biopsy may be associated with prostate cancer diagnosis in a subsequent biopsy. Specifically, we evaluated if global hypomethylation, measured through LINE-1 methylation, and GSTP1 hypermethylation detected in an initial negative biopsy are markers of the probability to detect prostate cancer in a rebiopsy.

The study was approved by the Local Ethical Committee.
Results of this study have been recently published [Zelic et al., 2015b].

3.1 STUDY DESIGN AND PARTICIPANTS SELECTION

We conducted a case-control study within an unselected cohort of men who underwent prostate biopsy, TURP or partial prostatectomy between 1993 and 2003 at the San Giovanni Battista Hospital, Turin, Italy, and whose archived formalin fixed paraffin embedded (FFPE) tissue samples were available at the two Pathology Wards of the Hospital. The two Pathology Wards, hereafter referred to as Ward 1 and Ward 2, were associated with different Urology Wards of the Hospital and were included in the study for replication purposes.
In total, in the two Wards, 8755 men underwent at least one procedure (i.e. biopsy, TURP or partial prostatectomy) during the study period, of whom 1105 underwent two or more consecutive procedures. We restricted the study to 737 subjects with a minimum of three months between the two procedures. For subjects with tissue samples available from more than three consecutive procedures, only the last three were considered.
Case subjects were patients with a histological confirmation of prostate cancer in the last biopsy, which was used as the index sample. Subjects with only one negative prostate tissue sample available prior to the positive index sample were counted as one case, while subjects with two available samples were counted as two cases, leading to 145 potential cases in Ward 1 and 99 potential cases in Ward 2. Original diagnostic slides from all the potential cases were traced and re-evaluated to assign a harmonized Gleason score. When Gleason score could not be re-evaluated the original Gleason score available from the pathology report was used or it was considered as missing. Cases without a matched control of for whom prostate cancer

diagnosis could not be confirmed were excluded, leaving 115 cases in Ward 1 and 84 cases in Ward 2 for further analysis.

Non-case subjects were patients who remained prostate cancer-free at the last prostate sampling. Non-case subjects with two negative prostate tissue samples available were counted as one potential control, while non-case subjects with three negative samples available were counted as two or three potential, thusleading to 293 potential controls in Ward 1 and 365 potential controls in Ward 2.

Within each Ward, actual controls were matched to cases (1:1 ratio) on calendar year of sampling (four-year groups), age (five-year groups) and time between the first and second sampling (six-months groups). Controls with high-grade prostatic intraepithelial neoplasia (HGPIN) or atypical small acinar proliferation (ASAP) on the index procedure were excluded: HGPIN is an abnormality of the prostatic gland considered to precede the development of prostate cancer, while ASAP generally is not considered a pre-malignancy, but it is an expression of diagnostic uncertainty. After the sampling procedure, 94 controls in Ward 1 and 84 controls in Ward 2 remained for analysis. 6 cases (4 in Ward 1 and 2 in Ward 2) and 2 controls (2 in Ward 1) were excluded from the study because their FFPE blocks did not contain sufficient amount of prostate tissue needed for the molecular analyses. Finally, we randomly excluded one case (14 in Ward 1 and 7 in Ward 2) and one control (6 in Ward 1 and 3 in Ward 2) whenever two cases/controls originated from the same case/control subject and, at the same time, we minimized loss of information by trying to preserve at least one case and one control in each matching stratum. Process of selection and exclusion of cases and controls is summarized in Figure 3.1.

For both cases and controls, the molecular analyses focused exclusively on the first negative sample, meaning that the index procedure used to define cases and controls (i.e. subsequent positive or negative biopsy) was not analyzed. This approach is consistent with the aim of the study, i.e. assessing molecular markers used to help the rebiopsy decision.

3.2 METHODS

3.2.1 Choice of FFPE blocks

Analyses were performed on one tissue sample for each case and control. If a case or a control had more than two FFPE blocks we randomly selected one block. If a case or a control had more than two tissue samples in the selected FFPE block we selected the largest tissue samples and cut three to five (10 μm thick) sequential sections avoiding areas of chronic inflammation, fibromuscular stroma, glandular atrophy and epithelial dysplasia.

3.2.2 DNA extraction

Tissue slices cut from FFPE blocks were first dewaxed three times in xylene and washed three times in 100% ethanol, then genomic DNA was extracted and purified using the commercially available QIAamp ® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany): after removing the residual ethanol, the tissue was dried at room temperature (or in thermal block at set 37°C to shorten the time) and was 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 variable volume of elution buffer (ATE): 42 µl of ATE for DNA extracted from biopsies, 72 µl for DNA extracted from TURPs and prostatectomies. The extracted DNA was then stored at -80°C. The concentration of each DNA sample was assessed by UV-visible spectrophotometer (NanoDrop Technology).

3.2.3 Sodium bisulfite modification

Genomic DNA samples, along with fully methylated [CpGenomeTM universal methylated DNA (Chemicon Co.)] and fully unmethylated controls [EpiTect Control DNA, unmethylated (Qiagen, Hilden, Germany)], underwent sodium bisulfite conversion using the commercially available kit EpiTect bisulfite kit (Qiagen, Hilden, Germany). 1000 ng of DNA were modified, when possible, in a maximum 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. Elution was performed in two steps, using 21 µl of EB

(elution buffer) in each step, then resulting in a total volume of 42 μl of bisulfite-converted DNA that was stored at -80°C.

3.2.4 Quantification of CpG methylation by Pyrosequencing

Analysis of LINE-1 (GenBank accession number X58075) and GSTP1 (GenBank accession number M24485) promoter methylation status were performed using PyroMark Q24 MDx (Qiagen, Hilden, Germany). Primers, which amplify a 98bp-long fragment of LINE-1 promoter containing three CpG sites (positions 819, 826 and 829), and a 72bp-long fragment of GSTP1 promoter containing four CpG sites (positions 1038, 1040, 1043, 1049), were designed outside the CpG sites. PCR and sequencing primers are listed in Table 3.1. We performed PCR reaction in a total volume of 30 μl containing 1X buffer (KCl), 2 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 μM of each primer, 0.05 U Taq polymerase and 6 μl of bisulfite-converted DNA with the following cycling profile: 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min for LINE-1 and at 50°C for 1 min for GSTP1, extension at 72°C for 1 min 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 0.15 μl of 50 μM sequencing primer (final concentration 0.3 μM) in a PyroMark Q24 MD instrument. Pyrosequencing methylation assays were created according to the manufacturer's instruction. Methylation quantification was achieved using the provided software, and expressed for each DNA locus as 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 [EpiTect Control DNA, unmethylated (Qiagen, Hilden, Germany)] were included in each pyrosequencing run. Analyses on LINE-1 methylation were conducted ensuring that the matched case and control samples were analyzed within the same batch; thus, if analyses for a case or control had to be re-run, we re-analyzed the whole corresponding stratum, including both the case and the matched control(s).

3.2.5 Statistical analyses

Analyses were first conducted in Ward 1 and then replicated in Ward 2. Thus, all analyses were conducted in the two Wards separately, with the exception of the subgroup analyses in which the two Wards were combined to increase statistical power.

Since LINE-1 and GSTP1 methylation analyses involved more than 1 CpG site, mean methylation levels across the CpG sites were used. In sensitivity analyses using the maximum methylation level, results were only marginally changed. We thus have reported only estimates based on the mean methylation. In order to facilitate interpretation of the results LINE-1 methylation was categorized into four categories (<70%, 70-74%, 75-79%, ≥80%) and analyzed using 70-74% as the reference. To investigate possible non-linearities, we additionally modeled LINE-1 methylation in both Wards using cubic splines with four internal knots based on the tertiles of the LINE-1 distribution, and reported the results graphically using a methylation value of 72.7% as the reference. GSTP1 methylation was dichotomized using an *a priori* selected cut-off of 5% (based on a detection limit of pyrosequencing technique), where subjects with GSTP1 methylation ≥5% were considered as hypermethylated and subjects with GSTP1 methylation <5% as unmethylated.

We used conditional logistic regression to estimate odds ratios (ORs), and corresponding 95% confidence intervals (95% CIs), of the risk of prostate cancer diagnosis on a rebiopsy for LINE-1 hypomethylation and GSTP1 hypermethylation. Strata were defined by the matching variables, and we further adjusted for time between the first and second sampling (continuous variable), number of cores sampled at the first biopsy (≤2, 3-5, ≥6 cores categories), number of samples prior to the index sample (1, 2 and ≥3) and, mutually, for GSTP1 and LINE-1 methylation. The two Wards were combined to estimate the amount of diagnostic information added independently by GSTP1 (<5%, ≥5) and LINE-1 methylation (modelled by restricted cubic splines), by comparing the models including these two markers with the model without them, and calculating the Akaike Information Criterion (AIC) for each of the models. After combining the two Wards, we also conducted subgroup analyses by stratifying on time between the first and the second sampling, <12 vs. ≥12 months, and Gleason score, ≤3+4 vs. ≥4+3. We chose not to treat Gleason score 7 as a homogenous group as this has been reported to lead to a loss of prognostic information [Stark et al., 2009; Amin et al., 2011]. However, we performed sensitivity analyses using Gleason score 8 as the threshold. In addition, we calculated the observed sensitivity in cases and specificity in controls for GSTP1 ≥5% and LINE-1 <70%. All statistical analyses were conducted using STATA 12 (STATA Corporation, College Station, TX, USA).

3.3 RESULTS

In total, 97 cases and 86 controls in Ward 1 and 75 cases and 81 control in Ward 2 remained for the molecular analyses (Figure 3.1). Preliminary analyses of LINE-1 methylation revealed

higher methylation levels in TURPs than in biopsy samples (Figure 3.2), suggesting that LINE-1 methylation levels are higher in the tissue sampled from the transition zone. We therefore restricted the study to biopsies only, and excluded cases and controls sampled by TURP and prostatectomy and subjects who were left without cases or controls within the matching strata. Overall, 67 cases and 62 controls in Ward 1 and 62 cases and 66 controls in Ward 2 remained for the final analyses (Figure 3.1).

Table 3.2 summarizes some selected characteristics of cases and controls divided by Ward. Mean LINE-1 methylation was lower in cases than controls in Ward 1 but not in Ward 2, while GSTP1 methylation was higher in cases than controls in both Wards. The two Wards included slightly different patients. Cases in Ward 1 had a higher proportion of high grade tumors (≥4+3) than those in Ward 2. Additionally, controls in Ward 1 had higher LINE-1 methylation and lower GSTP-1 methylation than controls in Ward 2. In both Wards, biopsies with at least 6 cores were sampled more frequently from controls than from cases.

Table 3.3 reports results for LINE-1 methylation, categorized into four categories. The adjusted OR of prostate cancer diagnosis on the rebiopsy for LINE-1 hypomethylation (<70% vs. 70-74%) was 2.1 (95% CI: 0.5-9.1) in Ward 1 and 1.6 (95% CI: 0.4-6.1) in Ward 2. When LINE-1 was modelled using spline regression (Figure 3.3) we found an increased risk of prostate cancer at low methylation levels in both Wards, but, while in Ward 1 the relationship flattened with increasing LINE-1 methylation, in Ward 2 there was a U-shaped relationship with an increased risk of prostate cancer diagnosis also at high levels of LINE-1 methylation. GSTP1 hypermethylation was associated with diagnosis of prostate cancer at the second biopsy in both Wards (Ward 1: OR=5.1, 95% CI: 1.7-15.0; Ward 2: OR=2.0, 95% CI: 0.8-5.3) (Table 3.3). As reported in Table 3.4, when we stratified by the time between the first and the second biopsy, neither for LINE-1 hypomethylation nor for GSTP1 hypermethylation there was clear evidence of heterogeneity with the time between the first and second biopsy. In the analysis stratified by Gleason score, associations were stronger for Gleason score ≥4+3 for both GSTP1 methylation (OR_2=9.2, 95% CI: 2.0-43.1) and LINE1 methylation <70% (OR_2=9.2, 95% CI: 1.4-59.3). When we used Gleason score 8 as the cut-off, the associations with prostate cancer diagnosis remained stronger for more aggressive tumors (data not shown).

When the two Wards were combined, GSTP1 (p=0.0068) independently improved the predictive capability of the model (Table 3.5) and the model associated with the lowest AIC included only GSTP1 methylation. However, when the analysis was restricted to cases with Gleason score ≥4+3 and corresponding controls, the model with the lowest AIC included both LINE-1 and GSTP1 methylation (Table 3.5). For both GSTP1 and LINE-1, the specificity was

higher than the sensitivity (86.7% and 25.2% for GSTP1; 88.3% and 15.5% for LINE-1). When analyses were restricted to cases with Gleason score ≥4+3, the sensitivity and the specificity were 85.5% and 26.5% for GSTP1 and 88.2% and 23.57% for LINE-1.

3.4 DISCUSSION

It has been proposed that global DNA hypomethylation and gene-specific hypermethylation coexist in prostate cancer tissue and therefore can be used as markers of prostate cancer diagnosis and prognosis [Ehrlich et al., 2009 Cho et al., 2007; Yegnasubramanian et al., 2008]. In this study we assessed the relationship between LINE-1 hypomethylation and GSTP1 hypermethylation in men with a histologically negative initial biopsy and prostate cancer detection in a subsequent tissue sample. While GSTP1 methylation alterations seem to be associated with prostate cancer diagnosis, and the results were replicated in two independent Wards, effects were weaker and less consistent for LINE-1, with a possible exception of extremely low LINE-1 methylation levels.

Previously, a number of studies with a study design similar to ours have evaluated the hypermethylation of GSTP1 promoter in the first negative prostate biopsies [Troyer et al., 2009; Trock et al., 2011; Stewart et al., 2013]; however, to our knowledge, any research group have analyzed global DNA hypomethylation in repeat prostate biopsies before. Three previous studies have evaluated the methylation status of some selected genes, that have been tested on a negative biopsy as a predictor of prostate cancer detection on a rebiopsy: GSTP1, APC and RAR-2β [Troyer et al., 2009], GSTP1 and APC [Trock et al., 2011], or GSTP1, APC and RASSF1 [Stewart et al., 2013]. In addition, the high negative predicted value obtained combining GSTP1, APC and RASSF1 [Stewart et al., 2013] have been validated in the recent DOCUMENT multicenter study [Partin et al., 2014].

The three studies used different approaches to calculate sensitivity and specificity; however, in all three studies, GSTP1 was found to have a rather high specificity (75-85%) and rather low sensitivity (36-52%), and in two studies APC had higher sensitivity (46-95%) but lower specificity (40-78%). While our results on GSTP1 methylation are consistent with previous studies, the results for LINE-1 methylation do not suggest that this is a strong candidate marker for prostate cancer diagnosis, although they offer a novel insight into the possible association of the extremely low levels of LINE-1 methylation (i.e. <70% category, or ≤67% as visible from spline regression in Figure 3.3) with the risk of prostate cancer diagnosis on a rebiopsy. However, these results were less conclusive due to small number of subjects in these categories, as 15.5% of cases had LINE-1 methylation <70% and only 6.2% of cases had

LINE-1 methylation ≤67%. For both GSTP1 hypermethylation and LINE-1 hypomethylation the relationship with prostate cancer was stronger in the more aggressive tumors (i.e. those with Gleason score ≥4+3). For GSTP1, these results are in line with the findings of a previous study [Stewart et al., 2013], where more aggressive tumors (Gleason score ≥7) were found to have higher methylation and more epigenetic abnormalities in the initial negative biopsy. In addition, when the two Wards were combined and the analysis was restricted to cases with Gleason score ≥4+3 and corresponding controls, the model with the best prediction of the probability of prostate cancer diagnosis included both GSTP1 and LINE-1 methylation, suggesting that global DNA hypomethylation, specifically extreme global hypomethylation, could be considered, in addition to gene-specific hypermethylation, in nomograms for the decision on whether to rebiopsy or not.

Our results revealed that LINE-1 methylation level is higher in TURPs compared to biopsies, therefore suggesting that, from the molecular point of view, tissue sampled from the transitional zone is different from that of the peripheral zone. This is in line with the results of previous studies, which found that peripheral and transitional tissue retrieved from normal prostate show differential gene expression profiles, therefore highlighting the profound molecular differences between these two zones [van der Heul-Nieuwenhuijsen et al., 2006; Noel et al., 2008].

Our study has strengths: the underlying population from which cases and controls originate was an unselected series of men who underwent repeat biopsies at the San Giovanni Battista hospital, Turin, Italy, with a 19% risk of being diagnosed with prostate cancer on a rebiopsy, which is in line with previous studies [Stewart et al., 2013]. For quantitative analysis of LINE-1 and GSTP1 methylation we used pyrosequencing which, in contrast to quantitative methylation specific PCR (qMS-PCR) used for GSTP1 analysis in prior studies [Troyer et al., 2009; Trock et al., 2011; Stewart et al., 2013], detects low levels of methylation as methylation in each CpG site is measured independently [Havik et al., 2012]. Pyrosequencing has also been reported to have a higher sensitivity and accuracy than qMS-PCR [Quillien et al., 2012]. A high sensitivity is particularly important for analyses of GSTP1, as GSTP1 methylation is typically low in non-tumor prostate tissue, while high accuracy is particularly relevant for LINE-1, as LINE-1 methylation is an indicator of global methylation and is associated with a rather low variance. Furthermore, we paid attention to the possible batch effect in LINE-1 methylation quantification by pyrosequencing by analyzing matched cases and controls within the same batch: we could not completely eliminate the possibility of misclassification due to the molecular analyses, but we ensured that the misclassification was non-differential.

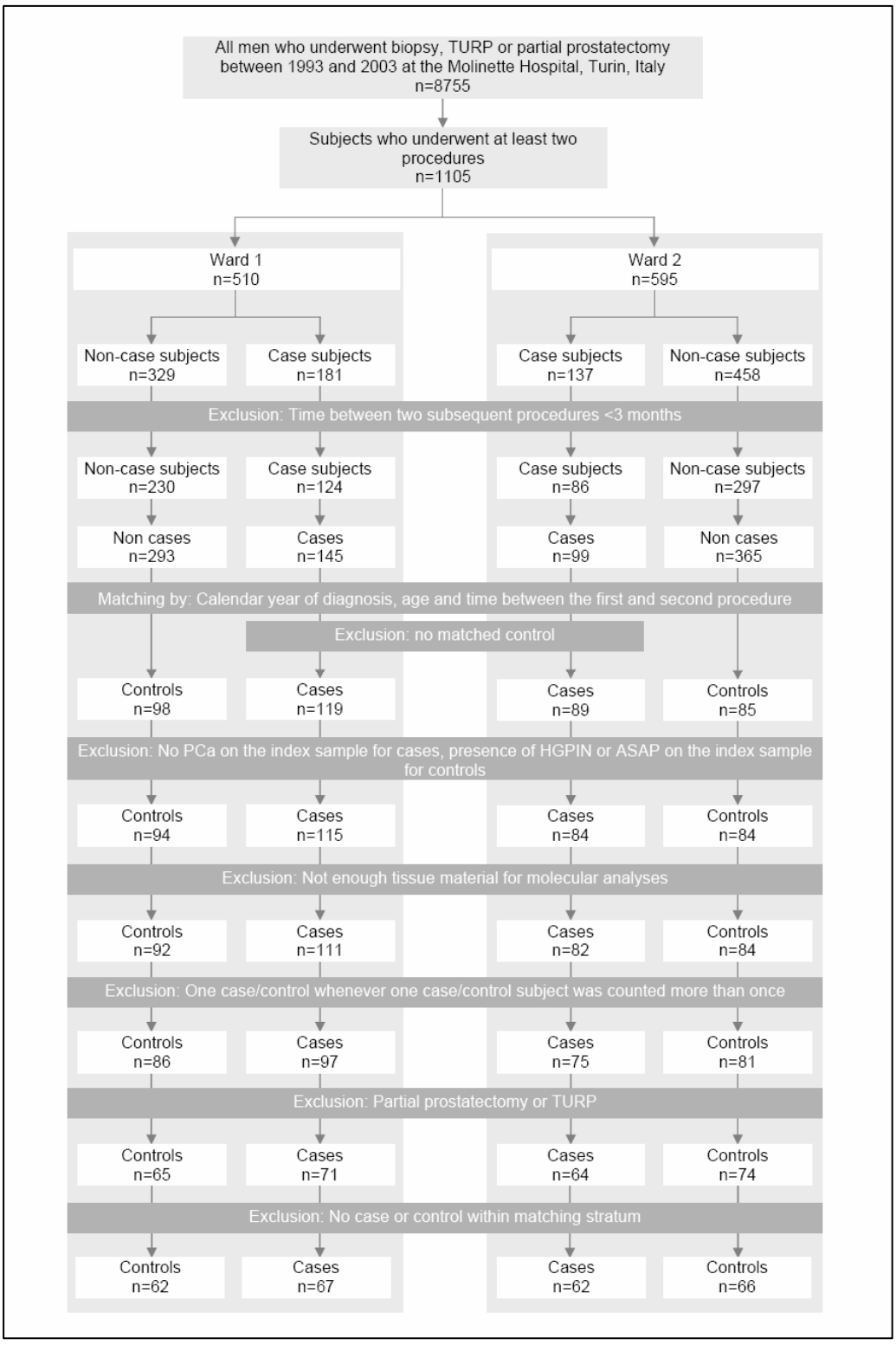
The main limitation of this study is that we lacked information on the various clinical and pathological parameters that are typically used to predict prostate cancer on a subsequent biopsy, including PSA and PSA derivatives, prostate volume, digital rectal examination finding and family history of prostate cancer. This hampers the estimation of the actual discrimination potentials of GSTP1 and LINE-1 methylation when added to the current nomograms. It is important to note, however, that our study was nested in a cohort of men who underwent a rebiopsy, which implies that the clinical parameters typically used to guide a rebiopsy decision were implicitly taken into account. Our markers therefore, to some extent, work in addition to these clinical variables. It should also be acknowledged that these two markers might not be enough to discriminate the disease on their own, but they could be used in addition to other previously suggested markers such as PSA levels, methylation in APC, RAR-2β, RASSF1, early prostate cancer antigen or gene hypermethylation in the urine samples collected at the time of the rebiopsy.

In our study, the quality of the DNA extracted from the FFPE blocks could potentially be suboptimal, as the samples included in this study were 10 to 22 years old; however, it has been shown that DNA, especially short target sequences of DNA suitable for methylation status analysis, can be efficiently extracted from FFPE blocks archived for more than 20 years [Gillio-Tos et al., 2007]. Finally, due to the small number of subjects at the extremes of the LINE-1 distribution (i.e. LINE-1 methylation <70% and >80%) and the observed non-linear relationship, our study did not have enough power to give precise estimates of the association between the extremes of LINE-1 methylation and the risk of prostate cancer diagnosis on a subsequent biopsy.

In conclusion, in two parallel analyses conducted among patients seen in two Wards of a large Hospital in Italy, we found that promoter methylation of GSTP1 measured in a negative biopsy tissue is associated with prostate cancer diagnosis on a rebiopsy, especially for more aggressive tumors. These results were consistent in the two Wards and they support prior findings that GSTP1 methylation is a specific predictor of malignancy on a prostate rebiopsy. Validation across Wards for LINE-1 was achieved only for low methylation levels and prior evidence is sparse. Its predictive ability, especially for more aggressive tumors, should thus be replicated in future studies including a larger number of patients with extremely low LINE-1 methylation values.

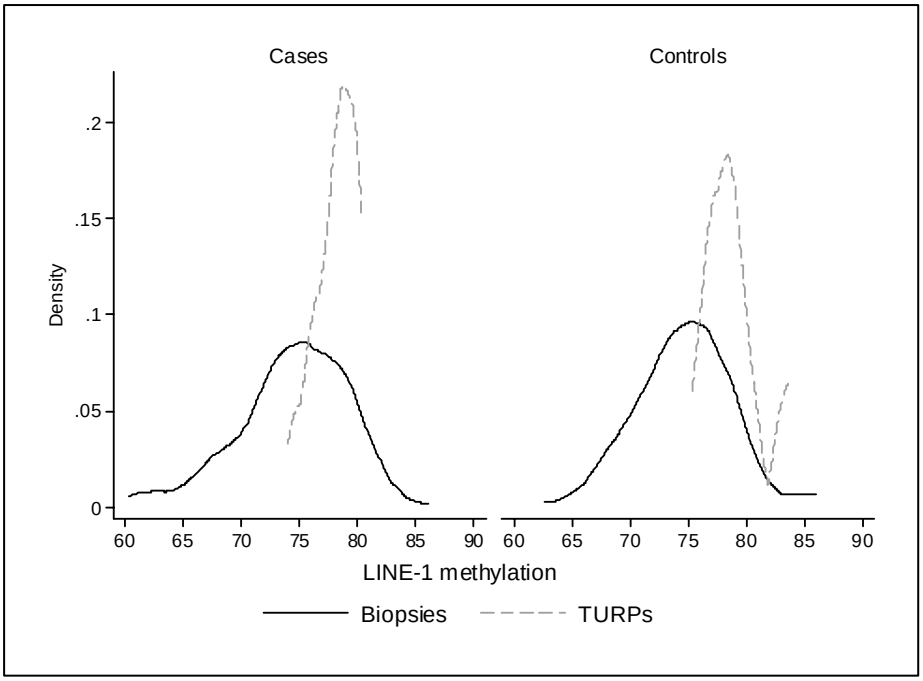
3.5 TABLES AND FIGURES

Figure 3.1. Flow chart of the case and control selection.



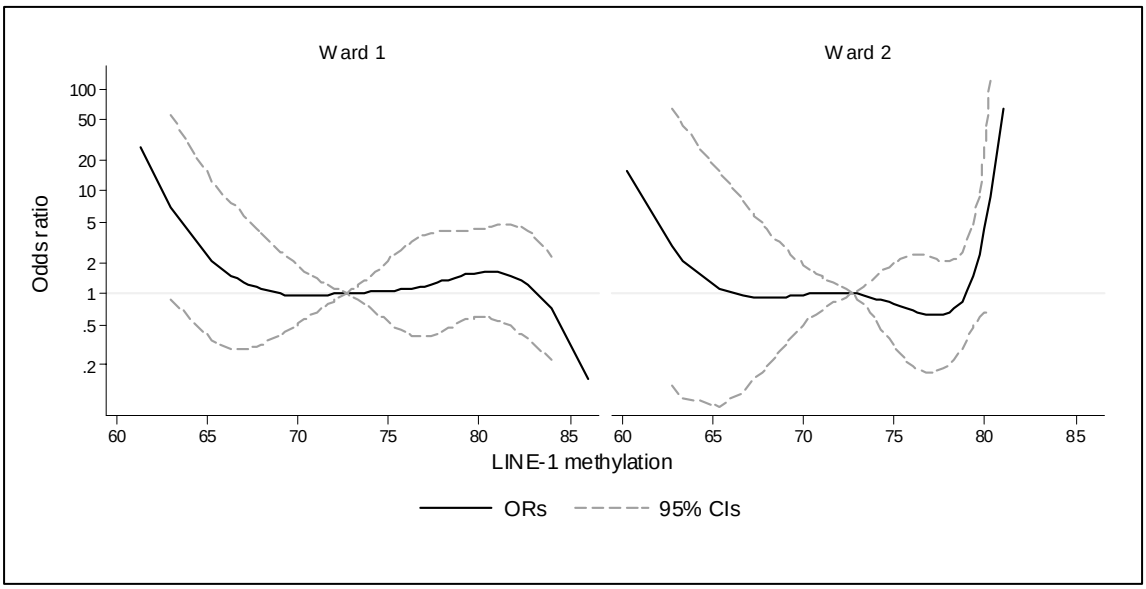
(Zelic et al., Clin Cancer Res 2015)

Figure 3.2. Kernel density estimate of LINE-1 methylation distribution in biopsies vs. TURP in the Ward 1 and Ward 2 combined. Cases and controls are presented separately.



(Zelic et al., Clin Cancer Res 2015)

Figure 3.3. Association between LINE-1 methylation on a negative biopsy and risk of prostate cancer diagnosis on a rebiopsy in the two Wards separately.



Estimates are, adjusted for matching variables and time between the two samples, number of cores sampled at the biopsy, number of biopsies prior to the index biopsy and GSTP1 methylation (the upper 95% limit is restricted to <200 and the lower 95% limit is restricted to >0.03).

(Zelic et al., Clin Cancer Res 2015)

Table 3.1. PCR and sequencing primers for amplification of a fragment of LINE-1 and GSTP1 promoter.

Primer	LINE-1 promoter	GSTP1 promoter
Forward	5'-TTTGAGTTAGGTGTGGGATATAGTT-3'	5'-GATTTGGGAAAGAGGGAAAGGT-3',
Reverse	5'-Biot-CACCTAAAAAATCCAATCACTCC-3'	5'-Biot-CAAAAAAACGCCCTAAAATCC- 3'
Sequencing	5'-TTAGGTGTGGGATATAGTTT-3'	5'-GGTTTTTTYGGTTAGTTG-3'

Table 3.2. Selected characteristics of cases and controls by Ward.

	Ward 1				Ward 2			
	Cases (n = 67)		Controls (n = 62)		Cases (n = 62)		Controls (n = 66)	
	n	%	n	%	n	%	n	%
Age (years)								
< 60	4	6.0	4	6.4	3	4.8	4	6.1
60 - 65	11	16.4	9	14.5	10	16.1	9	13.6
65 - 70	23	34.3	20	32.3	24	38.7	25	37.9
70 - 75	14	20.9	12	19.4	18	29.1	20	30.3
≥75	15	22.4	17	27.4	7	11.3	8	12.1
Calendar year								
1993 - 1996	8	11.9	8	12.9	11	17.7	11	16.6
1997 - 2000	31	46.3	31	50.0	20	32.3	24	36.4
2001 - 2003	28	41.8	23	37.1	31	50.0	31	47.0
Time between the first and second sampling (months)								
<6	7	10.5	7	11.3	11	17.7	14	21.2
6 - 12	16	23.9	16	25.8	14	22.6	14	21.2
12 - 18	9	13.4	9	14.5	5	8.1	7	10.6
18 - 24	8	11.9	7	11.3	3	4.8	3	4.6
24 - 36	14	20.9	10	16.1	16	25.8	16	24.2
36 - 48	5	7.5	5	8.1	7	11.3	5	7.6
≥48	8	11.9	8	12.9	6	9.7	7	10.6
Gleason score								
≤7 (3+4)	45	67.2			49	80.3		
≥7 (4+3)	22	32.8			12	19.7		
Missing	0	-			1	-		
Mean LINE-1 methylation (SD)								
%	74.7 (4.5)		75.3 (4.6)		74.5 (4.7)		73.8 (3.6)	
	p=0.461				p=0.388			
Categorized LINE-1 methylation								
<70%	9	13.4	6	9.7	11	17.7	9	13.6
70 - 74%	24	35.8	24	38.7	20	32.3	30	45.5
75 - 79%	29	43.3	24	38.7	24	38.7	25	37.9
≥80%	5	7.5	8	12.9	7	11.3	2	3.0
	p=0.666				p=0.181			
GSTP1 methylation (median and range)								
%	3.1 (1.0 - 27.5)		2.9 (0.8 - 34.0)		3.5 (0.5 - 22.0)		3.0 (1.3 - 12.8)	
	p=0.158				p=0.125			
Dichotomized GSTP1 methylation								
<5%	48	72.7	56	90.3	47	77.1	55	83.3
≥5%	18	27.3	10	9.7	14	22.9	11	16.7
Missing	1	-	0	-	1	-	0	-
	p=0.011				p=0.374			
Number of sampled cores								
≤2	56	83.6	51	82.3	37	59.7	40	60.6
3 - 5	10	14.9	6	9.7	22	35.5	12	18.2
≥6	1	1.5	5	8.0	3	4.8	14	21.2
	p=0.156				p=0.007			
Number of biopsies prior to the index sampling								
1	46	68.7	45	72.6	39	62.9	45	68.2
2	15	22.4	16	25.8	18	29.0	17	25.8
≥3	6	8.9	1	1.6	5	8.1	4	6.0
	p=0.180				p=0.801			

(Zelic et al., Clin Cancer Res 2015)

Table 3.3. LINE-1 methylation and GSTP1 hypermethylation on a negative biopsy and risk of prostate cancer diagnosis on a rebiopsy.

	Ward 1		Ward 2	
	OR1 95% CI	OR2 95% CI	OR1 95% CI	OR2 95% CI
LINE-1[a]				
<70%	1.6 0.5 - 5.0	2.1 0.5 - 9.1	1.9 0.6 - 6.0	1.6 0.4 - 6.1
70 - 74%	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
75 - 79%	1.3 0.5 - 3.1	1.5 0.5 - 4.3	1.4 0.6 - 3.2	0.8 0.3 - 2.0
≥80%	0.6 0.1 - 2.3 p=0.628	0.5 0.1 - 1.7 p=0.425	4.8 1.0 - 23.9 p=0.219	3.8 0.7 - 21.5 p=0.260
GSTP1[a]				
<5%	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
≥5%	2.8 1.0 - 7.6	5.1 1.7 - 14.9	1.5 0.6 - 3.7	2.0 0.8 - 5.3

OR1, odds ratio inherently adjusted for matching variables; OR2, odds ratio adjusted as OR1 and for time between the two biopsies, number of cores sampled at the biopsy, number of biopsies prior to the index biopsy and GSTP1/LINE-1 methylation; CI, confidence intervals.

[a]Cases without information on GSTP1 methylation and number of cores, and corresponding controls within the matching strata, were excluded from the analyses.

(Zelic et al., Clin Cancer Res 2015)

Table 3.4. LINE-1 methylation and GSTP1 hypermethylation on a negative biopsy and risk of prostate cancer diagnosis on rebiopsy stratified by time between the two samplings and Gleason score (two Wards combined).

	Time between the two samplings			
	<12 months		≥12 months	
	OR1 (95% CI)	OR2 (95% CI)	OR1 (95% CI)	OR2 (95% CI)
LINE-1[a]				
<70%	2.4 (0.6 - 9.0)	2.4 (0.5 - 12.1)	1.3 (0.5 - 3.6)	1.6 (0.4 - 5.9)
70-74%	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
75-79%	1.3 (0.5 - 3.4)	1.1 (0.4 - 3.0)	1.3 (0.6 - 3.0)	1.3 (0.5 - 3.3)
≥80%	2.8 (0.6 - 14.2)	5.0 (0.6 - 42.0)	1.3 (0.4 - 4.0)	1.0 (0.3 - 3.1)
GSTP1[a]				
<5%	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
≥5%	1.4 (0.5 - 4.0)	2.4 (0.9 - 6.4)	2.6 (1.1 - 6.1)	3.6 (1.4 - 9.2)
	Gleason score			
	≤3+4		≥4+3	
	OR1 (95% CI)	OR2 (95% CI)	OR1 (95% CI)	OR2 (95% CI)
LINE-1[a,b]				
<70%	1.2 (0.5 - 2.9)	0.8 (0.3 - 2.7)	4.3 (0.8 - 22.1)	9.2 (1.4 - 59.3)
70-74%	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
75-79%	1.3 (0.7 - 2.5)	1.2 (0.6 - 2.4)	1.1 (0.3 - 4.0)	0.8 (0.1 - 5.5)
≥80%	1.1 (0.4 - 3.5)	0.8 (0.3 - 2.5)	3.2 (0.6 - 16.5)	5.7 (0.4 - 76.1)
GSTP1[a,b]				
<5%	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
≥5%	1.8 (0.8 - 3.9)	2.2 (0.9 - 5.6)	2.7 (0.8 - 9.1)	9.2 (2.0 - 43.1)

OR1, odds ratio inherently adjusted for matching variables; OR2, odds ratio adjusted for matching variables and for time between the two biopsies, number of cores sampled at the biopsy, number of biopsies prior to the index biopsy and GSTP1/LINE-1 methylation; CI, confidence intervals.
[a]Cases without information on GSTP1 methylation and number of cores, and corresponding controls within the matching strata, were excluded from the analyses.
[b]Analysis based on subset of cases with specified Gleason score and all the controls within the matched stratum.

(Zelic et al., Clin Cancer Res 2015)

Table 3.5. Assessment of the predictive ability of models including GSTP1 methylation, LINE-1 methylation, or both markers, in comparison with the core model with no methylation markers.

Models	AIC	p-value[b]
All cases and controls		
Core model[a]	183.33	
Model 1 (LINE-1)	184.63	0.122
Model 2 (GSTP1)	177.99	0.007
Model 3 (LINE-1 + GSTP1)	178.94	0.012
Restricted to cases with Gleason score ≥4+3 and corresponding controls		
Core model	64.84	
Model 1 (LINE-1)	69.45	0.370
Model 2 (GSTP1)	63.24	0.058
Model 3 (LINE-1 + GSTP1)	62.25	0.024

AIC, Akaike Information Criterion.
[a]Core model includes time between the two biopsies, number of cores sampled at the biopsy and number of biopsies prior to the index biopsy.
[b]p-values for the comparison with the core model.

(Zelic et al., Clin Cancer Res 2015)

4. STUDY 2

"GLOBAL DNA HYPOMETHYLATION IN TUMOR TISSUE AND IN NON-NEOPLASTIC TISSUE ADJACENT TO TUMOUR AS A POSSIBLE PROGNOSTIC BIOMARKER FOR PROSTATE CANCER"

This study aimed at evaluating the potential of global DNA hypomethylation, measured in prostate tumor tissue as well as in non-neoplastic tissue adjacent to tumor, as a predictor of prostate cancer-specific mortality.

Specifically, we wanted to:

1. Estimate the association between decreased global DNA methylation level in tumor tissue and prostate cancer-specific mortality to explore its value as a possible prognostic marker;
2. Study the global DNA methylation level in non-neoplastic tissue adjacent to tumour to understand if this epigenetic alteration could be an early marker of prostate cancer development, and evaluate if it is correlated with risk of death from prostate cancer;
3. Evaluate if global DNA hypomethylation in tumor and in non-neoplastic tissue adjacent to tumor is associated with Gleason score.

We estimated the loss of global DNA methylation in terms of methylation levels of the promoter of the retrotransposon LINE-1, a widely used approach since LINE-1 elements are abundant, globally distributed along the genome and heavily methylated. Evaluation of LINE-1 methylation level can serve as a reliable surrogate marker for global genomic methylation [Lisanti et al., 2013].

The study was approved by the Local Ethical Committee.

4.1 PRELIMINARY DATA

This cohort study represent a further step of our ongoing project on the role of epigenetic alterations as possible prognostic biomarkers for prostate cancer. In our first study [Richiardi et al., 2009] we studied the methylation status of the promoter of three selected genes to understand their potential value as markers of prostate cancer progression. Genes have been selected on the basis of the previously reported association between their aberrant methylation status and clinical features of poor prognosis in prostate cancer patients. The study involved

two indipendent cohorts of, in total, 459 consecutive prostate cancer patients diagnosed between 1982 and 1988 (1980s cohort) or between 1993 and 1996 (1990s cohort), at the pathology ward of the San Giovanni Battista Hospital, Turin, Italy.

Patients were followed up from the date of the pathology report to February 2006 for the 1980s cohort, and to January 2007 for the 1990s cohort. Information on vital status at the end of the follow up and copies of the death certificates came from the demographic offices: information from death certificates were used to classify the cause of death as either prostate cancer, or other causes. Dead patients were censored on their date of death. For each patient we had available some clinical information (age, source of tumor tissue, place of residence and tumor grade) retrieved from pathology reports. We did not have information on PSA levels or other clinical characteristics. For each patient archival formalin-fixed paraffin-embedded tumor tissue, withdrawn through biopsy, TURP or prostatectomy procedure, was available; diagnostic slides were re-evaluated by a single pathologist in order to assign a uniform Gleason score according to current guidelines.

We studied the prostate cancer survival in association with promoter hypermethylation in GSTP1, APC, and RUNX3: we found that hypermethylation of APC promoter is associated with prostate cancer mortality, particularly among those with a highly to moderately differentiated tumor (Gleason score <8). A similar association was found for hypermethylation in RUNX3 in patients diagnosed during the 1990s and, in the two cohorts combined, the risk of death from prostate cancer increased with increasing number of methylated genes.

The second study [Richiardi et al., 2013] was nested in the first and involved 157 prostate cancer patients of the previous cohort who had available, in their FFPE tissue blocks, well-recognizable areas of non-neoplastic tissue adjacent to tumor (NTAT), identified and highlighted by the uropathologist by re-analyzing the diagnostic slides. In this study, the follow up of the patients has been extended until August 2010. Apart from patients for who we could not retrieve non-neoplastic tissue adjacent to tumor in any diagnostic slide, we also excluded a-priori patients diagnosed during the 1980s who underwent biopsy procedure. The assumption was that, since during the pre-PSA era prostate cancer diagnosed were bigger and more advanced that those diagnosed during the 1990s, their diagnostic slides included mainly neoplastic tissue and were associated with considerable technical problems in isolating NTAT without contamination from tumoral tissue.

We evaluated the methylation status of GSTP1 and APC promoter in NTAT to assess first if aberrant methylation is already detectable in the apparently healthy tissue near to the tumor, according to the hypothesis of the “field cancerization”, and also whether these molecular

changes are potential candidates as prognostic markers by testing their association with mortality from prostate cancer. We found that hypermethylation of APC and GSTP1 occurred in the 40-45% of NTAT, supporting the hypothesis that DNA hypermethylation is an early event in carcinogenesis that can be detected before the tumour becomes morphologically evident. Hypermethylation of APC and GSTP1 correlated with methylation pattern in prostate tumour tissue, in line with the notion of field cancerisation in prostate cancer. More importantly, gene-specific hypermethylation was strongly associated with mortality from prostate cancer, further supporting the hypothesis on the role of aberrant hypermethylation in prostate cancer progression.

4.2 STUDY POPULATION AND AVAILABLE DATA

This cohort study was carried out on the 157 prostate cancer patients who have been previously selected in the second nested study [Richiardi et al., 2013]. Specifically, the study involved patients with prostate cancer diagnosed between 1982 and 1988 who underwent TURP or radical prostatectomy procedures, and patients diagnosed between 1992 and 1996 who underwent biopsy, TURP or radical prostatectomy procedures, conducted at the Pathology Ward of the San Giovanni Battista Hospital, Turin, Italy.

All the clinical data available for the 157 prostate cancer patients involved in the study are summarized below:

- ✓ Clinical information (age, source of tissue, place of residence and tumor grade) from pathology reports;
- ✓ Gleason score re-evaluated by a single uropathologist according to current guidelines;
- ✓ Life status at the end of the follow up (until August 2010);
- ✓ Cause of death from death certificates.

In addition, molecular data on methylation status of the promoter of some selected genes have been provided by the previous studies:

- ✓ APC, GSTP1 and RUNX3 promoter methylation in tumor tissue;
- ✓ APC and GSTP1 promoter methylation in non-neoplastic tissue adjacent to tumor.

Since that, for a part of the subjects, DNA extracted from their NTAT tissue has been terminated during the previous study, we overcome the limitation of incomplete values of

LINE-1 methylation level in NTAT using the multiple imputation, an approach previously employed in literature to impute missing molecular data [van der Heijden, 2006; Leu et al., 2013].

4.3 METHODS

4.3.1 Molecular methods

Genomic DNA was previously extracted and purified from FFPE tissues of tumor [Richiardi et al., 2009] and correspondent NTAT [Richiardi et al., 2013] using the QIAamp ® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), and underwent fresh bisulfite modification using the Epitect Bisulfite Kit (Qiagen), along with fully methylated and unmethylated synthetic controls (CpGenomeTM universal methylated DNA and CpGenomeTM universal unmethylated DNA, Chemicon Co. Billerica, MA, USA.), that were included in each modification set. For 53 patients DNA extracted from NTAT was not available, therefore we performed molecular analyses on their -80°C-stored bisulfite modified DNA saved by the previous research [Richiardi et al., 2013]

After DNA conversion, analysis of LINE-1 (GenBank accession number X58075) promoter methylation status was performed using PyroMark Q24 MDx (Qiagen, Hilden, Germany). Primers, which amplify a 98-bp sequence of LINE-1 promoter containing three CpG sites (positions 819, 826 and 829), were designed outside the CpG sites using PyroMark Assay Design software version 2.0.6 (Qiagen) as follows: forward 5'-TTTGAGTTAGGTGTGGGATATAGTT-3', reverse 5'-Biot-CACCTAAAAAATCCAATCACTCC- 3' and sequencing 5'-TTAGGTGTGGGATATAGTTT-3'. LINE-1 promoter methylation status was analyzed both in tumoral tissue and in NTAT. PCR reaction was performed in a total volume of 30 µl containing 1X buffer (KCl), 2 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 µM of each primer, 0.05 U Taq polymerase and 6 µl of bisulfite-converted DNA with the following cycling profile: 95°C for 10' followed by 45 cycles of denaturation at 95°C for 30'', annealing at 55°C for 1', extension at 72°C for 1' and final extension at 72°C for 10'. 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 of 25 µl, including 24.85 µl of 20 mM Tris-Acetate, 5 mM $MgAc_2$ and 0.15 µl of 50 µM sequencing primer (final concentration 0.3 µM). Pyrosequencing methylation assay was created according to the manufacturer's instruction and was set up using as the sequence to analyze and the

dispensation order C/TGTGGTGC/TGTC/TG and GTCGTAGATAGTCAGATC, respectively. Positive controls for fully methylated and fully unmethylated status were included in each pyrosequencing run. Methylation quantification was achieved using the provided software, and expressed for each DNA locus as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines.

4.3.2 Statistical analyses

Correlation among the three CpG sites analyzed in LINE-1 promoter was high both in the tumor tissue and in the NTAT (pairways correlation coefficients were always > 0.83). We, therefore, combined the information for the three CpG sites using the mean level of methylation. Using factor analysis would have identified one principal component both in the tumor and in the NTAT explaining more than 90% of the variance.

LINE-1 methylation in the tumor tissue and in the NTAT were treated as a continuous variables, with no logarithmic transformation as this transformation did not improve normality. We also created categorical variables with three levels of LINE-1 methylation.

We used multivariate linear regression to estimate the association between selected characteristics and level of tumor tissue methylation in LINE-1. Normality was satisfied as indicated by tests based on skewness and Kurtosis (p=0.15) We used Cox proportional hazard regression models to estimate the hazard ratio (HR) of mortality from prostate cancer in association with LINE-1 methylation in the tumor tissue. Time from diagnosis was used as the time axis, while age and year of diagnosis were introduced as a continuous variable. The proportional hazard assumption was met as indicated by test based on Schoenfeld residuals (p =0.34). Models were always adjusted for the source of tumor tissue and progressively adjusted for tumor tissue methylation in APC and GSTP1 and Gleason score (variables categorized as shown in Table 4.1). Analogue analyses were performed by stratifying by tumor tissue and Gleason score (<8, 8+) respectively.

Analyses of LINE-1 methylation in NTAT were restricted to biopsies and TURPs (105 patients out of 157). Since data on LINE-1 methylation level in NTAT from freshly-modified stored DNA were available only for 50 patients (~50%), we performed a multivariate imputation by chained equations (MICE) approach by assuming the data. Specifically, MICE approach was adopted assuming the data were missing at random [Rubin, 1976; Raghunatan and Bondarenko; 2007]. The imputation model involved regression of the LINE-1 methylation level in NTAT from freshly-modified stored DNA on diagnosis year, age at diagnosis, source of tissue, Gleason score, number of methylated genes out of APC and GSTP1 in the tumour

tissue and in the NTAT, LINE-1 tumor methylation level, LINE-1 methylation level in NTAT from stored bisulfite-modified DNA and the survival data, including the event indicator, the cumulative hazard at the time of entry for the event actually experienced, and the difference between the estimated cumulative hazard at the exit time and that at entry [White and Royston, 2009]. After fitting the imputation model, imputed values for the missing data were created using predictive mean matching.

Similarly to the approach adopted for tumor tissue LINE-1 methylation, for each imputed dataset (n=20) we performed a multivariate linear regression to identify the factors associated with LINE-1 methylation in NTAT and Cox regression to estimate the association between NTAT LINE-1 methylation and prostate cancer-specific mortality. The survival analysis was further extended by estimating the association for different strata defined by Gleason score (<8,8+). The obtained estimates were then combined into overall estimates, with standard errors, confidence intervals and p-values calculated using Rubin's rule [Rubin, 1987]. Finally we compared the mean LINE-1 methylation level in NTAT and in tumor tissue restricted to subjects with biopsy or TURP by Wilcoxon test for paired data and the Spearman correlation coefficient. This analysis was furtherly stratified by tumor tissue.

4.4 RESULTS

Selected characteristics of the 157 prostate cancer patients are listed in Table 4.1. The majority of the study subjects have been diagnosed during the 1990s, with a median survival of 6.79 years. Of the 128 deaths that occurred during the follow up, 43 were prostate cancer-specific. The source of tissue was equally distributed among biopsy, TURPs and radical prostatectomy. An higher proportion of both APC and GSTP1 promoter hypermethylation in tumor tissue (73.2%) can be noticed, while the higher proportion of the NTAT (42.7%) presents an unmethylated status.

In Table 4.2 are reported the results of the analysis of association between selected characteristics of the tumor and LINE-1 methylation level measured in tumor tissue. Five patients have been excluded from this analysis due to missing data, thus leaving 152 prostate cancer patients for the following evaluations. LINE-1 methylation level was significantly higher in TURP tissue compared to biopsies and radical prostatectomies. Neither Gleason score nor hypermethylation of APC and GSTP1 in tumor tissue were associated with LINE-1 methylation, although the direction of the association with gene-specific hypermethylation was, if anything, inverse.

There was a weak inverse association between LINE-1 methylation level and mortality from prostate cancer (HR2=1.07; 95% CI, 0.99-1.15, per each decrease in 1% of LINE-1 methylation level) (Table 4.3), that remained also in analyses stratified by source of tumor tissue (Table 4.4), indicating no heterogeneity in different type of samplings.

When LINE-1 methylation value was categorized (Table 4.3) we found evidence, though not statistically significant, of increased risk for prostate cancer mortality especially for lower levels of LINE-1 methylation (methylation level <75%) (HR1=2.07; 95% CI: 0.71-6.02). This indication of association remained after adjustment for Gleason score and methylation of APC and GSTP1 (HR2=2.22; 95% CI: 0.75-6.58).

When we stratified the analyses by Gleason score (<8 and 8+) (Table 4.5), LINE-1 methylation level remained higher in TURPs.We found an indication of inverse association between LINE-1 hypomethylation and gene-specific hypermethylation, but only in high-risk prostate cancer patients (Gleason score ≥ 8) (adjusted β = -2.06; 95% CI: -5.17, 1.06) (Table 4.5). Inverse association beween LINE-1 methylation level and mortality from prostate cancer has been conserved only in analyses restricted to patients with Gleason score 8+, (HR2=1.12; 95% CI 1.00-1.24, for each decrease in 1% of LINE-1 methylation level), with a stronger effect for low levels of LINE-1 methylation (LINE-1 methylation level <75% HR2=4.68; 95% CI 1.03-21.34, adjusted); whereas LINE-1 methylation was not associated with prostate cancer-specific mortality of patients with Gleason score <8 (Table 4.6), as also well-described by the cubic splines (Figure 4.1). After exclusion of the 49 subjects who underwent prostatectomy, the analyses of association involving LINE-1 methylation level in NTAT have been performed on 105 prostate cancer patients. As reported in Table 4.7, LINE-1 methylation level was significantly higher in NTAT dissected by TURPs, compared to that retrieved from biopsies (adjusted β = 3.71; 95% CI: 1.73, 5.69), and was associated neither with Gleason score nor with hypermethylation of APC and GSTP1.

LINE-1 methylation level measured in NTAT was not associated with prostate cancer mortality (Table 4.8), and similar results were obtained in analyses stratified by source of tissue and by Gleason score (data not shown in tables). Mean LINE-1 methylation measured in tumor and in NTAT showed similar levels (79.20±4.13% and 79.27±3.87%, respectively), also when we stratified for source of tissue (data not shown in tables).

4.5 DISCUSSION

Hypermethylation of prostate cancer-related genes and LINE-1 hypomethylation, considered as surrogate of global DNA hypomethylation, have been reported as crucial epigenetic

mechanisms involved in development and progression of prostate cancer; therefore, they have been considered as potential biomarkers for prostate cancer diagnosis and prognosis [Valdés-Mora and Clark, 2015]. In this study, we evaluated the prognostic potential of LINE-1 methylation level, since multiple studies have repeatedly described its association with clinicopathological features of poor prognosis [reviewed by Zelic et al., 2015a].

We found an indication of association between low levels of LINE-1 methylation measured in tumor tissue and mortality from prostate cancer, that in patients with high-risk prostate cancer (Gleason score 8+) became stronger. This association was not present neither in patients with Gleason score <8 nor when LINE-1 methylation was measured in the NTAT.

Our results also pointed out a difference in LINE-1 methylation level among different sources of tissue. Both tumor tissue and NTAT retrieved from TURP showed higher mean percentages of LINE-1 methylation, compared to those measured in biopsies and radical prostatectomies, thus suggesting possible molecular differences among these types of samplings. In general, TURP procedure is performed to remove an obstruction of the lower urinary tract caused by a benign prostate hyperplasia, therefore it withdraws tissue portions from the anterior part of the prostatic gland through the urethra, namely transition zone [McVary et al., 2011].

Some previous investigations indicate that tumors originating from the transition zone show some differences in morphology [Greene et al., 1991], in biology (in terms of clinicopathological features) [Lee et al., 2015] and in molecular patterns, particularly for gene expression profiling [Sinnott et al., 2015] and for microRNA expression signature [Carlsson et al., 2013], compared to those growing in the peripheral zone. Now, our data suggest that tumors originating from the transition zone could also present a different epigenetic pattern, i.e. higher levels of LINE-1 methylation. A possible hypothesis could be that cancers arising from different zones of the prostatic gland may be differentialy susceptible to acquisition of genetic and epigenetic alterations, which might in part explain their consistent differences in prognosis, volume and other clinical variables, sugh as serum PSA level [Lee et al., 2015].

Higher LINE-1 methylation levels detected in NTAT from TURPs should further support the hypothesis that morphologically normal transition zone has a different molecular pattern from peripheral tissue [Van der Heul-Nieuwenhuijsen et al., 2006; Noel 2008]. Therefore, we recommend to take into account this difference in studies aiming to measure LINE-1 methylation level in prostate tissue: samplings coming from different zones should be considered separately in the analyses to avoid biases due to the source of tissue.

This was also the reason why, from the analyses of association involving LINE-1 methylation level in NTAT, the 49 subjects who underwent radical prostatectomy have been excluded. This

was due to the high suspiction that the prostatectomy tissue contained an indistinguishable admixture of both transition and peripheral tissue, that have significantly different LINE-1 methylation levels. Therefore, contamination of the prostatectomy by tissue coming from transitional zone could modify the levels of LINE-1methylation. This assumption was further confirmed by the quantification of the mean LINE-1 methylation level in NTAT retrieved by the prostatectomies, that was found to be intermediate between that measured in biopsies and that in TURPs (data not shown in tables). Since we could not exclude with certainty a possible contamination by transition tissue of the identified NTAT even re-evaluating the diagnostic slide, we had to exclude all the radical prostatectomies from the analyses.

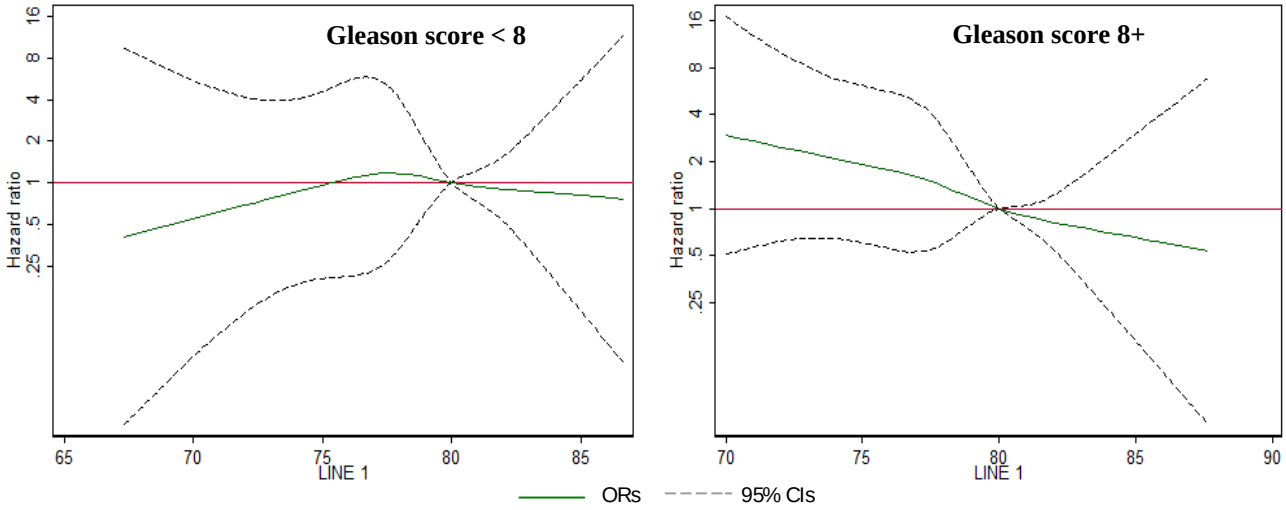
To our knowledge this is the first study that explored the relationship between LINE-1 methylation status and mortality from prostate cancer. Our data support the possible involvement of global hypomethylation in tumor progression. There is an indication of association between LINE-1 methylation level and prostate cancer-specific mortality, that is already visible for intermediate levels of LINE-1 methylation (75-80%), but is particularly evident for drastic reductions in methylation percentage (<75%): this estimate is not statistically significant, probably because of the limitated sample size (N=152) and of the low number of events (N=18) present in the subgroup with marked LINE-1 hypomethylation. We could hypothesize that only a high derepression, via hypomethylation, of LINE-1 promoter could induce general disregulation involving chromosomal instability, reactivation of repeated sequences and oncogenes, as already demostrated *in vivo* [Howard et al., 2008]. The direction of this association has been conserved after adjusting for Gleason score, indicating that LINE-1 hypomethylation might involved in prostate cancer progression through biological mechanisms that are in part different from that captured by the Gleason score. Furthermore, this effect on mortality from prostate cancer is relevant only for patients with high-risk prostate cancer: a possible explanation is that drastic LINE-1 hypomethylation is involved only in very later stages of the disease, even more late than those we previously thought, when many other molecular alterations (captured by the high Gleason score) have already occurred, thus leading to a wide dysregulation. On the contrary, in less aggressive tumors in which this global disregulation did not occurr, even low LINE-1 methylation level has any prognostic significance. According to this hypothesis, it might be speculated that low levels of LINE-1 methylation are not able to distinguish indolent from aggressive cancer, rather they seem to have a potential in distinguish lethal prostate cancer from aggressive disease. Furthermore, the observation that even low levels of LINE-methylation in NTAT were not associated with mortality from prostate cancer give further strenght to the hypothesis that LINE-1

hypomethylation is involved only in very later stages of the disease. Also, our results suggest that gene-specific hypermethylation and global DNA hypomethylation coexist in tumor tissue, expecially in the most aggressive, but not in NTAT. Our data reinforce the previously proposed model of prostate cancer as the results of an epigenetic catastrophe [Perry et al., 2010], where not only hypermethylation of specific genes involved in prostate tumorigenesis contributes to disease progression, but also global hypomethylation may play a relevant role.

In conclusion, our data suggest that in prostate cancer gene-specific hypermethylation and global DNA hypomethylation coexist, expecially in high-grade tumors. Our results did not suggest that LINE-1 hypomethylation is an early event in prostate tumor: rather, LINE-1 hypomethylation seems to be a late epigenetic mechanism implicated in prostate cancer progression. Specifically, low levels of LINE-1 methylation appear to have a strong effect on prostate cancer-specific mortality only in patients with high Gleason score. For these reasons, it could be speculated that LINE-1 hypomethylation might be useful to distinguish into the group of the high-risk prostate cancers those with high risk of death.

4.6 TABLES AND FIGURES

Figure 4.1. Association between LINE-1 methylation level in tumor tissue and mortality from prostate cancer by stratifying for Gleason score.



Cubic splines with four internal knots are defined by the 20,40,60,80 percentile. Results have been reported graphically using the methylation value of 80% as the reference.
Estimates have been adjusted for age at diagnosis, year of diagnosis and source of tumor tissue.

(Fiano et al., in preparation)

Table 4.1. Selected characteristics of the study patients.

Characteristics	Number	(%)
Year of diagnosis		
1982-1988	28	(17.8%)
1993-1996	129	(82.2%)
Range survival time (years)	0.03-24.11	
Median survival time (years)	6.79	
Age at diagnosis (years)		
40-64	26	(16.6%)
65-69	35	(22.3%)
70-74	40	(25.5%)
75+	56	(35.7%)
Mortality		
Overall	128	(81.5%)
From prostate cancer	43	
From other causes	85	
Source of tumour tissue		
Biopsy	54	(34.4%)
TURP	54	(34.4%)
Radical prostectomy	49	(31.2%)
Gleason score		
<7	59	(37.6%)
7	41	(26.1%)
≥8	57	(36.3%)
N. genes methylated in prostatic tumour tissue (out of APC and GSTP1)		
0	11	(7.0%)
1	31	(19.8%)
2	115	(73.2%)
N. genes methylated in NTAT (out of APC and GSTP1)		
0	67	(42.7%)
1	45	(28.7%)
2	45	(28.7%)

(Fiano et al., in preparation)

Table 4.2. Selected characteristics in association with LINE-1 methylation in the tumor tissue.

Characteristic	Mean Line-1 (Sd)	Coefficient*	95% CI
All patients (N=152)	78.55 (4.29)	-	-
Source of tumor tissue			
Biopsy	77.52 (4.32)	Ref	
TURP	80.72 (3.42)	2.91	1.23, 4.59
Radical prostectomy	77.32 (4.26)	-0.64	-2.38, 1.11
Gleason score			
<7	77.95 (3.63)	Ref	
7	78.26 (4.89)	0.37	-1.34, 2.08
≥8	79.36 (4.29)	0.41	-1.21, 2.04
APC and GSTP1 methylation in tumour tissue			
Continuous: 0,1,2 methylated genes	78.55 (4.29)	-0.37	-1.54, 0.80

*Adjusted for age at diagnosis, year of diagnosis, and variables listed in the Table.

(Fiano et al., in preparation)

Table 4.3. LINE-1 methylation level in tumor tissue in association with mortality from prostate cancer.

LINE-1 methylation	Prostate cancer deaths (N)	HR1 (95% CI)[a]	HR2 (95% CI)[a]
Decrease in 1% methylation	41	1.05 (0.97-1.14)	1.07 (0.99-1.15)
Categorical			
<75	18	2.07 (0.71-6.02)	2.22 (0.75-6.58)
75-80	6	1.47 (0.73-2.95)	1.60 (0.81-3.18)
80+	17	1.00	1.00

[a] HR1, hazard ratio adjusted for age and year of diagnosis and source of tumor tissue; HR2, adjusted as HR1, Gleason score and APC and GSTP1 methylation in the tumor tissue; CI, confidence intervals.

(Fiano et al., in preparation)

Table 4.4. LINE-1 methylation level in tumor tissue in association with mortality from prostate cancer, stratified by type of tumor tissue.

LINE-1 methylation	Prostate cancer deaths (N)	HR1 (95% CI)[a]	HR2 (95% CI)[a]
Biopsy			
Decrease in 1% methylation	14	0.98 (0.87-1.11)	1.04 (0.92-1.18)
Radical prostatectomy			
Decrease in 1% methylation	6	1.09 (0.95-1.26)	1.09 (0.95-1.26)
TURP			
Decrease in 1% methylation	21	1.09 (0.89-1.33)	1.10 (0.89-1.35)

[a] HR1, hazard ratio adjusted for age and year of diagnosis; HR2, adjusted as HR1, Gleason score and APC and GSTP1 methylation in the tumor tissue; CI, confidence intervals.

(Fiano et al., in preparation)

Table 4.5. Selected characteristics in association with LINE-1 methylation in the tumor tissue stratified by Gleason score.

Characteristic	Mean Line-1 (Sd)	Coefficient*	95% CI
Patients with Gleason score <8 (N=96)	78.08 (4.17)	-	-
Source of tumor tissue			
Biopsy	77.38 (4.31)	Ref	
TURP	80.61 (3.08)	3.04	0.80, 5.28
Radical prostectomy	77.27 (4.09)	-0.47	-2.50, 1.57
APC and GSTP1 methylation in tumour tissue			
Continuous: 0,1,2 methylated genes	78.08 (4.17)	-0.03	-1.28, 1.22
Patients with Gleason score 8+ (N=56)	79.36 (4.40)	-	-
Source of tumor tissue			
Biopsy	77.83 (4.46)	Ref	
TURP	80.80 (3.70)	2.57	-0.21, 5.35
Radical prostectomy	77.50 (5.11)	-2.03	-6.06, 2.00
APC and GSTP1 methylation in tumour tissue			
Continuous: 0,1,2 methylated genes	79.36 (4.40)	-2.06	-5.17, 1.06

*****Adjusted for age at diagnosis, year of diagnosis, and variables listed in the Table.

(Fiano et al., in preparation)

Table 4.6. LINE-1 methylation level in tumor tissue in association with mortality from prostate cancer stratified by Gleason score.

LINE-1 methylation	Prostate cancer deaths (N)	HR1 (95% CI)[a]	HR2 (95% CI)[a]
Patients with Gleason score<8 (N=96)			
Decrease in 1% methylation	13	0.98 (0.85-1.13)	0.99 (0.86-1.13)
Categorical			
<75	6	0.91 (0.15-5.38)	0.83 (0.14-4.96)
75-80	5	0.74 (0.20-1.72)	0.67 (0.17-2.59)
80+	2	1.00	1.00
Patients with Gleason score 8+ (N=56)			
Decrease in 1% methylation	28	1.11 (1.00-1.23)	1.12 (1.00-1.24)
Categorical			
<75	11	4.33 (1.02-18.39)	4.68 (1.03-21.34)
75-80	13	1.98 (0.85-4.64)	2.03 (0.85-4.86)
80+	4	1.00	1.00

[a] HR1, hazard ratio adjusted for age and year of diagnosis and source of tumor tissue; HR2, adjusted as HR1 and APC and GSTP1 methylation in the tumor tissue; CI, confidence intervals.

(Fiano et al., in preparation)

Table 4.7. Selected characteristics in association with LINE-1 methylation in the NTAT

Characteristic	Mean Line-1 (Sd)*	Coefficient*	(95% CI)
All patients (N=105)	79.27 (3.87)	-	-
Source of tumor tissue			
Biopsy	77.37 (3.32)	Ref	
TURP	81.12 (3.48)	3.71	1.73, 5.69
Gleason score			
<7	78.04 (3.58)	Ref	
7	79.67 (3.98)	1.19	-1.27, 3.65
≥8	79.95 (3.89)	0.34	-1.75, 2.43
APC and GSTP1 methylation in NTAT			
Continuous: 0,1,2 methylated genes	79.20 (4.13)	0.33	-0.89, 1.56

*Adjusted for age at diagnosis, year of diagnosis, and variables listed in the Table.

(Fiano et al., in preparation)

Table 4.8. LINE-1 methylation in the NTAT and mortality from prostate cancer**.**

LINE-1 methylation	Prostate cancer deaths (N)	HR1 (95% CI)[a]	HR2 (95% CI)[a]
Decrease in 1% methylation	37	0.96 (0.83-1.12)	0.97 (0.84-1.12)
Categorical	*N**		
<1st tertile (<75)	3.45	0.57 (0.05-6.60)	0.71 (0.06-7.89)
1st to 2nd tertile (75-80)	17.4	0.91 (0.33-2.47)	0.79 (0.30-2.09)
2nd+ tertile (80+)	16.15	1.00	1.00

[a] HR1, hazard ratio adjusted for age and year of diagnosis and source of tumor tissue; HR2, adjusted as HR1, Gleason score and APC and GSTP1 methylation in the NTAT tissue; CI, confidence intervals.
***** Failure's average among imputed datasets.

(Fiano et al., in preparation)

5. STUDY 3

"EVALUATION OF DNMT3b VARIANTS IN ASSOCIATION WITH GENE-SPECIFIC HYPERMETHYLATION, GLOBAL DNA HYPOMETHYLATION, GLEASON SCORE AND PROSTATE CANCER"

In this study we performed the genotyping of three selected DNMT3b genetic variants on two cohorts of prostate cancer patients with two specific aims:

1. To assess the associations between DNMT3b polymorphisms and LINE-1 methylation level, as well as with APC and GSTP1 hypermethylation, both measured in tumour tissue, to understand if these variants could affect the methylation pattern in prostate cancer cells;
2. To evaluate the direct effect of DNMT3b polymorphisms on tumour aggressiveness (as measured by the Gleason score) and on mortality from prostate cancer, to understand their possible involvement in prostate cancer progression.

The study was approved by the Local Ethical Committee.

5.1 PRELIMINARY DATA

In our ongoing project on epigenetic alterations and prostate cancer we have explored, in a large cohort of consecutive prostate cancer patients (for cohort characteristics see section 4.1), the effect of DNMT3b variant rs406193 (C>T) on methylation status of the promoter of APC and GSTP1 in tumor tissue, on the Gleason score of the tumor and on mortality from prostate cancer.

Specifically, to better understand the possible interplay between the variables under study, a model of causal relationships has been proposed, under some specific assumptions:

i) selected variants can affect DNMT3b enzymatic activity;

ii) DNMT3b activity can affect methylation status in tumor tissue;

iii) aberrant methylation status can affect tumor morphology and thus the Gleason score;

iv) aberrant methylation affects mortality both directly and via Gleason score.

Despite we did not found evidence of association between T carriers and number of hypermethylated genes, however, T carriers had a reduced risk of a Gleason score 8+ (OR=0.57, 95 % CI 0.39-0.85), and a hazard ratio of 0.81 (0.61-1.09) of dying from prostate cancer. These results not only provided new insights on the involvement of DNMT3b variants in prostate cancer progression, but they also suggested that to provide a reliable estimate of the

association between hypermethylation tumor tissue and prostate cancer mortality it is very important to consider the possible causal relationships between the involved variables [Gillio Tos et al., 2012].

In the current study, to increase the complexity of this model, we analysed three additional DNMT3b polymorphisms on two cohorts of patients belonging to the original cohort of 459 subjects with prostate cancer described in section 4.1.

5.2 STUDY DESIGN AND CHARACTERISTICS OF THE TWO COHORTS

This cohort study was carried out on two cohorts of prostate cancer patients for validation purposes. Subjects of these two cohorts come from the cohort of 459 consecutive prostate cancer patients who underwent biopsy, radical prostatectomy or TURP between 1982 and 1996, at the pathology ward of the San Giovanni Battista Hospital, Turin, Italy, described previously in details (Section 4.1). The first cohort, hereafter referred as "adjacent tissue cohort", is composed by the same prostate cancer patients involved in the STUDY 2 (for summary of the characteristics of the cohort see section 4.2). The second cohort, hereafter referred as "1980-biopsy cohort" included prostate cancer patients diagnosed during the 1980s at the San Giovanni battista Hospital, Turin, who underwent only prostate biopsy procedure, hence have been excluded from the STUDY 2. While for patients of the 1980-biopsy cohort only one type of sampling was available (i.e. biopsies), for the adjacent tissue cohort biopsy, TURP and radical prostatectomy samples for patients diagnosed during the 1990s, and TURPs and radical prostatectomies for those diagnosed during the 1980s were available. Clinical data for each patient have been listed before (section 4.1). For both cohorts methylation data of APC, GSTP1 and RUNX in tumor tissue have been provided by a previous study [Richiardi et al., 2009]. In addition, only for adjacent tissue cohort patients, LINE-1 methylation data in tumor tissue were available (STUDY 2).

5.3 METHODS

5.3.1 Selection of SNPs for genotyping

In our first study [Gillio Tos et al., 2012] the polymorphism rs406193 was chosen on the basis of the association of its variant T with a reduced risk of breast and colorectal cancer in women. DNMT3b maps at locus q11.2 on chromosome 20 and several polymorphisms affecting its expression, DNA methylation activity and suceptibility to cancer have been identified [reviewed by Duan et al., 2015]. The most extensively studied DNMT3b polymorphism is rs2424913 (-149C $\rightarrow$ T), an intron variant located -149 bp from the transcription start site

(TSS). The T variant has been shown to result in a 30% increase in promoter activity in lung cancer cells [Shen et al., 2002], and also in pancreatic cancer cells (3.8-fold), thus enhancing its transcription [Xiao et al., 2011] and inducing methylation changes at the promoters of different genes [Kawakami et al., 2006] Furthermore, carriers of the T allele have been reported to have significantly increased risk of several types of cancers [Duan et al., 2015], and it has been also correlated to poor prognosis for head and neck cancer [Azad et al., 2012]. However, as recently reviewed, published data on the role of rs2424913 in cancer susceptibility remain inconclusive [Zhu et al., 2015].

Rs1569686 (G → T) is an intron variant mapping in the promoter of DNMT3b, -579 bp from the TSS of exon 1B. The allele G has been shown to decrease susceptibility to colorectal, gastric and lung cancer, and to increase susceptibility to acute myeloid leukemia [Duan et al., 2015]. Furthermore, it has been associated with poor prognosis for gastric cancer [Wang et al., 2015]. The functional role of the DNMT3b rs1569686 polymorphism is not yet completely elucidated. Some authors suggest that it can directly impair promoter activity and gene expression levels by inducing changes in transcription factor binding affinity, and others observed a linkage disequilibrium (LD) between this SNP and other DNMT3b promoter polymorphisms, which have been functionally associated with promoter activity and gene expression levels. These observations suggest that this polymorphism might either have a functional role, or be a tag SNP of other functional haplotypes [Coppedè et al., 2013].

The third selected SNP, rs2424932 (G→A), is a 3' UTR variant repeatedly reported to increase susceptibility to developing schizophrenia and to suicide attempts in psychiatric patients. Its role in cancer has been poorly explored, however, it has been chosen as a Tagging SNP with predicted functionality in multiple studies [Mostowska et al., 2013; Murphy et al., 2013].

In summary, we performed the genotyping of three new SNPs in the DNMT3b gene, rs2424913, rs1569686 and rs2424932, located in noncoding regions: although they do not translate protein, these regions may have a predictive role in gene expression and transcription regulation. Indeed, apart from their correlations with a wide range of cancers and with other human pathologies, the previously described evidences suggest that these polymorphisms might be potentially able to change the DNMT3b gene expression by acting on the promoter activity and on the transcription factor binding, thus altering the enzymatic levels of the methyltransferase. Futhermore, these SNPs show strong evidences of historical recombination with the rs406193 polymorphisms, already studied by our group [Gillio tos et al., 2012].

Location of the selected variants along the DNMT3b gene is shown in Figure 5.1.

5.3.2 Molecular analyses

Genomic DNA extracted from FFPE blocks of prostate cancer patients of the two cohorts was already available from previous studies [Richiardi et al., 2009; Gillio Tos et al., 2012]. Genotyping of the three DNMT3b SNPs was carried on by pyrosequencing analysis using PyroMark Q24 MDx (Qiagen, Hilden, Germany). Primers for genotyping of each SNP, have been designed using PyroMark Assay Design software version 2.0.6 (Qiagen). Primer sequences used to analyze each SNP are reported in Table 5.1.We performed PCR reaction in a total volume of 30 µl containing 1X buffer (KCl), 2 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 µM of each primer, 0.05 U Taq polymerase and 1-2 µl of genomic DNA with the following cycling profile: 95°C for 10' followed by 45 cycles of denaturation at 95°C for 30'', annealing for 30'' at 62°C for rs2424913, at 54°C for rs1569686 and at 61°C for rs2424923, extension at 72°C for 1' and final extension at 72°C for 10'. 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 of 25 µl, including 24.85 µl of 20 mM Tris-Acetate, 5 mM $MgAc_2$ and 0.15 µl of 50 µM sequencing primer (final concentration 0.3 µM). Pyrosequencing genotyping assays were created according to the manufacturer's instruction setting a SNP assay for each polymorphism. Sequence to analyze and dispensation order used to analyze each SNP are reported in Table 5.1.

5.3.3 Statistical analyses

All analyses were first conducted in the 1980s biopsy cohort and then replicated in the adjacent tissue cohort (except for the analysis on LINE-1 methylation level, which was performed only in the adjacent tissue cohort).

For each of the three DNMT3b variants Hardy-Weinberg equilibrium was tested [Cleves, 2005], and the R-squared (R^2) statistics of linkage disequilibrium was calculated and plotted using *pwld* command (available from http://www-gene.cimr.cam.ac.uk/clayton) for both cohorts. We inferred haplotypes of DNMT3b by using the PHASE algorithm (version 2.1, Matthew Stephens Lab, University of Chicago, Chicago, IL, USA).

We used dominant and recessive models of inheritance to estimate the associations between single genotypes and haplotypes and different outcomes: i) number of methylated genes; ii) LINE-1 methylation level; iii) Gleason score; iiii) prostate cancer mortality. We used ordinal logistic regression to estimate the odds ratio and corresponding 95% confidence intervals of the

effect of each DNMT3b variant on the number of methylated genes. We used three levels for the dependent variable (0–1, 2 or 3 methylated genes out of APC, GSTP1, and RUNX3), and the odds ratios estimated in this model should be interpreted as the effect of single genetic variants/haplotypes on the increase in the number of methylated genes.

Linear regression was used to estimate the coefficients and corresponding 95% CIs of the effect of each SNP/haplotype on the LINE-1 methylation level: as LINE-1 methylation was analyzed at more than 1 CpG site, mean methylation level across the CpG sites was used in the analyses. Logistic regression was used to estimate the OR and the corresponding 95% CIs of having a high Gleason score (8+). Since the number of methylated genes was assumed to be an intermediate variable in the path between the DNMT3b variants and Gleason score, we did not adjusted for it in this analysis.

We evaluated the effect of DNMT3b genetic variants on prostate cancer mortality by using a Cox regression model to estimate hazard ratios and corresponding 95% CIs. The proportional hazard assumption was checked by the formal test based on Schoenfeld's residuals. The number of methylated genes and Gleason score were not included in the analysis as it was assumed that they were intermediate variables in the path between the DNMT3b variants and prostate cancer-specific mortality.

Age at diagnosis, calendar year of diagnosis and source of tumor tissue (biopsy, TURP or prostatectomy) were included as potential confounders when necessary. No correction for multiple comparisons was performed.

All statistical analyses were conducted using STATA 12 (STATA Corporation, College Station, TX, USA).

5.4 RESULTS

Characteristics of the prostate cancer patients involved in this study are reported in Table 5.2. The 1980-biopsy cohort included 176 patients with prostate cancer diagnosed during the 1980s who received prostate biopsy, while the adjacent tissue cohort (N=157) is composed by subject with prostate cancer diagnosed between 1982 and 1996 who received prostate biopsy (only those diagnosed during the 1990s), TURP or radical prostatectomy. Patients of the adjacent tissue cohort are younger at diagnosis and have a longer median survival time. In the 1980-biopsy cohort, out of 174 deaths, 111 were due to prostate cancer, while in the adjacent tissue cohort prostate cancer-specific mortality occurred in 43 out of 129 total deaths.

Genotyping was unsuccessful for 4 patients for the rs1569686, for 3 patients of the rs2424913 and for 7 patients of the rs2424932. Genotyping success rate ranged from 97,8 to 99%. All the

polymorphisms are in Hardy-Weinberg equilibrium in both cohorts (Table 5.3), and the LD relationships between the genetic variants, along with their recombination rates, are reported in Figure 5.2. rs1569686 and rs2424913 demonstrated strong LD both in the 1980-biopsy cohort (r^2=0.75) and in the adjacent tissue cohort (r^2=0.81).

Table 5.4 reports the results of the analysis of association between genotypes and haplotypes of DNMT3b and hypermethylation of selected genes in tumor tissue. Single polymorphisms did not give statistically significant results, however in the 1980 biopsy cohort we found a strong effect of the haplotype GCG in homozygosis in increasing the number of hypermethylated genes (OR=12.86; 95% CI 1.41-117.16), although the confidence interval was very large. Adjustment for age at diagnosis and year of diagnosis did not substantially alter the results (data not shown in tables).

Association between DNMT3b variants and LINE-1 methylation level was performed only within the adjacent tissue cohort: genotype AA of the polymorphism rs2424932, compared to AG+GG, was strongly associated with higher LINE-1 methylation levels (β=1.72; CI 95% 0.07-3.37, fully adjusted), whereas allele T of polymorphism rs1569686 in homozygosis, as well as TTG haplotype in homozygosis, show a weak inverse association with LINE methylation level. Moreover, subjects carrying two copies of haplotype GCA have higher LINE-1 methylation levels (β=2.32; CI 95% 0.56-4.08), adjusted for source of tissue and year of diagnosis) (Table 5.5). Further adjustment for age at diagnosis gave similar results (data not shown in tables).

Neither single DNMT3b genotypes nor haplotypes are associated with Gleason score of the tumor (Table 5.6).

In prognostic analyses (Table 5.7), any polymorphism showed noteworthy effects, apart from a weak inverse association between allele A of rs2424932 and prostate cancer mortality, noticeable in both cohorts. In haplotype analysis, GCA carriers (in both inheritance models) show a slightly decreased risk of dying for prostate cancer in both cohorts. Furthermore, carriers of two copies of haplotype GCG have showed a more than three-times higher risk of dying from prostate cancer, and this strong effect was replicated in both cohorts (HR=3.74; 95% CI 1.33-10.55 for the 1980-biopsy cohort; adjusted HR=3.04; 95% CI 1.16-7.94 for the adjacent tissue cohort; adjusted for source of tissue, age at diagnosis and year of diagnosis) (Table 5.7). Analyses restricted to the first 5 years of follow-up gave similar results (data not shown).

Finally, we used the database of the Prostate Adenocarcinoma (TCGA, provisional) study available through the cBioPortal (http://www.cbioportal.org) to perform an explorative analysis on

the association between DNMT3b expression levels and prostate cancer survival. The results obtained pointed out a strong association between DNMT3b expression level and prostate cancer-free survival (Logrank Test P-Value: 2.968e-4).

5.5 DISCUSSION

In this study, we evaluated selected genetic variants in the gene codifying for the DNA methyltransferase 3b in relation with Gleason score and prostate cancer prognosis, as well as with gene specific hypermethylation and global DNA hypomethylation measured in prostate tissue. Specifically, we wanted to assess the direct effect of selected variants on mortality from prostate cancer (irrispectively of the aberrant methylation status in tumor tissue), as well as to evaluate their effect on aberrant methylation (considered as intermediate variable) that, in turn, affect prostate cancer prognosis. Assumed causal relationships between the variables considered in this study have been previously discussed [Gillio-Tos et al., 2012].

We selected three polymorphisms mapping in DNMT3b gene sequence: our selection included both putative functional polymorphisms (rs1568696 and rs2424913) and a Tag-SNP (rs2424932), a strategy that has been already used in previous researches [Potter et al., 2013]. The putative functional SNPs, apart from their previously reported associations with other kinds of cancer, have been chosen because of their potential effect on DNMT3b gene transcription. The underlying hypothesis is that these variants could alter the transcription of the DNMT3b gene, thus affecting its expression levels and, in turn, influencing the enzyme production. Significantly higher expression levels of DNMT3b, as well as higher protein levels, has been repeatedly detected in cancerous over benign tissues [Hoffmann et al., 2007; Kobayashi et al., 2011; Gravina et al., 2013; Basu et al., 2015]. In addition, higher levels of expression of DNMT3b have been measured in high-tumorigenic prostate cancer cell lines compared to low-tumorigenic cells [Gravina et al., 2013], thus suggesting a possible association between increased DNMT3b expression and tumor progression. In order to corroborate these evidences, we used freely available DNMT3b expression data and survival data of a dataset of prostate cancer patients, accessible via the cBioPortal for Cancer Genomics. cBioPortal is a very useful resource that stores genomic data from large scale and integrated cancer genomic data sets, also allowing explorative data analyses. Specifically, we used dataset provided by the Prostate Adenocarcinoma (TCGA, Provisional) study: for 213 patients of this cohort both DNMT3b mRNA expression data and disease-free survival data were available. We found that prostate cancer patients with DNMT3b overexpression have a significantly shorter disease-free survival compared to subjects with normal levels of the

enzyme, thus strengthening the hypothesis of a relationship between DNMT3b overexpression and prostate cancer prognosis.

We evaluated if selected variants in DNMT3b gene are associated with prostate cancer-specific mortality in order to assess their direct effect on the outcome. GCG haplotype in homozygosis is strongly associated with a three-times higher risk of dying from prostate cancer, and this strong effect was replicated in both cohorts with a very similar magnitudo of the effect, although the confidence intervals of these estimates were wide. Our results suggest that prostate cancer-specific mortality may be affected by DNMT3b variants but, since the effects of single SNPs on prostate cancer prognosis seem to be too little to be detectable, a considerable effect could be obtained only in the form of the haplotype.

Gene-specific hypermethylation and global DNA hypomethylation have been widely studied in prostate cancer, and their strong association with tumor development and progression have been repeatedly observed. Nevertheless, the determinants of aberrant methylation are still poorly understood in this type of cancer. Since DNMT3b enzyme is considered to have a pleiotropic effect and its overexpression was correlated to hypermethylation of several tumor-related genes [Roll et al., 2008; Amara et al., 2010], in our analyses we did not consider the effect of the genetic variants on the hypermethylation of each gene separately, but, collectively, on increasing number of hypermethylated genes in tumor tissue.

GCG haplotype in homozygosis was strongly associated with increasing number of hypermethylated genes in 1980-biopsy cohort. Hypothesizing that this combination of genetic variants in DNMT3b sequence could cause an higher level of synthetized enzyme and, therefore, higher methylation levels in the promoter of the genes specifically involved in prostate cancer, it is plausible that the effect of GCG haplotype on prostate cancer mortality may be explainable by its effect on gene-specific hypermethylation. In the adjacent tissue cohort the association, although outlined, is very weak, likely due to the lower prevalence of hypermethylation in tumor tissue of these patients. We have data on the methylation status of only three genes in tumor tissue, likely adding other genes to this analysis these estimates might be more consistent. A good strategy to expand and to validate this analysis could be to draw, from the public databases, genotyping data and methylation data of other genes obtained on large cohorts of prostate cancer patients.

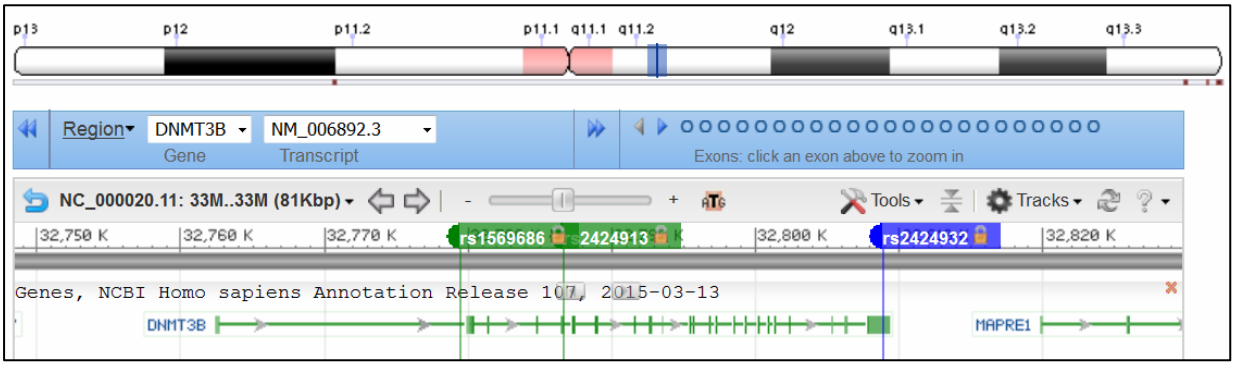
We evaluated the effect of DNMT3b variants also on LINE-1 methylation levels, since a significative correlation between DNMT3b expression levels and global hypermethylation in prostate tumor has been found [Kobayashi et al., 2011]. Interestingly, GCG haplotype has no effects on LINE-1 methylation, whereas opposite effects were noted for the GCA haplotype,

which is strongly associated with higher LINE-1 methylation levels when present in homozygosis. We could speculate that different DNMT3b haplotypes could exert different effects on DNMT3b gene expression levels, thus modulating the enzyme production and, as a consequence, leading to different effects on the methylation pattern. Specifically, since DNMT3b is preferentially a *de novo* methyltransferase but its role in the maintenance of the global DNA methylation pattern during each cellular replication was also demonstrated, we could hypothesized that a possible increased expression induced by GCG haplotype might have an effect on gene-specific hypermethylation, rather that in maintaining LINE-1 methylation at consistently high levels, while normal levels of enzyme (possibly induced by GCA haplotype) might not exert this *de novo* gene-specific effect but only a maintenance effect. This is in part supported by previous works that have demonstrated that DNMT3b overexpression induces DNA methylation in some CpG islands in a non-random manner [Linhart et al., 2007; Kobayashi et al., 2011]; analogously, depletion of DNMT3b have been reported to affect locus-specific DNA methylation, thus increasing the expression of distinct set of genes [Yaqinuddin et al., 2008; Wang et al., 2015]. Obviously, further investigations and experimental assays are needed to prove our hypothesis.

Our data show that the opposite effects observed for these different combinations of DNMT3b genotypes might be partly due to the polymorphism rs2424932. The effect of this genetic variant on cancer risk has been poorly investigated, and functional assays to test its consequence on enzyme expression and activity are still lacking; however, it could have functional significance. An in silico tool (SNP Function Prediction (FuncPred) (snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm)) revealed that presence of A allele adds an additional regulation to the gene through influencing hsa-mir-920 binding [Saradalekshmi et al., 2014]; also, the A/G polymorphism at this locus is predicted to create alternative transcription factor binding sites [Murphy et al., 2013].

In conclusion, in two indipendent cohorts of prostate cancer patients, we have found that an haplotype of the DNMT3b gene, when present in homozygosis, is strongly associated with mortality from prostate cancer. The same haplotype showed, in one of the two cohorts, to increase the gene-specific hypermethylation, leading to the hypothesis that different DNMT3b haplotypes could have different effects on gene expression and, consequently, on the enzyme production and activity. This hypothesis need to be tested in further investigations.

Figure 5.1. Graphical view of the location of the SNPs genotyped in the current study along the DNMT3b gene sequence



(Source: http://www.ncbi.nlm.nih.gov/variation/view/)

Figure 5.2. Linkage Disequilibrium plot for the polymorphisms under study in the 1980-biopsy cohort (left) and in the adjacent tissue cohort (right).

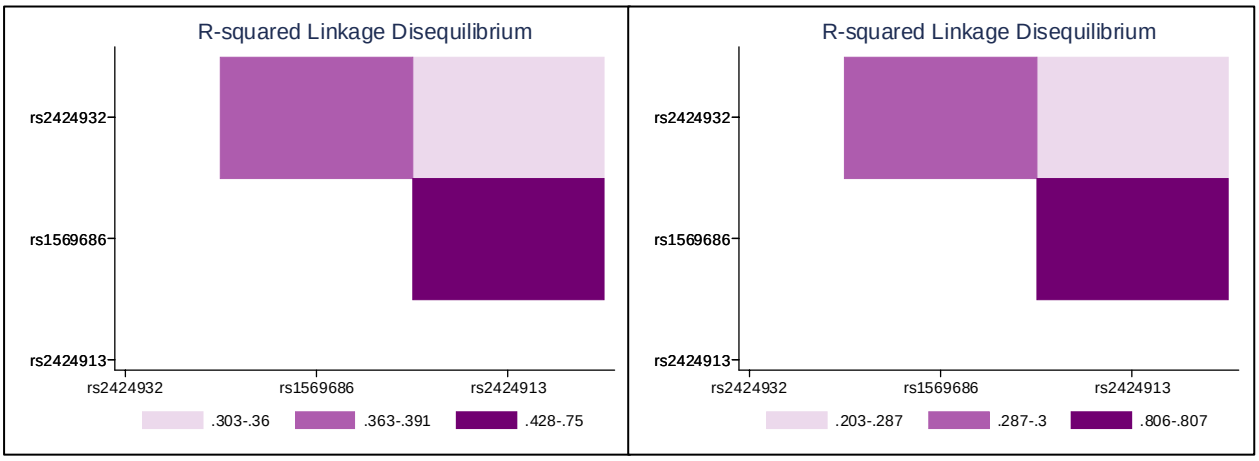
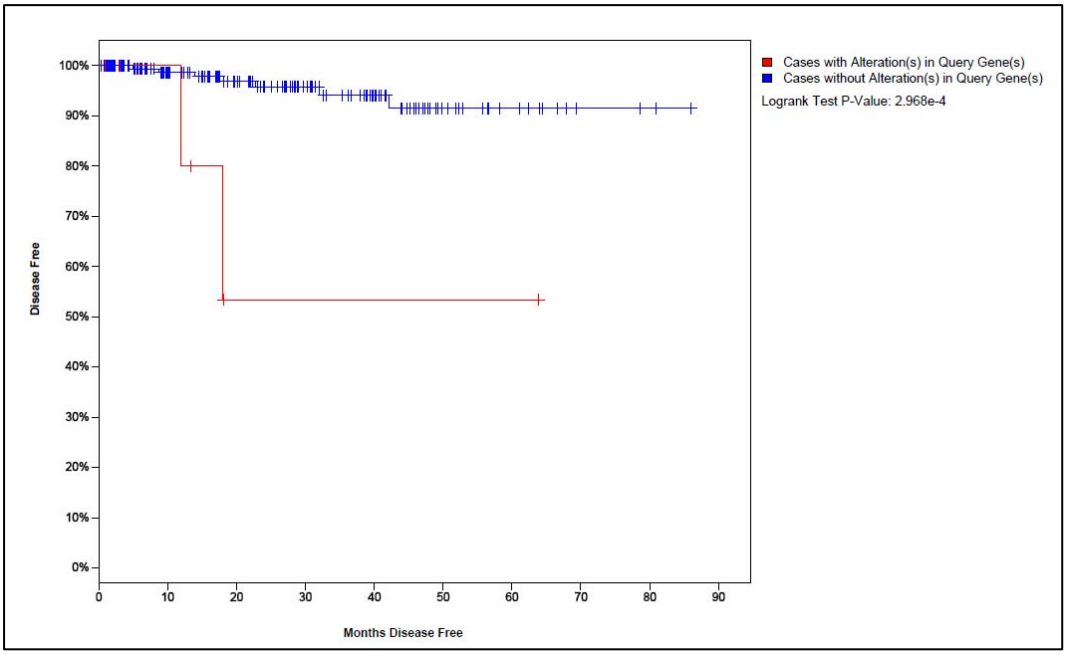


Figure 5.3. Association between DNMT3b expression level and disease-free survival in a cohort of patients with prostate adenocarcinoma



Source: pubblicly available data at cBioPortal for Cancer Genomics, Prostate Adenocarcinoma (TCGA, provisional) study. z-score threshold: ± 1.5.

Table 5.1. List of primers, sequence to analyze and dispensation order for each genotyped DNMT3b polymorphisms by pyrosequencing

	rs2424913	**rs1569686**	**rs2424932**
Primer FW	GCCACCTGAAGGCCTAATC	[Bnt] CAAAGGCAAGTGACTTGGAA	TTTTGTAGACAAGTATGGCTCCTC
Primer Rev	[Bnt]GACACTCACTGGGCCTTA	AGTAAAAAACTTCAGGGCATAAAT	[Bnt] GCTCCTTGCTTCACACTCCT
Primer Seq	GGCCCCGCCAGACCC	AAACTTCAGGGCATAAAT	CAAGTATGGCTCCTCC
Sequence to analyze	C/TAGGCCTCCAG	CCCA/CGCTGAAACCGA	A/GTATCTCCC
Dispensation Order	GCTAGCTC	GCCAGCTGA	CAGTATCT

Table 5.2. Selected characteristics of the two cohorts of prostate cancer patients

Characteristics	1980-biopsy cohort		Adjacent tissue cohort	
	N	%	N	%
Year of diagnosis				
1982-1988	176	100%	28	17.8%
1993-1996	0	0	129	82.2%
Range survival time (years)	0.02-26.8		0.03-24.11	
Median survival time (years)	3.21		6.79	
Age at diagnosis (years)				
40-64	33	19.0%	26	16.6%
65-69	23	13.1%	35	22.3%
70-74	36	20.5%	40	25.5%
75+	82	46.6%	56	35.7%
Mortality				
Overall	174	98.3%	129	81.5%
From prostate cancer	111		43	
From other causes	63		86	
Source of tumour tissue				
Biopsy	174	100%	53	33.8%
TURP	0	-	55	35.0%
Radical prostatectomy	0	-	49	31.2%
Gleason score				
<7	23	13.7%	59	37.6%
7	66	38.7%	41	26.1%
≥8	80	47.6%	57	36.3%
Missing	7	-	-	-
Number of methylated genes[#]				
0	2	1.27	18	12.68
1	35	22.29	44	30.99
2	79	50.32	58	40.85
3	41	26.11	22	15.49
Missing	19	-	15	-
LINE-1 mean (SD)	78.55 (4.29)		-	

[#]Methylation of GSTP1, APC and RUNX in tumor tissue

Table 5.3. Tests for Hardy-Weinberg Equilibrium

Polymorphism	1980-Biopsy cohort (N=174)	Adjacent tissue cohort (N=157)
	P value	P value
rs1569686	0.52	0.43
rs2424913	0.87	0.14
rs2424932	0.28	0.61

Table 5.4. Analysis of association between genotypes and haplotypes of DNMT3b and number of hypermethylated genes measured in tumor tissue

Variant	1980-biopsy cohort		Adjacent tissue cohort	
	N° of hypermethylated genes		N° of hypermethylated genes	
	OR (95% CI)	OR (95% CI)	OR (95% CI)[b]	OR (95% CI)[b]
	Dominant	Recessive	Dominant	Recessive
Genotype				
rs1569686_T	1.05 (0.58-1.92)	1.50 (0.61-3.67)	0.63 (0.34-1.18)	0.95 (0.28-3.23)
rs2424913_T	0.83 (0.46-1.52)	1.10 (0.45-2.69)	0.55 (0.29-1.03)	1.06 (0.34-3.34)
rs2424932_A	0.72 (0.37-1.40)	0.94 (0.42-2.08)	1.11 (0.58-2.13)	1.28 (0.57-2.86)
Haplotype[a] (>5%)				
TTG	1.06 (0.58-1.92)	1.12 (0.43-2.92)	0.61 (0.33-1.12)	1.28 (0.36-4.59)
GCA	0.75 (0.39-1.45)	1.46 (0.61-3.48)	0.98 (0.53-1.84)	1.92 (0.81-4.57)
GCG	0.81 (0.44-1.50)	12.86 (1.41-117.16)	1.25 (0.68-2.30)	1.76 (0.47-6.56)

Associations have been evaluated both in dominant and recessive models.
[a] rs1569686, rs2424913, rs2424932
[b]Adjusted for source of tissue
OR: Odds Ratios; CI: Confidence Intervals

Table 5.5. Association between genotypes and haplotypes of DNMT3b and LINE-1 methylation level measured in tumor tissue

Variant	Adjacent tissue cohort			
	LINE-1 methylation level in tumor tissue			
	$Coeff_1$ (95% CIs)		$Coeff_2$ (95% CIs)	
Genotype	**Dominant**	**Recessive**	**Dominant**	**Recessive**
rs1569686_T	-1.07 (-2.38 – 0.24)	-2.11 (-4.72 – 0.50)	-1.01 (-2.32 – 0.31)	-2.20 (-4.80 – 0.40)
rs2424913_T	-0.91 (-2.21 – 0.39)	0.04 (-2.41 – 2.48)	-0.83 (-2.14 – 0.48)	0.00 (-2.44 – 2.44)
rs2424932_A	0.42 (-0.96 – 1.81)	1.77 (0.12 – 3.42)	0.42 (-0.96 – 1.80)	1.72 (0.07 – 3.37)
Haplotype[a] (>5%)				
TTG	-1.14 (-2.41 – 0.14)	-1.44 (-4.17 – 1.29)	-1.08 (-2.35 – 0.20)	-1.40 (-4.13 – 1.32)
GCA	0.06 (-1.28 – 1.40)	2.41 (0.66 – 4.17)	0.06 (-1.28 – 1.40)	2.32 (0.56 – 4.08)
GCG	0.35 (-0.94 – 1.63)	-0.87 (-3.60 – 1.87)	0.40 (-0.89 – 1.69)	-1.12 (-3.87 – 1.63)

Associations have been evaluated both in dominant and recessive models.
[a] rs1569686, rs2424913, rs2424932
$Coeff_1$: adjusted for source of tissue; $Coeff_2$: adjusted for source of tissue and year of diagnosis
CI: Confidence Intervals

Table 5.6. Analysis of association between genotypes and haplotypes of DNMT3b and Gleason score

Variant	1980-biopsy cohort		Adjacent tissue cohort	
	Gleason 8+ vs. <8		Gleason 8+ vs. <8	
	OR (95% CI)[b]	OR (95% CI)[b]	OR (95% CI)[b]	OR (95% CI)[b]
	Dominant	**Recessive**	**Dominant**	**Recessive**
Genotype				
rs1569686_T	1.39 (0.75-2.57)	1.23 (0.52-2.88)	1.06 (0.53-2.15)	0.50 (0.12-2.12)
rs2424913_T	1.09 (0.59-2.01)	1.55 (0.62-3.90)	1.07 (0.53-2.15)	0.88 (0.23-3.33)
rs2424932_A	1.24 (0.64-2.39)	0.59 (0.25-1.37)	1.13 (0.54-2.37)	0.58 (0.22-1.48)
Haplotype[a] (>5%)				
TTG	1.24 (0.67-2.27)	1.40 (0.55-3.58)	1.06 (0.53-2.11)	0.69 (0.15-3.07)
GCA	1.27 (0.66-2.43)	0.67 (0.27-1.65)	0.98 (0.48-2.00)	0.53 (0.19-1.50)
GCG	0.74 (0.39-1.38)	0.54 (0.10-3.02)	1.36 (0.68-2.70)	2.19 (0.55-8.77)

Associations have been evaluated both in dominant and recessive models.
[a] rs1569686, rs2424913, rs2424932
[b] Adjusted by source of tissue
OR: Odds Ratios; CI: Confidence Intervals

Table 5.7. Analysis of association between genotypes and haplotypes of DNMT3b and mortality from prostate cancer

Variant	1980-biopsy cohort		Adjacent tissue cohort	
	HR (95% CI)[b]	HR (95% CI)[b]	HR (95% CI)[b]	HR (95% CI)[b]
	Dominant	Recessive	Dominant	Recessive
Genotype				
rs1569686_T	1.22 (0.83-1.80)	1.24 (0.72-2.13)	0.91 (0.49-1.70)	0.60 (0.14-2.57)
rs2424913_T	1.02 (0.69-1.51)	1.37 (0.78-2.42)	0.71 (0.38-1.31)	1.14 (0.39-3.28)
rs2424932_A	0.72 (0.48-1.09)	0.88 (0.50-1.55)	0.94 (0.49-1.82)	0.36 (0.13-1.00)
Haplotype[a] (>5%)				
TTG	1.10 (0.75-1.61)	1.44 (0.80-2.59)	0.89 (0.48-1.63)	0.59 (0.14-2.50)
GCA	0.84 (0.56-1.27)	0.79 (0.41-1.52)	0.84 (0.45-1.58)	0.47 (0.17-1.32)
GCG	0.91 (0.62-1.35)	3.74 (1.33-10.55)	1.71 (0.91-3.19)	3.04 (1.16-7.94)

Associations have been evaluated both in dominant and recessive models.
[a] rs1569686, rs2424913, rs2424932
[b] Adjusted by age at diagnosis, year of diagnosis, source of tissue.
HR: Hazard Ratios; CI: Confidence Intervals

6. CONCLUSIONS

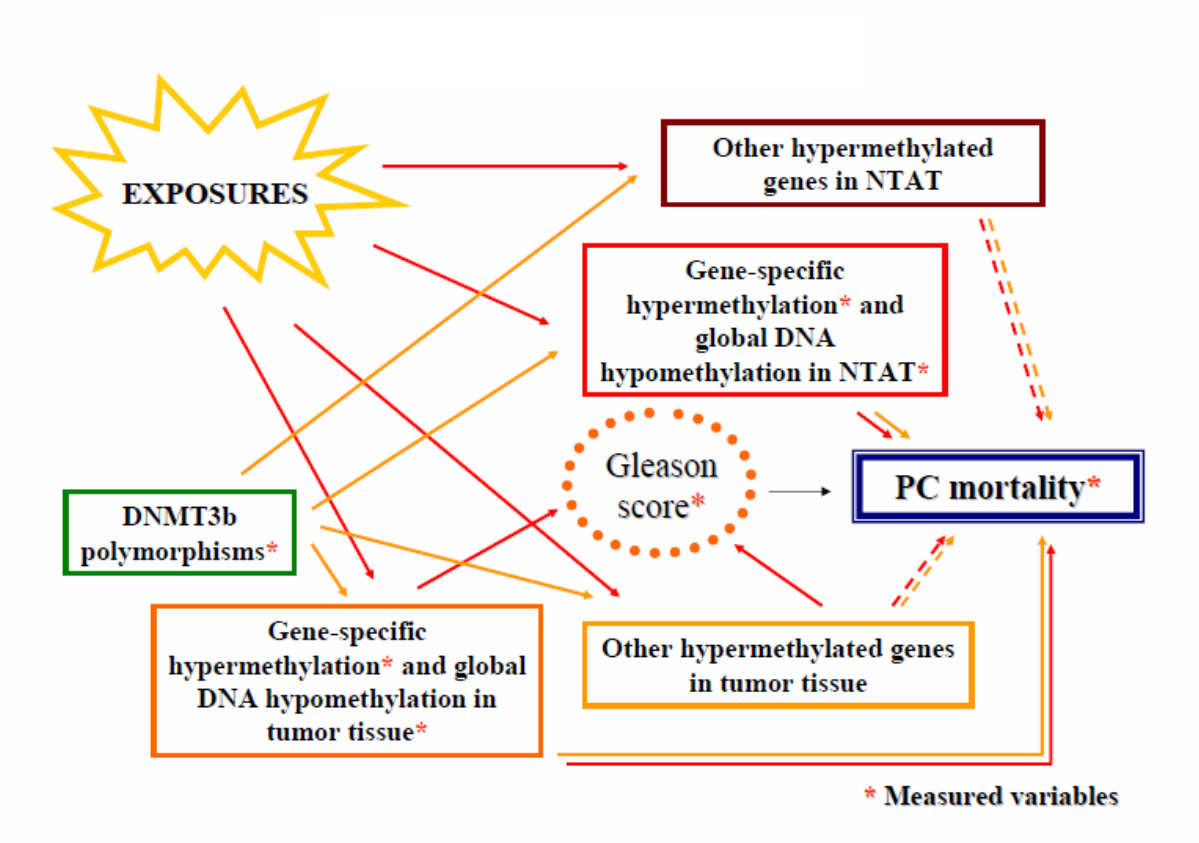
In our ongoing project investigating the role of epigenetic alterations in prostate cancer, we found that promoter methylation of GSTP1 measured in a negative biopsy tissue is associated with prostate cancer diagnosis on a rebiopsy, especially for more aggressive tumors; predictive value of LINE-1 methylation level is less consistent and is reported only for low methylation levels.

In prostate tumor tissue, gene-specific hypermethylation and global DNA hypomethylation coexist, expecially in high-grade tumors. We understood that LINE-1 hypomethylation, unlike gene-specific hypermethylation, is not an early event in prostate tumor: rather, it seems to be implicated in prostate cancer progression, but only in patients with more aggressive tumors. Low levels of LINE-1 methylation might be useful to distinguish into the group of the high-risk prostate cancers only those leading to death. Our results also suggested that combinations of polymorphisms of the DNMT3b gene may have a noteworthy effect on mortality from prostate cancer and on gene-specific hypermethylation, suggesting that they could increase the expression of DNMT3b gene, thus.enhancing the enzyme production. The relationships between DNMT3b variants, methylation status in tumor and in NTAT, Gleason score and prostate cancer-specific mortality that we have assumed in our research are summarized in Figure 6.1 (below).

Prostate cancer is a biologically and clinically heterogeneous disease, and new biomarkers to help avoid unnecessary biopsies and distinguish between aggressive and indolent disease are urgently requested. Epigenetic changes are hallmarks of prostate cancer and are associated with malignant initiation as well as tumor progression (Figure 6.1). DNA methylation is the most frequently studied epigenetic alteration in prostate cancer, and the diagnostic and prognostic potential of DNA methylation markers for this disease has been demonstrated in multiple studies.

Identification of clinically relevant biomarkers remains a major challenge for prostate cancer management, as at present there are no prognostic tools that can accurately predict tumor progression at the time of diagnosis and improve the management of the patients. Currently, the best strategy could be the use of multi-parametric marker panels with inclusion of molecular markers into algorithms of existing clinicopathological parameters, thus potentially generating more robust tools for predicting prostate cancer outcome.

Figure 6.1. Overall model



Red filled arrows indicate hypotetical causal relationships among currently unknown environmental exposures and the gene methylation status. Previously we evaluated the relationship among the hypermethylation of selected genes in tumor and non-neoplastic tumor adjacent to tumor (NTAT), tumor aggressiveness (Gleason score) and prostate cancer mortality [Richiardi et al., 2009; Richiardi et al., 2013]. In STUDY 2 we evaluated the relationship between LINE-1 hypomethylation in tumor and non-neoplastic tumor adjacent to tumor (NTAT), tumor aggressiveness (Gleason score) and prostate cancer mortality.

Dashed red arrows indicate that other possible unmeasured variables (e.g. hypermethylation in genes other that those evaluated in this research) can potentially contribute to explain the association we found with aggressiveness and mortality. Yellow arrows (filled and dashed) indicate possible implication of the variants of the DNA-methyltransferase-3b gene in the methylation activity of the enzyme, and its possible relationship with prostate cancer mortality. In this model, the Gleason score represents an effect mediator, that captures the effects of the other variables.

7. FUTURE PERSPECTIVES

We will expand the study on the potential prognostic value of methylation patterns in the tumour tissue and in the non-neoplastic tissue adjacent to tumor of prostate cancer patients within the "ProMort study", a large case-control study that will involved prostate cancer cases and controls from the Swedish National Prostate Cancer Register. The overall aim of this project is to develop prognostic models for prostate cancer mortality in low- and intermediate-prostate cancer to improve the selection of patients who should be offered curative treatment or active surveillance. Cases will be prostate cancer patients with low- or intermediate-risk disease who died from the disease, while controls will be subjects with low- or intermediate-risk prostate cancer who were alive at the time of death of the corresponding cases. Specifically, we will study a-priori specified methylation changes in the prostate tumour tissue and in NTAT in association with mortality from prostate cancer, to understand if these methylation markers, that have been previously identified in more heterogeneous and smaller series of patients, could have the predictive ability to further separate patients with intermediate Gleason score with respect to prognosis.

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9. APPENDIX

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Papers currently under review

- **Grasso C**, Trevisan M, Fiano V, Tarallo V, De Marco L, Sacerdote C, Richiardi L, Merletti F, Gillio-Tos A. Performance of different analytical software packages in quantification of DNA methylation by pyrosequencing. Under review on PloS One.

Manuscripts in preparation

- **Grasso C**, Zugna D, Fiano V, Robles N, Maule M, Gillio-Tos A, Ciuffreda L, Lista P, Segnan N, Merletti F, Richiardi L. Subfertility and risk of testicular cancer in the EPSAM case-control study.

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- Struijk EA, May AM, Beulens JWJ, Langenberg C, van der Schouw YT, Peeters PHM, **EPIC-authors**, Onland-Moret NC, Wareham N. New loci identified for macronutrient intake in the EPIC-Interact study.