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Clinical Cancer Research

Global Hypomethylation (LINE-1) and Gene-Specific Hypermethylation (GSTP1) on Initial Negative Prostate Biopsy as Markers of Prostate Cancer on a Rebiopsy

Renata Zelic[1], Valentina Fiano[1], Daniela Zugna[1], Chiara Grasso[1], Luisa Delsedime[2], Lorenzo Daniele[2], Diego Galliano[2], Andreas Pettersson[3], Anna Gillio-Tos[1], Franco Merletti[1], and Lorenzo Richiardi[1]

Abstract

Purpose: Men at risk of missed prostate cancer on a negative biopsy often undergo a rebiopsy. We evaluated whether global hypomethylation, measured through LINE-1 methylation, and GSTP1 hypermethylation on a negative biopsy are associated with subsequent prostate cancer diagnosis.

Experimental Design: We performed a case–control study nested in an unselected series of 737 men who received at least two prostate biopsies at least three months apart at the Molinette Hospital (Turin, Italy). Two pathology wards were included for replication purposes. The study included 67 cases and 62 controls in Ward 1 and 62 cases and 66 controls in Ward 2. We used pyrosequencing to analyze LINE-1 and GSTP1 methylation in the negative biopsies. Odds ratios (OR) of prostate cancer diagnosis were estimated using conditional logistic regression.

Results: After mutual adjustment, GSTP1 hypermethylation was associated with an OR of prostate cancer diagnosis of 5.1 (95% confidence interval: 1.7–14.9) in Ward 1 and 2.0 (0.8–5.3) in Ward 2, whereas an association was suggested only for low LINE-1 methylation levels (<70% vs. 70%–74%) with an OR of 2.1 (0.5–9.1) in Ward 1 and 1.6 (0.4–6.1) in Ward 2. When the two wards were combined the association was stronger for tumors with Gleason score ≥4+3 [GSTP1 hypermethylation: 9.2 (2.0–43.1); LINE-1 (<70% vs. 70%–74%): 9.2 (1.4–59.3)]. GSTP-1 alone improved the predictive capability of the model ($P = 0.007$).

Conclusions: GSTP1 hypermethylation on a negative biopsy is associated with the risk of prostate cancer on a rebiopsy, especially of high-grade prostate cancer. Consistent results were found only for extremely low LINE-1 methylation levels.
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Introduction

As a consequence of a high false-negative rate of prostate biopsy (1–3), men with a suspicion of missed prostate cancer after an initial negative biopsy are often subjected to one or more rebiopsies, leading to growing discomfort, adverse events (such as sepsis, with a risk of >2%; ref. 4) and increased costs.

Various clinical and pathologic parameters have been considered as predictive of prostate cancer on a rebiopsy, such as PSA, PSA density, percent free PSA, prostate volume, digital rectal examination finding, age, family history of prostate cancer, number of cores taken at biopsy, time between first biopsy and rebiopsy, and presence of high-grade prostate intraepithelial neoplasia (HGPIN) or atypical small acinar proliferation (ASAP) at first biopsy (5–9). Several nomograms containing some of these parameters have been developed to aid the rebiopsy decision, but they are hampered by low accuracy (76%–86%; 10, 11). Thus, it is important to identify additional factors predictive of malignancy on a prostate rebiopsy.

DNA methylation is the most investigated epigenetic alteration in prostate cancer. DNA hypermethylation represents increased CpG island methylation in normally unmethylated promoter regions of cancer-associated genes and glutathione *S*-transferase pi 1 protein (GSTP1) hypermethylation is the most intensely investigated gene-specific hypermethylation in prostate cancer (12). In a recent meta-analysis, GSTP1 was methylated in 82% of prostate cancer cases and in 5% of controls (12) making it a promising prostate cancer marker. Global DNA hypomethylation represents a genome-wide loss of DNA methylation in regions where it usually occurs and long interspersed nuclear elements-1 (LINE-1) methylation is often used as a surrogate of global DNA hypomethylation (13, 14). Both GSTP1 hypermethylation and LINE-1 hypomethylation can successfully differentiate tumor from nontumor prostate tissue (12, 15), and, in line with the field effect concept, they can be found in non-neoplastic tissue adjacent to tumor tissue (16–18). GSTP1 methylation alterations can also be detected in histologically negative biopsy samples and can be used to improve the sensitivity of the standard histology work-up for prostate cancer detection (19). These findings suggest

[1]Cancer Epidemiology Unit-CERMS, Department of Medical Sciences, University of Turin, and CPO-Piemonte, Turin, Italy. [2]Division of Pathology, A.O. Città della Salute e della Scienza Hospital, Turin, Italy. [3]Clinical Epidemiology Unit, Department of Medicine, Solna, Karolinska Institutet, Stockholm, Sweden.

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Corresponding Author: Renata Zelic, University of Turin, Via Santena 7, 10126 Turin, Italy. Phone: 39 01-1633-4629; Fax: 39 01-1633-4664; E-mail: renatazelic@yahoo.com

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Translational Relevance

Men with a suspicion of missed cancer after a negative initial biopsy often undergo one or more rebiopsies, which leads to growing discomfort, adverse events, and increased costs. DNA methylation alterations are the most investigated epigenetic alterations in prostate cancer and have been often considered as possible prostate cancer diagnostic markers. In two large series of men with repeat prostate samplings, we performed a matched case–control study and found that gene-specific hypermethylation (GSTP-1) is associated with prostate cancer diagnosis, especially for more aggressive tumors (i.e., tumors with Gleason score $\geq$4+3), while for LINE-1 methylation consistent results were found only for extremely low LINE-1 methylation levels. This finding suggests that including gene-specific hypermethylation into existing models could improve their accuracy, help identify men with a high risk of missed aggressive cancer and reduce the number of unnecessary rebiopsies.

that epigenetic alterations in histologically negative biopsy samples may serve as potential markers of prostate cancer diagnosis on a rebiopsy (20). However, while gene-specific hypermethylation, including GSTP1 hypermethylation, has previously been investigated as a possible marker of prostate cancer diagnosis on a rebiopsy (20–22), to the best of our knowledge, LINE-1 hypomethylation has not.

We conducted a case–control study in two different pathology wards to evaluate whether LINE-1 hypomethylation and GSTP1 hypermethylation in an unselected series of men with a histologically negative initial biopsy are associated with prostate cancer diagnosis on a rebiopsy.

Materials and Methods

Study design and participants selection

We conducted a case–control study within an unselected cohort of men who underwent prostate biopsy, transurethral resection of the prostate (TURP) or partial prostatectomy between 1993 and 2003 at the Molinette Hospital in (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, 8,755 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 tissue samples from at least two consecutive procedures and with a minimum of 3 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 histologic confirmation of prostate cancer, 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 (100 in Ward 1 and 76 in Ward 2), while subjects with two available samples were counted as two cases (45 in Ward 1 and 23 in Ward 2), 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 (1 case in Ward 2) or it was considered as missing (1 case in Ward 2). Cases without a matched control (26 cases in Ward 1 and 10 cases in Ward 2) and cases for whom prostate cancer diagnosis could not be confirmed from the diagnostic slides (4 cases in Ward 1 and 5 cases in Ward 2) 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 (188 in Ward 1 and 259 in Ward 2), while non-case subjects with three negative samples available were counted as two (2 in Ward 1 and 2 in Ward 2) or three potential controls (103 in Ward 1 and 104 in Ward 2), leading 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 (6 months groups). Controls with HGPIN or ASAP on the index procedure (4 controls in Ward 1 and 1 control in Ward 2) were excluded. After the sampling procedure, 94 controls in Ward 1 and 84 controls in Ward 2 remained for analysis.

For both cases and controls, the molecular analyses focused on the first negative sample, meaning that the index procedure used to define cases and controls was not analyzed. This approach is consistent with the aim of the study, namely assessing molecular markers used to aid the rebiopsy decision.

We analyzed one tissue sample per 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. When the FFPE blocks did not contain sufficient amount of prostate tissue needed for the molecular analyses, the corresponding cases (4 in Ward 1 and 2 in Ward 2) and controls (2 in Ward 1) were excluded from the study.

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.

Molecular analysis

Genomic DNA was extracted from the FFPE blocks and purified using the commercially available QIAamp DNA FFPE Tissue Kit (Qiagen). The Epitect Bisulfite Kit (Qiagen) was used to perform bisulphite modification of genomic DNA, along with positive controls for methylated [CpGenomeTM universal methylated DNA (Chemicon Co.)] and unmethylated status [EpiTect Control DNA, unmethylated (Qiagen)].

Analysis of LINE-1 (GenBank accession number X58075) and GSTP1 (GenBank accession number M24485) promoter methylation status were performed using PyroMark Q24 MDx (Qiagen). Primers, which produce a 98 bp amplicon containing three CpG

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sites for LINE-1 (positions 819, 826, and 829), and a 72 bp amplicon containing four CpG sites for GSTP1 (positions 1038, 1040, 1043, 1049), were designed outside the CpG sites as follows: for LINE-1 forward 5′-TTTGAGTTAGGTGTGGGATATAGTT-3′, reverse 5′-Biot-CACCTAAAAAATCCAATCACTCC- 3′ and sequencing 5′-TTAGGTGTGGGATATAGTTT-3′, for GSTP1 forward 5′-GATTTGGGAAAGAGGGAAAGGT- 3′, reverse 5′-Biot-CAAAAAAACGCCCTAAAATCC- 3′ and sequencing 5′-GGTTTTTTYGGTTAGTTG-3′. We performed PCR reaction in a total volume of 30 μl containing 1× buffer (KCl), 2 mmol/L $MgCl_2$, 0.8 mmol/L dNTPs, 0.5 μmol/L of each primer, 0.05 U Taq polymerase, and 6 μL of converted DNA with the following cycling profile: 95°C for 10 minutes followed by 45 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute for LINE-1 and at 50°C for 1 minute for GSTP1, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. 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 mmol/L Tris-Acetate, 5 mmol/L $MgAc_2$, and 0.15 μL of 50 μmol/L sequencing primer (final concentration 0.3 μmol/L). 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)] 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 rerun, we reanalyzed the whole corresponding stratum, including both the case and the matched control(s).

Statistical analysis

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.

As 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 report only estimates based on the mean methylation. 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 nonlinearities, 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; ref. 23), 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 (OR), and corresponding 95% confidence intervals (95% CI), 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 (modeled 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 (24). 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 (25, 26). 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).

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 (Fig. 1). Preliminary analyses of LINE-1 methylation revealed higher methylation levels in TURPs than in biopsy samples (Supplementary Fig. S1), 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 (Ward 1: 12 cases and 111 controls; Ward 2: 6 cases and 5 controls) and partial prostatectomy (Ward 1: 14 cases and 10 controls; Ward 2: 5 cases and 2 controls). In addition, we excluded 4 cases and 3 controls in Ward 1 and 2 cases and 8 controls in Ward 2 who were left without cases or controls within the matching strata (Fig. 1). Overall, 67 cases and 62 controls in Ward 1 and 62 cases and 66 controls in Ward 2 remained for the final analyses.

Table 1 lists characteristics of cases and controls by ward. Mean LINE-1 methylation was lower in cases than controls in Ward 1 but not in Ward 2. GSTP1 methylation was higher in cases than controls in both wards. In both wards, biopsies with at least 6 cores were sampled more frequently from controls than from cases. 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. In addition, controls in Ward 1 had higher LINE-1 methylation and lower GSTP-1 methylation than controls in Ward 2.

Table 2 reports results for LINE-1 methylation, when categorized into the four categories. The adjusted OR of prostate cancer diagnosis 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 modeled using spline regression (Supplementary Fig. S2), 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 also at high levels

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of LINE-1 methylation. GSTP1 hypermethylation was associated with diagnosis of prostate cancer at the second biopsy in both wards (Ward 1: OR2 = 5.1, 95% CI, 1.7–14.9; Ward 2: OR2 = 2.0, 95% CI, 0.8–5.3; Table 2).

As reported in Table 3, 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.

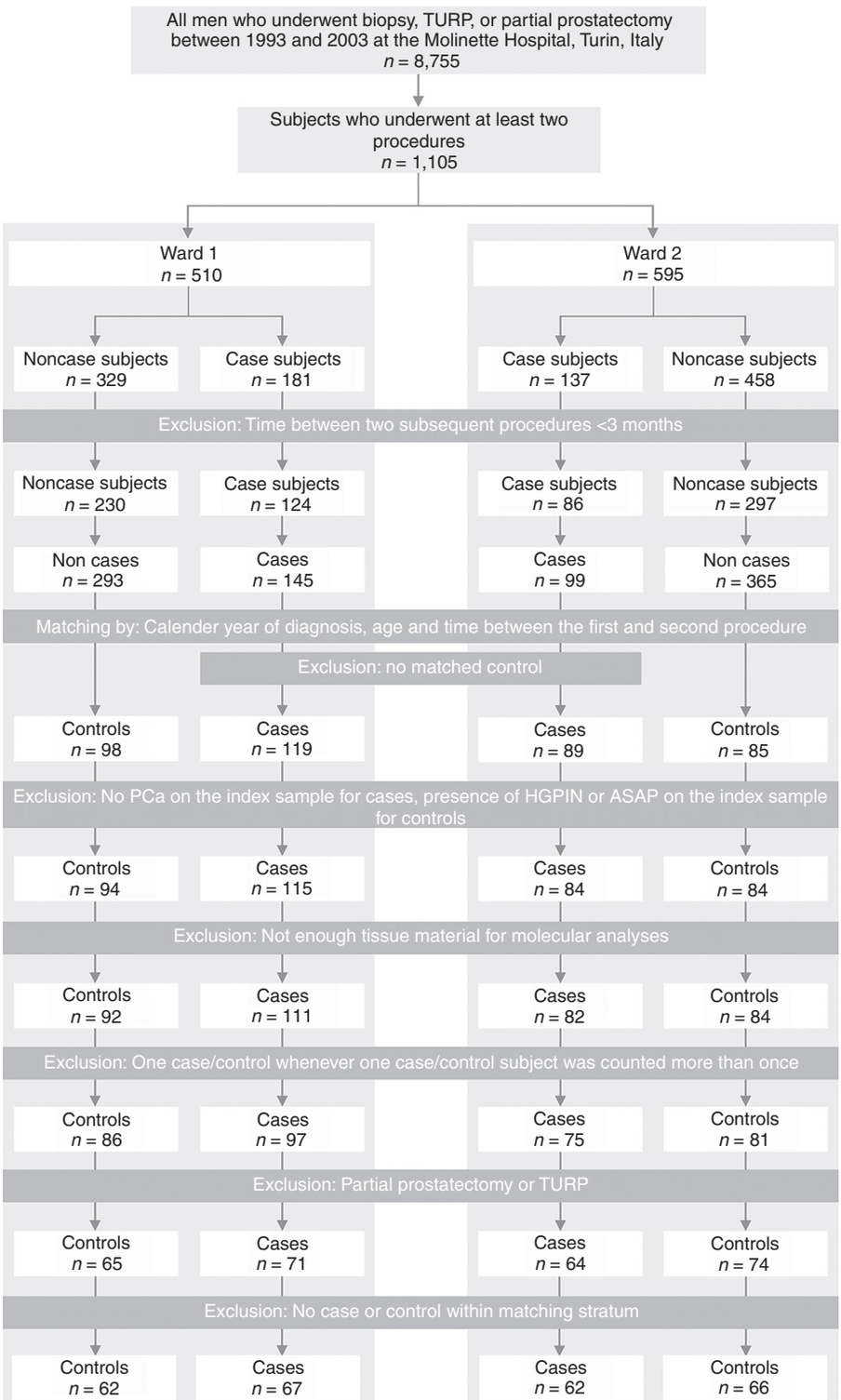

Figure 1.
Flow chart of the case and control selection. PCa, prostate cancer.

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Table 1. Characteristics of cases and controls by ward

	Ward 1		Ward 2	
	Cases ($n = 67$) *n* (%)	Controls ($n = 62$) *n* (%)	Cases ($n = 62$) *n* (%)	Controls ($n = 66$) *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)
	$P = 0.180$		$P = 0.801$	

In the analysis stratified by Gleason score, associations were stronger for Gleason score ≥4+3 for both GSTP1 methylation (OR2 = 9.2, 95% CI, 2.0–43.1) and LINE1 methylation <70% (OR2 = 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 GSTP-1 ($P = 0.007$) independently improved the predictive capability of the model (Table 4) 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 4). 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 23.5% for LINE-1.

Discussion

It has been proposed that global DNA hypomethylation and gene-specific hypermethylation coexist in prostate cancer and can be used as markers of prostate cancer diagnosis and prognosis (13, 15, 27). In this study, we assessed the relationship between LINE-1 hypomethylation and GSTP1 hypermethylation in men

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Table 2. LINE-1 methylation and GSTP1 hypermethylation on a negative biopsy and risk of diagnosis of prostate cancer 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)	0.5 (0.1–1.7)	4.8 (1.0–23.9)	3.8 (0.7–21.5)
	$P = 0.628$	$P = 0.425$	$P = 0.219$	$P = 0.260$
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)

NOTE: OR1, OR inherently adjusted for matching variables; OR2, OR 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.
[a]Cases without information on GSTP1 methylation and number of cores and corresponding controls within the matching strata were excluded from the analyses.

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, results were weaker and less consistent for LINE-1 with a possible exception of extremely low LINE-1 methylation levels.

While a study design similar to ours has been used in three previous studies to analyze GSTP1 methylation (20–22), to our

Table 3. LINE-1 methylation and GSTP1 hypermethylation on a negative biopsy and risk of diagnosis of prostate cancer on the rebiopsy stratified by time between the two samplings and Gleason score (the 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)

NOTE: OR1, OR inherently adjusted for matching variables; OR2, OR 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.
[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 was based on the subset of cases with specified Gleason score and all the controls within the matched stratum.

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Table 4. 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^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

[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.

knowledge, this is the first study to analyze global DNA hypomethylation in repeat prostate biopsies. Three previous studies have evaluated gene-specific methylation [GSTP1 and APC (22), GSTP1, APC and RAR-2β (21) or GSTP1, APC and RASSF1 (20)] on a negative biopsy as a predictor of prostate cancer on a rebiopsy. The three studies used different approaches to calculate sensitivity and specificity. However, in all three studies, GSTP1 was found to have a high specificity (75%–85%) and a low sensitivity (36%–52%), and in two studies APC had higher sensitivity than specificity (95% vs. 40% and 72% vs. 50%; refs. 21, 22). The clinical performance of combinations of multiple markers was evaluated in all three studies; however, improvement over a single marker was found in only one of them (20). 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 extremely low levels of LINE-1 methylation (i.e., <70% category, or ≤67% as visible from spline regression in Supplementary Fig. S2) with the risk of prostate cancer diagnosis on a rebiopsy. However, these results were less conclusive due to the small number of subjects included 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., Gleason score ≥4+3). For GSTP1, these results are in line with the findings of a previous study (20), 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 hypomethylation, specifically extreme global hypomethylation, could be considered, in addition to gene-specific hypermethylation, in models for the decision on whether to rebiopsy or not.

Our study has a strength in that the underlying population from which the patients and controls originate was an unselected series of men who underwent repeat biopsies at the Molinette hospital, Turin, Italy, with a 19% risk of being diagnosed with prostate cancer on a rebiopsy, which is in line with previous studies (20, 28). For quantitative analysis of LINE-1 and GSTP-1 methylation we used pyrosequencing which, in contrast to quantitative methylation specific PCR (qMS-PCR) used for GSTP1 analysis in prior studies (20–22), detects low levels of methylation as methylation in each CpG site is measured independently (29). Pyrosequencing has also been reported to have a higher sensitivity and accuracy than qMS-PCR (30). A high sensitivity is particularly important for analyses of GSTP1, as GSTP1 methylation is typically low in nontumor 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 nondifferential.

The main limitation of this study is that we lacked information on the various clinical and pathologic parameters that are typically used to predict prostate cancer on a subsequent biopsy (5–9), 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 rebiospy, 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. The selection of panel of markers is thus one of the priorities in prostate cancer research. Moreover, 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 (31). 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 nonlinear 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 rebiopsy.

In conclusion, in two parallel analyses conducted among patients seen in the two wards of a large Hospital in Italy, we found that GSTP1 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.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: F. Merletti, L. Richiardi

Development of methodology: R. Zelic, V. Fiano, D. Zugna, C. Grasso, L. Richiardi

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Daniele

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Zelic, D. Zugna, L. Delsedime, D. Galliano, A. Pettersson, F. Merletti, L. Richiardi

Writing, review, and/or revision of the manuscript: R. Zelic, V. Fiano, D. Zugna, C. Grasso, A. Pettersson, F. Merletti, L. Richiardi

Study supervision: L. Richiardi

Other (performing and interpretation of molecular analysis): V. Fiano, C. Grasso

Other (supervision of molecular analyses and interpretation of molecular results): A. Gillio-Tos

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Renata Zelic, Valentina Fiano, Daniela Zugna, et al.

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