

Risk Score Predicts High-Grade Prostate Cancer in DNA-Methylation Positive, Histopathologically Negative Biopsies

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BACKGROUND. Prostate cancer (PCa) diagnosis is challenging because efforts for effective, timely treatment of men with significant cancer typically result in over-diagnosis and repeat biopsies. The presence or absence of epigenetic aberrations, more specifically DNA-methylation of *GSTP1*, *RASSF1*, and *APC* in histopathologically negative prostate core biopsies has resulted in an increased negative predictive value (NPV) of ~90% and thus could lead to a reduction of unnecessary repeat biopsies. Here, it is investigated whether, in methylation-positive men, DNA-methylation intensities could help to identify those men harboring high-grade (Gleason score ≥ 7) PCa, resulting in an improved positive predictive value.

METHODS. Two cohorts, consisting of men with histopathologically negative index biopsies, followed by a positive or negative repeat biopsy, were combined. EpiScore, a methylation intensity algorithm was developed in methylation-positive men, using area under the curve of the receiver operating characteristic as metric for performance. Next, a risk score was developed combining EpiScore with traditional clinical risk factors to further improve the identification of high-grade (Gleason Score ≥ 7) cancer.

RESULTS. Compared to other risk factors, detection of DNA-methylation in histopathologically negative biopsies was the most significant and important predictor of high-grade cancer, resulting in a NPV of 96%. In methylation-positive men, EpiScore was significantly higher for those with high-grade cancer detected upon repeat biopsy, compared to those with either no or low-grade cancer. The risk score resulted in further improvement of patient risk stratification and was a significantly better predictor compared to currently used metrics as PSA and the prostate cancer prevention trial (PCPT) risk calculator (RC). A decision curve

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analysis indicated strong clinical utility for the risk score as decision-making tool for repeat biopsy.

CONCLUSIONS. Low DNA-methylation levels in PCa-negative biopsies led to a NPV of 96% for high-grade cancer. The risk score, comprising DNA-methylation intensity and traditional clinical risk factors, improved the identification of men with high-grade cancer, with a maximum avoidance of unnecessary repeat biopsies. This risk score resulted in better patient risk stratification and significantly outperformed current risk prediction models such as PCPTRC and PSA. The risk score could help to identify patients with histopathologically negative biopsies harboring high-grade PCa. *Prostate*

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KEY WORDS: DNA methylation; epigenetics; prostate neoplasms; significant cancer; high-grade; repeat biopsy; logistic regression

INTRODUCTION

Prostate cancer (PCa) patient management is challenging when trying to achieve high sensitivity, in order not to miss clinically significant cancer, while retaining high specificity, to avoid false positives. Effective, timely treatment of potential aggressive PCa can be achieved through early detection by adequate screening, for example, by means of prostate-specific antigen (PSA) [1,2]. However, these same first-line diagnostic techniques quite often result in over-diagnosis and over-treatment of patients with indolent disease and unnecessary biopsies [3–6]. In the US alone, over one million biopsies are performed each year, with ~25% of these resulting in a PCa diagnosis [7]. Furthermore, only a fraction of these would be considered at high risk for harboring clinically significant, aggressive PCa [8].

No single biomarker has proven to be efficient enough to be used as the sole diagnostic or prognostic tool. While serum PSA is easy to assess, there is no optimal cut point simultaneously resulting in high sensitivity and specificity as high-grade tumors can be missed even when applying low PSA cutoffs [1,9]. Histopathological examination of prostate biopsies, the diagnostic gold standard, suffers from a sampling bias, due to a limited amount of the prostate tissue being examined [10–12]. When, over time, risk factors persist and the risk for missed PCa is considered too high, those men will undergo one or more repeat biopsies. However, because the high false positive rate [9], these (repeat) biopsies can be an unnecessary patient burden and healthcare cost, and can also lead to complications [13,14].

Epigenetic profiling by determining the DNA-methylation status of *GSTP1*, *APC*, and *RASSF1* has been validated in two large, independent cohorts to be able to increase the negative predictive value (NPV) for men with PCa-negative biopsy tissue. When no methylation of either one of these three markers is detected in any of the residual tissues from

previously cancer-negative prostate biopsy cores, this biomarker panel has been shown to result in an NPV of 88–90% for all PCa [15,16]. This is a significant increase over the gold standard histopathological evaluation of these same biopsies and could result in a decrease in unnecessary repeat biopsies [17].

Due to the high rate of unnecessary (repeat) biopsies, attention has shifted towards identifying men with significant PCa, often characterized as the presence of Gleason pattern four or five, non-organ-confined disease and larger tumor volume [18]. In addition, patients with insignificant or low-risk disease under active surveillance, are at risk for disease reclassification, upgrading and upstaging, warranting faster radical treatment for these men [19–22]. A large, contemporary study in over 34,000 men found that Gleason score (GS) upgrading in GS6 patients is still very frequent when comparing the clinical and pathological scores [23,24].

In current clinical practice, multimodal approaches are used, with experts integrating several information sources to determine the best course of action for each patient. This entails both classical clinical risk factors, such as digital rectal examination (DRE) and histopathological examination of biopsy tissue, and traditional biomarkers, such as PSA. More recently, better molecular biomarkers with higher specificity for PCa have been introduced into clinical practice to improve patient management, in particular DNA-methylation profiling of *GSTP1*, *RASSF1*, and *APC* [25,26]. The goal of this study is to evaluate the performance of an existing DNA-methylation assay [15,16], to predict men at risk of harboring high-grade cancer. Interestingly, the three genes involved in this assay have all been associated with PCa prognosis and might therefore also be predictive of PCa aggressiveness [27,28]. Therefore, two main objectives were set; first, absence or low levels of DNA-methylation of the genes in the assay should reach a high NPV for high-grade cancer, and second, assay-positive patients should be further accurately stratified according to the risk of harboring high-grade cancer.

MATERIALS AND METHODS

Two previously published cohorts, of which all patients had two consecutive biopsies within 24–30 months, were combined into one set of 803 patients [15,16]. Each center received institutional review board approval, exemption, or waiver to use archived clinical samples for research purposes (Western General Hospital, Edinburgh, UK; University Hospital of Liège, Belgium; Institut de Pathologie et Génétique, Belgium; Cleveland Clinic, USA; Eastern Virginia Medical School, USA; Lahey Hospital and Medical Center, USA; Johns Hopkins University, USA; University of California Los Angeles, USA). Because this is a non-interventional, retrospective, subject-anonymized study, written patient consent was not required by the ethics committees. All men had a negative index biopsy followed by either a positive (179 men) or negative (624 men) repeat biopsy. The cohorts were joined and annotation was harmonized for histopathology of the first, PCa-negative biopsy, that is, benign, high-grade prostatic intraepithelial neoplasia (HGPIN) or atypia, and DRE, that is, normal or abnormal.

The DNA-methylation profile based on *GSTP1*, *RASSF1*, and *APC* was measured using quantitative real-time PCR as described before [15,16]. Besides the final assay result per patient (methylation positive or negative), the methylation intensity of each individual marker in each core of the index biopsy was evaluated.

Patients were classified according to the histopathological outcome of the repeat biopsy. Men with high-grade PCa (GS ≥ 7) detected upon repeat biopsy ($n=67$) were considered high-risk patients, while men with GS ≤ 6 disease ($n=106$) potentially/likely have indolent PCa. Six PCa patients (3.4%) were not classified due to incomplete Gleason scoring. Men without PCa detected, after repeat biopsy, are considered control patients, although cancer could be missed due to biopsy sampling error.

Patients are also stratified according to their overall methylation status (positive or negative) as determined in MATLOC and DOCUMENT [15,16]. Only 36.2% of all control patients are methylation positive, compared to almost the double (64.8%) for men with cancer detected upon repeat biopsy. A risk score was developed to improve stratification of methylation-positive patients according to their risk of harboring occult, high-grade cancer. In addition to the epigenetic profiling, the contribution of standard risk factors, that is, histopathology of the negative index biopsy, digital rectal examination, PSA, and age were considered. Clinical risk was also examined by the risk calculator (RC) of the prostate cancer prevention trial

(PCPT) [29]. Logistic regression models were optimized and the final selection was based on the overall predictive accuracy as measured by the area under the curve (AUC) of the receiver operating characteristic (ROC) and DeLong confidence intervals.

All statistical analyses were performed in R [30]. Continuous variables are compared with either Welch's *t*-test or the Mann–Whitney–Wilcoxon test for two samples, and ANOVA or Kruskal–Wallis test for more samples. The χ^2 or Fisher's exact test was applied to assess the significance of frequency distributions and a binomial test was applied when comparing proportions. *P*-values were corrected using the false discovery rate for multiple hypothesis testing, resulting in a *q*-value [31]. Calculations that are dependent on prevalence all made use of the overall cancer detection rate upon repeat biopsy observed in MATLOC, that is, 18%. Finally, clinical utility was determined using a decision curve analysis (DCA), and executed with the available R package [32].

RESULTS

Combined Cohort Description

A total of 7,899 prostate core biopsies from 803 patients in the unified cohort were epigenetically profiled. The most important clinical and demographic characteristics are shown in Table I. Each individual patient typically had 10 evaluable cores and the repeat biopsy often took place within 1 year of the index biopsy. The NPV of finding low levels of DNA-methylation in the combined cohort was 89.2% for all cancers. The positive predictive value (PPV) of the epigenetic assay performed on the index biopsies was 28.2% for detecting any cancer upon repeat biopsy. Of note, none of these cancers were identified at the time of the index biopsy, and based on the cancer detection rate after repeat biopsy, the epigenetic assay had a significantly increased PPV ($P < 0.001$) compared to current clinical practice. Of the traditional clinical risk factors, only histopathology was significantly different between the distinct groups, however, this did not allow a straightforward separation between controls, patients with low-grade cancer and men with high-grade cancer.

Limiting Delayed Diagnosis of High-Grade Cancer

While no tumors were found at time of the index biopsy, both high- and low-grade disease were found during repeat biopsy. Here, 106 out of 173 men with PCa had GS6 disease, thus 38.7% of all cancers identified at repeat biopsy were considered clinically

TABLE I. Main Clinical and Demographic Characteristics of the Combined MATLOC and DOCUMENT Cohorts

	Group			<i>P</i>
	Controls	GS ≤6	GS ≥7	
n	624	106	67	
PSA (ng/ml)				
Mean/median	6.85/5.6	7.19/5.0	8.26/6.0	0.117
DRE				
% Abnormal	31.3%	29.8%	38.8%	0.520
Histopathology				
%HGPIN	22.8%	33.0%	19.4%	<0.001
%Atypia	6.7%	17.9%	13.4%	
Age				
Mean/median	62.5/62.0	63.3/64.0	65.6/66.0	<0.001
Evaluable cores				
Mean/median	9.9/10	9.6/10	9.4/10	0.265
Time between biopsies (months)				
Mean/median	12.5/9.2	9.8/8.5	12.0/11.1	0.178

significant, based on the clinical grade. High-grade cancer is found in merely 7.0% (18% of men will have PCa detected upon repeat biopsy, of which 38.7% will have high-grade [GS ≥7] disease) of men undergoing repeat biopsy. Because frequent upgrading of GS6 patients, the NPV of high-grade cancer cannot easily be determined based on clinical GS. When including all patients with clinical GS6 as control, a lower boundary for the NPV for high-grade cancer of 95.7% was obtained. When GS6 patients were omitted from the calculations, the NPV was 95.9% for high-grade cancer.

Stratifying Methylation-Positive Men for High-Grade PCa Risk

From the entire cohort, a subset consisting of the 43 men with high-grade PCa and the 226 men without PCa detected in a repeat biopsy was taken, however, all of which had a DNA-methylation positive index biopsy. This subset was used to evaluate whether men with high-grade PCa can be identified by determining DNA-methylation intensities in their PCa-negative index biopsies. GS6 patients were not included due to the high reclassification risk of under-graded disease. Several methylation parameters were evaluated, that is, the relative number of methylation positive cores, the relative number of methylation events, and the number of distinct, methylated genes. These methylation-based metrics were compared with traditional risk factors in their ability to identify men with high-grade PCa, but with histopathologically cancer-

negative biopsies (Table II). DNA-methylation metrics and age at the time of the index biopsy were significantly higher in the men with high-grade PCa upon repeat biopsy. Pathology, PSA and DRE did not perform better than random (all $P > 0.05$; Table II).

EpiScore: Measuring Epigenetic Risk Via DNA-Methylation Intensity

Because the level of DNA methylation was the most significant and strongest predictor (Table II) of a methylation-positive man having high-grade cancer detected upon repeat biopsy, a general epigenetic risk score was developed based on methylation intensities of the three genes in individual cores. Per core, the methylation intensity of each gene was divided by a normalization factor, optimally weighing each gene's contribution. These normalized intensities were added per core and subsequently averaged over all evaluable cores per patient, to obtain one final epigenetic score. This EpiScore summarizes all available methylation signals that can help in identifying men with high-grade PCa detected upon repeat biopsy, that is, methylation intensity, number of methylated cores, and number of methylated genes.

A saturation parameter was applied to avoid overweighing a limited number of patients with very high methylation signals. Gene weights and the saturation

TABLE II. Univariate Analysis of All Available Traditional and Molecular Risk Factors

Risk factor	AUC	95%CI	<i>P</i> -value
PSA (continuous or log-transformed)	0.574	0.481–0.667	0.151
PSA (three categories: <4, ≥4 and <10, >10)	0.550	0.476–0.625	0.157
PSA (two categories: <10, ≥10)	0.493	0.432–0.554	1.000
PSA (continuous when ≥4, otherwise 0)	0.569	0.474–0.664	0.179
PSA (continuous when ≥10, otherwise 0)	0.497	0.433–0.561	0.924
DRE	0.529	0.432–0.626	0.549
Pathology	0.486	0.400–0.572	0.152
Pathology (only presence of atypia)	0.532	0.477–0.587	0.228
Age	0.632	0.544–0.720	0.006
#Cores methylated	0.635	0.541–0.730	0.005
#Methylation events	0.661	0.572–0.751	0.001
#Distinct genes methylated	0.596	0.522–0.671	0.002

Performance of the risk factor was measured as the AUC of the ROC and as the significance when comparing the controls to the GS ≥7 patients (Fisher's exact test for categorical variables and a Mann-Whitney-Wilcoxon test for numerical variables).

parameters were exhaustively optimized to reach a maximal AUC of 0.742 (Fig. 1). In addition to the AUC, the mean EpiScore for the control group was compared to that in the group of men with high-grade PCa during the optimization process, to assure robustness of the algorithm, and only models with $q < 0.001$ were retained. In the final model, EpiScore was significantly higher for those men with high-grade PCa detected upon repeat biopsy compared to those with a non-cancer diagnosis ($P < 0.001$; Fig. 2).

EpiScore and Potentially Indolent Cancer

As an additional test of robustness of the algorithm, EpiScore was calculated for methylation-positive men with likely indolent disease ($GS \leq 6$), detected at time of repeat biopsy. This confirmed the original hypothesis concerning these men, with intermediate EpiScores compared to the other two groups (Fig. 2). Indeed, overall there were significant differences between the three groups ($P < 0.001$). A more detailed analysis of the differences indicated significantly higher EpiScores for those men with high-grade disease versus the control group ($P < 0.001$) and the men with GS6 PCa detected upon repeat biopsy ($P < 0.001$), while the increase of EpiScore for GS6 patients versus the control patients was not significant ($P = 0.184$).

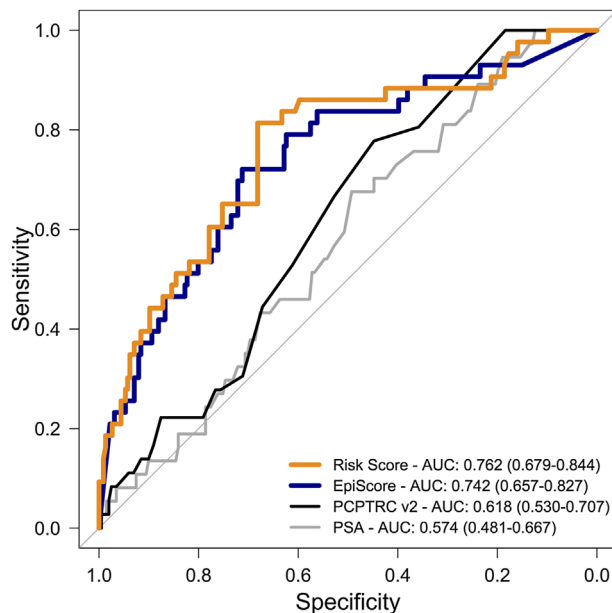


Fig. 1. ROC of EpiScore and the risk score in methylation positive men with either a negative repeat biopsy (controls) or $GS \geq 7$ repeat biopsy (cases). PSA and PCPTRC v2 are also depicted, serving as current references for clinical practice. AUC and 95% confidence interval (CI) are shown in the legend.

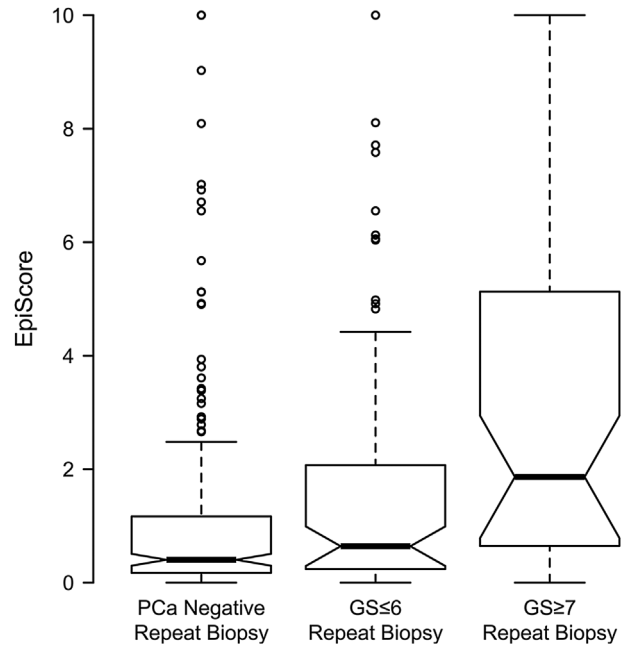


Fig. 2. Box-and-whisker plot of EpiScore in methylation-positive men, that is, for control patients with a negative repeat biopsy, for the group of men with potentially insignificant cancer detected upon repeat biopsy ($GS \leq 6$) and for those men with significant cancer ($GS \geq 7$) detected upon repeat biopsy.

Holistic, Multimodal Risk Score for Clinically High-Grade Cancer

It was evaluated whether the EpiScore logic could be improved further by adding classical risk factors to the algorithm. First a logistic regression model was built, including EpiScore, age, PSA, DRE, and histopathology of the PCa-negative index biopsy. When the logarithm (base 10) of PSA was used instead of the actual PSA value (in ng/ml), the relevance of PSA in the model increased, most likely due to the restricted weight of very high PSA values. EpiScore was the only significant factor in this model with an odds ratio (OR) of 9.80 (95%CI: 2.12–45.23) (Fig. 3). PSA was borderline significant and a positive trend was observed for the presence of atypia and age at time of the index biopsy (all $P > 0.05$). HGPIN was the only risk factor that inversely correlated with the detection of high-grade PCa upon repeat biopsy (OR < 1).

A stepwise forward selection procedure was implemented. When combining two risk factors, pathology of the cancer-negative index biopsy was added to the EpiScore and, next, age was selected as third factor. Adding more factors did not further improve the model, however, missing data for PSA (not available for 11.5% of patients) and DRE (not available for 23.4% of patients) could lead to an underestimation of their effects. In this final logistic regression model

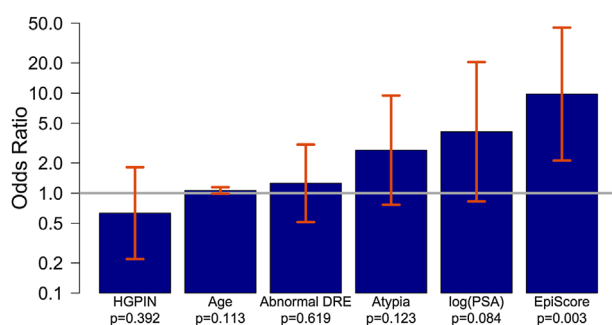


Fig. 3. Odds ratios (OR) of a logistic regression model containing EpiScore and classical risk factors. A horizontal line is drawn at OR = 1 above which the risk factors have a positive contribution.

containing EpiScore, age and histopathology, the trends for these risk factors remained unchanged relative to those depicted in Figure 3. However, now age was a significant contributor ($P = 0.010$) and the OR for EpiScore increased to 14.12 and appeared more robust (95%CI: 12.59–15.84; $P < 0.001$). The final model, based on EpiScore, histopathology of the first, cancer negative biopsy and age, reached an AUC of 0.762 (Fig. 1).

To further evaluate the role of missing clinical data, the risk score was generated using all available risk factors for each individual patient. The risk score was calculated based on EpiScore, pathology, age, DRE and PSA, however, models were also optimized for all combinations of missing data, that is, most often DRE and PSA in this cohort. With this strategy an AUC of 0.77 (95%CI: 0.69–0.84) was obtained, which was not significantly higher than the model only including EpiScore, pathology and age ($P = 0.688$).

Clinical risk was also calculated by means of the PCPTRC version 2. Due to the small, and sometimes counterintuitive, effect of DRE in this cohort, PCPT risk for high-grade cancer was calculated with and without DRE, but always including PSA, age and race. Because of missing values, the cohort was limited to those men with a valid PCPT risk score, since PSA is a necessary parameter for this algorithm. While EpiScore alone reached an AUC of 0.714 in this subset of the cohort, the PCPT risk was far less predictive, with an AUC of 0.618, regardless of DRE inclusion. Combining EpiScore with the risk predicted by the PCPT risk calculator increased the AUC to 0.742 (without DRE; or 0.741 with DRE). Relative to EpiScore, the single most significant parameter in the model, the addition of the PCPTRC traditional clinical risk represents an increase of 3.9% for the AUC, compared to 2.7% (increase from 0.742 to 0.762) with the addition of clinical risk as specifically optimized in this cohort. The risk score resulted in a significantly higher AUC compared to

currently used risk stratification algorithms, that is, PSA ($P = 0.004$) and PCPTRC ($P = 0.029$).

Clinical Utility

A DCA was executed to determine the clinical utility of the risk score and to obtain an accurate assessment of the net benefit, in terms of high-grade PCa detected corrected for performing unnecessary repeat biopsies, and net avoidance rate, that is, the reduction in repeat biopsies corrected for missing high-grade cancers. Test harm, that is, the fact that a larger number of men needs to be tested in order to identify a subset of men with high-grade PCa, was not included in the DCA, since no additional testing would be required. PSA and DRE (included in PCPTRC) were obtained at the time of the first biopsy, and EpiScore was calculated as the DNA-methylation intensity observed in the previous, cancer-negative biopsy. Hence, all information was already available at the time when a repeat biopsy was considered, with no additional testing required.

Compared to PSA and PCPTRC, the risk score clearly had the highest net benefit in terms of identifying men with high-grade PCa (Fig. 4A). Taking into account the 7.0% prevalence of high-grade PCa in the general repeat biopsy population, and 16.0% for those men with a methylation-positive prior biopsy, the risk score proved to have a large net benefit, even for those men who are very risk averse, that is, at low probability thresholds. The net benefit of the risk score was larger compared to a biopsy strategy where all men receive a repeat biopsy, as soon the accepted risk was $\geq 3\%$, that is, starting well below the overall risk of having high-grade PCa detected in either the general or the methylation-positive population repeat biopsy population. The risk score showed the largest net benefit over the entire range of clinically applicable and acceptable probability thresholds that high-grade PCa will be found upon repeat biopsy.

Importantly, the risk score also resulted in the largest reduction of unnecessary repeat biopsies compared to PSA and PCPTRC. If a risk, or the probability threshold below which an intervention is not considered desirable, of having high-grade PCa detected upon repeat biopsy of 15% is considered, that is, similar to the overall prevalence of high-grade cancer in the methylation-positive population, then the risk score resulted in a 3.3- and 5.0-fold net reduction in repeat biopsies compared to PCPTRC and PSA, respectively. This net reduction is the unnecessary repeat biopsy part of interventions avoided and hence does not come at the cost of additional high-grade PCa missed. In summary, in methylation-positive men, and applying the same probability

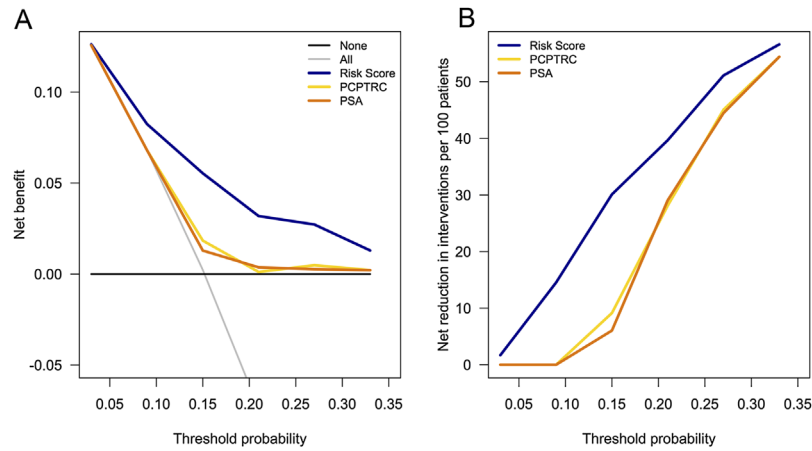


Fig. 4. DCA illustrating the overall clinical utility of the risk score compared to PCPTRC and PSA. Clinical utility of the risk score is demonstrated by the overall net benefit in detecting high-grade PCa corrected for unnecessary biopsies (**A**) and the net reduction in interventions corrected for missed high-grade cancers (**B**).

threshold of 15%, an additional 30 unnecessary repeat biopsies per 100 patients would be avoided with the risk score, compared to only nine and six for the PCPTRC and PSA, respectively.

DISCUSSION

PCa screening and diagnosis debates center around two goals that are often hard to reconcile. First, all men with high-grade cancer should be identified as early as possible, as these patients usually require radical treatment. Second, men with low-grade PCa should not be over-treated, especially because the treatment could cause more harm than benefit [14]. The absence of DNA-methylation of *GSTP1*, *APC*, or *RASSF1* in PCa-negative, residual biopsy tissue resulted in a NPV of 96% for high-grade cancer, successfully addressing the over-treatment issue.

To better stratify methylation-positive patients for the risk of harboring high-grade cancer missed by biopsy, a novel algorithm was developed. EpiScore weighs the DNA-methylation intensities of *GSTP1*, *RASSF1*, and *APC* across a patient's biopsy cores, with significantly higher intensities observed in men with high-grade PCa detected upon repeat biopsy. EpiScore successfully identified men with high-grade PCa that was missed by a prior biopsy, and stratified men who are likely in higher need of a repeat biopsy, due to an increased risk of occult, high-grade cancer.

An important aspect of current and future clinical research is a multimodal approach, integrating several information sources to obtain the best possible, most objective assessment for each individual patient. Therefore, known, traditional risk factors were combined with EpiScore into one holistic model, albeit

with the epigenetic component of this risk score being the most significant and important risk factor. The risk score consists of EpiScore, histopathology of the cancer-negative index biopsy (atypia, HGPIN, or benign) and a patient's age at time of the index biopsy. In this cohort the risk score resulted in an improved patient segregation, with a higher AUC than EpiScore alone. While the cohort was sufficiently complete for all risk factors, at least to get an idea about the potential contribution to the risk score, the missing data for PSA and DRE might have led to over- or under-interpretation of the actual effect for these two factors. When available, the addition of PSA or DRE to the risk score led to a minor, non-significant increase of the overall model's performance. However, in particular for DRE, inter-observer variability could have an unexpected impact. When the risk score was defined as the combination of EpiScore and the clinical risk as predicted by the PCPT risk calculator, EpiScore remained the most predictive and significant factor, however, a small benefit was again observed by adding clinical risk to the molecular, epigenetic risk. Finally, the risk score significantly outperformed currently used risk prediction models such as the PCPTRC and PSA. In summary, this risk score combines clinical risk factors with EpiScore, resulting in an improved risk stratification of high-grade PCa in histopathologically negative biopsies.

Unfortunately, due to the lack of sufficient long-term follow-up data, for example, pathological grades were not available, and more extensive clinical information, men with high-grade cancer were defined as those with PCa-positive, $GS \geq 7$ repeat biopsies. In addition, data on Gleason patterns were also not recorded, so a more detailed analysis of Gleason 3 + 4

versus 4+3 patients was not possible. While more accurate risk classification tools exists, such as the guidelines from the National Comprehensive Cancer Network, most of these are dependent, at least to some extent, on the clinical GS used here [33]. Men with GS6 cancer were not included in the cohort for the development of the risk score, because risk for men with clinical GS6 is harder to predict. This also reflects a clinical reality, since upgrading of clinical GS6 patients occurs frequently [23]. Inter-observer variability could also play a role, since centralized pathology review occurred only within the DOCUMENT sub-cohort.

While it can be debated whether GS6 patients should be detected by screening, such a statement would only hold value when knowing the true pathological GS. In addition, if disease progresses over time, it would be more efficient to have such patients monitored closely or predict who is at increased risk for disease progression. For these two reasons, men with clinical GS6 disease would still benefit from being identified, however with lower priority compared to men likely harboring high-grade cancer.

While unique, optimal solutions were found for the weighing factors in both EpiScore and the risk score, closely related algorithms resulted in a similar performance in terms of AUC. Therefore, cohorts for validation studies would benefit from enrichment for men with high-grade PCa detected upon repeat biopsy, making the risk score more robust. In addition, future studies would also benefit from including long-term follow-up, that is, radical prostatectomy results and pathological GS. The same or a similar algorithm could also be validated as an identification tool for those patients diagnosed with GS6 that are at risk of being under-graded. It remains to be evaluated whether these epigenetic-based algorithms or the applied molecular methodology could also help triage such patients in active surveillance programs and separate those who are likely under-graded or likely to progress, from those with stable, low-grade disease.

Finally, besides the clinical performance, the clinical utility of the risk score was investigated. A DCA was executed, evaluating clinically acceptable probability thresholds above which a repeat biopsy was warranted. Because this probability threshold is personal, it is important to note that the risk score resulted in a net benefit, and the largest benefit compared to PCPTRC and PSA, across the entire range of clinically relevant probability thresholds. In addition, the risk score also resulted in the largest reduction of unnecessary repeat biopsies, again over the entire range of clinically relevant probability

thresholds. This demonstrates the large clinical utility of the risk score for men with a PCa-negative, methylation-positive index biopsy.

CONCLUSIONS

Clinical practice is shifting towards more complex integrations of several risk factors, rather than relying on an individual (bio) marker. Here, a risk score was developed that combines EpiScore and known clinical risk factors into one algorithm, identifying men at risk of harboring high-grade PCa, despite a negative biopsy result. EpiScore is an epigenetic profiling algorithm based on the DNA-methylation intensities of *GSTP1*, *RASSF1*, and *APC* and was the most significant and best performing risk factor to identify men with occult, high-grade PCa based on residual tissue of a prior biopsy negative for PCa. A DCA indicated that the risk score was associated with the largest net benefit and the largest avoidance of unnecessary repeat biopsies, compared to two commonly used methods for decision-making, that is, the PCPTRC and PSA, demonstrating clinical utility.

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