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Circulating microRNAs combined with PSA for accurate and non-invasive prostate cancer detection

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Running head

Plasma microRNA analysis for PCa detection

Keywords: extracellular/circulating microRNA, plasma, prostate cancer, PSA, diagnosis

Abstract

The dosage of prostate specific antigen (PSA), a non-invasive biomarker easily evaluable, has made early detection of prostate cancer possible. However, PSA measurements lead to a high percentage of unnecessary biopsies and may miss aggressive tumors in men with antigen levels below the standard threshold of 4 ng/ml. We thus propose to combine circulating microRNAs with PSA, to improve the diagnostic route for prostate cancer.

Plasma microRNA profiling was performed to identify candidate diagnostic microRNAs in a discovery cohort of 60 tumors and 60 controls (men with benign prostatic hyperplasia or healthy donors). Linear models with an empirical Bayesian approach and multivariate penalized logistic regression were applied to select tumor-associated microRNAs and/or clinical variables. A classifier was developed and tested on a validation cohort of 68 tumors and 174 controls, consecutively collected, where microRNAs were evaluated by quantitative real-time polymerase chain reaction.

A classifier based on miR-103a-3p, let-7a-5p and PSA could detect both overall and clinically significant tumor better than PSA alone, even in 50-69 aged men with PSA ≤ 4ng/ml. In the validation cohort, the same classifier still performed better than PSA alone in terms of specificity and positive-predictive-value and allowed for a correct identification of 8 out of 9 tumors not detected by PSA, including three high-risk and three tumors in 50-69 years old men. 34% of carriers of non-malignant lesions with PSA in the 4-16 ng/ml interval, who may avoid unnecessary and harmful biopsies, were correctly identified.

Coupling the analysis of two circulating microRNAs with PSA dosage could be a useful strategy to diagnose clinically significant PCa and avoid an important fraction of unnecessary biopsies.

Summary

Circulating miRs and PSA were combined in a non-invasive blood-based test for PCa detection that allows for reduction of useless biopsies and for identification of prostate tumors with low PSA levels in the 50-69 age range.

Introduction

Prostate Cancer (PCa) is the most frequent neoplasia diagnosed in males and one of the most common causes of cancer-related death (1).

Currently, PCa is investigated by digital rectal examination (DRE) and/or Prostate Specific Antigen (PSA) levels. However, DRE has low sensitivity (2) while PSA is organ- but not tumor-specific and has a low positive predictive value (PPV=30-35%) (3). As a consequence, correct diagnosis relies on histopathological verification of adenocarcinoma by invasive multiple bioptic sampling (4). For a long time, standard biopsies (10-to-12-core transrectal ultrasonography-guided excisions) have been considered the best reference standard despite their low detection rate and the upgrading of a percentage of tumors upon surgical tissue examination. Multiparametric magnetic resonance imaging (mp-MRI), with or without targeted biopsy, is now an alternative with level 1 evidence to standard biopsy for PCa detection (5). However, there is still an urgent need of non-invasive markers for i) the early detection of aggressive cancers, especially when PSA is below the usual cut-off of 4 ng/ml, ii) the avoidance of unnecessary biopsies in men with high PSA due to benign prostatic hyperplasia (BPH) and iii) the identification of patients on active surveillance who have truly indolent prostate cancer (6).

For these purposes, in the age of "liquid biopsies", microRNAs (miRs) represent ideal candidates, especially for their proved stability in body fluids. miRs control major pathways such as cell growth, proliferation, differentiation and survival (7) and tumor-associated miRs participate in intercellular communication and disseminate through the extracellular fluid to reach and influence the phenotype of remote targets (8).

For these reasons, researchers have focused on the analysis of body fluids and have proposed miRs as possible biomarkers for several diseases, including PCa (6, 9). Nevertheless, little reproducibility

of results has been observed. Only few studies include biomarker discovery, validation and application on large independent prospective cohorts, carefully considering all the steps from sample collection to storage and analysis.

In the present work, miR profiling of 120 plasma samples was performed to identify candidate miRs able to detect PCa more accurately than PSA alone. A classifier was built and validated on an independent cohort of 242 consecutively collected samples.

Materials and methods

Samples

Samples from men with PCa, suspected symptoms of PCa but negative biopsies (labeled as BPH), high grade prostatic intraepithelial neoplasia (HGPIN) or atypical small acinar proliferation (ASAP) were collected at the S. Giovanni Battista hospital of Turin, prior to standard 12-core transrectal ultrasonography-guided biopsy. PCa were labelled, according to Gleason Score (GS) values, as GS6, GS7 or GS>7. According to PSA, GS and tumor size (cT) within clinical TNM staging, PCa were labelled as low risk, if GS=6, PSA<10, cT<2b; as intermediate risk, if 10≤PSA≤20 or GS=7 or cT2b-cT2c; as high risk, if GS=8-9-10 and/or PSA>20 and/or cT3-cT4. Clinically significant tumors comprised intermediate/high-risk PCa. Healthy donor's (HD) plasma was collected at Fondazione Edo and Elvo Tempia, in the same geographic area as patients. HD were in the same age-range as patients, had negative DRE and PSA<4ng/ml, were not under any pharmacologic treatment nor had any previous prostatic pathology. The study was approved by the Ethics Committee of Novara (Italy), protocol reference: NC-SERPROS, CE 149/11.

All human subjects provided written informed consent with guarantees of confidentiality. Plasma collection, processing and storage adhered to good practice rules. Haemolyzed samples or samples belonging to men with other cancer diagnosis were excluded from analyses.

Plasma isolation and storage

Plasma was isolated from EDTA or Lithium Heparin blood samples within 1 hour from collection, with a standard procedure to prevent haemolysis. Blood was centrifuged at 2500 rpm (1250g) at 4°C for 10 min. The supernatant was transferred into new tubes and subjected to a second centrifugation step at 2500 rpm (1250g) at 4°C for 10 min to remove cell debris and fragments. Plasma was stored in 4.5 ml cryovials at -80°C until transfer to the Cancer Genomics Lab. To calculate haemolysis score (HS) (10), 10 μl of plasma was centrifuged at 1000g for 5 min at room temperature and the absorbance at 385 and 414 nm was measured by a NanoDrop

spectrophotometer (Thermo Fisher) using the UV-VIS program. Finally, 220 µl aliquots were created for each sample and stored into 1.5 ml tubes at -80°C. Samples with HS<0.057 and/or 414nm/385nm absorbance ratio below 2 were kept for further processing.

Circulating RNA extraction

Before extraction, one 220 µl aliquot per sample was centrifuged for 5 min at 1000g at 4°C. Total RNA was extracted with miRNeasy serum/plasma kit (Qiagen) using Exiqon protocol, with the batteriophage MS2-RNA carrier (Roche Diagnostics) to promote RNA precipitation and purification on membranes. The *C. elegans* cel-miR-39-3p miR mimic spike-in (Qiagen) was added. RNA samples were eluted in 30 µl of nuclease-free water and stored at –80°C.

miR profiling

miR profiling was carried out on 138 samples, 120 of which (60 PCa, 51 BPH and 9 HD) were homogeneously collected in heparin tubes and constituted the discovery phase dataset (**Table I**). The other 18 HD samples were collected in EDTA tubes and were used for comparison purposes only.

The miRNA microarray protocol V2.4 (Agilent Technologies) was followed, starting from 4 µl of total RNA. Briefly: RNA was dephosphorylated and denaturated, then a ligation and labeling step was performed. Samples were hybridized to oligonucleotide glass arrays representing 2006 miRbase-V19 miRs (Agilent Technologies). After hybridization, slides were washed following the manufacturer's washing procedure and scanned with the dual-laser microarray scanner version C (Agilent Technologies). Images were analysed using Feature Extraction software v10.7.

Statistical analysis

Normalization

Raw data were processed using the limma R package for microarray analysis. Background correction and inter-array normalization were performed applying *normexp* (offset=20) and *quantile* methods, respectively. Raw and average normalized log_2Intensities are available in the GEO public functional genomics data repository (https://www.ncbi.nlm.nih.gov/geo/), with the following identifier: GSE113234.

Sample size calculation

An online tool (https://biostatistics.mdanderson.org/MicroarraySampleSize/) was used to compute sample size for class comparisons: knowing the total number of miRs analyzed (2006) and inputting 1% as acceptable percentage of false-positives, 0.8 as desired statistical power, 0.25 as standard deviation and 0.2 as minimum log2fold-change, the minimum number of samples per class should be 36.

Class comparison

To compare miR profiles between classes, linear model and empirical Bayesian analyses were combined using the limma R package. Top differentially expressed miRs were selected using 0.2 as cut-off for the log_2fold-change in PCa versus non-PCa samples and 0.05 for raw p-value.

To compare RT-qPCR data between PCa and non-PCa samples, Student t-test with Benjamini-Hochberg correction for multiple testing was applied, while to compare PSA levels among different disease classes, analysis of variance (ANOVA) with Dunnett correction for multiple testing was applied. In both cases, differences were considered statistically significant if adjusted p-values were <0.05.

Classifier

Since i) the log_2 PSA distribution density of the PCa-BPH cohort of more than 400 consecutively collected samples available at the San Giovanni Battista hospital in Turin showed an inflection at 4, that corresponds to PSA = 16 (**Figure 1A**, red curve), and ii) within PSA>16 ng/ml samples there was a strong PCa enrichment, then in our classifier the latter were directly considered as PCa. Only samples with PSA ≤16 ng/ml were used to build a score that combines PSA with other variables.

The appropriateness of this cut-off was further verified by observing the $\log_2$ PSA distribution density of another big cohort of nearly 14,000 asymptomatic men, over 50, who tested their PSA levels at our Foundation from 2012 to middle 2018 (**Figure 1**, black curve). This curve, representative of a voluntary adhesion context, from PSA = 16 on is very close to zero.

Using $\log_2$Intensities of differentially expressed miRs in PCa within PSA≤16 ng/ml samples, $\log_2$PSA and $\log_2$Age as input variables, a logistic regression model with LASSO penalty (11) was fitted to build a classifier able to discriminate PCa from non-PCa, using the *glmnet* R package. 5-fold cross-validation was applied to find the best tuning parameter. Following the usual standards of binary regression, the estimated log odds-ratios of these variables were multiplied by their values and then summed to build a score: if the score was higher than a selected cut-off, the sample was classified as PCa. This way, the resulting overall classifier turned out to be a combination of the initial PSA check (>16 ng/ml) and the score. The accuracy of the classifier was measured by the area under the ROC curve (AUC).

RT-qPCR

Seven miRs were evaluated first on the same EDTA-HD samples used for miR profiling, to validate expression changes using RT-qPCR, and then on an independent set of EDTA plasma samples (10 HD, 10 BPH, 10 PCa). Exiqon miRCURY LNA™ Universal RT microRNA PCR protocol (Exiqon) was followed, starting from 4 μl of total RNA, using cel-miR-39-3p as exogenous normalizer and UniSp6 as internal control for reverse transcription (RT). BioRad CFX96 real-time instrument was used to test all miR assays on each sample in the same 96-wells plate, with 3 replicated measurements for each test, RT and real-time negative controls for each miR.

The two validated miRs were then analyzed on a validation phase dataset (**Table I**): a larger set of 242 consecutively and prospectively collected plasma samples (68 PCa, 93 BPH, 8 HGPIN/ASAP, 73 HD), all collected in EDTA tubes. Exiqon pick&mix 384-wells plates were analyzed on a 7900 HT Fast real-time PCR platform (Life Technologies). UniSp6 was analyzed separately to check RT.

UniSp3 was used as inter-plate calibrator (and positive real-time control), cel-miR-39-3p as exogenous normalizer and each miR was analyzed in triplicate, as well as negative controls. Data transformation was done with the delta Ct (DCt) method (Ct = threshold cycle), where Ct miR = average Ct of the 3 replicates and DCt miR_of_interest = Ct miR_of_interest – Ct cel-miR-39. Final Ct (Ctn) was given by Ctn miR_of_interest = -DCt miR_of_interest + K, where K= 6.2 is a constant chosen to make –DCt ranges comparable with microarray $\log_2$ intensity ranges. To calculate K, only BPH and PCa samples were considered (since the two cohorts differed in terms of percentages of HDs and precancerous lesions were not included in the validation set). The means of microarray $\log_2$ Intensities for the two miRs were calculated and then averaged, yielding 6.6. Then K was calculated in order to make the average of mean Ctn_miR-103a-3p and mean Ctn_let-7a-5p equal to 6.6.

The independent set was used to test the classifier, using the same coefficients (estimated log odds-ratios) previously generated, Ctn instead of $\log_2$Intensities for miRs and the same classification rule.

Results

Discovery phase

138 not haemolyzed plasma samples were analysed by miR profiling. Log_2PSA distribution in HD, BPH and PCa groups according to risk class is shown in **Figure 1B**. PSA distinguished HD from BPH/PCa, high-risk from low/intermediate-risk tumors, but was incapable of discriminating between BPH and low/intermediate-risk tumors.

EDTA-HD versus Heparin-HD comparison revealed that the use of heparin-coated tubes did not affect miR profiling (**Suppl_Figure 1A-B**).

The 120 homogeneously collected samples (**Table I**) were used to highlight numbers and types of miRs detected in each class (**Suppl_Figure 1C-D** and **Suppl_Table I**) and compare their levels (**Suppl_Table II**).

PCa (n=60) versus HD+BPH (n=60) analysis evidenced one down-regulated (miR-4530) and 9 up-regulated miRs (let-7a-5p, miR-103a-3p, ~~let-7d-5p~~, let-7f-5p, miR-17-5p, miR-4454, ~~miR-26a-5p~~, miR-130a-3p, miR-15b-5p, miR-24-3p, ~~miR-199a-3p~~, miR-21-5p) in PCa (**Figures 2A-2B**).

On the other hand, the comparison between PCa (n=60) and BPH (n=51) resulted in only 2 up-regulated miRs (let-7a-5p and miR-103a-3p).

Restriction to PSA≤16 ng/ml samples (48 PCa vs 57 BPH+HD) resulted in 11 up-regulated miRs (miR-103a-3p, let-7a-5p, let-7d-5p, miR-17-5p, let-7f-5p, let-7b-5p, miR-24-3p, miR-26a-5p, miR-20a-5p, miR-130a-3p and miR-15b-5p), while analysis of 4-16 ng/ml PSA samples (39 PCa versus 40 BPH) yielded only down-regulated miRs (miR-4530, miR-1207-5p, miR-575, miR-4739, miR-1202, miR-3679-5p, miR-6085, miR-3656, miR-663a, miR-4687-3p, miR-5739).

Discovery phase: development of a classifier

In order to find a strategy to classify samples, individuals with PSA>16 were considered independently and directly classified as PCa: 12 were true PCa while 3 were false positives. Using only samples with PSA≤16, the most recurrent variables (out of the 11 up-regulated miRs, log_2Age

and log_2PSA) with coefficient ≠ 0, selected by the penalized logistic regression model, were: miR-103a-3p, let-7a-5p and log_2PSA, all associated with a positive coefficient (0.1994, 0.1294, 0.0385 respectively). **Figures 2C and 2D** depict log_2Intensity box-plots for the two miRs in each sample class. A score was built by summing the products of the coefficients by the variable values, and samples were ordered by increasing score. Samples with score > 2.02 (chosen to optimize accuracy) were classified as PCa. The final classifier was then built combining this score with the initial PSA check (**Figure 3**), obtaining an AUC of 0.68 (95%C.I.=0.59-0.78), whereas the AUC of PSA alone was 0.62 (95%C.I.=0.53-0.73) (**Figure 4**). 36/39 (92%) clinically significant PCa were detected by the final classifier, whereas 34/39 (87%) by PSA alone. Only 3 intermediate and 5 low-risk PCa were misclassified. All high-risk PCa were identified, as well as 7/9 (77%) PCa with PSA≤4 ng/ml, all in men falling in the 50-69 age range. Moreover, 9/40 (22.5%) BPH with PSA>4 ng/ml and all HD were correctly classified.

Validation of miR expression by an independent technique

Seven potentially interesting miRs, selected from the discovery phase, were further evaluated by RT-qPCR (**Suppl_Table II**) on the EDTA-HD samples profiled with microarrays and on 30 EDTA-collected independent samples (10 PCa, 10 BPH, 10 HD). Since heparin inhibits RT, RT-qPCR as independent technique to validate expression changes could be applied on EDTA-HD samples only. Correlation between array intensities and RT-qPCR relative expressions was positive and > 0.8 only for miR-103a-3p and let-7a-5p (the top 2 up-regulated miRs in all previous analyses, and the ones included in the classifier), reinforcing their robustness. Also miR-21-5p was detectable in all the samples, however correlation between the two techniques was not satisfactory (correlation coefficient for miR-21-5p = -0.08, versus 0.87 and 0.90 for let-7a-5p and miR-103a-3p, respectively). The other miRs were not detectable in all the samples, thus correlation coefficients were not calculated for them. However, they were further tested in the independent group of 30 samples including BPHs and PCas. Since they had either Ct higher than 40 or nonspecific

amplification products (as observed by melting curve analysis), or coefficient of variations always higher than 0.1, they were considered as undetectable. In this independent cohort, statistically significant up-regulation in PCa versus HD+BPH resulted for both miR-103a-3p and let-7a-5p (**Figure 5A-B**).

Validation of the classifier on an independent cohort

Additional 242 independent plasma samples (**Table I**), consecutively collected and checked for hemolysis, were used for the validation phase. **Figure 6A** shows log_2PSA box-plot among different sample classes, whereas **Figures 6B and 6C** refer to the log_2Intensities of the two miRs.

14 samples out of 242 had PSA >16 ng/ml and were directly classified as PCa: 10 were high-risk PCa while 4 were false-positives. The remaining 228 samples were classified using the same exact score coefficients and cut-off generated in the discovery step.

The final classifier yielded an AUC of 0.76 (95%C.I.=0.70-0.82) (**Figure 7A**), whereas the AUC of PSA alone was 0.74 (95%C.I.=0.68-0.80). In particular, our classifier correctly identified 8/9 (89%) patients with PCa and PSA ≤4ng/ml, 7 of which harbored clinically significant (3 high-risk and 4 intermediate-risk) tumors. Of note, three of them fell in the 50-69 age range and had negative DRE. 70/73 HD (96%) were correctly identified. The AUC of the final classifier, for PSA values lower than 4ng/ml, was 0.86 (95%C.I.=0.77-0.95) while PSA alone had an AUC of 0.79 (95%C.I.=0.59-0.98) (**Figure 7B**). In the 4-16 PSA range, the classifier yielded an AUC of 0.6 (95%C.I.=0.43-0.70) and correctly identified 38/49 (78%) PCa, 31 of which were clinically significant, and 25/74 (34%) non-PCa. The AUC of PSA alone, in the same interval, was only 0.47 (95%C.I.=0.36-0.57) (**Figure 7C**).

Discussion

Studies conducted on normal and (pre)tumoral prostate tissues showed patterns of differentially expressed miRs according to disease status and/or severity (9,12), with a certain degree of concordance among different laboratories.

Due to their stability in body fluids, and particularly in serum/plasma, miRs could also be ideal non-invasive biomarkers for PCa detection or prognosis prediction (6,9,13). However, circulating miR analysis is affected by variability in sampling procedures, RNA extraction and analysis methods, and high quality study designs are scarce. Only few published studies strictly checked haemolysis, discarded haemolysis-related miRs, optimized RNA extraction and miR quantification and used independent validation cohorts.

Most of the proposed miRs are able to distinguish HD from PCa or HD from BPH but perform very badly in distinguishing BPH from PCa. Some perform very well in specific datasets or have been found deregulated in more than one study, but the sign of deregulation is not always concordant (9) and their accuracy might suffer of overfitting. Indeed, validation of the results gained from a screening cohort on a prospective one, using techniques more suitable for diagnostic purposes such as RT-qPCR, remains still a hard task. Validation should be intended as applying the same classification rule, without any change to improve classification results according to the independent dataset. Moreover, several studies do not include BPH in the dataset, or the size of this class is limited and does not reflect a real representation of BPH incidence. Additionally, many prognostic miRs proposed so far derive from studies where only high-risk PCa are included in the analyses, without considering that these markers are aberrantly expressed in low-risk samples as well, questioning their use as prognostic markers.

We therefore believe that our study presents several added values, such as the quality of study design, adequate sample size, accurate sample collection and processing, and appropriate classifier validation. The two miRs included in our diagnostic score are miR-103a-3p and let-7a-5p. miR-103a-3p was proposed as a plasmatic endogenous normalizer even by the Exiqon protocol, but

several recent studies highlighted that its levels in body-fluids are not stable and it may work with other miRs as putative diagnostic (14) or prognostic (15) circulating biomarker. It was included into diagnostic/prognostic PCa serum scores by Mihelich (16), and was able to predict biochemical relapse together with PSA after prostatectomy (17). Another study, where miRs were analysed in expressed prostatic secretion, found miR-103 associated with prostatitis (18). Let-7a-5p belongs to the let7 family of tumor suppressors and is usually down-regulated in PCa versus normal or BPH tissues (19). Although it is now well-established that cancer cells may release tumor suppressor miRs in the blood stream to get rid of them and prevent their antitumor effect, let-7a-5p levels have been found either up- or down-regulated in PCa compared to controls (9), or positively associated with PCa reclassification upon active surveillance (6). Its plasma levels strongly vary depending on whether extracellular vesicle-incorporated or cell-free miRs are analysed (20).

Our study meant to translate results into clinical practice. Therefore, we chose not to enrich our samples in extracellular vesicle-incorporated miRs, as this analysis would have inserted other sources of variability and technical challenges, even though we were aware that this fraction of miRs might be informative for specific PCa diagnosis (21) or prediction of prognosis (22). Some urine RNA biomarkers are also under study (23-25), but they require post-DRE sampling and/or exosome isolation. Instead, our classifier is easily applicable as long as blood is carefully collected and plasma quickly isolated.

A classifier that combines two miRs with PSA was able to discriminate PCa from non-PCa and to identify clinical significant PCa, better than PSA alone in the discovery cohort. The same methodology, applied to the validation cohort, allowed for identification of all but one low-PSA tumors: 3 high-risk, 4 intermediate-risk and 1 low-risk PCa. This is an important improvement, given that three of them were found in men with negative DRE and age in the 50-69 range, that is the one for which screening for prostate cancer could provide benefits (26). Moreover, for two of them PSA was even less than 2.5 ng/ml, limit after which free-PSA is dosed (if free to total PSA ratio (%fPSA) is less than 0.2, further investigations are recommended). %fPSA was not available

for our cohorts so we could not compare it to our score. However, the use of %fPSA or of other PSA-related markers, that are currently under investigation (27-29), is still questionable. In the 4-16 ng/ml PSA range, our classifier identified 25/74 (34%) carriers of non-malignant lesions, who may avoid unnecessary and harmful biopsies, and correctly classified 78% of PCa.

While in the discovery phase the final classifier outperformed PSA alone in detecting overall (**Figure 3**) and clinically significant PCa, in the validation cohort its AUC, specificity and PPV were higher than those of PSA alone, at the cost of a lower overall sensitivity (**Figure 7A**). However, it strongly outperformed PSA sensitivity in the 0-4 PSA interval (**Figure 7B**) and PSA specificity (**Figure 7C**) in the 4-16 PSA interval. Indeed, all but one missed PCa fall in this critical interval for PSA, where intense research is still underway (30).

We also acknowledge that diagnosis was based on standard rather than mp-MRI targeted biopsy. To further validate and improve our classifier, we have already planned a large multicenter prospective study for men in the 50-69 age range where PSA and DRE will be coupled with circulating miR analysis and mp-MRI, and standard biopsy to mp-MRI targeted biopsy.

In summary, we propose an easily applicable blood-based classifier that requires testing of two miRs plus PSA and is a promising non-invasive diagnostic tool for PCa detection that can complement PSA test.

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Tables

Table I: Study populations for the discovery and the validation phases. Cases and controls were homogeneous for geographic area, collection times, plasma separation method, storage and haemolysis level. For the discovery phase, samples homogeneous for age, within each disease class, were selected. In the validation phase, samples were consecutively collected and included high grade prostatic intraepithelial neoplasia (HGPIN) or acinar small atypical proliferation (ASAP) lesions as well. This cohort is enriched in >65 years old men and in intermediate-risk PCa.

	Discovery phase dataset	Validation phase dataset
Samples	120	242
PCa	60	68
BPH	51	93
HGPIN/ASAP		8
HD[a]	9	73
Age		
Median	65	68
<=65	61	89
>65	59	153
PSA	5.96 (4.42 - 8.40)[b]	4.91 (1.80 - 7.26)[b]
PSA <= 4	26	105
4<PSA<=16	79	123
PSA>16	15	14
Gleason Score		
GS6	25	10
GS7	22	40
GS>7	13	18
Risk class		
PCa low risk	21	9
PCa intermediate risk	24	36
PCa high risk	15	23

[a]Only Heparin-collected HD are reported. Other 18 EDTA-collected HD were analysed by microarrays but they were not included in the classifier construction.

[b]Median (1st -3rd quartile)

Legends to Figures

Fig. 1: (A) Red curve: log_2PSA distribution density in the cohort of 430 BPH or PCa patients available at the San Giovanni Battista hospital of Turin. Black curve: log_2PSA distribution density in a large cohort of nearly 14,000 not symptomatic men over 50 who tested their PSA levels at our Foundation from 2012 to middle 2018, within a voluntary adhesion context. The value of 4, corresponding to PSA = 16, is highlighted. (B) Box-plots for log_2PSA values in the 138 plasma sample cohort profiled with microarrays, divided according to risk class. Age was homogeneously distributed among classes. 414nm/385nm absorbance ratio was < 2 for all samples and HS < 0.057 for 82% of them. Dunnett corrected p-values for comparisons between low-risk PCa or intermediate-risk PCa and BPH are equal to 0.99 and 0.91, respectively, and for comparisons between HD or high-risk PCa and BPH are <0.001.

Fig. 2: (A) Volcano plot showing log_2fold-changes (x axis) and $-log_{10}$p-values (y axis) of the miR probes analyzed, highlighting up-regulated (red circles) and downregulated (blue circles) miRs in the comparison between PCa (n=60) and BPH+HD (n=60). For miRs with more than one probe, the one with lower p-value is highlighted. (B) Unsupervised hierarchical clustering of the expression matrix of 120 plasma samples (columns) and the 10 miRs (rows) that are differentially expressed between PCa and BPH+HD. Squares represent microarray log_2 intensities after row median centering and division by standard deviation. Euclidean distance and average linking were applied. (C) Box-plots of log_2 intensities for miR-103a-3p in the discovery cohort, according to sample class. (D) Box-plots of log_2 intensities for let7a-5p in discovery cohort, according to sample class. Dunnett corrected p-values for comparisons of each sample class with HD are all higher than 0.05, except for intermediate-risk PCas (0.0096 and 0.0482 for miR-103a-3p and let-7a-5p, respectively). Dunnett corrected p-values for comparisons of each sample class with BPH are all higher than 0.05, except for intermediate-risk PCas (0.0197 and 0.0276 for miR-103a-3p and let-7a-5p, respectively).

Fig. 3: Decision tree of the final classifier developed in the discovery set and applied to the validation set.

Fig. 4: ROC curve for PSA (dotted line) or the final classifier (continuous line) in the discovery cohort of 120 samples (AUC PSA = 0.62, C.I.=0.53-0.73; AUC final classifier = 0.68, C.I.=0.59-0.78). Asterisks correspond to the thresholds for PSA (4ng/ml) and for the final classifier (2.02). Sensitivity, specificity, accuracy, PPV and NPV of the final classifier are 87%, 35%, 68%, 57% and 72%, respectively, all higher than those given by PSA alone.

Fig. 5: Box-plots of –DCt values for miR-103a-3p (A) and let7a-5p (B), showing statistically significant up-regulation for both miRs (log_2fold-change > 1 and Benjamini-Hochberg corrected t-test p-value < 0.05).

Fig. 6: (A) Log_2PSA in the 242 sample validation cohort, according to sample class. Dunnett corrected p-values for comparisons between HGPIN/ASAP or PCa low-risk and BPH are equal to 1, for comparison between intermediate-risk PCa and BPH is equal to 0.99 and for comparisons between HD or high-risk PCa and BPH are less than 0.0001. (B) Box-plots of –DCt values for miR-103a-3p in the 242 sample validation cohort, according to sample class. (C) Box-plots of –DCt values for let7a-5p in the 242 sample validation cohort, according to sample class.
Dunnett corrected p-values for comparisons of each sample class with HD are all less than 0.0001, while comparison with BPH yielded statistically significant p-values only for HD (<0.0001, both for miR-103a-3p and let-7a-5p).

Fig. 7: (A) ROC curve for PSA (dotted line) or the final classifier (continuous line) in the validation cohort of 242 samples (AUC_PSA = 0.74, CI = 0.68 - 0.80; AUC_final classifier = 0.76, CI = 0.70 -

0.82). Asterisks correspond to the thresholds for PSA (4ng/ml) and for the final classifier (2.02). Sensitivity, specificity, accuracy, PPV and NPV of the final classifier are 82%, 57%, 64%, 43%, 89%, respectively. (B) ROC curve for PSA (dotted line) or the final classifier (continuous line) in the validation cohort, considering 105 samples with PSA≤4 ng/ml (AUC_PSA = 0.79, 95% C.I. = 0.59 - 0.98; AUC_final classifier = 0.86, 95% C.I. = 0.77 - 0.95). (C) ROC curve for PSA (dotted line) or the final classifier (continuous line) in the validation cohort, considering 123 samples with 4<PSA<=16 ng/ml (AUC_PSA = 0.47, 95% C.I. = 0.36 - 0.57; AUC_final classifier = 0.6, 95% C.I. = 0.43 - 0.70).