Exosomal and Non-Exosomal Urinary miRNAs in Prostate Cancer Detection and Prognosis

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BACKGROUND. MicroRNAs (miRNAs) are non-coding small RNAs, involved in post-transcriptional regulation of many target genes.

METHODS. Five miRNAs that have been consistently found deregulated in PCa (miR-21, miR-141, miR-214, miR-375, and let-7c) were analyzed in urinary pellets from 60 prostate cancer (PCa) patients and 10 healthy subjects by qRT-PCR. Besides, urinary exosomes were isolated by differential centrifugation and analyzed for those miRNAs.

RESULTS. Significant upregulation of miR-21, miR-141, and miR-375 was found comparing PCa patients with healthy subjects in urinary pellets, while miR-214 was found significantly downregulated. Regarding urinary exosomes, miR-21 and miR-375 were also significantly upregulated in PCa but no differences were found for miR-141. Significant differences were found for let-7c in PCa in urinary exosomes while no differences were observed in urinary pellets. A panel combining miR-21 and miR-375 is suggested as the best combination to distinguish PCa patients and healthy subjects, with an AUC of 0.872. Furthermore, the association of miRNAs with clinicopathological characteristics was investigated. MiR-141 resulted significantly correlated with Gleason score in urinary pellets and let-7c with clinical stage in urinary exosomes. Additionally, miR-21, miR-141, and miR-214 were found significantly deregulated in intermediate/high-risk PCa versus low-risk/healthy subjects in urinary pellets. Significant differences between both groups were found in urinary exosomes for miR-21, miR-375, and let-7c.

CONCLUSIONS. These findings suggest that the analysis of miRNAs—especially miRNA-21 and miR-375- in urine could be useful as biomarkers in PCa. *Prostate*

KEY WORDS: **prostate cancer; miRNA; exosomes; urine**

INTRODUCTION

Prostate cancer (PCa) is currently the second most common frequently diagnosed cancer and the fifth leading cause of cancer-related death among males worldwide [1]. Prostate-specific antigen (PSA) has been extensively used in the early detection of PCa despite the problems related to its low specificity. On the other hand, PSA screening results in overdiagnosis and overtreatment of insignificant PCa. Consequently, it is an imperative to identify new accurate biomarkers that

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allow the detection of PCa at an early stage and can distinguish aggressive from insignificant tumors.

MicroRNAs (miRNAs) are small non-coding RNAs that modulate the expression of specific genes at post-transcriptional level and have emerged as novel promising biomarkers for PCa detection and prognosis. MiRNAs have been found involved in multiple biological processes and therefore in a variety of oncogenic pathways [2,3]. MiRNAs can either function as oncogenes or tumor-suppressors depending on their target genes [4].

Several profiling studies have already shown that the expression of miRNAs is commonly altered in PCa compared with normal tissues [5,6]. Numerous studies have demonstrated that miR-21, miR-141, and miR-375 were the most repeated significantly deregulated miRNAs in PCa in tissue as well as in blood [7,8]. On the other hand, let-7c and miR-214 were also found deregulated in PCa, playing an outstanding role in tumor progression and metastasis [9,10].

Urine appears as a challenging alternative to plasma/serum for PCa biomarker discovery. In the last years, several authors have defined miRNA profiles of PCa in urine [11–13] but no overlapping expression pattern exists between the different publications. Consequently, there is not at the moment any miRNA urine test for the early detection and prognosis of PCa. More studies in urine are necessary to validate those miRNAs that have been found repeatedly deregulated in tissue.

Moreover, exosomes emerge as a new source of cancer biomarkers [14]. They are small (30–150 nm) vesicles released by most cell types that contain proteins, lipids, and nucleic acids (e.g., miRNAs) that serve a variety of functions including intercellular communication [15]. The study of new biomarkers in exosomes is a promising field because they are remarkably stable in body fluids and their content is protected from enzymatic degradation by the exosomal lipid bilayer [16]. The presence of exosomes in urine was first reported in 2004 [17], and since then they have been shown to be of interest in several diseases [18] including PCa [19].

The goal of this work, was to study the potential of five cancer-associated miRNAs, miR-21, miR-141, miR-214, miR-375, and let-7c in urinary pellets and exosomes as biomarkers for PCa detection and prognosis.

MATERIALS AND METHODS

Patients and Clinical Samples

We collected freshly voided urine samples from 60 patients with PCa. Demographic data and medical history were obtained at the entry of each patient to the study. Digital rectal examination (DRE) was performed in all patients in order to determine the clinical stage. Patients with other malignancies than non-melanoma skin cancer and patients who had undergone any previous treatment related to PCa or benign prostatic hyperplasia (BPH) with 5-alpha-reductase inhibitors were excluded from the study. A group of 10 healthy volunteers was also included. PSA serum levels were lower than 4 μg/L (mean: 1.14 μg/L; standard deviation: 0.95) and DRE was negative in all healthy subjects.

The study was approved by ethical committees of involved medical centres, and written informed consent was obtained from all study participants.

Urinary Pellets Preparation

Urine samples (30–50 ml) were collected in containers without any preservative after a prostate massage. Samples were stored at 4°C and centrifuged within 4 hr after collection (2,000 g for 20 min at 4°C). The pellet was then washed with 2 ml of an ice-cold phosphate buffered saline solution (PBS) and again centrifuged (2,000g for 5 min at 4°C). Afterwards, 1 ml QIAzol (Qiagen®, Hilden, Germany) was added to the pellet, mixed, and left at room temperature for 5 min. The pellet with QIAzol was stored at −80°C until analysis. Sera from all participants were also obtained before prostate massage for the PSA measurement.

Urinary Exosomes Isolation

Exosomes were isolated by differential centrifugation from urine of 10 healthy subjects and 52 of the 60 PCa patients included in the study. After obtaining the urinary pellet, the supernatant was transferred to ultracentrifuge tubs (polycarbonate) and centrifuged at 17,000 g for 45 min at 4°C. Then the supernatant was transferred to another tub and again centrifuged at 2,00,000 g for 2 hr at 4°C. Afterwards, the supernatant was discarded. The pellet was recovered, reconstituted with PBS and stored at −80°C until the RNA isolation. The presence of exosomes in the pelleted sample was confirmed by electronic microscopy (Fig. 1).

Electronic Microscopy

For transmission electron microscopy, an aliquot of the pelleted exosomes was fixed in 3% paraformaldehyde. A drop of the sample was placed in a formvar coated grid for 10 min and after extracting the excess of fluid, it was stained with 2% uranyl acetate for 3 min. After that, a MilliQ water drop was added for

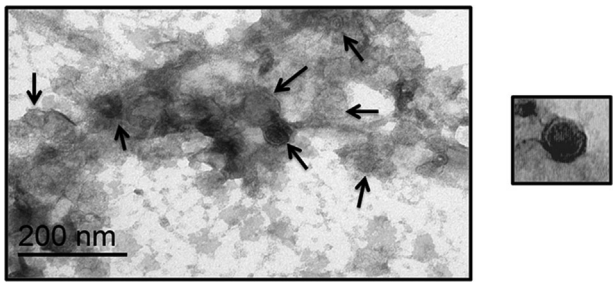


Fig. 1. Electron micrograph of urinary exosomes isolated by differential centrifugation. A drop of the pelleted exosomes fixed in 3% paraformaldehyde was placed in a formvar coated grid and stained with uranyl acetate. The sections were examined in a Jeol 1010 transmission electron microscope. At the right photo, the double membrane of exosome is appreciated with more detail. Scale bar = 200 nm.

3 min, then the excess of fluid was extracted and the sample was dried. Finally, the samples were examined in a Jeol 1010 transmission electron microscope at 80 kV supplied with CCD Gatan ORIUS camera.

Isolation of miRNAs from Urinary Pellets and Exosomes

Total RNA was isolated from frozen pellets and exosomes using miRNeasy serum/plasma kit (Qiagen®) according to the manufacturer's instructions. Previously to the addition of the chloroform, the spike-in control cel-miR-39 was incorporated. The concentration of RNA was quantified using NanoDrop ND-1,000 Spectrophotometer (Thermo Scientific®, Waltham, MA). RNA was kept at −80°C till its use in the reverse transcription polymerase chain reaction.

Reverse Transcription and Preamplification PCR

RNA was converted to cDNA using TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems®, Foster City, CA). Non reverse transcriptase controls were used to rule out the possibility of potential genomic DNA contamination. After reverse transcriptase PCR, a preamplification step using TaqMan® Preamp Mastermix (Applied Biosystems®) was performed in order to increase the amount of cDNA and to improve the sensitivity of the qRT-PCR.

Measurement of miRNA Expression With Quantitative Real Time PCR Reaction (qRT-PCR)

The expression of five mature miRNAs namely hsa-miR-21, hsa-miR-141, hsa-miR-214, hsa-miR-375, and hsa-let-7c (Table I) was quantified using TaqMan® single microRNA assays (Applied Biosystems®) in accordance with the manufacturer's protocol and the MIQE guidelines. Quantitative real time PCR (qRT-PCR) was carried out by Applied Biosystems® 7,300 system with the following cycling parameters: 95°C for 10 min, then 40 cycles of 95°C for 15 sec, followed by 60°C for 60 sec.

TABLE I. Sequences of Taqman® miRNAs Assays Chosen for Evaluation in the Present Study

miRBase ID	Sequence
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-141-3p	UAACACUGUCUGGUAAAGAUGG
hsa-miR-214-3p	ACAGCAGGCACAGACAGGCAGU
hsa-miR-375	UUUGUUCGUUCGGCUCGCGUGA
hsa-let-7c-5p	UGAGGUAGUAGGUUGUAUGGUU
cel-miR-39-3p	UCACCGGGUGUAAAUCAGCUUG

Relative miRNA expression levels were normalized against the spike-in control cel-miR-39. Relative gene expression was calculated using the $-2\Delta\Delta Ct$ method [20] with normalization to RNA input.

Statistical Analysis

Statistical analyses were performed with SPSS Statistics version 23 (IBM, Somers, NY). MiRNA expression data were assessed for the distribution of normality using Kolmogorov–Smirnov test. Mann–Whitney U or Kruskal–Wallis tests were used to identify significantly ($P < 0.05$) deregulated miRNAs and to compare miRNA expression levels in relation to clinicopathological characteristics. Receiver operating curve (ROC) analysis was used to calculate the area under the curve (AUC) of miRNAs expression levels. Models including several miRNAs were constructed by binary logistic regression analysis. AUCs for these models were calculated using ROC analysis.

RESULTS

Patients' Characteristics

Demographic and clinical data are summarized in Table II, including information regarding PSA serum levels, biopsy Gleason score, and clinical stage. PCa patients were classified according to the D'Amico criteria, including four patients with low-risk (clinical stage T1–T2a, Gleason score <7, and PSA ≤10 μg/L), 19 patients with intermediate risk (clinical stage T2b or Gleason score =7 or PSA 10–20 μg/L) and 37 patients with high-risk (clinical stage T2c or PSA >20 μg/L or Gleason score >7). Urinary exosomes were obtained in 52 of these patients (Table II).

TABLE II. Demographic and Clinicopathological Characteristics of the Patients Included in the Study

Number of patients	60 (52)
Age (years)	
Median	71 (71)
Range	60–83 (60–83)
PSA serum levels (μg/L)	
Median	7.7 (8.3)
Range	2.21–47 (4.18–47)
Biopsy Gleason score	
<7	14 (11)
7	34 (29)
>7	12 (12)
Clinical stage	
T1c	16 (16)
T2-T3	44 (36)
D'Amico risk groups	
Low-risk	4 (3)
Intermediate risk	19 (16)
High-risk	37 (33)

PCa, prostate cancer; PSA, prostate-specific antigen. In brackets are shown the data of patients in which urinary exosomes were also analyzed.

Comparison of miRNA Levels in Urinary Pellet Between PCa Patients and Healthy Subjects

We found that miR-21, miR-141, and miR-375 were upregulated in the urinary pellet of PCa patients in comparison with healthy subjects (P of 0.001, 0.033, and 0.038, respectively), whereas miR-214 was significantly downregulated in PCa patients ($P = 0.049$). The expression levels of let-7c showed no significant differences between both groups (Fig. 2A).

We further analyzed miRNAs by the receiver-operating characteristic curve, showing 95% confidence intervals. The AUC of urinary miR-21, miR-141, miR-214, miR-375, and let-7c were 0.817, 0.712, 0.716, 0.707, and 0.636, respectively (Fig. 3 A). In a multivariate study based on binary logistic regression, we showed that the higher AUC was obtained combining miR-21 and miR-375 (0.872).

Comparison of miRNA Levels in Urinary Exosomes Between PCa Patients and Healthy Subjects

The five miRNAs were also analyzed by qRT- PCR on the urinary exosomes. We found that miR-21, miR-375, and let-7c were significantly upregulated (P of 0.018, 0.001, and 0.048, respectively) in the PCa group compared with the healthy group (Fig. 2B). No significant differences for the other studied miRNAs were found comparing both groups, although miR-141 showed a trend to upregulation ($P = 0.091$).

The AUC of miR-21, miR-141, miR-214, miR-375, and let-7c in urinary exosomes were 0.713, 0.652, 0.542, 0.799, and 0.679, respectively (Fig. 3B). No improvement was found combining these miRNAs in a multivariate study based on binary logistic regression.

Association of Urinary miRNA Expression With Clinicopathological Factors

The expression value of each miRNA in tumors was tested in both urinary pellets and exosomes for association with clinicopathological parameters.

Urinary pellets. miR-141 was significantly ($P = 0.034$) differently expressed in relation to Gleason score, showing higher levels in patients with higher Gleason score (Fig. 4). No significant differences between Gleason score groups were found for miR-21, miR-214, miR-375, and let-7c. On the other hand, no significant differences were found for any miRNA comparing T1c versus T2/ T3 patients. MiRNAs levels in patients classified according to the D'Amico criteria are shown in Figure 5A. Significant differences were found comparing results in healthy subjects and low-risk PCa group versus intermediate and high-risk PCa groups for miR-21 ($P < 0.0001$), miR-141 ($P = 0.016$), and miR-214 ($P = 0.024$). No significant differences were found for miR-375 ($P = 0.098$) and let-7c ($P = 0.214$).

Urinary exosomes. No significant differences between Gleason score groups were found for any of the analyzed miRNA. On the other hand, let-7c levels were significantly associated with clinical stage ($P = 0.023$) comparing T1c versus T2/T3 patients (Fig. 6). No significant differences were found for the rest of the analyzed miRNAs. MiRNAs levels in patients classified according to the D'Amico criteria are shown in Figure 5B. Significant differences were found comparing results in healthy subjects and low-risk PCa group versus intermediate and high-risk PCa groups for miR-21 ($P = 0.003$), miR-375 ($P = 0.001$), and let-7c ($P = 0.048$). No significant differences were found for miR-141 ($P = 0.055$) and miR-214 ($P = 0.833$).

DISCUSSION

Previous findings have shown that the different expression patterns of miRNAs in tissues can be used to distinguish PCa from normal tissue [6,9]. Also, the diagnostic and prognostic value of circulating miRNAs has been suggested by several authors [11,13]. Urine appears as a challenging alternative to plasma/serum

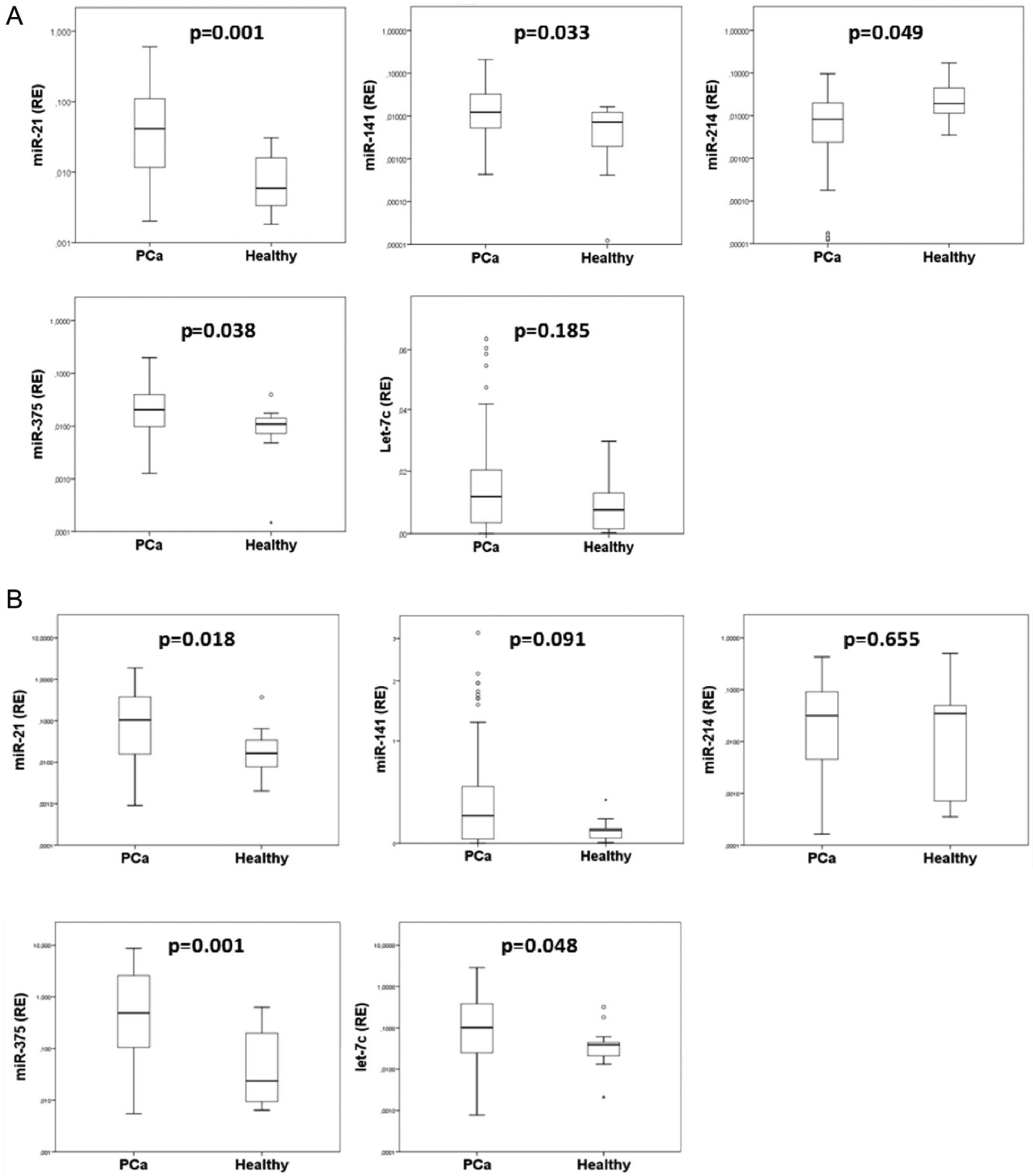
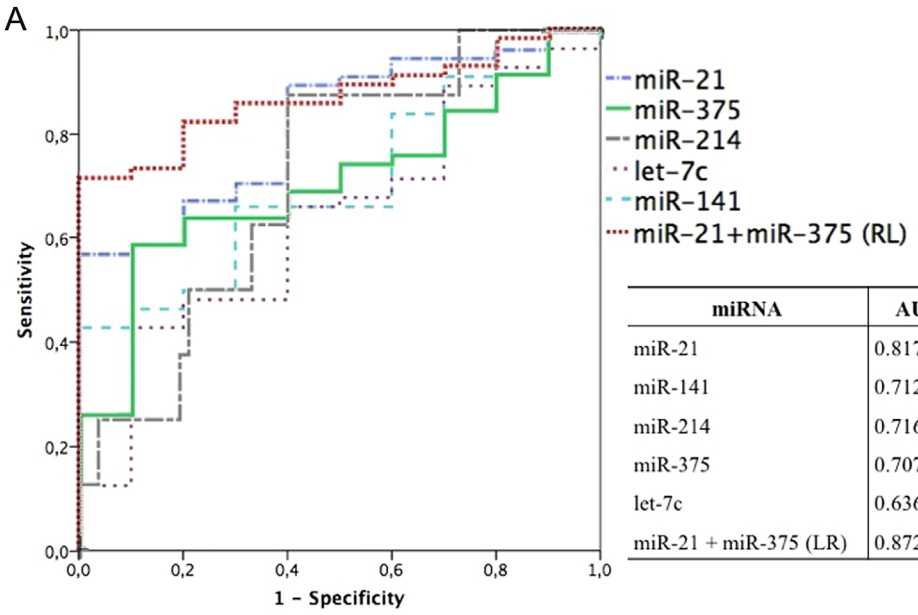


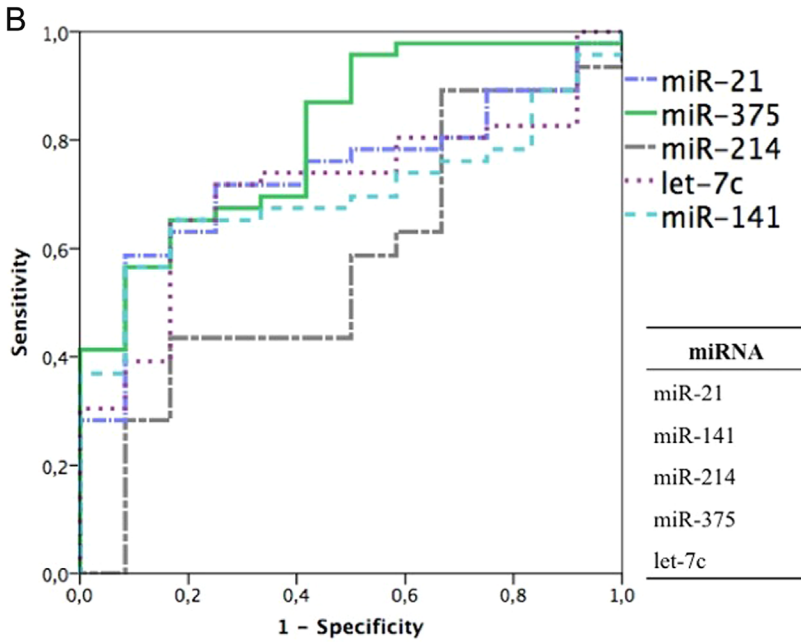
Fig. 2. Boxplots of the five microRNAs relative expression comparing PCa patients and healthy subjects groups in urinary pellets **(A)** and in urinary exosomes **(B)**. Statistical test: U Mann–Whitney. RE, relative expression; PCa, prostate cancer.

for PCa biomarker discovery. Urine contains exfoliated PCa cells and other secreted products, especially when prostate massage is performed. Besides, the protein content is lower in urine than in serum and plasma, reducing thus interferences in the RNA-derived biomarkers isolation [21].

In the present study, the levels of five miRNA in urinary pellets from 60 PCa patients and 10 healthy controls were analyzed. Furthermore, these miRNAs were studied in urinary exosomes from 52 patients of those PCa patients and from all healthy subjects. We found that miR-21, miR-141, and miR-375 were



miRNA	AUC (95% CI)	p value
miR-21	0.817 (0.696-0.938)	0.001
miR-141	0.712 (0.562-0.863)	0.033
miR-214	0.716 (0.545-0.886)	0.049
miR-375	0.707 (0.555-0.859)	0.038
let-7c	0.636 (0.461-0.812)	0.185
miR-21 + miR-375 (LR)	0.872 (0.786-0.958)	<0.0001



miRNA	AUC (95% CI)	p value
miR-21	0.713 (0.581-0.845)	0.018
miR-141	0.652 (0.520-0.785)	0.091
miR-214	0.542 (0.358-0.726)	0.655
miR-375	0.799 (0.675-0.923)	0.001
let-7c	0.679 (0.542-0.816)	0.048

Fig. 3. Receiver-operating characteristic curve analysis and area under the curve with 95% confidence interval of miRNAs analyzed in urinary pellets **(A)** and in urinary exosomes **(B)**. AUC, area under the curve; CI, confidence interval; LR, logistic regression analysis.

significantly upregulated in pellets of PCa patients, while miR-214 was significantly downregulated. A panel combining miR-21 and miR-375 is suggested as the best combination to distinguish PCa patients and healthy subjects, showing an AUC of 0.872. When we studied the expression of the same miRNAs in the urinary exosomes, we confirmed that the levels of miR-21 and miR-375 were also significantly increased in PCa patients. No significant differences were found for miR-141 and miR-214 in exosomes comparing both groups, although there was a trend to upregulation for miR-141. Let-7c was significantly upregulated in exosomes, but not in urinary pellet.

Urinary miRNAs showing diagnostic potential for PCa identification have been reported in several previous studies analysing different urine fractions [7,8]. Our data are in concordance with results recently published by Stuopelytė et al. [22], showing that miR-21 levels were higher in urinary pellets of PCa than in BPH patients. We also agree with Srivastava et al. [12] who found that miR-214 was downregulated in total urine samples of PCa

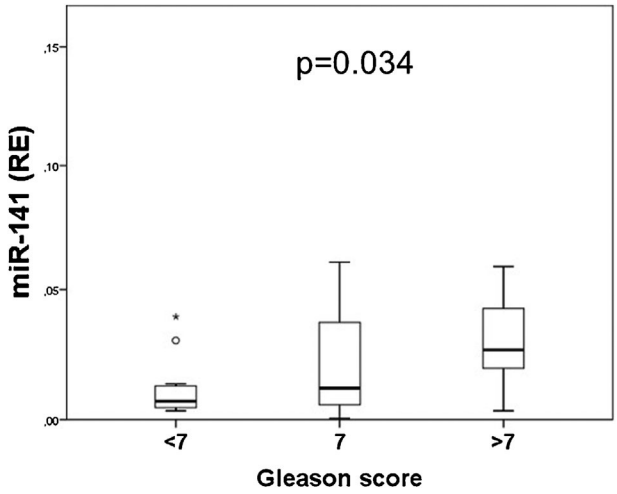


Fig. 4. Boxplots of miR-141 comparing the different Gleason score groups in urinary pellets. Statistical test: Kruskal–Wallis. RE, relative expression.

patients compared with healthy donors. According to Ahumada-Tamayo et al. [23], let-7c was upregulated in urinary pellets of PCa versus BPH patients, while we only found significant differences for let-7c in urinary exosomes. Finally, contrarily to our results, Bryant et al. [24] did not found significant differences for miR-375 and miR-141 in urinary pellets comparing PCa patients and benign controls. Differences in methodology could explain contradictory results between studies [25].

Faithfully, few authors have studied miRNAs in urinary exosomes in PCa patients until the moment. Bryzgunova et al. [19] have recently demonstrated promising results in the detection of PCa analysing miR-19b in urinary exosomes isolated by differential centrifugation. On the other hand, Samsonov et al. [26] showed that miR-21, miR-141, and miR-574 were upregulated in PCa patients compared with healthy controls in urinary exosomes isolated by a lectin-based exosomes agglutination method. However, in this study, only miR-141 was found significantly upregulated when urinary exosomes were isolated by differential centrifugation. Our results suggest the value of urinary exosomes in PCa detection, although the AUCs were in general lower than those obtained in urinary pellet. Our results confirm the aforementioned data about miR-21 and underline the value of miR-21 and miR-375, with AUCs of 0.713 and 0.799, respectively. Methodological differences in the isolation of exosomes could explain discordant results in relation to previous published data concerning miR-141.

Furthermore, miRNAs analyzed in this study have been already found significantly deregulated in PCa patients when they were evaluated in serum/plasma or in circulating exosomes. For instance, Cheng et al. [27] observed the significant upregulation of circulating miR-141 and miR-375 with AUCs of 0.842 and 0.660, respectively, comparing metastatic castration resistant PCa patients with healthy controls. Similarly, Mitchell et al. [11] found significant upregulation of miR-141 in serum of metastatic PCa patients compared with healthy donors, with an AUC of 0.907. Yaman Agaoglu et al. [28] obtained an AUC of 0.88 for miR-21, showing higher levels in plasma of PCa patients versus healthy controls. Selth et al. [29] also found higher levels of miR-141 and miR-375 in plasma of metastatic castration PCa patients compared with healthy individuals. Li et al. [30] studied miRNAs in serum exosomes of PCa patients and healthy controls and found that miR-375 and miR-21 were upregulated in PCa.

PCa is a very heterogeneous disease, including patients with low-risk of progression, in which cancer-specific survival rates exceeded 99% at 15-year follow-up. Specific gene-expression patterns have been identified for low-risk PCa. Recently, Rubin et al. [31] have reported specific genomic profiles related to Gleason score, showing fewer driver mutations and no polyploidy when Gleason score is lower than 7. Moreover, several authors argued that the label of cancer must be put into question for patients with Gleason score 6 or lower [32,33]. In fact, active surveillance, differing treatment in case of cancer progression, has been proposed for patients with low-risk of progression as an alternative to radical treatment.

Published data concerning the value of miRNAs to predict the aggressiveness of PCa are contradictory. According to Stephan et al. [34] miR-183 and miR-205 were not associated with Gleason score and serum PSA. Sapre et al. [35] showed that several miRNAs, including miR-21 and miR-375, were upregulated in patients with high-risk PCa. The authors selected a miRNAs signature combining miR-16, miR-21, and miR-222 to predict high-risk PCa. However, results were not confirmed when this signature was validated in an independent cohort of patients. On the other hand, Lewis et al. [36] found significant differences comparing patients with Gleason score higher than 8 versus patients with lower scores for miR-888, miR-34a, miR-205, let-7c, let-7d, miR-200b, miR-92a, miR-99a, miR-141, and miR-375. In our study a significant association of miR-141 expression in urinary pellet with Gleason score was shown, while let-7c in urinary exosomes was significantly lower in patients with T2-3 clinical stage. However, no significant differences were found for other miRNAs.

The D'Amico risk group classification, combining Gleason score, PSA serum levels, and clinical stage, was proposed to estimate the risk of biochemical recurrence for localized PCa [37]. It has been shown

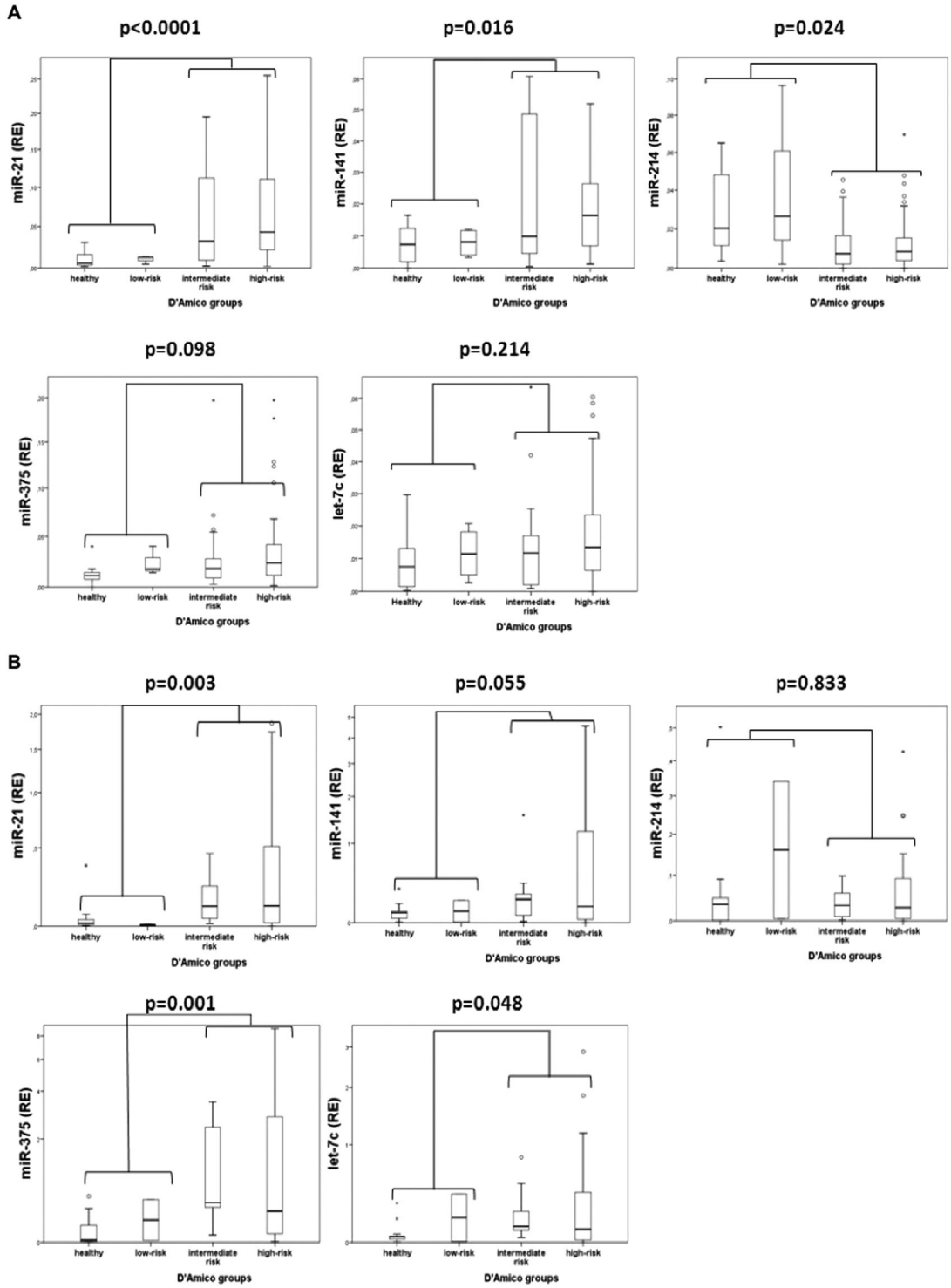


Fig. 5. Boxplots of the five microRNAs relative expression comparing the different D'Amico prostate cancer risk groups and healthy subjects in urinary pellets **(A)** and in urinary exosomes **(B)**. Statistical test: U Mann–Whitney. RE, relative expression.

that the prognosis for patients in the low-risk group is optimal. For this group of patients, the cancer-specific survival was 99.7% and local recurrence-free survival was 97% at 10 years, according to a Mayo Clinic validation of the D'Amico risk group classification [38].

In our study, we studied the association between the expression of miRNAs and the D'Amico risk groups, comparing patients with intermediate and high-risk against low-risk patients together with healthy subjects. We found that miR-21, miR-141, and miR-214 in

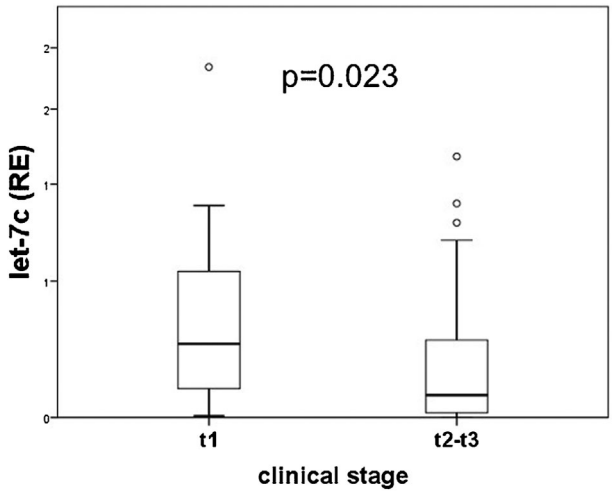


Fig. 6. Boxplots of let-7c comparing the different clinical stage groups in urinary exosomes. Statistical test: U Mann–Whitney. RE, relative expression.

urinary pellet showed significant differences between both groups, while a certain trend existed for miR-375 ($P = 0.098$). In urinary exosomes, we observed that miR-21 and miR-375 levels were higher in intermediate/high-risk PCa group and that the same trend was observed for miR-141 ($P = 0.055$). Finally, we encountered an irregular pattern for let-7c levels, showing higher levels in low-risk PCa than in the other groups.

Our preliminary results suggest that urinary miRNAs could distinguish aggressive PCa. Likewise, Huang et al. [39] showed that higher levels of miR-375 and miR-1290 in exosomes isolated from plasma were significantly associated with poor overall survival in metastatic castration resistant PCa patients. Notably, Wang et al. [40] proposed a miRNAs signature combining miR-19, miR-345, and miR-519c-5p in serum to predict adverse pathology in PCa patients eligible for active surveillance. Additional studies are necessary to demonstrate the value of miRNAs to predict PCa aggressiveness.

According to our data, miR-21 and miR-375 have diagnostic and prognostic value, suggesting their usefulness in PCa management. MiR-21 is usually upregulated in early PCa as well as in metastatic cancer [41,42], playing a crucial role in prostate carcinogenesis. It contributes to tumor growth, invasiveness, androgen insensitivity and metastasis through various pathways [42–44]. MiR-21 directly downregulates *PTEN*, reducing apoptosis significantly and promoting angiogenesis through HIF-1a signaling pathway [45,46]. It also regulates *MARCKS*, conferring apoptosis resistance [47]. Furthermore, the upregulation of miR-21 in PCa also plays an important role in epithelial-mesenchymal transition (EMT) by decreasing the *BTG2* levels and promoting the acquisition of luminal markers [44,48]. Besides, it has been shown that miR-21 and androgen receptor (AR) gene regulate each other in a positive feedback loop. Ribas et al. [49] reported that AR act as a direct transcriptional regulator and binds to a defined miR-21 promoter, miPPR-21, resulting in overexpression of miR-21, which enhances androgen-dependent PCa growth. Moreover, the abnormal expression of miR-375 in PCa has been involved in enhanced migration and invasion of the tumor. Recently, Pickl et al. [50] identified the Polycomb repressive complex I member CBX7 as a major target of miR-375 in PCa progression. The repression of *CBX7* by miR-375 leads to the activation of transcriptional programs related to malignant progression. Additionally, miR-375 also plays a role in mesenchymal–epithelial transition (MET), through a ZEB1-miR-375-YAP1 network, favouring the invasion and metastatic disease [51]. The involvement of miR-21 and miR-375 in key signaling pathways remarks their usefulness as PCa biomarkers. According to our results, both biomarkers are upregulated in intermediate and high-risk PCa patients, suggesting their participation in PCa progression.

The small sample size of the control group and the low-risk PCa group constitutes a limitation of this study. A larger study would be necessary to confirm the prognostic and diagnostic value of miR-21 and miR-375. Furthermore, our conclusions are limited because biopsy was not performed in the healthy control group. Additional studies including patients with negative biopsy and BPH patients would be necessary to support our data. Because of RNA in urine is scarce, a prostate massage previous to collect the urine was performed in our study. Further studies would be necessary to check if similar miRNAs results could be obtained collecting urine without a previous prostate massage.

CONCLUSIONS

Several challenges should be investigated before the use of miRNAs as PCa biomarkers in clinical practice. Contradictory results between studies can be caused by differences in the methodology used to analyze miRNAs or for the exosomes isolation as well as by the different urine fraction studied by each group. Optimization and standardization of the methodology used for miRNAs analysis is a previous requirement to obtain high quality results. Besides, more effective methods for isolation of exosomes in body fluids are required. Nevertheless, the data presented in this evaluation are encouraging. Urine obtained after prostate massage represent a reliable biological sample for a minimally invasive approach

to analyse miRNAs. Because of the anatomical localization of the prostate in relation to the urethra, urine appears as an ideal substrate to obtain biomarkers directly related with prostate carcinogenesis. Urinary exosomes and their cargo become an emerging source of biomarkers that reflects the intercellular communication, which promotes tumorogenesis. MiRNAs, and especially miR-21 and miR-375, come out as valuable biomarkers in the detection and prognosis of PCa. Further studies with larger cohorts are necessary to confirm the utility of urinary miRNAs as PCa biomarkers.

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