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Novel Solution Based on Detection of Mirs-410-3p and 141-5p for Diagnostic of Prostate Cancer Evolution

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Research Article

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ABSTRACT

To date, Prostate Cancer (PCa) is both the most common tumour diagnosed in males and the second most common cause of cancer-related mortality. Prevention programs and screening protocols have proven useful to detect the disease at population level, but they lack sensitivity and specificity in comparison to the molecular tests routinely available for screening of other types of cancer, leading to unnecessary biopsies and overtreatment in many cases. In this context, a new set of small RNA biomarkers are surfacing with promising results to predict tumour progression, risk reclassification and treatment response such as miR-410-3p and miR-141-5p. Former studies where these biomarkers were examined in prostate cancer tissues and cell lines by qRT-PCR have shown that high expression of miR-410-3p correlates to both a) accurate diagnosis in certain groups where the PSA levels do not match results from biopsy, surgery and/or digital rectal examination and b) poor prognosis of prostate cancer patients. Likewise, miR-141-5p shows a parallel behaviour suggesting a potential combo for fine molecular analysis of the ratios. In this sense, recent studies have demonstrated that miR-410-3p exert oncogenic functions through downregulating PTEN, proving that miR-410-3p inhibits prostate cancer progression *via* downregulating PTEN/AKT/mTOR signalling pathway. Curiously enough, different behaviour has been reported for the biomarker in both, peripheral blood from patients and cancer-cell line(s) models further pointing at the advantages of a dual gauging made possible by parallel semi-quantitation of miR-141-5p. In this sense, miR-141-5p has been

clearly identified as to be upregulated in large cohorts (n over 500) of prostate cancer patients confirming overexpression in multivariate analysis in tumour epithelium and tumour stroma. This overexpression taken into the context of a peripheral blood reduction of miR-410-3p appears to be associated with increased risk of biochemical cancer recurrence in an independent study over 500 patients. Here we present the design, molecular set up and preclinical assessment of a novel system that uses the discarded volume from PSA blood tests to predict prostate cancer progression and biochemical cancer recurrence *via* detection of the biomarkers. The method described could potentially eliminate the need of invasive means such as biopsy, surgery and digital rectal examination.

Keywords: RTqPCR; miR-410-3p; mir-141-5p; Diagnostic and prostate cancer progression

INTRODUCTION

Prostate Cancer (PCa) has become one of best characterised tumours in male under a molecular perspective [1-5]. Prostate Cancer (PCa) is now one of the most thoroughly understood tumors in males from a molecular perspective [6,7]. In recent years, the set of miRNAs playing regulatory function(s) in this type of cancer has been defined and subjected to scrutiny further revealing that miR-410-3p and mir-141-5p act as a key biomarker for diagnosis [8,9].

The involvement of miRNAs in gene regulatory processes and their availability for molecular detection in peripheral blood of cancer patients make them attractive candidates for refining diagnosis, prediction of tumour progression, risk reclassification and treatment response. miRNAs are a class of small noncoding RNA molecules that post-transcriptionally modulate gene expression by binding to the 3' Untranslated Region (3 ′ -UTR) of the target mRNA. This binding induces key processes for cancer progression such as silencing of the mRNA by the Argonaut (Ago) protein in the RNA-induced Silencing Protein Complex (RISC) [10]. Since certain miRNAs are deregulated in cancer patients, whose peripheral blood levels show a wide variation correlating to changes in the synthesis of the proteins necessary for tumorigenesis, disease progression, and metastasis [4], they have been recently regarded as key biomarkers for the oncologic condition. In the context of PCa, the recent study of Zhang, et al. [1] has shown that the elevated expression of miR-410-3p -3p correlates with the downregulation of Phosphatase and Tensin- Homolog On Chromosome Ten (PTEN) and results in an activation of the AKT/mTOR signaling pathway, showing that miR-410-3p -3p plays a major role as an oncogene in PTEN/AKT/mTOR pathway [11]. The parallel study of Liu, et al. [2] has provided further evidence that upregulation of miR-410-3p could promote a disease progression and a metastatic ability of PCa due to deactivation of PTEN protein, a natural inhibitor of PI3K/AKT/ mTOR as well as having similar significance in Clear Cell Renal Cell Carcinoma (CCRCC) [12]. Furthermore, evidence of MiR-410-3p in PCa and CCRC cancers being expressed at a high has been provided thus supporting for both types of cancer [1, 2]. In addition to this, miR-410-3p was confirmed an independent risk factor for the survival prognosis of patients with CCRCC, its high expression indicating poor prognosis. Besides, the inhibition of miR-410-3p in cancer-cell models including the two most widely used prostate cancer cell lines (PC3 and DU145) has proven to suppress cell proliferation, cycle progression,

migration, invasion and tumour growth *in vivo*, also promoting cell apoptosis. In this context of molecular characterisation, MiR-410-3p has been highlighted as a key biological indicator for the diagnosis and prognosis of PC and CCRCC, being considered an independent risk factor for the survival prognosis [13-15]. Mechanistically, analysis in other cell models such as lung cancer [15] has demonstrated that the oncogenic effects of miR-410-3p -3p are exerted through downregulating PTEN further confirming that miR-410-3p -3p acts in PC *via* downregulating PTEN/AKT/mTOR signalling pathway.

Parallel studies over large associates exceeding 500 patients have also proven that the expression of miR-141 is associated with an increased risk of biochemical cancer recurrence and biochemical and clinical failure [10]. Nevertheless, its role at different stages of cancer progression and how its expression changes during the multistep carcinogenesis, suggest that a second biomarker of known behaviour would be needed to better understand and gauge; hence the parallel definition of levels for miR-410-3p whose opposite behaviour under such conditions allows a double-check-point to finely tune accuracy of the molecular test. This strategy has been further confirmed by evidences that both biomarkers are reported detectable in studies from liquid biopsies such as urine, serum, plasma and whole blood reporting that the two tumour-derived miRNAs can enter the circulatory system and be measured in serum and plasma [16]. In this sense, different studies have supported that circulating miR-141-5p is significantly elevated in the sera of PC patients compared to healthy controls, being associated with more aggressive and advanced disease (high Gleason score and lymph node metastases). Besides, studies reporting a decreased circulating miR- 410-3p and a significantly higher circulating miR-141 in patients with locally advanced-stage disease point at this combination as one of the more promising marker-set for PC diagnostic and evaluation of progression.

In this work, a novel RTqPCR- based assay for the semi-quantitative simultaneous detection of MiR-410-3p and miR-141-5p is presented, showing the performance data from its closed preclinical assessment. Analysis of MiR-410-3p from subsets of primary plasma samples from patients attending the Urology Service of Hospital Universitario Puerta de Hierro has been performed using the discarded volume from PSA (formerly assessed within the week) tests on the novel system (MiRNAX Biosens ProstREACT©). Performance data from clinical specimens and comparison to *in vitro* results obtained in parallel further point at the use of this method as a promising option to potentially reduce the need of first-instance-invasive means such as biopsy, surgery and digital rectal examination.

MATERIALS AND METHODS

Between 2022 and 2023, 43 patients with a high probability of PCa or confirmed tumor and a positive PSA Enzyme Linked Immunosorbent Assay Test (ELISA) were recruited in the Urology Service of Hospital Universitario Puerta de Hierro (Madrid) to gauge the levels of MiR-410-3p and miR-141-5p on the novel system (MiRNAX Biosens ProstREACT©). Results were contrasted blindly with the PCa diagnostic formerly defined in accordance with the existing clinical guidelines. For this, patients underwent a biopsy test for prostate to confirm the cancer and the Gleason score was obtained in parallel to evaluate the microscopic features of the phenomenon found. Serum PSA was used as supposed biomarker of PCa-associated risk and treatment prognosis. Afterwards, risk stratification was evaluated according to the following groups defined by the urology service in compliance with the clinical guidelines. During the period, 43 patients were diagnosed to have PCa, which complied with the ethical and technical requirements for the study set this research (documentation available through the urology service and ethics committee of Hospital Universitario Puerta de Hierro). The controls included healthy volunteers and all attendants are listed in the Table 1.

Table 1. Sample data.

Samples	Group	PSA
HPH-01	EVA-01	5,98
HPH-02	EVA-02	5,22
HPH-13	EVA-03	10,4
HPH-18	EVA-04	7,55
HPH-37	EVA-05	7,19
HPH-38	EVA-06	1,04
HPH-41	EVA-07	3,03
HPH-46	EVA-08	5,87
HPH-48	EVA-09	0,15
HPH-03	EEP-01	7,52
HPH-04	EEP-02	4,6
HPH-10	EEP-03	11,7
HPH-14	EEP-04	0,02
HPH-15	EEP-05	0,06
HPH-16	EEP-06	0,04
HPH-27	EEP-07	6,69
HPH-28	EEP-08	10,96
HPH-29	EEP-09	9,75
HPH-05	CRB-01	0,05
HPH-06	CRB-02	9,66
HPH-08	CRB-03	0,01
HPH-22	CRB-04	1,38
HPH-23	CRB-05	0,7
HPH-24	CRB-06	0,03
HPH-25	CRB-07	0,18
HPH-39	CRB-08	0,01
HPH-40	CRB-09	0,03
HPH-42	CRB-10	0,02
HPH-43	CRB-11	0,14
HPH-45	CRB-12	0,01
HPH-07	ACT-01	0,13
HPH-09	ACT-02	155
HPH-11	ACT-03	0,31
HPH-12	ACT-04	7,96
HPH-17	ACT-05	4,11
HPH-21	ACT-06	9,74
HPH-30	ACT-07	74,11
HPH-34	ACT-08	12,2
HPH-35	ACT-09	0,68
HPH-36	ACT-10	9,17
HPH-44	ACT-11	5,2
HPH-47	ACT-12	4,48

Table 2. List of attendants diagnosed as per the existing clinical guidelines and tested for miR-4103p and miR-141-5p on the novel system (MiRNAX Biosens ProstREACT©). Sample names are encrypted so that they are not known to anyone outside the research group. De-anonymization will be made available upon request to the authors.

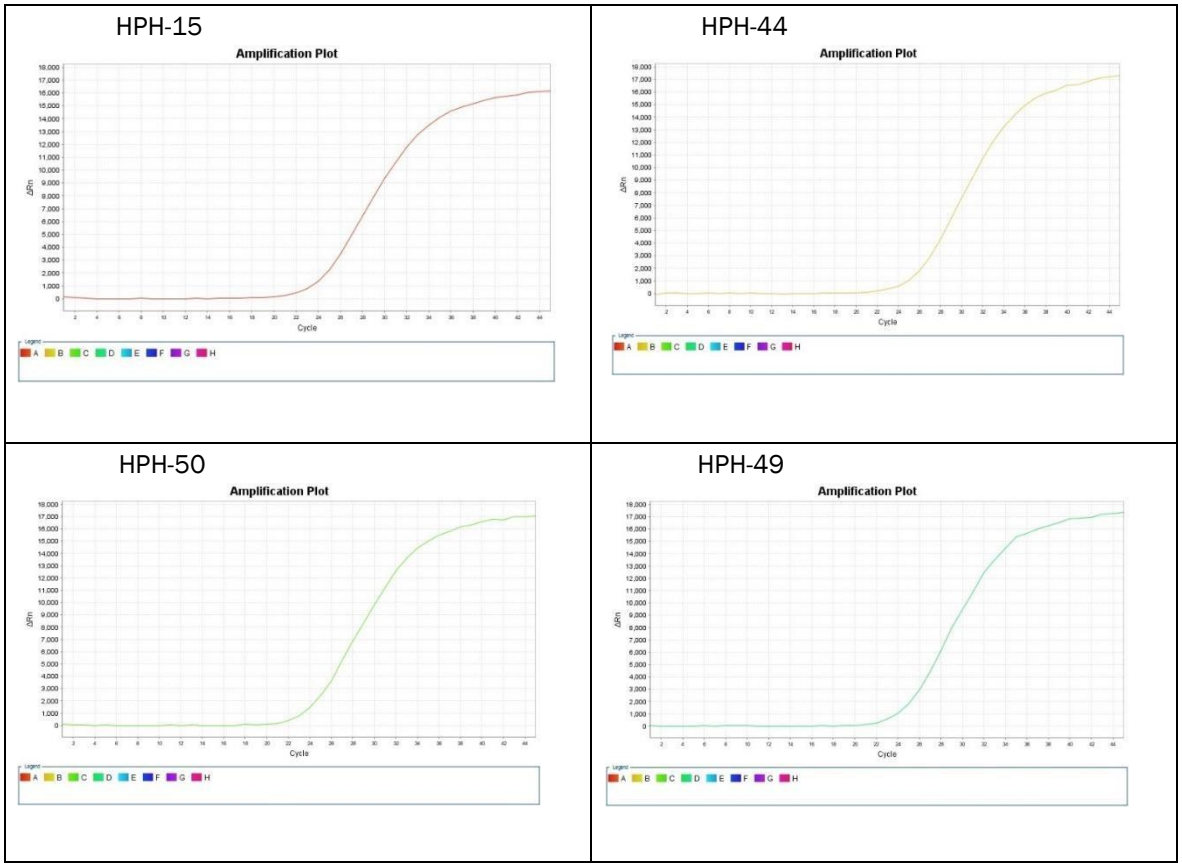
Pathological cohort	Description
EVA	Patients on active surveillance
EEP	Patients waiting for a prostactetomy
CRB	Confirmed patients in biochemical recurrence
ACT	High tumor burden
ESB	Suspicion pending biopsy

Serum sampling and total RNA isolation

Peripheral blood samples from donors were obtained at the urology service of Hospital Universitario Puerta de Hierro and allowed to stand for 1 hour before plasmas were obtained by separation from sera removing clots by centrifugation at room temperature for 10 minutes. The resulting supernatant (serum) was carefully removed to obtain the plasma from the whole blood samples collected in tubes with an as soon as the two phases were clearly observed. Resulting upper plasma aliquots (volumes ranging from 500 uL to 1 ml) were transferred to sterile 1.5 mL Eppendorf tubes labeled as "Primary Plasma" and stored at -80 °C. The remaining blood devoid of clot was thoroughly homogenized by gentle shaking and added to Falcon® tubes containing 5 mL of Ficoll. Addition was carried very slowly, avoiding a turbulent blood-Ficoll interaction while allowing the carbohydrate and metrizamide polymer to cause precipitation of erythrocytes and granulocytes, maintaining mononuclear cells afloat. Once the process was completed and cell separation due to the difference of densities was visually evident, a second centrifugation was performed at 2500 rpm for 30 min at room temperature yielding 4 phases: Surface Secondary Plasma (SSP), Peripheral Blood Mononuclear Cells (PBMC), Ficoll and Erythrocytes. SSP was then recovered (volumes ranging from 500 uL to 1 ml) and transferred to a 1.5 mL Eppendorf tube labeled as "Secondary Plasma" before storing at-80 °C. Samples thus obtained were kept frozen until cohorts were completed before progressing to manipulate for the detection of miR-410-3p -5p and miR- 141-5p. For this, total RNA was isolated using the miRNEasy® kit (Ref: 217184 Qiagen, USA) according to the manufacturer's instructions for use. Briefly, 200 µL plasma aliquots were used to perform total RNA isolation. Total RNA was eluted in 14 µL of RNAse-free water and subsequent RNA quantification was performed without freezing using a NanoDrop® recording the average of absorbance at 230, 260, 280 and 330 nm from four readings to calculate the concentration and purity of the purified products.

Once the concentration values were recorded, all RNA samples were analyzed for RNA integrity using MiRNAX-Biosens Kit for determination of hRNAse-P expression® (Ref. M001) which uses a stable endogenous reference small coding RNA used to accurately assess the yield of intact RNA fit for further amplification within the total RNA pool isolated. This system provides an estimation based on the Ct criteria with valid samples registering amplification in the range of 21-26 Ct. the Ct values were then used to make sure that proportionate amounts of primary plasma borne intact RNA were used in each reaction for reverse transcription and qPCR (Figure 1).

Figure 1. Example of primary and secondary plasmas analyzed for RNA integrity using miRNA X Biosens Kit for determination of hRNAse-P expression® (Ref. M001) for normalization as per the description above. **Note:** (▬) A; (▬) B; (▬) C; (▬) D; (▬) E; (▬) F; (▬) G; (▬) H

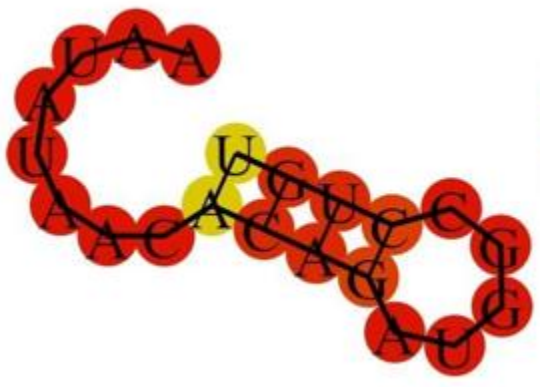


Quantification of miR-410-3p and miR 141-5p expression and statistical analysis

miR-410-3p and miR-141-5p were detected using the novel system (MiRNAX Biosens ProstREACT©), a patented RtqPCR assay [17] for the semi quantitative detection of both biomarkers based on the staggered priming of the forward and reverse oligonucleotides building an extended amplicon fit for perfect-match hybridization of a FAM-MGB fluorescent probes covering the entire MiR-410-3p and miR-141-5p mature sequences (Figure 2). The specific primers for amplification of MiR-4103p and miR-141-5p in this assay were subjected to *in silico* analysis for specificity and cross-reactivity before confirming the analytical sensitivity of the test (Figure 3).

Figure 2. Image of (A) miR-410-3p conformation and pair-base mating probability and (B) amplification scheme highlighting primers and probe positions. **Note:** (miRNA) AAUAUAACACAGAUGGCCUGU; (Probe) TAACACAGATGGC; (Forward) AATACTACATTAATGTCATAATATA; (Reverse) CGGACATACTGTAATTACATCATAA

A



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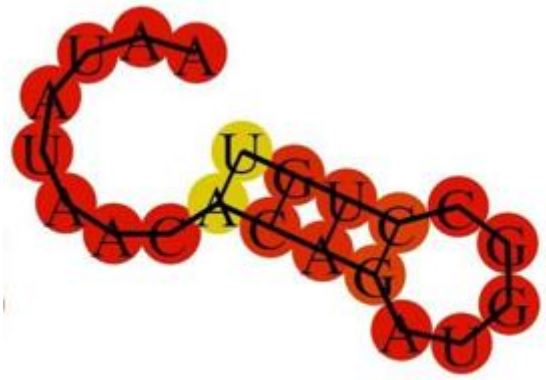
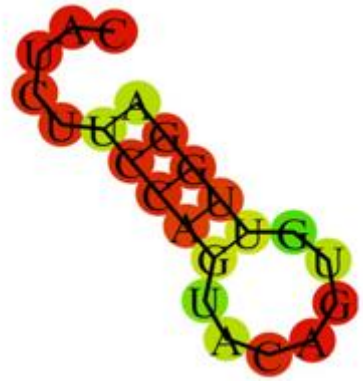


Figure 3. Image of (A) miR-141-5p conformation and pair-base mating probability and (B) amplification scheme highlighting primers and probe positions. **Note:** (miRNA) CAUCUUCCAGUACAGUGUUGGA; (Probe) TTCCAGTACAGTGT; (Forward) AATACTACATTAATGTCATCA; (Reverse) CAACCTTACTGTAATTACATCATAA

A



B

Refence curves for interpolation of Ct values obtained for miR-410-3p and miR-141-5p detected using the novel system (MiRNAX Biosens ProstREACT©) were generated under the following experimental conditions with a commercially available synthetic target from Integrated DNA Technologies (Ref: 229462048 IDT) across a set of dilution series (from 6 x 104-6 x 1012), to set the analytical sensitivity of the assay (Table 3).

Table 3. PCR conditions for the amplification using MiRNAX Biosens ProstREACT© assay.

Cycles	Temperature	Time	Activity
1	95°C	10 minutes	Polymerase activation
5	95°C	15 seconds	Denaturalization
	25°C	30 seconds	Hybridization
	60°C	30 seconds	Elongation
45	95°C	15 seconds	Denaturalization
	56°C	30 seconds	Hybridization
	60°C	30 seconds	Elongation
			Acquisition miR-410-3p
			FAM-NFQMGB
1	10°C	∞	Holding

The commercial Taqman miRNA Assay kits available from Applied Biosystems for amplification of miR-410-3p and miR-141-5p using nested RT-PCR amplification (Ref: 4427975 Applied Biosystems) were used for reference also and lastly, a similar dilution series was performed for comparison of both methods. Overlapping Ct values were obtained thus making both systems indistinct for subsequent Receiver Operating Characteristic (ROC) analysis. Therefore, Ct values obtained for miR-4103p and miR-141-5p detected using the novel system (MiRNAX Biosens ProstREACT©) were confirmed fit for the determination since no incremental or detrimental diagnostic value between the study methods was observed. One step RTqPCR programs fit for application of both biomarkers with the patented MiRNAX Biosens ProstREACT© assay were performed on Applied Biosystems Step-One Plus PCR System; the results were retrieved as Ct values and normalized to calculate the average Ct of each sample (ΔCt) and expressed as 2- ΔCt. All amplifications were run in duplicate to minimize the experimental error. Data characterized by the normal classification was expressed as the average and standard deviation so that miR-410-3p-5p and miR-141-5p content could be presented as a direct function of the Ct obtained using the double standard curve method. The Ct values of clinical samples were always compared with standards to calculate the copy numbers and provide a good estimation of the test's accuracy in terms of sensitivity, specificity, predictive value, and likelihood ratio. The sensitivity of the system as a potential clinical test was finally estimated using the proportion of subjects with the PCa who could be correctly identified by the test and provide a “positive” result under the thresholds defined.

RESULTS

Given the following three facts: 1) miRNAs are present in human plasma in a remarkably stable form that is protected from endogenous RNase activity, 2) miRNAs originating from human prostate cancer enter the blood circulation and 3) levels of miR-141-5p and MiR-410-3p have been readily measured in plasma to distinguish patients with PCa from healthy controls [18]: We initially opted to test secondary plasma for the presence of both miRNAs. The decision to follow this initial approach was based on the clearer nature and simpler composition of this fraction in relation to primary plasma. Therefore, a series of samples were processed as described and secondary plasma fractions were extracted using the MiR-410-3p miRNEasy kit® (Ref: 217184 Qiagen, USA). Total miRNAs were initially subjected to

amplification of MiR-410-3p and miR-141-5p with MiRNAX Biosens ProstREACT© assay, stratifying samples in agreement to a) the nanodrop quantification in relation to the total amount of RNA originally purified and b) the Ct values obtained for the hRNAse-P in relation to the integrity of targets readily available for quantification (Table 4). Purified total miRNA was subjected in parallel to amplification using the Taqman miRNA Assay kits available from Applied Biosystems for amplification of miR-410-3p and miR-141-5p using nested RT-PCR amplification (Ref: 4427975 Applied Biosystems). Ct data obtained with the nested RTqPCR system from Applied Biosystems for amplification of miR-410-3p and miR-141-5p were compared to those obtained with MiRNAX Biosens ProstREACT© (Table 5).

Table 4: RTqPCR results for the amplification of miR-410-3p and miR-141-5p from samples within cohort-1 (Ct value for hRNAse-P below 31,00) comparing Applied Biosystems nested RT-PCR amplification (Ref: 4427975 Applied Biosystems) and MiRNAX Biosens ProstREACT© assay.

Sample	Group	PSA level	Ct hRnase-P	Averaged Ct 410-3p Taqman	Averaged Ct 410-3p ProstREACT©	Averaged Ct 141-5p Taqman	Averaged Ct 141-5p ProstREACT©
HPH-13	EVA-03	10,4	27,74	32,8	33	39,78	39,99
HPH-18	EVA-04	7,55	28,53	34,27	34,07	39,08	39,02
HPH-15	EEP-05	0,06	30,54	34,02	34,09	38,99	39,72
HPH-08	CRB-03	0,01	28,76	35	34,02	UNDETECTED	UNDETECTED
HPH-09	ACT-02	155	27,5	33,9	34,3	UNDETECTED	UNDETECTED
HPH-17	ACT-05	4,11	29,19	31,73	32,03	39,96	38,29

Table 5. RTqPCR results for the amplification of miR-410-3p and miR-141-5p from samples within cohort-2 (Ct value for hRNAse-P above 31, 00) comparing Applied Biosystems nested RT-PCR amplification (Ref: 4427975 Applied Biosystems) and MiRNAX Biosens ProstREACT© assay.

Sample	Group	PSA level	Ct hRnase-P	Averaged Ct 410-3p Taqman	Averaged Ct 410-3p ProstREACT©	Averaged Ct 141-5p Taqman	Averaged Ct 141-5p ProstREACT©
HPH-01	EVA-01	5,98	34,83	UNDETECTED	UNDETECTED	UNDETECTED	UNDETECTED
HPH-02	EVA-02	5,22	36,1	38,29	39,01	UNDETECTED	UNDETECTED
HPH-03	EEP-01	7,52	33,57	36,91	38,01	UNDETECTED	UNDETECTED
HPH-04	EEP-02	4,6	32,55	UNDETECTED	UNDETECTED	UNDETECTED	UNDETECTED
HPH-10	EEP-03	11,7	34,78	UNDETECTED	38,99	UNDETECTED	UNDETECTED
HPH-19	GC-01	N.A.	32,39	35,96	35,29	UNDETECTED	UNDETECTED
HPH-20	GC-02	N.A.	34,65	35,9	36,76	UNDETECTED	UNDETECTED

Analysis of the data obtained from those clinical samples revealed that a) Ct values obtained for each kit were remarkably similar, making both systems apparently indistinct for subsequent Receiver Operating Characteristic (ROC) analysis and b) the cleanliness of this fraction in relation to primary plasma accounted for amplification values in cohort-1 for miR-410-3p and miR-141-5p to be >Ct32, which was the lowest threshold arbitrary fit for ROC analysis. Consistently, amplification values in cohort-2 for miR410-3p and miR-141-5p were significantly higher and not fit for purpose by a wider margin, correlating to the higher Ct values obtained for amplification of the h-RNAse-P marker of integrity with a low standard deviation (σ=0'8), thus indicating that data for amplification of the mi-RNA biomarkers were clustered tightly around the mean value for the marker of integrity.

Aiming to prove if amplification of the miRNA biomarkers and the integrity hRNAse-P gauge was possible below the Ct value set as minimum threshold for reliable ROC analysis (i.e. <Ct32), the richer primary plasma fraction was obtained and tested from a third cohort of samples now using the novel MiRNAX Biosens ProstREACT© assay as the results from Applied Biosystems nested RT-PCR amplification (Ref: 4427975 Applied Biosystems) and MiRNAX Biosens ProstREACT© assay were proven indistinct in all experiments performed so far. In this sense, it's worth noting that primary plasma is regarded as a dirtier sample than secondary plasma as it also contains the same 91% to 92% of water, but a higher amount of solids (8% to 9% compared to less than a half in secondary plasma [19] plus an amount of fibrinogen (Table 6). It is also reported to yield higher amount of tRNA from standard purification in a variety of works [20], hence the approach to test if primary plasma could act as a potential source for diagnostic of PCa using the amount of blood discarded after PSA analysis (Table 7).

Table 6. Results for the amplification of the marker for RNA integrity (hRNAse-P) in the primary plasmas obtained from clinical samples in Cohort-3 (Ct value for hRNAse-P below 30,00).

Sample	Group	PSA level	Ct hRnase-P
HPH-18	EVA-04	7,55	29,69
HPH-04	EEP-02	4,6	32,73
HPH-14	EEP-04	0,02	25,33
HPH-16	EEP-06	0,04	24,37
HPH-29	EEP-09	9,75	25,19
HPH-05	CRB-01	0,05	31,41
HPH-30	ACT-07	74,11	25,87
HPH-19	GC-01	N.A.	32,95
HPH-20	GC-02	N.A.	30,09
HPH-31	GC-03	N.A.	27,64
HPH-32	GC-04	N.A.	25,72
HPH-15	EEP-05	0,06	22,58
HPH-44	ACT-11	5,2	22,46
HPH-19	GC-01	N.A.	24,28
HPH-49	GC-06	N.A.	21,85
HPH-50	GC-07	N.A.	21,43

Table 7: Averaged RTqPCR results for the amplification of miR-410-3p and miR-141-5p using MiRNAX Biosens ProstREACT© assay from primary plasmas from Cohort-3 (Ct value for hRNAse-P below 30,00).

Cohort	Averaged Ct ProstREACT©	410-3p	σ in relation hRNAse-P	Averaged Ct ProstREACT©	141-5p	σ in relation hRNAse-P
3	31,97		0'75	30,93		0'72

Setting again the arbitrary limit of C<Ct32 for the amplification of the miRNA biomarkers to give a positive result in the amplification of miR-410-3p and miR-141-5p using MiRNAX Biosens ProstREACT© assay from the primary plasmas the Cohort-3, all determinations were found to sit below the threshold with no need to apply stratification in relation to the values obtained for the integrity hRNAseP gauge. This set of data proved that amplification for miR-410-3p and miR-141-5p in primary plasmas obtained from the blood volumes to be discarded after PSA

determination was possible using MiRNAX Biosens ProstREACT© assay within cohort-3 implementing the astringent Ct value set as minimum threshold for reliable ROC analysis (i.e. <Ct32).

After that, the MiRNAX Biosens ProstREACT© assay was tested for accuracy referring the Ct obtained from each donor within the cohort to the estimated copy number calculated using the reference curves for interpolation of Ct values obtained for MiR-410-3p and miR-141-5p using MiRNAX Biosens ProstREACT© with synthetic target from Integrated DNA Technologies (Ref: 229462048 IDT) across a set of dilution series (from 6x104-6x1012) (Figure 4).

Figure 4. Panel-1 Curves obtained across a dilution series for titration for miR-410-3p and miR-141-5p. **Note:** (■) A; (■) B; (■) C; (■) D; (■) E; (■) F; (■) G; (■) H

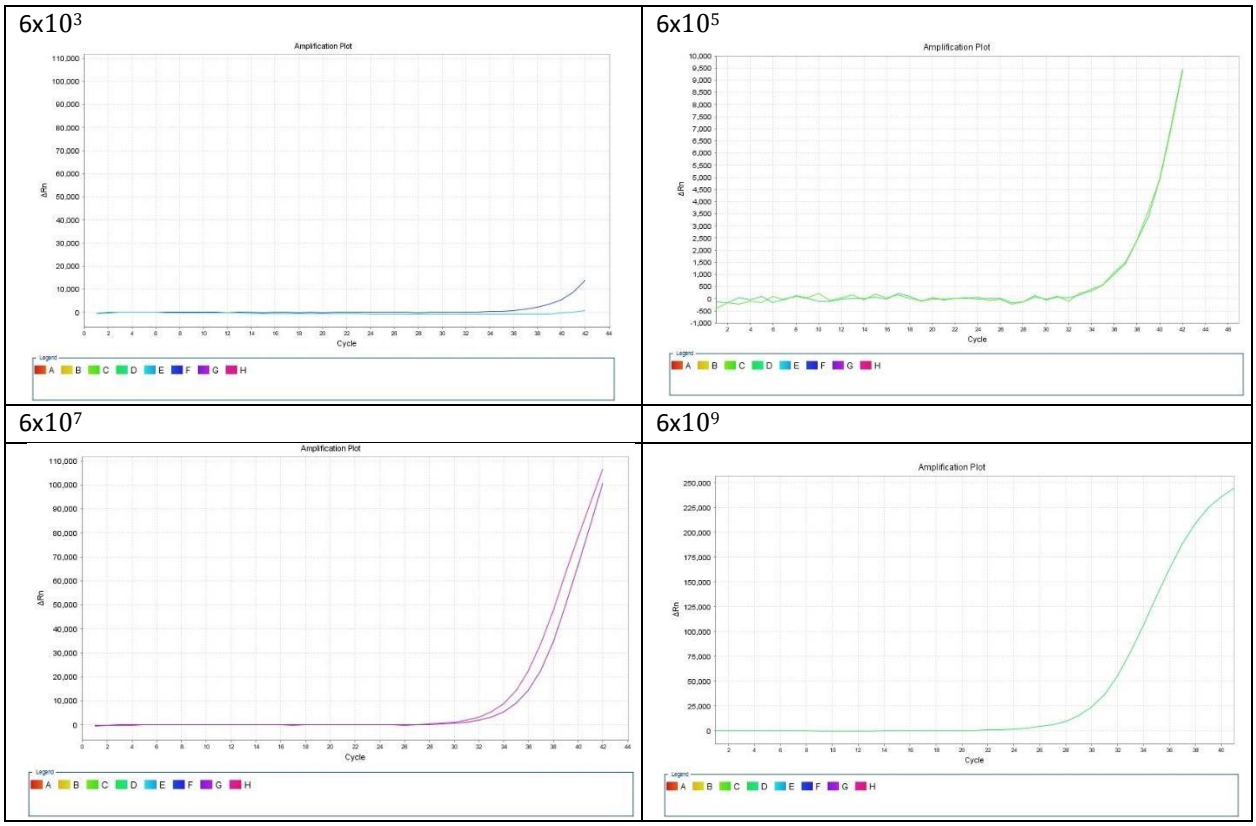
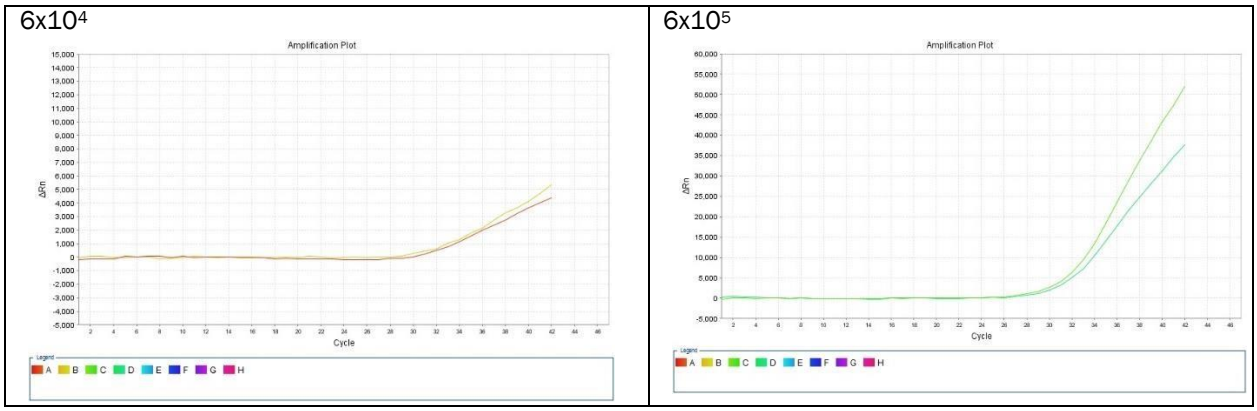
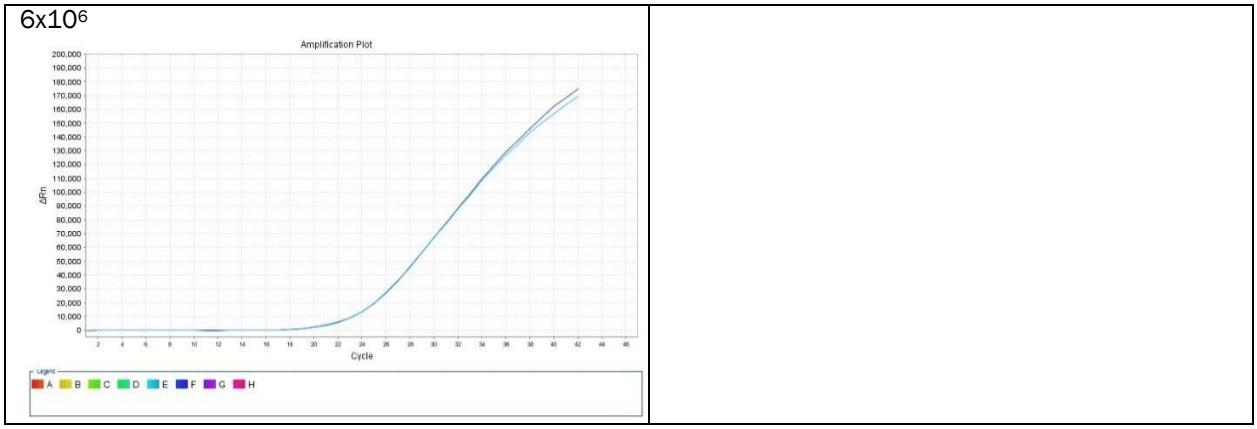


Figure-4. Panel-2 Amplification for miR-410-3p and miR-141-5p in samples from the cohort titrated by comparison to the dilution series obtained for synthetic targets. **Note:** (■) A; (■) B; (■) C; (■) D; (■) E; (■) F; (■) G; (■) H





Panel-1 Image of amplification for both biomarkers across a dilution series for synthetic miR410-3p and miR-141-5p used for normalization as per the description above. Perfect overlap at dilutions 6x109 and 6x105.

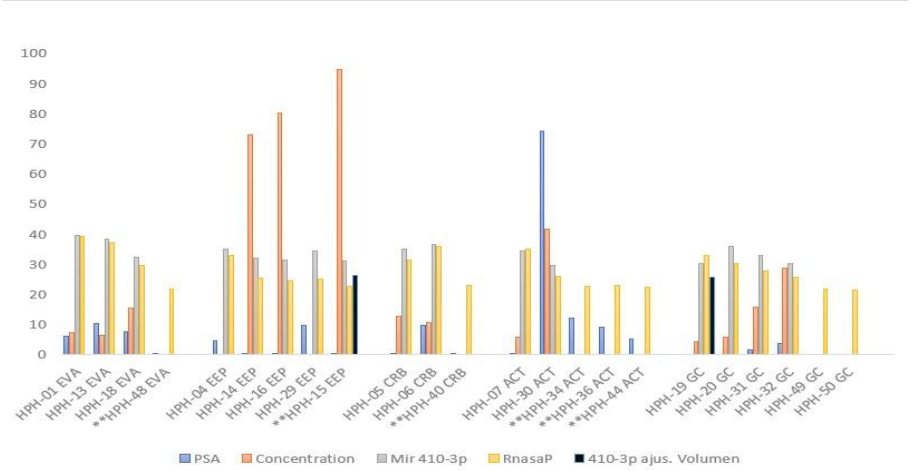
Panel-2 Image of amplification for both biomarkers in samples from the cohort showing detection at 6x106, 6x105 and 6 x 104 copies obtained for MiR-410-3p and miR-141-5p using the novel MiRNAX Biosens ProstREACT© Precision for Cohort-3 proved to be in the range of 97% for MiR-410-3p and 99 for miR141-5p as far as individual measurements taken in duplicated independent determinations for clinical samples were in accordance with the copy number interpolation and their equivalence to a potential positive or negative result regarding a yes/no blind answer to the question: Is patient X showing a quantifiable measurement for both MiR-410-3p and miR-141-5p biomarkers that correlates to confirmed PCa above the arbitrary threshold set for reliable ROC analysis (i.e. <Ct32)?. The comprehensive analysis of results from the three cohorts, prompted a clinical proof of concept with those samples that had still enough volume for the RTqPCR after the experimental so far described. Furthermore, having inferred that the two deciding factors for detection for the miRNA biomarker were a) integrity of the RNA extracted (Table 6) and concentration of the target miRNA target sequence (Figure 5), a decision was made to detect only MiR-410-3p using MiRNAX Biosens ProstREACT© but now comparing the protocol used for Cohorts 1 to 3 with a similar protocol where the final volume of extracted from primary plasma added to the RTqPCR reaction was solely calculated from the NanoDrop® (Ref:ND2000) records averaging the four readings of absorbance at 230, 260, 280 and 330 nm, initially used to calculate the concentration and purity of the purified products. This approach was used on a final Cohort-4 only when the material available from a given sample allowed and was christened "Correction by Volume" (CV). Unluckily, it required to decimate the samples to the point that there was not enough material to perform a parallel detection of MiR-141-5p using MiRNAX Biosens ProstREACT© (Table 8).

Table 8. Data compilation.

Sample	PSA	Nanodrop concentration	Mir 410-3p Ct std protocol	RNAse-P Ct std protocol	Mir 410-3p Ct CV protocol	Delta Ct miR410 *vs.* RnasaP
HPH-01 EVA	5.98	7.4	39.47	39.1		0.37
HPH-13 EVA	10.4	6.3	38.24	36.97		1.27
HPH-18 EVA	7.55	15.5	32.24	29.69		2.55
HPH-48 EVA	0.15			21.59		-21.59
						0
HPH-04 EEP	4.6		35.09	32.73		2.36
HPH-14 EEP	0.02	72.8	31.94	25.33		6.61
HPH-16 EEP	0.04	80.1	31.46	24.37	26.04	7.09

HPH-29 EEP	9.75		34.4	25.19		9.21
HPH-15 EEP	0.06	94.7	31.12	22.58	26.22	8.54
						0
HPH-05 CRB	0.05	12.7	34.92	31.41		3.51
HPH-06 CRB	9.66	10.6	36.36	36.02		0.34
HPH-40 CRB	0.03			23.04		-23.04
						0
HPH-07 ACT	0.13	5.9	34.46	34.9		-0.44
HPH-30 ACT	74.11	41.5	29.49	25.87		3.62
HPH-34 ACT	12.2			22.58		-22.58
HPH-36 ACT	9.17			23.08		-23.08
HPH-44 ACT	5.2			22.46		-22.46
						0
HPH-19 GC		4.4	30.24	32.95	25.63	-2.71
HPH-20 GC		5.8	35.77	30.09		5.68
HPH-31 GC	1.54	15.6	32.95	27.64	26,98	5.31
HPH-32 GC	3.67	28.8	30.29	25.72		4.57
HPH-49 GC				21.85		-21.85
HPH-50 GC				21.43		21.43

Figure 5. Graphical representation for samples in Cohort-4 comparing detection of MiR-410-3p using MiRNAX Biosens ProstREACT© understand and CV protocols. Note: ( ) PSA; ( ) Concentration; ( ) MiR-410-3p; ( ) 410-3p ajus. Volumen



DISCUSSION

The data obtained for amplification of MiR-410-3p and miR-141-5p biomarkers using the novel MiRNAX Biosens ProstREACT© system show a high correlation to confirmed PCa above the arbitrary threshold set for reliable ROC analysis (i.e. <Ct32) in cohorts 3 and 4 and are in good agreement to the integrity of the RNA present in the sample gauged by amplification of the hRNAse-P marker. Furthermore, the increase of input target using the CV protocol seems to further improve this correlation. Unluckily, no clinical conclusion can be drawn from the cohorts tested at the time of writing due to: a) the optimization nature of great part of the work described here as far as setting a definitive method (std. *vs.* CV) is respected, b) the limited number of clinical samples processed and c) the limitations in volume at the end of the processed tested in parallel. In this sense, a feasibility study has recently been completed as bigger study designed to re-confirm the data presented here and to show compliance with the regulations in EU market regarding IVD requirements. Results from this study will be also compiled for publication shortly [21].

Furthermore, a forthcoming clinical trial has been designed extending the framework program that is referred in this paper (see Materials and Methods) basing the recruitment in the Urology Service of Hospital Puerta de Hierro (Madrid) and parallel blind testing at the MBR&DU. This clinical trial has been subjected to review by the corresponding Ethics Committee (EC) to confirm if the levels of MiR-410-3p and miR-141-5p detected on the novel system (MiRNAX Biosens ProstREACT©) are fit for diagnostic of PC in patients with a moderate to high PSA value. The EC has approved this trial that will start in March 2024.

CONCLUSION

On a more general context, PC diagnostics using a non-invasive test such as MiRNAX Biosens ProstREACT© based on the innovative and patented RTqPCR technology may prove to be a unique tool for diagnostics as circulating tumor miRNA biomarker analysis has the potential to significantly improve patient stratification complementing the current criteria set by PSA and/or multi-parametric magnetic resonance imaging for biopsy and treatment. In this sense, extensive clinical trials as the one mentioned above need to be completed in order to confirm the potential of using circulating tumor miRNA biomarker analysis such as the described here in cancer diagnosis, particularly to predict clinically significant prostate cancer in pre-biopsy cases. Actually, the presentation of novel solutions such as MiRNAX Biosens ProstREACT© may relieve diagnostic pressure from the anticipated increase of PCa mortality rates that may rise in the years to come. As a result, systems showing promising data in the line presented here may end up forcing urologists to consider new paradigms in the disease management: Both to improve methods of detection and to be more effective in managing the likely increasing numbers of men with advanced disease.

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REFERENCES

1. Siegel RL, et al. Cancer statistics, 2020. CA: A cancer journal forclinicians. 2020; *70:7*-30.
2. Bevacqua E, et al. The potential of micrornas as non-invasive prostate cancer biomarkers: A systematic literature review basedon a machine learning approach. Cancers. 2022;14: 5418.
3. Wang J, et al. MicroRNA-410-5p as a potential serum biomarker for the diagnosis of prostate cancer. Cancer Cell Int. 2016;16:12.
4. Fredsøe J, et al. Profiling of circulating micrornas in prostate cancer reveals diagnostic biomarker potential. Diagnostics *(Basel, Switzerland)*. 2020;*10*:188.
5. Parra-Medina R. miRNA expression profile in early-onset prostate cancer and identification of its possible target genes related to carcinogenesis. 2020.
6. Wang J, et al. Cancer-derived circulating micrornas promote tumor angiogenesis by entering dendritic cells to degrade highly complementary micrornas. Theranostics. 2017; 1407-1421.

7. Richardsen E, et al. MicroRNA 141 is associated to outcome and aggressive tumor characteristics in prostate cancer. Sci Rep. 2019; 9: 386.
8. Fernández-Turizo MJ, et al. Role of microRNAs in the pathogenesis of prostate cancer. The role of microRNAs in the pathogenesis of prostate cancer. 2021; 35: 26-36.
9. Li W, et al. Plasma exosomal miR-125a-5p and miR-141-5p as non-invasive biomarkers for prostate cancer. Neoplasma. 2020; 67:1314-1318.
10. Richardsen E, et al. MicroRNA 141 is associated to outcome and aggressive tumor characteristics in prostate cancer. Sci Rep. 2019; 9:386.
11. Zhang Y, et al. miR-410–3p promotes prostate cancer progression *via* regulating PTEN/AKT/mTOR signaling pathway. Biochem Biophys Res Commun. 2018; 503: 2459-2465.
12. Liu Z, et al. Expression of miR- 410 in peripheral blood of patients with clear cell renal cell carcinoma and its effect on proliferation and invasion of Caki-2 cells. J BUON. 2021; 26:2059-2066.
13. Zhang Y, et al. miR-410-3p promotes prostate cancer progression *via* regulating PTEN/AKT/mTOR signaling pathway. Biochem Biophys Res Commun. 2018;503.
14. Liu Z, et al. Expression of miR-410-3p in peripheral blood of patients with clear cell renal cell carcinoma and its effect on proliferation and invasion of Caki-2 cells. Oxid Med Cell Longev. 2021;2022:5983629.
15. Yuan Y, et al. miR-410-3p induces both epithelial–mesenchymal transition and radioresistance through activation of the PI3K/mTOR pathway in non-small cell lung cancer. Signal transduct target ther. 2020; 5.
16. Bevacqua E, et al. The potential of micrornas as non-invasive prostate cancer biomarkers: A systematic literature review based on a machine learning approach. Cancers. 2022;14:5418.
17. Castan P, et al. International Patent - EP4055189A1-Bivalent Reverse Primer.2021.
18. Rana S, et al. MicroRNAs as biomarkers for prostate cancer prognosis: a systematic review and a systematic reanalysis of public data. Br J Cancer. 2022; 126:502-513.
19. Tanidir Y, et al. Radical prostatectomy is a valuable treatment alternative in patients with high risk prostate cancer. J Urol Surg. 2015; 2.
20. Cooperberg MR, et al. The changing face of prostate cancer. J Clin Oncol. 2005;23:8146-8151
21. Cooperberg MR, et al. Time trends and local variation in primary treatment of localized prostate cancer. J Clin Oncol. 2010;28:1117-1123.