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Abstract

This work describes the development and the application of a lateral flow biosensor for the detection of the prostate specific antigen in urine (uPSA). The biosensor allowed uPSA detection in 10 minutes with a limit of detection and a range of quantification respectively of 20 ng mL^{-1} and 37 – 420 ng mL^{-1}, showing within and between-day coefficients of variation ≤ 13%. It showed 92% of accuracy and an almost perfect concordance with the reference electrochemiluminescence immunoassay method. The biosensor design provides the disappearance of the Test line signal at the cut-off concentration. This was achieved with a double layer sensing strategy, in which gold nanoparticles were functionalized with Staphylococcal protein A – a mediator – instead of anti-PSA antibody. This strategy allow making a fine-tune on the concentration of the specific antibody, obtaining an on/off switch of the Test line at the cut-off value. The cut-off value was also established in this work, based on the distribution of uPSA levels from 140 patients, who were suspected of prostate cancer and who underwent to first biopsy. The clinical application of the biosensor to predict repeat biopsy outcome in 28 patients showed sensitivity, specificity, positive and negative predictive values of 100%, 64%, 74% and 100%, respectively.

Keywords: Lateral Flow Immunoassay, Paper-based biosensors, Immunochromatographic strip test, Prostate cancer, Repeat biopsy, Urinary prostate specific antigen

1. Introduction

Prostate cancer (PCa) is the second most common tumor among men worldwide [1] and, respectively, the second and the third most frequent cause of cancer-related death in Europe and United States [2,3]. PCa is generally asymptomatic until it reaches an advanced stage. Therefore, the possibility to screen for its early detection is of utmost importance in order to reduce the chance of death from PCa.

Routine screening for PCa relies on the measurement of the prostatic specific antigen (PSA) level in blood and on the digital rectal examination (DRE) [4]. Men with a positive PSA test result (serum PSA higher than 4 ng mL^{-1}) usually undergo a transrectal ultrasound-guided biopsy of the prostate, which is the gold standard for PCa diagnosis [5].

Results of several clinical trials suggested that PSA screening can reduce prostate cancer mortality, thanks to its high diagnostic sensitivity in detecting PCa at an early stage [6-8]. However, PCa diagnosis based on serum PSA lacks sufficient diagnostic specificity [9] because elevated PSA levels in blood may be related to other conditions, e.g., benign prostatic hyperplasia (BPH), prostatitis and infections of the urinary tract [10]. For this reason, screening based on PSA is still controversial as can be observed from the different recommendations for and against, among the several guidelines existing on the subject [11]. All the guidelines agree that the identification of asymptomatic cancer that would never cause symptoms or contribute to death is one of the most important damage of PSA-based screening programs [12].

Another important issue in PCa diagnosis is related to the different aggressiveness of the tumor. According to aggressiveness, the physician and the patient will decide for the immediate treatment, for monitoring the progression of the tumor by re-measuring PSA level and by DRE (observation), or looking for changes in cancer growth through biopsies on a regular basis (active surveillance). Usually the aggressiveness of PCa is assessed by means of the Gleason score (GS), which requires the execution of the biopsy and the assignment by a pathologist. GS ranges from 2 to 10, but most PCa are scored 6 to 10 [13]. Because biopsy does not guarantee 100% accuracy, in case of first negative biopsy the subject is still considered at risk and the European guidelines suggest recurring to rebiopsy after six-twelve months [9].

Overtreatment of indolent PCa, which aggressiveness is overestimated negatively impacts patients' life, causing urinary retention, urinary incontinence and erectile dysfunction. Therefore, the availability of reliable PCa biomarkers and accurate and non-invasive tests to reduce the number of unnecessary biopsies and avoid overtreatment is highly demanded. Recently, the possible use of multi-parametric Magnetic Resonance Imaging (MRI) for screening for PCa has been suggested, since the very high specificity showed by this technique [14]. However, difficulty of results interpretation combined with high cost and limited access are major obstacles to the widespread implementation of MRI as a viable screening tool.

As an alternative, several risk prediction models based on blood and urine biomarkers (e.g., Prostate Cancer Antigen 3 [15], Prostate Health Index [16], 4KScore [17], Stockholm3 test [18] and iXip index [19]) and risk

calculators based on the European Randomized Study of Screening for Prostate Cancer trial data [20], have been proposed in the last decades. Some of these models increased the accuracy in predicting PCa and reduced the number of false-positive results. However, no unanimous consensus has been expressed about the use of these tests in routinely clinical practice, some of them are extremely expensive and time-consuming and therefore hardly applicable on a large scale. For this reason, the level of serum PSA is still widely used by the health care system as the standard screening test for PCa early diagnosis, during observation and in the active surveillance. PSA has also been revalued in combination with other parameters for the risk stratification in the PCa management. In its last update (March 2020 [21]), the National Comprehensive Cancer Network® Guideline on PCa reported 10 ng mL^{-1} as the PSA upper limit to identify subjects with low risk of PCa progression.

Physiologically, PSA is present in urine due to the anatomic proximity of the prostate to the bladder and urethra [22]. Tremblay et al. measured a urinary excretion of PSA (µg/24 hr, mean ± standard error of the mean) equals to 195 ± 26, 410 ± 87, 97 ± 29 in normal subjects (10-45 years old), patients with BPH and patients with PCa, respectively [23]. Bolduc and co-workers [24] measured urinary PSA (uPSA) in patients that have been diagnosed PCa and BPH, through an enzyme-linked immunosorbent assay (ELISA). They supported uPSA as a useful marker in the differential diagnosis of PCa and BPH, especially when serum PSA is between 2.5 ng mL^{-1} and 10 ng mL^{-1}. Moreover, they stated that low uPSA points toward prostate cancer and that an uPSA threshold of > 150 ng mL^{-1} may be used to decrease the number of prostatic biopsies.

In this study, we retrospectively evaluated the use of uPSA as a biomarker for predicting outcomes of repeat biopsies and developed a lateral flow biosensor for the point-of-care detection of uPSA. Urinary levels of PSA from 168 subjects who were scheduled for biopsy according to Prostate Cancer Guidelines of European Association of Urology [25] were quantified by means of an electrochemiluminescence immunoassay employing a Cobas® laboratory-based platform. All the subjects underwent to biopsy and were classified as PCa positive (PCa+) or negative (PCa-) according to the GS. Twenty eight patients classified as PCa-, after the first biopsy, were still considered at risk and were scheduled for repeat biopsy.

Based on the receiver operating characteristic (ROC) curve from 140 patients that were not rescheduled for biopsy, we calculated a threshold value for uPSA corresponding to 90% diagnostic sensitivity. According to the established cut-off value, we developed a simple and fast biosensor for the detection of PSA in urine and verified its suitability for predicting repeat biopsy outcomes. Results were validated on the group of 28 subjects who were submitted to the second biopsy.

The lateral flow immunoassay (LFIA) technology is widely recognized as a convenient and efficient tool for monitoring and self-monitoring health status. The advantages of point-of-care diagnosis based on LFIA are rapidity, cost-effectiveness, affordability, and capability of providing accurate identification and/or quantification of different kinds of biomarkers, including pathogens, proteins, hormones, metabolites, etc.

[26]. Therefore, we designed a biosensor based on the LFIA technology for detecting PSA in urine. The LFIA biosensor comprised: a reactive membrane where two lines were drawn, one (Test) line responsive to the uPSA level and the second (Control) line to confirm validity of the device; a sample membrane suitable for the direct application of the urine sample; and an absorbent membrane to guide the capillary flow from the sample to the reactive membrane (Fig. 1a). Gold nanoparticles (AuNPs) were employed as the colorimetric probe and linked to Staphylococcal protein A (Au-SpA), which is able to bind immunoglobulins from various species (in this case, a specific mouse monoclonal anti-PSA antibody), while the anti-PSA antibody was added to the sample. To comply with the clinical significance of the uPSA biomarker, the assay format was designed to provide a positive outcome (i.e. the appearance of a visible signal at the Test line) for those subjects with increased risk of developing PCa and a negative outcome (i.e. no signal in correspondence of the Test line) for patients with low risk of evolving the malignancy. In fact, according to the inverse relationship found between urinary PSA and the risk of evolving the tumor, uPSA exceeding the cut-off value corresponded to healthy or low-risk subjects (negative outcome) while subjects with uPSA below the cut-off value should be tested as positive. The parameters of the assay (AuNp-SpA, anti-PSA antibody and antigen amount, capillary flow rate, etc.) were tailored to reach the disappearance of the Test line color in correspondence of the cut-off value established for urinary PSA.

The uPSA measurement through the proposed POCT device as an affordable and rapid tool for the follow up of patients considered at risk of developing PCa would dramatically cut operating costs and times to treatment and would be a great ally in the management and monitoring of subjects with different levels of risk to incur PCa.

2. Materials and methods

2.1 Immunoreagents, chemicals and materials

Staphylococcal protein A (SpA), bovine serum albumin (BSA), rabbit immunoglobulins (r-IgG) and Tween 20® were obtained from Sigma–Aldrich (St. Louis, MO, USA). Urea, creatinine and other chemicals were purchased from VWR International (Milan, Italy). Synthetic urine was prepared as follows: 0.65 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0,65 g L^{-1} $MgCl_2 \cdot H_2O$, 4.6 g L^{-1} NaCl, 2.3 g L^{-1} Na_2SO_4, 0.65 g L^{-1} Na_3 citrate$\cdot 2H_2O$, 2.8 g L^{-1} KH_2PO_4, 1.6 g L^{-1} KCl, 1.0 g L^{-1} NH_4Cl, 25 g L^{-1} urea and 1.1 g L^{-1} creatinine, with pH solution adjust to 6.5. PSA and mouse monoclonal antibodies against PSA (anti PSA Ab) were kindly supplied by Nib biotec Srl (Turin, Italy). Nitrocellulose membranes (HF180 plus card), glass fiber conjugate pad and cellulose absorbent pads were obtained from Merck Millipore (Billerica, MA, USA). Glass fiber sample pads Standard 14 were obtained from Whatman (Little Chalfont, UK). Plastic cassettes were furnished by Kinbio (China).

187 **2.2 Study population**

188 One hundred and sixty-eight men scheduled for prostate biopsy at the urology clinic, Città della Salute e
189 della Scienza di Torino - Molinette Hospital, Turin, Italy were included in the study. Subjects received biopsy
190 indication based on PSA level, DRE, prostate volume, and family history.

191 A group of 140 men who were classified for suspected prostate cancer according to the result of the first
192 biopsy were considered as the training dataset. In this population, 53 subjects (38%) were diagnosed PCa+
193 (clinically significant PCa, GS≥7), while 87 (62%) were PCa- (no cancer and indolent PCa, GS=6).

194 The validation set included 28 men whose first biopsy was negative (PCa-), but were still considered at risk
195 basing on other parameters (i.e. family history, PSA level, etc.) and who had one repeat biopsy. According
196 to the second biopsy 14 (50%) subjects were confirmed as PCa- while 14 subjects (50%) were re-classified
197 as PCa+.

198 The clinical characteristics of the study population (training and validation data set) are shown in Table 1.

199 **2.3 Quantification of urinary PSA**

200 Urine samples were collected before the biopsy after a standardized DRE. After a gentle shake of the
201 sample, 15 mL were collected and stored in Falcon tubes at -80 °C within 5 minutes from collection.

202 Urinary measurements of PSA were performed by means of an electrochemiluminescence immunoassay
203 (ECLIA) employing a Cobas® laboratory-platform (Roche Diagnostic GmbH, Mannheim, Germany) in the
204 Biochemical and Clinical Laboratory (Baldi and Riberi), Città della Salute e della Scienza di Torino - Molinette
205 Hospital, Turin, Italy. The measuring range of the ECLIA is 0.003 to 100 ng mL^{-1} (defined by the lower limit of
206 quantification, LLOQ, and the upper limit of quantification, ULOQ). Samples with PSA concentrations above
207 the measuring range can be diluted with Elecsys Diluent Universal (furnished within the kit assay) up to a
208 recommended dilution of 1:50. Values below the detection limit are reported as < 0.003 ng/mL, while
209 values above the measuring range are reported as > 100 ng mL^{-1} (or up to 5000 ng mL^{-1} for 50-fold diluted
210 samples).

211 Considering the physiological levels of uPSA, urine samples were diluted 1:50 with the recommended
212 diluent and analyzed.

213 **2.4 Statistical data treatment**

214 SigmaPlot v.14.0 (Systat Software, Inc) was used to perform the statistical analyses. For statistical
215 comparisons, a p value < 0.05 was accepted as statistically significant. Samples with urinary PSA above the
216 ULOQ (5000 ng mL^{-1}) were assigned the ULOQ value. No samples contained uPSA below the LLOQ. For the
217 definition of the cut-off level 140 subjects were considered (training set). Statistic descriptive of the PCa-

and PCa+ groups for the training set are shown in Table S1. Normality was tested by the Kolmogorov-Smirnov and the Shapiro-Wilk tests. Both distributions deviated from the normal distribution, then groups were compared using the Wilcoxon rank-sum test (Mann-Whitney U test) and differences were shown using the Box-Whisker plot. To evaluate the diagnostic performance of uPSA, the ROC curve was constructed and the area under the curve (AUC) was calculated.

The discriminating value of uPSA was established as the concentration allowing to obtain 90% diagnostic sensitivity on the training data set. Then, we checked its predictivity on the validation set of data (n=28), considering the response of the second biopsy to classify subjects.

2.5 Preparation and characterization of gold nanoparticles and of the colorimetric probe

Gold nanoparticles (AuNPs) with a diameter of about 30 nm were synthesized through the tetrachloroauric acid reduction with sodium citrate, as previously reported [27, 28]. Briefly, 1 mL of 1% w/v sodium citrate was added to 100 mL of boiling 0.01% tetrachloroauric acid under vigorous stirring. The color of the solution changed gradually from light yellow to ruby red, indicating the successful formation of AuNPs. Finally, the colloidal gold was cooled down to room temperature and stored at 4 °C for subsequent conjugation to SpA.

The AuNPs were used to label SpA (AuNPs-SpA), through passive adsorption, according to previous reports [27, 29]. Briefly, 12 µg of SpA and 1 ml of borate buffer (20 mM, pH 7.4) were mixed with 10 ml of colloidal gold and incubated for 30 min at 37 °C. Next, 1 ml of 1% BSA in borate buffer was added and reacted for 10 min at 37 °C to saturate the uncovered AuNPs surface. AuNPs-SpA conjugates were recovered by centrifugation (13000 g, 15 min) and washed twice with borate buffer supplemented with 0.1% BSA. Finally, the pellet was reconstituted in AuNPs storage buffer (borate buffer 20 mM pH 7.4 with 1% BSA, 0.25% Tween 20®, 2% sucrose and 0.02% sodium azide) and stored at 4°C until use.

AuNPs and AuNPs-SpA were characterized by visible spectroscopy, transmission electron microscopy (TEM) and dynamic light scattering technique (DLS). Spectrophotometric analyses were carried out by Varian Cary 1E spectrophotometer (Agilent Technologies, USA). For TEM imaging, a drop of the GNPs aqueous suspension was put on a copper grid covered with a lacey carbon film for the analysis using a Jeol 3010-UHR (Jeol Ltd, Japan) high resolution transmission electron microscope (HR-TEM) equipped with a LaB_6 filament operating at 300 kV and with an Oxford Inca Energy TEM 300 X-ray EDS analyzer. The hydrodynamic diameter distribution of the suspensions prepared was evaluated using a Zetasizer instrument (Zetasizer Nano-ZS, Malvern Instruments, U.K.). The measurements were performed after checking that the automatic attenuator was between 6 and 9. The results were expressed as hydrodynamic diameter distribution in intensity (average of mean values of 5 measurements).

2.6 Lateral flow biosensor fabrication and uPSA detection

The biosensor developed in this work to detect uPSA is composed by i) a glass fiber sample pad, ii) a glass fiber conjugate pad, iii) a nitrocellulose membrane (NC), iv) a cellulose absorbent pad and v) a plastic cassette.

PSA (0.5 mg mL^{-1}) and rabbit IgG (1 mg mL^{-1}) solutions prepared in 20 mM phosphate buffer pH 7.4, were applied on the NC card, by means of XYZ3050 dispenser platform (Biodot, Irvine, CA, USA), to form the Test and the Control line, respectively. The reagents were non-contact dispensed at 1 µL cm^{-1}, keeping a distance of 4 mm between the lines. The NC card were dried at 37°C for 45 minutes under vacuum. The conjugate pad was saturated with the AuNPs-SpA conjugate (optical density 1) and dried for 3 hours at room temperature. Finally, we cut the laminated cards by means of CM400 guillotine (Biodot, Irvine, CA, USA) to obtain 5-mm width strips, inserted them into plastic cassettes and stored the final biosensor at room temperature in the dark in plastic bags containing silica.

To perform the test, 70 µL of solution, containing patient urine (or PSA standard solution) and anti-PSA antibody (prepared in 20 mM phosphate buffer pH 7.4 with 1% BSA and 0.1% Tween 20®) in 1:1 ratio, was applied to the sample pad. The immunoreactions take place and the immunocomplexes between anti-PSA and PSA immobilized on NC were revealed by the AuNPs-SpA probe that is capable to bind to immunoglobulins from various species with high affinity (Fig. 1b). If the sample contained PSA, the binding of the antibody to the immobilized PSA was increasingly inhibited, until reaching the complete inhibition when PSA level is above the cut-off value (Fig. 1c). The result was assessed after 10 minutes by the naked-eye as the absence/presence of the Test line. Strip images were also acquired by a scanner and processed by a software (QuantiScan 3.0, Biosoft, Cambridge, UK) in order to quantify the Test line intensity (for biosensor development).

The assay was considered invalid in the absence of the Control line. The Control line signal was generated by the interaction between AuNPs-SpA and the rabbit IgG.

2.7 Set-up of the lateral flow biosensor parameters for uPSA detection

It is well known that several parameters deeply affect the analytical performance of lateral flow immunoassay devices. The parameters evaluated to tune the lateral flow biosensor were: a) PSA and rabbit IgG concentrations to form Test and Control lines, respectively; b) the amount of anti-PSA; c) the optical density of AuNPs-SpA and d) the volume of sample to be analyzed. The modulation of these parameters was conducted to obtain a lateral flow biosensor in which the Test line signal disappeared when uPSA was equal or greater than the established cut-off level.

2.8 Analytical evaluation of the lateral flow biosensor for uPSA detection

The biosensor was calibrated between 0 and 5000 ng mL^{-1} by using PSA-fortified synthetic urine [30] and measuring each analyte concentration in triplicate. The limit of detection (LOD) was calculated as the

concentration of the analyte that corresponds to the signal of the blank minus three standard deviation of the blank. The range of quantification was considered as the PSA level giving signals between 80% (IC_{80}) and 20% (IC_{20}) of the signal of the blank.

For comparing with the reference ECLIA, the 168 samples were also classified as positive/negative on the basis of the measured uPSA below /above the cut-off value. We calculated the biosensor accuracy in comparison to the reference ECLIA as the number of agreeing results divided by the number of samples measured (multiplied by 100). Concordance between the two methods was estimated by the Cohen's K.

We evaluated the within-day, between-day, within-batch and between-batch reproducibility of the uPSA biosensor by analyzing 10 different samples (5 samples with uPSA < cut-off and 5 samples with uPSA > cut-off) on 3 different days and using strips from 3 different batches (n=3).

Finally, we evaluated the shelf life of the biosensor performing real-time stability experiments. For stability experiments, fully assembled test strips were sealed in a foil pouch with desiccant and stored at room temperature. On each testing day, the necessary strips were removed from storage and used to test 3 uPSA concentrations. The shelf life was studied during a 6 months period, testing the strips on month 0, 1, 3 and 6.

2.9 Diagnostic validity of the lateral flow biosensor for repeat prostate biopsy outcome

The biosensor was applied to detect uPSA in the whole study population. Results were visually judged by three operators, who provided agreeing results for all tested samples. Samples were assigned as positive/negative according to the presence/absence of the test line.

The diagnostic sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) of the biosensor were calculated on the validation set (n=28) by considering the diagnosis from the second biopsy.

3. Results and discussion

3.1 Urinary PSA cut-off level

Urine samples were collected from patients suspected of PCa at the time of the biopsy. The uPSA levels were measured by means of a quantitative *in vitro* diagnostic test based on the ECLIA method routinely employed in a major clinical laboratory of Torino (Italy). The measuring range of the ECLIA is 0.003 to 100 ng mL^{-1} (0.15-5000 ng mL^{-1} considering that urine samples were 50-fold diluted before analysis). Samples with PSA concentrations above the measuring range were assigned to 5000 ng mL^{-1} (22 samples out of 140). No samples were measured below the LLOQ. Urinary PSA mean, median and interquartile range of the training population were 1708 ng mL^{-1}, 1024 ng mL^{-1} and 202 ng mL^{-1} - 2871 ng mL^{-1}, respectively.

The uPSA levels distribution of patients diagnosed PCa+ and PCa- at the biopsy were compared using the Mann-Whitney U test and differences were shown using the Box-Whisker plot (Fig. 2).

The difference in the median values between PCa+ and PCa- groups is greater than would be expected by chance ($P < 0.001$), thus confirming a statistically significant difference between uPSA levels in the PCa+ and PCa- population.

To evaluate the diagnostic performance of uPSA in distinguish PCa+ and PCa-, the ROC curve was constructed, and the area under the curve (AUC) was calculated as 0.70 (Fig. 3). The AUC confirmed the diagnostic power of the uPSA that was significantly higher than serum PSA (AUC 0.54) and similar to the combination of serum PSA, age and DRE (AUC 0.73), which is the model currently in use for PCa screening [25].

Considering the use of uPSA as a first level screening tool, the diagnostic threshold to distinguish the group was set at 90% diagnostic sensitivity. The corresponding level of uPSA was calculated as ca. 2800 ng mL^{-1}.

To evaluate the diagnostic performance of uPSA in predicting re-biopsies outcomes, we compared the ROC curves for serum PSA, uPSA (from ECLIA) and the combination of age, serum PSA and DRE for the validation set of subjects and the AUCs were calculated as 0.65, 0.97 and 0.76, respectively (Fig. 3b). The comparison pointed out an outstanding improvement in the diagnostic performances of uPSA, which showed Se, Sp, PPV and NPV of 100%, 50%, 64% and 100%, respectively.

3.2 Characterization of gold nanoparticles and of the colorimetric probe

The AuNPs resulted almost spherical in shape as reported by TEM analysis that allowed visualizing AuNPs morphology (Fig.4 b).

Visible spectrophotometric measurements were used to verify the effective adsorption of SpA onto AuNPs, since the formation of a protein layer onto AuNPs surface causes the modification of the refractive index and a shift of the localized surface plasmon resonance (LSPR) band. A λ_{max} shift from 525 nm to 529 nm was recorded and was considered as indicating successful conjugation (Fig. 4a).

The effective adsorption of the protein layer was also confirmed by DLS measurements resulted in a greater hydrodynamic size for AuNPs-SpA (Fig. 4c).

3.3 Principle of the lateral flow biosensor

Ideally, the sensitive detection of PSA can be achieved by applying a sandwich-type immunoassay format, using a pair of specific antibodies (i.e. a capture Ab on the NC and a detection Ab labelled to provide the signal when the ternary complex forms). In this format, the signal formed in correspondence of the Test line means that PSA is present in the sample and increases proportionally to the increase of the analyte

amount. The coloring of the Test line is commonly attributed to a positive status, e.g., confirmed pregnancy, positivity to infections, etc.

In the case of uPSA, the positivity, intended as the high risk of evolving the cancer, is associated with decreasing levels of the biomarker that can lead to a misleading interpretation of the result. In order to facilitate result interpretation, we opted for a 'reverse' format to detect uPSA (i.e. the indirect competitive format). In this format, the antigen (PSA) is used as the capture reagent and one PSA-specific antibody is used (detection antibody). PSA immobilized on the NC and the one in the urine sample compete for binding to the detection antibody, resulting in inverse correlation between the signal and the analyte level. The immunocomplexes are revealed thanks to the AuNPs-SpA probe. The strategy to functionalize AuNPs with a mediator (SpA) and not directly with the anti-PSA antibody, allowed us to tailor the amount of the specific antibody to achieve the disappearance of the test line due to the complete binding inhibition of the anti-PSA antibody to the immobilized PSA, since the available anti-PSA sites were saturated by the PSA in the sample at the exact cut-off value.

3.4 Set-up of the lateral flow biosensor parameters for uPSA detection

Amounts of the antigen used to form the Test line, the anti-PSA antibody and the AuNPs-SpA optical density were established by the checkerboard titration approach, based on requirements of reaching intense signals for uPSA below 2800 ng mL^{-1} and a sharp decrease of the Test line color in correspondence of the cut-off value. We prepared calibrators by fortifying simulated urine [30] with known amount of PSA in the interval between 0 and 5000 ng mL^{-1} and analyzed them with different AuNPs-SpA and anti-PSA combinations (Fig. S1). We also checked the optimal sample volume to be used and the time required to achieve clearly visible and stable colors at the Test and Control lines. The best performance was obtained using the following conditions: 0.5 mg mL^{-1} PSA and 1 mg mL^{-1} rabbit IgG as Test and Control line, respectively; 50 ng anti PSA Ab; AuNPs-SpA optical density 1; 70 µL as sample volume; 10 min as the running time. Extensive discussion about the study on parameters is reported in the supplementary materials (table S2, Fig. S1 and S2).

As already mentioned, several parameters affect the analytical performance of lateral flow assay devices. One of the most important, especially for competitive immunoassay based on a cut-off readout, is the amount of the specific antibody used. We decided to apply a double layer sensing strategy, in which gold nanoparticles were functionalized with Staphylococcal protein A and used to stain the anti-PSA antibody. The double layer sensing strategy has several advantages and allowed us to i) stabilize AuNPs with a protein different from the specific antibody and therefore avoiding restriction regarding the anti-PSA amount; ii) use a high amount of probe without affecting the immunoreactions and ensuring the revelation of the immunocomplexes even using low amount of specific antibody; iii) use a precise and accurate amount of

anti-PSA; iv) have superior capacity of giving specific binding since the antibodies are well oriented thanks to the SpA high affinity for the Fc portion of IgG; v) avoid loss in specific binding capacity due to possible steric hindrance and antibodies multilayer formation phenomena when AuNPs are directly functionalized with specific antibodies.

The aforementioned advantages enabled the calibration of the specific antibody in order to switch the signal off/on at the exact cut-off value.

3.5 Analytical performance of the lateral flow biosensor for uPSA detection

A typical calibration curve using the optimal conditions is shown in Fig. 5. We obtain a LOD and a quantification range of 20 ng mL^{-1} and 37 – 420 ng mL^{-1}, respectively. However, our main goal was to obtain the disappearance of the Test line signal at the cut-off level (Fig. 5a, b). Accordingly, the parameters were defined to switching off the color at the Test line in correspondence of the clinically relevant uPSA level.

The ability of adequately measuring PSA level in human urine was assessed by comparing the results obtained from the biosensor with those furnished by the reference ECLIA method. Two out of the 168 urine samples provided invalid results, as they were unable to flow along the device and to complete the run in the due time (1.2% invalid test rate). Then, the following analyses were conducted on 166 sample. We obtained 92.2 % of accuracy and an almost perfect concordance between the two methods, expressed as the Cohen's kappa coefficient ($\kappa = 0.82$) [27].

The reproducibility of the test was assessed by analyzing 10 urine samples (five scoring as negative and 5 as positive) in triplicate on each day, on three days, and by using three batches of strips. Each sample was analyzed 9 times in total each day. The analytical response of the test was qualitatively the same: no samples were misclassified, confirming the reliability of the biosensor (Fig. 6). To quantitatively evaluate the reproducibility of the test we compared the mean values of the signals (T/C) by a one way analysis of variance (ANOVA). For each sample, we compared the mean values obtained in the same day using strips from the same batch and strips from two other batches. For each batch, we compared the mean values obtained in different days using the same strips batch. We also compared the overall means value for each sample using three different batches on three different days. No significant statistical difference was observed and we concluded that the precision of the test was adequate to allow the correct classification of samples, considering within-day, between-day, within-batch and between-batch variability.

We assessed the real-time stability of the strips for 6 months at room temperature. On each testing day, we measured in triplicate PSA-fortified synthetic urine (0, 100 and 1000 ng mL^{-1}). The measurements evidenced good stability since the results were acceptably comparable to those of the freshly prepared device, with coefficient of variation ranging from 2 to 12 % (Fig. S3). In detail, we calculated the mean

values of the signal (T/C) obtained as measurements for each uPSA concentration on the various months (n=3) and compared the mean values with the ones obtained using freshly prepared strips (month 0) by a one way ANOVA. The differences in the mean values among the groups were not statistically significant (P ranging from 0.086 to 0.977) even if the powers of performed tests with alpha = 0.050 were below the desired power of 0.800.

3.6 Clinical application of the lateral flow biosensor

The lateral flow biosensor was applied to detect uPSA in samples belonging to all subjects involved in the study (training and validation data set).

The diagnostic validity of the developed test was assessed calculating the diagnostic Se, Sp and accuracy, PPV and NPV for the validation data set. Se is the proportion of subjects with confirmed PCa+ who were tested positive; Sp is the proportion of subjects with PCa- who were tested negative; the diagnostic accuracy is the proportion of true results over the total samples of the validation data set; PPV is the probability that an individual with a positive test result is PCa+; and NPV is the probability that an individual with a negative test result is PCa-. Both PPV and NPV are related to Se and Sp through the disease prevalence. The diagnostic figures of merit of the lateral flow biosensor, with the corresponding 95% confidence interval (CI), is reported in Table 2.

The application of the lateral flow biosensor to measure uPSA would have avoided 64% of unnecessary re-biopsies (9 out 14 PCa- subjects), saving adverse effects of the invasive procedure and reducing cost for the health system. More relevant, 100% of subjects who were re-classified as PCa+ by the repeated biopsy were correctly identified by the lateral flow biosensor as positive, based on the urinary PSA level measured at the time of the first biopsy. This finding also confirmed the lack in accuracy of the gold standard in PCa diagnosis (biopsy).

The results of this preliminary study allow us to make a speculative, but fairly well founded consideration about the possible use of uPSA as a biomarker in the management process of PCa diagnosis, not only as a tool to predict the outcome of repeat biopsy, but also to classify patients even before the first biopsy, allowing for the non-invasive and rapid stratification of PCa risk (Fig. 7a, b).

In this broader context, the availability of a point-of-care test for uPSA measurement would further enable screening analyses in an easy and rapid way, at low cost and directly at the physician's office.

4. Conclusion

We evaluated uPSA as possible biomarker for predicting re-biopsies outcomes. We established a threshold for uPSA value to distinguish PCa+ and PCa- on a training population of 140 subjects scheduled for biopsy

because of the high risk of PCa. Based on the established cut-off value, we developed and reported the first POCT to detect uPSA exploiting an intuitive immunoassay format for the straightforward result interpretation. The lateral flow biosensor showed a LOD and a range of quantification respectively of 20 ng mL^{-1} and 37 – 420 ng mL^{-1}. In the analysis of human urine samples, the biosensor showed coefficients of variation and test invalid rate of ≤ 13% and 1.2%, respectively. We applied the lateral flow biosensor for PCa diagnosis in a validation population and we found that the extremely simple, non-invasive and cost-effective detection of uPSA by a portable and rapid biosensor can be used to improve accuracy of PCa detection and to avoid 64% unnecessary re-biopsies without losing diagnostic sensitivity. Future work is focusing on increasing the study population in order to improve the diagnostic validity of the test and to confirm the use of the uPSA biosensor as a convenient tool to guide clinical decision-making in assessing PCa risk stratification.

Ethics statement

The study was conducted according to the Declaration of Helsinki and approved by the Scientific Ethics Committee of A.O.U. Città della Salute e della Scienza di Torino, A.O. Mauriziano, A.S.L. TO1 (Prot. No. 0110644). All patients provided written informed consent and specimens were anonymized.

Acknowledgments

The authors are grateful to the Biochemical and Clinical Laboratory (Baldi and Riberi), Città della Salute e della Scienza di Torino - Molinette Hospital, Turin, Italy, for the quantification of urinary PSA. S.O. acknowledges Fondazione Umberto Veronesi for “Postdoctoral Fellowship 2018, 2019 and 2020”. The authors acknowledge the Ministry of Education, University and Research of Italy (PRIN: 2017Y2PAB8) for financial support.

Competing Interests

The authors declare the following competing interests: S.O and F.D.N are shareholders of NIB biotec srl.

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Table 1. Clinical characteristic of the training and validation data set

Parameters	patients (total enrolled 140)	Patients (total enrolled 28)
mean age, yrs (median, IQR)	68 (69, 64-75)	70 (71, 65-75)
mean serum PSA, ng/ml, (median, IQR)	13 (5.9, 4.5-8.5)	6.2 (5.7, 4.5-8.3)
DRE abnormal, n (%)	61 (44)	10 (36)

Table 2. Diagnostic figures of merit of the biosensor

Parameters	Value (95% CI)
Se	100.0% (76.8% - 100.0%)
Sp	64.3% (35.1% - 87.2%)
LR	2.8 (1.4 - 5.6)
PPV	73.7% (58.1% - 85.0%)
NPV	100.0% (100.0% - 100.0%)
accuracy	82.1% (63.1% - 93.9%)

LR: Likelihood ratio

Figure 1

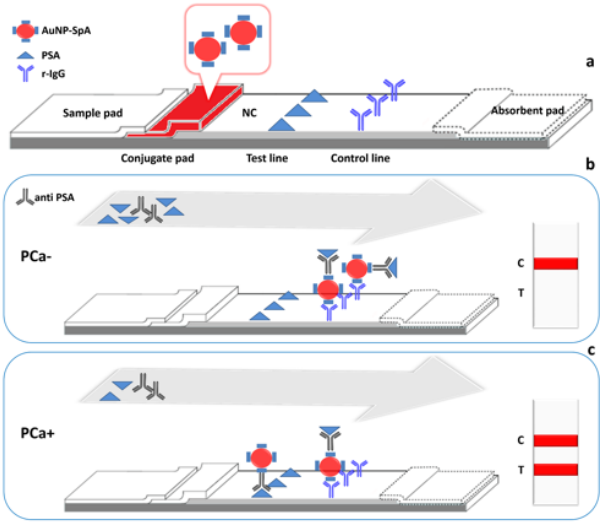


Fig. 1. Schematic diagram of the lateral flow biosensor. (a) Components of the lateral flow biosensor. (b) Negative test outcome for uPSA > cut-off level ('low risk PCa'). (c) Positive test outcome for uPSA < cut-off level ('high risk PCa').

Figure 2

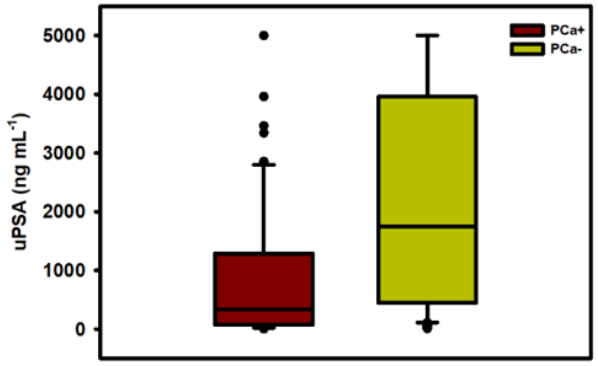


Fig. 2. Box-Whisker plot for uPSA levels of PCa+ and PCa- groups. The whisker above the box includes the data within up to Q_3 + 1.5 IQR while the whisker below the box includes data down to Q_1 – 1.5 IQR. The data outside the whiskers are potentially outliers and are displayed as dots.

Figure 3

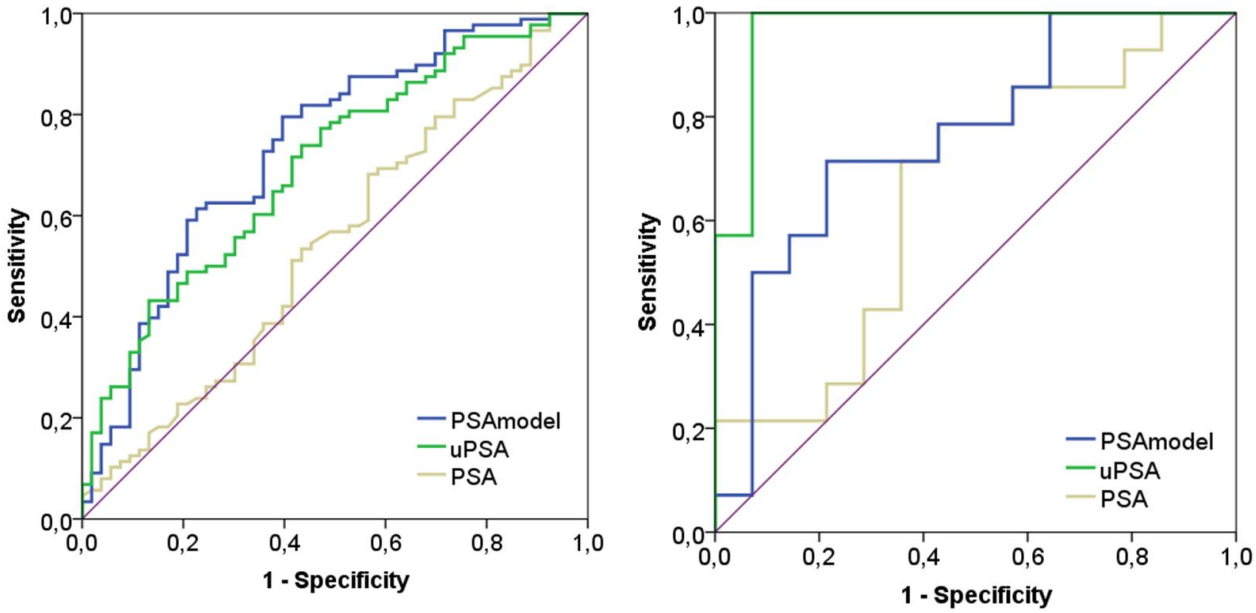
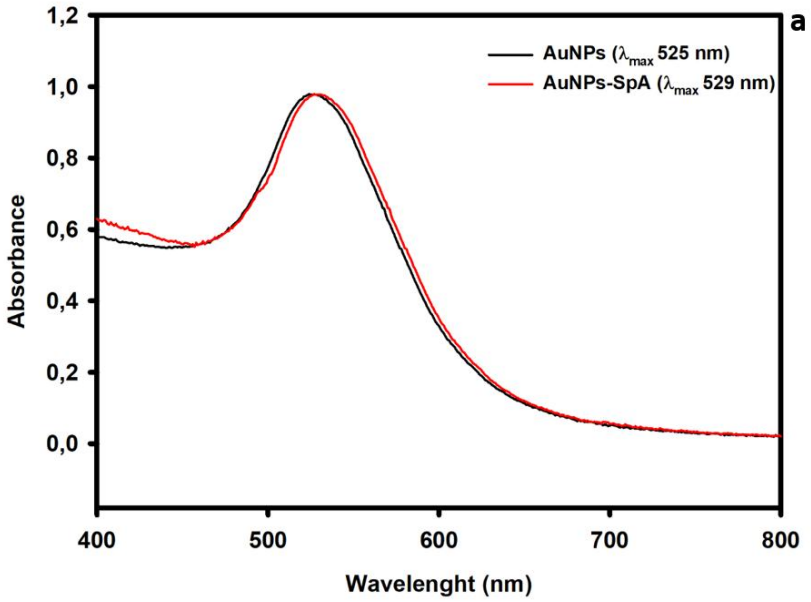
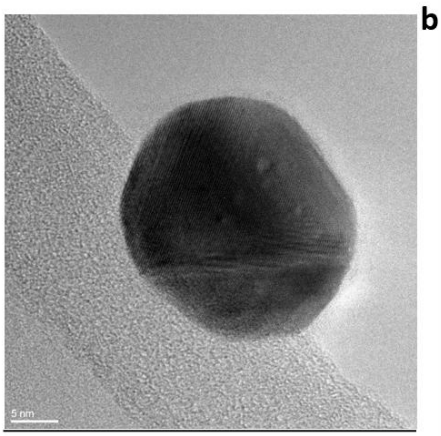


Fig. 3. ROC curves for uPSA, serum PSA and the combined model (age, serum PSA and DRE) recommended as diagnostic indicator [25] from: (a) the training set (n=140, subjects classified according to the first biopsy) and (b) the validation set (n=28, patients classified according to the repeat biopsy).

Figure 4





c

Probe	Hydrodynamic diameter (nm)
AuNPs	38
AuNPs-SpA	64

Fig. 4. Characterization of gold nanoparticles and of the colorimetric probe: (a) Vis spectra of AuNPs and AuNPs-SpA, (b) TEM image of AuNPs obtained through the citrate reduction method. (c) Hydrodynamic diameters of AuNPs and AuNPs-SpA.

Figure 5

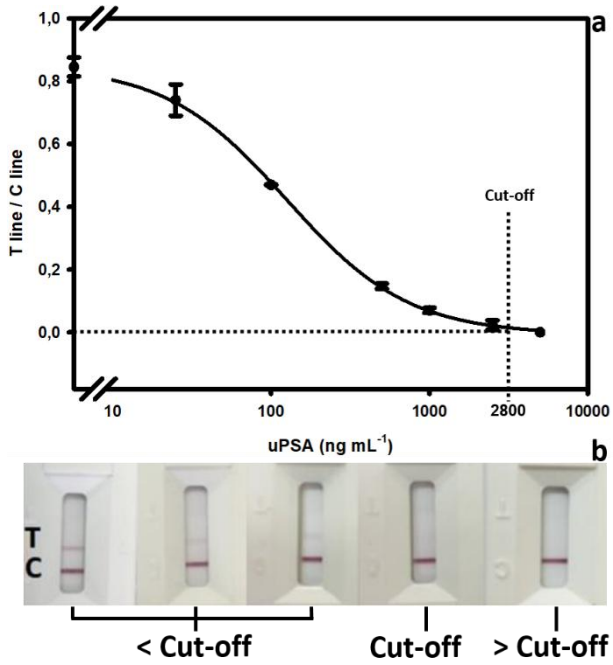


Fig. 5. uPSA detection by the lateral flow biosensors. (a) Plot of calibrator signals obtained at the Test line normalized by the color at the Control line towards uPSA concentrations in fortified synthetic urine. (b) The naked-eye readout, in which the color intensity of the Test line progressively decreased as uPSA increased and completely disappeared in correspondence of the cut-off level. The uPSA concentrations are 0, 500, 1000, 2800 and 5000 ng mL^{-1}.

Figure 6

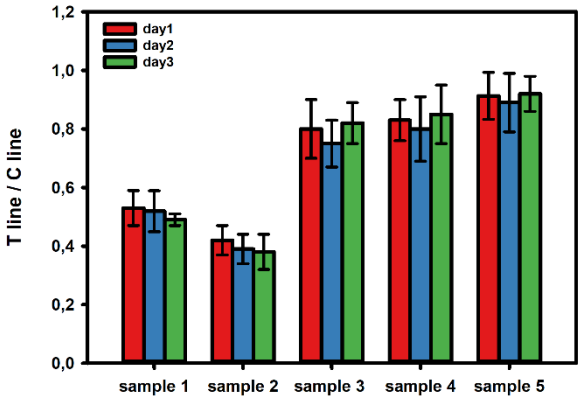


Fig. 6. Assessment of the reproducibility of the biosensor as measured on different days and from different strip batches. Bars represent standard deviation of nine measurements repeated on the same day (three for each batch). Samples 6 to 10 did not show the Test line signals as their uPSA concentrations were above the cut-off level.

Figure 7

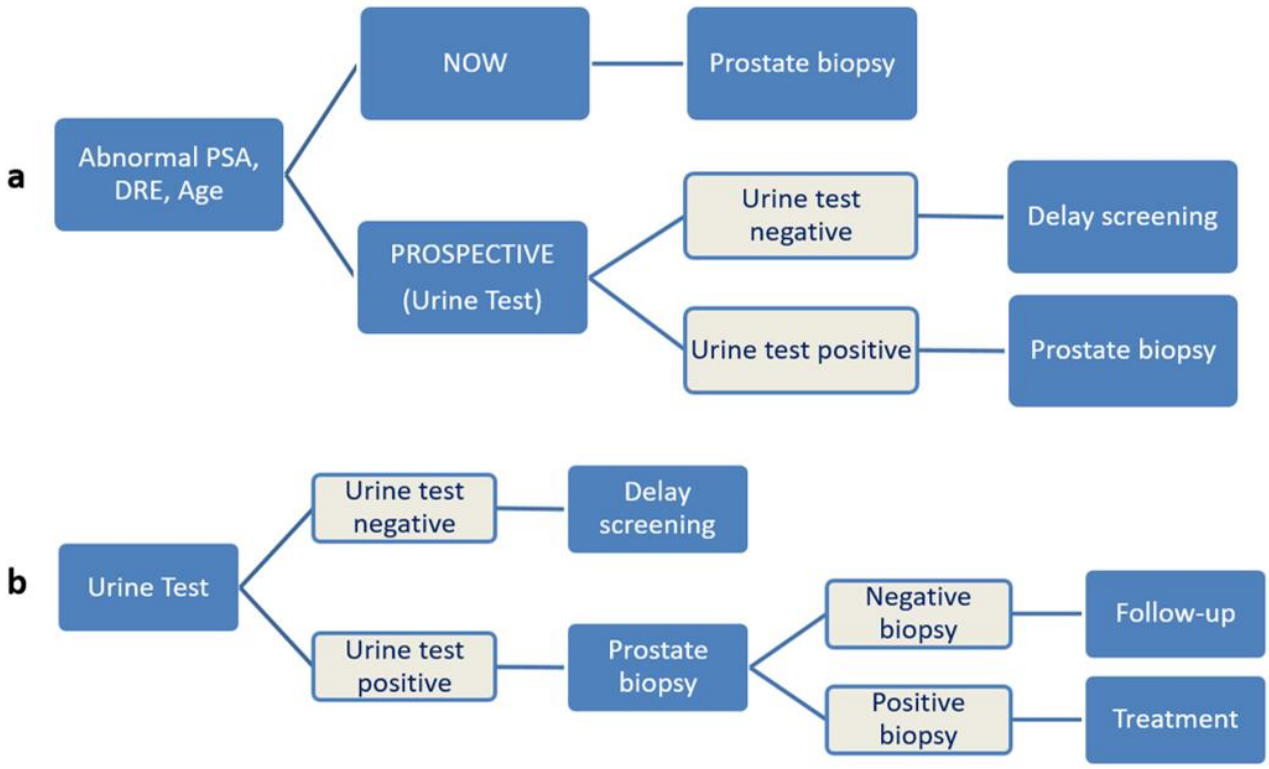


Fig. 7. Urine test will help to improve clinical decision making in detection and management of PCa. (a) Decision tree currently used to identify patients with significant prostate cancer and prospective introduction of the urine test to reduce repeat biopsy. (b) Alternative decision tree based on the use of uPSA as a first level screening biomarkers of PCa.