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# Immuno-based detection of extracellular vesicles in urine as diagnostic marker for prostate cancer

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# ABSTRACT

#### Introduction

Extracellular vesicles (including the subclass exosomes) secreted by cells contain specific proteins and RNA that could be of interest in determining new markers. Isolation/ characterization of PCa-derived exosomes from bodily fluids enables us to discover new markers for this disease. Unfortunately, isolation with current techniques (ultracentrifugation) is labor intensive and other techniques are still under development. The goal of this study was to develop a highly sensitive time resolved-fluorescence immunoassay (TR-FIA) for capture/detection of PCa-derived exosomes.

#### **Material and methods**

In our assay, biotinylated capture antibodies against human CD9 or CD63 were incubated on Streptavidin-coated wells. After application of exosomes, Europium-labeled detection antibodies (CD9 or CD63), were added. Cell medium from 37 cell lines was taken to validate this TR-FIA. Urine was collected (after digital rectal exam) from patients with PCa (n=67), men without PCa (n=76). As a control, urine was collected from men after radical prostatectomy (n=13), women (n=16) and patients with prostate cancer without digital rectal exam (n=16). Signal intensities were corrected for urinary PSA and creatinine.

### Results

This TR-FIA can measure purified exosomes with high sensitivity and minimal background signals. Exosomes can be measured in medium from 37 cell lines and in urine. DRE resulted in a pronounced increase in CD63-signals. After DRE and correction for urinary PSA, CD9 and CD63 were significantly higher in men with PCa.

# Conclusions

This TR-FIA enabled us to measure exosomes with high sensitivity directly from urine and cell medium. This TR-FIA forms the basis for testing different antibodies directed against exosome membrane markers to generate disease-specific detection assays.

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#### **INTRODUCTION:**

Prostate specific antigen (PSA, KLK3) is a protein that is commonly used in daily practice to aid urologists in diagnosing prostate cancer (PCa). Although PSA has a high sensitivity, it lacks specificity and therefore causes unnecessary biopsies. Furthermore, PSA is a poor prognostic marker.<sup>1</sup> In order to increase specificity and distinguish between the clinically insignificant cancers and the ones that are clinically relevant, novel markers have to be identified. Recent studies have shown that extracellular vesicles and particularly the vesicles from endosomal origin, referred to as exosomes, could help us in identifying novel tissue-specific markers. Moreover, also their presence and number might be indicative of disease.<sup>2-4</sup>

Quantifying the number of exosomes and characterizing them on single particle level remains challenging. To determine the number of exosomes in body fluids or measure an exosomal marker of interest, purification and concentration steps are often needed. Isolation of exosomes is most commonly performed by ultracentrifugation, filtration, precipitation or antibody-based capture technologies. Most of these protocols are still under development, labor intensive and limited with respect to efficient isolation or purity of the final exosomal preparation.<sup>5</sup> Measuring the number and size distribution of the exosomes is a next challenge and technologies such as nanoparticle tracking analysis, tunable resistive pulse sensing and flow cytometry (FACS) are utilized, although again, each with their own set of limitations.<sup>6</sup>

In order to count exosomes or measure an exosomal protein marker in bodily fluids in a clinical setting, novel or optimized assays have to be developed. One technique that is of special interest is an immunoaffinity assay (e.g. ELISA or TR-FIA) directed against exosomal transmembrane proteins. With a sandwich-structure, exosomes can be captured without cumbersome preprocessing from body fluids (e.g. plasma, serum or urine) and characterized with antibodies directed against disease-specific markers. Although the quantity of the protein of interest is reliably measured, the immunoassays in this format do not provide an exact number for the concentration of exosomes.

Current efforts to establish such an immunoassay have room for improvement.<sup>7-9</sup> Published reports have shown a sandwich ELISA (CD9-based) with which it is possible to isolate and detect exosomes from plasma.<sup>7</sup> Exosomes had to be purified first and the minimum amount of exosomes that could be detected was 3-10 µg exosomal protein. We developed a TR-FIA (time resolved-fluorescence immunoassay) against the transmembrane proteins CD9 and CD63. Both proteins are known to be commonly expressed on the membrane of exosomes derived from different cell types. Antibodies against these proteins were biotinylated for optimal capture and another batch labeled with a Europium-chelate for time resolved fluorescence detection. As compared to ELISA, the TR-FIA typically has a higher sensitivity and dynamic range and does not require an en-

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zymatic reaction.<sup>10</sup> With this TR-FIA we showed that extracellular vesicles can directly be measured in cell medium and urine and can be used as a marker for the presence of PCa.

# MATERIAL AND METHODS

# **Cell Culture**

Prostate cancer cell lines (DU145, VCaP, PC3 and LNCaP), two immortalized prostate epithelial cell lines (PNT2C2 and BPH-1) and the hepatocellular carcinoma cell line (Hep3B) were used for exosome isolation. All cell lines were cultured in ten T175 (175 cm2) culture flasks (Greiner Bio-One, Frickenhause, Germany) up to 80-100% confluency. DU145 (androgen independent)<sup>11</sup>, LNCaP (androgen dependent)<sup>12</sup>, PC3 (androgen independent)<sup>13</sup> and PNT2C2<sup>14</sup> were cultured in RPMI 1640 (Lonza, Verviers, Belgium) and supplemented with 5% fetal calf serum (FCS) and 500 U penicillin/ 500 U streptomycin (P/S). BPH-1<sup>15</sup> and VCaP<sup>16</sup> (androgen dependent) were cultured in the same medium and supplements, only with 10% FCS. Hep3B<sup>17</sup> was cultured in Alpha MEM (Lonza) supplemented with 5% FCS and P/S. When 80-100% confluency was reached, cells were 48 h incubated with FCS-free medium. Cell medium was collected for exosomes isolation. All other cell lines tested were cultured under their optimal conditions in regular medium, containing various concentrations of serum. When 80-100% confluency was reached, cell medium, cell medium was centrifuged 3000 x g and the supernatant stored for short term at 4°C or long term at -80°C.

# Urine collection

Whole urine from men with PCa (n=67) and without PCa (n=76) was collected at the out patients clinic from the Erasmus Medical Center Rotterdam after written consent (Medical Ethics Approval number 2005-077 and 2010-176). Urine samples from men were collected after digital rectal exam (DRE) at the time of day most convenient to the person. DRE was performed to increase prostate fluid secretion and therefore the number of prostate-derived vesicles in the urine. From another 16 men with prostate cancer, urine was collected without DRE. From each prostate cancer patient, PSA-levels and prostate biopsy results (Gleason score) are known. Gleason scores varied from 6 (3+3) up to 9 (5+4). Furthermore, whole urine from women (n=16) and men after radical prostatectomy (n=12) was collected at the person's convenience and were used as controls. Patient characteristics are shown in Table 1. All urine samples were centrifuged at 3,000 x g (20 min) in order to remove cellular debris. Subsequently, urine samples were stored in 1.5 mL aliquots at -80 °C.

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#### **Isolation of exosomes**

Exosomes were isolated according to a protocol that was previously described.<sup>4,18</sup> Briefly, cell culture medium was subjected to consecutive centrifugation steps (3,000 x g and 10,000 x g) to remove cellular debris and large vesicles. Exosomes were then pelleted with ultra-centrifugation at 64,000 x g (2 h) and at 100,000 x g (1 h, in a 0.32 M sucrose solution). Sucrose supernatant was removed and pellets resuspended in 100  $\mu$ L PBS and stored at -80 °C. Total amount and concentration of exosomal proteins was measured with a BCA-assay (Pierce, Rockford, IL, USA).

#### Time resolved fluorescence immunoassay (TR-FIA)

#### Biotinylation of capture antibodies

A streptavidin-coated 96-well plate (KaiSA96, Kaivogen, Turku, Finland) was used to bind biotinylated capture antibodies. CD9 (mouse monoclonal against human CD9, clone MAB1880, R&D systems, Abingdon, UK) and CD63 (mouse monoclonal against human CD63, clone 556019, BD Bioscience, Breda, Netherlands) antibodies were biotinylated.<sup>19</sup> Biotin isothiocyanate (BITC) was dissolved in ethanol to a final concentration of 10 mM. Before adding biotin, the antibody solution had to be adjusted with 0.5 M carbonate buffer to a pH of 9.8. For the most optimal final concentration of 2 mg/ml biotinylated antibodies, a 40-fold excess of biotin was used. Antibodies and BITC were incubated for 4 hours at room temperature. Unreacted BITC was removed by gel filtration with a NAP-5 column (GE-Illustra, Diegem, Belgium).

MaxiSorp plates (Thermo Scientific, Amsterdam, Netherlands) were used to bind exosomes directly to the plate (without capture antibodies) or to capture exosomes with unbiotinylated antibodies (CD9/CD63).

#### Labeling detection antibodies with Europium (Eu)

Antibodies used for Eu-labeling were CD9 (same as above), CD63 (same as above), and Human Kallikrein 2 (KLK2). 2 mg Eu-chelate (Perkin-Elmer, Turku, Finland) was dissolved in 200  $\mu$ L sterile water and filtered through a 0.22  $\mu$ m filter. From this, 100 nM Eu-chelate was dissolved in Enhancement solution (Product number 1244-105, Perkin-Elmer) to reach 1 nM. For optimal results the final concentration of labeled antibodies should be 2 mg/ml. A 100-fold excess of Eu-chelates was added to the antibody. pH was adjusted to 9.8 with 0.5 M carbonate buffer. The antibodies and Eu-chelates were incubated overnight at 4 °C. Purification of the labeled antibodies was performed by gel filtration (FPLC), using a Superdex 200 column (GE Healthcare Europe, Helsinki, Finland) with a flow rate of 30 ml/h. Fractions containing the protein were pooled. BSA was added to a final concentration of 0.1% and filtered through a 0.22 um filter and stored at 4 °C.

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# **TR-FIA protocol**

The streptavidin-coated plates were incubated with 200 ng biotinylated CD9 or CD63 in 100  $\mu$ L per well for 1 h with shaking (750 rpm) at room temperature. Supernatant was removed and the plates were washed with Wash buffer (Product nr 42-02, Kaivogen, Turku, Finland) three times with an automatic plate washer (TECAN Columbus). Subsequently, samples (in triplicate) were diluted in the sample buffer and added to the wells and incubated for 1 h with shaking at room temperature. The plates were washed again three times. 25 ng of Eu-labeled antibodies was added per well (suspended in 100  $\mu$ L sample buffer) and incubated for 1 hour at room temperature. Excess antibody was removed and the plates washed again three times. 100  $\mu$ L Enhancement solution (Perkin-Elmer) was added and incubated for 15 min at room temperature with slow shaking. Europium (time resolved fluorescence) was measured by the Wallac Victor 2, 1420 multilabel counter (Perkin-Elmer) at a wavelength of 615 nm. For analysis of the patient samples the assay was constructed with either biotinylated CD9 with Europium-labeled CD9 (CD9-assay) or the combination of biotinylated CD63 with Europium-labeled CD63 (CD63-assay).

LNCaP cell culture medium was collected in a large batch, centrifuged at 3000 x g, aliquoted and stored at -80°C for the 0, 25, 50 and 100  $\mu$ L control concentration series for each 96-well plate we performed. These concentration series were used to correct signal level variability between plates.

#### Urinary PSA and creatinine

As a measure for the amount of prostate fluid present in the urine sample and abnormal kidney function, urinary total PSA and creatinine were used for normalization. Both urinary proteins were measured in urine with Roche-developed assays for creatinine (CRE2U, ACN 8152) and PSA (total-PSA, 04491734 016) by the Erasmus MC Department of Clinical Chemistry using a Roche Cobas 8000 Modular Analyzer (Roche, Woerden, the Netherlands).

#### Statistical analyses

GraphPad Prism 6 was used to visualize results and for statistical analyses. Unpaired T-tests were used to calculate p-values of expression values between different patient groups. Furthermore, this software was used to calculate correlation coefficients (r<sup>2</sup>) and ROC-curves.

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#### RESULTS

For optimization of the assay, several experiments were conducted. First, capturing exosomes via biotinylated antibodies to a streptavidin-coated plate versus direct exosome coating to a MaxiSorp 96-well plate was compared. Using biotinylated antibodies on streptavidin-coated plates increased signal intensity and lowered background signals (Figure 1A). Second, unconjugated CD9 or CD63 antibodies were directly coated on MaxiSorp plates. This resulted in up to 3 times higher background signals and limited signal increase when an increasing amount of exosomal proteins was added (Figure 1B). Third, we tested whether the detergent/emulsifier Tween-20 could reduce background TR-FIA signals. Despite the theory that detergent might dissolve exosomal lipid membranes and impair the TR-FIA exosome detection, washing with Tween-20 0.05% improved Europium signal intensity (Figure 1C). Fourth, as a negative control for Europium labeling, exosome-specificity was tested using Europium-labeled KLK2 which is not present on exosomal membranes. As expected, no signals were observed above background (Figure 1D). Fifth, FCS is rich in proteins and bovine-derived exosomes and therefore could interfere with the capture and detection of cell-derived exosomes. We spiked purified VCaP exosome samples (1 µg protein) in culture medium containing different concentrations (0-40%) of FCS. We observed no statistical difference, indicating that our assay does not detect exosomes in FCS and that FCS does not affect humanspecific detection of vesicles (Figure 1E).

The conditions we chose for the capture and detection of exosomes included a sandwich assay with streptavidin coated plates, biotinylated CD9/CD63 antibodies and Europium-labelled CD9/CD63 antibodies. Tween 20, at a concentration of 0.05%, was added to wash buffers to reduce background and increase CD9/CD63 signals.

#### Sensitivity analysis of the TR-FIA with purified exosomes

After optimization of the TR-FIA, sensitivity was tested with purified exosomes from the cell lines PC346C and VCaP. A minimum of 9.39 ng exosomes per well in 100  $\mu$ L (measured by amount of protein present in purified exosome preparation) was enough to reliably measure CD9 signal. Capturing with biotinylated CD9 antibody and detection with the Europium-labeled CD9 antibody (CD9-assay) showed highest sensitivity for these samples after background correction (Figure 2). For capture with biotinylated CD63 antibody and detection with Europium-labeled CD63 antibody (CD63-assay), the lowest measurable amount of protein was 18.75 ng with VCaP exosomes and 37.5 ng with PC346C exosomes (Figure 2).

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**Figure 1.** Development of the TR-FIA (data on the CD63-assay not shown). (A) direct application of PC346C exosomes to uncoated plates without the capture antibody (direct) versus using streptavidin-coated plates with the biotinylated capture antibody (bCD9). Both exosomes were measured with europium-labelled CD9 antibody. (B) MaxiSorp plates were coated with non-biotinylated CD9 antibodies and compared with streptavidin plates that were coated with biotinylated capture antibody (bCD9). Both exosomes were measured with europium-labelled CD9. (C) Addition of 0.05% tween 20 in the wash buffer was tested in the CD9-assay. (D) Application of a Europium-labelled KLK2 in our assay as a control for membrane-specific binding. KLK2 is a protein secreted in a different cellular pathway and normally not present in exosomal membranes. (E) We spiked purified VCaP exosome samples (1 µg exosomal protein) in culture medium with 0-40% FCS and performed the CD9 TR-FIA. All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

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**Figure 2.** CD9-assay (A) and CD63-assay (B) on purified exosomes from the PC346C and VCaP cell culture medium. The amount of protein in purified exosome preparation is used as a measure for the number of exosomes (ng exosomal protein). All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

# **TR-FIA with cell culture medium**

The next step was to test whether minimally processed (only centrifuged once at 3000 x g) cell culture medium and urine samples could directly be used for TR-FIA analysis. Serum free cell culture medium of the LNCaP cell line was collected after 48 h exposure to cells (when 80-100% confluency was reached) and tested in the CD9 and CD63 TR-FIAs (Figure 3). High Europium signals were found within 100  $\mu$ L cell culture medium and dilution series revealed a high linear correlation. After dilution of LNCaP cell culture medium, samples were aliquoted and used as calibration curve in all following experiments. Subsequently, we tested 100  $\mu$ L cell culture medium from 37 cell lines (Figure 4). In almost all cell lines both the CD9 and CD63 assay showed a signal above background, but levels varied dramatically. Some human cell lines had extremely low signals for CD9 but were positive for CD63 (e.g. PNT2C2, TOV21G, H460, H295R and U2OS2-G3) or vice versa (e.g. EVSA-T, MOA MB453M and BPH1). Overall, the correlation between CD9 and CD63 signals among these cell lines was weak (r2=0.1816).

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FCS (bovine origin), which is often supplemented in cell culture medium, showed no signals in the assay (Figure 4, control medium). Also, no or very low signal was measured in non-human (mouse and dog) cell line medium. These findings are in agreement with the antibody specificity against human CD9 and human CD63.



**Figure 3.** Dilution-assays with CD9 (A) and CD63 (B) of LNCaP cell culture medium. 100  $\mu$ L cell culture medium was two-fold diluted for 4 times. As a control (0) medium was taken that was not exposed to cells. This control was used to correct for background signals. All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

# **TR-FIA** with urine samples

A total of 135 patient urine samples (100  $\mu$ L per well in triplicate) were analyzed with the CD9 and CD63 TR-FIA. Urine that was collected after DRE showed significantly higher Europium signals (Figure 5). Urine from men without a DRE or treated by radical prostatectomy did not significantly differ in CD9 and CD63 levels. Women had the lowest signals compared to any other group tested. Figure 5 also depicts Europium counts between the samples (after DRE) with or without PCa in the two different assays. No significant difference was found between men with PCa and without PCa (CD9 p=0.166, CD63 p=0.223).

Since variability in DRE and urine volume will result in fluctuating concentrations of prostate (cancer) fluid in the voided urine, a correction factor is needed. We have chosen the urinary prostate-specific antigen PSA (UPSA) as a measure for the contribution of prostate fluid in the urine. In addition, urinary creatinine (UCr) was measured in each sample to analyze potential effects of differences in renal function. Distribution of UPSA (mean 3446.2, range 0.16-57100) and UCr (mean 10.4, range 1.7-30.2) is shown in supplemental Figure 1. No significant difference was observed in UPSA and UCr between men with and without PCa. UCr was lower in women, which is well known (data not shown).<sup>20</sup> As expected, UPSA was much higher in urine from men that underwent DRE (on average 6171.3 ng/ml) and significantly lower in urine from men without DRE

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**Figure 4.** Cell culture medium from cell lines (n=37) from different human malignancies was tested in the CD9 and CD63-TR-FIA. Two mouse cell lines and one dog cell line were taken as control. Cell lines were cultured up to 80-100% confluency. When this was reached, medium was taken and centrifuged at 3000 x g to remove cellular debris. Measurements were performed twice with 100  $\mu$ L independently from each other. Individual signal intensities were corrected for background (unexposed medium) and differences between measurements was corrected based on the same LNCaP control concentration series that was applied in both measurement. All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

(on average 317.2 ng/ml (p=0.032). Men treated by radical prostatectomy and women mostly had undetectable UPSA levels (data not shown).

After normalization for UCr the CD9 and CD63 assay showed no significant difference (Figure 6) between urine from men with and without PCa (both after DRE). However, when normalized for UPSA, the CD9 and CD63 signals were significantly higher in urine from PCa patients (p=0.0006; p<0.0001). The mean of the Eu-signal/UPSA ratio increased from 6.6 to 18.1 for CD9, whereas the mean ratio for CD63 increased from 16.1 to 58.2. The Receiver operating characteristic (ROC) curve for the CD63 assays showed an Area under curve (AUC) of 0.68 (Figure 7), indicating that within this small cohort, the TR-FIA has higher diagnostic accuracy in detecting prostate cancer than CD9 (AUC = 0.58) and serum PSA (AUC = 0.61).

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**Figure 5.** CD9-assay (A) and CD63-assay (B) on urine samples after DRE (DRE+) from men with (n=67) or without PCa (n=76), men with PCa (n=16) without DRE (DRE-), patients after radical prostatectomy (n=13) and women (n=16). Average of triplicate measurements is shown. All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

# DISCUSSION

The goal of this study was to design a non-invasive, sensitive assay for detection of (prostate cancer) exosomes in body fluids, which could be easily implemented in a clinical setting. We have reached most of these aims by constructing a TR-FIA using streptavidin-coated plates, biotinylated capture antibodies and Europium-labeled detection antibodies. This TR-FIA enabled us to detect prostate-derived vesicles with high sensitivity and over a broad dynamic range in shortly centrifuged (at low speed) post-DRE urine.

Since we use the same monoclonal antibody for capture and detection we do not measure single CD9 or CD63 molecules but distinct molecules kept together in membrane structures of, for example vesicles. Shortly centrifuged urine supernatant will not only contain the 30-150 nm (diameter) size exosomes, but also larger extracellular vesicles

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**Figure 6.** CD9-assay (A and B) and CD63-assay (C and D) on urine samples from men with (n=67) or without PCa (n=76), after DRE. Assays were corrected for urinary creatinine (UCr. A and C) or urinary PSA (UPSA, B and D). All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).



**Figure 7.** A Receiver-operating curve (ROC) for the CD63 and CD9-assay after correction for urinary PSA (UPSA). Serum PSA and urinary PSA from patients were also analyzed with a ROC.



and membrane debris.<sup>21,22</sup> As long as multiple CD9 or CD63 molecules are present, any membrane fragment can give a positive TR-FIA signal.

The TR-FIA signal measured is determined by the number of exosomes present, the number of antigens per vesicle and the affinity of the antibody to the epitope. For example, the high CD9 and CD63 signals in medium from COLO205 could mean that many exosomes were secreted and/or a high concentration of the antigens on each exosome. With the currently available techniques for quantifying exosomes such as flow cytometry, nanoparticle tracking analysis and tunable resistive pulse sensing, it is possible to estimate the number of vesicles. Unfortunately these techniques still have many restrictions such as vesicle size detection limits, quantitation accuracy and laborious procedures for regular and high throughput use.<sup>6</sup> Whether these technologies correlate to the TR-FIA outcome, needs to be established. Regarding the clinical use of the TR-FIA for detection of prostate cancer, the difficulty to quantify vesicles is not necessarily a limiting factor as long as the signal is reliably measured and significant for the presence of disease. A complicating factor that arises within our TR-FIA and could influence results is the presence of viruses that use outward-budding from host cell membrane to form their envelope.<sup>23</sup> For the HIV-1 virus it is known that this envelope contain the transmembrane proteins CD9 and CD63.<sup>24</sup> We used exosomes from the VCaP and PC346C cell line that were provided from xenografts, which produce XMRV-like viruses. If these viruses also produce envelopes containing CD9 and CD63 is unknown, but if this is the case it could produce a signal in the TR-FIA.

In order to be able to check and correct for variability between TR-FIA plates, we used the high CD9 and somewhat lower CD63 signals measured in LNCaP cell culture medium. A large batch of medium was prepared and stored and new aliquots were used for each experiment. In the future, a more robust common universal standard, such as synthetic double-epitope peptides, will have to be devised for assay calibration.

One of the major improvements of the described assay over currently available conventional ELISAs is the use of Europium chelate used as a label for time resolved fluorescence.<sup>25</sup> The main advantage of Europium is that the fluorescence emitted after excitation is long lived as compared to auto-fluorescence. Therefore, this technique reduces background and enables us to measure with high sensitivity. Furthermore, Europium can be measured over a much broader dynamic range (signal intensity 50 – 300,000) as compared to conventional ELISAs (e.g. HRP-based). Other strategies to improve immunoassays include the adapted proximity ligation assay (PLA) and amplified luminescent proximity homogeneous assay using photosensitizer-bead.<sup>26,27</sup> Also these technologies show high sensitivity and the ability to measure vesicles in low volumes of bodily fluids but are relatively more labor intensive.

CD9 and CD63 are transmembrane proteins that are 7-10 times enriched in exosomes and are used as a general marker for exosomes.<sup>28,29</sup> CD9 was shown to be higher ex-

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pressed in exosomes from prostate cancer, but also in other types of malignancies. Furthermore, CD9 was reported to have a specific role in metastasis.<sup>4,30-32</sup> We showed that CD9 and CD63 are present on exosomes from almost all cell lines with varying signal intensities. Although CD9 and CD63 are considered common extracellular vesicle markers, we showed that these markers are detectable on exosomes from almost all cell lines (n=37) but with highly varying expression levels.

Control medium with FCS and animal cell lines such as those derived from mouse and dog showed no or very low signals. This demonstrates that our assay based on antibodies against human CD9 and CD63 is indeed human-specific. The spike-in experiments revealed that high concentrations of FCS do not interfere in the detection of markers in the TR-FIA.

Although CD9 and CD63 are not prostate (cancer)-specific, we could use their presence as a marker for prostate-derived exosomes in urine, since levels were very low in urine from women, men after prostatectomy and men before DRE. This indicates that the number of exosomes and/or the levels of CD9 and CD63 on exosomes from kidney and bladder are low in urine as compared to prostate-derived exosomes after DRE. This is particularly evident for CD63. Initially, no statistical difference was observed in CD9 and CD63 signals between men with and without PCa. Urinary creatinine, a marker for kidney function showed no additional value as a correction factor in our assay. Although serum PSA can be elevated in prostate cancer, urinary PSA itself has shown not to be different between healthy men and men with prostate cancer and therefore could function as correction factor for DRE in our assay.<sup>33-36</sup> After correction for the relative amount of prostatic fluid after DRE using UPSA, a statistically significant difference was observed for both markers. These observations show that a DRE is currently an essential element of the urine collection. One could argue that a DRE is not necessary anymore if the CD9 and CD63-assays would be more sensitive. However, as a consequence of not performing a DRE, the lower ratio of prostate (cancer) exosomes among other urinary exosomes (from bladder and kidney) might result in loss of distinction between men with and without PCa. This issue could be resolved by using antibodies that specifically recognize prostate (cancer)-derived exosomes. Antibodies against known prostate (cancer)-specific transmembrane proteins need to be tested in the TR-FIA. Exosomal transmembrane proteins have been identified using mass spectrometry, but so far few of these proteins have been found higher expressed in cancer and none to be cancer-specific.<sup>4</sup>

The assay we developed reveals that measurements of urinary vesicles can indicate the presence of PCa. Because we only measured two markers in urine of men with or without PCa, it is unclear whether this assay is affected in men with other diseases of the urinary tract and in particular in diseases of the prostate such as benign prostate hyperplasia or prostatitis. Other steps to take include determining the assay robustness and intra-person variability at different time-points. In addition, we will further have to

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select the optimal capture and detection markers as discussed above. The CD63 TR-FIA has an AUC of 0.68 and although this is already better than serum PSA alone in our cohort, it still does not fully address the clinical needs.

In this study we only assessed this assay as a diagnostic test. The limitations of the PSA assay results in taking unnecessary biopsies in approximately 68% of men with PSA higher than 3 ng/mL. In addition, with the cutoff of 3 ng/mL, approximately 13% of prostate cancers are missed.<sup>37</sup> In our cohort, the CD63 TR-FIA outperforms the PSA assay in diagnosing prostate cancer. Logistic regression analysis showed no independence between the two assays (p>0.05). Even more relevant would be to predict whether the identified PCa should be treated or is insignificant and active surveillance is a valid strategy to follow the patient. The main marker for prognosis currently is Gleason score. Since our cohort is based on men entering our clinic for their first consult, there is a strong bias towards Gleason score 6. In order to determine whether the TR-FIA has diagnostic and prognostic value, a larger cohort balanced for different Gleason scores needs to be analyzed. In conclusion, the CD63 TR-FIA could influence patient management with respect to making decisions on taking biopsies, while a role in tracking patients on active surveillance and therapy selection is still to be investigated.

#### CONCLUSION

The presented TR-FIA enabled us to measure transmembrane proteins on vesicles directly in urine and cell culture medium with low background signals and high sensitivity. CD9 and CD63 are exosomal markers that show higher signals in men with PCa after DRE and correction for urinary PSA. More antibodies need to be tested using this TR-FIA to discover the most optimal combination of diagnostic and prognostic PCa markers.

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# SUPPLEMENTARY DATA

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