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KeepEX, a simple dilution protocol for improving extracellular vesicle yields from urine

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ABSTRACT

Urinary extracellular vesicles (EVs) are a promising source of biomarkers, which can be obtained in a non-invasive manner. However, the yield of EVs from urine samples may be insufficient for various analyses due to the entrapment of EVs by the Tamm-Horsfall protein (THP) meshwork.

Here, we developed a simple dilution protocol to increase the urinary EV yield by disrupting the interaction between THP filaments and EVs with the help of alkaline pH and lowered ionic concentration. The integrity of the EVs and THP was assessed by electron microscopy. The effect of the protocol on the EV yield was quantified against an undiluted control by western blotting of four EV markers, nanoparticle tracking analysis and measuring of the RNA/miRNA concentration of the EV samples.

The average EV yield from the dilution protocol was 2–7 fold the yield from the undiluted control i.e. increased by 130–624% as measured by western blotting and NTA. The yield increased most from samples with a high THP to EV ratio. The morphology and size range of the EVs were unaltered by the protocol. However, RNA/miRNA yields were the same as from the undiluted control and THP filaments could still be detected in EV samples.

The dilution protocol, that we named KeepEX, provides a simple and efficient way to prevent loss of EVs thus increasing their yield from urine. Since KeepEX does not require individual adjustment of sample pH nor extra centrifugation steps, it could be used on its own or in combination with other EV purification protocols to improve EV isolation particularly from small urine volumes.

1. Introduction

Extracellular vesicles (EVs) including exosomes and microvesicles are small secreted membrane enclosed entities involved in various biological, physiological and pathological phenomena (Yáñez-Mó et al., 2015). Since EVs protect a wide range of biomolecules originating from the secreting cells and their molecular cargo change in diseases or other physiological states, EVs are regarded as a highly promising source of biomarkers (Witwer et al., 2013; Duijvesz et al., 2011; Franzen et al., 2016; McKiernan et al., 2016; Fais et al., 2016).

EVs can be isolated from all body-fluids including urine (Yáñez-Mó et al., 2015; Pisitkun et al., 2004). The urine sample collections for biobanks and routine diagnostics in the clinics preferably involve small volumes and minimum handling time. Even if EVs are a rich source of biomarkers and present in $>10^8$/ml of urine (Li et al., 2014), they are very small: the total quantity of EV-biomarkers may be below the detection limit of the used analytical methods. Thus, it is important to develop methods which maximize the EV yield.

Urine as an EV source has many advantages, such as non-invasive collection, but also one major drawback. Tamm-Horsfall protein (THP), the most abundant protein in human urine (Tamm and Horsfall, 1950), forms long filaments that aggregate into networks or gel-like matrices effectively entrapping EVs (Fernández-Llama et al., 2010). THP forms aggregates under conditions found in normal urine: acidic pH and the presence of Ca^{2+} and Na^{+} ions (Kobayashi and Fukuoka, 2001; Phillips et al., 2007). For solubilization of THP, raising the pH, chelating Ca^{2+} ions, dilution and detergent treatment have been reported to be effective (Kobayashi and Fukuoka, 2001).

EVs that are trapped in THP aggregates are lost in the low speed centrifugation step that is usually done to remove cellular debris or large vesicles before EV isolation. On the other hand, after low speed centrifugation, THP filaments remaining in the supernatant cosediment with the EVs during isolation by ultracentrifugation. Abundant THP has been shown to hamper proteomic/glycan studies of EVs (Kosanović

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and Janković, 2014; Raj et al., 2012). Due to these problems, multiple methods have been applied to disrupt THP filaments for improved EV yield including treatment with a reducing agent DTT (Fernández-Llama et al., 2010; Pisitkun et al., 2004) that can be coupled with size-exclusion chromatography or precipitation (Lozano-Ramos et al., 2015; Alvarez et al., 2012), treatment with CHAPS detergent (Musante et al.,

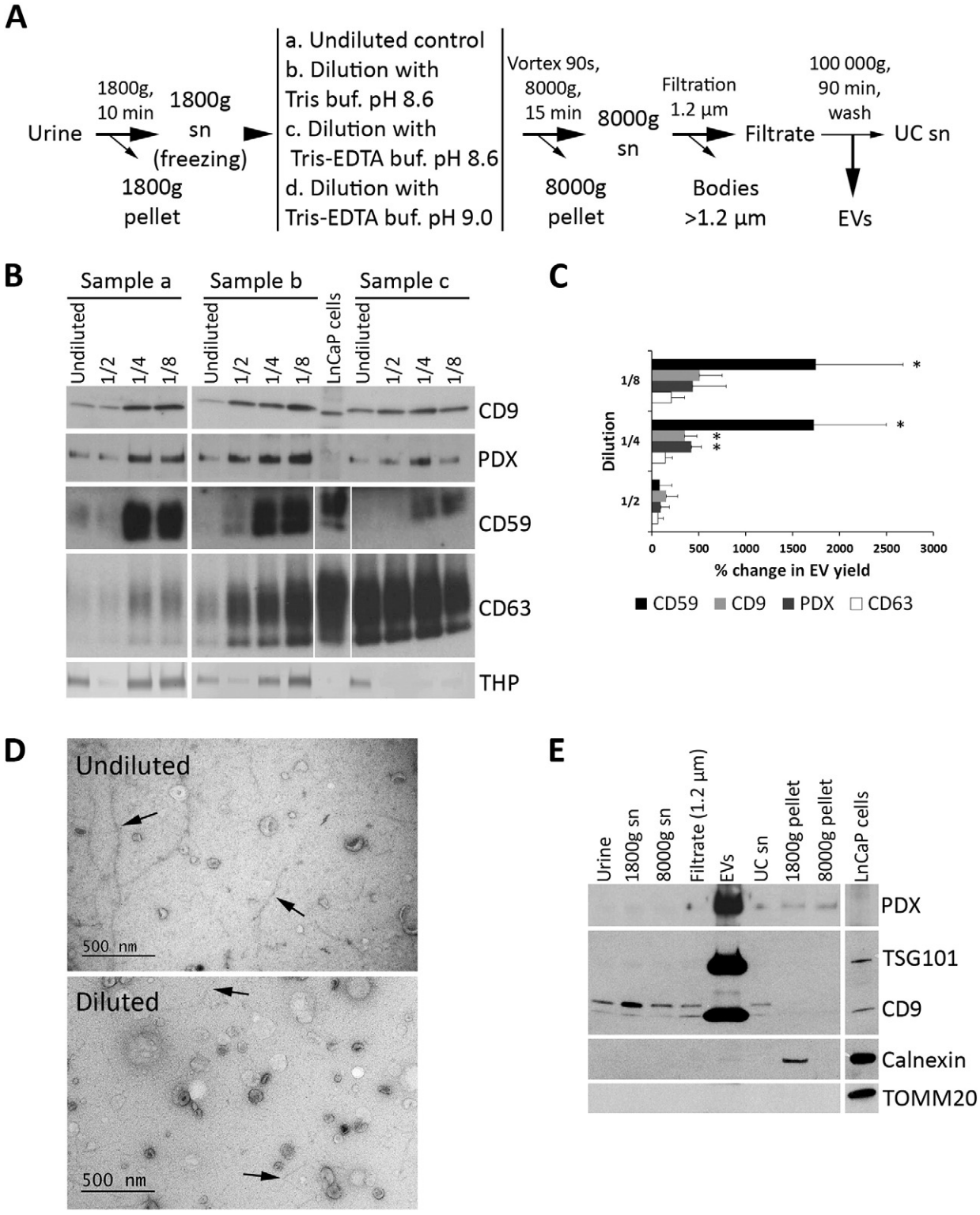

Fig. 1. The dilution protocols increase EV yield and retain morphology and purity of the EV samples. A. shows the outline of the tested protocols (a–d). B–C. Dilution series of a pilot set of urine samples (n = 3, samples a–c) in Tris buffer (pH 8.6) increased the EV yield most with 1/4 dilution as compared to undiluted control based on western blotting of multiple EV-enriched markers. C shows the average increase (%) ± SEM of the EV yield based on western blotting of the four EV-markers (see color key below the plot). D. The morphology of the EVs from undiluted control and Tris (1/4 dilution, pH 8.6) protocols appeared similar in EM. Both samples still contained some THP filaments (arrows). E. Western blotting of the samples from each step of the purification (A) with Tris protocol (1/4 dilution, pH 8.6) revealed the enrichment of CD9, TSG101 and PDX as well as the lack of organelle proteins calnexin and TOMM20 in the EVs. Equal quantities of protein were loaded from EVs and urine samples. Podocalyxin (PDX), Tamm-Horsfall protein (THP), ultracentrifugation (UC), supernatant (sn), buffer (buf.). Star (*) denotes $p < 0.05$ compared to undiluted control.

2012) or use of hydrostatic dialysis (Musante et al., 2014). For removal of THP from the final EV preparation without the aim of improving EV yield, use of sucrose cushion together with alkaline conditions (Raj et al., 2012) or salting out (Kosanović and Janković, 2014) have been previously reported. While all of the above protocols achieved their aims with a varying amount of THP remaining in the EV preparations, their effect on EV yield has not been systematically quantified. In addition, many of the methods are long, tedious and/or unsuitable for large scale EV studies involving small urine volumes.

Here, we tested a simple protocol with the aim to increase EV yield via the release of EVs from THP aggregates by dilution, alkaline pH and lowering of ionic concentration. With our systematic quantitative approach, we show for the first time, that the dilution protocol significantly increased the EV yield from urine samples independent of a wide range of initial pH. The results suggest that the protocol could

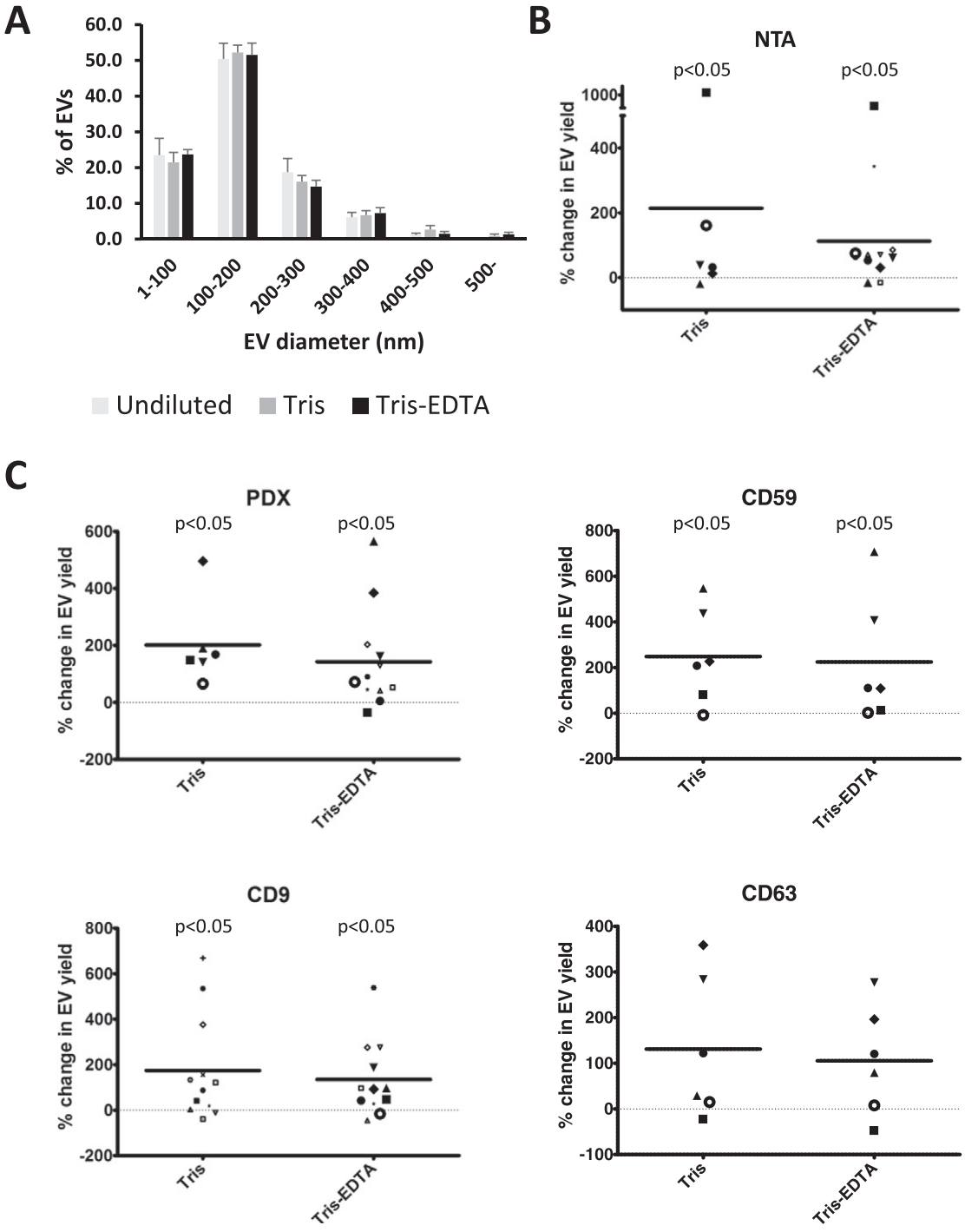

Fig. 2. Both Tris and Tris-EDTA (pH 8.6) dilution protocols give superior yields of EVs based on NTA and western blotting. A. Size distribution of EVs by nanoparticle tracking analysis (NTA) was similar with all protocols. The plot shows average EV size distribution ± SEM from samples processed with all compared protocols (n = 6, samples U1-6). B. NTA showed that the EV yield elevated upon dilution with both protocols as compared to the undiluted control (n = 6 for Tris, samples U1-6 and n = 12 for Tris-EDTA, samples U1-12). C. EV yield increases measured by Western blotting were similar to increases measured with NTA (n = 6–12, samples U1-6 were quantified in all, U7-12 in Tris-EDTA/CD9 and PDX and U13-18 in Tris/CD9). Yield changes in B and C are shown as % change compared to the undiluted control in individual samples. The line marks the average increase. Podocalyxin (PDX), nanoparticle tracking analysis (NTA).

serve as a basis for a simple and efficient EV isolation from limited urine volumes.

2. Materials and Methods

2.1. Isolation of EVs

EVs were isolated from frozen (−80 °C or liquid N_2) cell-free urine from untimed collections prepared by Helsinki Urological Biobank by centrifugation at 1800*g* for 10 min at +4 °C. Protease inhibitors (Calbiochem® EDTA-free Protease Inhibitor Cocktail Set III, Merck Millipore, San Diego, CA, USA) were added 1/2000 during thawing in a 37 °C water bath. Where indicated, the 1800*g* urine supernatant samples were diluted in cold 20 mM Tris-HCl buffer, pH 8.6 (Raj et al., 2012) or 20 mM Tris-HCl, 20 mM EDTA buffer, pH 8.6 or pH 9.0 at + 4 °C. All samples were vortexed for 90 s and subjected to centrifugation at 8000*g* for 15 min at +4 °C using a fixed angle AG-6512C rotor (Kubota Corp. Tokyo, Japan), filtered with Whatman™ 1.2 μm cellulose acetate filters (GE Healthcare, Buckinghamshire, UK) and subjected to ultracentrifugation (L-70) in polyallomer thick-wall tubes using a swing-out rotor SW-28 with 27,500 rpm for 90 min (average 100,073*g*, k-factor 254.5) at +4 °C (all Beckman Coulter, Inc., CA, USA). The pellets were resuspended in 30 ml of cold PBS, and the ultracentrifugation step was repeated. Before final resuspension of the EV pellet in cold PBS, the walls of the tubes were wiped with cotton swabs to remove all supernatant. EVs were stored in protein LoBind tubes (Eppendorf, Hamburg, Germany) at −80 °C. Urine samples were processed with the protocols under comparison (Fig. 1A) simultaneously to avoid bias caused by variation due to handler or day. Samples U1-6 were centrifuged at the 8000*g* step using equal volumes of the diluted and undiluted samples, whereas all other samples were centrifuged using unequal volumes of the diluted and undiluted samples at this step, i.e. they contained the same volume of urine with or without addition of the dilution buffer.

2.2. Western Blotting

For Western blotting, EV samples in reducing or non-reducing Laemmli sample buffer (Bio-Rad, USA) were denatured at 95 °C for 5 min, loaded to Mini-PROTEAN® TGX™ 4–20% gradient SDS-PAGE gels (Bio-Rad) and blotted on Immobilon-FL or Immobilon-P membranes (Millipore, Bedford, MA, USA). Blocking (1 h, RT) and antibody incubations (primaries o/n, +4°; secondaries 1 h, RT) were performed in 3% non-fat powdered milk or BSA (Amresco LLC, OH, USA) in TBS and TBS with 0.1% Tween-20, respectively. For detection, HRP-conjugated secondary antibodies (Jackson ImmunoResearch) and Pierce® ECL Western Blotting substrate (Thermo Fisher Scientific, Rockford, IL, USA) were used. Antibodies applied with reduced samples were against CD9 (SC-13118), podocalyxin (PDX, clone 3D3, Novus Biologicals), TSG101 (SC-7964), THP (SC-20631), calnexin (ADI-SPA-860, Enzo Life Sciences), GM130 (ab52649, Abcam) and TOMM-20 (ST1705, Millipore). Anti-CD59 (clone MEM-43, Thermo Scientific) and anti-CD63 (M1544, Sanquin) were applied to non-reduced samples. To compare EV yields from the different protocols, EVs from equal volumes of urine were loaded to gels. For visualization of EV isolation process (Fig.1E and Supplementary Fig. 1), raw urine, urine supernatants and filtrate samples were concentrated with Amicon Ultra-15 concentrators (3 kDa MWCO, Millipore). Proteins were extracted from the concentrate with ProteoSpin™ Urine Protein Concentration Micro Kit (Norgen Biotek Corp., Ontario, Canada). From each sample, 4.0 μg protein was loaded to gels except pellets from 1800*g* and 8000*g* centrifugations were loaded based on urine volume i.e. all pellet material derived from 10.0 ml of urine. For Supplementary Fig. 1, 7.5 μg (non-reduced samples) or 10.0 μg (reduced samples) of urine sample proteins were loaded to gels. Pellets from 1800*g* and 8000*g* centrifugations and EV pellet were loaded based on urine volume i.e. all pellet material derived from 5.0 ml of urine. For EVs, this corresponded to 2.0 μg of protein. For the measurement of CD9 and THP in urine samples (1800*g* supernatants, Fig. 5C), urine proteins were extracted as explained above and loaded based on equal urine volumes. Protein concentrations were measured with Pierce® BCA protein assay or Qubit (both Thermo Fisher Scientific). Quantification of protein bands was done with Fiji Image J 1.49C (Schindelin et al., 2012).

2.3. Nanoparticle Tracking Analysis (NTA)

Particle number and size distribution of the EV samples was analyzed with NTA instrument LM14C (NanoSight LTD., London, UK) equipped with blue (404 nm, 70 mW) laser and sCMOS camera (Hamamatsu Photonics K.K., Hamamatsu, Japan), as described (Aatonen et al., 2014). Videos of 3 × 90 s were recorded using camera level 14, screen gain 1.0 and set temperature of 22 °C. EV samples were diluted with filtered (0.2 μm) PBS to obtain optimal concentration for detection, and measured three times. Data were analyzed with Nanosight software v3.0, using threshold 5 and gain 10.

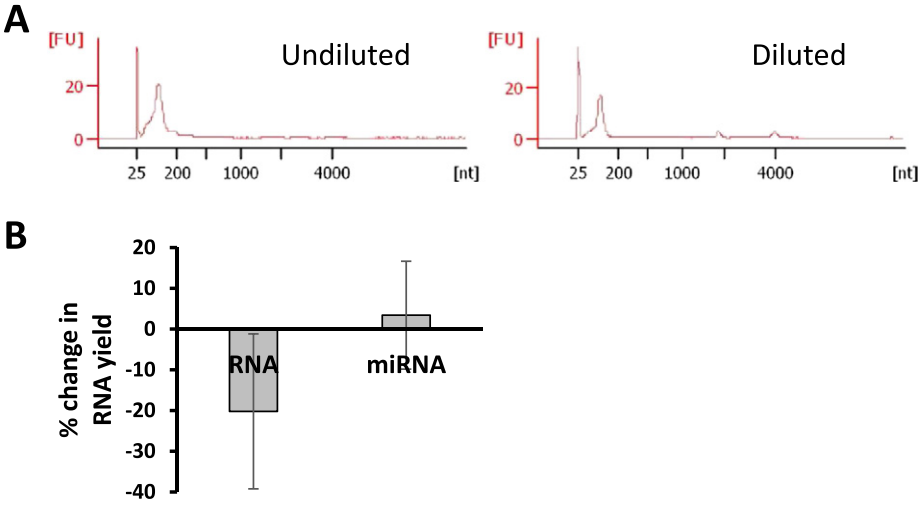

Fig. 3. The Tris-EDTA (pH 8.6) dilution protocol does not change the RNA size or yield from EV samples. A. The EV-RNA size in Bioanalyzer Pico chip appeared similar with or without dilution of the urine sample using Tris-EDTA buffer. A representative pair of RNA profiles is shown. B. RNA and miRNA yields from EV samples did not differ significantly from undiluted control samples (n = 6, samples U1-6). Yield changes are expressed as average % change ± SEM compared to the undiluted control.

2.4. Electron Microscopy (EM)

EVs were loaded on 200 mesh pioloform- and carbon-coated glow-discharged copper grids. Samples were fixed with 2% PFA (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M $NaPO_4$ buffer (pH 7.0), stained with 2% neutral uranyl acetate, embedded in methyl cellulose uranyl acetate mixture (1,8/0,4%) and viewed with transmission EM using Tecnai 12 (FEI Company, Eindhoven, The Netherlands) operating at 80 kV. Images were taken with Gatan Orius SC 1000B CCD-camera (Gatan Inc., USA) with 4008 × 2672 px image size and no binning. To quantitate the size distribution of EVs, we measured >150 EV profiles (max diameter) in 2–7 randomly sampled images of EVs from urine samples U1-6 prepared with Tris-EDTA pH 9.0 dilution and undiluted control protocols.

2.5. RNA Isolation and Measurements

Total EV-RNA was isolated from EVs pelleted from 7.5 ml of urine with miRNeasy Micro Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA samples were analyzed with 2100

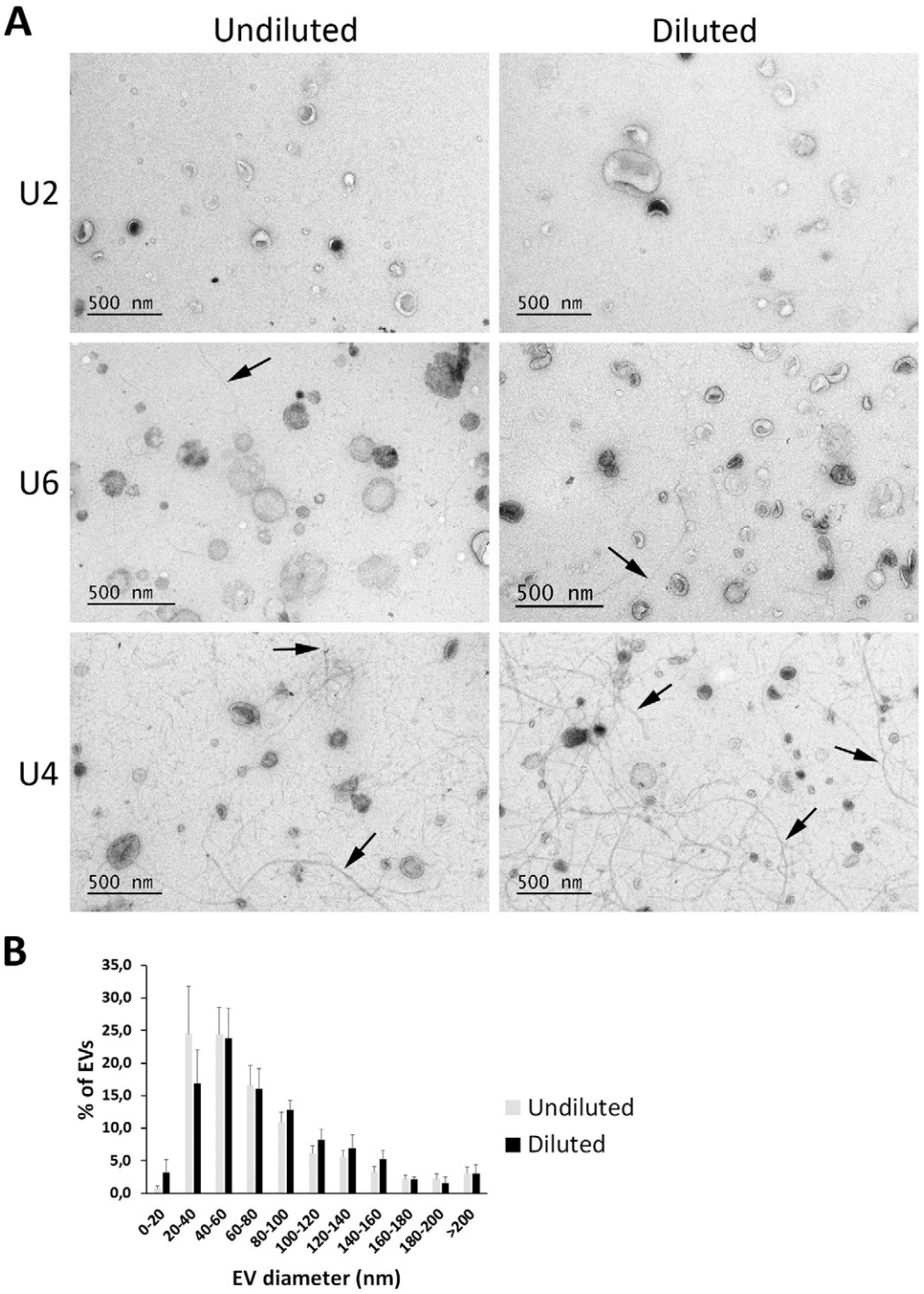

Fig. 4. Dilution with Tris-EDTA buffer (pH 9.0) retains EV morphology and size distribution. A. Three pairs of samples (U2, 4, 6) indicate the preservation of the EV morphology upon the use of Tris-EDTA dilution protocol and sample to sample variation in the amount of Tamm-Horsfall protein filaments (arrows). B. There were no significant changes in the size distribution of the EVs in EM images (n = 6, samples U1-6). The plot shows average EV size distribution ± SEM.

BioAnalyzer using BioAnalyzer Pico kit (Agilent Technologies, Santa Clara, CA, USA) and with Qubit® 2.0 using microRNA Assay kit (Thermo Fisher Scientific) according to manufacturer's instructions.

2.6. Statistics

Normality testing of the data was done with Shapiro-Wilk's test. Differences between undiluted control and the different dilution protocols (Fig. 1A) was tested for statistical significance with either paired student's *t*-test or with Wilcoxon signed rank test depending on the normality of the data distribution. The results were expressed as average ± SEM.

2.7. Ethical Issues

Samples were obtained from patients participating in the urological biobank initiative (Helsinki Urological Biobank, HUB, Dnro 263/13/03/02/2011; 379/13/03/02/2012 and Dnro § 212).

3. Results

3.1. Pilot Testing Indicates That Dilution of Urine With Alkaline Tris-buffer Increases EV Yield and Retains EV Integrity and Purity

Raj et al. (2012) reported the protocol using dilution of EVs with Tris-HCl buffer at pH 8.6 for removing of THP from EV preparations. We wanted to test, whether dilution of urine samples with this buffer would help to disrupt THP aggregates. The aim here was to release EVs and thus they would not be lost with the THP aggregates during the low speed centrifugation prior to the EV isolation by ultracentrifugation.

As a first pilot experiment, we subjected three urine samples to a dilution series (undiluted, 1/2, 1/4, 1/8) with Tris buffer (pH 8.6) and isolated the EVs by differential centrifugation (Fig. 1A, protocols a and b). On average, the EV yield, as determined by the density of bands in western blotting of typical urinary EV-enriched markers CD9, PDX, CD59 and CD63, was increased with all dilutions compared to the undiluted control (Fig. 1B and C). The increases were statistically significant with the higher dilutions. For 1/4 dilution, the increase of the western blot signals was 145–1722%. However, the high yields did not correlate with a systematic change of THP in the EV pellet (Fig. 1B). The presence of the characteristic THP filaments could also be detected in the EM images of both diluted and undiluted EV samples (Fig. 1D).

Since 1/8 dilution did not further improve the results from 1/4 dilution, we decided to continue the testing of the protocol with 1/4 dilution, which was more practical considering the limited volume of the ultracentrifugation tubes. To ascertain that the quality of the EVs did not decline by dilution, we analyzed the EVs from the diluted samples (1/4 in Tris-buffer, pH 8.6) by EM and Western blotting. The morphology of the isolated EVs from both the undiluted control and the diluted samples were similar based on EM (Fig.1D). With Western blotting, we visualized the intensities of the EV markers, albumin or cellular organelle proteins from the different steps of the isolation protocol, including the final EV sample (Fig. 1E and Supplementary Fig. 1). We first loaded equal protein amounts of EVs and urinary proteins (Fig. 1E). The EV markers CD9, TSG101 and PDX were enriched in the EV samples relative to the other samples. Probing the EV samples for common contaminant markers indicated that organelle markers calnexin (endoplasmic reticulum) and TOMM20 (mitochondria) were

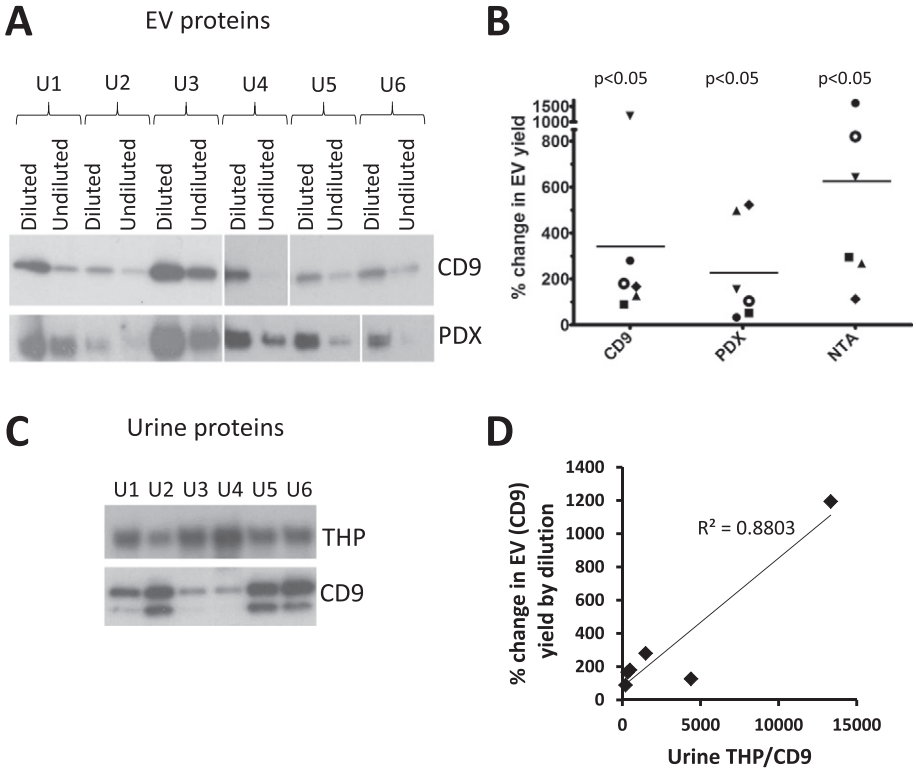

Fig. 5. Dilution with Tris-EDTA buffer (pH 9.0) increases EV yield significantly in all samples, although more in samples with high initial THP/CD9 ratio of urine. A–B. Dilution of urine samples with Tris-EDTA buffer improved the EV yields in all samples based on western blotting (PDX, CD9) and NTA as compared to undiluted control (n = 6, samples U1-6). Yield increases are shown as % change compared to the undiluted control in individual samples. The line marks the average increase. C–D. Positive correlation between the magnitude of EV yield increase based on CD9 and the initial urinary THP/CD9 ratio suggests that the dilution protocol improves the yield most in samples with high THP load relative to EV number. Podocalyxin (PDX), nanoparticle tracking analysis (NTA), Tamm-Horsfall protein (THP).

absent. Next, to allow quantification of the samples from the same blots, we loaded higher amounts of urinary (7.5–10 μg) than EV (2 μg) proteins (Supplementary Fig. 1). Here, CD9, podocalyxin and TSG101 showed higher enrichment (20–150 fold) in the EVs relative to the urine samples than CD63 and CD59 (2–8 fold) due to the relatively high amount of CD63, 55%, remaining in the ultracentrifugation supernatant and the presence of CD63 and CD59 in the 8000*g* pellet. Albumin, a soluble protein, and organelle proteins including GM130, a Golgi protein, were not detected in the EVs. However, all of the samples still contained THP. The dilution protocol thus seemed to purify EVs and increase the EV yield, but it was not able to completely depolymerize THP filaments.

3.2. EV Yields Increase Upon Dilution Based on NTA and Western Blotting

To further test the dilution protocol, we isolated EVs from six urine samples (U1-6) using our control protocol (undiluted), 1/4 dilution with Tris buffer alone or with added EDTA (both pH 8.6) in an attempt to lower the Ca^{2+} concentration (Fig. 1A, protocols a–c). EDTA concentration (20 mM) was chosen based on the previously reported buffer

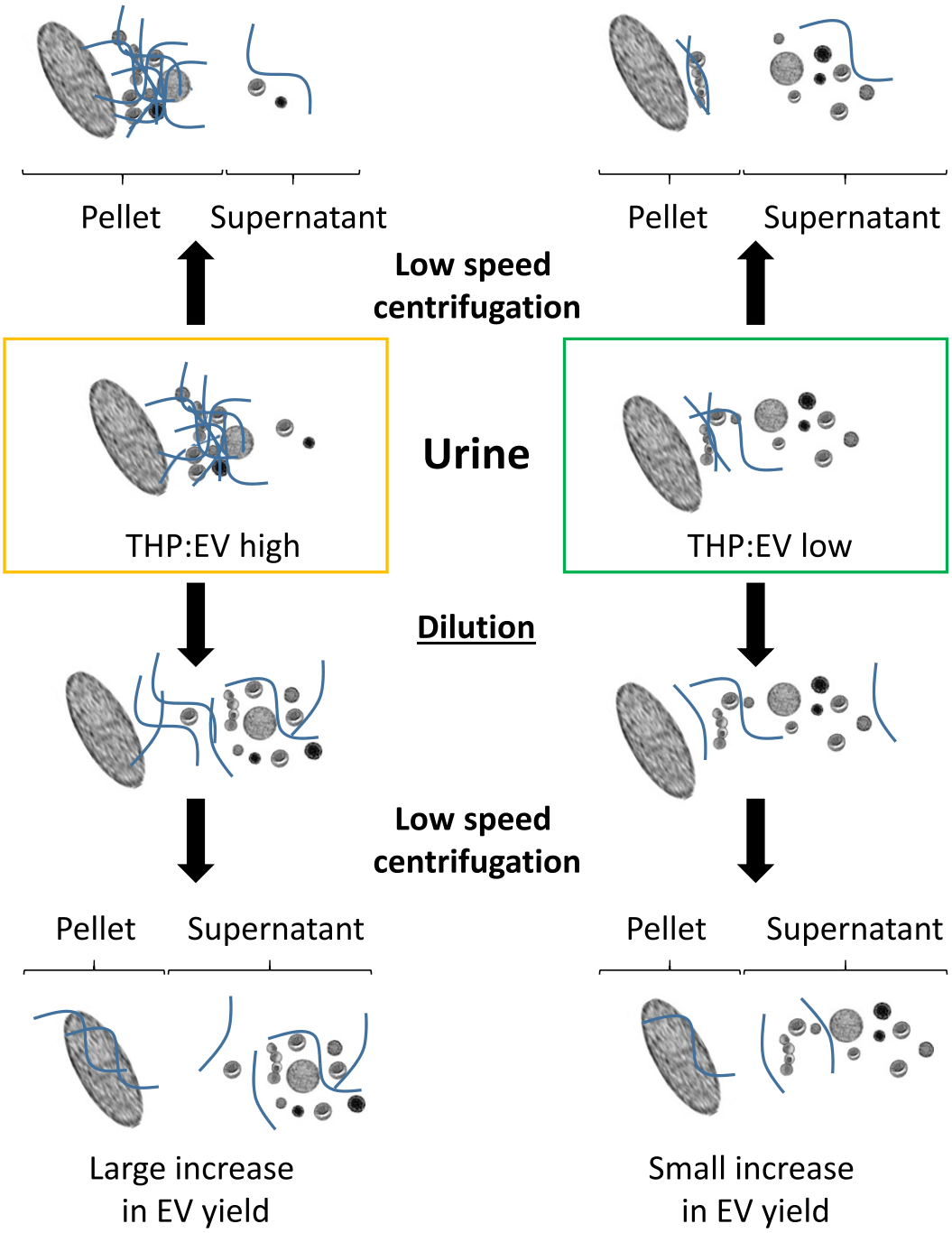

Fig. 6. A model depicting the action of the dilution protocol and benefits of EV release from THP networks in urines containing low or high THP/EV ratio. The urine samples can contain a high or low amount of THP relative to the EVs in the sample (orange and green boxes). In high THP/EV conditions, low speed centrifugation will remove a large part of THP-trapped EVs (arrow up from orange box) unless the THP network is loosened by dilution before the low speed centrifugation (arrow down from orange box). THP networks release EVs and the size of THP aggregates decrease upon dilution. THP filaments are not completely removed by the low speed centrifugation, but some remain in the supernatant together with EVs. The supernatant will be used in the following EV collection step. Urines with lower THP to EV ratio benefit from the dilution less, since THP does not bind a significant part of EVs.

conditions for the solubilization of THP and typical Ca^{2+} concentrations in urine (Kobayashi and Fukuoka, 2001). There was no difference in the size distribution of EVs isolated with either dilution or undiluted control protocols when analyzed by NTA (Fig. 2A). The EV yields were analyzed by NTA and Western blotting and by RNA quantity (Figs. 2, 3 and Supplementary Fig. 2). Both NTA and Western blotting indicated a large variation between EV yields from the different samples ranging from over 1000% increase to 48% decrease of EV yield compared to the undiluted control. In all, EV yield from 5/6 samples increased and 1/6 showed a relatively minor decrease compared to the average increases. Similar results were obtained irrespective of using equal or unequal sample volumes in the 8000*g* centrifugation step of the protocols under comparison (see Materials and Methods).

According to NTA, the average EV yield from samples U1-6 increased by 213 ± 171% using Tris or by 158 ± 118% using Tris-EDTA buffer for dilution compared to the undiluted controls (both $p < 0.05$, Fig. 2B). The two dilution buffers did not significantly differ from each other. This result was validated by processing six additional samples (U7-12) using the Tris-EDTA dilution protocol, which resulted in an average increase of EVs by 130 ± 62% in all 12 samples ($p < 0.05$, Fig. 2B).

Western blotting of the U1-6 EV samples showed that the yield of EVs increased by dilution with Tris and Tris-EDTA relative to undiluted controls by >150% based on PDX, CD59 and CD9 and >100% based on CD63 (Fig. 2C and Supplementary Fig. 2). The results were statistically significant ($p < 0.05$) for all other comparisons except CD9/Tris-EDTA ($p = 0.059$) and CD63/Tris and Tris-EDTA ($p > 0.1$). Validation of the results with additional six samples using the Tris-EDTA (samples U7-12) or Tris (samples U13-18) dilution protocols produced an average yield increase of 135 ± 47% (Tris-EDTA, CD9), 142 ± 50% (Tris-EDTA, PDX) and 174 ± 66% (Tris, CD9) across all 12 samples ($p < 0.05$, $n = 12$ for all, Fig. 2C). Surprisingly, we detected a similar increase in the remaining THP in the EV samples obtained with dilution vs. undiluted control protocols: the THP signal increase was 242 ± 115% for Tris-EDTA protocol ($p < 0.05$, $n = 4$) and 209 ± 108% ($p < 0.05$, $n = 6$) for Tris protocol, when excluding one sample, where THP signal disappeared upon dilution (Fig. 1B). There was no significant difference between Tris and Tris-EDTA protocols with any of the markers.

After these results, we wanted to analyze, whether the RNA yield would also be significantly increased with the dilution protocol. For this, we used EVs obtained with the Tris-EDTA dilution protocol from U1-6 and measured the EV-sample derived RNA and miRNA yields using Bioanalyzer Pico kit and Qubit miRNA assay, respectively (Fig. 3). The bioanalyzer profiles from the diluted and undiluted control samples appeared the same: the majority of the RNAs were small and small ribosomal peaks were occasionally visible. Despite the consistent increase of yield observed with NTA and Western blotting, the RNA or miRNA yields did not significantly differ from the yield produced by the undiluted control protocol.

3.3. Both the pH of the Dilution Buffer and the Amount of THP Influence the Yield of EVs

Since the dilution protocol improved the yield of EVs in >80% of samples, but not in all, we wanted to test whether pH of the buffer played a role in the success of the protocol. The initial pH of the urine samples varied between 5.0 and 7.5 and was raised to 7.0–8.5 by diluting 1/4 with both Tris and Tris-EDTA pH 8.6 buffers ($n = 20$). Since our aim was to develop a simple protocol that would not require measurement/adjusting of the pH in individual samples, we next tested whether the protocol could be improved by simply raising the pH of the dilution buffer to 9.0 (Fig. 1A, protocol d). In our test urine set U1-6, the initial pH ranged between 5.5 and 7.5 and 1/4 dilution with Tris-EDTA buffer (pH 9.0) resulted in the final pH between 7.5 and 8.75. By EM, the morphology and the size range of the EVs isolated by 1/4 dilution with Tris-EDTA buffer pH 9.0 and by undiluted control protocol were similar (Fig. 4, samples U1-6). Variable amounts of THP filaments were observed in both sample types. Analyzed by Western blotting and NTA, the EV yield was increased by the higher pH buffer in all samples averaging 340 ± 173% for CD9 ($p < 0.05$), 228% ± 91% for PDX ($p < 0.05$) and 624% ± 223% by NTA ($p < 0.01$) compared to the yield from the undiluted control protocol (Fig. 5A and B). Nevertheless, there was no correlation between the increase of the yield and the final pH of the samples (data not shown).

The improved yield could also depend on the initial ratio of THP to EV in the urine. Even if the dilution protocol did not remove all THP, we hypothesized that it could loosen the networks of THP thereby aiding the release of EVs especially in samples with high THP/EV ratios. We thus tested this hypothesis by quantifying the amount of THP and CD9 in the urine samples (1800*g* supernatants, Fig. 5C) and correlated their ratio with the improvement of CD9 yield in the EV samples isolated with the pH 9.0 Tris-EDTA dilution protocol vs. the undiluted control protocol. The THP/CD9 ratio of the urine samples correlated well with the increase in the EV (CD9) yield ($R^2 = 0.8803$, Fig. 5D) suggesting that the Tris-EDTA pH 9.0 dilution protocol improved the EV yield more in the samples which had a high THP/EV ratio than in the samples where the THP/EV ratio was low.

4. Discussion

Urine as a source for EV-based biomarkers has many advantageous properties. First, it can be obtained non-invasively. Second, the EVs in urine seem to be relatively stable resisting, for example, conditions with high salinity and alkaline pH (Zhou et al., 2006; Mitchell et al., 2009; Raj et al., 2012; Musante et al., 2013). Third, apart from THP, urine contains only minor amounts of soluble proteins compared to for example plasma. However, the loss of EVs due to the entrapment within THP aggregates is a problem which has required development of counter measures (Fernández-Llama et al., 2010; Musante et al., 2012, 2014). However, although these improvements have released EVs from THP aggregates, the achieved increase in EV yield per urine volume has not been systematically quantified until now.

Release of EVs from the THP aggregates is important due to variable excretion of THP, which leads to variable entrapment of EVs in urine samples (Fernández-Llama et al., 2010). In addition, there is a possibility that THP could selectively bind specific EV subtypes. Thus, the choice of not releasing EVs from THP aggregates might skew the gained results. Since the loss of EVs does not occur to a great extent during the initial centrifugation producing cell-free urine (at 1800–2000*g*, Fig.1E, Supplementary Fig. 1 and Musante et al., 2014), we concentrated our efforts on improving the EV yield from the cell-free urine typically stored in biobanks.

Our optimized protocol was developed from the combination of approaches reported by Raj et al. (2012) and Kobayashi and Fukuoka (2001). We chose a relatively high pH alkaline buffer (8.6–9.0) to ensure that pH ≥ 7.0 was reached in all diluted urine samples, which have a highly variable initial pH and buffering capacities. Ionic concentration of Ca^{2+} and Na^{+} were lowered both by dilution and chelation with EDTA. With this protocol, we show for the first time a quantitative effect on the EV yield evaluated by multiple EV-markers, NTA and RNA. With our best buffer (pH 9.0), the achieved increase in yield varied between 33 and 1604% based on western blotting and NTA. Remarkably, in a urine sample with a high THP load the EV yield improved over six fold (>650%) on average. Since the increase in yield of EVs (CD9) correlated positively with the THP to CD9 ratio in urine (Fig. 5D), the variable benefit from the dilution protocol could be explained by the conditions in urine: a large amount of THP relative to EVs will entrap, and therefore release upon dilution, more EVs than a small amount of THP relative to EVs (Fig. 6). In our model, we hypothesize that the alkaline dilution is loosening the THP network and releasing EVs thus preventing their loss during the low speed centrifugation step (Fig. 6). Since dilution changes the pH and ionic strength in urine, EVs may also be released due to the disruption of the ionic interactions between THP and EVs as

has been detected in the case of THP and extracellular matrix interactions (Lambert et al., 1993). These two mechanisms are by no means mutually exclusive and could together explain the EV yield increase.

Unlike EV-markers and particle count, the dilution protocol did not improve the yield of EV-RNA. Similar phenomenon has been observed in other type of EV samples, when RNAses were not used (Sáenz-Cuesta et al., 2015). We also omitted the RNAse treatment. However, since the dilution protocol adds an efficient washing step, the result could mean that soluble contaminating RNAs have been removed and replaced with EV-RNAs. A further study would be needed to address this issue.

While removing albumin, the dilution protocol failed to remove all THP from EV pellets (Fig. 1 and Supplementary Fig. 1). However, total removal of THP has not been achieved with other methods aiming to improve the yield of urinary EVs via solubilization of THP or alternatively, the improvement in EV yield has not been reported against a non-treated control (Fernández-Llama et al., 2010; Alvarez et al., 2012; Musante et al., 2012, 2014; Lozano-Ramos et al., 2015). It is also good to keep in mind that a complete removal of THP is not imperative for all downstream applications and that the methods to decrease THP interference are not limited to measures during EV isolation (Hiemstra et al., 2011).

Because the dilution protocol is very simple and a potential alternative to for example DTT or CHAPS treatments (Fernández-Llama et al., 2010; Musante et al., 2012), it could be combined with multiple EV isolation strategies. For example, ultracentrifugation could be replaced with less tedious and faster EV collection methods (Musante et al., 2014; Zhao et al., 2016). In addition, EV samples for applications requiring a stringent removal of THP could be processed further with other methods (Raj et al., 2012; Kosanović and Janković, 2014; Lozano-Ramos et al., 2015). These developments would further expedite the protocol and its applicability for large-scale EV studies utilizing low volume urine samples.

5. Conclusions

We have developed a method to prevent loss of EVs during isolation – hence we named the method "KeepEX". KeepEX, i.e. dilution of urine with alkaline buffers, is a simple, efficient and low cost method to improve EV yields from limited sample volumes. Because it does not require individual adjustment of sample pH nor extra centrifugation steps, it could be applied on its own or coupled with other EV purification protocols to achieve efficient EV isolation suitable for large-scale EV studies. The improvement of EV yield with the dilution protocol also gives hope that complicated sample collections, such as urine samples collected after digital rectal examination to increase prostate-derived EVs in urine (Duijvesz et al., 2015), could be avoided in the future.

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