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Serum and urinary levels of CD222 in cancer: origin and diagnostic value

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The mannose 6-phosphate/insulin-like growth factor 2 receptor (CD222, M6P/IGF2R) is a multifunctional transmembrane type I receptor, mostly localized intracellularly and less on the surface of all types of mammalian cells. It is known to both transport lysosomal enzymes through their mannose 6-phosphate moieties and to internalize extracellular ligands such as insulin-like growth factor 2 or plasminogen. CD222 is involved in regulation of cell proliferation, migration, T cell activation and apoptosis, and soluble CD222 has been found in higher concentrations in the sera of liver disease patients. Herein, we analyzed the level of CD222 present in cancer patient serum and urine body fluids and found significantly elevated soluble CD222 levels in cancer patients' sera compared to healthy controls, irrespective of disease type. The urinary CD222 levels were specifically increased in breast cancer and multiple myeloma. CD222 was present in CD222-positive exosomes in urine, but not in sera, thus identifying different CD222 origins present in human body fluids. These results inspire us to propose serum soluble CD222 as a general biomarker for tumorigenesis.

Key words: mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R; CD222), cancer, exosomes, biomarker, serum, urine

There are two known mannose 6-phosphate binding receptors (MPRs) in mammals: the approximately 46 kDa cation-dependent MPR and the approximately 250 kDa cation-independent mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R, CD222). Both are in the p-type lectin family [1]. CD222 is a multifunctional protein which can bind and transport various ligands; either through their mannose 6-phosphate (M6P) moieties as soluble acid hydrolases [2-4], or independent of M6P such as insulin-like growth factor 2 (IGF2) and plasminogen [5, 6]. Only 5–10% of CD222 is located on the cell surface [7]; with most found intracellular in the trans-Golgi network and less in early, late and recycling endosomes. CD222 is a type I transmembrane protein with an extracellular domain consisting of 15 cysteine rich, partly homologous repeats, each composed of approximately 147 amino acids. There are four distinct M6P-binding sites [8] and a single IGF2-binding site [5] in this domain and the urokinase plasminogen activator receptor and plasminogen binding sites are localized in the N-terminal portion [9]. A short cytoplasmic tail encompasses various sorting signals, some of which are modified by phosphorylation or palmitoylation [1, 10]. Although CD222 appears to be a dimer in the plasma membrane, it is solubilized as a monomer in detergents [11, 12].

We have previously shown that the tumour necrosis factor-α-converting enzyme TACE (ADAM17), a protease from the ADAM family (A Disintegrator And Metalloprotease), mediates CD222 shedding. In addition, the β-site APP cleaving enzyme 1 also sheds CD222 [14]. Soluble CD222 (sCD222) has the molecular weight of approximately 205 kDa and lacks the carboxy terminal cytoplasmic and transmembrane domains but retains the ectodomain with the ligand binding sites [13]. Higher levels of sCD222 have been found in rat serum following liver damage [15] and human patients with liver diseases [13], and it is also reported that sCD222 transports IGF2 and mannose 6-phosphated proteins in the circulation [16] and neutralizes plasminogen [13]. While the 46 kDa cation-dependent MPR may be released from cells as a component of extracellular vesicles [17], CD222-positive exosomes have not yet been identified.

Loss of heterozygosity (LOH) in the CD222 gene has been described in many tumors, including hepatocellular carcinoma and breast cancer, and it was shown that LOH in the CD222 gene predicts poor clinical outcome in hepatocel-

lular carcinoma patients [18]. This loss in the CD222 gene could result either in attenuated expression or eventual lack of intact receptor protein expression. These effects support the ideas that CD222 is considered a tumour suppressor and that CD222 lack is implicated in tumour development by impaired protein transport, deregulated peri-cellular proteolysis and imbalanced apoptosis [19–21].

Despite these ideas, little is certain about sCD222's association with cancer [16], and this inspired us to analyze the serum and urine in a cohort of cancer patients with different malignancies.

Materials and methods

Chemicals and reagents. Glycine, methanol, Rotiphorese® GRI 30 acrylamide, SDS (sodium dodecyl sulphate) pellets and Tris-(hydroxymethyl)-aminomethane (Tris) were purchased from Carl Roth (Karlsruhe, Germany). 2-isopropanol, sodium deoxycholate, protease inhibitor cocktail (inhibitor of a broad spectrum of proteases), tetramethylethylenediamine (TEMED), thimerosal, tween, Human IGF2R/Cation-independent mannose-6-phosphate Receptor ELISA Kit for serum, plasma and cell culture supernatants, fetal calf serum (FCS) and transforming growth factor-β (TGF-β) were from Sigma-Aldrich (St. Louis, MO). Ammonium persulfate (APS) was from Serva (Heidelberg, Germany), Bradford solution from BioRad (Hercules, CA). Both Carestream GBX fixer and replenisher and Kodak GBX developer and replenisher were from Carestream Health (Rochester, NY). The kit for exosome isolation, ExoQuick-TC Exosome Precipitation Solution, was purchased from System Biosciences (Mountain View, CA). NHC-X-Biotin was from Calbiochem (San Diego, CA), 0.25% Trypsin-EDTA, Glutamine, Penicillin-Streptomycin (10 000 U/ml), RPMI 1640 medium from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA). Western Bright Chemilumineszenz Substrat Sirius for CCD system was from Biozym Scientific GmBH (Hessisch Oldendorf, Germany). Finally, Page Ruler Prestained Protein Ladder #26616 was from Thermo Fisher Scientific (Waltham, MA).

Antibodies and conjugates. MEM-61 – the exosome specific mAb (CD9), MEM-238 – mAb to CD222, MEM-240 mAb to CD222, and PPV-06 – anti-plant pathogen – an isotype control, were purchased from EXBIO (Prague, Czech Republic). Biotinylation of MEM-238 was performed in our laboratory. The streptavidin-horse radish peroxidase (HRP) complex was from GE Healthcare (Piscataway, NJ) and the secondary antibody Anti-Mouse IgG-Peroxidase antibody produced in rabbit and Anti-Rabbit IgG-Peroxidase produced in goat came from Sigma-Aldrich. Finally, the rabbit polyclonal antibody to Fibronectin was from Abcam (Cambridge, United Kingdom).

SDS-polyacrylamide gel electrophoresis and Western blot. Samples equilibrated with Laemmli buffer were loaded in 7.5% polyacrylamide gel for SDS-electrophoresis and proteins were then transferred onto a PVDF membrane. CD222 was first detected with biotinylated mAb MEM-238 and then with streptavidin-HRP conjugate. The cell lysate of the monocytic cell line THP-1 was used as positive control to monitor the full-length membrane form of CD222.

ELISA. CD222 concentrations in sera were measured by ELISA as previously described [13] – mAb MEM-240 was used as a binding antibody and the biotinylated mAb MEM-238 as a detection antibody to differentiate epitopes [22]. The calibration curve was performed with sCD222 purified as also described [13], and absorbance was measured at 405 nm.

The CD222 concentration in urine was then determined by the ELISA Kit (Sigma-Aldrich), with absorbance measured at 450 nm. Before the experiment, all urine samples were adjusted with Tris solution to pH = 6.8.

Cell cultures. The TCL-598; HUVECtert, MDA-MB-231 cells were plated on 48-well plates, starved for 24 hours in RPMI medium with 0.1% FCS and incubated with cytokines, including TGF-β, in RPMI 1640 medium without FCS for 4 hours. After this time, medium with cytokine medium was then replaced with clean medium without FCS and incubation continued for 18 hours when cells were harvested and samples prepared with Laemmli buffer.

Analysis of exosomes. The urine samples were centrifuged at 515×g and supernatants were filtered through 0.20 μm-pore filters. Individual fractions were analyzed for CD222 by immunoblotting and exosomes were isolated using the ExoQuick – TC™ kit (System Biosciences) from 5 ml of urine and analyzed by immunoblotting to detect CD222 and also CD9 which is an exosomal marker.

Sera and urine samples. The sera and urine samples were obtained from healthy donors and patients with various types of cancer after their informed consent that part of the routine body fluid samples could be used in research approved by the Ethics Committee of the Medical University of Vienna. All samples were stored at –20 °C before use, and equal volumes of serum were later loaded for Western blot analysis.

Results

sCD222 in sera. We analyzed sera of the cohort of 87 cancer patients with various types of tumours by measuring the CD222 concentrations with ELISA. Results showed increased levels of CD222 in sera of all cancer patients (12–67 μg/ml) compared to healthy donors (up to 7 μg/ml); this increase however was not specific for any cancer type (Figure 1A). Western blot analysis of sera samples revealed that the size of serum CD222 was approximately 205 kDa (Figure 1B). This corresponded to sCD222, the shed form of CD222 which we described in our previous sera analysis in liver disease patients [13]. The increase in CD222 shedding most likely corresponds to the tumorigenic or fibrotic processes involved in cancer progression; thus promoting sCD222 as a general biomarker of tumorigenesis.

We incubated the following cell cultures to investigate the mechanics of CD222 shedding; kidney epithelial

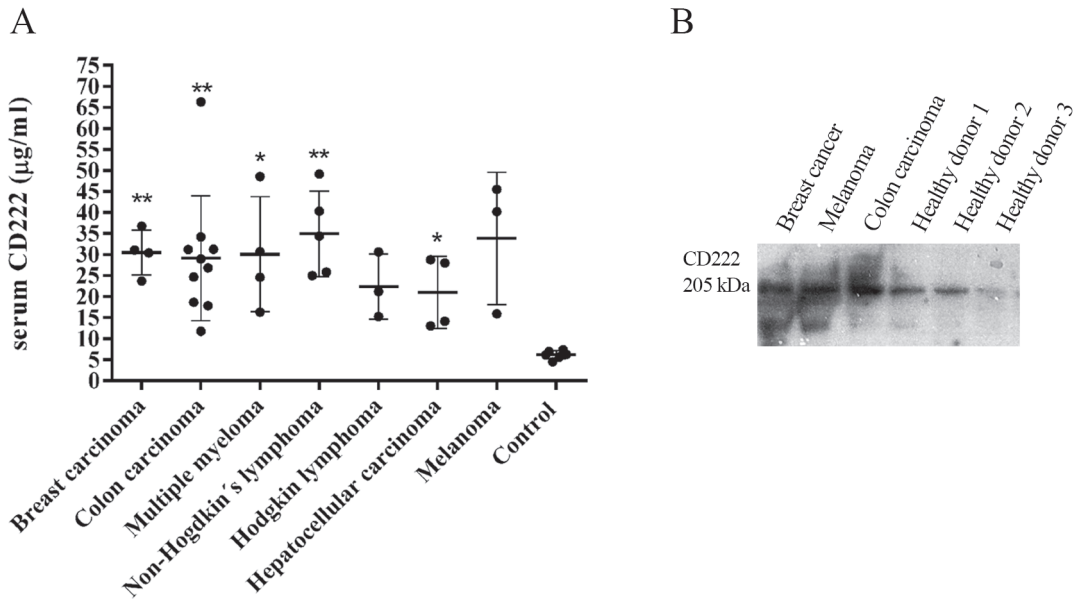

Figure 1. CD222 in human blood serum. A) CD222 concentrations were measured using ELISA. The absorbance was measured at 405 nm. Data show higher concentrations of CD222 in sera of all analyzed cancer patients (*p<0.05, **p<0.005). B) By Western blotting, the same samples were analyzed with the biotinylated CD222 mAb MEM-238 as a primary antibody. Proteins were visualized by streptavidin-HRP as conjugate and chemiluminescence. Equal volumes of sera samples prepared with Laemmli buffer were loaded on gel.

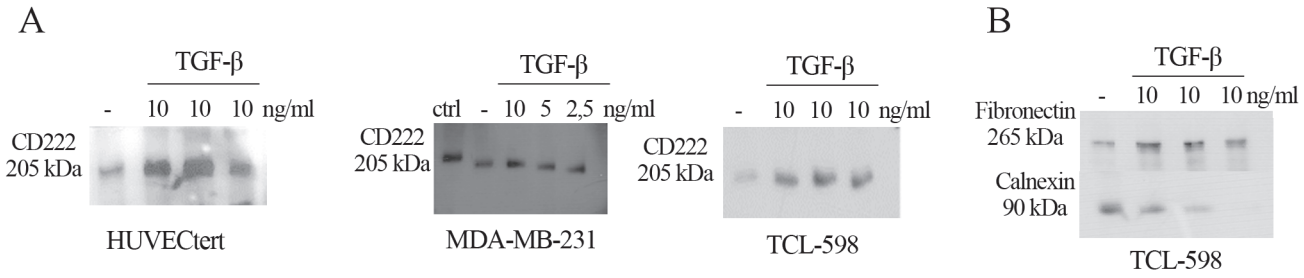

Figure 2. TGF-β as a potential player in CD222 shedding. A) By Western blotting, the media of TCL-598, HUVECterts and MDA-MB-231 cells were analyzed with the CD222 MEM-238 mAb as a primary antibody. Proteins were visualized by Anti-Mouse IgG-Peroxidase antibody and chemiluminescence. sCD222 (205 kDa) increase was observed when TGF-β (10 ng/ml) was added to cells. Equal volumes of cell media prepared with Laemmli buffer were loaded on gel. In addition, we provide the cell lysate of the monocytic cell lines THP-1 showing the full-length 250 kDa membrane form of CD222. B) By Western blotting, the TCL-598 cell lysates were analyzed with the anti-fibronectin mAb primary antibody. Calnexin was used as loading control Proteins were visualized by anti-rabbit IgG-HRP as a conjugate and chemiluminescence. Fibronectin increase was observed when TGF-β (10 ng/ml) was added to cells. Equal volumes of cell lysates prepared with Laemmli buffer were loaded on gel.

tumour TCL-598 cell line, immortalized human umbilical vein endothelial cells (HUVECtert) [23] and the MDA-MB-231 breast carcinoma cell line. Different cytokines identified their influence on CD222 shedding. The conditioned media and corresponding cell lysates were then analyzed for CD222 presence, and also fibronectin which marks fibrosis. Results showed significant increase in sCD222 (205 kDa) in media after transforming growth factor-β treatment (TGF-β, 10 ng/ml – Figure 2A). TGF-β also produced significant increase in fibronectin concentration in the cell lysates (Figure 2B), thus suggesting fibrosis triggering.

CD222-positive exosomes in urine. We then analyzed the corresponding urine samples from the same cohort of patients. ELISA identified significant increase in the CD222 concentration in urine samples of patients with breast carcinoma (up to 12.5 ng/ml; $p<0.05$) and with multiple myeloma (up to 8 ng/ml; $p<0.05$) compared to other urine samples and the control samples from healthy donors (up to 5 ng/ml) (Figure 3A). Total protein concentrations in the corresponding samples were also increased, but not significantly (Figure 3B). We also evaluated CD222 % protein concentration (Figure 3C) in breast cancer and multiple myeloma samples and compared percentages with CD222:protein concentration in healthy donors. We observed higher significance in multiple myeloma samples (up to 0.0017%; $p<0.005$) than in breast cancer samples (up

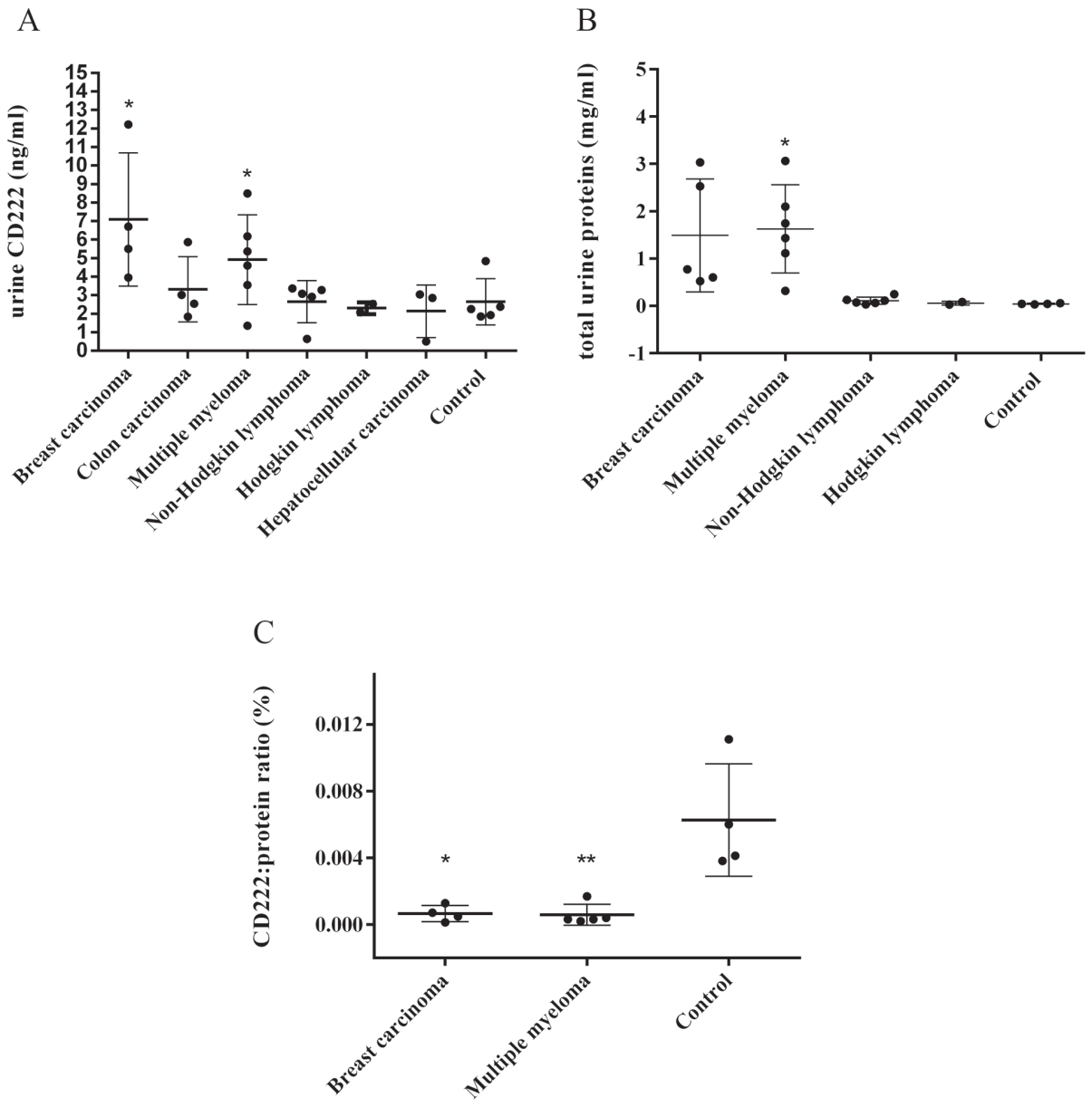

Figure 3. CD222 in human urine. A) To determine the concentration of CD222 in urine we used the ELISA Kit from Sigma Aldrich. The absorbance was measured at 450nm. Increased concentrations were detected in the samples of patients (patients in every subgroup of cancer were distinguished according to numbers) with breast cancer and multiple myeloma (*p<0.05, **p<0.005). B) The total protein concentrations in the urine samples were measured by the BIORAD Protein Assay kit. Absorbance was measured at 595 nm. C) CD222:protein concentration ratio (%) in breast cancer and multiple myeloma urine samples was evaluated and compared with CD222:protein concentration ratio in urine samples of healthy donors (*p<0.05, **p<0.005).

to 0.0007%; p<0.05). Thus, the higher CD222 concentrations detected specifically in the urine of patients with breast carcinoma and multiple myeloma most likely results not only from disease progression but also from general pathological processes affecting urine protein concentration such as kidney insufficiency, inflammation and physical activity.

Finally, we analyzed the CD222-positive urine samples by Western blotting. In contrast to the sCD222 present in sera with a molecular weight of approximately 205 kDa (Figure 1B), the molecular weight of the urine CD222 was higher – approximately 250 kDa (Figure 4A); and this confirmed the presence of the full-length form of CD222. We therefore sought the origin of CD222 in urine. When the CD222-positive urine sample was run through a 0.20 μm pore filter, we detected CD222 in the filtrate fraction but not in the pellet or the filter *retenate* (Figure 4B). Since particles

sized up to 0.20 µm passed through the filter, we concluded that CD222 could be present in small vesicles, such as exosomes. We therefore analyzed the CD222-positive urine samples with the ExoQuick – TC™ kit to isolate exosomes and confirmed that the CD222-positive exosomal fraction was positive for tetraspanin CD9; an exosomal marker (Figure 4C).

These results indicate that CD222 is present in urine as a component of exosomes and not in a soluble shed form as in sera. Thus, different mechanisms must be responsible for producing CD222 in sera and urine.

Discussion

Proteins in serum and urine are commonly used as biomarkers for diagnosing diseases; including cancer [24]. Herein, we analyzed the presence of the mannose 6-phosphate/insulin like growth factor 2 receptor (CD222) in serum and urine of patients with different types of cancer.

We found that the CD222 levels were increased in sera of all cancer patients irrespective of the type of tumour. In serum, CD222 was present in its soluble form – sCD222 (~205 kDa). As previously described [13] sCD222 is released from endothelial cells by TACE. Although sCD222 is significantly increased in cancer patient sera, it can only be a general biomarker of tumorigenesis. Concomitant phenomena in cancer progression, such as hormone level changes, inflammation and fibrosis may contribute to the higher serum sCD222, while CD222 shedding is involved in general bodily physiological responses to cancer progression. For example, in our previous study we suggested endothelial cell-produced sCD222's role as an anti-angiogenic agent [13]. But sCD222 can also be produced by tumors and other cells, including immune cells. As shown herein, shedding can be influenced by TGF-β which plays an important role in the control of cell growth, migration and cell survival [25]. Moreover, TGF-β is involved in activation and up-regulation of TACE, which is phosphorylated and subsequently translocated onto the cell surface in TGF-β receptor occupation [25]. Subsequently, the increased activity of TACE induced by TGF-β could result in increased CD222. Therefore, sCD222 may be considered a biomarker of tumorigenesis.

We then analyzed the urine samples of the same cohort of patients. In contrast to serum, urine does not require invasive medicine when used as a biomarker source, and this is therefore better for the patient. The results showed a significant increase in CD222 concentration in breast carcinoma and multiple myeloma patients compared to the control donors.

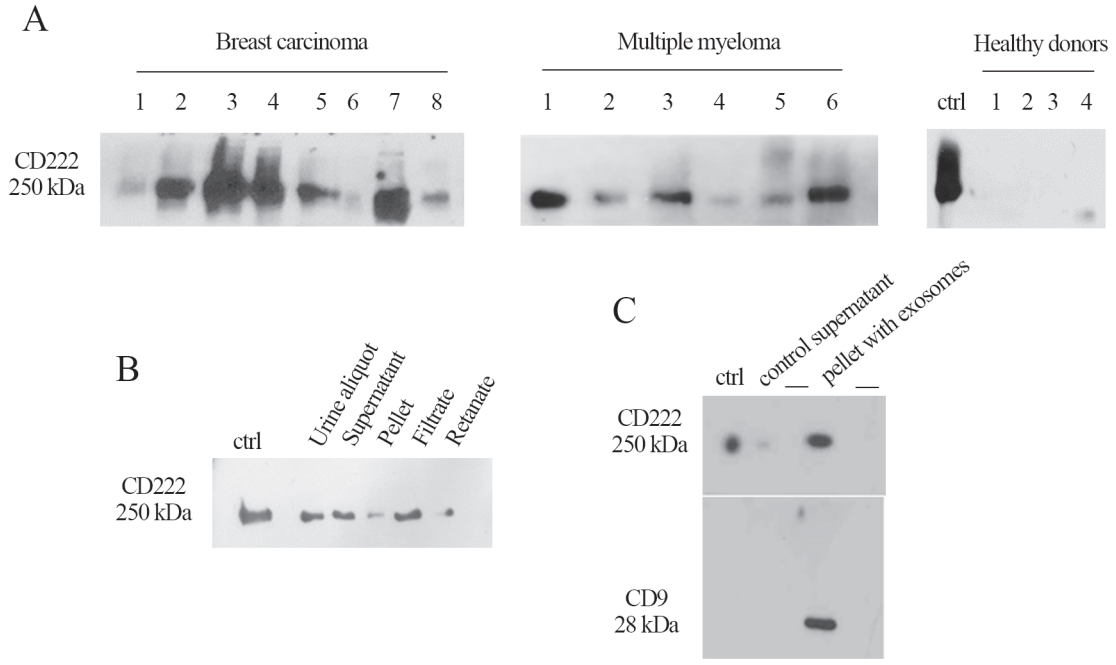

Figure 4. CD222-positive exosomes in human urine. A) CD222 was detected through Western blotting in urine of cancer patients and in healthy control donors. As a control, the cell lysate of the monocytic cell lines THP-1 was used; showing the full-length membrane form of CD222. Equal volumes of urine samples prepared with Laemmli buffer were loaded on gel. B) The CD222-positive urine sample of a healthy person was centrifuged to remove cells and debris. Then, the supernatant was filtered through a pore size of 0.20 µm. Aliquots from each step were evaluated by Western blotting. [Urine aliquot – a urine aliquot before analysis; supernatant – an aliquot of supernatant after centrifugation; pellet – an aliquot of pellet after centrifugation; filtrate – an aliquot of filtrated supernatant; retenate – an aliquot of fraction retained on and in the filter washed out with 1.2 × Laemmli buffer.] C) Exosomes isolation was performed using the ExoQuick – TC™ kit (System Biosciences) from 5 ml of a CD222-positive urine sample of a healthy person. A supernatant fraction and a pellet exosomal fraction were loaded on the gel; ctrl – cell lysates from THP-1 cells. CD222 was detected as described in Figure 3. CD9 was detected using CD9 mAb followed by Anti-Mouse IgG-Peroxidase antibody as secondary antibody.

However, phenomena indirectly connected to disease progression could contribute to this result

Although we identified soluble CD222 present in sera, we found the full-length 250 kDa form in urine as an exosome component. In addition to soluble forms, proteins can be found in body fluids in extracellular vesicles such as exosomes, membrane vesicles and apoptotic bodies [26]. Typically, 50–400 nm bilayered-membrane vesicles [26] are released from many cell types, including red blood cells, platelets, lymphocytes, and dendritic endothelial and tumor cells [27–29]. They originate from internal vesicles of multivesicular bodies in various cell types and are released to the extracellular environment by fusion with the plasma membrane [30]. During exosome detection, researchers identified extracellular vesicle markers including the CD9, CD63 and CD81, tetraspanin family membrane proteins and also cytosolic proteins including heat shock protein 4 (HSP70) and the tumor susceptibility gene 101 [26]. This could explain the presence of such a large protein as CD222 at 250 kDa in urine, because exosomes can either pass through the tubular-glomerular system or be produced directly by urinary tract epithelium [30].

Finally, it is not yet determined which cells produce urine CD222-positive exosomes and what functions these vesicle perform, and therefore all proteins, not only CD222, should be evaluated with caution as urinary diagnostic biomarkers.

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