The Phosphorylated Sodium Chloride Cotransporter in Urinary Exosomes Is Superior to Prostasin as a Marker for Aldosteronism

Nils van der Lubbe, Pieter M. Jansen, Mahdi Salih, Robert A. Fenton, Anton H. van den Meiracker, A.H. Jan Danser, Robert Zietse, Ewout J. Hoorn

Abstract—Urinary exosomes are vesicles derived from renal tubular epithelial cells. Exosomes often contain several disease-associated proteins and are thus useful targets for identifying biomarkers of disease. Here, we hypothesized that the phosphorylated (active) form of the sodium chloride cotransporter (pNCC) or prostasin could serve as biomarkers for aldosteronism. We tested this in 2 animal models of aldosteronism (aldosterone infusion or low-sodium diet) and in patients with primary aldosteronism. Urinary exosomes were isolated from 24-hour urine or spot urine using ultracentrifugation. In rats, a normal or a high dose of aldosterone for 2, 3, or 8 days increased pNCC 3-fold in urinary exosomes (P<0.05 for all). A low-sodium diet also increased pNCC in urinary exosomes approximately 1.5-fold after 4 and after 8 days of treatment. The effects of these maneuvers on prostasin in urinary exosomes were less clear, showing a significant 1.5-fold increase only after 2 and 3 days of high-aldosterone infusion. In urinary exosomes of patients with primary aldosteronism, pNCC was 2.6-fold higher (P<0.05) while prostasin was 1.5-fold higher (P=0.07) than in patients with essential hypertension. Urinary exosomal pNCC and, to a lesser extent, prostasin are promising markers for aldosteronism in experimental animals and patients. These markers may be used to assess the biological activity of aldosterone and, potentially, as clinical biomarkers for primary aldosteronism. (Hypertension. 2012;60:00-00.)

Key Words: aldosterone • epithelial sodium channel • hypertension • primary aldosteronism

Urinary proteins originate from various sources. They may be derived from glomerular filtration, tubular secretion, shedding, glycosylphosphatidylinositol anchored protein detachment (eg, Tamm-Horsfall protein), or exosome secretion. Exosomes are low-density membrane vesicles that originate from multivesicular bodies. Urinary exosomes have sparked interest as potential biomarkers for human disease.1–3 The presence of urinary exosomes and a reproducible method for their isolation were reported in 2004 by Pisitkun and colleagues.4 Proteomic analysis of these exosomes showed that they contain many disease-related proteins; however, the question remained whether the presence of a given protein in urinary exosomes could provide information on physiological or disease processes in the kidney. Studies addressing this question analyzed urinary exosomes in patients with monogenic diseases, resulting in inactivity or overactivity of renal sodium transport proteins. For example, in Bartter and Gitelman syndrome, in which the sodium potassium chloride cotransporter and the sodium chloride cotransporter (NCC) are genetically inactivated, these proteins were also found to be absent or reduced in urinary exosomes.5,6 Conversely, Mayan et al found the abundance of NCC to be increased in patients with familial hyperkalemic hypertension, in which mutations in NCC-regulating kinases cause overactivity of this cotransporter.7 Thus, in these homogeneous groups, the expression of sodium-transport proteins in urinary exosomes correlated with what one would expect from their renal expression. The next step in assessing the potential of exosomes as urinary biomarkers is to analyze their performance in acquired disease. Therefore, in this study, we asked whether various forms of aldosteronism resulted in increased expression of aldosterone-sensitive proteins in urinary exosomes. To address this question, we used animal models of primary and secondary aldosteronism and also studied patients with primary aldosteronism. In the kidney, the 2 main sodium transporters activated by aldosterone are NCC and the epithelial sodium channel (ENaC).8,9 It therefore appears logical to study NCC and ENaC in urinary exosomes in different forms of aldosteronism. Esteva-Font et al, however, found no difference in the abundance of NCC in urinary exosomes...
exosomes of patients with salt-sensitive hypertension. Recently, it has become clear that the phosphorylated form of NCC (pNCC) represents the active form of NCC and that trafficking and phosphorylation of NCC can be regulated independently. Therefore, we propose that pNCC is a better reflection of NCC’s biological activity. ENaC is difficult to study in urinary exosomes because it is present in very low quantities. Instead, prostasin has emerged as an interesting surrogate marker of ENaC activity. Prostasin is a serine protease that can increase the activity of ENaC and is also sensitive to aldosterone. Here, we test the hypothesis that pNCC and prostasin in urinary exosomes are markers for aldosteronism.

Methods

Animal Studies
All animal protocols were approved by the Animal Care Committee of the Erasmus University Medical Center Rotterdam (EUR 127-11-01 and EUR 127-10-11). Three studies were conducted. In the first study, ten 15-week-old male Sprague-Dawley rats (Charles River) were adrenalectomized via a bilateral lumbodorsal incision and randomized to receive either high-dose aldosterone (100 μg/kg/d) or vehicle via osmotic minipump (subcutaneous insertion, Alzet). All rats also received glucocorticoid replacement (dexamethasone, 5 μg/kg/d) and an angiotensin receptor blocker (losartan, 10 mg/kg/d) to inhibit the effects of angiotensin II on pNCC and prostasin.

The second study was similar to the first but now included a third group of 3 rats that received a normal dose of aldosterone (50 μg/kg/d). In addition, this study lasted for 8 days instead of 3 days. In the second study, we also harvested the right kidney for immunoblotting analysis. In the third study, 12 rats were randomly assigned to receive a normal (0.001%) or low (0.002%) sodium chloride diet for 8 days (Harlan diets, Harlan Laboratories). In all 3 experiments, animals were housed in metabolic cages to collect 24-hour urine for isolation of urinary exosomes.

Studies in Patients
Five patients with primary aldosteronism and 4 patients with essential hypertension were randomly selected from an ongoing study on primary aldosteronism. Briefly, patients were eligible to participate in this study when they had uncontrolled hypertension despite the use of at least 2 antihypertensive drugs. All patients were subjected to volume expansion (2 liters of 0.9% sodium chloride [NaCl] during 4 hours) to analyze whether aldosterone was suppressible. Primary aldosteronism was defined as insuppressible plasma aldosterone (>235 pmol/L) after volume expansion. The patients whose posttest aldosterone was below 235 pmol/L were considered to have essential hypertension. In all patients, spot urine was collected under controlled circumstances for isolation of urinary exosomes. Urine sodium, potassium, and creatinine concentrations were measured as well.

Isolation and Immunoblot Analysis of Urinary Exosomes
Urinary exosomes were isolated as reported previously, with a more detailed protocol is provided as an online-only Data Supplement. Briefly, all urine samples were treated with a protease inhibitor before storage at −80°C; no phosphatase inhibitors were used. Urinary exosomes were isolated using a 2-step centrifugation process. First, urine was centrifuged at 17,000×g for 15 minutes at 37°C to remove whole cell membranes and other high density particles. Dithiothreitol was used to disrupt the Tamm-Horsfall polymeric network. Subsequently, the samples were subjected to ultracentrifugation at 200,000×g for 105 minutes at 25°C. The pellet that formed during ultracentrifugation was suspended in isolation buffer. Finally, the suspended pellets were solubilized in Laemmli buffer for immunoblot analysis. Immunoblotting of the urinary exosomes and kidney samples was performed as described previously (see also online-only Data Supplement). The antibody against NCC phosphorylated at threonine 38 was generated by 1 of the investigators (R.A.F.) and has been characterized previously. All other antibodies were purchased: prostasin (BD Biosciences) and NCC (Stessmarq Biosciences). For the animal studies, the complete volume of the 24-hour urine was used to isolate urinary exosomes, and, therefore, no normalization was used in the analysis. Coomassie blue staining was used to confirm that there were no differences in total protein contents. Conversely, for the patient study, the amount of sample loaded during immunoblotting was normalized by the urinary creatinine concentration.

Statistics
All data are expressed as mean ± standard error of the mean. Group comparisons were made using the unpaired Student t test or analysis of variances with a post hoc test, as appropriate. For analysis, the natural logarithm of the plasma aldosterone concentration was used to yield a normal distribution. P<0.05 was considered statistically significant.

Results

Three-Day Infusion of Aldosterone Increased pNCC and Prostasin in Urinary Exosomes
Adrenalectomized rats were infused with high-dose aldosterone or vehicle for 3 days. The differences in plasma aldosterone and urinary sodium between the 2 groups confirmed that both the adrenalectomy and the infusion of aldosterone were successful (Figure 1A). The infusion of aldosterone significantly increased pNCC, NCC, and prostasin in urinary exosomes on days 2 and 3 (Figure 1B). The abundance of pNCC in urinary exosomes increased 2.9±0.4-fold on day 2 and 3.2±0.3-fold on day 3 (P<0.05 for day 2 and P<0.01 for day 3). The abundance of NCC in urinary exosomes also increased 2.3±0.4-fold on day 2 and 1.8±0.4-fold on day 3 (P<0.05 for both). The abundance of prostasin in urinary exosomes increased 1.8±0.2-fold on days 2 and 3 (P<0.05 and P<0.01, respectively, Figure 1C).

Effects of an 8-Day Infusion of Aldosterone on pNCC and Prostasin in Kidney and Urinary Exosomes
Adrenalectomized rats were infused with vehicle, a normal or a high dose of aldosterone for 8 days. The plasma aldosterone concentrations were significantly different among the 3 groups (Figure 2A). Both the normal and high-aldosterone dose increased the abundance of pNCC and NCC in urinary exosomes (Figure 2B). pNCC increased 3.0±0.4-fold with the normal dose and 2.5±0.5-fold with the high dose (P<0.01 and P<0.05, respectively); NCC increased only with the normal dose (1.5±0.2-fold, P<0.05). Although pNCC and NCC in exosomes showed similar responses to aldosterone compared with the abundances of these proteins in the kidney, no direct correlation was observed (data not shown). In contrast to the 3-day infusion (Figure 1C), the 8-day infusion of aldosterone did not increase prostasin in urinary exosomes (Figure 2C). In the kidney, only the
high-aldosterone dose increased prostasin significantly (1.5±0.1-fold, *P*<0.05).

**A Low-Salt Diet Increased pNCC but Not Prostasin in Urinary Exosomes**

To induce a physiological increase in plasma aldosterone, 2 groups of rats were fed a normal sodium diet, after which 1 group was switched to a low-sodium diet. The low-sodium diet caused higher plasma renin activity and higher plasma aldosterone after 8 days (Figure 3A). When both groups were on the normal-sodium diet, the abundance of pNCC and NCC in urinary exosomes was similar (Figure 3B). The low-sodium diet increased pNCC in urinary exosomes on day 4 (1.7±0.2-fold) and day 8 (1.4±0.1-fold, *P*<0.05 for both). The low-sodium diet increased NCC in urinary exosomes on day 4 (1.5±0.1-fold) and day 8 (2.0±0.3-fold, *P*<0.05 for

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**Figure 1.** Effects of chronic aldosterone infusion in rats on the abundances of the phosphorylated form of sodium chloride cotransporter (pNCC) and prostasin in urinary exosomes. Adrenalectomized rats were treated with high-dose aldosterone (Aldo-H, 100 μg/kg/d) or vehicle (control) for 3 days. **A**, Plasma aldosterone and urine sodium. **B**, pNCC and NCC in urinary exosomes. **C**, Prostasin in urinary exosomes. *P*<0.05, **P**<0.01, ***P***<0.001 by Student t test.
pNCC and Prostasin Are Increased in Urinary Exosomes of Patients WithPrimary Aldosteronism

The characteristics of 5 patients with primary aldosteronism and 4 patients with essential hypertension are shown in Figure 4. Both groups had a similar degree of hypertension but had significant differences regarding the plasma aldosterone-to-renin ratio and the urine sodium-to-potassium ratio. The abundance of pNCC in urinary exosomes of patients with primary aldosteronism was higher in patients with essential hypertension (2.6±0.3-fold, *P*<0.05, Figure 4). The abundance of prostasin in urinary exosomes showed a trend toward being higher in the patients with primary aldosteronism (1.5±0.3-fold, *P*=0.07).

**Discussion**

In this study, we asked whether NCC and prostasin in urinary exosomes can be used as markers for primary and secondary aldosteronism. In our hands, both total and phosphorylated NCC were superior to prostasin as a marker for aldosteronism. In fact, the abundance of prostasin in urinary exosomes only increased during high-dose, short-term treatment with aldosterone (Figure 1C), whereas the abundance of pNCC in urinary exosomes was higher during aldosteronism, regardless of the duration, dose, or stimulus (Figure 5). Because the changes in pNCC were more pronounced than for NCC, pNCC appears the better marker. In addition, pNCC was increased significantly in urinary exosomes of patients with primary aldosteronism compared with patients with essential hypertension (Figure 4). In contrast, the increase in the abundance of prostasin in urinary exosomes of patients with primary aldosteronism was of borderline significance.

Several observations regarding the dynamics of pNCC and prostasin excretion in urinary exosomes during aldosteronism in this study merit discussion. For example, the results of the short-term infusion of aldosterone in rats suggest that it takes at least 1 day for both pNCC and prostasin to increase in urinary exosomes (Figure 1). This may be related to the time it takes aldosterone to increase transcription or posttranslational modification of these proteins. The long-term infusion of aldosterone in rats showed that a lower (physiological) dose of aldosterone was already sufficient to increase pNCC (Figure 2B). In fact, pNCC expression in urinary exosomes was slightly lower with the high dose. This suggests that the stimulatory effect of aldosterone on pNCC in urinary exosomes either saturates or that aldosterone escape has occurred, a defense mechanism known to reduce NCC in the kidney. Although the induction of secondary aldosteronism by a low-sodium diet also increased pNCC in urinary exosomes (Figure 3B), the magnitude of this effect was less than with aldosterone infusion (Figure 5). One could have predicted even higher pNCC in urinary exosomes during a low-sodium diet because this maneuver is likely to increase both plasma angiotensin II and aldosterone. We recently showed that angiotensin II can increase renal pNCC independently of aldosterone and that the combination of angiotensin II and aldosterone leads to an additive effect. Apparently, other factors limit pNCC excretion in urinary exosomes during a low-sodium diet. Furthermore, differences in the plasma aldosterone concentrations in the control groups should be taken into consideration (virtually absent in adrenalectomized rats versus ∼100 pg/mL in rats on a normalsodium diet, Figures 1A and 3A). Of interest, the increase in NCC in urinary exosomes we observed in this study with a low-sodium diet was similar to the increase Esteva-Font et al found for NCC in a separate study.

Our study suggests that pNCC was better than prostasin as a marker of aldosteronism, which could be explained by...
several factors. First, although prostasin is sensitive to aldosterone and can activate ENaC, this does not necessarily render it a direct marker of ENaC activity and, as such, it may not be a direct marker of distal sodium reabsorption. Second, because prostasin is also present in prostate epithelial cells, urinary exosomes may also contain prostasin from this source. Third, by using 2-dimensional electrophoresis, Olivieri and colleagues have previously shown that several subunits of ENaC increase in urinary exosomes during aldosterone exposure.

**Figure 3.** A low-salt diet increased phosphorylated form of sodium chloride cotransporter (pNCC) but not prostasin in urinary exosomes of rats. **A,** Plasma renin activity and plasma aldosterone. **B, pNCC** and NCC in urinary exosomes. **C,** Prostasin in urinary exosomes. *P*<0.05 by Student t test.
prostasin exist, only some of which are aldosterone-sensitive.\textsuperscript{12} Our prostasin results differ from those reported by Narikiyo and coworkers.\textsuperscript{13} They found that rats continued to increase urinary prostasin during 7 days of aldosterone infusion (from 1.5- to 4-fold), whereas we were unable to detect increased prostasin in urinary exosomes after 8 days of treatment (Figure 2). This difference may be explained by the use of a 3-fold higher aldosterone dose in the previous study and that their analysis of prostasin was performed on whole urine instead of urinary exosomes.

In recent years, the potential to use proteins in urinary exosomes as markers of diseases affecting the kidney has attracted much interest.\textsuperscript{1–3,26} Progress has been somewhat hindered by technical and normalization issues, but the proposal of uniform protocols has been a step in the right direction.\textsuperscript{18,27} We believe that the strength of this study was to combine a controlled experimental setting with a clinical setting for the analysis of urinary exosomes in aldosteronism. Because of the well-characterized actions of aldosterone on distal tubular sodium transport, aldosteronism appears especially suitable for analysis with urinary exosomes. Clinically, primary aldosteronism is important because recent studies suggest it to be a common condition among patients with resistant hypertension that is often difficult to diagnose.\textsuperscript{28} We do emphasize, however, that the primary aim of this study was to provide proof of principle that the abundance of aldosterone-sensitive proteins in urinary exosomes is increased during aldosteronism. The question whether pNCC in urinary exosomes has diagnostic potential in patients with primary aldosteronism remains to be determined. This will require larger and well-characterized groups of patients to be tested against a golden standard. In addition, the overall differences in NCC and pNCC between conditions were relatively mild even under controlled experimental conditions. Even if the sensitivity and specificity of pNCC in urinary exosomes would outweigh existing tests such as the ARR, the current method of exosome isolation is not suitable for clinical use.\textsuperscript{29} Instead, development of

\textbf{Figure 4.} Patients with primary aldosteronism show increased abundance of phosphorylated form of sodium chloride cotransporter (pNCC) and prostasin in urinary exosomes. \textbf{A}, Characteristics of patients with essential hypertension (EH) or primary aldosteronism (PA). \textbf{B}, Patients with PA show increased abundance of pNCC and prostasin in urinary exosomes. *$P<0.05$ by Student $t$ test or Mann-Whitney.

\textbf{Figure 5.} Summary of the performance of urinary exosomal prostasin and phosphorylated form of sodium chloride cotransporter (pNCC) as markers of aldosteronism. The densitometry values of the immunoblot analyses are shown. A value of 1 represents no difference compared with the control group. *$P<0.05$, **$P<0.01$ vs control.
an enzyme-linked immunoassay for pNCC would be an attractive alternative. This study suggests several directions for future research. One obvious next step will be to evaluate how pNCC and prostatin in patients with primary aldosteronism respond to treatment with either mineralocorticoid receptor antagonists or adrenalectomy. Narikiyo et al showed that urinary prostatin decreased in 3 patients with primary aldosteronism who had undergone adrenalectomy.13 Similarly, Olivieri et al showed that prostatin decreased in normotensive subjects with aldosteronism owing to a low-sodium diet who were subsequently treated with spironolactone.12 It would also be informative to know whether other commonly used antihypertensive drugs such as diuretics, angiotensin-converting enzyme inhibitors, or angiotensin receptor blockers affect urinary exosome excretion. Because total NCC increased, it would be important to know whether NCC mRNA is also present in urinary exosomes and if it behaves similarly. If so, it could be a more sensitive marker because the signal can be amplified. In the kidney, however, previous studies found that the protein and mRNA abundances of NCC did not always correlate.21,22 Finally, given the recent insights into the effects of angiotensin II on distal tubular sodium transport, it would be important to compare urinary exosomal pNCC and prostatin in patients with primary and secondary aldosteronism.

In conclusion, in urinary exosomes of animals and patients, pNCC was superior to prostatin as a marker of aldosteronism. These results justify further evaluation of the applicability of urinary exosomes as a diagnostic tool in primary aldosteronism and, possibly, other forms of hypertension. Furthermore, pNCC and, to a lesser extent, prostatin may be used experimentally as a noninvasive method to analyze the biological action of aldosterone in the kidney.

Perspectives

In hypertension, urinary exosomes could be applied as a diagnostic test for primary aldosteronism, to evaluate salt-sensitivity or the response to antihypertensive drugs acting on the kidney. Our study can be regarded as proof of principle that analysis of urinary exosomes can be applied to aldosteronism. Subsequent validation studies will be necessary to define their use in experimental or clinical settings.30

Sources of Funding
Dr Hoon is supported by grants from the Erasmus Medical Center, Dutch Kidney Foundation (KJBP 08.004), and Netherlands Organisation for Scientific Research (Veni). Dr van der Lubbe is supported by The Netherlands Foundation for Cardiovascular Excellence. Dr Jansen is supported by a grant from the Dutch Kidney Foundation (C05-2151).

Disclosures
None.

References


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**Novelty and Significance**

**What Is New?**
- The phosphorylated form of the sodium chloride cotransporter, a salt transporter in the kidney, is increased in so-called urinary exosomes in experimental models of aldosteronism and in patients with the hypertensive disorder primary aldosteronism.

**What Is Relevant?**
- pNCC in urinary exosomes may be used as a noninvasive urinary biomarker for aldosteronism and, potentially, as a diagnostic test in primary aldosteronism.

**Summary**
Disease-associated proteins in the kidney are excreted in the urine in so-called exosomes. We tested 2 of these proteins in experimental and clinical aldosteronism. In urinary exosomes of animals and patients, we found that the phosphorylated sodium chloride cotransporter was superior to prostasin as a marker of aldosteronism.
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Hypertension. published online July 30, 2012;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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ONLINE SUPPLEMENT

The phosphorylated sodium chloride cotransporter in urinary exosomes is superior to prostasin as a marker for aldosteronism

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Protocol for urinary exosome isolation

Collection and storage of urine

- To collect 24 hour urine from rats, they were placed in metabolic cages and mineral oil (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the collection tubes to prevent evaporation.
- The total 24 hour urine volume was used for isolation of urinary exosomes.
- The following protease inhibitor cocktail was added per 50 ml of urine (all purchased from Sigma-Aldrich):
  - 1.67 ml 100mM NaN3
  - 2.5 ml (2,75 mg/ml) 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)
  - 50 µl (1 mg/ml) Leupeptin
- In patients, second morning urine samples were collected and a Complete Protease Inhibitor Tablet (Roche Diagnostics, Mannheim, Germany) was added. Twelve ml of urine was used for urinary exosome isolation.
- Urine samples were stored immediately after collection in –80°C.

Isolation of urinary exosomes

- The following isolation solution was used (all compounds were purchased from Sigma-Aldrich):
  - 10 mM triethanolamine (0.5ml of 1M stock)
  - 250 mM sucrose(25ml of 0.5M Stock)
  - ddH2O was added to 45ml
  - pH was adjusted to 7.6 with 1M NaOH
  - ddH2O was added to 50 ml
- To remove whole cell membranes and other high density particles, urine samples were centrifuged at 17,000 x g for 15 minutes at 24°C in 70 ml polycarbonate centrifuge bottles (Beckman Coulter, Woerden, The Netherlands) in an ultracentrifuge (Beckman L8-70M ultracentrifuge, Rotor 45 Ti).
- The supernatant was stored at room temperature (Supernatant 1).
- The pellet was resuspended in 360 µl (dithiothreitol 500 mg/ml) and 450 µl isolation solution and incubated for 5 minutes at 37°C and vortexed until the pellet was dissolved.
- The dissolved pellet was centrifuged again in at 17,000 x g for 15 minutes at 24°C in 10 ml polycarbonate tubes (Rotor 70.1 Ti).
- This supernatant was pooled with Supernatant 1 and centrifuged in 70 ml polycarbonate tubes at 200,000 x g for 1:54 minutes at 24°C.
- Th supernatant was discarded and the pellet was resuspended in 1.5 x Laemmli buffer and stored at –20°C for immunoblotting.

Immunoblotting

- Electrophoresis and immunoblotting were carried out using the Mini-PROTEAN Tetra Electrophoresis system (Bio-Rad laboratories, Hercules, CA, USA)
- Samples were preheated at 60°C for 15 min.
- For the animal studies 10 µl of sample was used. In the patient study the samples were adjusted for the urine creatinine concentration with 1 x Laemmli buffer to a final volume of 20µl.
- SDS/PAGE was carried out on a 8% polyacrylamide gel, and proteins were transferred to Immobilon®-P membranes (Millipore, Amsterdam, The Netherlands).
- The membranes were blocked in 5% milk and were probed overnight at 4°C with the antibody. The antibody against NCC phosphorylated at threonine 58 (1:500 in 5% Bovine Serum Albumin) was generated by one of the investigators (dr. R.A. Fenton, University of Aarhus). The other antibodies were obtained: NCC (Stressmarq biosciences, Victoria, Canada, 1:500 in 5% Milk) and Prostasin (BD biosciences, Breda, the Netherlands 1:500 in 5% Milk).

- After three washes (3 x 10 minutes in TBS-Tween 20), the membranes were incubated with the secondary antibody in 5% milk (Thermo Scientific, Rockford, IL USA, 1:3000) for 1 hour and washed again.

- For visualization blots were exposed to Pierce® enchanced chemiluminescent substrate and exposed to X-ray film (Kodak Biomax MR Film, Rochester, NY, USA).