

Syndrome de Gordon-like induit par les anticalcineurines : une approche clinique et biochimique

Ludovic Di Ascia

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Syndrome de Gordon-like induit par les anticalcineurines:

Une approche clinique et biochimique

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ABREVIATIONS

CHU : Centre Hospitalier Universitaire

BMI: Body Mass Index

CNI: Calcineurin inhibitor

CsA: Cyclosporin A

CYROI : Cyclotron Réunion Océan Indien

DBP: Diastolic Blood Pressure

DTT: Dithiothreitol

(e)GFR: (Estimated) Glomerular filtration rate

kDa: kilodalton

KT: Kidney transplant patients

L: Liter

M.A.N.G.U.E: Major Activation of NCC in Graft Urinary Exosomes

min: Minutes

ml: Milliliter

mmol: Millimole

NODAT: New Onset Diabetes After Transplantation

OS: Optional Step

PPI: Proton Pump Inhibitor

pNCC: phosphorylated NCC

SBD: Systolic Blood Pressure

TMP-SMX:Trimethoprim/sulfamethoxazole

THP: Tamm-Horsfall protein

TSG101: Tumor Susceptibility Gene 101

WB: western blot

WNK: With No Lysine Kinase

µl: Microliter

GENESE DU PROJET

L'histoire du projet débuta le mercredi 3 février 2016. Je présentais, lors de la séance hebdomadaire de bibliographie du CHU de Bordeaux, un article de Hoorn et al paru dans Nature en 2011[1]. Cet article liait, chez la souris, prise d'anticalcineurines, augmentation d'expression des transporteurs rénaux NCC et pNCC, et hypertension artérielle. Ces données récapitulaient le phénotype clinique et la physiopathologie d'une maladie génétique appelée syndrome de Gordon. Dans cette dernière, l'activation de NCC entraine une hypertension artérielle, avec acidose métabolique et hyperkaliémie. Est-ce que ce « Gordon-like » existe également chez les patients greffés sous anticalcineurines ? Dans ce cas, devait-on privilégier chez les greffés hypertendus un traitement par diurétiques thiazidiques, inhibiteur de NCC, dont l'effet hypertensif pourrait être particulièrement efficace dans ce contexte ? Autant de questions qui ne trouvaient pas de réponse dans la littérature, et qui conduisirent le Pr Couzi à me dire à la fin de la présentation « eh bien, tu n'as qu'à en faire ta thèse ! ».

Je contactai le Pr Eladari, néphrologue à Paris et futur professeur réunionnais, qui se trouva idéalement intéressé par la problématique : Sa femme, le Dr Chambrey, et lui travaillaient sur le syndrome de Gordon. Courant 2016, nous posâmes les premiers jalons de ce qui constitua, plus tard, notre étude. A partir du flou d'un projet initial qui aurait testé les diurétiques thiazidiques chez les patients transplantés rénaux, nous avons progressivement préciser la nécessité de mieux identifier et caractériser ce syndrome « Gordon-like » sur lequel n'existaient, finalement, que peu de données. En décembre 2016, peu après mon retour à la Réunion, une orientation décisive fut prise lorsque le Dr Chambrey proposa d'utiliser les exosomes urinaires des patients afin de mesurer l'expression de NCC. Nous rajoutâmes à notre démarche l'évaluation de la pendrine, échangeur Cl^-/HCO_3^- dont le rôle dans l'acidose métabolique du patient Gordon venait juste d'être démontré par leur équipe.

Nous validâmes le projet auprès du comité de protection des personnes et inclûmes des patients transplantés rénaux à trois mois de greffe à Bordeaux et à la Réunion. Les techniques d'isolation d'exosomes et de quantification furent réalisées lors de mon année recherche qui s'acheva fin octobre dernier.

Ce projet s'intitule M.A.N.G.U.E (Major Activation of NCC in Graft Urinary Exosomes).

WHAT ARE EXOSOMES?

Extracellular vesicles originating from cells have been identified around 30 years ago. Most classifications use the mode of biogenesis to distinguish extracellular vesicles into apoptotic cell-derived vesicles, microvesicles or exosomes. Exosomes are the smallest ones, with a size ranging from 30 to 150 nanometers[2]. They are generated by reverse budding of the limiting membrane of early endosomes, termed as multivesicular bodies, and secreted by fusion of the multivesicular bodies with the cell membrane[3]. Exosomes contain numerous factors issued from the parent cells, such as proteins, lipids, and even genetic materials (miRNA, mRNA or DNA)[3]. The membrane, composed of a bilayer of lipids, contain surface receptors that can mediate organ-specific release of this material[4]. Consequently, they may play a key role in cell to cell communication, either in healthy or disease state[2]. Several studies report exosomes influences on physiological processes, such as immune modulation, hemostasis, angiogenesis, matrix modulation or tissue regeneration, and diseases, from cancer to kidney diseases[5].

As they can carry markers of the parent's cells, urinary exosomes have been found very interesting to study diseases involving kidney transporters[6]. Indeed, collection of urine is non invasive and urinary exosomes can directly inform upon the state of expression of these transporters in the upward tubular cells[7]. For example, the abundance of the renal NaCl cotransporteur NCC has been found to be increased in urinary exosomes from Gordon syndrome patients[8], from patients with primary aldosteronism[9], and decreased in Gitelman patients[10], who have genetic defect in NCC.

ABSTRACT

Calcineurin inhibitors (CNI) are immunosuppressive treatments widely used in transplantation. In mice, CNI activate the renal NaCl cotransporter (NCC), and thereby they cause high blood pressure, metabolic acidosis and hyperkalemia. This association of symptoms is also observed in patients suffering Gordon syndrome, a genetic disease also caused by inappropriate stimulation of NCC. Recently, it has been reported that acidosis of Gordon syndrome also involves stimulation of the Cl^-/HCO_3^- exchanger pendrin. In humans, little is known about the prevalence of Gordon-like syndrome phenotype in patients treated with CNI. Interestingly, NCC and pendrin expression can be assessed by immunoblots using urinary exosomes, small extracellular vesicles that reflect the composition of the plasma membrane of their parental cells. We designed two studies including incident CNI-treated kidney transplants 3 months after transplantation: a retrospective study was designed to determine the prevalence of Gordon-like phenotype, then a prospective one to evaluate NCC and pendrin expression in urinary exosomes. 172 patients were included in the retrospective study: 26% exhibited Gordon-like syndrome phenotype. Then, 58 patients were prospectively included and compared to 58 healthy volunteers. Again ~ 24% of kidney transplant recipients exhibited the Gordon-like phenotype. We show, however, that KT with Gordon-like phenotype do not exhibit a significant increase in NCC, pNCC or pendrin abundance. Furthermore, we show that CNI-treated KT have a marked down regulation of NCC, pNCC protein abundance in urinary exosomes compared to healthy volunteers.

Key words: Calcineurin inhibitors - Gordon like syndrome – Urinary exosomes – NCC – Pendrin

Les anticalcineurines sont les immunosuppresseurs les plus utilisés en transplantation rénale. Chez la souris, ils activent le co-transporteur rénal sodium chlore NCC, entrainant un phénotype associant hypertension, acidose métabolique et hyperkaliémie. Ce phénotype est également observé dans une maladie génétique appelée syndrome de Gordon, causée par une stimulation inappropriée de NCC. Récemment, il a été montré que l'acidose dans le Gordon impliquait une stimulation de l'échangeur Cl^-/HCO_3^- pendrine. Chez l'homme, il existe peu de données sur la prévalence d'un phénotype Gordon-like syndrome chez les patients traités par anticalcineurines. De manière intéressante, l'expression de NCC et/ou de pendrine peut être appréhendée par immunoblot en utilisant les exosomes urinaires, qui sont des vésicules extracellulaires dont le contenu dépend de l'activité des cellules émettrices. Nous avons mené deux études qui ont inclus des patients greffés rénaux incidents trois mois après la greffe : Une étude rétrospective pour déterminer la prévalence du phénotype Gordon-like, puis une prospective pour évaluer l'expression de NCC et de pendrine dans les exosomes urinaires. 172 patients ont été inclus dans l'étude rétrospective, dont 26 % présentait le phénotype. Nous avons ensuite recruté 58 patients et 58 volontaires sains. A nouveau, 24 % des greffés présentait le phénotype Gordon-like. Nous montrons, cependant, que les greffés ayant ce phénotype ne présentent pas d'augmentation d'abondance de NCC, pNCC ou pendrine. De plus, nous montrons que les patients greffés sous anticalcineurines ont une abondance significativement moindre de NCC, pNCC dans les exosomes urinaires comparativement aux volontaires.

Mots clefs : Anticalcineurines – Syndrome de Gordon-like – Exosomes urinaires – NCC – Pendrine

INTRODUCTION

Since the 80's, calcineurin inhibitors (CNI) are the key drugs used in immunosuppressive protocol[11]. CNI include two molecules: Cyclosporin A (CsA), which binds to cytoplasmic cyclophilin, and tacrolimus, which binds to cytoplasmic FKBP12. After formation, the complex binds to calcineurin, a calcium- and calmoduline-dependent serine-threonine phosphatase. Calcineurin is implicated in the activation and nuclear migration of NFAT, a transcription factor involved in the transcription of genes regulating the immune response of T lymphocytes. The calcineurin-NFAT axis is, however, not specific of immune cells[12] and FKBP12 is involved in various cellular processes[13]. Thus, the use of CNI is associated with numerous adverse events, including high blood pressure and metabolic acidosis[14].

High blood pressure and metabolic acidosis are important complications for physicians, because both of them are associated with higher risk of allograft failure and reduced patient survival[15], [16]. New onset or aggravated high blood pressure after kidney transplantation is observed in more than 80% patients, with reduced incidence in patients with CNI-free regimen[15],[17]. Metabolic acidosis has been less frequently reported in studies with ~ 58% of patients affected[18]. Numerous studies have been performed to elucidate the mechanisms of CNI toxicity in kidney transplant patients (KT). Recent findings from animal models showed that, CNI-induced high blood pressure involves activation of the thiazide-sensitive NaCl cotransporter NCC in the distal convoluted tubule[1],[19]. Interestingly, CNI-treated mice also exhibited metabolic acidosis and trend to hyperkalemia, mimicking the phenotype of Gordon syndrome[1]. Gordon syndrome, also known as pseudohypoaldosteronism type II, or familial hyperkalemic high blood pressure, is characterized by the association of high blood pressure, metabolic acidosis, hyperkalemia and high sensitivity to thiazides diuretics[20]. It is a rare genetic disorder caused by mutations in genes coding for two serine threonine kinases from the With-No-lysine Kinase family (WNK) WNK1 and WNK4 or in genes modulating the degradation of WNKs. These mutations all lead to a gain of function of NCC by increasing its phosphorylation[21]. The latter favors renal NaCl retention and ultimately increases blood pressure. Interestingly, tacrolimus, in mice, alters the WNK pathway with enhanced expression of WNK4[1],[22]. Hence, the author concluded that CNI provoke an "acquired" Gordon syndrome[1]. Recently, using a mouse model of WNK4-dependent Gordon syndrome[23], our group has demonstrated that while abnormal regulation of NCC causes high blood pressure of Gordon syndrome, metabolic acidosis is caused by abnormal upregulation of pendrin, a Cl^-/HCO_3^- exchanger of the collecting duct involved in renal excretion of bicarbonate[24]. Whether pendrin is abnormally regulated and might cause metabolic acidosis in humans or mice treated with CNI is unknown.

It is not clear whether CNI in humans, like in mice, increase phosphorylation of NCC causing a “Gordon-like syndrome”. This is an important question because thiazides are not generally used in KT, while they are the most effective treatment of Gordon syndrome.

Our hypothesis was that NCC, pNCC and pendrin are abnormally regulated in KT treated with CNI, provoking thus high blood pressure, metabolic acidosis and hyperkalemia. To test our hypothesis, we first assessed in a retrospective cohort of CNI-treated KT 3 months after transplantation the prevalence of the typical features of Gordon-like syndrome. Then, we performed a prospective study evaluating NCC, pNCC and pendrin abundance in urinary exosomes isolated from the urine of KT treated with CNI three months after transplantation.

METHODS

Retrospective cohort of kidney transplants

We included all kidney transplants using CNI who underwent medical visit three months after transplantation from April 2017 to April 2018 in Bordeaux Transplant Center. Blood pressure was recorded once in routine clinical practice, blood and urines tests were performed in the morning. Patients with estimated Glomerular Filtration Rate (eGFR) < 15 ml/min/1.73m2 were not included. High blood pressure was defined as Systolic Blood Pressure (SBP) ≥ 140 mmHg and/or Diastolic Blood Pressure (DBP) ≥ 90 mmHg or need of antihypertensive medications. Metabolic acidosis (also called indifferently acidosis in this manuscript) was defined as a blood [HCO_3^-] < 22 millimole (mmol)/L and/or the need of an alkaline supplementation either as alkaline pills or prescription of alkaline water. Hyperkalemia was defined as plasma potassium > 4.5 mmol/L or by the need of a potassium chelating agent. “Gordon-like” syndrome phenotype was the association of high blood pressure along with metabolic acidosis and hyperkalemia. Uncontrolled high blood pressure was defined as SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg at inclusion despite antihypertensive medication. Cardiovascular disease included history of myocardial infarction, congestive heart failure, peripheral artery disease, abdominal aortic aneurysm or stroke. Body Mass Index (BMI) was expressed in kg/m². A diagnosis of diabetes after transplantation was defined as New Onset Diabetes After Transplantation (NODAT). Expanded criteria donor was defined as age ≥ 60 years, or age 50-59 years plus two of the following: cerebrovascular accident as the cause of death, preexisting high blood pressure, or terminal serum creatinine greater than 1.5 milligrams/deciliter. Delayed graft function was defined as the requirement of dialysis within the first week of transplantation. Proteinuria was defined as a proteinuria/creatininuria ratio > 50milligrams/mmol.

Prospective cohort: M.A.N.G.U.E study

Patients, design and setting

M.A.N.G.U.E study was a prospective multicentric observational study conducted in two sites: Bordeaux university hospital, France and Saint Denis university hospital, la Réunion, France. Enrollment occurred from April 14, 2018 through August 31, 2018.

All participants gave their informed consent and the study was approved by institutional review board (Comité de Protection des Personnes Ile de France X, clinical trial number NCT03503461).

In our center, kidney transplants undergo three months after transplantation either a hospitalization for a protocol biopsy or a systematic routine day hospital evaluation. At that time, all patients who were using CNI were eligible for enrollment. We excluded patients who had been using thiazide diuretics or aldosterone antagonists after transplantation, which can interfere with NCC quantification, and patients with significant artery stenosis of the graft previously diagnosed at systematic doppler ultrasonography. Additional exclusion was done for patient with nephrostomy, or acute disease at the time of inclusion. Healthy volunteers with no treatment and no history of high blood pressure, kidney or metabolic disorders, were recruited as controls in Reunion island.

For kidney transplants, clinical evaluation, blood pressure record, according to European Society of Cardiology guidelines, blood and urine testing were performed in the morning. Routine blood and urine analysis were performed in each center, accordingly to the protocol of these centers. Samples of ~50 ml of urine were also collected for exosomes study. For healthy volunteers, samples of ~50 ml of urine were collected in the morning. Collection of urine in the morning could avoid bias from circadian rhythm[25]. Pierce protease inhibitors tablets were immediately added to the urine to prevent degradation of proteins in exosomes, and samples were stored at -80 °C until use. Urine collected in Bordeaux were transferred to la Reunion center for exosome analysis.

Urinary Exosome Isolation

Urinary exosome isolation was performed using variations of the technique of Pisitkun *et al.*[26]. Procedure of optimization of our exosome's isolation protocol is shown in supplemental data section (see ancillary study).

Frozen urine samples were thawed and vigorously vortexed before transfer of 50-ml sample in Nalgene tubes for analyses. Another 1ml was aliquoted for creatinine measurement and frozen at −20°C until use. The 50-ml samples were subjected to 17 000 g (11 498 rpm) centrifugation (Eppendorf Centrifuge 5810R, Rotor F34-6-38) at 4°C for 12 min to eliminate whole cells and debris and supernatants were kept. Pellet was resuspended in isolation buffer (10 mM Triethanolamine, 250 mM Sucrose, distilled water) for 5mn. This resuspension was followed by a new 17 000 g (13 145 rpm) centrifugation (Eppendorf Centrifuge 5424R; rotor FA-45-24-11) for 4 min at 4°C. The supernatant was kept and pooled with the first one. We then performed two ultracentrifugation (Optima L-80XP; Beckman Coulter, Inc., 70 Ti rotor) in 25 ml high-speed tube (thick wall, polycarbonate tube #355618, Beckman) at 52 000 rpm (277 820 g) for 60 min at 4°C. Pellet were then resuspended in 100 µl of 1X Laemmli buffer during 30 min and frozen at −20°C until use.

Immunoblotting

Loading of urinary exosomes sample of each subject on SDS-PAGE gel was normalized to urinary creatinine concentration. Validation of the normalization procedure is shown in supplemental data section (see ancillary study). Two Internal standards were loaded on each blot (Figure 1). SDS-PAGE was carried out with 7.5% polyacrylamide gels using Xcell SureLock electropheris apparatus for mini gels (Novex) under a constant voltage of 125 V for 2 hours or Criterion cell electrophoresis apparatus for midi gels (BioRad) under a constant current of 200 mA for 55 min. Proteins were then transferred to nitrocellulose membranes (Protran premium 0.45 µm, Amersham, USA) using XCell blot module for mini gels (Novex) under a constant voltage of 25 V for 1.5 hours at 4°C or Criterion blotter for midi gels (BioRad) under under a constant current of 100 mA for 45 min at 4°C. Midi gels Ponceau Red staining was performed to ensure good quality protein transfer. Membranes were blocked with 5% non-fat dry milk in PBS for 1 hour at room temperature and probed with antigen-specific primary antibodies diluted in PBS containing 1% non-fat dry milk overnight at 4°C. Blots were rinsed twice then washed 5 x 5 min with PBS containing 0.1% tween 20. Blots were incubated with species-specific secondary antibodies conjugated to horse-radish-peroxydase in PBS containing 5% non-fat dry milk for 2 hours at room temperature. Blots were rinsed twice then washed 5 x 5 min with PBS containing 0.1% tween 20. Signals were visualized using enhanced chemiluminescence (Supersignal West pico, Thermo Fischer

Scientific, Waltham, MA, USA) using the Imager 600 (GE Healthcare). Both the dimeric and monomeric forms of the NCC bands on the blots were quantified together (around 260 and 130 kDa) as well as pNCC (320 and 150 kDa). Pendrin was measured as the single dominant band at 130 kDa. The major bands of Tumor Susceptibility Gene 101 (TSG101) were measured at 45 kDa. Relative quantitation of the band densities from immunoblots was carried out with ImageQuant TL software (GE Healthcare) as previously described[27].

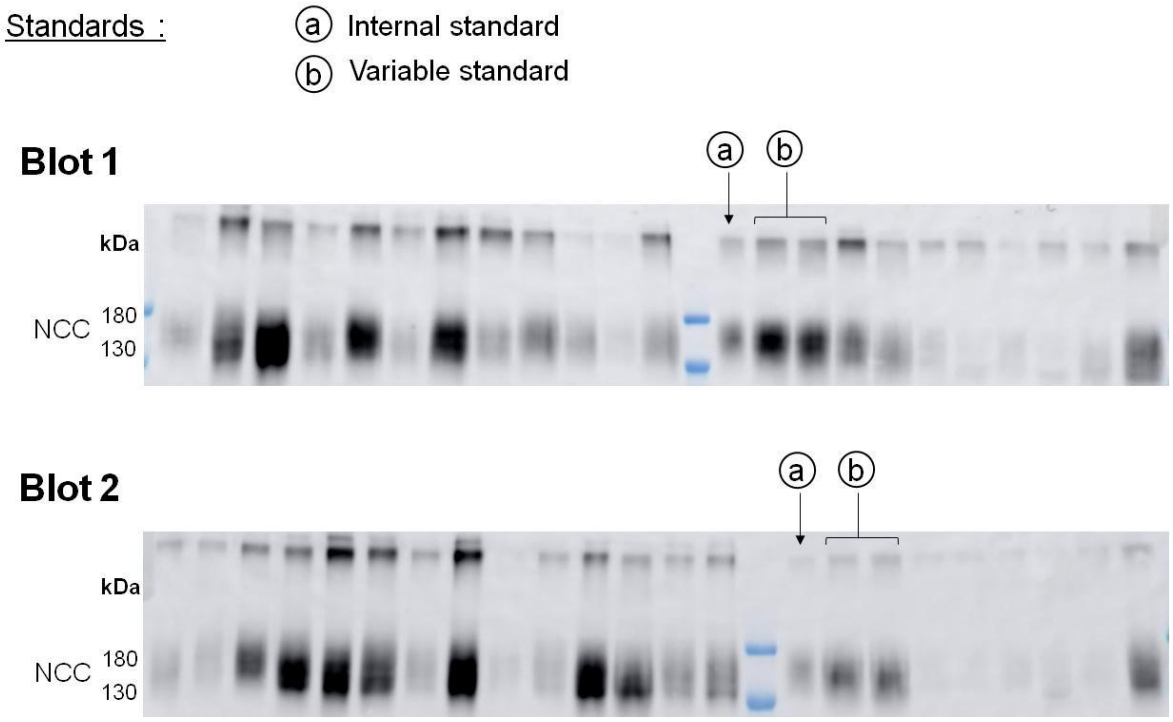


Figure 1: *Description of the internal standards used to take into account the variability of the western blot procedure from one membrane to another, and the wide range of urine dilution between samples of KT and volunteers.*

a. 5µL of exosomes of the same control were loaded on each blot.

b. A variable volume of exosomes of the same control was loaded twice on each blot according to the lowest creatininuria of the blot.

- Use of an internal standard

Signal intensity of the band recorded using enhanced chemiluminescence depends on the time of exposure, which is automatic and depends on the average signal intensity of the membrane. To adjust for this, we loaded on each blot 5 µL of exosomes issued from one sample used as “internal standard”. For this sample, different band intensities were obtained from one membrane to another. The highest intensity recorded was defined as equivalent to 1, and a fold change of this band intensity on each blot was calculated. We then applied the fold change of the internal standard of each blot to the band intensities of the whole blot to all other samples. This adjustment corrects for the variability of the western blot and

chemoluminescence techniques and allow comparison of densitometric analyses of data from the different samples analyzed in different blots.

- Second normalization with variable standard.

In order to ensure loading of similar amount of proteins the volume of each sample to load was determined according to the urinary concentration of creatinine assuming that the amount of creatinine excreted by 24h is constant. Since the value of urinary creatinine concentration was wide (from 16 to 230 mg.dl) reflecting very variable urine output. And since the volume that can be loaded by well is limited to a minimum of 2µl and a maximum of 15 µl, samples with similar range of creatininuria were runned on same blot. For each blot, the sample with the lowest creatininuria was assigned to a loading volume of 15 µL and the volume of the other samples was determined according to their urinary creatinine volume. To adjust between blots, each blot included internal standard run in duplicate. This standard had a creatininuria of 101 mg.dl and, depending of the creatininuria of the other samples, was loaded from 2.37 µl to 15 µl. For each band the mean of the two bands was calculated. The highest intensity recorded, which, expectedly was for the volume loading of 15 µl, was defined as equivalent to 1, and densitometric analyses of data were expressed as a fraction of this internal standard.

Antibodies

The following antibodies were used: rabbit anti-total human NCC (Millipore AB3553, 1: 2000); rabbit anti-phosphorylated NCC (pT53NCC) 1: 10000 (kindly provided by Dr J Loffing, University of Zurich, Switzerland), rabbit anti-mouse pendrin 1: 1000 (kindly provided by Dr P Aronson, Yale University, New Haven, USA), TSG101 (rabbit monoclonal anti human TSG 101 1: 1000 (Abcam Ab125011, USA). Secondary antibodies used were peroxidase-conjugated goat anti-rabbit 1: 5000.

Measurement of urinary creatinine

All urinary creatinine were measured in same laboratory in La Réunion using Jaffe's method[28].

Statistical analysis

Data were expressed as medians (first quartile, third quartile). Qualitative data were expressed as frequency and percentages. Bivariate comparisons of quantitative data were assessed using Mann–Whitney test. Bivariate comparisons of qualitative data were assessed using Fisher's test. For multivariate analysis, we performed logistic regression with backward selection. All covariates with a *p*-value < 0.20 in the bivariate analysis were entered in the

model and removed if they did not reached the statistical significance. Densitometric analyses of data were normalized to the mean of controls that was defined as 100% and results were expressed as mean ± SD. Statistical significance was assessed by analysis of variance (ANOVA) after normalization using natural logarithm. All analyses were performed using Prism 5 software (GraphPad Software Inc, La Jolla, CA, USA) or SAS 9.4 software (SAS Cary Inc USA). A two-tailed alpha level of 5% was considered as significant.

RESULTS

Gordon-like syndrome features in a large retrospective cohort of kidney transplant patients

To test whether CNI induce a Gordon-like syndrome, we first assessed in a retrospective cohort of KT the prevalence of the combined parameters high blood pressure/metabolic acidosis/hyperkalemia.

Characteristics of the patients:

Between 04/04/2017 and 04/13/2018, we screened all KT using CNI who had an eGFR ≥ 15 ml/min/1.73 m2 and underwent medical visit three months after transplantation in Bordeaux. Among 194 KT, 172 were included (Figure 2). Characteristics are shown in Table 1. KT were mostly men (70.9%) with a median age of 57.5 (47-68) years, BMI was 23.7 kg/m² (21.1-27.8). At inclusion, around 30% were diabetics. eGFR was 44.5 (30-55) ml/min/1.73 m². Most patients received a beta-blocker as antihypertensive treatment (44.8% of patients), only 7% received a thiazide diuretic.

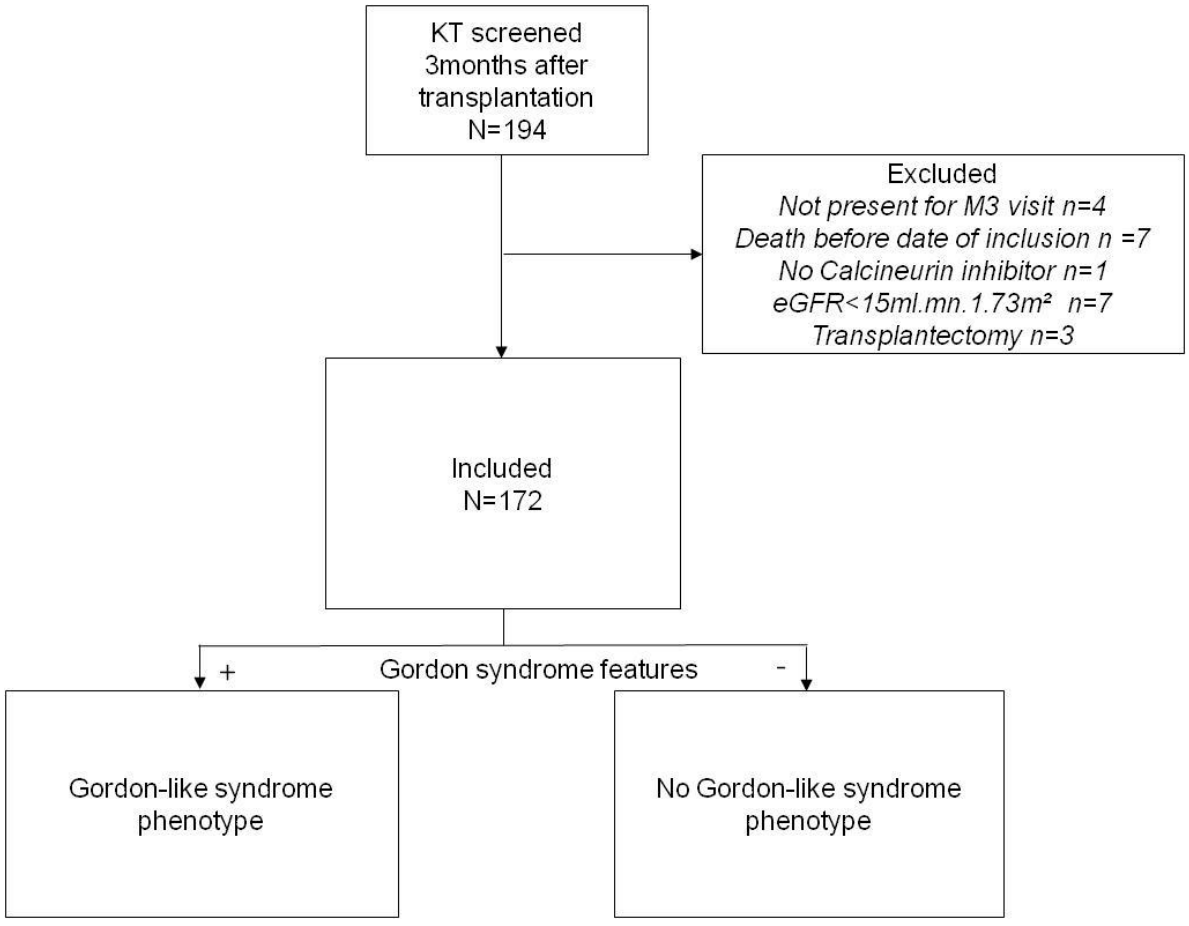


Figure 2: Flow chart of the retrospective cohort of kidney transplants

Table 1: Clinical and laboratory characteristics of the retrospective cohort at inclusion (n=172)

Demographic characteristics Δ	
Age, median (Interquartile Range (IR)), years	57.5 (47-68)
Sex ratio, M/F, N (%)	122/50 (70.9/29.1)
BMI, median (IR), kg/m²	23.73 (21.1-27.8)
Rank of transplantation, N (%) **1** **>1**	 145 (84.3) 27 (15.7)
Nephropathy, N (%) Glomerular Tubulo-interstitial Vascular Diabetes Polycystic kidney disease Others (including unknown**)**	 47 (27.3) 23 (13.3) 17 (9.8) 18 (9.8) 32 (18.6) 35 (20.3)
Living donors, N (%)	36 (20.9)
Delayed Graft function, N (%) ε	24 (13.9)
Expanded criteria donors, N (%) κ	82 (47.7)
Cardiovascular disease, N (%) ϕ	42 (24.4)
High blood pressure before transplantation, N (%)	121 (70.3)
Diabetes (including NODAT)	51 (29.6)
Immunosuppressive treatment CNI-based (CsA/Tacrolimus), N (%) Mycophenolic acid, N (%) Corticosteroids, N (%)	 14/158 (8.1/91.9) 156 (90.7) 125 (72.7)
TMP-SMX use, N (%)	141 (82)
PPI use, N (%)	94 (54.7)
Biopsy-proven rejection until inclusion, N (%)	8 (4.7)
SBP, median (IR), mmHg	135 (127-146)
DBP, median (IR), mmHg	79 (70-84)
High blood pressure, N (%) Untreated high blood pressure, N (%)	145 (84.3) 9 (5.2)
Uncontrolled high blood pressure, N (%) η	72 (41.8)
Antihypertensive pills use, N (%) Calcium channel blocker angiotensin- converting enzyme inhibitors or angiotensin receptor blockers Beta-blocker Diuretic except thiazide Thiazide Others	 74 (43) 61 (35.5) 77 (44.8) 34 (19.8) 12 (7) 18 (10.5)
Plasma bicarbonate, median (IR), mmol/L	21 (19-23)
Acidosis, N (%) Plasma bicarbonate < 22 mmol/L, N (%) Plasma bicarbonate ≥ 22 mmol/L with alkaline supplementation, N (%)	127 (73.8) 88 (51.2) 39 (22.7)
Plasma potassium, median (IR), mmol/L	4.3 (4.05, 4.6)
Hyperkalemia, N (%) Plasma potassium >4.5 mmol/L, N (%) Plasma potassium ≤4.5 with potassium chelating agent use, N (%)	66 (38.4) 65 (37.8) 1 (0.6)
Potassium chelating agent use, N (%)	8 (4.7)
eGFR, median (IR), ml/min/1,73m2	44.5 (30-55)
Presence of proteinuria, N (%) γ	56 (33.7)

Δ Data not shown include previous transplantation (except kidney), HLA immunization, Induction protocol, Diabetes before transplantation, number of antihypertensive pills before transplantation, total ischemia of the graft, hemoglobin, blood urea, creatinine, residual tacrolimus.

Interquartile range were expressed as median (1^{st} and 3^{rd} quartiles)

ε Delayed graft function was defined as the requirement of dialysis within the first week of transplantation.

κ Expanded criteria donor was defined as age > or = 60 years, or age 50-59 years plus two of the following: cerebrovascular accident as the cause of death, preexisting High blood pressure, or terminal serum creatinine greater than 1.5 milligrams/deciliter

ϕ Cardiovascular disease included history of myocardial infarction, congestive heart failure, peripheral artery disease, abdominal aortic aneurysm or stroke

η SBP $\geq$ 140 mmHg and/or DBP $\geq$ 90 mmHg at inclusion

γ Proteinuria was defined as a proteinuria/creatininuria ratio > 50milligrams/mmol.

IR denotes Interquartile Range, BMI Body Mass Index, CNI CalciNeurin Inhibitors, CsA Cyclosporin A, TMP-SMX Trimethoprim/sulfamethoxazole, PPI Proton Pump Inhibitors, SBD Systolic Blood Pressure DBP Diastolic Blood Pressure and eGFR estimated Glomerular Filtration Rate.

Gordon-like syndrome is frequent in a retrospective cohort of kidney transplants:

High blood pressure was observed in 84.3% of patients, with only 9 patients having untreated high blood pressure at inclusion. Acidosis was found in 73.9% of patients, with 51.2% of patients exhibiting biological acidosis and 22.7% with normal plasma bicarbonate values but receiving alkaline supplementation. 38.4% of patients had hyperkalemia, and only one patient had plasma potassium $\leq$ 4.5 mmol/L but with the use of a chelating agent.

Among the 172 KT, 45 (26.2%) exhibited the full Gordon-like syndrome phenotype (high blood pressure, acidosis and hyperkalemia). The association of high blood pressure and acidosis without hyperkalemia was also found in 63 (36.6%) of patients. Interestingly, only 16.8%, 5.2% and 0% had high blood pressure, acidosis or hyperkalemia alone, respectively (Table 1 and Figure 3).

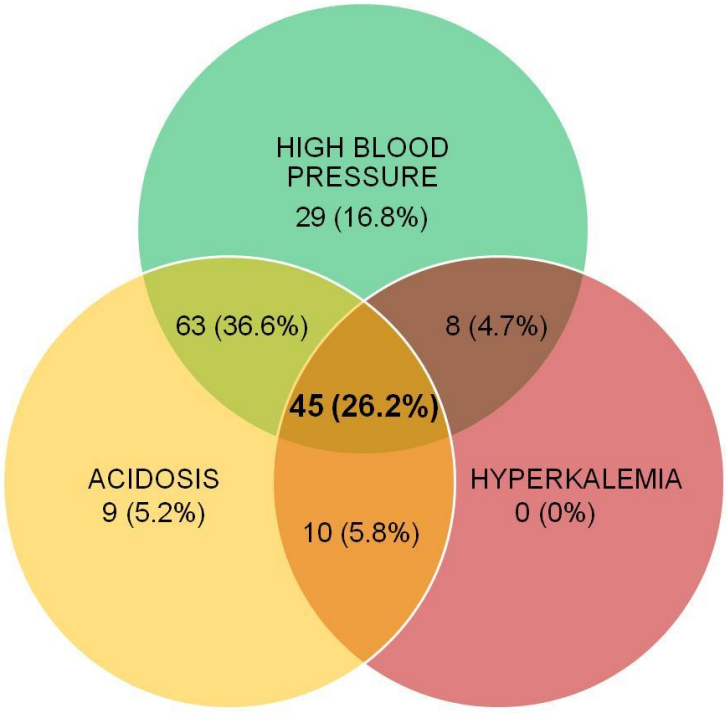


Figure 3: Distribution of high blood pressure, acidosis and hyperkalemia in the retrospective cohort of 172 kidney transplants 3 months after transplantation.

Gordon-like syndrome phenotype is associated with medications but remains independent from most of classical high blood pressure or acidosis-causing factors

We separated the KT according to the presence or not of Gordon-like syndrome phenotype and analyzed the distribution of the previously described characteristics. Data are shown in Table 2. In unadjusted analyses, the demographic characteristics of the two groups were similar with no difference in age, sex ratio, BMI. Patients with Gordon-like syndrome phenotype had more severe high blood pressure or acidosis. Hypertensive patients with Gordon-like syndrome phenotype had higher DBP (p=0.04) than hypertensive patients without the phenotype. Patients with Gordon-like syndrome phenotype were taking higher number of antihypertensive medications (p=0.04). Patients with acidosis and Gordon-like syndrome phenotype had lower plasma bicarbonate than patients with acidosis and without the phenotype (p=0.02). Gordon-like syndrome phenotype was associated with the use of beta-blocker (p=0.02), angiotensin converting enzyme inhibitors, or angiotensin receptor blockers (p=0.03) and diuretic (except thiazides) (p=0.01). After multivariate analysis, use of beta-blockers (p=0.02), angiotensin converting enzyme inhibitors or angiotensin receptor blockers (p=0.03) and diuretic (except thiazides) (p=0.01) remained associated with Gordon-like syndrome phenotype. All others factors known to classically cause high blood pressure, acidosis or hyperkalemia, including type of nephropathy, diabetes, delayed graft function,

cardiovascular disease, rejection, corticoids use, low eGFR or use of TMP-SMX, were not associated with Gordon-like syndrome.

Table 2: Univariate and multivariate analysis of factors associated with Gordon-like syndrome phenotype in the retrospective study, using logistic regression with backward selection.

Univariate analysis			
Characteristics	No Gordon-like syndrome phenotype* N=122	Gordon-like syndrome phenotype* N=45	P value
Age, median (interquartile range), years	58 (43-68)	56 (49-68)	0.92
Sex ratio, M/F, N (%)	85/42 (66.9-82.2)	44/8 (33.1-18.8)	0.05
Diabetes, N (%)	40 (31.5)	11 (24.4)	0.37
Biopsy-proven rejection, N (%)	5 (3.9)	3 (6.7)	0.43
Corticoides use, N (%)	94 (74)	31 (68,9)	0.51
TMP-SMX use, N (%)	106 (83.5)	35 (77.8)	0.39
Patients with high blood pressure, N (%) SBP, median (interquartile range), mmHg **DBP, median (interquartile range), mmHg**	122 (84.1) 137 (128-146) 77 (70-81)	20 (95.2) 142 (130-152) 80 (70-88)	0.32 0.06 **0.04**
Antihypertensive pills use, median (interquartile range), number **Beta-blocker** **angiotensin- converting enzyme inhibitors or angiotensin receptor blockers** **Diuretic except thiazides**	1 (0–2) 50 (39.4) 39 (30.7) 31 (24.4)	2 (1–2) 27 (60) 22 (48.9) 3 (6.7)	**0.04** **0.02** **0.03** **0.01**
Patients with acidosis, N (%) **Plasma bicarbonate, median (interquartile range)**	82 (64.6) 20 (19-23)	45 (100) 19 (18-21)	**<0.0001** 0.02
Patients with hyperkalemia, N (%) **Plasma potassium, median (interquartile range)**	20 (15.7) 4.7 (4.6-4.83)	45 (100) 4.8 (4.6-5)	**<0.0001** **0.42**
eGFR, (median (interquartile range))	45 (30-55)	42 (33-53)	0.67
Multivariate analysis			
Characteristics	Odds ratio (95% CI)		P value
Beta-blocker **angiotensin- converting enzyme inhibitors or angiotensin receptor blockers** **Diuretic except thiazides**	2.1 (1–4.5) 2.6 (1.3–5.4) 0.2 (0.1–0.7)		**0.04** **0.009** **0.01**

Quantitative data were expressed as median (1st and 3rd quartiles). Qualitative data were expressed as frequency and percentages. Bivariate comparisons of quantitative data were assessed using Mann–Whitney test. Bivariate comparisons of qualitative data were assessed using Fisher's test. Multivariate analyses were performed logistic regression with backward selection. All covariates with a p-value below 0.20 in the bivariate analysis were entered in the model and removed if they did not reached the statistical significance. P<0.05 was considered as significant.

**Gordon like syndrome phenotype: high blood pressure/acidosis/hyperkalemia*

TMP-SMX stands for Trimethoprim/sulfamethoxazole and eGFR estimated Glomerular Filtration Rate

Exosomes evaluation in kidney transplants: M.A.N.G.U.E cohort

Characteristics of the M.A.N.G.U.E cohort and Volunteers

Between April and august 2018, 78 patients were screened and eligible three months after transplantation. 12 were excluded, and 60 patients were initially enrolled in the study. 6 samples were lost during the exosome isolation procedure, thus, 6 additional KT were included to reach the initial goal. Of 60 KT, 2 couldn't be analyzed: one because of the presence of heavy proteinuria interfering with the immunoblot procedure, and one because no exosomes were detected. In 2 KT, pendrin was not detected. Of 60 healthy volunteers, 2 were not analyzed because they exhibited a polyuria (Figure 4).

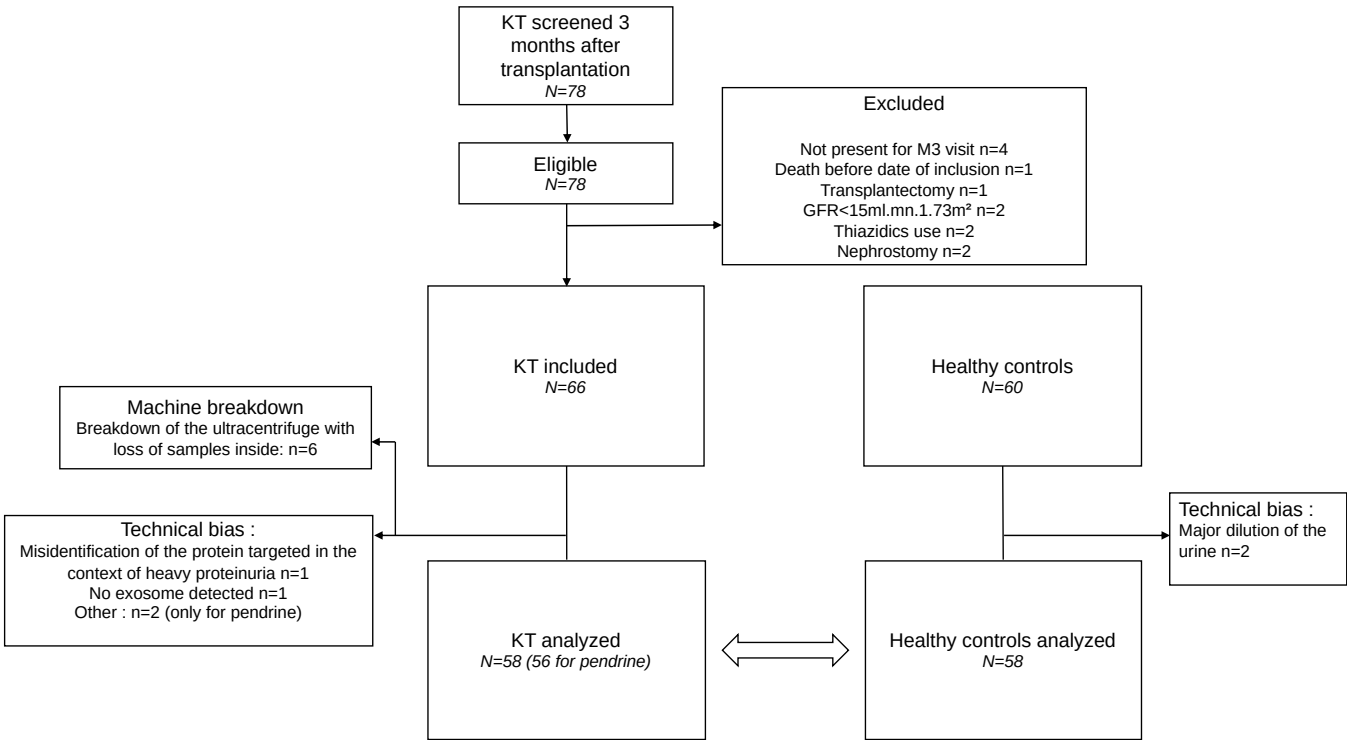


Figure 4: Flow chart of the M.A.N.G.U.E study

The baseline characteristics of the participants are presented in Table 3. One potassium value was discarded because the blood sample was hemolyzed.

Table 3: Clinical and laboratory characteristics of the MANGUE cohort at inclusion (n=58)

Demographic characteristicsΔ	
Age, median (Interquartile Range (IR)), years	48 (36-62)
Sex ratio, M/F, N (%)	35/23 (60.3/39.7)
BMI, median (IR), kg/m²	24.3 (22-28.5)
Rank of transplantation, N (%) **1** **>1**	 49 (84.5) 9 (15.5)
Nephropathy, N (%) Glomerular Tubulo-interstitial Vascular Diabetes Polycystic kidney disease Others (including unknown)	 17 (29.3) 6 (10.3) 3 (5.1) 6 (10.3) 7 (12.1) 19 (32.8)
Donor Age, median (interquartile range), years	54 (36-66)
Living donors, N (%)	6 (10.3)
Delayed Graft function, N (%) ε	6 (10.3)
Expanded critera donor, N (%) κ	22 (37.9)
Cardiovascular disease, N (%) ϕ	6 (10.3)
High blood pressure before transplantation, N (%)	44 (75.9)
Diabetes (including NODAT)	10 (17.2)
Immunosuppressive treatment CNI-based (CsA/Tacrolimus), N (%) Mycophenolic acid, N (%) Corticosteroids, N (%)	 1/57 (1.7/98.3) 53 (91.4) 35 (60.3)
TMP-SMX use, N (%)	47 (81)
PPI use, N (%)	27 (36.5)
Biopsy-proven rejection until inclusion, N (%)	2 (3.5)
SBP, median (IR), mmHg	134 (125-145)
DBP, median (IR), mmHg	83 (74-89)
High blood pressure, N (%) Untreated high blood pressure, N (%)	48 (82.8) 2 (3.4)
Uncontrolled high blood pressure, N (%) η	25 (43.1)
Antihypertensive pills use, N (%) Calcium channel blocker ACE or ARB Beta-blocker Diuretic except thiazide Others	 25 (46.6) 18 (31.03) 14 (24.1) 8 (13.8) 11 (19)
Plasma bicarbonate, median (IR), mmol/L	21 (19-23)
Acidosis, N (%) Plasma bicarbonate < 22 mmol/L, N (%) Plasma bicarbonate ≥ 22 mmol/L with alkaline supplementation, N (%)	43 (74.1) 33 (56.9) 10 (17.3)
Plasma potassium, median (IR), mmol/L	4,18 (3,9-4,7)
Hyperkalemia, N (%) $ Plasma potassium >4,5 mmol/L, N (%) Plasma potassium ≤4,5 with potassium chelating agent use, N (%)	22 (37.9) 22 (37.9) 0 (0)
Potassium chelating agent use, N (%)	2 (3.4)
eGFR, median (IR), ml/min/1.73m2	43.5 (31-60)
Presence of proteinuria, N (%) γ	9 (15.5)

Δ Data not shown include previous transplantation (except kidney), HLA immunization, Reactive panel antigen, Induction protocol, CMV status, Diabetes before transplantation, number of antihypertensive pills before transplantation, Donor sex, total ischemia of the graft, donor specific antibodies at inclusion, infection after transplantation and type of infection, tobacco use, white blood cells, neutrophils, lymphocytes, CD4 lymphocytes, CD8 lymphocytes, $\gamma\Delta$ lymphocytes, hemoglobin, platelets counts, blood sodium, chloride, proteins, venous pH, blood urea, creatinine, uric acid, total calcium, ionized calcium, phosphorus, magnesium, parathormone, vitamin d, residual tacrolimus, urine electrolytes.

Quantitative data were expressed as median (1^{st} and 3^{rd} quartiles).

ε Delayed graft function was defined as the requirement of dialysis within the first week of transplantation.

κ Expanded criteria donor was defined as age > or = 60 years, or age 50-59 years plus two of the following: cerebrovascular accident as the cause of death, preexisting high blood pressure, or terminal serum creatinine greater than 1.5 milligrams/deciliter

ϕ Cardiovascular disease included history of myocardial infarction, congestive heart failure, peripheral artery disease, abdominal aortic aneurysm or stroke

η SBP $\geq$ 140 mmHg and/or DBP $\geq$ 90 mmHg at inclusion

γ Proteinuria was defined as a proteinuria/creatininuria ratio > 50milligrams/mmol.

BMI denotes Body Mass Index, CNI CalciNeurin Inhibitors, PPI Proton Pump Inhibitors, CsA Cyclosporin A, TMP-SMX Trimethoprim/sulfamethoxazole, SBD Systolic Blood Pressure DBP Diastolic Blood Pressure, ACE or ARA2 angiotensin-converting enzyme inhibitors or angiotensin receptor blockers and eGFR estimated Glomerular Filtration Rate.

KT were mostly men (60.3%). Median age was 48 years (36-62). BMI was 24.3 kg/m² (22-28.5). 84.5% of patients were undergoing their first transplantation. Initial nephropathy was either not identified (32.8%), 30% was glomerular nephropathy and 10% had diabetic nephropathy. Around 10% of patients had living donors and 10% expanded criteria donors. Median age of donor was 54 (36-66). After transplantation, around 10% experimented delayed graft function. At inclusion, nearly 18% of patients were diabetics and 10% had a previous history of cardiovascular disease. More than 90% were taking Mycophenolic acid with CNI, and 60% corticosteroids. 81% were using Bactrim, and 36.5% Proton Pump Inhibitor (PPI). Antihypertensive medication included most frequently calcium channel blocker (46.6%). Patients had a eGFR of 43.4 ml/min/1.73m2 (31-60) and more than 15% had proteinuria. SBP and DBP were 134 mmHg (125-145) and 83 mmHg (74-89), respectively. Plasma bicarbonate concentration was 21 mmol/L (19-23) and potassium was 4.18 mmol/L (3.9-4.7).

Characteristics of the retrospective cohort and the M.A.N.G.U.E cohort were similar except for younger age (p=0.03), higher diastolic pressure (p=0.01), and less proteinuria (p=0.01) in the M.A.N.G.U.E cohort.

60 healthy volunteers from la Reunion were also included in our study. Both testified that they have no history of high blood pressure, kidney disease or metabolic disorders, and none were taking medications. Compared to M.A.N.G.U.E, median age was 3.5 years lower in volunteers (p=0.03) but there were no difference of sex and BMI (Table 4).

Table 4: Demographic characteristics of healthy volunteers and kidney transplants of M.A.N.G.U.E cohort

Characteristics	**Healthy volunteers N= 58**	**Kidney transplants N=58**	**P value**
Age, median (interquartile range), years Age < 39 years, N (%) Age 40-59 years, N (%) Age > 60 years, N (%)	44.5 (34-52) 22 (37.9) 34 (58.6) 2 (3.5)	48 (36-62) 17 (29.3) 24 (41.4) 17 (29.3)	**0.03**
Sex ratio, M/F, N (%)	35/23 (60.3/39.7)	35/23 (60.3/39.7)	1
BMI, median (interquartile range), kg.m2	22.9 (20.9-25.7)	24.3 (22-28.5)	0.34

BMI: Body Mass Index

Gordon-like phenotype in the M.A.N.G.U.E cohort

High blood pressure was recorded in 82.2% of patients, with only 2 patients having untreated high blood pressure at inclusion. Acidosis was found in 74.1% of patients, with 56.9 who had biological acidosis and 17.3% plasma bicarbonate>22mmol/L with alkaline supplementation. 37.9% of patients had hyperkalemia, both of them with plasma potassium>4.5 mmol/L. Among the 58 KT, 14 (24.1%) exhibited the Gordon-like syndrome phenotype (high blood pressure, acidosis and hyperkalemia). The association of high blood pressure and acidosis without hyperkalemia were also found in 20 (34.4%) of patients. Only 15.5%, 10.3% et 0% had high blood pressure, acidosis or hyperkalemia alone. Gordon-like features are summarized in Table 5 and Figure 5.

Table 5: Prevalence of Gordon-like syndrome features in the prospective cohort of 58 kidney transplants 3 months after transplantation.

Characteristics	
High blood pressure, N (%) Untreated high blood pressure at inclusion, N (%)	48 (82.8) 2 (3.4)
Acidosis, N (%) Plasma bicarbonate < 22 mmol/L, N (%) Plasma bicarbonate ≥ 22 mmol/L with alkaline supplementation, N (%)	43 (74.1) 33 (56.9) 10 (17.3)
Hyperkalemia, N (%) $ Plasma potassium >4.5 mmol/L, N (%) Plasma potassium ≤4.5 with potassium chelating agent use, N (%)	22 (37.9) 22 (37.9) 0 (0)

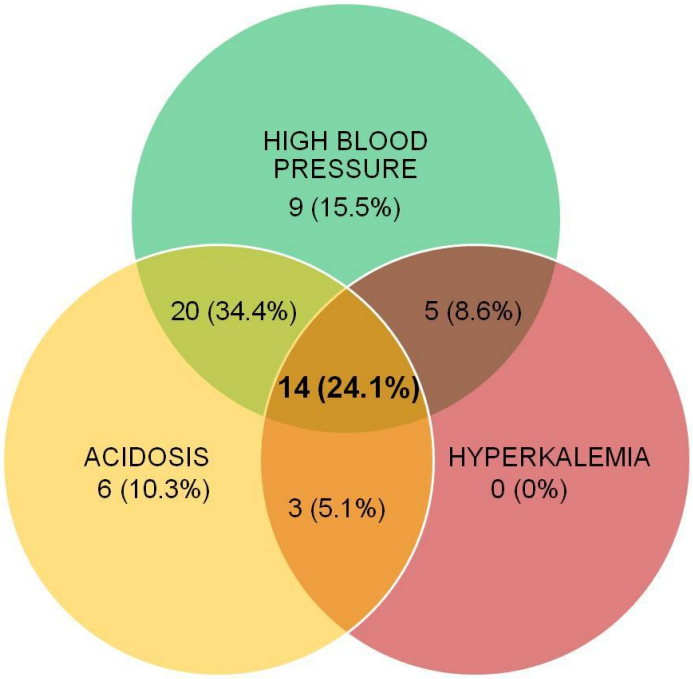


Figure 5: Distribution of high blood pressure, acidosis and hyperkalemia in the prospective cohort of 58 kidney transplants 3 months after transplantation. Data are shown as N (%).

Of note, prevalence of high blood pressure, acidosis and hyperkalemia of the M.A.N.G.U.E cohort was similar to the retrospective cohort (p=0.78, p=0.96 and p=0.91, respectively).

Gordon-like syndrome phenotype is associated with recipient's age

We separated the KT according to the presence of Gordon-like syndrome phenotype and analyzed the distribution of the previously described characteristics (Table 6). In unadjusted analysis, patients with Gordon-like syndrome phenotype were significantly older (p=0.002). Donor age was significantly older (p=0.03) and they had less preemptive transplantation (p=0.04). Hypertensive patients with Gordon-like syndrome phenotype had higher SBP than

those without Gordon-like syndrome phenotype (p=0.03). They were using more PPI (p=0.01), diuretic (0.006), eGFR was also significantly lower (p=0.01). In multivariate analysis adjusted with all these variables, only age was still associated with Gordon like syndrome phenotype (OR = 1.9 (IC95%: 1.2-3.0) p=0.0058).

Table 6: Univariate and multivariate analysis of factors associated with Gordon-like syndrome phenotype in the M.A.N.G.U.E study, using logistic regression with backward selection.

Univariate analysis			
Characteristics	No Gordon-like phenotype* N= 44	Gordon-like phenotype* N=14	P value
Age, median (interquartile range), years	41.5 (34-56)	56.5 (49-70)	**0.002**
Sex ratio, M/F, N (%)	24/20 (54.5/45.5)	11/3 (78.6-21.4)	0.05
Donor Age, median (interquartile range), years	48 (32-64)	60.5 (50-77)	**0.03**
Preemptive, N (%)	10 (23.2)	0 (0)	**0.04**
Diabetes, N (%)	11 (25)	3 (21.4)	0.78
Rejection, N (%)	2 (4.5)	0 (0)	0.42
Corticoides use, N (%)	27 (61.4)	8 (57.1)	0.78
TMP-SMX use, N (%)	38 (86.4)	9 (64.2)	0.06
PPI use, N (%)	17 (38.6)	10 (71.4)	**0.03**
Patients with high blood pressure, N (%) **SBP, median (interquartile range), mmHg** DBP, median (interquartile range), mmHg	34 (77.3) 134.5 (125-145) 83 (75-89)	14 (100) 144.5 (134-156) 83.5 (76-92)	0.05 **0.03** 0.9
Antihypertensive pills use, N (%) Beta-blocker angiotensin-converting enzyme inhibitors or angiotensin receptor blockers **Diuretic except thiazides**	 9 (20.5) 11 (25) 3 (6.8)	 5 (33.7) 7 (11) 5 (35.7)	 0.24 0.08 **0.006**
Patients with acidosis, N (%) Plasma bicarbonate, median (interquartile range)	29 (65.9) 20 (18-21)	14 (100) 21 (19-24)	**0.01** 0.34
Patients with hyperkalemia, N (%) Plasma potassium, median (interquartile range)	8 (18.2) 4.75 (4.54-4.89)	14 (100) 4.91 (4.7-5.2)	**<0.001** 0.11
eGFR, median (interquartile range) ml/min/1.73m2	52 (34.5-66.5)	34 (30-32)	**0.01**
Multivariate analysis			
Characteristics	Odds ratio (95% CI)		P value
Age (1)	1.9 (1.2-3.0)		**0.0058**

Quantitative data were expressed as median (1st and 3rd quartiles). Qualitative data were expressed as frequency and percentages. Bivariate comparisons of quantitative data were assessed using Mann–Whitney test. Bivariate comparisons of qualitative data were assessed using Fisher's test. Multivariate analysis was performed logistic regression with backward selection. All covariates with a p-value below 0.20 in the bivariate analysis were entered in the model and removed if they did not reached the statistical significance. P<0.05 was considered as significant.

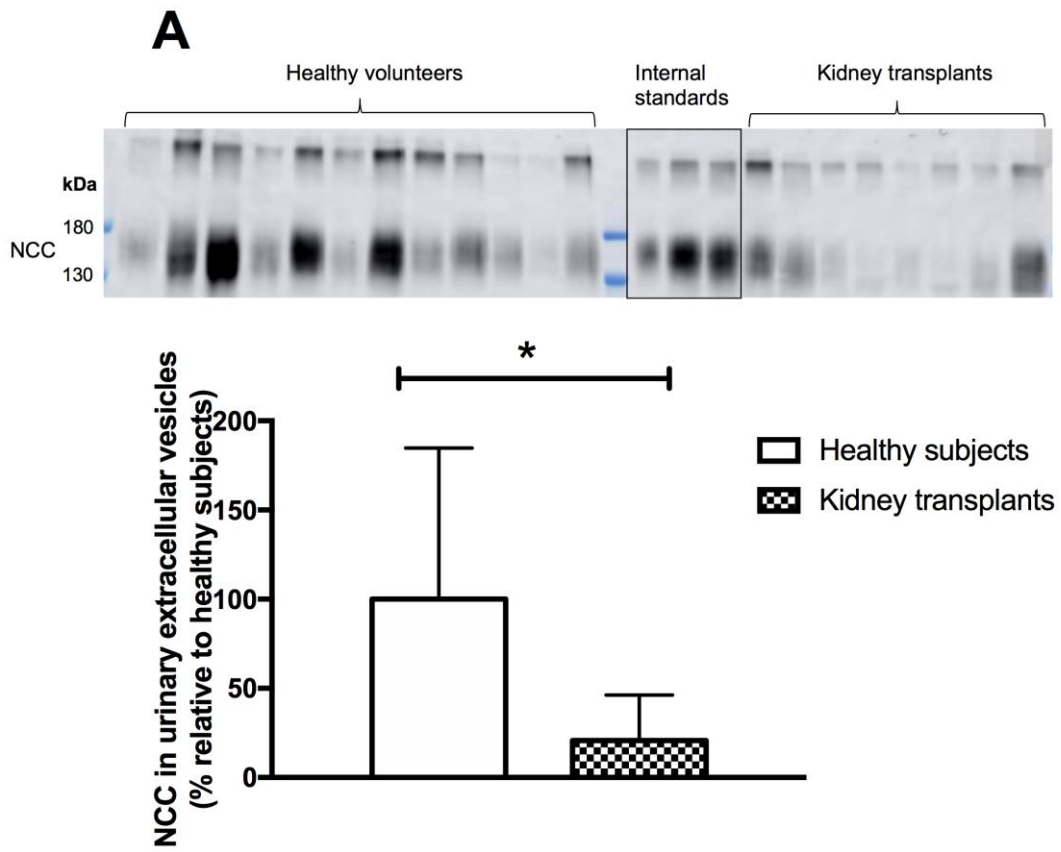
**Gordon-like syndrome phenotype: high blood pressure/acidosis/hyperkalemia*
(1) Each ten-year increase

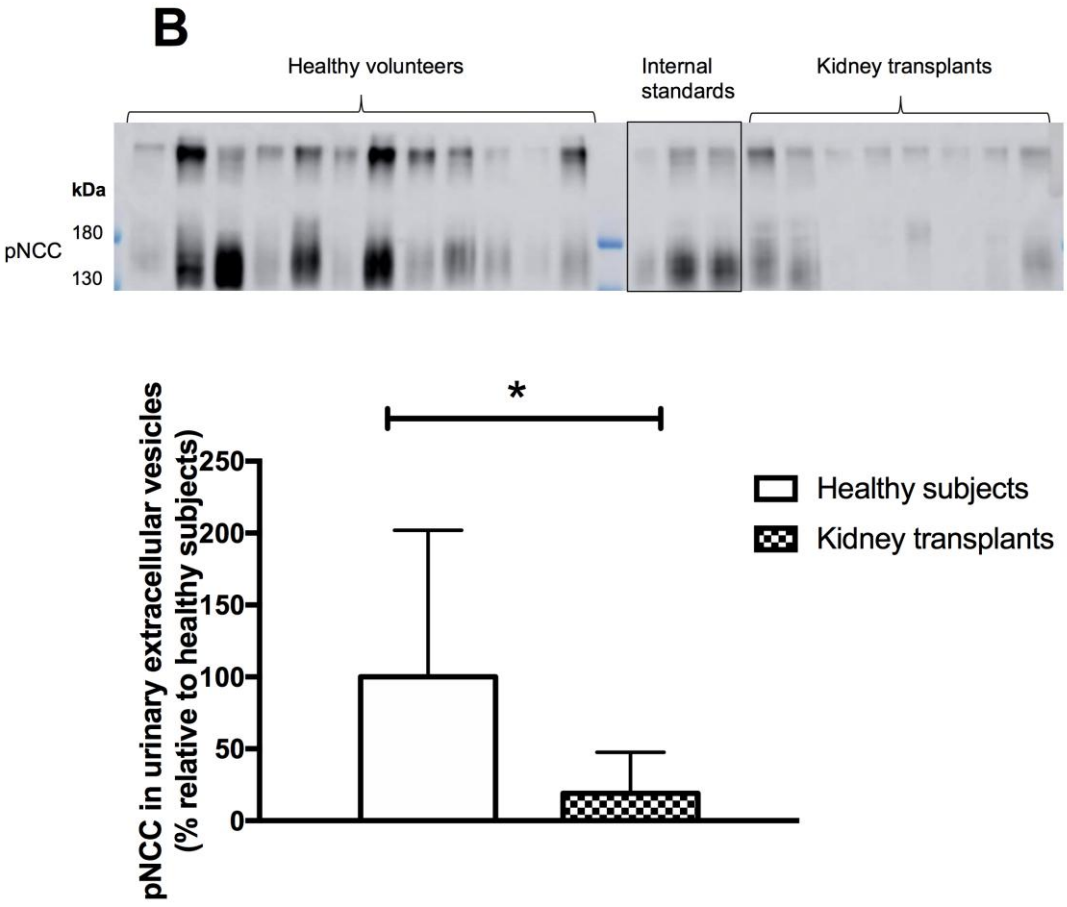
TMP-SMX denotes Trimethoprim/sulfamethoxazole, PPI Proton Pump Inhibitors, SBD Systolic Blood Pressure DBP Diastolic Blood Pressure and eGFR estimated Glomerular Filtration Rate

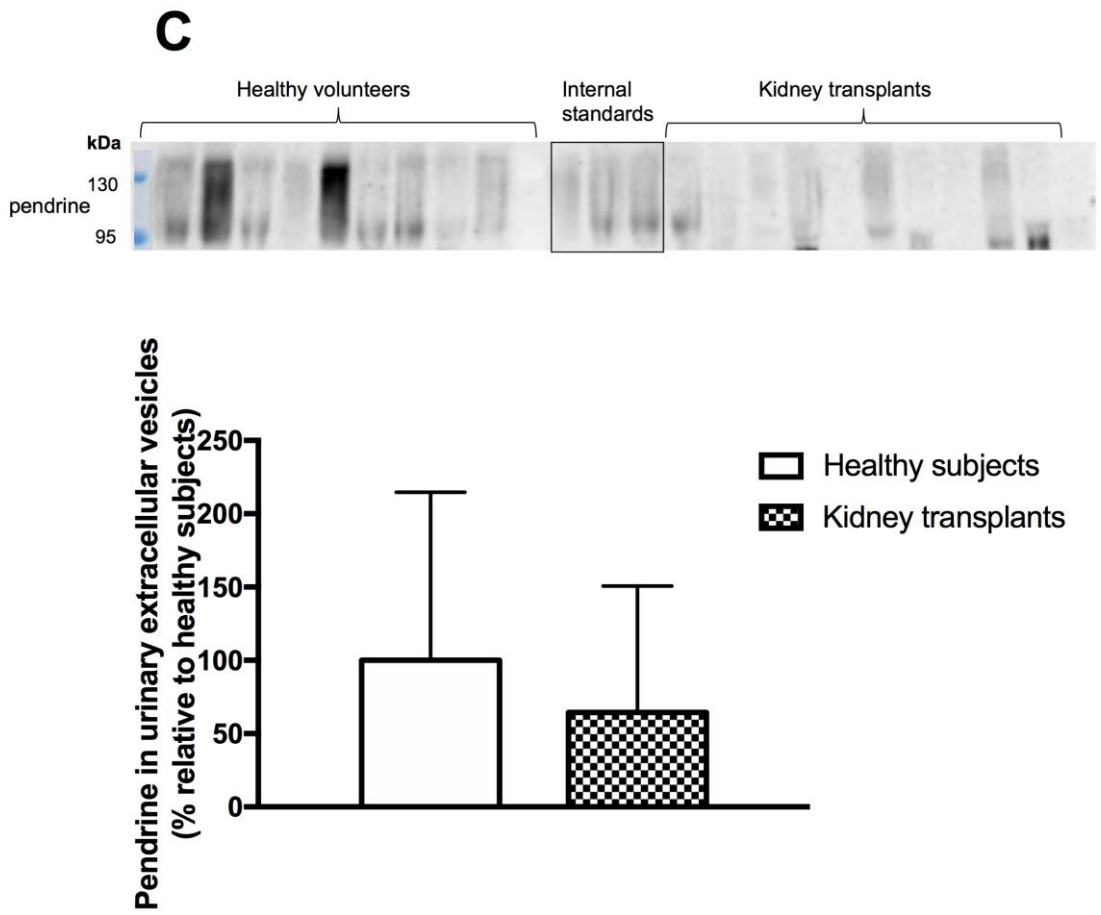
CNI-treated kidney transplants don't have increased NCC, pNCC or pendrin abundance in exosomes compared to CNI-free healthy volunteers.

NCC, pNCC, pendrin and TSG101 abundance were quantified in KT and volunteers by immunoblot analyses. As shown in Figure 6, NCC and pNCC abundance in urinary exosomes from CNI-treated KT were significantly lower when compared to exosomes isolated from healthy volunteers ($p<0.05$). Pendrin abundance was not different between KT and healthy volunteers ($p=0.05$). Surprisingly, TSG101 abundance was lower in KT patients when compared to healthy volunteers suggesting that KT excrete less exosomes. Thus, we tested again the effects of CNI on the different transporters after normalization for TSG101 to take into account the decrease in exosome excretion. These additional experiments yielded exactly same results (*i.e.*, NCC and pNCC were decrease in CNI treated KT vs controls and pendrin was unchanged) (not shown).

Also, we found no differences in NCC, pNCC, pendrin and TSG101 abundance between KT from Bordeaux and la Reunion (not shown).


A
Healthy volunteers
Internal standards
Kidney transplants
kDa
180
NCC
130
*
NCC in urinary extracellular vesicles (% relative to healthy subjects)
200
150
100
50
0
Healthy subjects
Kidney transplants


B
Healthy volunteers
Internal standards
Kidney transplants
kDa
180
pNCC
130
*
pNCC in urinary extracellular vesicles (% relative to healthy subjects)
250
200
150
100
50
0
Healthy subjects
Kidney transplants


C
Healthy volunteers
Internal standards
Kidney transplants
kDa
130
pendrine
95
Pendrine in urinary extracellular vesicles (% relative to healthy subjects)
250
200
150
100
50
0
Healthy subjects
Kidney transplants

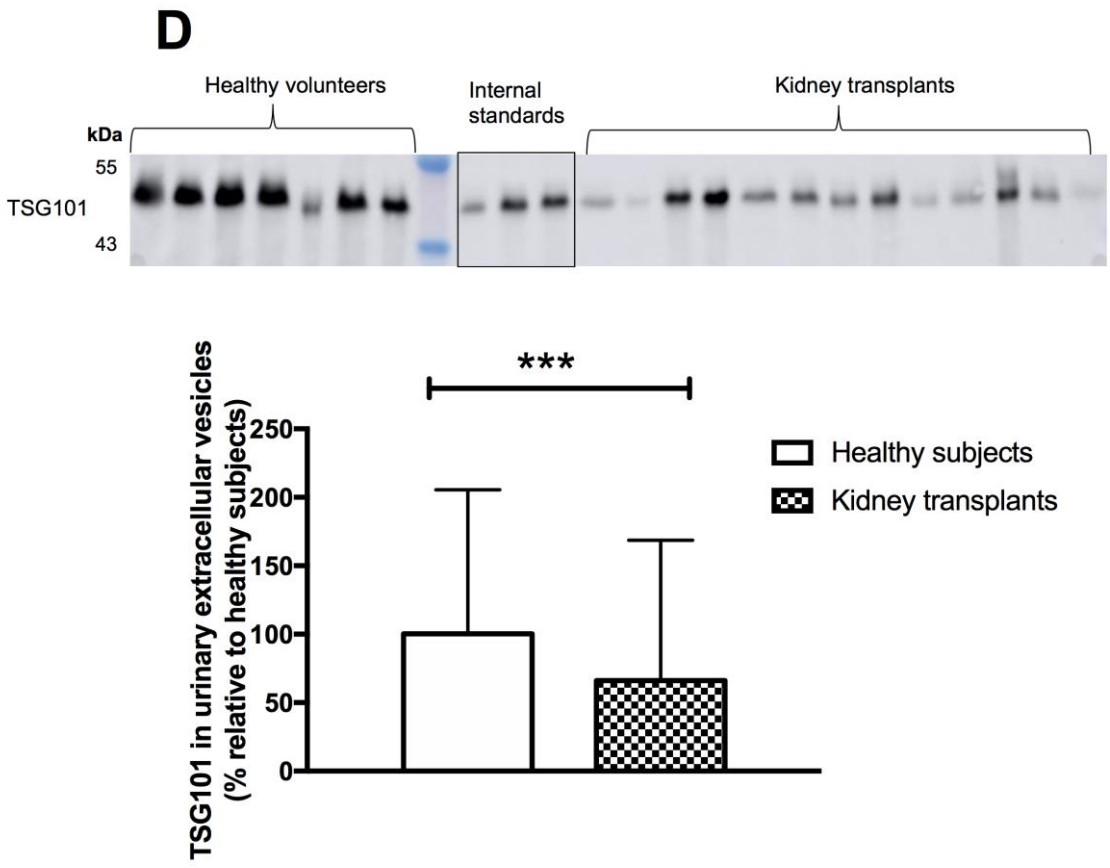

D
Healthy volunteers
Internal standards
Kidney transplants
kDa
55
TSG101
43
***
TSG101 in urinary extracellular vesicles (% relative to healthy subjects)
250
200
150
100
50
0
Healthy subjects
Kidney transplants

Figure 6*: Densitometric analysis of the western blot of N-terminal sodium-chloride cotransporter (NCC), phosphorylated NCC (pNCC), pendrin and Tumor Susceptibility Gene 101 protein (TSG101) in urinary exosomes of healthy subjects and kidney transplants.*

*NCC (A) and pNCC (B) abundance were significantly lower in urinary exosomes of kidney transplants compared to healthy volunteers. There was no difference in pendrin abundance (C) in urinary exosomes of kidney transplants and healthy volunteers. TSG101 (D) was also slightly significantly reduced in urinary exosomes of kidney transplants but additional normalization of NCC, pNCC and pendrin with TSG101 didn't modified our results. Values are mean ± SD normalized to healthy subjects. Blots shown above are representative of individual experiments. n=58 in each group except for pendrin in kidney transplants (n=56). Statistical significance was assessed by analysis of variance (ANOVA) after normalization using natural logarithm *P < 0.05 for NCC and pNCC, 0.05 for pendrine and ***p=0.0007 for TSG101.*

Kidney transplants with Gordon-like syndrome phenotype don't have an increase in NCC and pNCC abundance. Pendrin is appropriately downregulated in response to acidosis

When abundance of the renal transporters in urinary exosomes from KT harboring a complete Gordon-like syndrome was compared to the one from KT without Gordon-like phenotype we found no significant difference (p=0.78 for NCC, 0.85 for pNCC, 0.97 for pendrin, respectively) (Figure 7). No difference in TSG101 abundance between groups was detected, thus, normalization with TSG101 was not necessary (p=0.45).

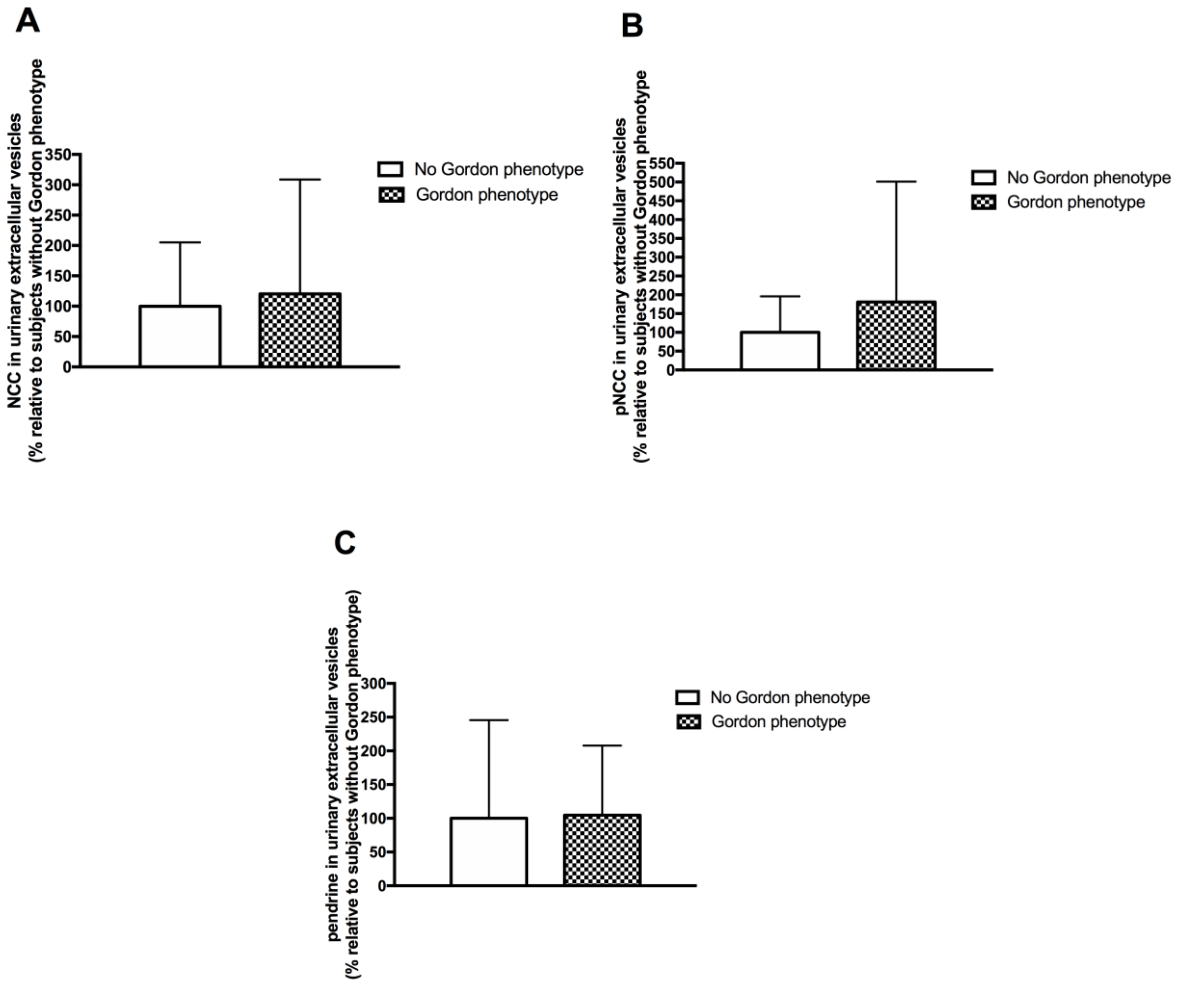


Figure 7: *Densitometric analysis of the western blot of N-terminal sodium-chloride cotransporter (NCC), phosphorylated NCC (pNCC), pendrin in urinary exosomes of kidney transplants according to the presence of Gordon-like syndrome phenotype.*

Abundance of NCC (A), pNCC (B) and pendrin (C) was not different between patients with or without Gordon-like syndrome phenotype (p=0.78 for NCC, 0.85 for pNCC, 0.97 for pendrin). Values are mean ± SD normalized to subjects without Gordon-like phenotype. Gordon-like phenotype was defined as the simultaneous presence of high blood pressure, acidosis and hyperkalemia. Subjects without Gordon-like phenotype n=44 and subjects with Gordon-like phenotype n=14. Statistical significance was assessed by analysis of variance (ANOVA) after normalization using natural logarithm

We next compared NCC, pNCC and pendrin abundances in urinary exosomes from patients exhibiting either high blood pressure, or acidosis, or hyperkalemia. There was no difference in TSG101 abundance between KT hypertensive or not, with or without acidosis and with or without hyperkalemia, so normalization with TSG101 wasn't necessary

As shown in Figure 8, NCC and pNCC abundances were not significantly altered in patients with acidosis (p=0.16 and 0.08). As expected, pendrin abundance was significantly lower in patients with acidosis (p=0.006) indicating that patients treated with CNI keep a normal regulation of pendrin.

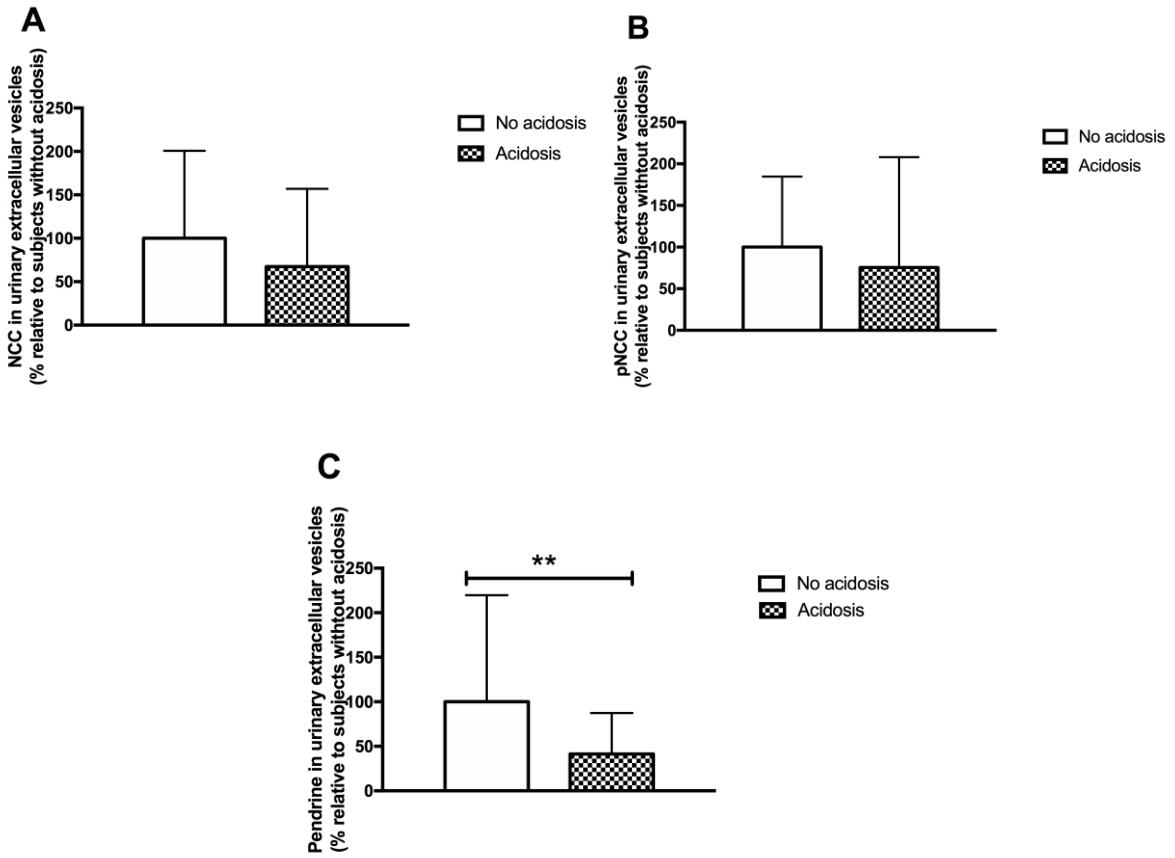


Figure 8: *Densitometric analysis of the western blot of N-terminal sodium-chloride cotransporter (NCC), phosphorylated NCC (pNCC), pendrin in urinary exosomes of kidney transplants according to the presence of acidosis.*

*Pendrin abundance (C) was significantly lower in KT with acidosis, whereas there was no difference in NCC (A) and pNCC (B) abundance. Values are mean ± SD normalized to subjects without acidosis. n=15 in subjects without acidosis and 43 in subjects with acidosis except for pendrin (n=14 in subjects without acidosis and 42 in subjects with acidosis). Statistical significance was assessed by analysis of variance (ANOVA) after normalization using natural logarithm. P=0.16 for NCC, 0.08 for pNCC, **p=0.006).*

As shown in Figure 9, there was no difference in NCC, pNCC and pendrin abundance between normotensive and hypertensive KT (p=0.29; 0.74; and 0.38, respectively).

When comparing KT according to the presence of hyperkalemia, NCC, pNCC and pendrin abundance were unchanged in hyperkalemic patients (p=0.74, 0.83 and 0.52, respectively).

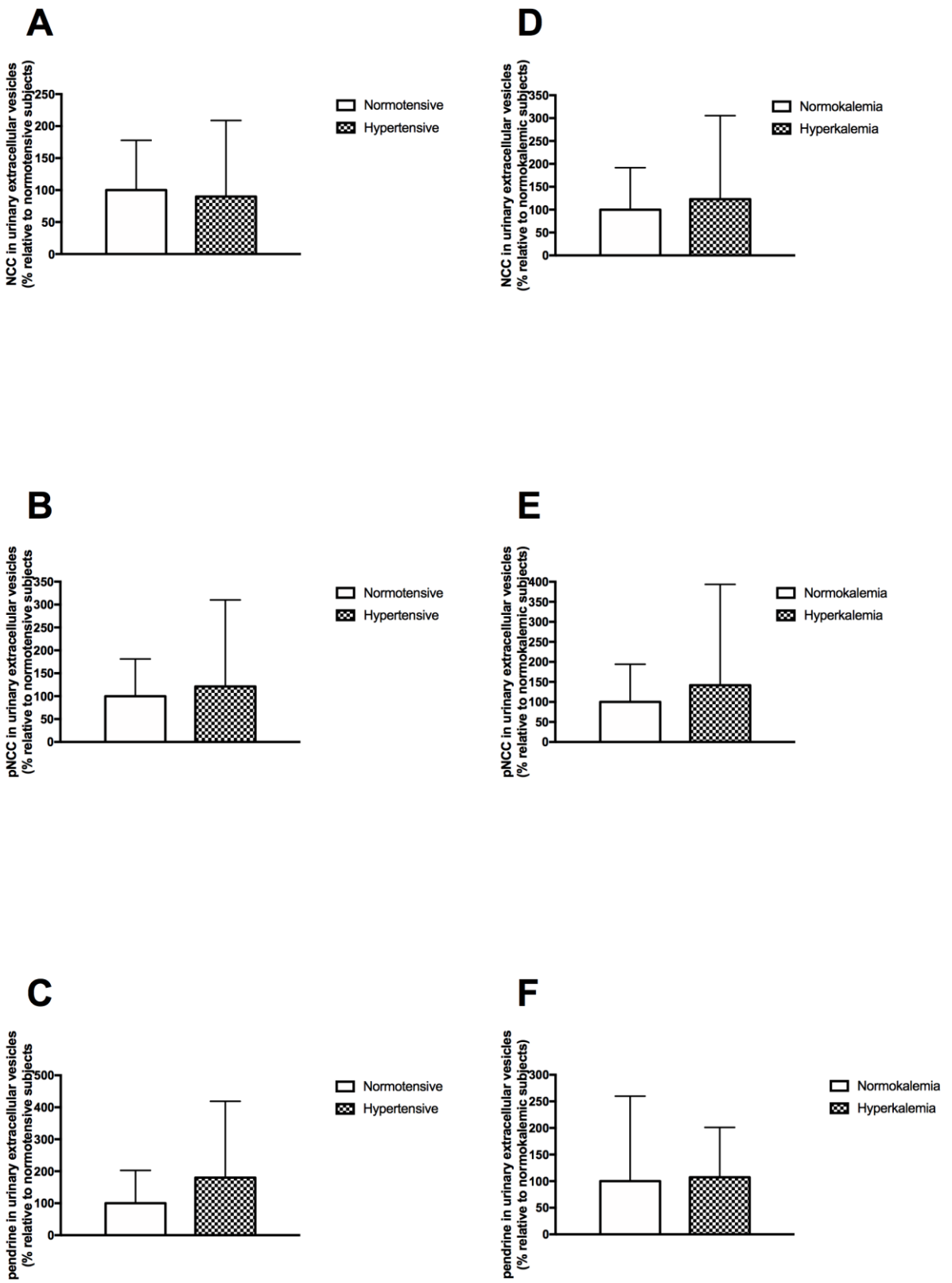


Figure 9: Densitometric analysis of the western blot of N-terminal sodium-chloride cotransporter (NCC), phosphorylated NCC (pNCC), pendrin in urinary exosomes of kidney transplants according to the presence of high blood pressure and hyperkalemia.

There was no difference of NCC, pNCC and pendrin abundance in hypertensive patients compared to normotensive (A, B and C), as well in hyperkalemic patients compared to normokalemic (D, E and F). Values are mean ± SD normalized to normotensive or normokalemic subjects. A and B: n=10 in normotensive and 48 in hypertensive subjects. p=0.29 and 0.74. C: n=10 in normotensive and 46 in hypertensive subjects. p=0.38. D and E: n=35 in normokalemic subjects and 22 in subjects with hyperkalemia. p=0.74 and 0.83. F: n=33 in normokalemic subjects and 22 in subjects with hyperkalemia. p=0.52. Statistical significance was assessed by analysis of variance (ANOVA) after normalization using natural logarithm.

DISCUSSION

A study performed in mice has proposed that CNI can activate NCC to cause high blood pressure, hyperkalemia and acidosis, mimicking a genetic disease called Gordon syndrome[1]. Mutations causing Gordon syndrome lead to NCC activation, but, as recently shown, also implicate inappropriate pendrin activation[23]. Despite the wide use of CNI, particularly in transplantation, the possibility that CNI can induce Gordon-like syndrome in humans is not clear. We speculated that CNI-induced Gordon-like phenotype in human is frequent and might account for the high prevalence of high blood pressure and metabolic acidosis observed in transplant recipients. Here, we confirm that the combination of high blood pressure, acidosis and hyperkalemia, features of Gordon syndrome affect up to 24% of patients 3 months after renal transplantation. We show, however, that KT with Gordon-like phenotype do not exhibit significant increase in NCC, pNCC or pendrin abundance but rather exhibit a marked downregulation of NCC and pNCC protein abundance in urinary exosomes when compared to healthy volunteers.

Few studies have tried to determine if CNI in humans can induce a Gordon-like syndrome. Here, we report the prevalence of the combination of high blood pressure, metabolic acidosis, and hyperkalemia in a cohort of KT. As already reported previously[15], prevalence of high blood pressure was high. The prevalence of acidosis was high in our study, while others have reported a prevalence of low plasma bicarbonate concentration from 11 to 58%[29]. Hyperkalemia was less common than high blood pressure and acidosis, but data in the literature are too scarce to compare this rate[30]. The high prevalence of Gordon-like phenotype in renal transplant recipients treated by CNI prompted us to test the possible effects of CNI on NCC and pendrin, the two renal transporters which are central to the pathophysiology of Gordon syndrome.

We therefore included 60 prospective KT 3 months after transplantation and 60 healthy volunteers to evaluate NCC, pNCC and pendrin expression in urinary exosomes. Surprisingly we found that CNI-treated KT have a marked a down regulation of NCC pNCC protein abundance in urinary exosomes. These results contrast with one of the two studies that used urinary exosomes to compare CNI-treated to CNI-free KT or healthy volunteers. If one study found, like us, no difference in NCC abundance between CNI-treated KT and CNI-free[31], one other study reported that CNI-treated KT exhibit an up regulation of NCC when compared to CNI-free KT and healthy volunteers[27]. The latter compared 9 KT treated with ciclosporin and 23 KT treated with tacrolimus to 13 CNI-free KT and 6 male volunteers (supplemental table 1). Our two cohorts were quite similar in term of age, BMI, SBP, DBP, or eGFR. However, we noticed that NCC and pNCC abundance exhibit a great variability within exosomes samples

from our healthy volunteers. Numerous factors like dietary habits, age, sex, and treatments are known to modulate NCC expression[32]. Thus, one possible explanation of our opposite result could be that they have too few volunteers. Noteworthy, NCC is a transporter that plays a key role in renal regulation of extracellular fluid volume and blood pressure[33]. Therefore, downregulation of NCC and decreased phosphorylation of NCC might reflect renal adaptation to volume-dependent high blood pressure in the setting of kidney transplantation.

We also show that KT with Gordon-like phenotype do not exhibit an increase in NCC, pNCC or pendrin abundance. We did not found difference in NCC, pNCC or pendrin expression associated with the presence of high blood pressure. This contrast with one study that showed that tacrolimus-treated men KT with high blood pressure have a slight increase in NCC abundance compared to those without high blood pressure[34]. However, the number of normotensive patients (only 3) in our study was to small to allow to test for differences in transporter abundances between normotensive and hypertensive patients. Our study assessed the effects of CNI only 3 months after transplantation which is shorter than the other studies that have tested the effects of CNI on renal transporters. However, the prevalence of high blood pressure, acidosis and hyperkalemia in our study was similar to what has been reported in other studies after one year or more. It is thus unlikely that we failed to detect the molecular effects of CNI because the design of our study was too short.

Finally, many alternative factors other than NCC and pendrin dysregulation can explain the presence of high blood pressure, hyperkalemia and metabolic acidosis in KT [35,36]. Thus, it would be interesting to test again the effects of CNI on NCC, pNCC and pendrin regulation in patients also treated with CNI but with a normal renal function like patients treated for dermatological diseases, uveitis, rheumatologic diseases. Also, exquisite sensitivity to thiazide diuretics is one of the key feature of Gordon syndrome, thus, it would be interesting to test whether thiazide diuretics can alleviate Gordon-like phenotype in KT. We predict from the results of the present study that KT patients won't be more sensitive to these compounds than other hypertensive patients. Accordingly, one prospective trial has compared thiazide diuretics to calcium channel blockers in CNI-treated KT and found that chlortalidone was only non inferior to amlodipine in term of blood pressure reduction[38]. Finally, we found that pendrin abundance was significantly lower in KT with acidosis. This indicate that abnormal upregulation of pendrin does not cause acidosis in KT patients, further, this in agreement with many studies by us and others showing that metabolic acidosis decreases pendrin protein abundance in rodents and humans[39].

Design of the study was performed by Dr Chambrey, Pr Eladari and myself. I was in charge of the writing of the original script and information sheets submitted to Comité de protection des personnes under the supervision of Dr Chambrey and Pr Eladari. I was involved in all part of the establishment of the study in Bordeaux and Saint Denis de La Réunion, in coordination with la Direction de la Recherche Clinique and le Centre de Ressource Biologique. As a holder of the « attestation de formation à la pratique clinique », I was in charge of the inclusion of the patients in La Réunion. I collected the clinical and biological data in Bordeaux and la Réunion and performed all the experiments presented in this manuscript. Statistical analyses were performed by Dr Ferdynus from l'Unité de Soutien Méthodologique, CHU Saint Denis, la Réunion.

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SUPPLEMENTAL MATERIALS

Supplemental tables

Supplemental table 1:

Clinical trials using urinary exosomes to study NCC expression in KT patients: Methods						
Reference	**Initial urine volume**	**Procedure of exosomes isolation**		**Heating before immunoblot**	**Exosome volume loading**	**Internal standards**
		OS 1	**OS 2**			
Esteva-Font C et al. [31],	100ml	No	No	No	24h urine volume based	Yes
Rojas-Vega L et al. [34]	30ml	Yes	No	No	Creatininuria based	Not mentionned
Tutakhel OAZ et al. [27]	10 to 40ml	Yes	Yes	Yes	Creatininuria based	Not mentionned
MANGUE	50ml	Yes	No	No	Creatininuria based	Yes

OS denotes Optionnal Step.

OS1: Its goal is to higher the yield of exosomes OS2: Its goal is to remove THP

Clinical trials using urinary exosomes to study NCC expression in KT patients: Protocol and results

Reference	Trial design	Modality of selection	Patients	Time since transplantation	Main findings	
					NCC fold change / if normalization with exosomal marker	**pNCC fold change** / if normalization with exosomal marker
Esteva-Font C et al.[31],	CNI (CsA) KT vs CNI free KT	Random KT >1year after transplantation	39 9	> 1 year (2,5-25 years)	No	Not tested
Rojas-Vega L et al.[34]	Male CNI KT with high blood pressure vs Male CNI KT without high blood pressure	Random KT 6months after transplantation	18 17	6 months	↑ 1,7 vs. normotensive	↑ 1,5 vs. normotensive
Tutakhel OAZ et al.[27]	CNI (Cs/Tacrolimus) KT vs CNI-free KT vs volunteers	Random KT >6 months with the same treatment	9/23 13 6	Not precised	↑ 4-5 vs. CNI free and vs volunteers / ↑ 6-7 vs. CNI free, No vs volunteers	↑ 4-5 vs. CNI free and vs volunteers / ↑ 8-9 vs. CNI free, No vs volunteers
MANGUE	CNI KT vs volunteers	All CNI KT 3months after transplantation	58 58	3 months	↓ 4 vs volunteers / ↓ 4 vs volunteers	↓ 4 vs volunteers / ↓ 4 vs volunteers

CNI denotes CalciNeurin Inhibitors, CsA Ciclosporin A, KT Kidney Transplants.

Ancillary study: Development of NCC, pNCC and pendrin analyses using human urinary exosomes

A Optimization of the procedure of exosomes isolation

Urinary exosome isolation technique of Pisitkun *et al.* was first described in 2004 (Figure 1)[1]. This technique uses two differential centrifugation steps. The first step, a low speed centrifugation, pellets and eliminates whole cells and debris. The pellet is discarded while the supernatant is used for the second step. Noteworthy, the pellet can also contain calcium-containing precipitates that co-sediment proteins such as Tamm-Horsfall protein (THP). Polymerization of THP form network that can trap exosomes[2]. Therefore, after step 1, a first optional step (OS1), sometimes called “enrichment procedure”, can be performed. Its goal is to increase the yield of exosomes in the supernatant by releasing the exosomes trapped in this pellet. Options to dissolve this network include resuspension of the pellet, eventually completed to 2 ml with isolation solution, followed by a second centrifugation, with or without use of dithiothreitol (DTT). DTT is a strong reducing agent capable of disrupting the zona pellucida disulphide of THP[2]. The second step, which is ultracentrifugation of the previous supernatant, pellets the exosomes. Then, a second optional step (OS2) can be done to try to remove THP because THP can disturb the migration of the different polypeptides present in exosomes. Procedure to remove THP include use of reducing agents, mostly DTT[3].

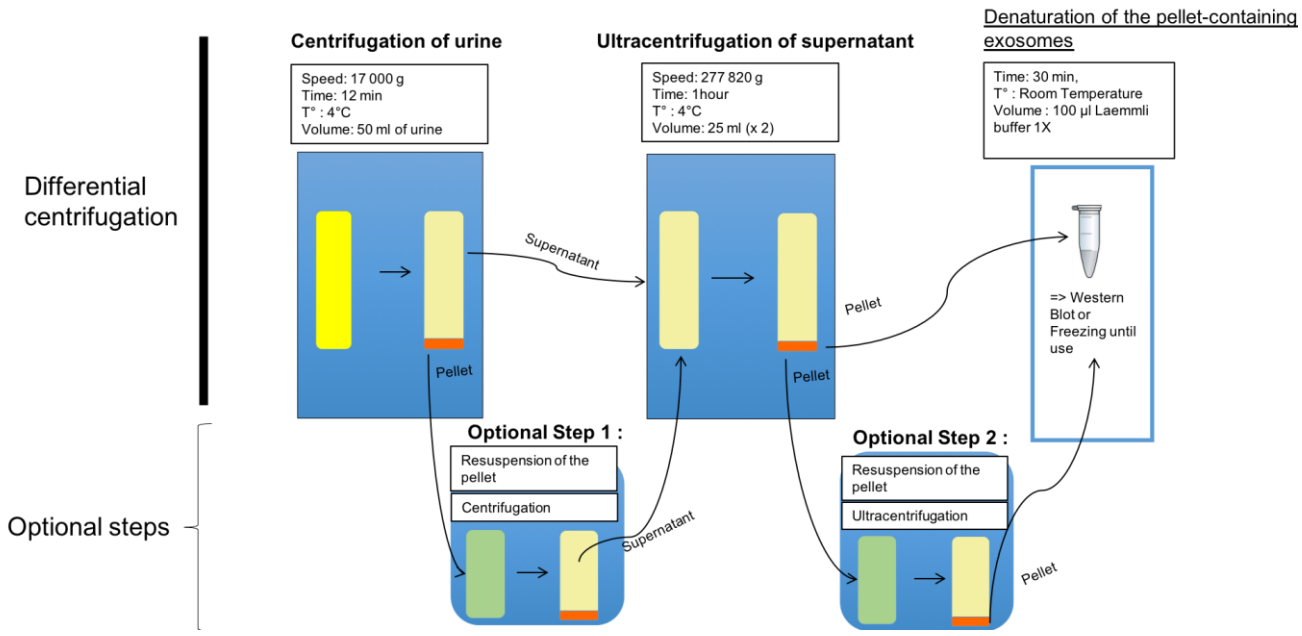


Figure 1: Protocol of isolation of human urine exosomes from Pitsikun et al [1] : 2 steps differential centrifugation and two optional steps

We tested several variations of these OS to find the best compromise between enrichment in exosomes, depletion of THP and detection of the membranous proteins NCC, pNCC and

pendrin. No normalization between conditions was needed for the following experiments because exosomes originated from the same initial sample (unique or mixed individuals). We used alix and TSG101 as exosomal markers.

- OS 1: Enrichment procedure

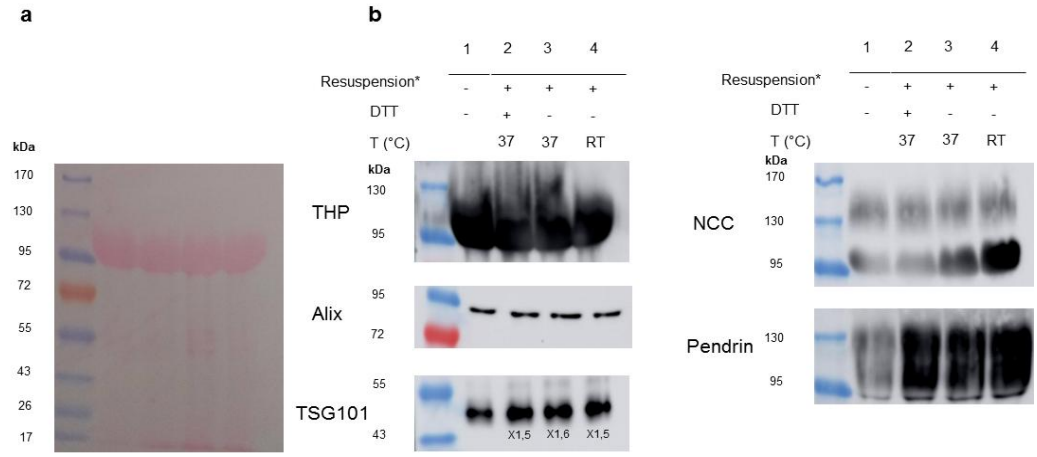


Figure 2: Analysis of the relevance of optional step 1 on enrichment of exosome samples.

***a** Protein staining after transfer (Ponceau red staining) **b** Immunoblots of exosome samples with or without optional step 1 and probed against NCC, pendrin, THP and exosomal proteins alix and TSG101. Relative quantitation of the band densities of TSG101 from immunoblots was carried out with ImageQuant TL software. RT indicates room temperature.*

** Resuspension of the pellet in 1ml of isolation buffer for 5 min followed by a second centrifugation*

Resuspension of the pellet increases the yield of exosomes

To investigate the effect of resuspension of the pellet on release of exosomes, we loaded 10 µl of the same exosome samples and compared condition 1: without resuspension, to condition 4: resuspension of the pellet in isolation liquid at room temperature for 5 min, followed by a centrifugation at 17000 g for 5 min at 4°C. The remainder of the procedure is written in material and methods (no OS2). We looked at the abundance of NCC, pendrin, THP and exosomal markers alix and TSG101. As it is shown in Figure 2, resuspension of the pellet followed by a second centrifugation step leads to a 1.6- fold increase in TSG101 signal while NCC and pendrin signals were increased by 1.5- to 2- fold. Of note, resuspension of the pellet at 37°C instead of room temperature didn't improved our results (condition 3).

DTT in OS1 doesn't improve the yield of exosomes and impairs NCC detection

As DTT is often used in OS1 to increase the yield in exosomes[2], we added DTT (200 mg/ml) in the isolation liquid at 37°C for 5 min (condition 2) and performed the same experiment than previously. We compared condition 2 to condition 1 and 4. As shown in Figure 3, DTT didn't

increase enrichment in exosomes, and altered NCC detection. We validated the use of an OS1 with resuspension of the pellet in liquid isolation for the remainder of the experiments.

- OS 2: Depleting THP

DTT in OS2 depletes THP…but prevents NCC detection

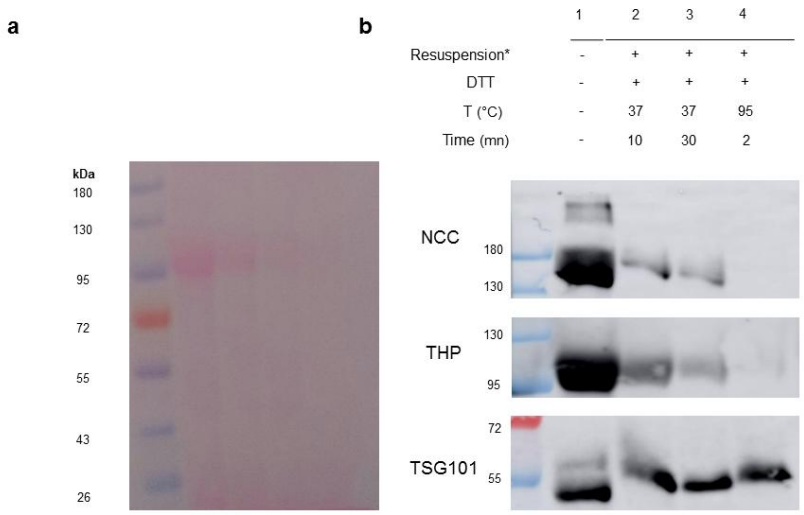


Figure 3: Analysis of the relevance of an optional step 2 with DTT on THP depletion

***a** Staining of proteins after transfer (Ponceau red staining) **b** Immunoblots of exosome samples with or without optional step 2 and probed against THP, NCC and exosomal protein TSG101*

** Resuspension of the pellet in 500 µl of isolation buffer*

THP is by far the most abundant protein in human urine and is highly present in exosomes (see Ponceau red staining, Figure 2 or 3). To remove THP, we resuspended the pellet resulting from ultracentrifugation with another 500 µl 200 mg/ml DTT, at 95°C for 2min, according to Gonzales protocol[4]. As shown in Figure 3, DTT depleted THP but not TSG101 in exosome samples (condition 4, compared to control: Condition 1, no OS2), but this results in a default of detection of NCC.

As proposed previously, we hypothesized that temperature could be a factor that impairs detection of these membrane proteins (membrane proteins are prone to aggregate at high temperature and aggregates don't run well into the gel). So, we resuspended and incubated pellets resulting from ultracentrifugation with DTT for 30 min or 10 min at 37°C (Figure 3, conditions 2 and 3), which are other protocols used in the literature. As shown, these temperatures were less effective to deplete THP than the previous one. Furthermore, even at these lowest temperatures, DTT (or temperature) still altered NCC identification. Another attempts with resuspension of the pellet with DTT at 4°C for 30 min was completely ineffective for depleting THP (not shown). To conclude, use of DTT in OS2 depleted THP

only at high temperature, but altered identification of membranous proteins even at lowest temperature. THP depletion with DTT in urinary exosomes can't be used for western blot detection of NCC.

In conclusion, we used a variation of the two steps differential centrifugation described by Pisitkun et al. to measure NCC, pNCC and pendrin protein abundances in human urinary exosomes. We validated an OS1 with resuspension of the pellet, and no OS2. Resuspension of the pellet followed by a second centrifugation step leads to a better enrichment of urinary exosome preparations. For OS2, THP depletion can be done with DTT but prevent NCC, pNCC and pendrin detection.

B Normalization between samples

Use of urinary creatinine concentration is a validated method to normalize spot urine collection for study of protein (and ions) excretion and facilitate comparison of subjects with widely different hydration states or urinary flow rates[5]. In exosomes studies, creatininuria, is assumed to reflect the concentration of the sample[6], with the number of exosomes correlating with this concentration. Creatininuria is often used as a method of normalization, but it hasn't been validated yet. One other way to normalize exosomes quantity between samples is to use antibodies against protein markers of exosomes and express their result as a ratio to this protein [7]. Among them, most promising proteins include TSG101 and alix[6]. Surprisingly, alix couldn't be identified in some women samples (in different women from different experiences), whereas we could always identify it in men. Thus, this sex discrepancy precludes to use this marker from the analysis. As we didn't have enough women samples for one of the experience, we decided to present results only issued from men experiences.

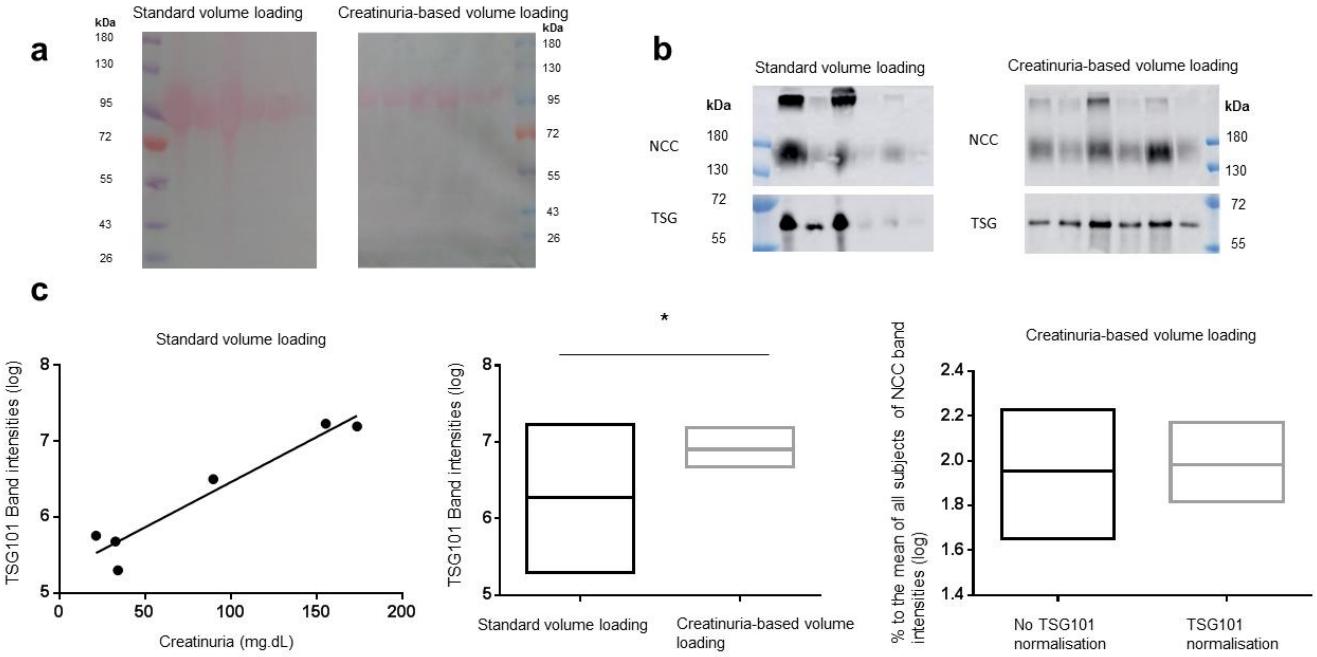


Figure 4: Analysis of the impact of normalization procedures with creatininuria or exosomal marker TSG101 on variability between subjects.

***a**: Proteins staining after transfer on nitrocellulose membranes (Ponceau red staining). Left: Standard volume loading of 8 µl for each subject. Right: Creatininuria-based volume loading **b** Immunoblots of exosomes from six male volunteers probed against NCC and exosomal marker TSG101. Left: Standard volume loading of 8µl for each subject. Right: Creatininuria-based volume loading **c** Left: Correlation between creatininuria and exosomal marker TSG101 band intensity. Each dot represents one subject. Correlation was assessed by spearman test. Correlation is r=0.71, p=0.11 (not significant). Middle: Influence of creatin*

*inuria-based volume loading on variability of exosomal markers TSG101 band intensity in six male volunteers. Data are presented as log of TSG band intensities (mean with minimal and maximal value). Statistical significance was assessed by likelihood ratio test between two mixed linear models. Firstly, a model with an unstructured variance-covariance matrix was fitted to take into account correlation between the two measures and assuming different variances for the two measures. (i.e. 3 random parameters estimated). Secondly, a model with a compound symmetry matrix was fitted to take into account the correlation between the two measures and assuming a common variance for the two measures (i.e. 2 random parameters estimates). The difference of likelihood between these two models followed a Chi square distribution with one degree of freedom. *p=0.003. Right: Influence of exosomal marker TSG101 normalization on variability of NCC band intensity in 6 men volunteers. Data are presented as log of the percentage of the mean of NCC band intensities (left) or NCC/TSG band intensities (right) (mean with minimal and maximal value).*

- TSG101 correlates well with creatininuria.

Creatininuria, is assumed to reflect the concentration of the sample[6], with the number of exosomes correlating with this concentration. To determine if the theoretical relationship between creatininuria and exosomes quantity is valid, we loaded 8 µl of exosomes samples from 6 men according to our previous validated protocol. As shown in Figure 4b, TSG 101 was detectable in both men. As shown in Figure 4c left, TSG101 had a good correlation with creatininuria, despite it wasn't statistically significant (r=0.71, p=0.11, spearman correlation).

- Load sample according to creatininuria diminishes the variability between samples.

We decided to compare a standard volume-based loading to a creatininuria-based loading of the exosome samples. We loaded in one hand a standard volume of 8µl from 6 men samples, and, in the other, a volume according to the creatininuria (a maximum of 8 µl of the more diluted sample was loaded, for all other samples loaded volume was inversely proportional to the creatininuria of these samples). As shown in Figure 4c middle, load sample according to creatininuria significantly diminished the variability between samples (likelihood ratio test of two mixed linear model, p=0.03).

- Normalization of the band intensity of NCC by exosomal marker TSG101 doesn't diminish the variability between samples

Exosomes from 6 men samples were loaded according to creatininuria. We then compared within each group if adding a normalization of the NCC band intensity by TSG101 diminished the variability between samples. NCC and TSG101 was easily detectable in all men (Figure 4b). As shown in Figure 4c right, normalize our result with TSG101 didn't diminish the variability between samples (likelihood ratio test, p=0.25).

To conclude, we assumed that loading exosomal samples on western blot according to urinary creatinine is the best method to quantify differences in protein expression not due to differences in exosomes quantity between samples. Adding a second normalization TSG101 wasn't beneficial.

C References

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APPENDIX



Image 1 : “Joe le frigo”.

Cryoporteur portable (-80°C) qui servit au transports des échantillons des patients depuis le CHU vers le laboratoire.



Image 2 : Ultracentrifugeuse

Utilisée pour l’isolation des exosomes.Tomba en panne à un très mauvais moment.

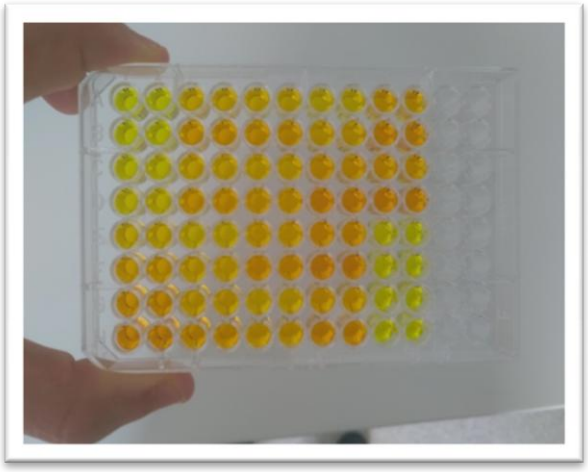


Image 3 : Dosage colorimétrique de la créatinurie

Plus l’échantillon est concentré en créatinine, plus la couleur sera foncée. Les 2 colonnes de gauche correspondent aux standards à différents degrés de dilution.

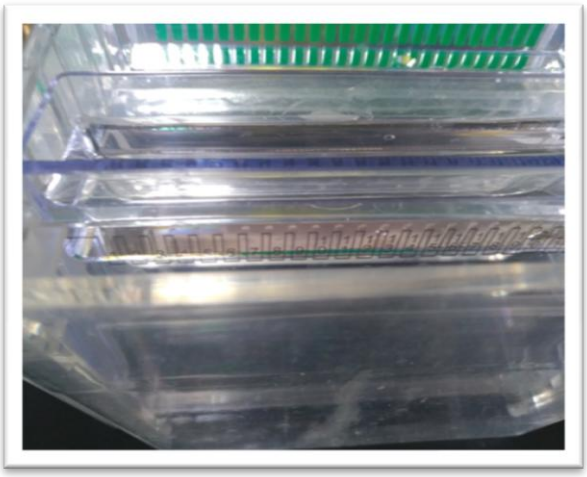


Image 4 : Western blot

On peut deviner en relief les puits du gel dans lesquels sont déposées les exosomes avant migration



Image 5 : Paillasse du laboratoire de recherche



Image 6 : Incubation des membranes avec l'anticorps primaire.



Image 7 : Tenue de confinement laboratoire P3.

Lorsque l'ultracentrifugeuse est tombée en panne, les chercheurs du laboratoire d'infectiologie ont accepté de me prêter leur ultracentrifugeuse. Malheureusement dans un laboratoire P3.

CONCLUSIONS GENERALES ET PERSPECTIVES DU PROJET

Au cours de ce travail nous avons inclus des patients greffés sous anticalcineurines dans une étude rétrospective puis une étude prospective observationnelle afin de caractériser une entité qui était jusqu'alors essentiellement décrite chez la souris : Le syndrome de Gordon-like induit par les anticalcineurines. Cette volonté était sous tendue par une visée thérapeutique, puisque les diurétiques thiazidiques, rarement utilisée en transplantation rénale, sont particulièrement efficaces chez les patients atteints de syndrome de Gordon.

En dépit de la constatation d'une forte prévalence du phénotype Gordon-like, nous n'avons pas observé d'augmentation d'expression des transporteurs NCC et pendrine dans les exosomes urinaires des patients greffés présentant ce phénotype. De plus, comparativement aux sujets sains, les patients greffés présentaient une moindre abondance de NCC et pNCC dans les exosomes urinaires. Ces données ne sont pas en faveur de l'existence d'un syndrome de Gordon-like induit par les anticalcineurines. Cela n'exclue pas la nécessité de tester les diurétiques thiazidiques dans cette population à la recherche d'une réponse accrue, ce qui permettra de compléter les données de notre travail.

Étant donné que nos résultats vont à l'encontre d'une autre étude de la littérature, il nous semble important de les confirmer pas d'autres manières. Une première pourrait être l'utilisation de techniques d'immunohistochimie pour identifier l'expression de NCC, pNCC et pendrine sur des biopsies de transplantés rénaux présentant le phénotype. Nous pourrons les comparer à celles de sujets sains (néphrectomie) afin de confirmer une moindre expression des transporteurs chez les greffés. Il pourrait également être judicieux de réaliser une étude observationnelle chez les patients sous anticalcineurines pour des indications non rénales (affections dermatologiques, rhumatologiques, ophtalmologiques) afin d'évaluer si l'effet tensinogénique des anticalcineurines, bien décrit, est associé à une acidose et une hyperkaliémie. En cas d'une prévalence quasi exclusive d'hypertension isolée, cela permettrait de détacher l'acidose et l'hyperkaliémie d'un effet propre aux anticalcineurines.

SERMENT d'HIPPOCRATE

Au moment d'être admis(e) à exercer la médecine, je promets et je jure d'être fidèle aux lois de l'honneur et de la probité.

Mon premier souci sera de rétablir, de préserver ou de promouvoir la santé dans tous ses éléments, physiques et mentaux, individuels et sociaux.

Je respecterai toutes les personnes, leur autonomie et leur volonté, sans aucune discrimination selon leur état ou leurs convictions. J'interviendrai pour les protéger si elles sont affaiblies, vulnérables ou menacées dans leur intégrité ou leur dignité. Même sous la contrainte, je ne ferai pas usage de mes connaissances contre les lois de l'humanité.

J'informerai les patients des décisions envisagées, de leurs raisons et de leurs conséquences.
Je ne tromperai jamais leur confiance et n'exploiterai pas le pouvoir hérité des circonstances pour forcer les consciences.

Je donnerai mes soins à l'indigent et à quiconque me les demandera. Je ne me laisserai pas influencer par la soif du gain ou la recherche de la gloire.

Admis(e) dans l'intimité des personnes, je tairai les secrets qui me seront confiés. Reçu(e) à l'intérieur des maisons, je respecterai les secrets des foyers et ma conduite ne servira pas à corrompre les moeurs.
Je ferai tout pour soulager les souffrances. Je ne prolongerai pas abusivement les agonies. Je ne provoquerai jamais la mort délibérément.

Je préserverai l'indépendance nécessaire à l'accomplissement de ma mission. Je n'entreprendrai rien qui dépasse mes compétences. Je les entretiendrai et les perfectionnerai pour assurer au mieux les services qui me seront demandés.

J'apporterai mon aide à mes confrères ainsi qu'à leurs familles dans l'adversité.

Que les hommes et mes confrères m'accordent leur estime si je suis fidèle à mes promesses ; que je sois déshonoré(e) et méprisé(e) si j'y manque.

Calcineurin inhibitors-induced Gordon-like syndrome: A clinical and biochemical approach

Calcineurin inhibitors (CNI) are immunosuppressive treatments widely used in transplantation. In mice, CNI activate the renal NaCl cotransporter (NCC), and thereby they cause high blood pressure, metabolic acidosis and hyperkalemia. This association of symptoms is also observed in patients suffering Gordon syndrome, a genetic disease also caused by inappropriate stimulation of NCC. Recently, it has been reported that acidosis of Gordon syndrome also involves stimulation of the Cl^-/HCO_3^- exchanger pendrin. In humans, little is known about the prevalence of Gordon-like syndrome phenotype in patients treated with CNI. Interestingly, NCC and pendrin expression can be assessed by immunoblots using urinary exosomes, small extracellular vesicles that reflect the composition of the plasma membrane of their parental cells. We designed two studies including incident CNI-treated kidney transplants 3 months after transplantation: a retrospective study was designed to determine the prevalence of Gordon-like phenotype, then a prospective one to evaluate NCC and pendrin expression in urinary exosomes. 172 patients were included in the retrospective study: 26% exhibited Gordon-like syndrome phenotype. Then, 58 patients were prospectively included and compared to 58 healthy volunteers. Again ~ 24% of kidney transplant recipients exhibited the Gordon-like phenotype. We show, however, that KT with Gordon-like phenotype do not exhibit a significant increase in NCC, pNCC or pendrin abundance. Furthermore, we show that CNI-treated KT have a marked down regulation of NCC, pNCC protein abundance in urinary exosomes compared to healthy volunteers.

Key words: Calcineurin inhibitors - Gordon like syndrome – Urinary exosomes – NCC – Pendrin

Les anticalcineurines sont les immunosuppresseurs les plus utilisés en transplantation rénale. Chez la souris, ils activent le co-transporteur rénal sodium chlore NCC, entrainant un phénotype associant hypertension, acidose métabolique et hyperkaliémie. Ce phénotype est également observé dans une maladie génétique appelée syndrome de Gordon, causée par une stimulation inappropriée de NCC. Récemment, il a été montré que l'acidose dans le Gordon impliquait une stimulation de l'échangeur Cl^-/HCO_3^- pendrine. Chez l'homme, il existe peu de données sur la prévalence d'un phénotype Gordon-like syndrome chez les patients traités par anticalcineurines. De manière intéressante, l'expression de NCC et/ou de pendrine peut être appréhendée par immunoblot en utilisant les exosomes urinaires, qui sont des vésicules extracellulaires dont le contenu dépend de l'activité des cellules émettrices. Nous avons mené deux études qui ont inclus des patients greffés rénaux incidents trois mois après la greffe : Une étude rétrospective pour déterminer la prévalence du phénotype Gordon-like, puis une prospective pour évaluer l'expression de NCC et de pendrine dans les exosomes urinaires. 172 patients ont été inclus dans l'étude rétrospective, dont 26 % présentait le phénotype. Nous avons ensuite recruté 58 patients et 58 volontaires sains. A nouveau, 24 % des greffés présentait le phénotype Gordon-like. Nous montrons, cependant, que les greffés ayant ce phénotype ne présentent pas d'augmentation d'abondance de NCC, pNCC ou pendrine. De plus, nous montrons que les patients greffés sous anticalcineurines ont une abondance significativement moindre de NCC, pNCC dans les exosomes urinaires comparativement aux volontaires.

Mots clefs : Anticalcineurines – Syndrome de Gordon-like – Exosomes urinaires – NCC – Pendrine