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Magnetic bead-based proteomic technology to study paricalcitol effect in kidney transplant recipients

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ABSTRACT

Secondary hyperparathyroidism is a common complication in patients with chronic kidney disease and frequently persists after kidney transplantation. Paricalcitol, a selective vitamin D receptor activator, is indicated in the management of this disorder and recent evidences have suggested that this drug has other beneficial effects. Aiming to elucidate these effects, our study included 52 stable kidney transplant recipients randomized 2:1 to treatment with paricalcitol or to no treatment. Bone mineral parameters, kidney function and inflammatory status were assessed at baseline, at 3 and at 12 months. Moreover, a proteomic approach, based on magnetic beads technology coupled to MALDI-TOF mass spectrometry readout, was used to determine changes in patients' plasma peptidome. Patients treated with paricalcitol showed a significant decrease in parathyroid hormone and alkaline phosphatase levels, and an increase of bone mineral density and glomerular filtration rate. The proteomic analysis revealed a decrease in bradykinin after paricalcitol treatment, whereas 2 peptides identified as fragments of the complement factor C4 decreased only in those patients not treated with paricalcitol. These findings suggest that paricalcitol may offer additional benefits due to immunomodulatory effects via the kallikrein-kinin and complement systems.

1. Introduction

Secondary hyperparathyroidism occurs frequently in patients with chronic kidney disease and usually persists after successful kidney transplantation (Messa et al., 1998; Mitterbauer and Oberbauer, 2008; Sprague et al., 2008; Torres et al., 2002). Persistent hyperparathyroidism can affect the outcome of kidney transplant recipients in several ways. The excessive release of parathyroid hormone (PTH) leads to hypercalcemia due to an excessive bone turnover, which has been associated with increased fracture risk, graft dysfunction and vascular calcifications, thus increasing the risk of cardiovascular morbidity and mortality in kidney transplant recipients (Egbuna et al., 2007; Gwinner et al., 2005; Marcucci et al., 2000; Stavroulopoulos et al., 2007; Torres et al., 2002). For these reasons, prevention and management of hyperparathyroidism are important therapeutic goals after kidney transplantation.

Paricalcitol (19-nor-1,25-dihydroxyvitamin D_2), a selective vitamin D receptor activator, effectively and rapidly suppresses PTH secretion with reduced calcemic and phosphatemic effects (Martin et al., 1998). Several studies have suggested that its benefits may extend beyond the PTH-lowering effect, and it has even been related with greater survival in patients with chronic kidney disease (Cozzolino et al., 2012; Kovesdy and Kalantar-Zadeh, 2008; Teng et al., 2005; Tentori et al., 2006).

In the last years, proteomics has been used in many studies aiming to identify new biomarkers for the diagnosis and prognosis of diseases, as well as biomarkers to predict the response of a patient to a pharmacological treatment. While proteomics studies proteins, peptidomics is focused on the peptidome, which comprises all the peptides and low-molecular-weight proteins contained in a biological sample (Richter et al., 1999; Schrader and Schulz-Knappe, 2001). The combination of mass spectrometry (MS) with functionalized magnetic beads for peptidome enrichment enables the simultaneous study of thousands of peptides and proteins with only a small amount of sample, rapidly and with high sensitivity (Nilsson et al., 2010; Yao et al., 2008).

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Furthermore, this process can be automated in a liquid-handling platform to ensure reproducibility. This proteomic approach has been successfully used to profile the peptidome of different biological fluids (Baumann et al., 2005; Fiedler et al., 2007; Perez et al., 2011, 2010; Villanueva et al., 2004).

To unravel the supposed pleiotropic effects described for paricalcitol, this study examined its effect on bone mineral parameters, kidney function and inflammatory status of a group of kidney transplant recipients. Moreover, because most studies evaluating the effects of paricalcitol have analyzed biochemical or bone mineral parameters, and because the study of changes in blood peptidome due to a pharmacological treatment may help to shed light on the pleiotropic effects of a drug (Perez et al., 2011, 2010), an innovative proteomic technology was used in this study to determine whether 12 months of treatment with paricalcitol modified the plasma peptidome of kidney transplant recipients.

2. Patients and methods

This prospective study included 52 patients with stable kidney transplant who were receiving a triple immunosuppressive regime consisting of prednisone, calcineurin inhibitor (cyclosporine or tacrolimus) and mycophenolate mofetil. Oral paricalcitol, at a dose of 1 μg/day, was prescribed for 31 of them (defined as paricalcitol group) and the other 21 patients did not receive paricalcitol (defined as control group). The demographic and clinical characteristics of kidney transplant recipients are presented in Table 1.

A group of 14 healthy individuals (defined as healthy group) was included (6 males, 34 ± 12 years).

The study protocol was approved by the local Ethics Committee and all patients and healthy subjects gave their informed consent prior to their inclusion in the study.

2.1. Biochemical estimations

Blood and urine samples of kidney transplant recipients were obtained, after an overnight fast, at baseline and at 3 and 12 months. Biochemical variables were determined immediately after extraction.

Serum concentration of 25-hydroxyvitamin D_3 and intact PTH in serum were measured by electrochemiluminescence immunoassays on the Roche Elecsys Modular Analytics E170 (Roche Diagnostics, Basel, Switzerland). Serum levels of alkaline phosphatase were measured by colorimetric assay. Total calcium concentration was determined using a colorimetric end-point assay procedure (Roche Diagnostics) and converted into albumin-corrected calcium (Ca-alb) using the following formula: Ca-alb (mg/dl)=total serum calcium (mg/dl)+[0.8 × (4−albumin) (g/dl)]. Total phosphorous concentration was determined using a phosphomolybdate end-point method (Roche Diagnostics). Bone mineral density measurements of lumbar spine and femoral neck were performed, at baseline and after 12 months, by dual-energy X-ray absorptiometry using a Lunar Prodigy densitometer (GE Healthcare, Madison, WI, USA).

Serum creatinine levels were determined using a modified Jaffe kinetic reaction (Roche Diagnostics). Twenty-four hour proteinuria was measured spectrophotometrically using a Cobas u 711 analyzer (Roche Diagnostics). Glomerular filtration rate was calculated using the Modification of Diet in Renal Disease (MDRD) formula.

Serum levels of ultra sensitive C-reactive protein were measured using a BN ProSpec nephelometer (Siemens, GMBH, Marburg, Germany) with an interassay variation coefficient of 3.7% and 3.5% for C-reactive protein concentrations of 2.38 mg/l and 52.2 mg/l, respectively.

Table 1
Demographic and clinical characteristics of kidney transplant recipients.

	Paricalcitol group	Control group
No. of subjects	31	21
Age (years)	60 (53–65)	55 (42–61)
Male gender (%)	84	81
BMI (kg/m^2)	28 (25–29)	28 (22–29)
Hypertension (%)	87	95
Diabetes mellitus (%)	23	19
Statins (%)	39	24
ACEI (%)	39	38
CyA (%)	43	29
FK (%)	57	71

Data are shown as median (interquartile range). BMI means body mass index; ACEI, angiotensin-converting enzyme inhibitor; CyA, Cyclosporine A; FK, Tacrolimus.

2.2. Peptidome isolation

The proteomic analysis of samples from kidney transplant recipients was performed at baseline and after 3 months. After 12 months, only samples from 22 and 18 patients of the paricalcitol group and the control group, respectively, were available for the proteomic analysis. Peptidome isolation of blood samples from 14 healthy individuals was also performed.

Blood samples were collected into EDTA-containing tubes (BD Vacutainer Plus, Plymouth, UK) and centrifuged at 2200 g for 10 min at room temperature. Plasma was pipetted out of the collection tubes, aliquoted and immediately frozen at −80 °C until processing.

Plasma aliquots were thawed and immediately pre-fractionated with immobilized metal ion affinity chromatography (IMAC-Cu) magnetic beads (Bruker Daltonics, Bremen, Germany). Samples were purified, in duplicate, through several steps of washing, binding and elution, according to the manufacturer's recommended protocol but modified with regard to sample volume for optimization purposes, as described previously (Perez et al., 2011). The eluted peptide fraction obtained was diluted 1:5 with LC-grade water (Lab-Scan, Gliwice, Poland), and then mixed 1:2 with matrix solution (1.84 mg/ml 2,6-dihidroxyacetophenone, 20% acetonitrile, 40 mmol/l ammonium citrate dibasic). One microliter of the resulting mixture was spotted, in duplicate, onto a MALDI target plate (AnchorChip 600/384, Bruker Daltonics) and allowed to air-dry at room temperature to let the matrix crystallize. In this manner, 4 spots of each sample were analyzed by MS. Automation of the analysis was achieved with a liquid-handling platform (Freedom Evo, Tecan, Männedorf, Switzerland), which improved throughput and ensured assay reproducibility.

2.3. Mass spectrometry

Mass spectrometry analyses were performed in an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). Ionization was achieved by irradiation with a 337-nm nitrogen laser operating in linear positive ion mode geometry, with a repetition rate of 1000 Hz. Each spectrum was acquired manually with 1000 laser shots delivered randomly over the surface of the spot. Operating conditions were as follows: ion source voltages, 25 and 22.40 kV; reflector 1, 26.45 kV; reflector 2, 13.40 kV; pulsed ion extraction time, 300 ns; laser energy, 70%. Spectra were externally calibrated with a commercially available mixture of protein/peptide calibrators (ClinProt Peptide Calibration Standard I, Bruker Daltonics), achieving a mass accuracy lower than 10 ppm. Peaks with a signal-to-noise ratio > 3 in the m/z range 1–10 kDa were recorded with the FlexControl acquisition software (version 2.0, Bruker Daltonics).

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2.4. Data analysis

Four spectra were obtained from each sample and a detailed analysis was performed with the DataAnalysis software (version 3.4, Bruker Daltonics), seeking to choose the best spectrum for each sample. Spectra with the highest number of peaks and the highest intensity were selected. ClinProTools software (version 2.2, Bruker Daltonics) was used for the recalibration of the selected spectra, normalizing them to their total ion count and calculating their peak area. For statistical operations, spectral data were converted to ASCII files, exported to Excel spreadsheets and finally analyzed with SPSS software for Windows (version 15.0, SPSS Inc., Chicago, Ill., USA).

2.5. Tandem mass spectrometry analysis

Selected peaks were identified directly by MALDI TOF/TOF analysis as follows. Peptides were purified by use of IMAC-Cu magnetic beads, as described above. Eluates were further trapped and desalted using ZipTip C18 pipette tips (Millipore, Bedford, Mass., USA) according to manufacturer's instructions. Resulting peptides were mixed 1:1 with matrix α-cyano-4-hydroxy cinnamic acid and 1 μl of the mixture was spotted onto a MALDI target plate (Ground Steel, Bruker Daltonics), and analyzed by MALDI-TOF/TOF MS in a MALDI-TOF UltrafleXtreme analyzer (Bruker Daltonics). Spectra were acquired in positive reflector mode with acceleration voltage of 25 kV. Spectra were calibrated using external calibrants (Bruker Bruker Daltonics). The MS/MS data were searched using the MASCOT program (Matrix Science, London, UK). Only proteins identified with a score confidence interval % $\geq$ 95 were accepted.

2.6. Correlation between peptide concentration and peak area

From a bradykinin peptide stock solution (Phoenix Pharmaceuticals, Burlingame, CA) of known concentration (1 ng/μl) different dilutions were made: 3/4 (0.75 ng/μl), 1/2 (0.5 ng/μl), 1/4 (0.25 ng/μl), 1/8 (0.125 ng/μl), 1/16 (0.0625 ng/μl) and 1/80 (0.0125 ng/μl). For each dilution, a capture of the peptide with IMAC-Cu magnetic beads and a posterior analysis by MALDI-TOF MS was conducted, as described above. ClinProTools software was used to calculate the area of the peak *m/z* 1060, which corresponds to bradykinin, for each dilution.

2.7. Statistical analysis

Due to the low number of subjects included in the study, continuous variables were expressed as medians with interquartile ranges. Differences in biochemical variables between groups at baseline were calculated using the nonparametric Mann–Whitney U test. Biochemical variables and plasma peptidome were compared within the different time points by using Wilcoxon or Friedman test, as appropriate. When comparing variables at baseline, at 3 and at 12 months, a testing correction was made following Bonferroni, by multiplying each *P* value by the number of comparisons. Associations among variables were estimated using the Spearman's correlation coefficient. Differences in plasma peptidome according to gender, age and immunosuppression were calculated with the Mann–Whitney U test. All analyses were performed using SPSS software. A *P* value <0.05 was considered to be significant.

3. Results

3.1. Effect of paricalcitol on biochemical variables of kidney transplant recipients

Biochemical variables of kidney transplant recipients at baseline and at 3 and 12 months are presented in Table 2. No differences in baseline values were found between paricalcitol group and control group.

For patients who received paricalcitol, levels of PTH decreased significantly with a median reduction after 12 months of −32.9% (−53.8% to −21.1%). Moreover, paricalcitol treatment resulted in a significant reduction in serum levels of alkaline phosphatase after 12 months of −11.1% (−17.3% to −0.6%). Levels of PTH and alkaline phosphatase remained unchanged in the control group. In parallel, at the end of the study period a small increase in serum levels of calcium was observed in paricalcitol and control group, although only 9.7% and 9.5% of patients reached levels above 10.5 mg/dl, respectively. Serum phosphorus and calcium phosphorous product remained unchanged in both groups.

There were no differences in baseline values of bone mineral density of the lumbar spine and femoral neck between groups. After the study period, 91.3% of patients of the paricalcitol group showed an increase in bone mineral density of femoral neck (Table 2).

An increase of the estimated glomerular filtration rate of 7.7% (1.7% to 18.9%), and a decrease in serum levels of creatinine of −5.1% (−14.0% to −1.1%) was detected after 12 months of paricalcitol treatment. No changes were observed in the control group with respect to kidney function (Table 2).

The significant decrease in PTH, alkaline phosphatase and creatinine serum levels, as well as the increase in bone mineral density was independent of the type of immunosuppression received by patients of the paricalcitol group, including the type of calcineurin inhibitor.

After the study period, the marker of inflammation C-reactive protein showed no significant change in any group (Table 2).

3.2. Effect of paricalcitol on plasma peptidome of kidney transplant recipients

Statistically significant changes in patients' plasma peptidome were observed in both groups during the study period (Table 3). Considering those peaks in which there was a change only in the paricalcitol group, after 12 months of treatment we observed that in 6 of them there was a significant increase in their peak area (*m/z* 1011, 1865, 2044, 2053, 2059 and 2084) and in 5 there was a decrease (*m/z* 1060, 1076, 2311, 2485 and 4964).

In the control group, 4 peaks showed an increase (*m/z* 1098, 1505, 1719 and 1847) and 2 peaks showed a decrease (*m/z* 1740 and 1896) in their area after 12 months; the area of these peaks remained unchanged in the paricalcitol group.

Statistically significant differences were found in the area of another set of peaks after 12 months, but these changes were observed in both groups, excluding the suggestion that they resulted from paricalcitol treatment.

Peaks with *m/z* 1865 and 2022 were identified as fragments of the complement factor C3 (SwissProt accession No.: P01024; *Homo sapiens*; amino acid sequences 1304–1319 and 1304–1320, respectively). After 12 months, the area of the peak with *m/z* 2022 increased in both groups. The area of the peak with *m/z* 1865 increased in both groups although only in the paricalcitol group the change was statistically significant (Fig. 1). In the healthy group, the area of these peaks was statistically significantly lower [12.65 (8.78–19.09) and 47.55 (32.32–77.40), respectively] than in kidney transplant recipients ($P<0.001$).

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Table 2
Biochemical variables of kidney transplant recipients at baseline, at 3 and at 12 months.

	Paricalcitol group						Control group					
	Baseline	3 months	12 months	P0-3	P3-12	P0-12	Baseline	3 months	12 months	P0-3	P3-12	P0-12
25OHVitD (ng/ml)	21.35 (16.30–25.90)	19.90 (13.96–23.05)	21.80 (14.30–28.38)	0.549	0.237	0.504	20.00 (9.74–23.21)	18.00 (7.05–26.97)	10.80 (8.90–17.65)	2.925	1.122	0.099
iPTH (pg/ml)	110.00 (74.80–140.30)	74.25 (47.35–93.88)	59.70 (40.30–90.60)	< 0.001	0.033	< 0.001	92.80 (71.13–118.65)	89.20 (70.13–120.85)	79.15 (63.78–134.05)	2.184	1.110	1.725
ALP (U/l)	70.00 (60.00–84.00)	62.00 (52.00–74.00)	64.00 (54.00–81.50)	< 0.001	0.837	0.021	70.50 (55.50–94.50)	68.50 (53.75–91.25)	66.50 (53.50–79.50)	1.970	0.333	0.186
Serum Ca-alb (mg/dl)	9.34 (9.06–9.57)	9.44 (9.08–9.68)	9.64 (9.28–9.96)	0.408	0.003	< 0.001	9.60 (9.35–9.87)	9.63 (9.00–10.02)	9.85 (9.55–10.18)	2.289	0.015	0.027
Serum phosphorous (mg/dl)	3.10 (2.79–3.44)	3.25 (2.94–3.56)	3.13 (2.71–3.47)	0.387	0.273	2.223	2.94 (2.52–3.39)	2.87 (2.64–3.22)	2.87 (2.50–3.33)	0.939	2.151	1.764
Calcium × phosphorous	29.76 (26.97–33.42)	30.59 (27.62–34.03)	30.38 (25.28–34.41)	0.591	1.881	1.662	28.97 (25.07–33.06)	28.36 (25.41–31.65)	28.48 (26.33–34.28)	0.696	0.537	1.242
BMD lumbar spine	1.13 (0.95–1.25)	–	1.10 (0.97–1.25)	–	–	0.346	1.23 (1.01–1.36)	–	1.18 (1.04–1.36)	–	–	0.569
BMD femoral neck	0.79 (0.71–0.93)	–	0.85 (0.75–0.92)	–	–	0.002	0.93 (0.79–1.06)	–	0.93 (0.80–1.08)	–	–	0.569
Serum creatinine (mg/dl)	1.51 (1.22–1.93)	1.53 (1.28–2.07)	1.47 (1.19–1.80)	0.171	0.003	0.189	1.40 (1.10–1.70)	1.37 (1.10–1.74)	1.24 (1.12–1.52)	2.733	1.263	0.651
MDRD (ml/min/1.73 m^2)	44.00 (33.00–60.00)	42.00 (31.00–57.00)	46.00 (37.00–60.00)	0.951	0.001	0.108	52.00 (39.00–60.00)	52.00 (36.00–66.00)	57.00 (42.00–67.00)	0.741	1.335	0.348
Proteinuria (g/24 h)	0.18 (0.13–0.35)	0.14 (0.10–0.27)	0.21 (0.13–0.39)	0.363	2.460	1.884	0.20 (0.13–0.41)	0.21 (0.15–0.36)	0.16 (0.12–0.50)	2.148	2.328	2.925
CRP (mg/l)	3.28 (1.11–7.06)	2.35 (1.31–5.91)	2.47 (0.76–7.91)	2.946	1.008	2.310	2.93 (1.08–5.38)	1.70 (0.86–5.27)	2.47 (0.84–4.67)	1.842	1.599	2.712

Data are shown as median (interquartile range). To assess differences the nonparametric Wilcoxon Test, adjusted by Bonferroni when multiple comparisons, was performed. P0-3 shows *P* value between baseline and 3 months; P3-12 between 3 and 12 months; and P0-12 between baseline and 12 months. A *P* value < 0.05 was considered significant.
25OHVitD means 25-hydroxyvitamin D; iPTH, intact parathyroid hormone; ALP, alkaline phosphatase; Ca-alb, albumin-corrected calcium; BMD, bone mineral density; MDRD, Modification of Diet in Renal Disease formula; CRP, C-reactive protein.

Table 3
Peak area of plasma peptides of kidney transplant recipients, at baseline and after 3 and 12 months.

m/z	Paricalcitol group						Control group					
	Baseline	3 months	12 months	P0-3	P3-12	P0-12	Baseline	3 months	12 months	P0-3	P3-12	P0-12
1011	5.3 (3.9–10.2)	7.1 (4.2–13.9)	24.1 (12.5–41.2)	0.954	< 0.001	0.003	6.2 (3.4–12.8)	8.3 (5.8–12.9)	10.2 (7.1–23.1)	1.770	0.212	0.143
1060	116.9 (16.5–473.8)	58.4 (19.7–295.6)	45.3 (3.9–145.7)	1.041	0.066	0.006	22.8 (2.8–79.5)	11.6 (3.2–51.5)	68.4 (6.8–101.9)	0.867	1.047	2.637
1076	32.4 (7.2–78.6)	25.8 (4.9–100.5)	5.8 (2.8–46.6)	2.172	0.060	0.009	6.1 (0.8–58)	3.8 (1.8–31.1)	19.5 (2.3–36.0)	1.017	0.307	2.948
1098	2.7 (1.0–19.8)	1.9 (1.2–16.3)	3.5 (1.5–8.6)	1.593	0.789	0.093	0.9 (0.6–1.6)	1.2 (0.8–3.5)	3.7 (1.0–7.0)	0.032	0.067	0.005
1505	8.8 (6.0–12.8)	8.5 (6.1–14.1)	12.8 (4.9–20.5)	1.791	0.447	0.201	5.8 (4.2–10.2)	10.2 (5.5–15.4)	20.7 (11.5–33.9)	0.039	0.116	0.004
1719	10.5 (7.0–16.0)	11.2 (8.0–15.1)	15.0 (10.9–19.2)	1.554	0.600	0.117	10.7 (6.2–14.7)	12.1 (7.5–16.8)	19.2 (10.9–26.7)	0.256	0.212	0.005
1740	19.5 (13.6–28.8)	17.5 (12.4–31.1)	13.0 (8.6–23.1)	2.907	0.831	0.345	23.4 (13.4–32.0)	20.8 (13.3–39.7)	12.6 (8.0–21.1)	2.545	0.010	0.053
1847	28.4 (23.8–44.5)	35.9 (26.0–55.1)	38.0 (23.6–53.7)	0.816	2.199	0.117	31.3 (23.8–51.0)	33.3 (27.2–56.7)	76.6 (35.8–111.5)	0.916	0.059	0.003
1865	40.1 (15.9–162.7)	85.7 (48.2–202.0)	187.2 (94.0–314.4)	0.125	0.670	0.017	99.7 (41.3–165.0)	151.2 (60.2–277.5)	222.5 (74.5–352.7)	0.126	1.417	0.281
1896	939.5 (468.4–1,524.0)	749.5 (360.8–1,244.7)	385.5 (250.7–655.3)	2.085	0.874	0.051	1,165.5 (621.5–1,682.0)	1,175.0 (563.4–1,971.2)	595.8 (405.4–1,049.2)	2.545	0.042	0.075
2044	6.3 (4.5–8.6)	6.5 (4.1–8.6)	12.4 (8.8–16.5)	2.217	0.018	0.002	6.1 (3.5–9.1)	6.4 (4.1–9.2)	7.0 (5.4–12.7)	2.710	1.187	0.158
2053	9.5 (6.6–15.6)	10.8 (7.5–15.2)	21.9 (14.1–34.6)	1.592	0.001	0.002	12.5 (7.6–16.6)	11.6 (8.0–17.8)	16.6 (8.3–26.2)	2.303	0.233	0.093
2059	8.7 (7.0–13.5)	9.3 (5.7–19.0)	75.6 (9.1–104.7)	2.813	0.005	0.001	5.7 (4.1–12.1)	6.0 (4.3–8.1)	5.1 (3.8–8.6)	0.691	1.670	2.331
2084	8.6 (5.0–14.4)	10.3 (7.1–19.2)	32.2 (14.7–47.0)	1.041	0.006	0.002	15.7 (8.9–36.1)	16.8 (8.0–37.6)	45.2 (17.9–97.5)	1.242	0.116	0.059
2311	16.5 (9.1–29.6)	17.3 (7.7–31.8)	8.1 (3.3–23.0)	2.042	0.078	0.004	11.7 (5.0–31.5)	6.9 (3.2–20.1)	11.2 (2.7–18.8)	0.220	1.849	1.116
2485	52.0 (32.4–145.0)	46.3 (24.8–106.7)	28.9 (14.4–62.8)	2.766	0.032	0.005	49.0 (18.8–99.5)	30.7 (22.1–51.4)	48.0 (18.4–91.5)	0.082	0.918	1.187
4964	207.6 (84.6–357.9)	247.5 (102.6–479.4)	35.0 (26.3–163.0)	0.651	0.004	0.043	192.9 (79.2–532.1)	131.9 (37.5–353.5)	142.6 (25.8–422.2)	0.318	2.432	0.800

Data are expressed, in arbitrary units (AU), as median (interquartile range). The nonparametric Wilcoxon test, with Bonferroni adjustment, was performed to assess differences. P0-3 shows *P* value between baseline and 3 months; P3-12 between 3 and 12 months; and P0-12 between baseline and 12 months. A *P* value < 0.05 was considered significant.

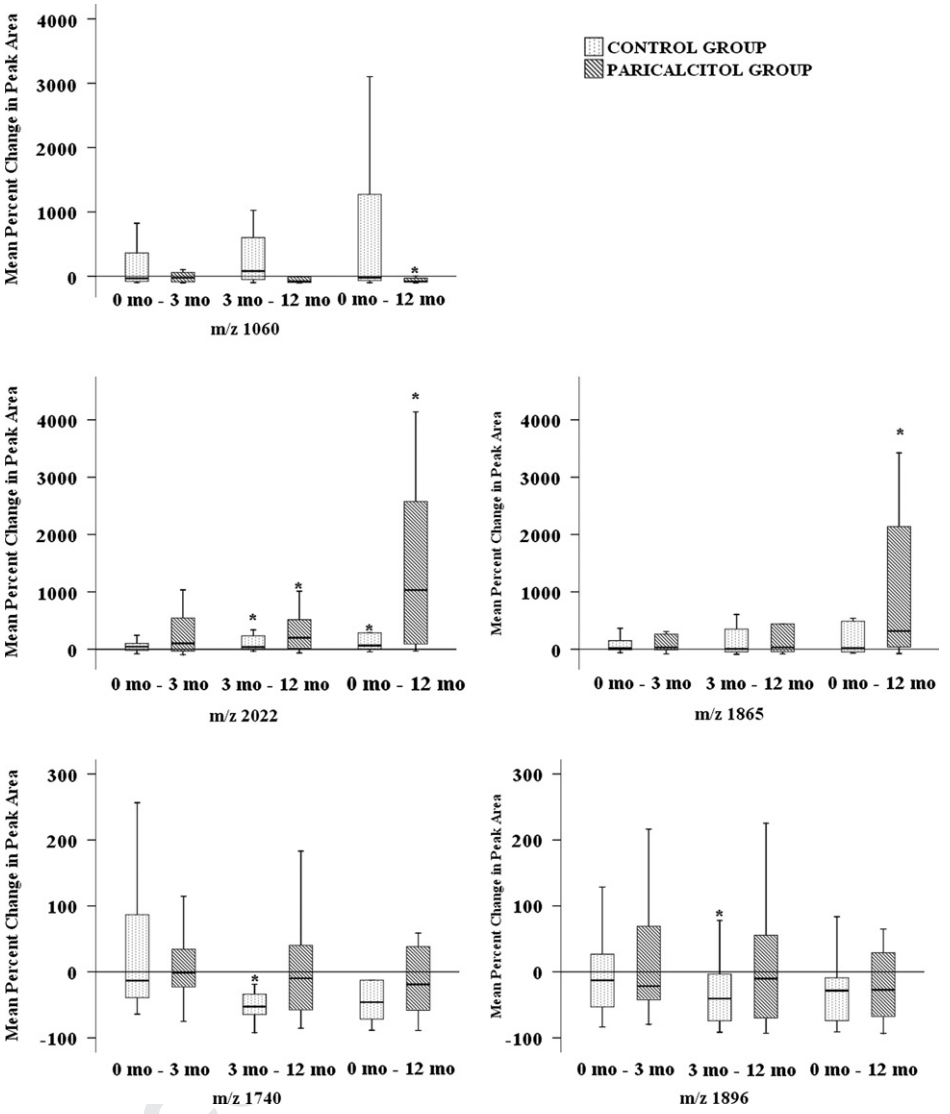

Fig. 1. Percentage change in peak area of plasma peptides, in control group and paricalcitol group. To assess differences the nonparametric Wilcoxon Test, adjusted by Bonferroni was performed. * Indicates a P value <0.05.

Peak with m/z 1060 was identified as bradykinin, a nonapeptide derived from the protein high-molecular-weight kininogen (SwissProt accession no.: P01042; *Homo sapiens*). In the paricalcitol group, its area showed a decrease of –78.1% (–90.8% to –24.5%) (Fig. 2) after 12 months of treatment, while it remained unchanged in the control group. After the study period, the area of this peak in healthy individuals [12.45 (8.34–17.25)] was statistically significantly lower than in patients of the control group ($P=0.045$), but no differences were observed when compared with paricalcitol group ($P=0.150$).

Peaks with m/z 1740 and m/z 1896 were identified as fragments derived from the complement factor C4 (SwissProt accession no.: P0C0L4; *Homo sapiens*; amino acid sequences 1337–1351 and 1337–1352, respectively). A significant decrease in their peak area of –46.1% (–72.6% to 12.8%) and –28.4% (–74.2% to –2.5%), respectively, was observed after the study period only in the control group. In the healthy group, the area of the peak with m/z 1740 [20.62 (11.49–33.18)] showed no differences compared with kidney transplant recipients. The area of the peak with m/z 1896 in healthy individuals [411.67 (264.80–870.85)] was statistically significantly lower compared with kidney transplant recipients at baseline and at 3 months, although after 12 months no differences were observed.

There were no significant differences in the area of peaks m/z 1060, 1740 and 1896 between groups at baseline. The decrease of these peaks was independent of patients' sex and age, as well as of other drugs received, such as statins and angiotensin converting enzyme inhibitors. With respect to immunosuppression, the type of calcineurin inhibitor affected the decrease in the area of peaks m/z 1740 and 1896; patients treated with tacrolimus showed a higher decrease in the area of these peaks [–49.8% (–69.8% to –16.2%) ($P=0.001$) for m/z 1740, and –49.1% (–75.4% to –19.1%) ($P=0.002$) for m/z 1896] than those patients treated with cyclosporine [–11.1% (–50.3% to 58.9%) ($P=0.955$) for m/z 1740, and –12.4% (–39.7% to 30.2%) ($P=0.281$) for m/z 1896].

3.3. Correlation between peptide concentration and peak area

Different concentrations made from a bradykinin stock solution positively correlated with the area of the corresponding peak (m/z 1060) when analyzed by MALDI-TOF MS ($P<0.001$, $r=0.964$) (Fig. 3).

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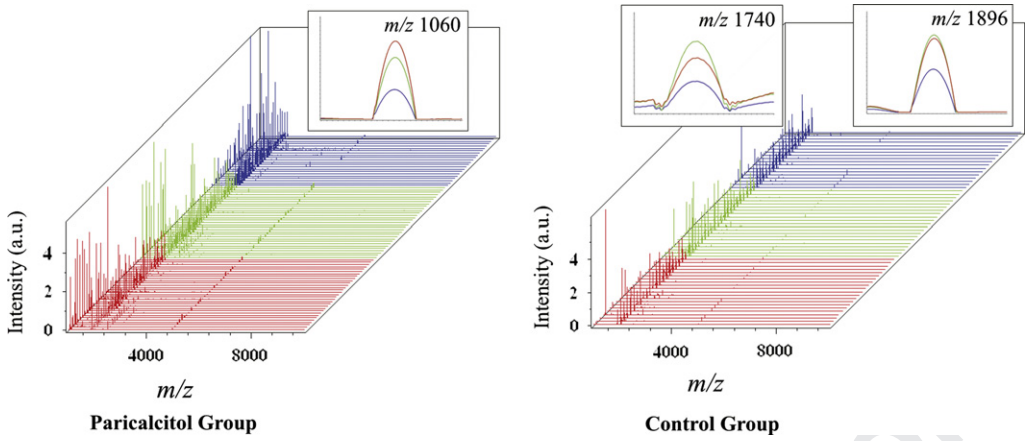

Q4 **Fig. 2.** Stack view, offered by ClinProTools software, of the aligned mass spectra of plasma samples of kidney transplant patients, at baseline (red) and after 3 (green) and 12 months (blue). The enlarged boxes show in detail the average intensity, in arbitrary units, of the signals *m/z* 1060, *m/z* 1740 and *m/z* 1896. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

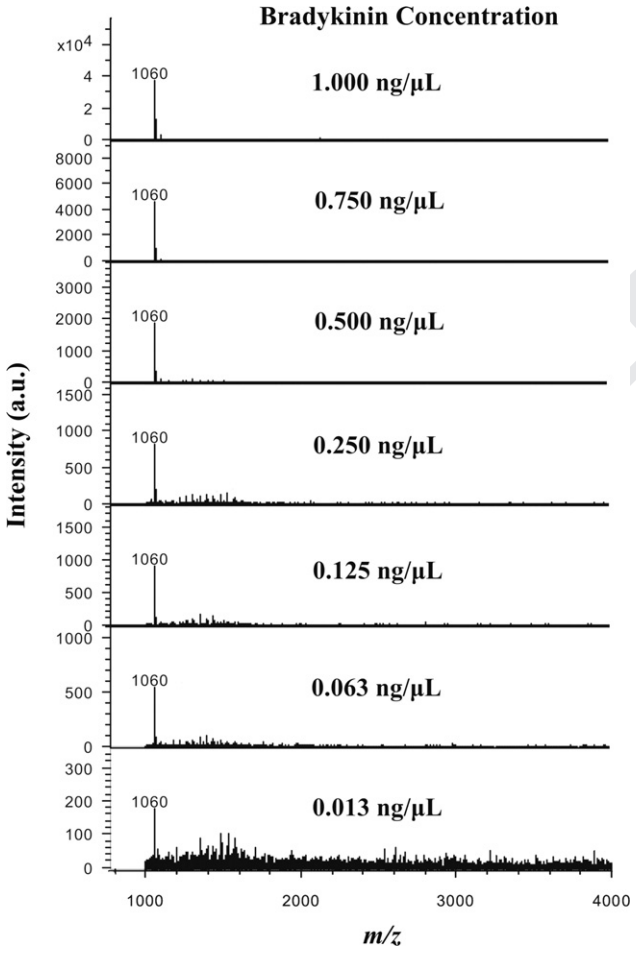

Fig. 3. Intensity (in arbitrary units) of peptide signal *m/z* 1060, corresponding to the peptide bradykinin, when analyzing different solutions of known concentration of the peptide. The nonparametric Wilcoxon test, with Bonferroni adjustment, was performed to assess differences. A P value < 0.05 was considered significant.iPTH means intact parathyroid hormone; ALP, alkaline phosphatase; BMD, bone mineral density.

4. Discussion

The results of our study showed the effectiveness of paricalcitol in controlling secondary hyperparathyroidism in kidney transplant recipients, with minimal hypercalcemic effects. In contrast to other studies, we observed an improvement in kidney function after treatment, although proteinuria remained unchanged (Agarwal et al., 2005, 2011). Furthermore, we used proteomics in an attempt to unravel the supposed pleiotropic effects described for paricalcitol.

Abnormalities of bone and mineral metabolism persist after successful kidney transplantation with persistent hyperparathyroidism (Mitterbauer and Oberbauer, 2008; Sprague et al., 2008). Paricalcitol is commonly used to manage hyperparathyroidism due to its effect suppressing PTH secretion, with minor effects on calcium and phosphorus levels (Martin et al., 1998). As expected, our results showed that treatment with paricalcitol effectively reduced PTH levels. Incidences of hypercalcemia and hyperphosphatemia were not different when comparing with a control group that did not receive paricalcitol, with an increase in serum levels of calcium in both groups that, nevertheless, remained inside the normal range.

Serum alkaline phosphatase is a biochemical marker of bone turnover used to monitor the metabolic bone disease associated with renal insufficiency. In our study, paricalcitol reduced serum levels of this enzyme, and it may be related with improved survival described for this drug, since elevated levels in serum of alkaline phosphatase have been associated with increased mortality in hemodialysis patients (Blayney et al., 2008; Regidor et al., 2008).

Moreover, our results showed that 12 months of treatment with 1 µg/day of paricalcitol slightly recovered femoral bone mineral density of kidney transplant recipients, which may be advantageous decreasing the high incidence of bone fractures suffered by this population (Sprague and Josephson, 2004). A previous experimental study concluded that paricalcitol might be able to ameliorate renal insufficiency-induced loss of bone mineral and mechanical competence of bone (Jokihaara et al., 2006).

Other clinical benefits have been attributed to paricalcitol, not related with its effect inhibiting PTH secretion. Recent clinical and experimental studies have postulated that paricalcitol exerts renoprotective and antiinflammatory actions (Alborzi et al., 2008; Park et al., 2009; Tan et al., 2008). With respect to kidney function, vitamin D receptor activators have been associated with significant reductions in proteinuria and albuminuria in patients with chronic kidney disease (Agarwal et al., 2005; Alborzi et al., 2008; Cheng et al., 2012), but this conclusion cannot be drawn from our findings. Regarding this topic, it should be noted that patients were not required to have proteinuria out of the normal range to be included in our study, so it would be difficult to reduce

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a relative normal level of proteinuria. Results of some clinical studies have shown an effect of paricalcitol increasing serum creatinine levels (Agarwal et al., 2011), but instead of this, we observed a decrease in serum creatinine levels and an increase in glomerular filtration rate.

Similarly, although antiinflammatory effects have been described for paricalcitol (Alborzi et al., 2008), C-reactive protein levels remained stable during the study period, maybe because in both groups about three quarters of patients had normal levels of this marker of inflammation at baseline (<5 mg/L).

In an attempt to understand the pathways affected by the supposed pleiotropic effects of paricalcitol, we used a proteomic approach based on magnetic beads for peptidome isolation coupled to MALDI-TOF MS analysis, and ClinProtTools software for peak area calculation. This combination allowed us to analyze a considerable number of peptides in a small volume of sample. This is, to our knowledge, the first study screening the effect of paricalcitol on plasma peptidome.

The analysis revealed that, although paricalcitol did not produce a decrease of C-reactive protein levels, it caused a decrease of bradykinin, another inflammatory mediator. Bradykinin is a polypeptide released from precursor molecule kininogen by action of enzymes called kallikreins at those sites of tissue injury and/or inflammation. Bradykinin is involved in a number of pathophysiological conditions, mainly by stimulation of bradykinin B2 receptors (Hall, 1997); it causes pain by activating sensory nerve terminals, induces the release of proinflammatory and hyperalgesic mediators such as neuropeptides, leukotrienes and cytokines, increases vascular permeability and induces vasodilatation, smooth muscle contraction and cell proliferation (Bhoola et al., 1992; Ueno and Oh-ishi, 2003). Regarding the proinflammatory actions of bradykinin, it has been reported that injecting the polypeptide into the skin of experimental animals and human volunteers resulted in the classical signs of inflammation, which are redness, pain, heat, swelling and the accumulation of leukocytes (Ellis and Fozard, 2002).

A progressive decrease in the peak area of the peptide bradykinin was observed only in the group treated with paricalcitol, achieving values similar to those observed in a group of healthy individuals. Changes in the area of this peak were independent of the treatment with angiotensin converting enzyme inhibitors, which increase levels of bradykinin in blood by inhibiting its enzymatic breakdown by angiotensin converting enzyme.

We found that the area of the peak *m/z* 1060, which corresponds to bradykinin, positively correlated with its concentration, and consequently it might be stated that after paricalcitol treatment there was a decrease of bradykinin in plasma of kidney transplant recipients. The way in which paricalcitol reduces blood levels of bradykinin remains unknown.

A decrease in the peak area of peptides identified as part of the complement factor C4 was observed in the control group but not in those patients that received paricalcitol. The complement system is a host defense system composed of a network of proteins that play an important role in innate and adaptive immunity. Moreover, recent evidences have demonstrated that the complement system is also involved in a variety of physiological and pathophysiological processes, such as promoting inflammation, enhancing the adaptive immune response, removing damaged cells, and it is involved in tissue regeneration and in angiogenesis (Markiewski and Lambris, 2007). Paricalcitol may be beneficial for kidney transplant recipients because deficiency in complement components levels predisposes to infections and autoimmune syndromes (Figueroa and Densen, 1991). Moreover, paricalcitol may be beneficial in kidney transplantation since earlier studies on soluble complement components C3 and C4 conducted in kidney transplant recipients reported a decrease in levels of these proteins during rejection episodes (Barnes et al., 1983; Sonkar and Singh, 2011).

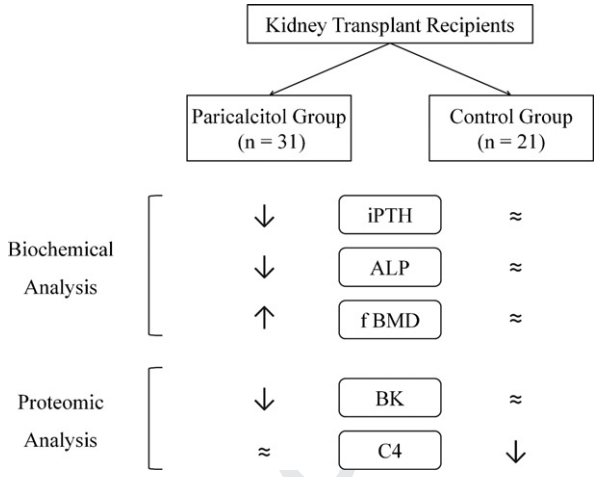

Fig. 4. Schematic diagram showing the statistically significant changes in biochemical variables and in the plasma peptidome of kidney transplant recipients after the study period. iPTH means intact parathyroid hormone; ALP, alkaline phosphatase; f BMD, bone mineral density of femoral neck.

Worth noting that the decrease in peptides of the complement factor C4 also depended of the type of calcineurin inhibitor received, with higher reductions observed in those patients treated with tacrolimus instead of cyclosporine. The mechanism of action of these immunosuppressive drugs is similar, but tacrolimus is up to 100 times more potent than cyclosporine. Tacrolimus prevents cytokine transcription and lymphocyte activation and, although the mechanism by which it affects the complement cascade remains unknown, our findings suggest a greater immunosuppressive effect of tacrolimus Fig. 4.

Of particular concern would be to identify the rest of peaks found in this study, arriving to the name of their parent proteins, i.e., those to which these peptides belong, as this would help to know more about the possible pleiotropic effects of paricalcitol.

5. Conclusions

Paricalcitol administration efficiently ameliorated mineral bone loss of kidney transplant recipients, who are at increased risk of fractures. Moreover, our study suggested that paricalcitol may provide other additional benefits due to immunomodulatory effects via the kallikrein-kinin and complement systems.

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