CLINICAL STUDY

Analysis of the 206M polymorphic variant of the *SLC26A6* gene encoding a Cl^- oxalate transporter in patients with primary hyperparathyroidism

S Corbetta, C Eller-Vainicher¹, M Frigerio², R Valaperta², E Costa², L Vicentini³, A Baccarelli⁴, P Beck-Peccoz¹ and A Spada¹

Endocrinology and Diabetology Unit, Department of Medical-Surgical Sciences, University of Milan, IRCCS Policlinico San Donato, 20097 San Donato Milanese, Milan, Italy, ¹Endocrine Unit, Department of Medical Sciences, University of Milan, IRCCS Fondazione Ospedale Maggiore, Mangiagalli e Regina Elena, Milan, Italy, ²Laboratory of Molecular Biology, IRCCS Policlinico San Donato, San Donato Milanese, Milan, Italy, ³Endocrine Surgery, IRCCS Fondazione Ospedale Maggiore, Mangiagalli e Regina Elena, Milan, Italy and ⁴Department of Preventive Medicine, University of Milan, IRCCS Fondazione Ospedale Maggiore, Mangiagalli e Regina Elena, Milan, Italy

(Correspondence should be addressed to S Corbetta; Email: sabrina.corbetta@unimi.it)

Abstract

Objective: Primary hyperparathyroidism (PHPT) is often complicated by kidney stones. Hypercalciuria and urine oxalate excretion are considered risk factors for urolithiasis in PHPT as well as in idiopathic stone-formers. Recently, the anion-exchanger *SLC26A6* has been involved in the oxalate metabolism. **Design and methods:** We tested the hypothesis that the 206M polymorphic variant of *SLC26A6* gene might contribute to the risk of kidney stones in PHPT. DNA samples from 145 PHPT patients and 129 age- and sex-matched healthy subjects were genotyped.

Results: The homozygous 206V genotype was the most frequent both in PHPT patients and controls (79.3 and 74.4%), while heterozygosity for the 206M allele was detected in 20.0 and 23.3% respectively. The homozygous 206M genotype was extremely rare, occurring in 0.7 and 2.3% of PHPT and healthy subjects respectively. In the PHPT cohort, the prevalence of urolithiasis did not differ between the V/V and V/M+M/M groups and urine oxalate excretions did not correlate with the genotype. Considering the subset of PHPT stone formers ($n=74$), calciuria was lower in V/M+M/M patients with respect to V/V stone-formers (4.40 ± 1.88 vs 5.92 ± 2.62 mg/kg per 24 h; mean $\pm$ s.d., $P=0.034$). Finally, the *SLC26A6* 206M alleles were significantly related to the presence of hypertension (73.3 vs 47.8%), showing an OR of 4.8.

Conclusions: Though the *SLC26A6* 206M polymorphism did not correlate with kidney stone development in PHPT patients, PHPT stone-formers harbouring the M allele had a lower hypercalciuria. This observation and the high prevalence of hypertension associated with the 206M polymorphism need further investigation.

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Introduction

The incidence of calcium oxalate nephrolithiasis is increasing (1) and primary hyperparathyroidism (PHPT) has been recognised as a well known, removable cause in up to 5% of stone formers (2). Kidney stones occur in PHPT with a prevalence ranging 10–40% in the different series (3–6). Massive hypercalciuria and urine oxalate excretion have been considered risk factors for the development of kidney stones both in idiopathic and in PHPT stone-formers (6, 7). As far as the genetic factors favouring hypercalciuria are concerned, polymorphic variants of the gene encoding the calcium-sensing receptor, which regulates PTH secretion from parathyroid cells and calcium reabsorption in the renal distal convolute tubule, have been

extensively investigated in PHPT patients (8, 9) as well in idiopathic stone-formers (10).

The role of urine oxalate excretion is poorly defined, though urine oxalate excretion has been indicated as a risk factor for nephrolithiasis (6, 7). With the exception of the few conditions genetically determined, hyperoxaluria is due to excessive intestinal absorption. Recently, the anion-exchanger *SLC26A6* (also known as Cl^- formate exchanger (CFEX) and putative anion transporter (PAT)1 has been recognised as a key molecule in oxalate metabolism (11, 12). *SLC26A6* is a member of the *SLC26* ‘sulphate transporter’ family, a family of multifunctional anion exchangers. In the intestine, *SLC26A6* mediates oxalate excretion and thereby limits the net absorption of ingested oxalate, a process critical for the prevention of hyperoxaluria and calcium oxalate

nephrolithiasis (12, 13). In human kidney, SLC26A6 is expressed in distal segments of proximal tubules, where it mediates oxalate-dependent NaCl absorption (11), in parts of the thin and thick ascending limbs of Henle's loops, macula densa, distal convoluted tubules and a subpopulation of intercalated cells of collecting ducts (14). Aronson *et al.* reported that mice lacking the anion exchanger *Slc26a6* showed hyperoxalemia and hyperoxaluria, which ameliorated under an oxalate-free diet, and a high incidence of calcium oxalate urolithiasis (12). This genetic manipulation clearly demonstrates that intestinal oxalate excretion plays a critical role in decreasing oxalate filtered load, preventing hyperoxalemia and reducing the risk of stone disease (15).

The human *SLC26A6* gene resides on chromosome 3p21.3 and has several known splice variants encoding different isoforms. Up to now no mutation has been identified in humans, while valine replacement by methionine at amino acid 206 position of the long N-terminal isoform, which corresponds to the amino acid 185 position of the short N-terminal isoform, is the only non-synonymous polymorphism reported for this gene in the human SNP database up to now (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=13324142). Recently, the human V206M variant has been demonstrated *in vitro* to exhibit reduced Cl⁻/oxalate exchange activity (20). In the present study, we first investigated the clinical impact of the human V206M polymorphic variants in a population at high risk of nephrolithiasis, such as PHPT patients.

Methods

Subjects

The study included 145 consecutive unrelated Italian patients (121 females and 24 males; mean age at presentation 61.7 ± 12.8 years, 50 of them described in a previous study (6)) referred to our Departments between 2000 and 2007 for diagnosis and management of PHPT. As controls, 129 age- and sex-matched subjects recruited among subjects referred to our outpatient Endocrine Units for subclinical thyroid diseases. Subjects were eligible as controls if they were Caucasian, had no personal or family history of kidney stones, had normal serum creatinine, calcium and phosphate, and were not taking any drug. Diagnoses of PHPT were made on the basis of high ionised calcium levels in the presence of elevated or inappropriately normal serum PTH levels (plasma ionised calcium 1.51 ± 0.15 mmol/l (1.31–2.10), n.v. 1.15–1.29 mmol/l; total serum calcium 2.76 ± 0.23 mmol/l (2.29–3.61), n.v. 2.20–2.60 mmol/l; serum PTH 172.0 ± 118.0 pg/ml (38–620), n.v. 10–65 pg/ml; mean $\pm$ s.d. (range)). The diagnosis of PHPT was confirmed by surgery in 77 patients (57% of the cohort). The series included 11 unrelated patients

with familial PHPT. Genetic analysis of genomic DNA revealed nine different *MEN1* gene mutations in nine patients (primers available on request).

All patients had normal serum creatinine. Fifty-three percent ($n=77$) of the patients presented with a previously established diagnosis of arterial blood hypertension according to the World Health Organization (SBP > 135 and DBP > 85 mmHg) that was appropriately treated. Anti-hypertensive therapy was modified in order to avoid administration of diuretics, with a washout period of at least 3 months. Nephrolithiasis, defined as a history of renal colics with stone expulsions and/or imaging identification or asymptomatic ultrasound imaging of stones, was identified in 51% of the patients ($n=74$). Stone composition was available in a minority of cases and revealed calcium oxalate stones. Urine sediment analysis performed in 50 PHPT subjects as previously described (6) showed calcium oxalate crystals in 82% of cases of crystalluria. Osteoporosis, defined as a *T*-score lower than -2.5 at least in one of the sites evaluated with dual energy X-ray absorptiometry (DEXA at femur and lumbar spine level) was present in 50% of the patients ($n=73$). No patients have been taking anti-fracture drugs (bisphosphonates or vitamin D) in the 3 months preceding the study.

Patients or controls with known bowel disease or resection as well as with cystic fibrosis have been excluded.

The study complies with the Helsinki Declaration revised in 2000 and was approved by the local Ethical Committee (IRCCS Fondazione Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milan, Italy). All participants signed an informed consent form, which included consent for genetic testing.

Laboratory tests

Venous blood samples after overnight fasting were obtained from all patients under a free diet for measurement of ionised calcium, total calcium, phosphate, intact PTH and creatinine, as previously reported (6). Plasma ionised calcium was measured by a potentiometric method (Radiometer ABL System 625, Copenhagen, Denmark) on heparinised blood samples within 30 min from blood collection. Serum intact PTH was measured by a chemiluminescent method (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), with intra- and interassay coefficients of variation of < 4.5 and < 10.0% respectively. Serum 1,25-dihydroxyvitamin D₃ was assessed by RIA kit (Immunodiagnostic Systems Ltd, Boldon, UK), with an intra- and interassay coefficients of variation of < 8 and < 10% respectively. Calcium, phosphate and creatinine were measured in 24 h urine collections (from 0700 h of the day before to 0700 h of the examination day) in all patients, while oxalate were determined in 50 randomly selected subjects (32 stone-formers and 18 non-stone formers) by enzymatic commercial kit using oxalate

oxidase (Greiner Diagnostic GmbH, Bahlingen, Germany; CV 5.6%) in a 24 h urine sample. Glomerular filtration rates were estimated by creatinine clearance calculation for all patients. All patients underwent an ultrasound examination of the urinary tract. All had bone mineral density evaluation by DEXA of the lumbar spine L2–L4 and the proximal femur (femoral neck).

DNA extraction and genotyping

Genomic DNA was extracted from 200 µl peripheral blood of each patients and control samples by High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's specifications.

The SNP at position 804 CT of SLC26A6 mRNA long N-terminal variant was genotyped using the TaqMan MGB allelic discrimination system assessed with the ABI PRISM 7900HT sequence detector machine and its correlated software SDS 2.3 (Applied Biosystems, Foster City, CA, USA) (17). Polymerase chain reaction was performed with a total volume of 25 µl, which contained 10 ng DNA, 1X TaqMan Universal PCR Master Mix (Applied Biosystems), each primer at a concentration of 36 µM, and each probe at a concentration of 8 µM. Primers and Probes of the 804 CT polymorphism were obtained from Applied Biosystems (Assay ID: C_25627247_10). The amplification was done under the following conditions: 95 °C for 10 min to activate the AmpliTaq Gold enzyme, followed by 40 cycles of 15 s at 92 °C (denaturation) and 1 min at 60 °C (annealing/extension). The fluorescence levels were measured with an ABI PRISM 7900HT (Applied Biosystems) before and after the PCR amplification. The allelic discrimination results were determined after the amplification by performing an end-point read. Quality control samples were included in the genotyping assay: each allelic discrimination session contained two water (first and last negative controls) and three unblinded controls for the three different genotypes.

Statistical analysis

Quantitative variables in the text were described as mean $\pm$ s.d. Differences among means were tested by using *t*-test, or one-way ANOVA with Bonferroni post-test where data were normally distributed. Otherwise, Mann–Whitney rank-sum test was performed. Chi-squared analysis test was used to test the hypothesis of no difference between patients and controls alleles frequency. Fisher's exact test was applied whenever expected values were <5 . Correlations between variables were analysed by Pearson's coefficient. A multiple logistic regression model was used to define independent risk factors for the occurrence of hypertension in PHPT patients. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS version 12.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

The frequency of the different alleles of the SLC26A6 gene in patients with PHPT ($n=145$) was similar to that observed in age- and sex-matched healthy subjects ($n=129$) and to that reported in other healthy populations previously evaluated with the same method (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=13324142). The homozygous 206V genotype was the most frequent both in PHPT patients and healthy controls (79.3 and 74.4% respectively), while heterozygosity for the 206M allele was detected in 20.0 and 23.3% respectively. The homozygous 206M genotype was rare, 0.7 and 2.3%, in PHPT and healthy cohorts respectively, with no significant difference (Table 1). The distribution of genotype in healthy controls was in Hardy–Weinberg equilibrium ($P=0.71$, exact HWE test). Owing to the very low prevalence of homozygous 206M genotype both in patients and controls, associations between SLC26A6 variants and quantitative variables were evaluated by grouping heterozygotes with relative homozygotes in the V/M+M/M group. No significant difference in biochemical parameters, such as plasma ionised calcium, serum total calcium and phosphorus, PTH, 1,25-dihydroxyvitamin D₃ levels, urine calcium levels, creatinine clearance and glomerular filtration rate was detected in PHPT patients according to the different SLC26A6 genotypes (Table 2). Similarly, the prevalence of kidney stone and the prevalence and severity of osteoporosis did not differ between the V/V and V/M+M/M groups (Table 2).

The majority of PHPT patients in whom urine oxalate excretion was evaluated ($n=50$) showed urine oxalate levels within the normal range (<40 mg/24 h, (18)). No significant difference in the mean urine oxalate levels between V/V and V/M+M/M groups was detected (Table 2).

Considering the subset of stone-formers patients ($n=74$), the V/V group did not differ from the V/M+M/M group for age, sex, severity of hyperparathyroidism (plasma ionised and serum total calcium and phosphorus levels, serum PTH levels and serum 1,25-dihydroxyvitamin D₃) and prevalence of osteoporosis (Table 3). Conversely, urine calcium excretion levels were significantly lower in the V/M+M/M with respect

Table 1 Comparison of genotype and allele frequencies for the V206M polymorphism of the SLC26A6 gene in primary hyperparathyroid patients and age and sex-matched healthy controls.

SLC26A6 codon 206 polymorphism	PHPT patients ($n=145$)	Healthy controls ($n=129$)	Significance
V/V, n (%)	115 (79.3)	96 (74.4)	NS
V/M, n (%)	29 (20.0)	30 (23.3)	NS
M/M, n (%)	1 (0.7)	3 (2.3)	NS
V allele frequency	0.893	0.860	NS
M allele frequency	0.107	0.140	NS

Table 2 Biochemical and clinical parameters observed in 145 patients with primary hyperparathyroidism according to the *SLC26A6* genotypes.

	nv	V/V	V/M+M/M	P
Number of patients		115	30	
Ionised Ca ²⁺ (mmol/l)	1.15–1.29	1.51±0.15	1.50±0.13	NS
Serum Ca (mg/dl)	8.4–10.4	11.2±0.9	11.3±0.8	NS
Serum PTH (pg/ml)	10–65	163.0±107.2	195.8±144.0	NS
Serum P (mg/dl)	2.7–4.5	2.24±0.43	2.24±0.37	NS
1,25(OH) ₂ D ₃ (pg/ml)	15–50	60.0±33.0	45.2±33.5	NS
Serum Cr (mg/dl)	0.7–1.1	0.84±0.27	0.83±0.26	NS
Cr Cl (ml/min)	70–100	87.6±30.2	83.4±26.4	NS
Urine Ca (mg/kg per 24 h)	<4.00	4.79±2.96	4.37±2.09	NS
Urine Ox (mg/24 h)	<40.0	22.6±11.6	27.9±13.3	NS
Nephrolithiasis (%)		50.4	53.3	NS
Osteoporosis (%)		51.3	46.7	NS
Femoral T-score		−1.73±1.04	−1.87±1.14	NS
L2–L4 T-score		−2.20±1.44	−1.82±1.63	NS
Hypertension (%)		47.8	73.3	0.013

Owing to the very low prevalence of 206M allele, patients homozygous or heterozygous for this allele were grouped (V/M+M/M). Data are reported as mean±s.d. V/V, homozygotes for 206V; V/M+M/M, heterozygotes and homozygotes for 206M. Nephrolithiasis was defined as a history of renal colics with stone expulsions and/or imaging identification or asymptomatic ultrasound imaging of stones. Osteoporosis was defined as a T-score lower than -2.5 at least in one of the sites evaluated with dual energy X-ray absorptiometry (DEXA at femur and lumbar spine level). Arterial hypertension was defined as systolic and diastolic blood pressure >135 and >85 mmHg respectively. Ox, oxalate; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; Cr, creatinine; Cr Cl, creatinine clearance; nv, normal values; NS, not statistically significant.

to V/V stone-formers (4.40 ± 1.88 vs 5.92 ± 2.62 mg/kg per 24 h; $P=0.034$; Table 3). A trend to such a difference in mean urine calcium excretion levels between the V/M+M/M and the V/V stone formers was present also in the subset of PHPT patients evaluated for the urine oxalate excretion.

Finally, the *SLC26A6* 206M alleles were significantly associated to the presence of hypertension in the overall cohort of PHPT patients and in the subset of stone formers (73.3 vs 47.8% and 75.0 vs 43.1%, $P=0.013$ and 0.048 respectively; Tables 2 and 3). After correction for age, BMI and creatinine clearance levels, PHPT patients harbouring the genotype 206M showed an odds ratio for hypertension of 4.8 (95% CI 1.5–14.9, $P=0.006$).

Discussion

In this study, we tested the hypothesis that polymorphic variants of the human *SLC26A6* gene might be associated with the development of kidney stones in PHPT patients. This hypothesis was supported by a number of considerations. Urine oxalate excretion has been identified as a risk factor for the development of kidney stones in both idiopathic and PHPT stone-formers (7, 19). In particular, relatively high oxaluria was found to be associated with a sevenfold increased risk for kidney stones in a similar cohort of PHPT patients (6, 7). Accordingly, calcium oxalate crystals occurred frequently in stone formers PHPT patients (6). The recently identified Cl[−] oxalate exchanger,

Table 3 Clinical and biochemical parameters observed in primary hyperparathyroidism (PHPT) patients complicated with nephrolithiasis ($n=74$) according to the *SLC26A6* genotypes.

	nv	V/V	V/M+M/M	P
<i>n</i>		58	16	
F/M		37/19	12/4	NS
Age (years)		58.6±12.7	60.4±13.6	NS
Ionised Ca ²⁺ (mmol/l)	1.15–1.29	1.56±0.17	1.52±0.10	NS
Serum Ca (mg/dl)	8.4–10.4	11.5±1.1	11.5±0.5	NS
Serum PTH (pg/ml)	10.0–65.0	188.6±131.4	239.2±145.7	NS
Serum P (mg/dl)	2.7–4.5	2.24±0.43	2.24±0.37	NS
1,25(OH) ₂ D ₃ (pg/ml)	15.0–50.0	65.5±34.1	49.3±41.1	NS
Serum Cr (mg/dl)	0.7–1.1	0.81±0.19	0.85±0.27	NS
Cr Cl (ml/min)	70.0–100.0	97.5±27.4	83.7±28.0	NS
Urine Ca (mg/kg per 24 h)	<4.00	5.92±2.62	4.40±1.88	0.034
Urine Ox (mg/24 h)	<40.0	23.7±11.8	26.7±7.8	NS
Osteoporosis (%)		46.5	43.7	NS
Hypertension (%)		43.1	75.0	0.048

Data are reported as mean±s.d. Ox, oxalate; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; Cr, creatinine; Cr Cl, creatinine clearance; NS, not statistically significant.

encoded by the *SLC26A6* gene, provided the molecular key of oxalate excretion regulation. The crucial role of this exchanger is clearly documented by the phenotype of *Slc26a6*-null mice that is characterised by the presence of hyperoxalemia, hyperoxaluria and calcium oxalate urolithiasis (12). In this model, the absence of the Cl^- exchanger converts the intestinal oxalate excretion in net oxalate absorption (20). The search for polymorphic variants rather than inactivating mutations of *SLC26A6* gene in patients with PHPT was in accordance with the observation that PHPT patients generally show urine oxalate excretion levels similar to those reported in healthy subjects and well below the levels observed in idiopathic stone formers (21, 22). Indeed, minimal changes in the intestinal and renal oxalate excretion potentially determined by *SLC26A6* gene polymorphisms might have been associated with increased susceptibility to kidney stone formation in PHPT patients. So far, the valine replacement by methionine at amino acid 206 position of the long N-terminal isoform of the *SLC26A6* protein is the only polymorphic change identified. This change is located in the domain indicated as 'sulphate transporter' that is highly conserved through the different *SLC26* family members as well as through other transporter proteins (<http://www.ncbi.nlm.nih.gov/blast/>). Importantly, the human V206M variant transfected in *Xenopus* oocytes, has been demonstrated to exhibit reduced Cl^- /oxalate exchange activity (16). In particular, the 206M variant showed slightly reduced Cl^- influx driven by injected oxalate, slightly reduced oxalate uptake, and a Cl^- concentration dependence slightly shifted towards higher Cl^- concentrations, physiologically relevant for the human intestinal lumen. This small shift has been suggested to reduce intestinal oxalate secretion enough in the setting of urinary calcium oxalate supersaturation to accelerate lithogenesis (16).

The present study failed to find any significant association between the polymorphic variants of the human *SLC26A6* gene and the development of kidney stones in this cohort of PHPT patients. In particular, the urine oxalate excretions were not related to the V206M genotypes. Admittedly, three major points need to be considered about the discrepancy between clinical negative data presented here and the available experimental data about *SLC26A6* V206M variant (16). First, functional data about the human 206M were relative to its homozygotic condition. In the present study, the M/M genotype was extremely rare both in PHPT and healthy cohorts, namely one PHPT patient and three healthy controls, making difficult to evaluate its actual clinical impact. Secondly, as far as the renal oxalate excretions are concerned, several acquired conditions might influence urine oxalate concentrations, overcoming the effect of the genetic background (23–26). In particular, high levels of 1,25-dihydroxyvitamin D_3

might induce high calcium and consequently oxalate intestinal absorption (23, 24), although in the present series of PHPT patients serum 1,25-dihydroxyvitamin D_3 levels were not significantly correlated with urine oxalate concentrations. Third, it has been suggested that oxalate can affect stone formation and growth independently from the direct effect of elevated urine concentrations, for example via interactions with the renal epithelium (25).

Although nephrolithiasis was present with the same frequency in PHPT patients independently from *SLC26A6* genotype, we investigated the potential effect of the 206M allele in the subset of PHPT stone-formers. The present cohort of PHPT patients showed a high prevalence of kidney stones. Interestingly, stone-formers harbouring the 206M allele showed a less severe urine calcium excretion, in the absence of differences in sex, age, renal function and PHPT severity. Therefore, though there is no experimental evidence that *SLC26A6* could be involved in urine calcium excretion, it is tempting to speculate that the *SLC26A6* M allele might contribute to the occurrence of kidney stones in PHPT patients with relatively low urine calcium excretion.

Finally, the study showed that 206M allele was significantly related to the presence of hypertension and was associated with a fourfold increased risk for hypertension also after correction for age, BMI and GFR. *In vitro* data suggested a model by which oxalate might facilitate NaCl entry across the apical membrane of proximal tubule cells (26). In particular, the *SLC26A6* transporter appears to primarily contribute to transtubular NaCl absorption by mediating Cl^- oxalate exchange in the intact proximal tubule under physiological conditions (12, 13, 27). Since *Slc26a6*-null mice were normotensive and showed no significant difference in plasma and urine concentrations of urea, Na^+ , Cl^- in comparison with the wild-type animals, the association between 206M allele and hypertension in PHPT patients was an unexpected finding. However, it should be considered that the NaCl facilitating function of the Cl^- exchanger has been demonstrated in the proximal tubule, while the exchanger might be involved in the NaCl reabsorption in other segment of nephron where it has been demonstrated to be expressed and where its function is still obscure (14). Moreover, other genes as well as PTH itself might interact and modulate the function of the *SLC26A6* exchanger at renal tubule level. The unexpected association of 206M variant with hypertension deserves further study in order to better understand the role of the Cl^- oxalate exchanger in the physiology of renal NaCl absorption in humans.

In conclusion, this study reports that the 206M polymorphic variant of *SLC26A6* gene is not associated with an increased risk of nephrolithiasis in PHPT patients. However, the observation that PHPT stone-formers harbouring the M allele showed relatively lower renal calcium excretion suggests a potential, though

minor, role for the SLC26A6 activity in the risk of kidney stones development. Further studies are needed to confirm the association of 206M variant with high prevalence of hypertension in other populations and to understand the molecular and biological events responsible for this relationship.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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