Impact of Natriuretic Peptide Clearance Receptor (*NPR3*) Gene Variants on Blood Pressure in Type 2 Diabetes

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OBJECTIVE—Hypertension in diabetes is characterized by abnormal sodium homeostasis, suggesting a particular role of natriuretic peptide pathway. Natriuretic peptides can affect blood pressure (BP) through their plasma concentrations, which are dependent on their receptor activities. We thus assessed the association between nine *NPR3* gene polymorphisms and BP levels in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS—Nine single nucleotide polymorphisms (SNPs) tagging the haplotype structure of the *NPR3* gene were genotyped in the 3,126 French Non-insulin-dependent Diabetes, Hypertension, Microalbuminuria or Proteinuria, Cardiovascular Events, and Ramipril (DIABHYCAR) trial participants. We then used a second population (Diabete de type 2, Nephropathie et Genetique [DIAB2NEPHROGENE]/Survie, Diabete de type 2 et Genetique [SURDIAGENE] study) of 2,452 patients for the purpose of replication. Finally, we separately investigated subjects selected according to their rs 2270915SNP genotypes for their BP response to salt restriction.

RESULTS—In DIABHYCAR patients, three SNPs (rs6889608, rs1173773, and rs2270915) were significantly associated with systolic BP (SBP). The effect of the rs2270915 was replicated in the second step population: AA homozygotes had a lower SBP than G carriers (137.4 ± 19.1 vs. 140.0 ± 20.2 mmHg, $P = 0.004$). The rs2270915 influenced the response of SBP to salt reduction, with AA homozygous patients showing greater reductions after restriction of salt intake compared with G carriers: −20 mmHg (−43 to −8) vs. −3 (−20 to +7); $P = 0.006$.

CONCLUSIONS—We found a consistent and significant association between the rs2270915 polymorphism of the *NPR3* gene and SBP in diabetic patients. This genetic variation may affect pressure response to changes in dietary sodium.

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Hypertension and type 2 diabetes are two chronic conditions associated epidemiologically and pathophysiologically. Hypertension is a well-known risk factor for diabetic vascular complications in type 1 diabetes and type 2 diabetes, and is a complex trait with an established heritability (1). Genome-wide association studies are complementary to a candidate gene approach and have helped to identify new genetic pathways involved in blood pressure (BP). However, only a few genetic associations have been clearly identified and replicated to date, leaving some impetus to search for new candidate genes.

Diabetic patients differ from nondiabetic patients by having an increase in total body sodium (2,3), renal tubular sodium reabsorption, and an impaired ability to excrete a sodium load (4). These factors suggest that natriuretic peptides (NPs) may play a key role in the pathophysiology of hypertension in the diabetic population.

The NP system regulates BP and fluid homeostasis by modifying glomerular filtration rate (GFR) and sodium urinary excretion. This system consists of a family of three peptidic hormones (A-type NP, B-type NP [BNP], and C-type NP) that interact with three receptors (NP receptor A, NP receptor B, and NP clearance receptor [NPRC]). The NPRC is encoded by the *NPR3* gene and is a determinant of NP plasma concentration. Local expression of NPRC is linked to NP activity in the kidney (5,6). The intracellular domain of NPRC modulates the contraction/relaxation properties of smooth muscle cells (5). Genetic variation in the *NPR3* may therefore affect the activity of NP through affecting plasma levels or NPRC-mediated vascular effects. The *NPR3* is located on chromosome 5p14-p13. It spans ~70 kb and contains eight exons and seven introns. Within the general population, allelic variants of the *NPR3* gene have been reported to be associated with hypertension (6–8).

This study aimed to assess the influence of genetic polymorphisms within *NPR3* on BP in patients with type 2

diabetes. We first used a candidate gene approach in two independent large populations. We then completed our investigation with a sodium restriction functional study.

RESEARCH DESIGN AND METHODS

Study protocols and subjects

First-step population: DIABHYCAR study. The design and results of the Non-insulin-dependent DIABetes, HYpertension, microalbuminuria or proteinuria, CARdiovascular events, and ramipril (DIABHYCAR) study have been reported (9). Briefly, the DIABHYCAR study is a clinical trial comparing the effect of a low dose of ramipril with placebo on cardiovascular and renal complications of patients with type 2 diabetes with micro- or macroalbuminuria. The main selection criteria were as follows: patients with type 2 diabetes undergoing treatment with oral antidiabetic drugs, high urinary albumin excretion, age ≥50 years, and serum creatinine ≤150 μmol/L. This first step population included 3,413 patients recruited in France. From the first step cohort data, we selected the single nucleotide polymorphisms (SNPs) that were most significantly associated with differences in systolic BP (SBP).

Second-step population: DIAB-2-NEPHRO-GENE and SURDIAGENE studies. This cohort, dedicated to replication, was previously described (10). Briefly, patients were recruited for the DIABete de type 2, NEPHROpathie et GENEtique (DIAB2-NEPHRO-GENE; D2NG) or the SURvie, DIAbete de type 2 et GENEtique (SURDIAGENE; SDG) study. The D2NG is a French multicenter case-control study of patients with type 2 diabetes, designed to assess the genetic determinants of diabetic nephropathy in type 2 diabetes. The SDG study is an inception cohort with ongoing recruitment of patients with type 2 diabetes regularly attending the diabetes department at Poitiers University Hospital, France. Altogether, the second step population comprised 2,635 unrelated patients (10). The design of these studies was approved by University Hospital Ethics Committees (Angers and Poitiers, respectively). All participants in the study gave their written informed consent.

Sodium restriction functional study

A subset of SDG micro- and macroalbuminuric patients selected for their rs2270915 genotypes were invited to participate in a "sodium-restriction functional" study (study design detailed in Supplementary Data and Supplementary Fig. 1). We included seven AA-genotype and seven AG- or GG-genotype patients, matched for age (±5 years), urinary albumin status (micro- or macroalbuminuria), and antihypertensive therapy: renin-angiotensin-aldosterone system blocker use (angiotensin receptor blocker or ACE inhibitor), and diuretics. Exclusion criteria included uncontrolled hypertension, estimated GFR <30 mL/min (Modification of Diet in Renal Disease formula), and prior low sodium diets. The study design was approved by the Poitiers University Hospital Ethics Committee. All patients gave informed consent before starting the study.

Polymorphism determination

On the basis of the Caucasian European population data available in the HapMap phase II panel (http://www.hapmap.org), at least one SNP was selected in each haplotype block of this large gene, focusing on SNPs associated with relevant end points in the literature. Thus, we selected one SNP (rs9716700) in the promoter region (6,7,11), three in haplotype block 1 (rs1421811, rs1252246, rs6889608) (11), two in haplotype block 2 (rs700923, rs16890196) (12), one in haplotype block 3 (rs1173773) (11), and two in haplotype block 4 (rs1173743, rs2270915) (11,13,14) (Supplementary Fig. 2).

The full set of nine SNPs was genotyped in the first step cohort analysis. SNPs were determined by quantitative PCR performed using the Taqman method (ABI/PRISM 7700; Applied Biosystems, Foster City, CA) or allele-specific PCR-based KASPar SNP genotyping system (KBiosciences, Hoddesdon, U.K.).

The three of the nine SNPs with nominal association with SBP were selected for the replication step and determined with the same methods. Genotyping errors were checked on 96 randomly selected samples with both TaqMan and KASPAR methods with a 100% concordance rate.

Laboratory methods

Serum creatinine and urinary albumin were measured by nephelometry on a Modular System P (Roche Diagnostics GmbH, Mannheim, Germany). Renal function was estimated by GFR using the four-variable Modification of Diet in Renal Disease formula. Urinary creatinine was measured on a Hitachi911 automatic analyzer (Roche Diagnostics, Meylan, France). Glycated hemoglobin (A1C) was determined by using a high-performance liquid chromatography method with an ADAMS A1C HA-8160 analyzer (normal values 4.0–6.0%; Menarini, Florence, Italy). Plasma *N*-terminal pro B-type natriuretic peptide (NT-proBNP) concentration was determined using ElecsysproBNP sandwich immunoassay on a Modular System E (Roche Diagnostics, Meylan, France). Measures were duplicated on each sample, and mean was considered for statistical analyses.

Statistical analysis

Statistical analyses were performed with StatView 5.0 (SAS Institute, Cary, NC). Patient characteristics were expressed as means ± SD or medians (interquartile range) for skewed distributions. Groups were compared using the χ^2 test for categoric variables or parametric (ANOVA) or nonparametric (if not normally distributed, Kruskal-Wallis or Mann-Whitney *U* tests) tests for continuous variables.

Allele frequencies were estimated by the gene-counting method, and Hardy-Weinberg equilibrium (HWE) was tested by the χ^2 test. We used THESIAS software for pairwise linkage disequilibrium (LD) between *NPR3* polymorphisms (expressed in terms of the D' and r^2 statistics) and haplotype analysis (http://genecanvas.ecgene.net/downloads.php?cat_id=1). Associations between BP and genotypes were evaluated by linear regression analysis and ANOVA.

Meta-analysis of our two cohorts was performed using the METAL software (http://www.sph.umich.edu/csg/abecasis/Metal/index.html) based on the weighted *z* score method.

The Bayesian method implemented in BIMBAM (http://quartus.uchicago.edu/~yguan/bimbam/index.html) was used with the HapMap CEU data to impute the genotype of other HapMap SNPs in the *NPR3* gene. The imputed SNPs were subsequently subjected to association testing with BP as well.

P values ≤ 0.05 were considered statistically significant.

RESULTS

First-step population: DIABHYCAR study—univariate analysis

DNA samples were available from 3,126 French patients in the DIABHYCAR study. Clinical and biological characteristics of the

patients are presented in Supplementary Table 1. Genotyping success ranged from 99.1 to 98.3%. The genotype distribution was in HWE for all SNPs except for rs6889608 (P = 0.046) and rs1173743 (P = 0.049). Pairwise LD is shown in Supplementary Table 2.

Of the nine studied SNPs, three (rs6889608, rs1173773, rs2270915) were significantly associated with SBP (Table 1) and six were not (Supplementary Table 3). AA homozygotes for the rs2270915 SNP had an SBP of 143.9 ± 13.0 vs. 144.9 ± 13.6 mmHg for G carriers (P = 0.03). Stratification on presence or absence of obesity (defined as BMI >30 kg/m^2) showed an association between SBP and rs1173773 in obese patients (P = 0.003) but not in nonobese patients (Supplementary Table 4).

Second-step population: D2NG and SDG studies—univariate analysis

Baseline characteristics of replication study participants are presented in Supplementary Table 1. DNA samples were available for 2,452 patients. Genotyping success ranged from 97.02 to 99.71%. There were 2,043 Europid and 409 non-Europid patients, including 88 subjects of African origin. We restricted our analyses to Europid patients. In this group, all the selected SNPs (rs6889608, rs1173773, rs2270915) were in HWE.

SBP was significantly associated with rs2270915 but not with rs6889608 and rs1173773 (Table 2). In agreement with the DIABHYCAR study, AA homozygotes for the rs2270915 SNP had a significantly lower SBP than G carriers (137.4 ± 19.1 vs. 140.0 ± 20.2 mmHg, P = 0.004). Stratification on obesity did not replicate in this population (Supplementary Table 5).

Multivariate and conjunct analysis

Multiple linear regression analysis showed that both rs1173773 and rs2270915, age, and BMI were independent predictive factors of SBP in each cohort (Table 3).

In addition, we combined the genotype analysis for SBP in a meta-analysis of our two cohorts, confirming the significant association between SBP and rs2270915 (z score = −3.109, P = 0.002) but not rs1173773 and rs6889608 (P = 0.111 and 0.519, respectively).

The haplotype analysis on conjunct cohorts evidenced that one haplotype containing the rs2270915 G allele was significantly associated with SBP (Supplementary Table 6).

Sodium reduction study

No differences were observed regarding duration of diabetes, medical history, or baseline BP between rs2270915AA genotype and G-carrier participants.

The clinical and biological responses to the sodium restriction functional study are presented in Table 4. Urinary sodium excretions did not differ between groups at baseline or in response to sodium restriction.

SBP, urinary albumin excretion, and NT-proBNP significantly decreased under restricted salt diet. The decrease was more marked in AA than in G carriers for SBP and urinary albumin, suggesting a significant gene-environment interaction. However, no such association was found for NT-proBNP. The responses to the salt restriction of SBP, according to genotype group, are plotted in Supplementary Fig. 3. The absolute difference in SBP after salt restriction was −20 mmHg (−43 to −8) vs. −3 (−20 to 7) (P = 0.006) in AA versus G carriers, respectively.

No significant modification of body weight, heart rate, and serum creatinine was observed in either of the two genotype groups (Supplementary Table 7).

CONCLUSIONS

We performed a large-scale analysis of several genetic variants within the *NPR3* gene in more than 5,500 patients with type 2 diabetes. We found that the rs2270915 SNP was associated with differences in SBP in patients with type 2 diabetes; in the DIABHYCAR and D2NG/SDG studies, the G allele was associated with a higher adjusted SBP of 1.1 and 1.7 mmHg, respectively. This G allele was also present in a haplotype significantly associated with higher SBP. We found that salt intake strongly influenced the relationship between BP and this genetic polymorphism, with a decreased salt sensitivity in G carriers compared with AA homozygotes.

The choice of the different SNPs was based on the haplotype structure of the *NPR3*, as detailed in RESEARCH DESIGN AND METHODS. We tested the association between imputed genotypes and SBP in the DIABHYCAR population. Graphically this analysis further supported the choice of SNPs rs6889608, rs1173773, and rs2270915 (Supplementary Fig. 4).

All the studied SNPs were in HWE, suggesting no selection bias in the replication study for which ethnic background was available. The current study mainly found that the rs2270915 was associated with SBP in the DIABHYCAR population, with positive replication in the D2NG/SDG studies. Other reports from the general population were not in accordance with our finding in type 2 diabetes. Iemitsu

Table 1—*Relationship between SBP and SNPs of* NPR3 *in patients from the DIABHYCAR study*

	Genotype			*P* value codominant model*/§		*P* value A1 dominant model†		*P* value A2 dominant model‡
SNP	A_1A_1	A_1A_2	A_2A_2		A_1 carriers		A_2 carriers	
rs6889608	CC	CT	TT		C carriers		T carriers	
	n = 231	*n* = 1,151	*n* = 1,715		*n* = 1,382		*n* = 2,866	
	145.2 ± 12.3	144.9 ± 13.0	143.7 ± 13.4	0.034/0.012	145.0 ± 12.9	0.010	144.2 ± 13.3	0.26
rs1173773	GG	AG	AA		G carriers		A carriers	
	n = 332	*n* = 1,397	*n* = 1,369		*n* = 1,729		*n* = 2,766	
	142.4 ± 13.2	144.4 ± 13.5	144.7 ± 12.9	0.018/0.019	144.1 ± 13.5	0.16	144.5 ± 13.2	0.005
rs2207915	GG	AG	AA		G carriers		A carriers	
	n = 140	*n* = 987	*n* = 1,971		*n* = 1,127		*n* = 2,958	
	145.8 ± 13.9	144.8 ± 13.5	143.9 ± 13.0	0.08/0.024	144.9 ± 13.6	0.035	144.2 ± 13.2	0.17

Data are means ± SD. */§ Upper *P* values correspond to ANOVA statistics: A_1A_1 vs. A_1A_2 vs. A_2A_2 /lower *P* values correspond to simple regression analysis: 0, 1, or 2 minor alleles. †A_1 carriers vs. A_2A_2. ‡A_2 carriers vs. A_1A_1.

Table 2—*Relationship between SBP and SNPs of* NPR3 *in Europid patients from the D2NG/SDG studies*

SNP	Genotype			*P* value codominant model*/†	A_1 carriers	*P* value A1 dominant model‡	A_2 carriers	*P* value A2 dominant model§
	A_1A_1	A_1A_2	A_2A_2					
rs6889608	CC	CT	TT		C carriers		T carriers	
	n = 116	*n* = 685	*n* = 1,160		*n* = 801		*n* = 2,230	
	135.5 ± 20.1	139.0 ± 19.2	138.4 ± 19.7	0.20/0.56	138.5 ± 19.3	0.93	138.6 ± 19.5	0.09
rs1173773	GG	AG	AA		G carriers		A carriers	
	n = 234	*n* = 864	*n* = 860		*n* = 1,098		*n* = 2,190	
	140.0 ± 19.1	138.9 ± 20.1	137.6 ± 18.9	0.16/0.056	139.1 ± 19.9	0.08	138.2 ± 19.5	0.19
rs2207915	GG	AG	AA		G carriers		A carriers	
	n = 91	*n* = 659	*n* = 1,271		*n* = 750		*n* = 2,440	
	137.0 ± 19.8	140.4 ± 20.2	137.4 ± 19.1	0.004/0.031	140.0 ± 20.2	0.004	138.4 ± 19.5	0.48

Data are means ± SD. */†Upper *P* values correspond to ANOVA statistics: A_1A_1 vs. A_1A_2 vs. A_2A_2./lower *P* values correspond to simple regression analysis: 0, 1, or 2 minor alleles. ‡A_1 carriers vs. A_2A_2. §A_2 carriers vs. A_1A_1.

et al. (14) found that the G allele was not related to SBP in the general population. These Japanese patients had a similar genotype frequency as our patients, but only 291 nondiabetic subjects were studied with a focus on arterial stiffness. Lanfear et al. (13) did not find a relationship between this SNP and BNP concentrations on the 100 genotyped Caucasian patients. The findings from Sarzani et al. (6), suggesting that the rs9716700 was associated with BP, were not replicated. In our study, the rs9716700 was in HWE, at variance with the data from Sarzani et al. (6). Genotypic frequencies were different in the two studies, suggesting different recruitments. We also evidenced a relationship between SBP and rs1173773 in obese patients (not reported by Sarzani et al. [6]) but not for rs9716700 as documented by others (6,8). However, we focused on patients with type 2 diabetes, who are well known to be particularly salt sensitive (15), possibly explaining the discrepancy for these results.

The conjunct analysis showed that a haplotype containing the rs2270915 G allele was associated with an increased BP compared with the reference haplotype. In multivariate analysis, the rs2270915 dose effect had an impact on SBP ranging from 1.1 to 1.7 mmHg in these two different populations. This effect seems to be modest, but it could be clinically relevant. We can speculate from data of clinical trials on BP that the rs2270915 effect on SBP could translate into a lower cardiovascular mortality risk by 2.5% over 4 years (16).

Our data from the functional salt restriction study, although generated on a small group, provide a better understanding of the effect of this genetic polymorphism on BP in patients with type 2 diabetes. We found that salt restriction was associated with a decrease in SBP and albuminuria, as demonstrated by others (17). As previously reported, the salt-sensitive AA genotype responded more strongly to salt restriction on albumin excretion than the salt-resistant G carriers (18). This result seems counterintuitive because it is generally accepted that salt-sensitivity is associated with hypertension (19), and we found that salt-sensitive patients (AA genotype) had a lower SBP than salt-resistant patients (G carriers). However, it has been reported that salt supplementation downregulated NPRC expression in the kidney (20). The gene interaction found in the current study therefore leads us to hypothesize that the influence of *NPR3* variants on BP could be masked in the presence of high sodium intake.

The speculations about the biological impact of the rs2270915 SNP are exciting. It is a nonsynonymous polymorphism resulting in the replacement of an asparagine by an aspartate residue in the COOH-terminal region of the NPRC, N521D. In silico analysis using PolyPhen2 (http://genetics.bwh.harvard.edu/pph2) predicted the consequence of this rs2270915 to be "probably damaging" to the protein. This residue is conserved in various species, including humans, rodents, and cows (21). This intracellular truncated domain has affinity for pertussis toxin-sensitive G proteins and is able to activate/inhibit various enzymes in the cell, such as adenylyl cyclase and isoforms of phospholipase C (21,22). Synthetic polypeptides generated by mutational study of this 37-amino acid intracellular domain were used to identify the homologous 17-amino acid sequence, R^{513}RNHQEESN^{521}IGKHRELR^{529}, responsible for these binding capacities (21) and were necessary and sufficient for the activation of G proteins and effector enzymes (23). Recent studies suggest that changes to this sequence, such as that introduced by the missense mutation in the current study, are likely to modify the tone of smooth muscle cells, as suggested by recent studies (23). NPRC is widely expressed in vascular smooth muscle cells and mesangial cells (23,24). Changes

Table 3—*Impact of NPR3 genetic variants and clinical variables on SPB (mmHg) from the DIABHYCAR and D2NG/SDG studies (multivariate analysis)*

	DIABHYCAR		D2NG/SDG	
	Estimate ± SE	*P* value	Estimate ± SE	*P* value
Sex (compared with male)	1.9 ± 0.5	0.0004	−1.5 ± 0.9	0.1058
Age (for each increment of 1 year)	0.2 ± 0.1	<0.0001	0.3 ± 0.04	<0.0001
BMI (for each increment of 1 kg/m^2)	0.3 ± 0.1	<0.0001	0.3 ± 0.1	0.0003
rs6889608*	1.2 ± 0.5	0.0087	−0.5 ± 0.7	0.5189
rs1173773*	−0.9 ± 0.4	0.0141	1.4 ± 0.7	0.0296
rs2270915*	1.1 ± 0.5	0.0163	1.7 ± 0.8	0.0306

*For each addition of a minor allele (i.e., C, G, and G for rs6889608, rs1173773, rs2270915, respectively).

Table 4—*Clinical and biological response to salt reduction in the functional study*

Variable	Salt diet	AA (n = 7)	P value	G carriers (n = 7)	Genotype effect (P value)	Global genotype effect (P value)
UNa (mmol/24 h)	Usual	215 ± 91		232 ± 85	0.749	0.51
	Low	103 ± 65		121 ± 69	0.565	
Treatment effect (P value)		0.018		0.018		
Global treatment effect (P value)			0.001			0.949*
SBP (mmHg)	Usual	144.6 ± 14.3		137.4 ± 7.7	0.338	0.51
	Low	124.2 ± 11.0		134.2 ± 7.7	0.096	
Treatment effect (P value)		0.018		0.40		
Global treatment effect (P value)			0.006			0.006*
Ualb (mg/24 h)	Usual	233 (419)		282 (405)	0.655	0.97
	Low	178 (239)		239 (456)	0.655	
Treatment effect (P value)		0.018		0.40		
Global treatment effect (P value)			0.013			0.048*
NT-proBNP (pg/mL)	Usual	97 (141)		147 (651)	0.338	0.07
	Low	46 (149)		95 (181)	0.227	
Treatment effect (P value)		0.128		0.0425		
Global treatment effect (P value)			0.013			0.338*

Data are mean ± SD or median (interquartile range). Treatment effect corresponds to the effect of the dietary salt restriction. UNa, urinary sodium excretion; Ualb, urinary albumin excretion. *P values for estimated genotype-treatment interaction.

in the *NPR3* primary sequence might therefore affect systemic and renal hemodynamics (natriuresis, vascular and mesangial tone) via at least two different mechanisms: changes in NP clearance by internalization and changes in the activation/inhibition capacities of the intracellular domain. Measuring atrial natriuretic peptide response in kidney cells of patients with type 2 diabetes according to rs2270915 genotype was beyond the scope of this article.

Some limitations must be acknowledged in this study. We had no information regarding salt intake in the DIABHYCAR and D2NG/SDG studies. Salt intake may have affected the relationship between BP and the genetic variants of *NPR3*. Our functional study on salt restriction is exciting but must be regarded as a pilot study. Although not performed on patients with type 2 diabetes, adequately powered studies on the genetic component of salt sensitivity, such as the recently released DASH trial, are mandatory to replicate our findings (25).

Finally, the question of multiple testing is an important issue. We found a replicated effect of the rs2270915 SNP in two independent cohorts. Associations were of borderline signification after Bonferroni correction in the DIABHYCAR population for any of the selected SNPs. However, the LD between the SNPs suggests that the tests were not independent, and thus Bonferroni method may overcorrect the multiplicity of testing. The borderline P values nevertheless deserved additional testing in a replication population. In the D2NG/SDG study, the significant association of rs2270915 with SBP was maintained after Bonferroni correction. The consistency of the results across the studies, including the functional study, strongly suggests that the association is not a pure chance finding. Further studies, particularly in patients from other ethnic origins, are warranted.

The effect of this *NPR3* variant observed in two independent populations was substantiated in a functional study. Homozygous AA patients for the rs2270915 SNP could be identified as a low-risk genotype and "salt restriction responders." Conversely, G carrier patients could be classified as "high-risk" because they seem to have higher SBP and are less sensitive to salt reduction. Our data suggesting a gene-environment interaction support the speculation of an individual tailoring of antihypertensive therapeutics according to its *NPR3* genotype. This point deserves further confirmation before routine clinical use.

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