The Functional c.-2G>C Variant of the Mineralocorticoid Receptor Modulates Blood Pressure, Renin, and Aldosterone Levels


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The Functional c.-2G>C Variant of the Mineralocorticoid Receptor Modulates Blood Pressure, Renin, and Aldosterone Levels


Abstract—The mineralocorticoid receptor (MR) is essential in the regulation of volemia and blood pressure. Rare mutations in the MR gene cause type 1 pseudohypoaldosteronism and hypertension. In this study we characterized the common MR polymorphism c.-2G>C (rs2070951) in vitro and tested its influence on parameters related to blood pressure regulation and the renin-angiotensin system. In vitro studies showed that the G allele was associated with decreased MR protein levels and reduced transcriptional activation compared with the C allele. Association studies were performed with several outcome variables in 3 independent cohorts: a mild hypertensive group subjected to a salt-sensitivity test, a healthy normotensive group included in a crossover study to receive both a high and low Na/K diet, and a large cohort (The Netherlands Study of Depression and Anxiety), in which blood pressure was measured. Subjects with the GG genotype had significantly higher plasma renin levels both in the mild hypertensive group and in normal volunteers compared with homozygous C carriers. The GG genotype was also correlated with higher plasma aldosterone levels in healthy subjects. In both the mild hypertensive group and The Netherlands Study of Depression and Anxiety cohort the genotype GG was associated with higher systolic blood pressure in males. In conclusion, the G allele of the common functional genetic polymorphism c.-2G>C in the MR gene associates with increased activation of the renin-angiotensin-aldosterone axis and with increased blood pressure, probably related to decreased MR expression. (Hypertension. 2010;56:995-1002.)

Key Words: mineralocorticoid ■ aldosterone ■ hypertension ■ nuclear receptor ■ sodium balance

The mineralocorticoid receptor (MR) mediates aldosterone effects on electrolyte balance and blood pressure (BP). Classical MR-expressing tissues include the distal parts of the nephron, colon, salivary glands, and sweat glands, where MR regulates transepithelial sodium transport. However, MRs are also expressed in nonepithelial tissues, including the cardiovascular system and the central nervous system; in these tissues, glucocorticoids represent the predominant endogenous ligand.1 The MR belongs to the nuclear receptor superfamily and acts as a ligand-activated transcription factor regulating expression of a coordinate set of genes ultimately eliciting physiological aldosterone and cortisol responses. The gene coding for the human MR, NR3C2, is composed of 10 exons and spans over ∼400 kb. By means of alternative promoter use, alternative splicing, use of different translational start sites, and genetic polymorphisms, considerable variability in MR function has been observed.2,3

Sodium handling is highly variable between individuals, and genetic factors are involved in the development of hypertension.4 Rare mutations of the MR are responsible for mendelian disorders of renal salt handling associated with high or low BP. Loss of function mutations of the MR lead to
type 1 pseudohypoaldosteronism, whereas the rare activating mutation S810L leads to juvenile hypertension exacerbated by pregnancy. Two recent studies showed associations between more common genetic variations, single nucleotide polymorphisms (SNPs), in the MR and BP. Previously we tested the amino acid changing SNP in exon 2, MR1180V (rs5522), in vitro and showed that the rs5522 G allele leads to a lower transactivational capacity. However, in a group of mild hypertensive individuals we did not find an association with BP and MR1180V, and the frequency of the same polymorphism was similar between hypertensive subjects and controls from a Brazilian birth cohort.

MRc.-2G>C (rs2070951) is a frequent SNP located in the 5’-untranslated (UT) region of the NR3C2 gene, 2 nucleotides upstream of the first translation start site. The C allele of MRc.-2G>C has been associated with lower basal cortisol levels and a decrease in MR-dependent transcriptional activa-
tions from a Brazilian birth cohort. Finally, we tested for an association between BP measures in included in a crossover study to receive a low Na-high K or 2-well phenotyped groups, a normotensive healthy group then assessed the effect of this polymorphism on sodium transactivational activity in vitro with different ligands. We investigated the functionality of MRc.-2G>C by testing its effect on MR protein expression, its influence on the MR-A/MR-B protein ratio, and the transactivational activity in vitro with different ligands. We then assessed the effect of this polymorphism on sodium handling and regulation of the renin-angiotensin system in 2-well phenotyped groups, a normotensive healthy group included in a crossover study to receive a low Na-high K or a high Na-low K diet and in a group of mildly hypertensive patients exposed to a salt sensitivity (Weinberger) test. Finally, we tested for an association between BP measures in a large multisite cohort for depression and anxiety.

Materials and Methods

Transactivation Assays
Rabbit RCSV3 cells derived from a kidney cortical collecting duct (kindly provided by Prof P. Ronco, Hôpital Tenon, Paris, France) were grown as described previously and transfected using lipofectamine 2000 (Invitrogen) with 0.25 μg of pcDNA3 plasmid containing either MR-2G or MR-2C, 0.625 μg of a GRE2_TATA_luc reporter plasmid and 0.25 μg of pSVβgal. The day after transfection, steroids were added at different concentrations, and 48 hours after transfection, luciferase and β-galactosidase activities were assayed using the Dual-Light System and the Galacton-Plus Substrate (Applied Biosystems). Results were standardized for transfection efficiency and expressed as the ratio of luciferase activity over β-galactosidase activity in arbitrary units.

Recombinant plasmids used in this study are presented in Figure 1. For construction of recombinant plasmids and transactivation assays in COS-1 cells, please see the online Data Supplement at http://hyper.ahajournals.org.

Protein Expression Studies
For studies investigating the effect of the c.-2G>C SNP on protein synthesis, rabbit RCSV3 cells and COS-7 cells were seeded in 6-well plates at a density of 3 × 10⁵ cells per well and 24 hours before transfection in fresh medium without any added steroid. Cells were transfected by the calcium phosphate method with 0.66 μg of plasmids pcDNA3 1α2-galuc, pcDNA3 1α2C-luc, pcDNA3 1β2-galuc, or pcDNA3 1β2C-luc. Cotransfection of 0.16 μg of pSVβgal (Clontech) was performed to normalize for transfection efficiencies. Cellular extracts were assayed for luciferase and β-galactosidase activities as described previously. Results were expressed as the ratio of luciferase activity over β-galactosidase activity in arbitrary units.

For Western blot, Cos-1 cells were seeded in 6-well plates (Greiner Bio-One) at 2 × 10⁵ cells per well. The cells were transfected the next day using Transit Cos transfection reagent (Mirus). Plasmids containing one of the MR variants, for example, MR-2G, MR-2C, mutated MR only expressing MR-A, mutated MR only expressing MR-B, or no MR (control), were used at 2 μg per well. Cells were harvested 48 hours after transfection. The Western blots using primary antibodies MR 1D5, detecting amino acid 1 to 18 and, therefore, only MR-A, and 2B7, detecting amino acid 64 to 82 and, therefore, both MR-A and MR-B, (a generous gift by C.E. Gomez-Sanchez, Division of Endocrinology, University of Mississippi, Jackson, MS) were performed as described previously. The differences in intensity of the MR bands were quantified with ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/).

RNA Isolation and Real-Time Quantitative PCR
For RNA isolation and real-time quantitative PCR procedures, please see the online Data Supplement.
Subjects
In all of the studies, respondents provided written informed consent, and all of the studies were performed in accordance with the Declaration of Helsinki guidelines.

Mild Hypertensive Group
Ninety Italian white patients (34 women and 56 men; mean age: 46.0 years, mean body mass index: 26.8) with mild essential hypertension, that is, mean systolic BP (SBP) 152 mm Hg and mean diastolic BP 97.5 mm Hg were recruited by 9 medical centers. The patients were taken off antihypertensive medication 14 days before testing. After a normal sodium diet (150 mmol/d) for 3 days, patients were subjected to an acute salt-loading (constant rate intravenous infusion of 2 L of 0.9% NaCl carried out over 4 hours) and salt-depletion protocol (sodium restriction: 50 mmol plus 3 doses of 37.5 mg of furosemide) to evaluate the distribution of BP sensitivity to salt.14 If the difference between the mean arterial pressures at the end of the salt-loading and salt-depletion periods was greater than the median (10 mm Hg), the patient was classified as salt sensitive; otherwise, the patient was considered salt resistant. Twenty-four–hour urinary sodium excretion, upright plasma aldosterone (2 hours of orthostatism), and plasma renin activity were measured after 3 days of normal sodium diet (150 mmol/d) just before the salt load. Postload plasma aldosterone and renin activity were measured 4 hours after the beginning of the salt load. Urine electrolytes analyses, measurements of plasma renin activity, and plasma aldosterone concentration were performed as described previously.18

Healthy Group
Forty healthy French white normotensive (BP <140/90 mm Hg in the supine position after 5 minutes of rest) men (18 to 35 years of age) were included in a crossover study to receive both a low Na⁺ (<20 mmol of NaCl per day) and high K⁺ (>140 mmol of KCl per day; low Na⁺+high K⁺ diet) or high Na⁺ (>250 mmol of NaCl per day) and low K⁺ (<50 mmol of KCl per day; high Na⁺−low K⁺ diet) diet for 1 week. The study design has been described previously in detail.19 Procedures were in accordance with institutional guidelines. Controlled Na⁺/K⁺ diet periods were separated by a 7-day washout period. On the ad libitum Na⁺ and K⁺ diet at baseline and on day 7 of each controlled Na⁺/K⁺ diet period, blood was sampled at 9:00 AM in the fasting state after 1 hour of rest in the sitting position for plasma immunoreactive active and total renin and plasma aldosterone and atrial natriuretic peptide (ANP) determinations. Urine was collected in two 12-hour periods from 8:00 AM to 8:00 PM and from 8:00 PM to 8:00 AM and was used for hormone and electrolyte determinations. The methods used for collecting blood samples and for quantifying plasma active renin, total renin, ANP, and aldosterone were as described previously.18

Multisite Cohort for Depression and Anxiety
Data were obtained from The Netherlands Study of Depression and Anxiety, an 8-year longitudinal cohort study that includes 2981 Dutch white participants, aged 18 through 65 years. A detailed description of the study design and sample has been published previously.20 Netherlands Study of Depression and Anxiety is a multisite cohort study to describe the long-term course and consequences of depressive and anxiety disorders in which cardiovascular parameters, such as BP, were analyzed.16 Participants were recruited from different locations in The Netherlands (Amsterdam, Leiden, and Groningen). For the current study, data were used from the baseline interviews conducted between September 2004 and February 2007. Of the 2981 participants, 1860 subjects have been genotyped, and after quality control (described previously21), 1754 subjects (67.9% women; mean age: 42.35 years; SD: 12.49 years) were included in the study. The cohort consisted predominantly of subjects with current or remitted anxiety and/or depressive disorders at time of BP assessment. However, the presence of psychiatric diagnosis was not associated with BP.

BP was registered by the OMRON Intellisense Professional Digital Blood Pressure Monitor, HEM-907XL (Omron Healthcare, Inc); SBP and diastolic BP were measured twice during supine rest on the right arm and were averaged over the two measurements. A correction was made for all of the individuals on hypertensive medication, which was considered as being used if subjects frequently (50% of days in last month) used antihypertensives (Anatomical Therapeutic Chemical [ATC] code C02), diuretics (ATC code C03), β-blocking agents (ATC code C07), or calcium channel blockers (ATC code C08). In accordance with earlier studies and based on the efficacy of antihypertensive drugs in randomized trials,22−24 we added 10 mm Hg to SBP and 5 mm Hg to diastolic BP for subjects who used antihypertensives.

Determination of Genotypes
For genotyping procedures, please see the online Data Supplement.

Statistical Analysis
For detailed description of the statistical analyses, please see the online Data Supplement.

Results
MRC-2G>C Influences the Transactivation Activity of the MR
The influence of the MR-2G>C SNP on MR function was measured with an in vitro transactivation assay. RCSV3 and Cos-1 cells were transfected with plasmids containing either MR with the −2C nucleotide or MR with the −2G nucleotide (Figure 1). Dose-response curves in the presence of aldosterone or cortisol showed significantly higher transcriptional activity in the presence of the −2C allele than with the −2G variant in both cell models (RCSV3 cells: P<0.001 for both aldosterone [Figure 2, left] and cortisol [Figure 2, right]; Cos-1 cells: P<0.05 for aldosterone [Figure S1, left], P<0.001 for cortisol [Figure S1, right]; please see online Data Supplement).

MRC-2G>C Affects Protein Expression Independent of the 5’-UT Region
In the human NR3C2 gene, two 5’-UT exons are alternatively transcribed and generate 2 different mRNAs coding for a unique MR protein.3 The c.-2G>C SNP is located in exon 2, 2 nucleotides upstream of the translation initiation site in the middle of the Kozak consensus sequence for translational initiation,24,25 which is highly conserved among the NR3C2 genes from several species (Figure 1A). We have investigated the influence of the c.-2G>C SNP on translational efficiency, in the context of both 5’-UT exons 1α and 1β. Chimeric constructs were generated, with exon 1α or 1β inserted together with the Kozak sequence containing either G or C immediately upstream of the coding sequence of the luciferase gene (Figure 1B). Transient transfections were performed in renal RCSV3 cells and in COS-7 cells, the amount of luciferase activity representing the amount of protein generated. In the presence of comparable mRNA levels (please see Figure S2A, available in the online Data Supplement), the C allele was associated with significantly higher protein levels compared with the G allele, both at 12 and 24 hours posttransfection in RCSV3 cells (P<0.0001; Figure 3A); this effect was observed in the presence of both the UT exons 1α or 1β. Interestingly, luciferase activity in the presence of the UT region 1β was ≈40% of that observed with exon 1α (P<0.0001). The same difference between the
2 alleles was observed in COS-7 cells \( (P < 0.0001; \text{Figure } 3B) \), but only in the presence of the UT region 1α.

We then investigated whether the −2G>C SNP may affect alternative translation of the previously described human MR isoforms MR-A and MR-B, generated through the use of alternative translation initiation sites26 (Figure 1C). Expression vectors containing MR with either −2C or −2G and control constructs expressing only MR-A or MR-B were transiently transfected in Cos-1 cells. The MR mRNA levels after transfection were similar for MR-2C and MR-2G (Figure S2B). Two different primary antibodies were used in Western blots: 1D5 directed against amino acids 1 to 18 to visualize MR-A, whereas 2B7 (amino acids 64 to 82) detects both MR-A and -B (Figure 1C). Specificity of the antibodies was confirmed with the control constructs expressing only MR-A or only MR-B (Figure S3A and S3B). In Cos-1 cells transfected with MR-2G or MR-2C, only MR-A was expressed but not MR-B (Figure S3B). Western blots confirmed the differences in MR-A protein expression between −2G and −2C containing constructs (Figure S3C), MR-2C resulting in significantly increased MR-A expression (ratio of MR/tubulin: MR-2G, 0.300 ± 0.014; MR-2C, 0.362 ± 0.022).

**Consequences of MRc.-2G>C on Renal Sodium Handling and BP Regulation**

The MRc.-2G>C SNP was determined in subjects from 3 independent groups of patients. Allele and genotype frequencies were not significantly different among the 3 groups \( (\chi^2 = 5.36, P = 0.07 \text{ and } \chi^2 = 6.57, P = 0.16, \text{respectively; Table S1}) \).

**Mild Hypertensive Group**

After 3 days of high-salt diet (150 mmol/d), plasma renin activity was significantly higher in mild hypertensive GG patients compared with the other genotypes (Table 1). In addition, the GG genotype was associated with significantly higher plasma renin activity compared with the other genotypes (Table 1).
higher SBP levels in men but not in women (Table S2). No association of the MRc.-2G>C genotypes was found with other parameters tested in the cohort, such as 24-hour urinary sodium excretion and plasma aldosterone or plasma aldosterone and plasma renin activity levels after salt loading (P = 0.42 and P = 0.49, respectively). After the Weinberger test, the ratio of salt-sensitive/salt-resistant subjects was not significantly different among genotypes (χ² = 1.69; P = 0.43), and there was no relationship between the genotypes and BP response to the salt-sensitivity test (P = 0.21; data not shown).

**Normotensive Subjects**

On the ad libitum Na⁺/K⁺ diet, 156 mmol of Na⁺ (interquartile range: 126 to 187 mmol) and 68 mmol of K⁺ (interquartile range: 59 to 81 mmol) were excreted in the urine in 24 hours. By controlling the Na⁺/K⁺ intakes, it was possible to achieve the desired Na⁺ and K⁺ balances, as reflected by 24-hour urinary NaCl and KCl excretion rates (Table 2). The 24-hour urinary Na⁺ and K⁺ excretion levels were identical for subjects of the 3 genotypes for all of the diets (Table 2).

Plasma active and total renin and aldosterone, as well as ANP, on the ad libitum Na⁺/K⁺ diet were within the physiological ranges and did not differ according to genotype (Table 2). As expected, plasma active and total renin and aldosterone concentrations increased with the low Na⁺-high K⁺ diet and decreased with the high Na⁺-low K⁺ diet (P < 0.0001 between diets for all of the parameters). The changes in plasma ANP concentrations were in the opposite directions (Table 2; P < 0.0001 between diets). On the high Na⁺-low K⁺ diet, GG subjects had significantly higher levels of plasma active and total renin and plasma aldosterone concentrations than CC subjects, with heterozygous GC subjects presenting intermediate values. A similar trend was observed on the low Na⁺-high K⁺ diet, but differences between GG and CC genotypes were not significant.

**Netherlands Study of Depression and Anxiety Cohort**

There was a significant association between SBP and MRc.-2G>C (P = 0.041), even after adjustment for confounding factors (Table S3). GG subjects had significantly higher SBP than GC or CC subjects (mean SBP for GG: 138.2 ± 1.9 mm Hg; GC: 137.3 ± 1.8 mm Hg; CC: 135.2 ± 1.9 mm Hg). Although we did not detect a sex × genotype interaction (P = 0.36), we performed a separate analysis for both men and women. The association with MRc.-2G>C and SBP was significant for men but not for women (Table 3). Men with the GG genotype had significant higher systolic pressure (5.17 mm Hg) than those with the CC genotype (P = 0.046). SBP among men was 147.0 ± 21.2 mm Hg for the GG genotype, 143.8 ± 17.6 mm Hg for the GC genotype, and 141.9 ± 20.6 mm Hg for the CC genotype.

**Discussion**

In this study we have undertaken the functional analysis of the c.-2G>C polymorphism, a frequent SNP in the NR3C2 gene coding for the MR, and its selective genotyping in subjects from different groups of subjects. The c.-2G>C variant was associated with differential expression of the MR in vitro; importantly, in vivo, this SNP influences circulating levels of plasma aldosterone and renin.

A possible role of the MRc.-2G>C polymorphism on translational efficiency had been suggested based on its location in the middle of the Kozak consensus sequence for translational initiation.24 First, we showed with 2 different endogenous ligands and in 2 different cell lines that MR translated from a construct carrying a C at position −2 was associated with a higher transcriptional response in vitro. These results are in contrast to previous work describing lower transactivation of a reporter gene by the −2C allele using aldosterone13; differences in methodology or the cell line used might explain this discrepancy. Second, using 3 different approaches and 2 different cell lines, including a kidney collecting duct epithelial cell line, we showed that the C allele results in more abundant protein expression than the G allele. Because mRNA expression is not modified by MRc.-2G>C, it is concluded that this polymorphism influences translation. This is in accordance with data showing that a C at position −2 is probably more favorable for translation; comparison of 1534 human transcripts has shown that the sequence surrounding the initiation codon contains a C at position −2 in 40% of cases, whereas a G nucleotide is present in only 18%.25 Furthermore, our results demonstrate that the 5’-UT has no influence on the observed effect of the SNP. In the presence of both UT exons 1α and 1β, the −2C allele was associated with higher protein translation. However, protein expression in the presence of the UT region 1β was ≈40% of that observed with exon 1α, indicating that the sequence of exon 1β is less optimal for translation. Finally, Western blot experiments showed that the MR-A isoform, translated from the first translation start, was more abundant.
The physiopathological relevance of our in vitro results was tested by studying the association of the MRc-2G>C polymorphism with parameters of BP regulation and electrolyte homeostasis in 2 groups of patients well phenotyped for BP and the renin-aldosterone axis under different experimental settings. Individuals carrying the CC genotype in either a healthy cohort under a high Na+ diet or a mild hypertensive cohort had significantly lower plasma renin concentration/renin activity levels, respectively. In parallel with the lower renin levels, the CC individuals in the healthy group also presented lower plasma aldosterone levels. The observed lower active renin, total renin, and lower aldosterone levels suggest a more efficient tubuloglomerular feedback in individuals with the CC genotype. According to the in vitro results obtained in this study, this effect might be attributed to more efficient sodium reabsorption because of higher levels of MR in the distal tubule, which was unmasked in conditions of low aldosterone synthesis and concentration, that is, on a high Na+ diet.27 There were trends in similar directions for these parameters in the healthy group at baseline or during the low Na+ diet, but this did not reach significance. The absence of significant difference between genotypes may be attributed to a low power of the study, which was not initially designed to test the effect of the MR polymorphism. Finally, both in the mild hypertensive group and in a large Dutch cohort, male GG carriers had a higher SBP. Taken together, these results indicate that the CC concentration/renin activity levels, respectively. In parallel with the lower renin levels, the CC individuals in the healthy group also presented lower plasma aldosterone levels. The observed lower active renin, total renin, and lower aldosterone levels suggest a more efficient tubuloglomerular feedback in individuals with the CC genotype. According to the in vitro results obtained in this study, this effect might be attributed to more efficient sodium reabsorption because of higher levels of MR in the distal tubule, which was unmasked in conditions of low aldosterone synthesis and concentration, that is, on a high Na+ diet.27 There were trends in similar directions for these parameters in the healthy group at baseline or during the low Na+ diet, but this did not reach significance. The absence of significant difference between genotypes may be attributed to a low power of the study, which was not initially designed to test the effect of the MR polymorphism. Finally, both in the mild hypertensive group and in a large Dutch cohort, male GG carriers had a higher SBP. Taken together, these results indicate that the CC
carriers may have a more favorable cardiovascular profile as compared with the GC and GG carriers.

Aldosterone has emerged as a key hormone determining cardiovascular and renal damage and risk prognosis, in addition to its role in BP regulation and potassium and sodium homeostasis. Within the last 10 years, blocking its effects with MR antagonists has been shown to have beneficial effects in congestive heart failure, especially after myocardial infarction, and proteinuric nephropathies.\(^\text{28,29}\) Although our results need to be replicated in larger cohorts well phenotyped for BP and the renin-angiotensin-aldosterone axis, our data suggest that functional variants of the MR may be associated not only with different cellular responses to aldosterone but also, indirectly, with increased aldosterone levels that may activate both genomic and nongenomic pathways in nonepithelial target tissues to promote deleterious cardiovascular effects.

Only male carriers of the MRc.-2G>C GG genotype showed higher BP compared with men with the other genotypes in the mild hypertensive cohort, the polymorphism not being associated with BP in women. The same sex-dependent association was found with SBP in the large-scale Dutch cohort, suggesting a sexual dimorphism. Many studies have reported sex-related differences in occurrence and severity of cardiovascular diseases related to the hormonal status.\(^\text{30}\) Interestingly, it has been shown that ovarian hormones positively affect salt sensitivity, protecting premenopausal women from the development of hypertension. After menopause, responsiveness of renin-angiotensin-aldosterone system changes, with a net increase in salt sensitivity.\(^\text{31}\) In addition, cortisol responses to stressors are different between men and women, and, as discussed above, cortisol might have an effect on epithelial MR as well.

**Perspectives**

Finding genetic variants involved in the regulation of BP offers mechanistic insights into the development of hypertension in the general population and helps in identifying novel targeted therapeutic strategies to prevent cardiovascular disease. Rare mutations of the NR3C2 gene result in monogenic diseases of sodium homeostasis and BP regulation. Our study provides evidence that frequent polymorphisms of the MR may exert quantitative effects on the activity of the renin-angiotensin-aldosterone axis and BP in the general population, modulating vulnerability for hypertension. Genotyping the common MR polymorphism c.-2G>C could help in identifying patients prone to develop hypertension and vascular disease, opening new strategies for prevention or targeted pharmacological treatment.

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**Disclosures**

None.

**References**


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Table 3. Association Analysis of the MR MRc.-2G>C Genotype With Diastolic BP and SBP in the Dutch Cohort

<table>
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<td>GG (295)</td>
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*Genotype effect by general linear model adjusted for age, presence of life events, years of education, smoking, alcohol abuse/dependence, use of tricyclic antidepressant, use of noradrenergic serotonergic antidepressant, No. of chronic diseases, and body mass index in each sex separately.


On-line supplement

THE FUNCTIONAL c.-2G>C VARIANT OF THE MINERALOCORTICOID RECEPTOR MODULATES BLOOD PRESSURE, RENIN AND ALDOSTERONE LEVELS

**Data supplement**

**Construction of the plasmids**

The recombinant pRSV human MR (hMR) plasmid, containing the last 30 base pairs of exon 1α to the untranslated region of exon 9, was obtained from Dr. R. Evans (gene expression laboratory and HHMI, The Salk Institute for Biological Studies, La Jolla, Ca). The MR c.-2G>C site was mutated from G to C with the Quick Change Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) using the primers 5’-GGCCGAGGCAGCGATGGAGACCAAAG-3’ and 5’-GCTGCCTCGGCCCCCTTTGGTCTCCAT-3’ according to the manufacturer’s protocol. The plasmids expressing only MR-A or MR-B were generated by mutating the second or the first ATG into ATC by using the primers 5’ -CTGAAGGTCTAGATACGGAAAGACGGTGG-3’ and 5’-CCACCGCTTTTCCGCTCCACCTTCCAG-3’ or 5’-CCGAGGCAGGGACGGAGACCAAAGG-3’ and 5’-CTTTTGCTGTCCGCTCCCTCGG-3’ respectively (Fig. 1c). After mutagenesis the hMR insert of the plasmid was sequenced to assure absence of other mutations. Plasmids were purified from DH5α E. coli bacterial cultures using the Pure Yield purification system (Promega, Leiden, The Netherlands).

To generate pcDNA3_1α2G-luc and pcDNA3_1β2G-luc (Fig. 1a and b), human kidney and hippocampus (for pcDNA3_1β2G-luc) cDNA was amplified to generate fragments containing exon 1α or 1β and 60 bp of exon 2 of the NR3C2 gene. The cDNA coding for luciferase was amplified, starting from codon 2, using the pGL2-basic vector (Promega, Madison, WI) as a template. Primers were designed containing specific restriction sites for subsequent cloning. All amplification fragments were subcloned into pGEMTeasy (Promega). The intermediate constructs pGEMTeasy_1α2 and pGEMTeasy_1β2 were digested with SpeI/XbaI, and ligated to the luciferase cDNA previously excised from pGEMTeasy_luc by digestion with SpeI. The chimeric construct 1α2luc was excised from pGEMTeasy_1α2luc by digestion with BamHI and Xhol and inserted into pcDNA3 (Invitrogen, Paisley, Scotland) to obtain pcDNA3_1α2-luc and pcDNA3_1β2-luc. Oligonucleotides used for amplification of the different fragments are the following:

1α2 : 5’-ATGGATCCAGAGGAAGCCCGTGCA 3’ and 5’-CCCACCGTCTTTCCATATCT-3’
1β2 : 5’-ATGGATCCCGCCGCCGTGCCTGGCCGCTTCC-3’ and 5’-CCCACCGTCTTTCCATATCT-3’
Luciferase: 5’-GCACGTAGCGAGGACCGCCAAAAACATAAAGA-3’ and 5’-CCCTCGAGCATTTTACAATTGGACCTTTC-3’

Different pcDNA3_1α2-luc and pcDNA3_1β2-luc clones were sequenced to verify the nucleotide rs2070951 (MR c.-2G>C). The G to C change was created by site-directed mutagenesis using the Quick change site-directed mutagenesis kit (Strategene, La Jolla, CA) on the recombinant plasmids. The following sense primer was used together with their corresponding antisense oligonucleotide.

**Transactivation assay COS-1 cells**

Cos-1 cells (African green monkey kidney cells) were cultured as described previously¹ and seeded in 24-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at 3 x 10⁴ cells/well in DMEM supplemented with charcoal-stripped serum. The cells were transfected the next day using SuperFect (Qiagen, Venlo, The Netherlands). Plasmids containing MR-2G or MR-2C or no MR (control) and the reporter plasmid TAT3-Luc (tyrosine amino transferase triple hormone response element) were used at 200 ng/well. Construction of the plasmids is described in the data supplement available online. The control plasmid pCMV-Renilla (Promega, Leiden, The Netherlands) was used at 2 ng/well. To exclude variation due to impurity or concentration of the plasmid each plasmid was cultured 3 times, purified and tested. In each of the three experiments the plasmids were tested in quadruplicate. One day after transfection, the cells were treated with different concentrations of either aldosterone or cortisol (both Sigma-Aldrich, Zwijndrecht, the Netherlands). After 24h of incubation the cells were harvested in passive
lyses buffer (Promega) and firefly and Renilla luciferase activity was determined using the dual label reporter assay (Promega) and a luminometer (CentroXS, Berthold, Bad Wildbad, Germany).

**RNA isolation and real time quantitative PCR**

All reagents used were from Invitrogen unless otherwise specified. Transfections with plasmids containing one of the hMR variants, pcDNA3_1α2G-luc, pcDNA3_1α2C-luc, pcDNA3_1β2G-luc, pcDNA3_1β2C-luc and pSVβgal were performed as described under the protein expression section. After transfection, total RNA was extracted in Trizol reagent according to manufacturer’s recommendations. Total RNA was treated with DNase I and quantified with the Ribogreen RNA quantitation kit as previously described. 1μg or 500 ng of RNA were used to generate cDNA. Amplification of the cDNA was performed using SYBRgreen (qPCR MasterMix Plus for SYBR® green I, Eurogentec, Seraing, Belgium) on a Chromo4 Continuous Fluorescence Detector (MJ Research, Bio-Rad laboratories, Waltham, MA), according to the manufacturers instructions. Primer sequences are available upon request. Controls without reverse transcriptase and without template were included to verify that fluorescence was not overestimated by residual genomic DNA amplification or from primer dimer formation. Moreover RT-PCR products were analyzed in a post-amplification fusion curve to ensure that a single amplicon was obtained. Ribosomal 18S RNA was used to normalize for RNA quality, quantity and RT-efficiency. Quantification of βgal was used to normalize for transfection efficiency. Quantification was done by the standard curve method. Standard curves were generated by serial dilutions of a linearized plasmid containing the specific amplicon, spanning six orders of magnitude, yielding a correlation coefficient of at least 0.98 in all experiments. For all experiments, PCR efficiency was close to 2 indicating a doubling of DNA at each PCR cycle, as expected.

**Determination of genotypes**

Patients from the Italian cohort were genotyped by direct sequencing after PCR amplification using an ABI BigDye termination sequence kit v1.1 (Applied Biosystems Foster City, USA) on an ABI 3700 DNA analyzer and the reaction was performed according to the manufacturer’s specifications. Patients from the French cohort were genotyped by direct sequencing of PCR products using the ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit on an ABI Prism 3700 DNA Analyzer. Primers for PCR amplification are Primers used for genotyping are:

Fw 5’-ATA TGT TTT GTG GCT TAG CAA AT-3’
Rv 5’AAC TTA GAG TGG AAG GAC GAT GG-3”

Subjects from the Dutch NESDA cohort were genotyped by Perlegen Sciences (Mountain View, CA, USA) using a set of four proprietary, high-density oligonucleotide array, which had a call rate of 98.9% for this SNP. A detailed description of the genotype process is described elsewhere.

**Statistical analysis**

In cell culture experiments the difference between the two alleles was analyzed with a two-way ANOVA with Bonferroni post-hoc tests. For western blot the difference in protein expression was analyzed with t-test. In vitro results are shown as the mean ± SD or mean ± SEM. Statistical analysis of all the in vitro results was performed with Graph Pad Prism version 5 (GraphPad software Inc, San Diego, CA).

In the Italian mild hypertensive group, the MR genotype effects were assessed using two-way (genotype/sex) ANCOVA with age and BMI as covariates. Skewed variables were log-transformed before statistical testing. When the interaction between genotype and sex was significant (blood pressure), analyses were separately performed in each sex. The statistical analysis was performed using Systat 11 statistical software.

In the healthy volunteers group, differences between MR genotype groups were assessed by ANOVA for a crossover design for plasma parameters and by non parametric Kruskal-Wallis test for urinary parameters. Statata Statistical Software (version 7.0; StataCorp.) was used for statistical analysis.
In the Dutch NESDA cohort, differences between MR genotype groups in SBP and DBP were analysed using general linear model (GLM). We tested for gene gender interaction and performed the GLM for the entire sample and split for gender. The previous identified confounders related to BP in this cohort: gender, age, years of education, alcohol use, smoking, tricyclic antidepressants, noradrenergic and serotonergic working antidepressants, number of chronic disease, body mass index, depression and anxiety comorbidity and presence of significant life events were included as covariates. SPSS 15.0 was used for the statistical analysis. Association study data are expressed as means ± SD or medians and interquartile ranges.

Allele frequencies in the three cohorts were calculated and analyzed for deviation from Hardy-Weinberg equilibrium (HWE) using Haploview. The groups consisting of mild hypertensive individuals and of healthy individuals did not deviate from HWE, as well as the healthy individuals from the NESDA cohort. However, deviation from HWE was observed in the patients with mood and/or anxiety disorders from the NESDA cohort (p=0.0053). Deviation from HWE in patients can be interpreted as an indication of association of particular genes with disease. Under these circumstances, the lack of HWE arises as a result of selection according to the phenotype that results in allele and genotype distributions that are nonrandom.

Data are expressed as mean ± SD or otherwise specified. A P-value of less than 0.05 was considered to be significant.
Reference List


<table>
<thead>
<tr>
<th>group</th>
<th>Allele frequencies</th>
<th>Genotype frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>0.59</td>
<td>0.41</td>
</tr>
<tr>
<td>Healthy</td>
<td>0.51</td>
<td>0.49</td>
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<tr>
<td>NESDA</td>
<td>0.51</td>
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**Table S2.** Association analysis of the MR c.-2G>C genotype with diastolic and systolic blood pressure in 90 mild hypertensive men and women from the Italian cohort

<table>
<thead>
<tr>
<th>Blood pressure</th>
<th>Group</th>
<th>MR-2G&gt;C</th>
<th>Mean</th>
<th>SD</th>
<th>P*</th>
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<tbody>
<tr>
<td>Systolic (mmHg)</td>
<td>Men (n=56)</td>
<td>CC (22)</td>
<td>151.5</td>
<td>11.6</td>
<td>0.004</td>
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<tr>
<td></td>
<td></td>
<td>GC (26)</td>
<td>147.8</td>
<td>12.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG (8)</td>
<td>165.8</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Women n=34</td>
<td>CC (7)</td>
<td>151.0</td>
<td>9.8</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GC (22)</td>
<td>158.1</td>
<td>18.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG (5)</td>
<td>141.0</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>Diastolic (mmHg)</td>
<td>Men (n=56)</td>
<td>CC (22)</td>
<td>99.1</td>
<td>5.1</td>
<td>0.053</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GC (26)</td>
<td>96.5</td>
<td>6.3</td>
<td></td>
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<td>GG (8)</td>
<td>102.0</td>
<td>5.3</td>
<td></td>
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<tr>
<td></td>
<td>Women n=34</td>
<td>CC (7)</td>
<td>95.6</td>
<td>8.3</td>
<td>0.056</td>
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<td>GC (22)</td>
<td>98.1</td>
<td>9.7</td>
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<td>GG (5)</td>
<td>90.0</td>
<td>3.5</td>
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BP: blood pressure; *genotype effect by ANCOVA adjusted for age and BMI in each sex separately.

Interaction genotype X sex: P = 0.001 and P = 0.005 for PAS and PAD respectively.
Table S3. Results of General Linear Model with mean systolic pressure (medication adjusted) as outcome in the whole cohort (n=1754 subjects), and stratified for gender

<table>
<thead>
<tr>
<th>Parameter</th>
<th>complete cohort</th>
<th>Gender</th>
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<th></th>
<th></th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>F test</td>
<td>P</td>
<td>R²</td>
<td>F test</td>
<td>P</td>
<td>R²</td>
<td>F test</td>
<td>P</td>
<td>R²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td></td>
<td>0.263</td>
<td></td>
<td></td>
<td>0.145</td>
<td></td>
<td></td>
<td>0.207</td>
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</tr>
<tr>
<td>MR c.-2G&gt;C</td>
<td>3.202</td>
<td>0.041</td>
<td></td>
<td>3.088</td>
<td>0.046</td>
<td></td>
<td>1.639</td>
<td>0.195</td>
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<tr>
<td>Negative life events, yes vs. no</td>
<td>1.104</td>
<td>0.294</td>
<td></td>
<td>0.327</td>
<td>0.568</td>
<td></td>
<td>0.895</td>
<td>0.344</td>
<td></td>
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<tr>
<td>Gender, female vs. male</td>
<td>141.204</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>198.771</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>Age, per 1-year increase</td>
<td>246.499</td>
<td>&lt;0.001</td>
<td></td>
<td>46.926</td>
<td>&lt;0.001</td>
<td></td>
<td>5.556</td>
<td>0.019</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Years of education, per 1-year increase</td>
<td>5.799</td>
<td>0.016</td>
<td></td>
<td>0.398</td>
<td>0.528</td>
<td></td>
<td>5.556</td>
<td>0.019</td>
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<tr>
<td>Smoking, yes vs. no</td>
<td>9.603</td>
<td>0.002</td>
<td></td>
<td>8.915</td>
<td>0.003</td>
<td></td>
<td>2.966</td>
<td>0.085</td>
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<tr>
<td>Alcohol abuse or dependence, yes vs. no</td>
<td>0.728</td>
<td>0.394</td>
<td></td>
<td>0.187</td>
<td>0.665</td>
<td></td>
<td>0.417</td>
<td>0.519</td>
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<tr>
<td>Use of a TCA, yes vs. no</td>
<td>4.241</td>
<td>0.040</td>
<td></td>
<td>1.439</td>
<td>0.231</td>
<td></td>
<td>2.638</td>
<td>0.105</td>
<td></td>
<td></td>
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<tr>
<td>Use of NS working antidepressant, yes vs. no</td>
<td>2.711</td>
<td>0.100</td>
<td></td>
<td>0.333</td>
<td>0.564</td>
<td></td>
<td>2.739</td>
<td>0.098</td>
<td></td>
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<td>Number of chronic diseases, per 1 disease increased</td>
<td>11.376</td>
<td>0.001</td>
<td></td>
<td>5.885</td>
<td>0.016</td>
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<td>6.001</td>
<td>0.014</td>
<td></td>
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<tr>
<td>BMI, per 1kg/m² increase</td>
<td>9.427</td>
<td>0.002</td>
<td></td>
<td>4.747</td>
<td>0.030</td>
<td></td>
<td>5.584</td>
<td>0.018</td>
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</table>

Abbreviations: TCA, tricycle antidepressant; NS, noradrenergic serotonergic; BMI, Body Mass Index
Figure S1. Aldosterone and cortisol-driven transactivation by MRc.-2C and MRc.-2G in COS-1 cells. Aldosterone (a) and cortisol (b) driven transactivation of the MRc.-2G>C variants on a TAT-3 promoter in COS-1 cells displayed as percentage of maximal induction obtained with -2G (±SEM), Results represent at least two independent experiments performed in triplicate. *, p<0.05; **, p<0.01; ***, p<0.001.
**Figure S2.** Effects of MRC-2G>C on mRNA expression levels of recombinant proteins used in this study. a) Chimeric constructs containing -2G (white and light gray bars) showed no significant difference in mRNA expression compared to chimeric constructs containing -2C (dark gray and black bars) after either 12 or 24 hours. The mRNA expression was normalized against total RNA and transfection efficiency. b) The MR-2G variant (white bar) showed no significant difference in mRNA expression compared with the MR-2G variant (dark gray bar); the mRNA expression was normalized against total RNA.
Figure S3. Effects of MRc.-2G>C on the expression of MR isoforms. Constructs containing MR with either -2C or -2G and control constructs expressing only MR-A or MR-B were transiently transfected in Cos-1 cells. The effect of the -2G or -2C allele on MR-A and B protein expression was measured by Western blot using two different antibodies recognizing aminoacids 1 to 18 (visualizing MR-A, Fig. S3a), or aminoacids 64 to 82, detecting both MR-A and B (Fig. S3b). c) Quantification of MR expression of multiple experiments using the MR-A detecting antibody (1D5).