Mineralocorticoid receptor gene variants : implications for stress, blood pressure and personality

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The functional c.-2G>C variant of the Mineralocorticoid receptor modulates blood pressure, renin and aldosterone levels

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Abstract

Context: 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.
Objective: Characterize the common MR polymorphism c.-2G>C (rs2070951) in vitro, and test its influence on parameters related to blood pressure regulation and the renin-angiotensin system.
Design: In vitro studies were performed to assess the influence of the polymorphism on MR expression and transcriptional activity. Association studies were performed with several outcome variables in three 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 (NESDA)), in which blood pressure was measured.
Results: The G allele was associated with decreased MR protein levels and reduced transcriptional activation compared to the C allele. Subjects with the GG genotype had significant higher plasma renin levels both in the mild hypertensive group and in normal volunteers compared to 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 NESDA cohort the genotype GG was associated with higher systolic blood pressure in males.
Conclusions: 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.

Keywords: mineralocorticoid, aldosterone, hypertension, nuclear receptor, sodium balance

Introduction

The mineralocorticoid receptor (MR) mediates aldosterone effects on electrolyte balance and blood pressure (BP). Sodium handling is highly variable between individuals and genetic factors are involved in the development of hypertension (Halushka et al., 1999). Genetic variation in the MR might be responsible for the variability in sodium handling and vulnerability for hypertension. Classical MR-expressing tissues include the distal parts of the nephron, colon, salivary and sweat glands. In these tissues the MR regulates trans-epithelial sodium transport. However, MR are also expressed in non-epithelial tissues, including the cardiovascular system, the central nervous systems and adipose tissue (Caprio et al., 2007); in these tissues, glucocorticoids represent the predominant endogenous ligand (Funder, 2009).

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 physiologic 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 utilization, alternative splicing, use of different translational start sites and genetic polymorphisms, considerable variability in MR function has been observed (Pascual-Le Tallec and Lombes, 2005)(Zennaro et al., 1995). Rare mutations of the MR are responsible for Mendelian diseases characterized by disorders of renal salt handling associated with high or low BP. Loss of function mutations of the MR lead to type I pseudohypoaldosteronism (Pujo et al., 2007; Fernandes-Rosa et al., 2006; Sartorato et al., 2004; Riepe et al., 2003; Geller et al., 1998), while the rare activating mutation S810L (rs41511344) leads to juvenile hypertension exacerbated by pregnancy (Fagart et al., 2005; Pinon et al., 2004). Two recent studies showed associations between more common genetic variations, single nucleotide polymorphisms (SNPs), in the MR and BP (Martinez et al., 2009; Tobin et al., 2008). Previously we tested the amino acid changing SNP in exon 2, MRI180V (rs5522) *in vitro* and showed that the rs5522 G allele leads to a lower transactivational capacity (DeRijk et al., 2006). However, in a group of mild hypertensive individuals we did not find an association with BP and MRI180V (DeRijk et al., 2006) , and the frequency of the same polymorphism was similar between hypertensive subjects and controls from a Brazilian birth cohort (Fernandes-Rosa et al., 2009).

MRc.-2G>C (rs2070951) is a frequent SNP located in the 5'-untranslated region of the NR3C2 gene, two nucleotides upstream of the first translation start site. The C allele of MRc.-2G>C has been associated with lower basal cortisol levels (Kuningas et al., 2007) and a decrease in MR-dependent transcriptional activation in the presence of aldosterone (Arai et al., 2003). However MRc.-2G>C has not been tested for associations with salt handling and the precise mechanism of action of this SNP is currently unclear.

In this study, we first 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 normotensive healthy subjects 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's) test. Finally, we tested for an association between BP measures in a large multi-site cohort for depression and anxiety.

Materials and Methods

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'-GCTGCCTCGGCCCTTTGGTCTCCAT-3' according to the manufacturers protocol (Fig. 1c). 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'TGAAGGTCTAGATACGGAAAGACGGTGG-3' and 5'CCACCGTCTTTCCGTATCTAGACCTTCAG-3' or 5'CCGAGGCAGGGACGGAGACCAAAGG-3' and 5'CCTTTGGTCTCCGTCCCTGCCTCGG-3' respectively. 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_1α2G-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 XhoI 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:

5'-ATGGATCCAGAGGAAGCCCGTGCAGTCA 3' and 5'-CCCACCGTCTTTCCATATCT-3'

1β2 : 5'-ATGGATCCCGCCGCTGCCTCGCCGCCTC-3' and 5'-CCCACCGTCTTTCCATATCT-3'

Luciferase: 5'-GCACTAGTCGAAGACGCCAAAAACATAAAGA-3' and 5'-CCCTCGAGCATTTTACAATTTGGACTTTC-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 assays

Rabbit RCSV3 cells derived from kidney cortical collecting duct (Vandewalle et al., 1989) (kindly provided by Pr. P. Ronco, Hôpital Tenon, Paris) were grown as described previously (Zennaro et al., 2001) and transfected using lipofectamine 2000 (Invitrogen) with 0.25 µg of plasmid containing either MR-2G or MR-2C pcDNA3, 0.625 µg of a GRE2_TATA_luc reporter plasmid plasmid (Asselin-Labat et al., 2004) 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.

Cos-1 cells (African green monkey kidney cells) were cultured as described previously(van Leeuwen et al., 2010) and seeded in 24-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at 3 x 10^4 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).

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 six-well plates at a density of 3x10^5 cells per well at least 6 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_1 β 2G-luc, pcDNA3_1 β 2C-luc, pcDNA3_1α2G-luc or pcDNA3_1 α 2C-luc. Co-transfection of 0.16 µg pSV β gal (Clontech, Palo Alto, CA) was performed to normalize for transfection efficiencies. Cells were rinsed twice with cold PBS, lysed in lysis buffer and cellular extracts assayed for luciferase and β -galactosidase activities as previously described(Zennaro et al., 2001). 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 x 10^5 cells/well. The cells were transfected the next day using Trans-it Cos transfection reagent (Mirus, Madison, USA). Plasmids containing one of the hMR variants, e.g. hMR-2G, hMR-2C, mutated hMR only expressing MR-A and mutated hMR only expressing hMR-B or no hMR (control) were used at 2µg/well. Cells were harvested 48 hours after transfection. The Western blots using primary antibodies MR 1D5, detecting amino acid 1-18 and therefore only MR-A, and 2B7, detecting amino

acid 64-82 and therefore both MR-A and MR-B, (a generous gift by Gomez-Sanchez, Division of endocrinology, University of Mississippi, Jackson, MS) were performed as described previously(Conway-Campbell et al., 2007) The differences in intensity of the MR bands were quantified with Image J (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/)

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_1β2C-luc, pcDNA3_1β2G-luc, pcDNA3_1β2C-luc and pSVgal were performed as described under the protein expression section. For the hMR transfections 24 hours and for other transfections both 12 and 24 hours 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 (Zennaro et al., 2001). For the transfections with hMR 1μg and for the others 500 ng of RNA was 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 β gal 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.

Subjects

In all studies respondents provided written informed consent and all studies were performed in accordance with the Declaration of Helsinki guidelines.

Mild hypertensive group. Ninety Italian patients (34 females, 56 males, mean age 46.0 yrs, mean body mass index 26.8) with mild essential hypertension , i.e. mean systolic blood pressure (SBP) 152 mm Hg and mean diastolic blood pressure (DBP) 97.5 mm Hg were recruited by 9 medical centres. The patients were taken off antihypertensive medication 14 days prior to testing. After a normal sodium diet (150 mmol/day) 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 three doses of 37.5 mg of furosemide) to evaluate the distribution of BP sensitivity to salt (Strazzullo et al., 2000a). If the difference between the mean arterial pressures at the end of the salt-loading and salt-depletion period was greater than the median (10 mmHg), the patient was classified as "salt-sensitive" otherwise the patient was considered "salt-resistant". Twenty-four hours urinary sodium excretion, upright plasma aldosterone (after two hours orthostatism) and plasma renin activity were measured after three days of normal sodium diet (150

mmol/day) just before the salt-load. Post-load plasma aldosterone and renin activity were measured 4 h after the beginning of the salt-load. Urine electrolytes analyses, measurements of plasma renin activity and plasma aldosterone concentration were performed as previously described (Strazzullo et al., 2000b).

Healthy group

Forty healthy French Caucasian normotensive (BP less than 140/90 mmHg in the supine position after 5 minutes of rest) males (18–35 years) were included in a crossover study to receive both a low Na+ (less than 20 mmol NaCl/day) and high K+ (more than 140 mmol Kcl/day; low Na+–high K+ diet) or high Na+ (more than 250 mmol NaCl/day) and low K+ (less than 50 mmol Kcl/day; high Na+–low K+ diet) for 1 week. The study design has been previously described in detail (Azizi et al., 2005) and all subjects completed the study after giving written informed consent. 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 0900 in the fasting state after 1 hour of rest in the sitting position for plasma immunoreactive active and total renin and plasma aldosterone and ANP determinations. Urine was collected in two 12-hour periods from 0800 to 2000 and from 2000 to 0800 and was used for hormone and electrolyte determinations. The methods used for collecting blood samples and for quantifying plasma active renin, total renin, atrial natriuretic peptide (ANP) and aldosterone were as described previously (Azizi et al., 2005).

Multi-site cohort for depression and anxiety

Data were obtained from the Netherlands Study of Depression and Anxiety (NESDA), an 8-year longitudinal cohort study that includes 2,981 Dutch Caucasian participants, aged 18 through 65 years, A detailed description of the study design and sample has been published previously (Penninx et al., 2008). NESDA is a multi-site cohort study to describe the long-term course and consequences of depressive and anxiety disorders in which cardiologic parameter such as blood pressure were analysed (Penninx et al., 2008). 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 (previously described (Sullivan et al., 2009)), 1754 subjects (67.9% woman, mean age 42.35, 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 DBP were measured twice during supine rest on the right arm and were averaged over the 2 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 (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 (Mancia and Parati, 2004; Cui et al., 2003) we added 10 mm Hg to SBP and 5 mm Hg to DBP for subjects who used antihypertensives.

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 (Sullivan et al., 2009).

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. Stata 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 (Licht et al., 2009) 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 (Lee, 2003; Deng et al., 2000). 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.

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. Cos-1 cells and RCSV3 cells were transfected with plasmids containing either MR with the -2 C nucleotide or MR with the -2 G nucleotide (Fig. 1c).

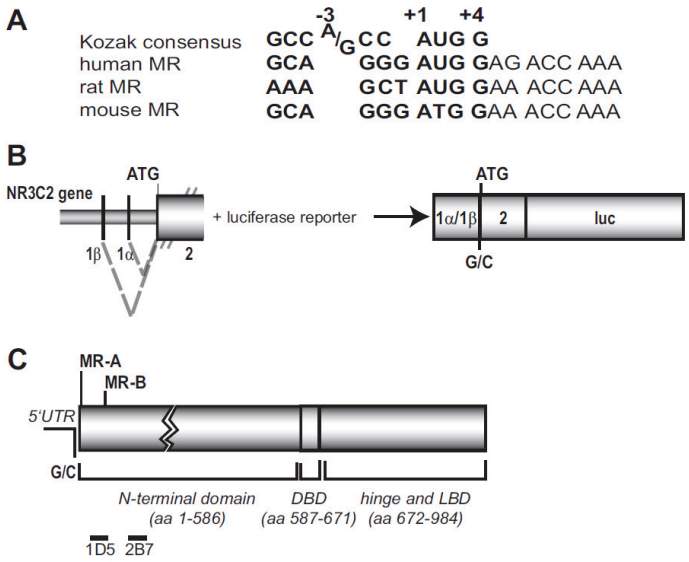

Figure 1. Presentation of the sequence context surrounding the SNP c.-2G>C, schematic representation of the chimeric gene constructs and schematic representation of hMR isoforms MR-A and MR-B . a) Alignment of the Kozak consensus sequence for translation initiation and the sequence upstream of the principal AUG codon (pos. +1) of the MR mRNA. The SNP MRc.-2G>C is located at position -2 with respect to the AUG. The human MR mRNA sequence is aligned with the rat and mouse MR mRNAs. b) Schematic representation of the chimeric constructs 1α2luc and 1β2luc (c) schematic representation of hMR isoforms MR-A and MR-B and the location of the antibodies used in westenblot for detection of the two isoforms.

After incubation with aldosterone (concentration range 0 to 10^{-7}M) or cortisol (concentration range 0 to 10^{-6} M) luciferase activity was measured. The -2C allele showed significantly higher transcriptional activity than the -2G variant using either aldosterone or cortisol in both cell models (Cos-1 cells: $p<0.05$, $p<0.001$ S12a and S1b; RCSV3 cells: $p<0.001$ and $p<0.001$ for aldosterone and cortisol respectively, (Fig. 2).

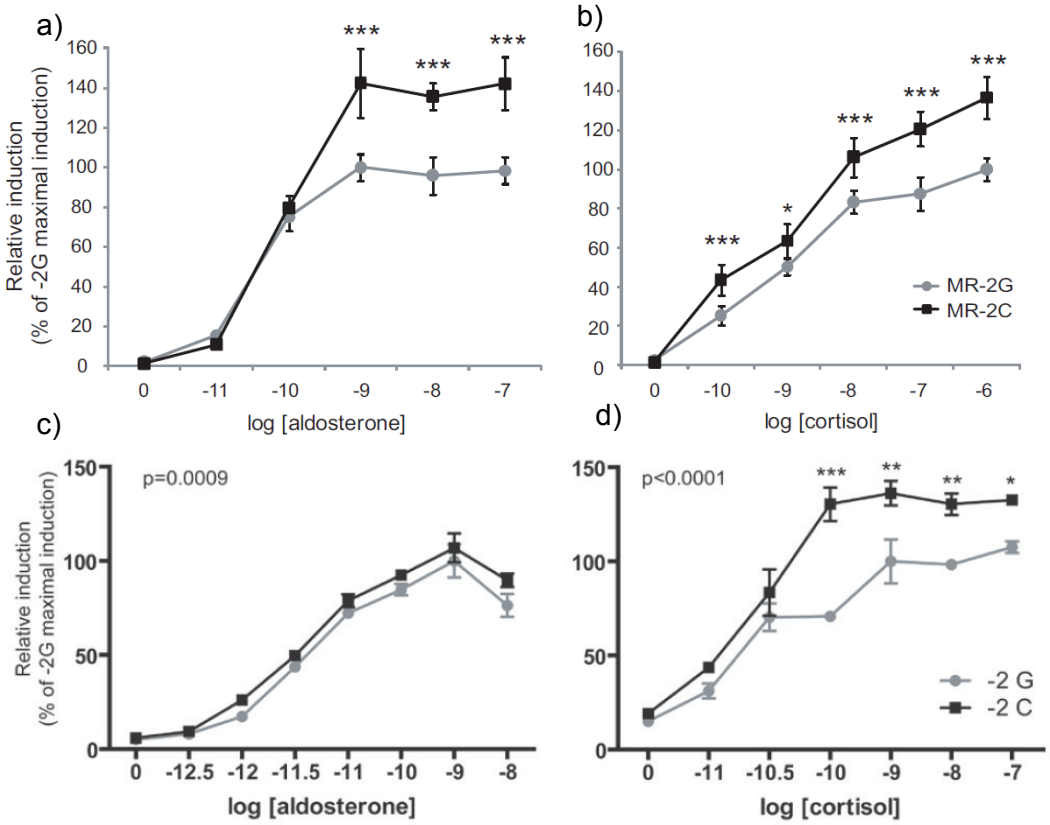

Figure 2. Aldosterone and cortisol-driven transactivation by MR c.-2C and MRc.-2G. Aldosterone (2a and 2c) and cortisol (2b and 2d) driven transactivation capacity of the MRc.-2G>C variants on a TAT-3 promoter in Cos-1 cells (2a and 2b) and RCSV3 cells (2c and 2d) displayed as percentage of maximal induction obtained with -2G (±SEM, c) and d)). The MR-2G variant showed a lower transactivation compared with the MR-2C variant. Results represent at least two independent experiments performed in triplicate.

MRc.-2G>C affects protein expression independently of the 5'untranslated region.

In the human NR3C2 gene, two 5'-untranslated (UT) exons are alternatively transcribed and generate two different mRNAs which code for a unique MR protein, since the translational start site is located at the beginning of exon 2. The c.-2G>C SNP is located 2 nucleotides upstream of the translation initiation site in the middle of the Kozak consensus sequence for translational initiation (Kozak, 1986) (Peri and Pandey, 2001) which is highly conserved among the NR3C2 genes from several species (Fig. 1a). In order to test a possible functional role of the c.-2G>C SNP, we have investigated its influence on translational efficiency, in the context of both 5'-UT exons 1α and 1 β. Using chimeric constructs, with exon 1 α or 1β inserted together with the Kozak sequence containing either G or C immediately upstream of the coding sequence of the luciferase gene (Fig. 1b), transient transfection assays were performed in renal RCSV3 cells and in COS cells, the amount of luciferase activity representing the amount of protein generated. In the presence of comparable mRNA levels 12 hs post-transfection, the C allele was associated with significantly higher protein levels compared to the G allele, both at 12 and 24h post-transfection in RCSV3 cells ($p<0.0001$, Fig. 3a) and Cos-7 cells ($p<0.0001$, Fig. 3b). 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 1 β was ~ 40% of that observed with exon 1 α ($p<0.0001$).

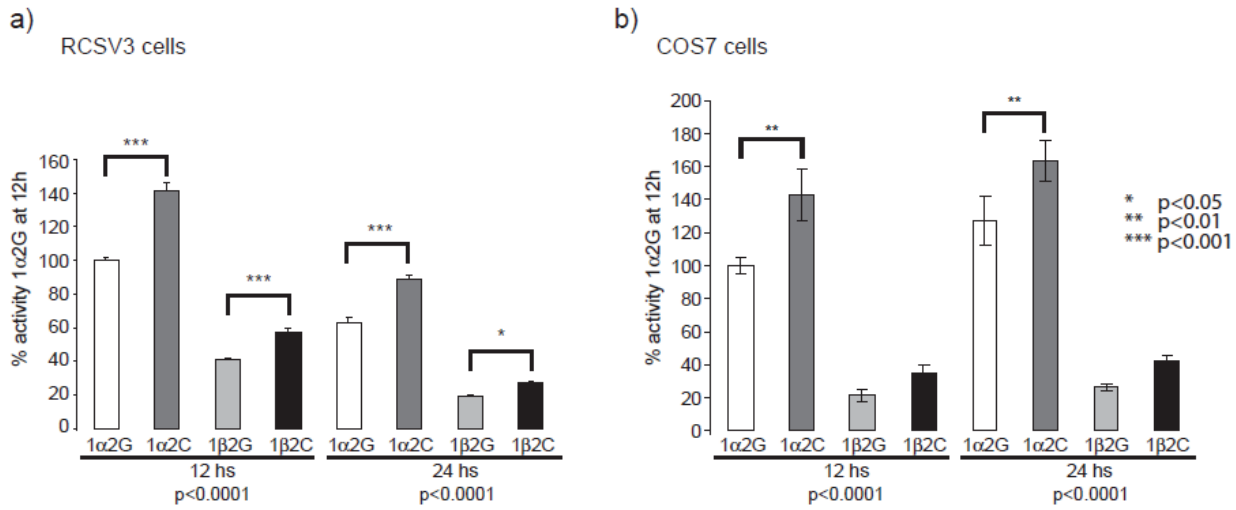

Figure 3. Effects of MRc.-2G>C on protein expression**.** Effects of -2G>C variants on protein expression were measured with a recombinant MR-luciferase construct. Chimeric constructs containing exon 1α (white and dark gray bars) showed a higher luciferase expression compared to chimeric constructs containg exon 1β (light gray and black bars) in either RCSV3 cells (3a) or COS-7 cells (3b). The MR-2C variant (dark gray and black bars) showed a higher luciferase expression compared with the MR-2G variant (white and light gray bars) in either RCSV3 cells (3a) or COS-7 cells (3b).

We then investigated the hypothesis that the -2G>C SNP may affect alternative translation of the previously described human MR isoforms MR-A and MR-B. Constructs containing either MR with the -2 C nucleotide or MR with the -2 G nucleotide 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 4).

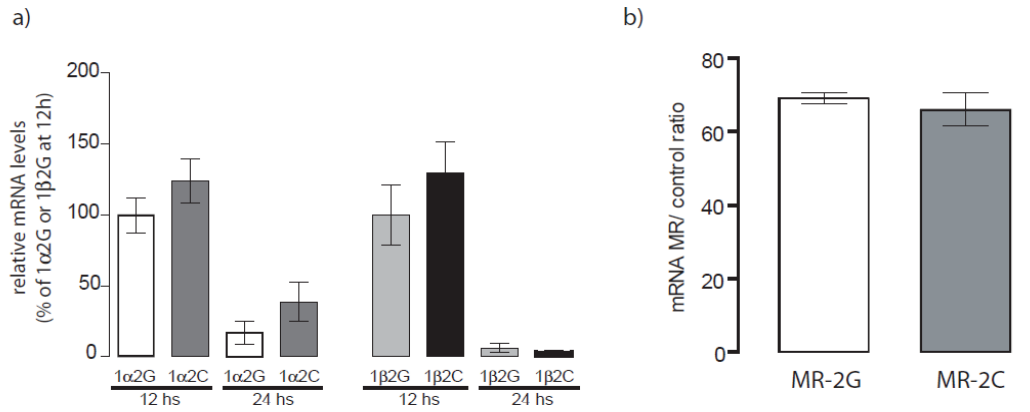

Figure 4. Effects of MRc.-2G>C on the expression of MR isoforms. Constructs containing MR with either -2C or -2G and control constructs expressing only MR-A or MR-B were transiently transfected in Cos-1 cells. The effect of the -2G or -2C allele on MR-A and B protein expression was measured by Western 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. S2b). c) Quantification of MR expression of multiple experiments using the MR-A detecting antibody (1D5).

Two different primary antibodies were used in Western blots: 1D5 directed against aminoacids 1 to 18 was used to visualize MR-A, while 2B7 directed against aminoacids 64 to 82 was used to detect MR-A and B (fig. 1c). Specificity of the antibodies was confirmed with the control constructs expressing only MR-A or only MR-B In Cos-1 cells transfected with the constructs MR -2G or MR

-2C only MR-A was expressed but not the MR-B. Western blots also confirmed the differences in MR-A protein expression between -2G and -2C containing constructs, the MR-2C resulted in significantly increased MR-A protein expression, the ratio between MR and the control tubulin was 0.300 (±0.014) for MR-2G and 0.362 (±0.022) for MR-2C (Fig. 5)

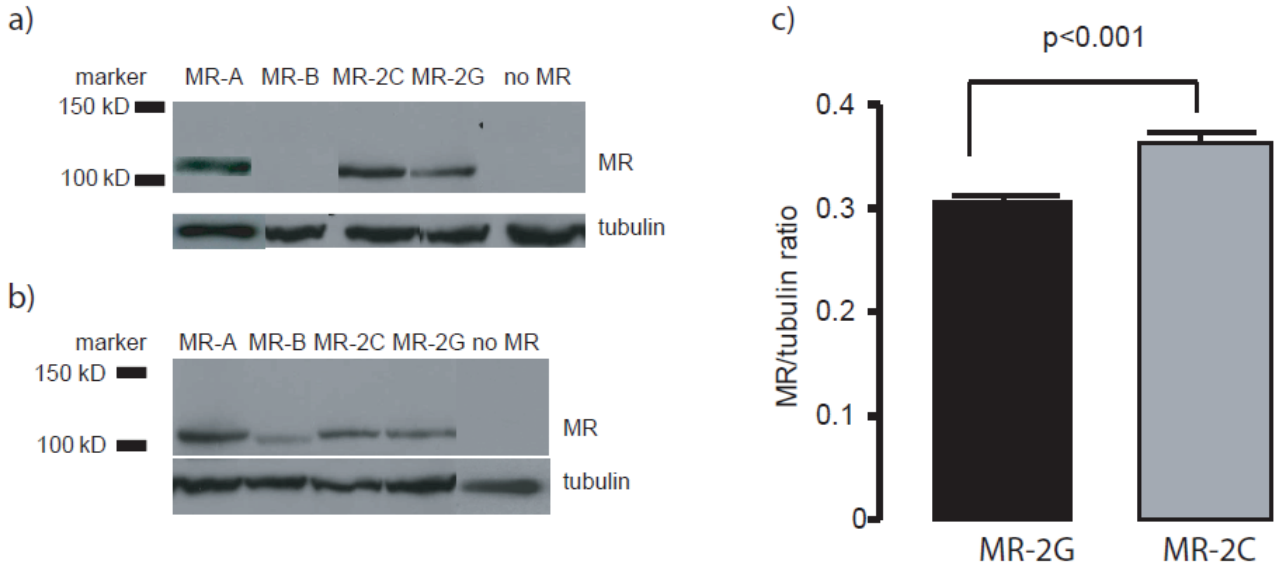

Figure 5. Effects of MRc.-2G>C on the expression of MR isoforms. Effect of -2G>C variants on MR-A and B protein expression was measured by Western bot using antibodies directed against a) the first 18 aminoacids of the MR, therefore only detecting MR-A and b) aminoacid 64-82 detecting MR-A and MR-B. c) Quantification of MR expression.

Consequences of MRc.-2G>C on renal sodium handling and blood pressure regulation

The MRc.-2G>C SNP was determined in subjects from the three independent cohorts. Allele and genotype frequencies were not significantly different between the three groups (χ^2_2= 5.36, p=0.07 and χ^2_4=6.57, p=0.16, resp.) (Table 1).

Table 1 Comparison of allele and genotype frequencies in the three different cohorts

group	Allele frequencies		Genotype frequencies		
	C	G	CC	GC	GG
Hypertensive	0.59	0.41	0.32	0.53	0.15
Healthy	0.51	0.49	0.25	0.53	0.22
NESDA	0.51	0.49	0.24	0.53	0.23

Mild hypertensive group. After 3 days of high salt diet (150 mmol/day), plasma renin activity was significantly higher in mild hypertensive GG patients compared to the other genotypes (Table 2). In addition, the GG genotype was associated with significantly higher SBP levels in men, but not in women (Table 3). No association of the MRc.-2G>C genotypes was found with other parameters tested in the cohort, such as 24-hours urinary sodium excretion and plasma aldosterone or plasma aldosterone and plasma renin activity levels after salt loading. Following the Weinberger's test, the ratio of salt sensitive to salt resistant subjects was not significantly different among genotypes (χ^2_1=1.69, P=0.43) and there was no relationship between the genotypes and blood pressure response to the salt sensitivity test (p=0.21, data not shown).

Table 2. Characteristics (mean ± SD) or geometric mean (95%CI) in 90 mild hypertensive men and women from the Italian cohort according to the MR MR-2G/C genotype

Italian cohort		3 days controlled salt intake (150 mmol/day)			Weinberger's test	
90 subjects	Freq (%)	24hr Urinary sodium excretion/creatine (mEq)	Aldosterone (ng/dl)	Plasma renin activity (ng/ml/h)	Salt Resistant (ΔBP< 10mm Hg) n	Salt Sensitive (ΔBP >10 mm Hg) n
MR c.-2CC (n=29)	32.2	155.1 ± 54.3 (n=25)	12.8 (10.1-16.2) (n=28)	1.42 (1.07-1.88) (n=28)	17	12
MR c.-2GC (n=48)	53.3	172.7 ± 83.7 (n=39)	12.8 (10.6-15.4) (n=47)	1.46 (1.20-1.79) (n=47)	21	27
MR c.-2GG (n=13)	14.8	180.1 ± 50.6 (n=11)	12.9 (9.5-17.6) (n=11)	2.25 (1.33-3.79) (n=11)	7	6
P-value:		0.69*	0.65*	0.029†	0.43‡	

* genotype effect from 2-way (genotype, sex) ANCOVA adjusted for age, BMI. There was no interaction between sex and genotype for any variable. For aldosterone and renin, log values were used in the statistical tests. †Global test (2df): 0.10, GG vs CC+GC. ‡$\chi^2 = 1.69$, df = 2

Table 3. Association analysis of the MR MR-2G/C genotype with diastolic and systolic blood pressure in 90 mild hypertensive men and women from the Italian cohort

Blood pressure	Group	MR-2G/C	Mean	SD	P*
Systolic (mmHg)	Men (n=56)	CC (22)	151.5	11.6	0.004
		GC (26)	147.8	12.6	
		GG (8)	165.8	9.8	
	Women n=34	CC (7)	151.0	9.8	0.21
		GC (22)	158.1	18.9	
		GG (5)	141.0	8.9	
Diastolic (mmHg)	Men (n=56)	CC (22)	99.1	5.1	0.053
		GC (26)	96.5	6.3	
		GG (8)	102.0	5.3	
	Women n=34	CC (7)	95.6	8.3	0.056
		GC (22)	98.1	9.7	
		GG (5)	90.0	3.5	

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.

Normotensive subjects. On the *ad libitum* Na^+/K^+ diet, 156 mmol of Na^+ (interquartile range, 126–187 mmol) and 68 mmol of K^+ (interquartile range, 59–81 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 4). The 24-hour

urinary Na^+ and K^+ excretion levels were identical for subjects of the 3 genotypes for all of the diets (Table 4).

Plasma active and total renin and aldosterone, and ANP on the *ad libitum* Na^+/K^+ diet were within the physiological ranges and did not differ according to genotype (Table 4). 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. The changes in plasma ANP concentrations were in the opposite directions (Table 4). On the high Na^+ – low K^+ diet, GG subjects had significantly higher levels of plasma active and total renin and 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.

Table 4. Biological characteristics of normal volunteers according to the MR MRc.-2G>C genotype at baseline and after 7 days of High Na-Low K and Low Na-High K diet.

	Baseline	High Na+ - Low K+	p	Low Na+ - High K+
Plasma active renin (pg/ml)				
CC (n=10)	13.8 [9.7;19.7]	5.8 [4.3;7.8]	*	30.7 [24;39.2]
GC (n=21)	14.3 [11.6;17.6]	8.4 [6.5;11]		38.5 [32.6;45.4]
GG (n=9)	15.8 [11.7;21.4]	10.9 [6.7;17.7]		43.6 [30;63.3]
Plasma total renin (pg/ml)				
CC (n=10)	108.3 [83.2;141.1]	62.4 [47.4;82]	*	142.3 [116;174.5]
GC (n=21)	125.4 [106.6;147.5]	91.7 [73.8;113.9]		169.3 [141.8;202.1]
GG (n=9)	112.5 [94.9;133.4]	83.2 [62.9;110]		156.9 [119.3;206.5]
Plasma ANP (pg/ml)				
CC (n=10)	23.3 ± 4.6	26.7 ± 8.9		17.9 ± 4.2
GC (n=21)	20.6 ± 4.6	25 ± 10.4		16.4 ± 3.3
GG (n=9)	20.3 ± 5.5	22.6 ± 8.4		16.4 ± 3.8
Plasma aldosterone (pg/ml)				
CC (n=10)	63.7 [47.7;85.1]	25.7 [18.3;36.1]	*	295.1 [216.4;402.5]
GC (n=21)	73 [64.2;83.1]	38.6 [31.6;47.2]		319.9 [262;390.4]
GG (n=9)	69.3 [52.8;90.8]	38.5 [30.7;48.3]		338.9 [266.1;431.5]
24 h urine volume (mL)				
CC (n=10)	1663 (1227;1980)	2092 (1896;3110)		2415 (1777;3463)
GC (n=21)	1496 (1187;1943)	1910 (1540;2610)		2123 (1781;2711)
GG (n=9)	1563 (1188;2286)	2448 (1918;2662)		2401 (2259;2976)
24h urinary sodium excretion (mmol/24h)				
CC (n=10)	177 (153.6;237.5)	277.4 (253.5;300.4)		17.2 (13.9;20)
GC (n=21)	138.9 (108;167.1)	246.2 (218.8;278.5)		14.1 (11.7;21.9)
GG (n=9)	158.2 (133;188)	242.9 (230;298)		17.4 (11.3;30.6)
24h urinary potassium excretion (mmol/24h)				
CC (n=10)	72.2 (65.8;86.6)	36.9 (26;47.2)		132.1 (96.5;144.9)
GC (n=21)	68.8 (55.3;83.1)	39.6 (34.3;43.1)		119.9 (106.1;140.8)
GG (n=9)	67.4 (57.6;72.2)	39.3 (29.9;42.6)		111.2 (94.5;132)

* : $p<0.05$ vs GC and vs GG. Data are mean [95% CI], mean ± SD or median (interquartile range)

NESDA cohort. There was a significant association between SBP and MRc.-2G>C (p= 0.041), even after adjustment for counfounding factors (Table 5). GG subjects had significant higher systolic blood pressure than GC or CC subjects (mean SBP for GG: 138.2±1.9 mmHg, GC: 137.3±1.8 mmHg and CC: 135.2±1.9 mmHg). Although we did not detect a gender x 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 5). Men with the GG genotype had significant higher systolic pressure (5.17 mm Hg) than those with the CC genotype (p= 0.15). SBP among men was 147.0±21.2 mmHg for GG genotype, 143.8±17.6 mmHg for GC genotype and 141.9 ± 20.6 mmHg for CC genotype.

Table 5. Association analysis of the MR MR c-2G>C genotype with diastolic and systolic blood pressure in Dutch cohort

Blood pressure	Group	MR-2G/C	Mean	SD	P*
Systolic (mmHg)	Men (n=563)	CC (137)	141.86	20.64	0.046
		GC (304)	143.81	17.57	
		GG (122)	147.03	21.23	
	Women (n=1191)	CC (262)	129.53	22.04	0.195
		GC (634)	131.26	18.96	
		GG (295)	131.03	17.76	
Diastolic (mmHg)	Men (n=563)	CC (137)	83.38	12.24	0.232
		GC (304)	84.83	11.07	
		GG (122)	85.49	12.71	
	Women (n=1191)	CC (262)	79.29	13.17	0.301
		GC (634)	80.01	11.41	
		GG (295)	80.26	11.08	

BP: blood pressure; *genotype effect by GLM adjusted for age, presence of life events, years of education, smoking, alcohol abuse/dependence, use of tricycle antidepressant (TCA), use of noradrenergic serotonergic (NS) antidepressant, number of chronic disease and BMI in each gender separately.

Table 6. Results of General Linear Model with mean systolic pressure (medication adjusted) as outcome in the whole cohort (n=1754 subjects), and stratified for gender

Parameter	complete cohort			Gender					
				Male (n=563)			Female (n=1191)		
	F test	P	R^2	F test	P	R^2	F test	P	R^2
Model			0.263			0.145			0.207
MR c.-2>C	3.202	0.041		3.088	0.046		1.639	0.195	
Negative life events,	1.104	0.294		0.327	0.568		0.895	0.344	
Gender	141.204	<0.001							
Age	246.499	<0.001		46.926	<0.001		198.771	<0.001	
Years of education	5.799	0.016		0.398	0.528		5.556	0.019	
Smoking	9.603	0.002		8.915	0.003		2.966	0.085	
Alcohol	0.728	0.394		0.187	0.665		0.417	0.519	
Use of a TCA	4.241	0.040		1.439	0.231		2.638	0.105	
NS antidepressant,	2.711	0.100		0.333	0.564		2.739	0.098	
chronic diseases	11.376	0.001		5.885	0.016		6.001	0.014	
BMI	9.427	0.002		4.747	0.030		5.584	0.018	

Abbreviations: TCA, tricycle antidepressant; NS, noradrenergic serotonergic; BMI, Body Mass Index

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 cohorts. The c.-2G>C variant is 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 2 nucleotides upstream of the first translation initiation site in the middle of the Kozak consensus sequence for translational initiation (Kozak, 1986). First, we showed with two different endogenous ligands and in two 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 aldosterone (Arai et al., 2003). Differences in methodology or the cell line that was used might explain this result. Second, using three different approaches and two different cell lines, we showed that the C allele results in more abundant protein expression than the G allele. Since mRNA expression and the affinity of the MR are 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 efficient for translation, since in 1534 human transcripts the sequence surrounding the initiation codon contains a C at position -2 in 40% of cases, while a G nucleotide is present in only 18% of transcripts (Peri and Pandey, 2001). Third, transcription of two different exons from alternative promoters on the NR3C2 gene can lead to different transcripts, with two different 5'-UT regions that can precede MRc.-2G>C. Our results demonstrate that the 5'UT has no influence on the observed effect of the SNP. With both exons 1α and 1β the -2C allele was associated with higher protein translation. However, protein expression in the presence of the UT

region 1 β was ~ 40% of that observed with exon 1α, indicating that the sequence of exon 1 β is less optimal for translation. Finally, the influence of MR -2G>C on the MR-A/MR-B ratio was investigated. In Western blot experiments the MR-A isoform, translated from the first translation start, was more abundant in the presence of the C allele. Transient transfection of MR containing either the -2 C or -2 G allele did not result in any detectable MR-B protein. As far as we know, only one study reported the MR-B isoform (Pascual-Le Tallec et al., 2004); in that study MR-B was detected with an *in vitro* translation assay. Our results suggest that MR-B is not translated, at least not in Cos-1 cells. Given that the Kozak region preceding the translation start of MR-B is weak, the existence of MR-B *in vivo* needs further clarification. Taken together, we conclude that the C allele increases MR-protein expression and thereby the *in vitro* transcriptional activity of the MR.

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. 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 two cohorts under different experimental settings. Individuals carrying the CC genotype in either a healthy cohort under a high Na^+ - low K^+ 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 tubulo-glomerular feed-back in individuals with the CC genotype. This effect might be due to more efficient sodium reabsorption due to higher levels of MR in the distal tubule which was unmasked in conditions of low aldosterone synthesis and concentration, i.e. on a high Na^+ - low K^+ diet (Menard et al., 2006). Indeed the C allele results in higher MR levels after transfection in a kidney collecting duct epithelial cell line (RCSV3) or a kidney fibroblast-like cell line (COS). There were trends in similar directions for these parameters in the healthy group at baseline or during the low Na^+- high K^+ diet but this did not reach significance. The absence of significant difference between genotypes may be due 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 favourable cardiovascular profile as compared to the CG 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 ten 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 (Mehdi et al., 2009; Pitt, 2005). Our data suggest that functional variants of the MR may be associated not only to different cellular responses to the hormone, but also, indirectly, to increased aldosterone levels that may activate both genomic and non-genomic pathways in non-epithelial target tissues to promote deleterious cardiovascular effects.

Only males carriers of the MRc.-2G>C GG genotype showed higher BP compared to males with the other genotypes in the mild hypertensive cohort, the polymorphism not being associated with

BP in women. The same gender dependent association was found with SBP in the large-scale Dutch cohort suggesting a sexual dimorphism. Many studies have reported gender-related differences in occurrence and severity of cardiovascular diseases, related to the hormonal status (Rosano et al., 2007). Interestingly, it has been shown that ovarian hormones positively affect salt sensitivity, protecting pre-menopausal women from the development of hypertension. After menopause, responsiveness of renin-angiotensin-aldosterone system changes, with a net increase in salt sensitivity (Schulman et al., 2006). 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. We hypothesise that the effect of the MR c-2G>C G allele, which leads to less MR, correlates with higher blood pressure only in men and not in women because of the interference of ovarian hormones. To further elucidate the role of sex hormones or differences in cortisol levels a cohort containing post menopausal women should be tested for an association with this SNP.

In conclusion, the MRc.-2G>C C allele increases translation of the MR protein resulting in an increase in MR protein expression and a higher transactivational activity *in vitro*. In two independent studies, GG carriers of MRc.-2G>C showed higher renin and aldosterone levels compared to individuals with the other genotypes. Finally, male GG carriers showed higher SBP. We propose that the MR -2C va