Arora et al., MicroRNA miR-425 is a Negative Regulator of Atrial Natriuretic Peptide

DETAILED METHODS

Study sample for genotype-directed study. Inclusion criteria for study subjects were as follows: age between 18 and 40 years old, blood pressure (BP) at two study visits ≤ 140/90, 18 < BMI < 25 kg/m^2, estimated creatinine clearance > 60 cc/min, and willingness to comply with the study diet. Subjects were excluded if they had any of the following: history of hypertension, history of cardiovascular, renal, or liver disease, treated diabetes, use of vasoactive or diuretic medications, atrial fibrillation, anemia, abnormal serum sodium or potassium, urine human chorionic gonadotropin level consistent with pregnancy, abnormal liver function tests, current smoking, use of stimulant medications in the past month, or inability to adhere to the study diet due to pre-existing dietary requirements (vegan, gluten-free, high-calorie athletic training diet).

Study Protocol. Individuals were genotyped for rs5068 (A/G; minor allele frequency, 4%). From 699 individuals who were screened, 31 were selected for further physiologic assessment based on genotype (attempting to recruit at least 1 AG individual for every 3 AA individuals). Only the study statistician had access to the unblinded genotype data.

The sodium contents of the high- and low-salt diets were 200 mEq/day and 10 mEq/day, respectively. Other micronutrients were standardized, including 100 (± 2) mEq potassium and 1000 (± 50) mg calcium per day. The macronutrient composition of

both diets was the same (protein 10-20%, fat 20-35%, and carbohydrate 50-65%). All meals were prepared on site at the metabolic kitchen in the Massachusetts General Hospital Clinical Research Center (MGH CRC). Subjects picked up the prepared, frozen meals and snacks directly from the CRC. They were provided with bottled distilled water to consume while on the diet protocol and during the inpatient visits. Subjects were also provided with low-sodium toothpaste during the low-sodium portion of the protocol. On day 5 of the diet, they performed a 24-hour urine collection (for urine sodium and creatinine) so that adherence to the study diet could be assessed. Women were studied during the first week of the menstrual cycle. After a week of standardized diet, subjects were admitted for an overnight visit. Subjects were kept supine overnight, and ambulatory BP monitoring was performed using a Space Labs system (Issaquah, WA). The following morning two intravenous catheters were placed for phlebotomy and infusion of 0.25 ml/kg/min normal saline (0.9 mEq/mL) over 2 hours. Venous blood was sampled hourly over 8 hours beginning immediately prior to the start of the infusion. Specifically, just prior to the initiation of the infusion, participants were asked to empty their bladder. Every 30 minutes during the infusion and afterwards up to 4 hours, we asked the participants to urinate. We measured urine volume and sodium concentration. The protocol was subsequently repeated using the alternate diet for one week. The first 13 individuals were also studied in a separate echocardiographic investigation (1).

Genotyping and measurement of natriuretic peptide levels. DNA was extracted from whole blood using the AutoGenFlex STAR system (AutoGen, Holliston, MA). A

TaqMan assay (C_11644811_10) was used to genotype SNP rs5068 (Applied Biosystems, Foster City, CA). The genotyping call rate was 99.5%.

Plasma Nt-proANP levels were measured by ELISA (proANP 1-98, Biomedica Medizinprodukte GmbH & Co KG, Austria). Plasma mature BNP was measured using CENTAUR immunoassay (Siemens, New York, NY) and Nt-proBNP levels were measured using an electrochemiluminescence immunoassay (Elecsys proBNP, Roche, Indianapolis, IN). Plasma cyclic guanosine 3′,5′-monophosphate (cGMP) levels were measured using an ELISA (Biomedical Technologies, Stoughton, MA).

Cultured cells. Epstein Barr virus (EBV)-transformed B cells from 5 individuals homozygous for the major allele of rs5068 (GM07019, GM07048, GM07349, GM10843, GM10850) and 4 individuals heterozygous for rs5068 (GM10852, GM10853, GM12864, GM07348) were obtained from Coriell Repositories (Coriell Institute for Medical Research, Camden, NJ). COS-7 cells (derived from monkey kidney) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Human cardiomyocytes (97% purity) derived from iPS cells were purchased from Cellular Dynamics International (Madison, WI).

miRNAs. Double-stranded RNAs designed to mimic the endogenous mature candidate miRNAs including miR-425 (AAUGACACGAUCACUCCCGUUGA, underline denotes rs5068), miR-4770 (UGAGAUGACACUGUAGCU), and miR-196a* (CGGCAACAAGAAACUGCCUGAG), as well as a negative control miRNA, were purchased from Applied Biosystems (Foster City, CA). A single-stranded RNA

designed to inhibit the endogenous mature miR-425 and single-stranded negative control miRNA were obtained from Applied Biosystems. A modified miR-425 mimic (AGUGACACGAUCACUCCCGUUGA), the seed sequence of which matched the *NPPA* minor allele mRNA, was purchased from Applied Biosystems.

RNA extraction. Total RNA was extracted from human tissues and cultured cells using TRIzol, according to the manufacturer's protocol (Life Technologies, Grand Island, NY). Peripheral whole blood samples (2.5 ml) from fasting participants were collected in PAXgene™ tubes during Framingham Heart Study (FHS) offspring cohort examination 8 (2005-2008). Total RNA was isolated from frozen PAXgene blood tubes by Asuragen, Inc., according to the company's standard operating procedures for automated isolation of RNA. The RNA Integrity Number (RIN) for the RNA samples used in this study was 7.2 ± 0.9 (mean and SD) indicating high RNA quality.

Measurement of mRNA and miRNA levels. *NPPA* mRNA levels in RNA extracted from FHS whole blood samples were measured using the Affymetrix Hu_Ex_1.0_st Exon array according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Log_2 expression levels, adjusted for technical covariates, were analyzed in relation to rs5068 genotype.

Levels of *NPPA* mRNA and 18S ribosomal RNA in RNA extracted from extracted from lymphoblasts and cardiac myocytes were measured using quantitative RT-PCR and Taqman primers. Relative *NPPA* mRNA levels normalized to 18S ribosomal RNA levels were determined using the relative cycle threshold method.

TaqMan microRNA reverse transcription and real-time assay kits were used to detect mature miR-425 and U6 snRNA (RNU6B). Differences in miR-425 levels normalized to RNU6B were determined using the relative Ct method (Applied Biosystems).

Evaluating the ability of miRNAs to modulate NPPA 3'UTR function.

Luciferase-NPPA 3' UTR reporter constructs: The *NPPA* 3' UTR sequences (299 bp) corresponding to the major A and minor G alleles of rs5068 were amplified by PCR from a de-identified human HapMap genomic DNA sample obtained from Coriell Repositories (Coriell Institute for Medical Research, Camden, NJ) using two primers (GCGGAGCTCAGATAACAGCCAGGGAGGACAAG and GCTCTAGATTGTCTTCTGTCCATGGTGCTGAAG). The 3' UTR *NPPA* sequences were cloned into the pISO vector (Addgene) 3' of the sequence encoding firefly luciferase to generate major (A) allele and minor (G) allele constructs (major-LUC and minor-LUC, respectively).

cDNA expression constructs: A full-length human *NPPA* major allele cDNA expression plasmid was purchased from OriGene (Rockville, MD, catalog # SC122740). A minor allele *NPPA* cDNA construct was created using site-directed mutagenesis using QuickChange II XL site-directed mutagenesis kit (Stratagene, Santa Clara, CA) and synthetic oligonucleotides, GTCATCTTGTTGCCATAGAGTTGTGATCATCCC and GGGATGATCACAACTCTATGGCAACAAGATGAC, according to the manufacturer's protocol.

Transient transfection of reporter plasmids into heterologous cells:

Luciferase activity: COS-7 cells were transfected with major-LUC or minor-LUC constructs, as well as a plasmid directing constitutive expression of renilla luciferase (as a transfection control), using FuGene 6 transfection reagent (Roche, Indianapolis, IN). Twenty-four hours later, miRNA mimics or anti-miRNAs were transfected using RNAi Max (Life Technologies, Grand Island, NY) at a final concentration of 5 nM. After an additional 24 hours, cells were harvested, and firefly and renilla luciferase activities in cell extracts were measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI).

ANP production: *NPPA* expression plasmids and the plasmid specifying renilla luciferase (as a transfection efficiency control) were transfected into COS-7 cells, followed 24 hours later by transfection of miRNAs, as described above. Cells and media were collected 48 hours after plasmid transfection for measurement of Nt-proANP levels by ELISA and renilla luciferase activity.

***Transfecting miRNAs into human cardiomyocytes*:** Human cardiomyocytes were transfected with either miR-425 or a negative control miRNA using RNAi Max. After 24 hours, cells were washed and then incubated in serum-free medium for an additional 48 hours. Cells were harvested for RNA extraction and measurement of *NPPA* mRNA and 18S ribosomal RNA levels. Nt-proANP immunoreactivity in the culture media was measured using the Nt-proANP ELISA kit (as described above).

Supplementary Table 1. Baseline characteristics of the genotype-directed study sample

Characteristics	**All subjects (N=31)**	**Major Homozygotes (AA) (N=23)**	**Heterozygotes (AG) (N=8)**
Age (years)	24±4	21±2	25±6
Male (%)	94	95	88
Body mass index (kg/m^2)	23.1±1.7	23.0±1.6	23.5±1.9
Systolic BP (mm Hg)	114±10	115±9	114±9
Diastolic BP (mm Hg)	73±7	74±4	72±8

Values are mean±SD.

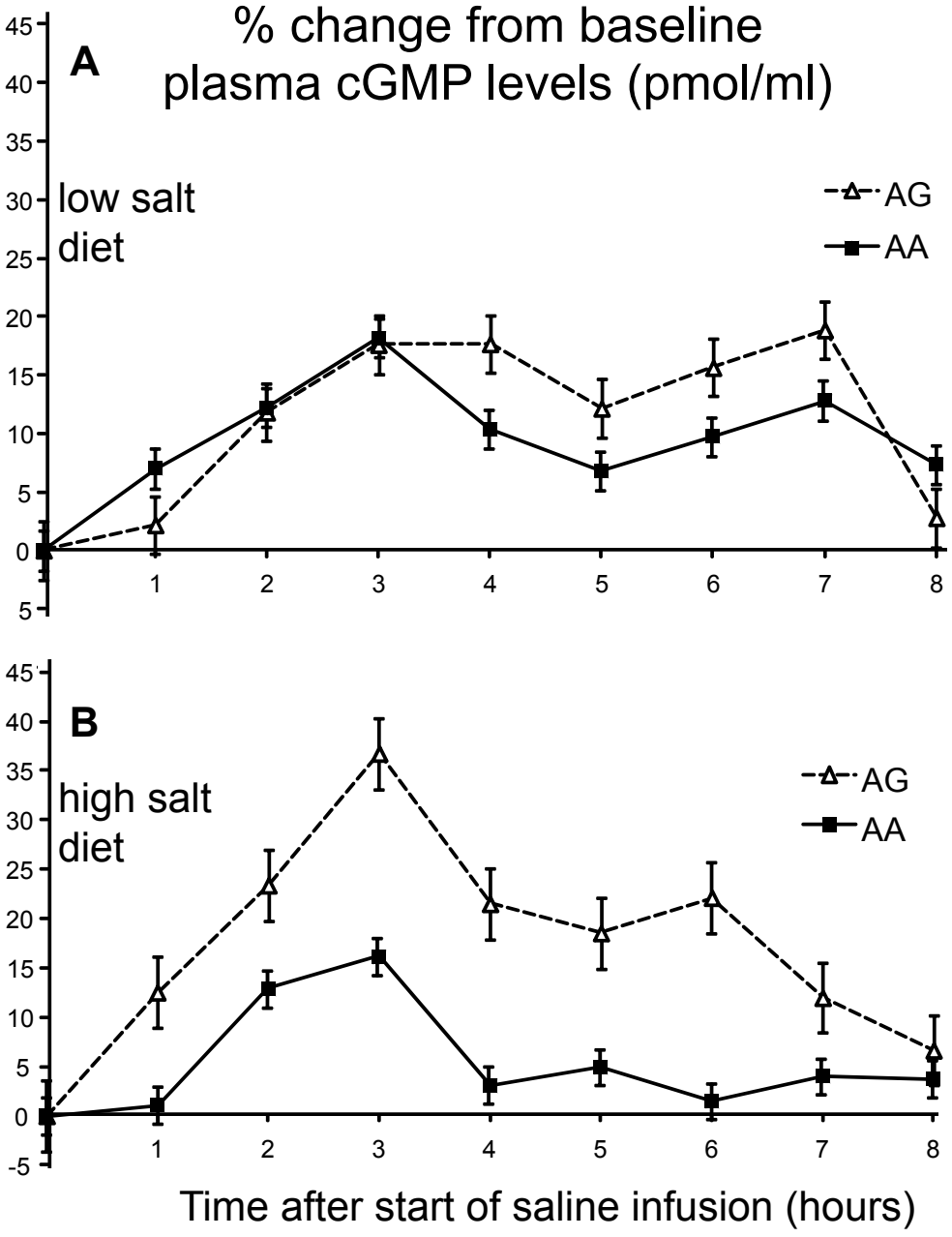
Supplementary Table 2. Blood pressure and 24-hour urine measurements

Diet	Variable	AA (N=23)	AG (N=8)
Low Salt	Overnight SBP (mm Hg), on day 6	117±10	117±11
	Overnight DBP (mm Hg), on day 6	70±6	71±6
	Post-Infusion SBP (mm Hg)	114±8	109±8
	Post-infusion DBP (mm Hg)	67±5	65±6
	Total urine sodium (mmol)	22±13	19±19
	Total urine creatinine (grams)	1.6±0.5	1.8±1.7
High Salt	Overnight SBP (mm Hg), on day 6	117±8	112±7
	Overnight DBP (mm Hg), on day 6	71±7	68±6
	Post-Infusion SBP (mm Hg)	114±6	111±8
	Post-infusion DBP (mm Hg)	66±6	65±6
	Total urine sodium (mmol)	146±53	133±49
	Total urine creatinine (grams)	1.5±0.4	1.5±0.5

Values are (mean±SD). SBP, systolic blood pressure; DBP, diastolic blood pressure. Blood pressure was measured using an ambulatory blood pressure cuff (Space Labs, Issaquah, WA) overnight while supine and then following infusion of intravenous saline.

REFERENCES

1. Mak, G.S., Sawaya, H., Khan, A.M., Arora, P., Martinez, A., Ryan, A., Ernande, L., Newton-Cheh, C., Wang, T.J., and Scherrer-Crosbie, M. 2013. Effects of subacute dietary salt intake and acute volume expansion on diastolic function in young normotensive individuals. *Eur Heart J Cardiovasc Imaging*.



Supplementary Figure 1. Samples for measurement of plasma cGMP levels were obtained at baseline (time 0) and at 1, 2, 3, 4, 5, 6, 7 and 8 hours (h) after the start of saline infusion. AA denotes rs5068 major homozygote, and AG denotes rs5068 heterozygote. The effect of genotype on the change in plasma cGMP levels induced by saline challenge after one week on a low-salt diet (**Panel 1A**) or a high-salt diet (**Panel 1B**) is shown ($p=0.03$ for difference by genotype, $p<0.001$ for effect of saline).

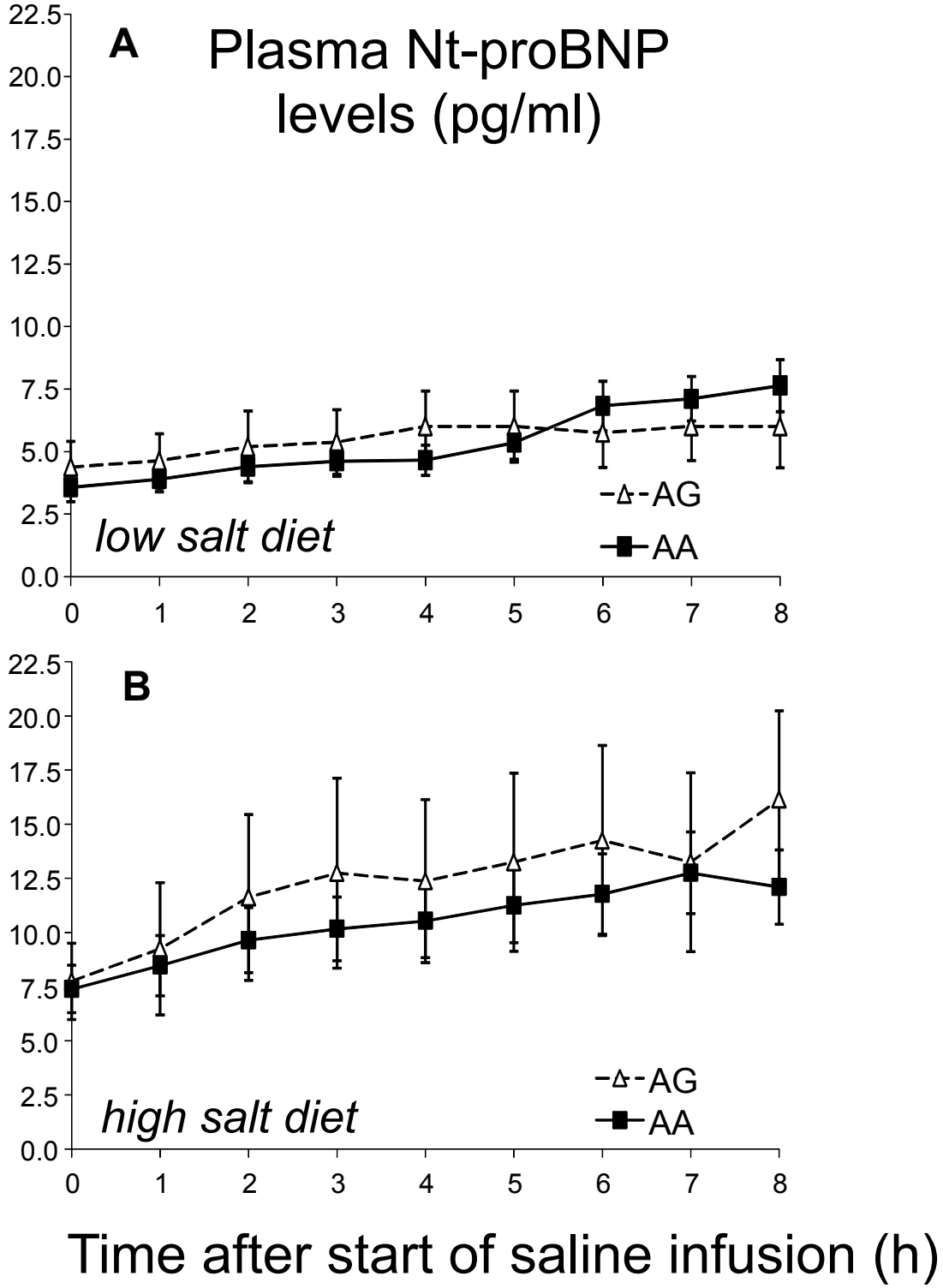


Figure 2. Samples for measurement of plasma Nt-proBNP levels were obtained at baseline and at 1, 2, 3, 4, 5, 6, 7 and 8 hours (h) after the start of saline infusion. AA denotes rs5068 major homozygote, and AG denotes rs5068 heterozygote. The effect of genotype and dietary sodium on plasma Nt-proBNP levels during saline challenge, after one week of a low-salt diet is shown in **Panel 2A** or high-salt diet in **Panel 2B (P = 0.5 for genotype effect, P < 0.001 for diet and saline effects).**

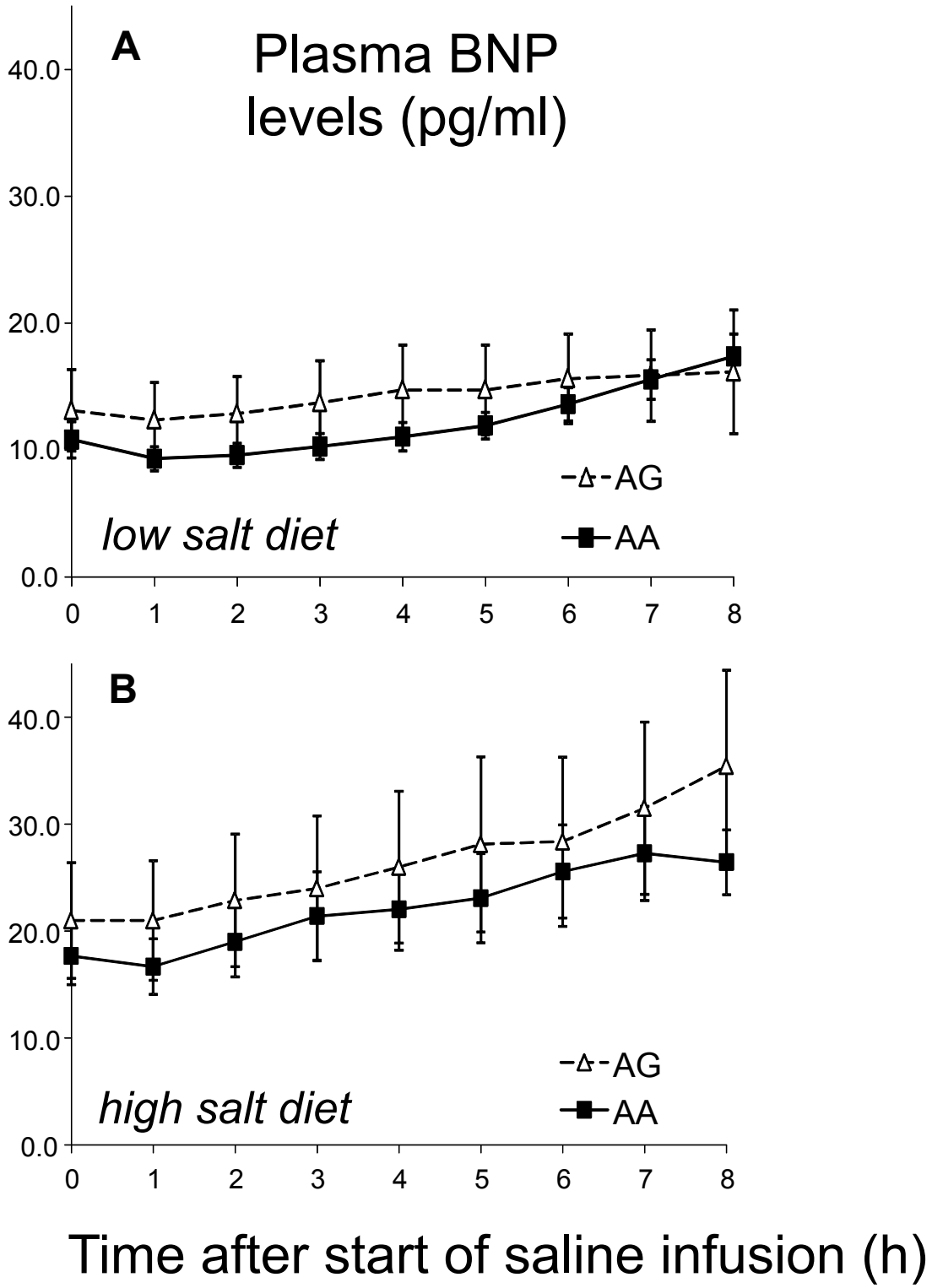
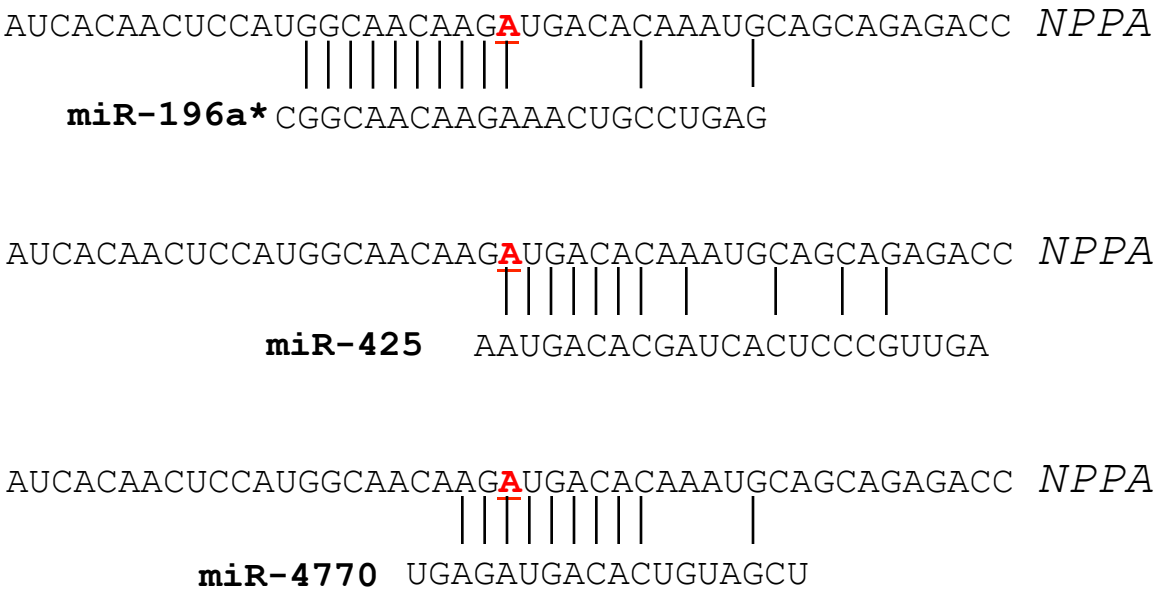
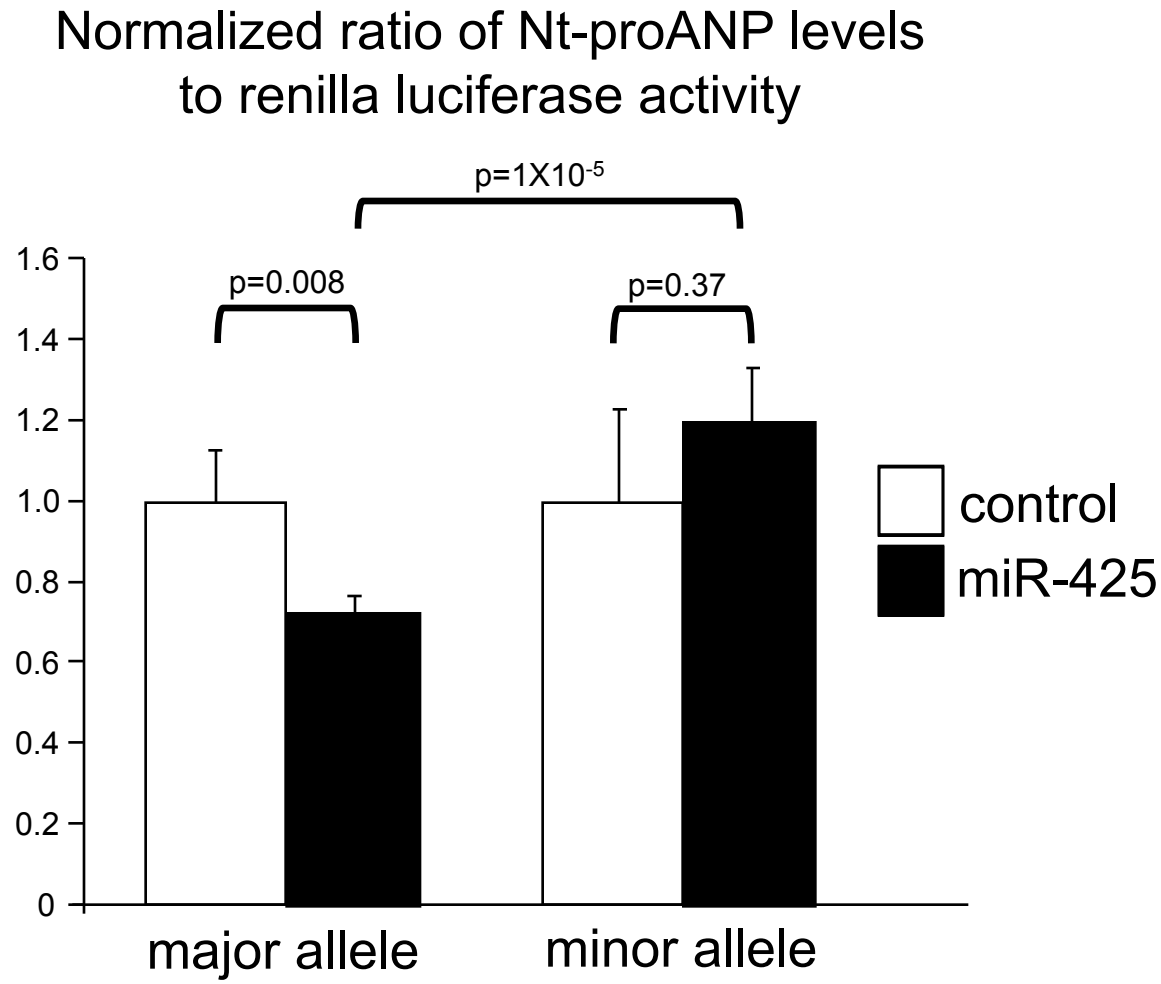


Figure 3. Samples for measurement of plasma BNP levels were obtained at baseline and at 1, 2, 3, 4, 5, 6, 7 and 8 hours (h) after the start of saline infusion. AA denotes rs5068 major homozygote, and AG denotes rs5068 heterozygote. The effect of genotype and dietary sodium on plasma BNP levels during saline challenge, after one week of a low-salt diet is shown in **Panel 3A** or high-salt diet in **Panel 3B (P = 0.6 for genotype effect, P < 0.001 for diet and saline effects).**



Supplementary Figure 4. miRNAs that are predicted to target the major but not minor allele of rs5068. The position of rs5068 is shown in red. The sequence complementary to the *NPPA* mRNA is shown in the figure to highlight the binding of the miRNAs to sequences around the rs5068 DNA variant.



Supplementary Figure 5. miR-425 suppresses Nt-proANP production by heterologous cells transfected with a plasmid directing expression of a human *NPPA* major allele cDNA. Plasmids specifying the human *NPPA* cDNA (major and minor alleles) were transfected into COS-7 cells together with a plasmid directing expression of renilla luciferase (as a control for transfection efficiency). Media was collected for measurement of Nt-proANP, and cells were collected for measurement of renilla luciferase activity. Data are expressed as the ratio of Nt-proANP measured in the culture medium normalized to the renilla luciferase activity (mean ± SEM; n=6). Experiments were repeated three times with similar results.