

BRIEF REPORT

Atrial Natriuretic Peptide Frameshift Mutation in Familial Atrial Fibrillation

Denice M. Hodgson-Zingman, M.D., Margaret L. Karst, B.A., Leonid V. Zingman, M.D., Denise M. Heublein, C.L.T., Dawood Darbar, M.D., Kathleen J. Herron, B.A., Jeffrey D. Ballew, M.S., Mariza de Andrade, Ph.D., John C. Burnett, Jr., M.D., and Timothy M. Olson, M.D.

SUMMARY

Atrial fibrillation is a common arrhythmia that is hereditary in a small subgroup of patients. In a family with 11 clinically affected members, we mapped an atrial fibrillation locus to chromosome 1p36-p35 and identified a heterozygous frameshift mutation in the gene encoding atrial natriuretic peptide. Circulating chimeric atrial natriuretic peptide (ANP) was detected in high concentration in subjects with the mutation, and shortened atrial action potentials were seen in an isolated heart model, creating a possible substrate for atrial fibrillation. This report implicates perturbation of the atrial natriuretic peptide–cyclic guanosine monophosphate (cGMP) pathway in cardiac electrical instability.

From the Department of Internal Medicine, University of Iowa, Carver College of Medicine, Iowa City (D.M.H.-Z., L.V.Z.); and the Cardiovascular Genetics Laboratory, Departments of Internal Medicine (M.L.K., D.D., K.J.H., J.D.B., T.M.O.) and Pediatric and Adolescent Medicine (T.M.O.), the Cardiorenal Research Laboratory, Departments of Physiology and Internal Medicine (D.M.H., J.C.B.), and the Department of Health Sciences Research (M.A.) — all at the College of Medicine, Mayo Clinic, Rochester, MN. Address reprint requests to Dr. Olson at Mayo Clinic, 200 First St. SW, Rochester, MN 55905, or at olson.timothy@mayo.edu.

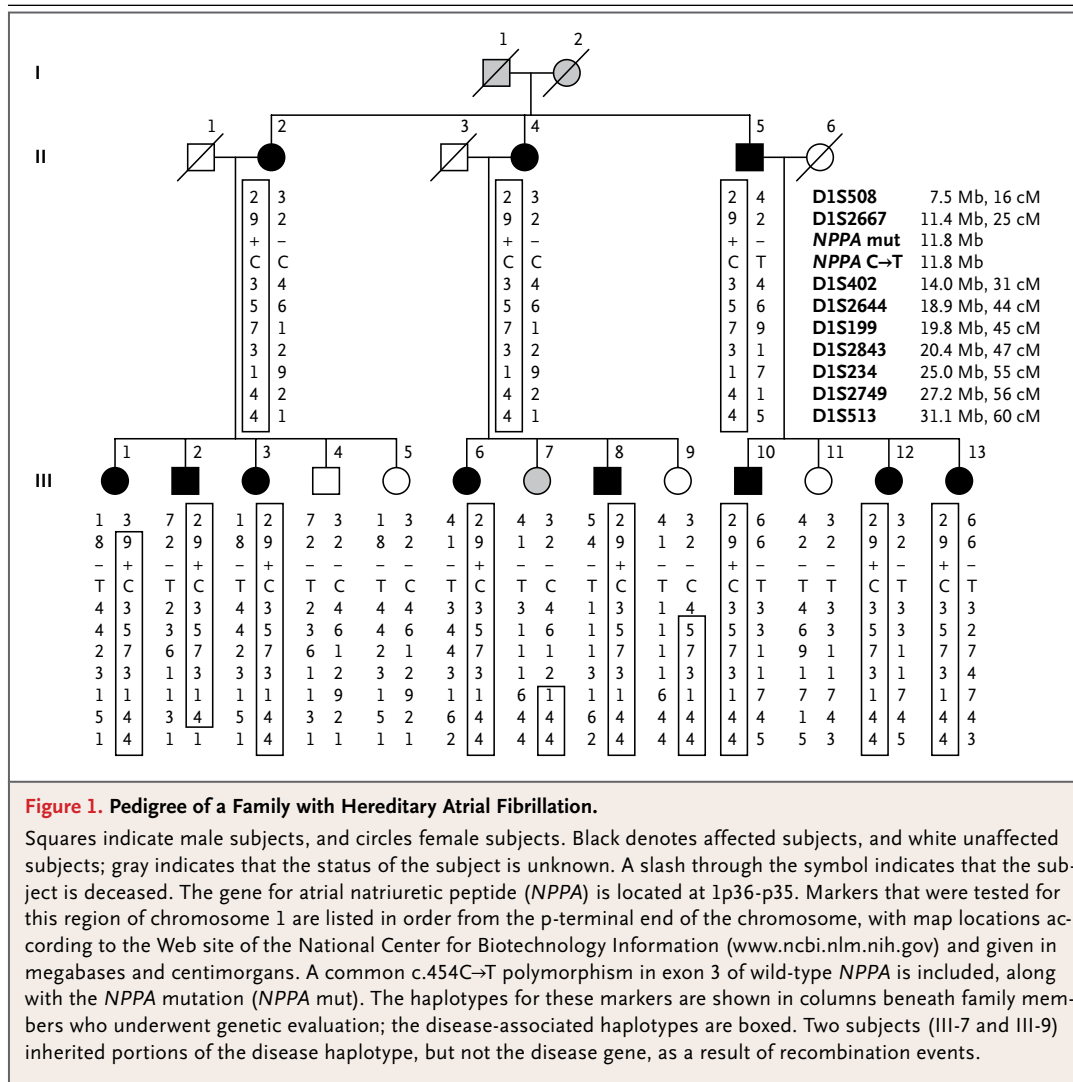
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ATRIAL FIBRILLATION IS THE MOST COMMON SUSTAINED CARDIAC ARRHYTHMIA. Since the lifetime risk of the condition is 25%, it constitutes a growing epidemic in the aging population.^{1,2} Atrial fibrillation develops as a paroxysmal disorder characterized by rapid, irregular electrical activation of the atria and can be associated with palpitations, syncope, thromboembolic stroke, and congestive heart failure. Valvular, ischemic, hypertensive, and myopathic heart diseases are the most common causes of acquired atrial fibrillation. However, a genetic basis for atrial fibrillation is evident in population-based studies^{3,4} and in a subgroup of patients with familial disease.^{5,6} Human genetics investigations have identified atrial fibrillation–associated mutations in cardiac ion channels⁷⁻⁹ and gap junction proteins,¹⁰ findings that implicate myocellular derangements in ion flux. However, the molecular basis of atrial fibrillation remains unknown in a majority of cases. We used linkage analysis to identify a novel mutation in the natriuretic peptide precursor A gene (*NPPA*), which encodes ANP. This mutation segregates with familial atrial fibrillation, thereby uncovering an unexpected association between a defect in a circulating hormone and susceptibility to arrhythmia.

METHODS

STUDY SUBJECTS

We studied members of a white family of northern European ancestry who had atrial fibrillation segregating as an autosomal dominant trait (Fig. 1). In addition, we randomly selected a group of 560 control subjects from a population-based white cohort of northern European ancestry who had normal results on electrocardiography and echocardiography. Family members and control subjects provided



written informed consent under a research protocol approved by the institutional review board at the Mayo Clinic.

We reviewed medical records of all the study subjects. The phenotypic classification of a subject as having familial atrial fibrillation (“affected”) required documentation of atrial fibrillation on electrocardiography and the absence of clinical risk factors for arrhythmia, such as uncontrolled hypertension and primary structural heart disease. Subjects with normal findings on electrocardiography who did not have symptoms of frequent palpitations, racing heart rate, dizziness, or syncope were classified as “unaffected.”

For genetic analysis, genomic DNA was isolated from peripheral-blood white cells. For protein studies, additional blood samples were obtained

from three family members. Samples were collected in EDTA tubes and centrifuged at 2500 rpm at 4°C for 10 minutes.

LINKAGE ANALYSIS AND MAPPING

For primary genome scanning, we used the ABI PRISM Linkage Mapping Set MD10, version 2.5 (Applied Biosystems), which consisted of fluorescently labeled polymerase-chain-reaction (PCR) primer pairs for 400 tandem repeat markers with average spacing of 10 cM. After PCR amplification of genomic DNA samples, amplified fragments were resolved on an ABI PRISM 3100 Genetic Analyzer and analyzed with GeneScan Analysis and Genotyper software. Two-point and multi-point linkage analyses were performed with the use of the FASTLINK program and specification

of the following variables: a disease allele frequency of 0.001, a phenocopy rate of 0.001, equal marker allele frequencies, and dichotomous liability classes (“affected” and “unaffected”). Lod scores were determined for affected subjects only and for 80% and 100% penetrance models at recombination frequencies of 0.0 to 0.4.

Fine mapping was performed with additional closely spaced microsatellite markers that were localized on genetic and physical maps, accessible on the Web site of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Genotyping was accomplished by PCR amplification of genomic DNA radiolabeled with [α - 32 P] deoxycytidine triphosphate, resolution of alleles by polyacrylamide-gel electrophoresis, and visualization by autoradiography. Scored genotypes were assembled as haplotypes to define the critical region of complete linkage on the basis of recombination events in affected subjects.

MUTATION DETECTION

Primer pairs for exon-specific PCR amplification of the three translated exons of *NPPA* were designed with the use of Oligo Primer Analysis Software, version 6.51 (National Biosciences) (for details, see the Supplementary Appendix, available with the full text of this article at www.nejm.org). Amplified products were treated with the PCR Product Presequencing Kit (USB) and sequenced by the dye-terminator method in a core facility with the use of an ABI PRISM 3730 XL DNA Analyzer (Applied Biosystems). DNA sequences were viewed and analyzed with the Sequencher computer program (Gene Codes), and a mutation was identified that segregated with atrial fibrillation. For single-allele sequencing of DNA from heterozygotes, the mutant allele was separated from the wild-type allele by polyacrylamide-gel electrophoresis. Samples from control subjects were screened for the mutation by denaturing high-performance liquid chromatography heteroduplex analysis with the use of the WAVE DNA fragment analysis system (Transgenomic).

MUTANT ANP PEPTIDE AND ANTIBODY

A custom-designed polyclonal antibody against the mutant form of ANP (mANP) was manufactured commercially (21st Century Biochemicals). The peptide Ac-CYRITAREDKQGWA-OH, corresponding to the anomalous residues of mANP, was synthesized, purified to 96% by high-performance liquid chromatography, and verified by peptide se-

quencing and mass spectroscopy. Conjugated peptide was injected into rabbits, serving as an epitope for the generation of affinity-purified polyclonal antibody. Full-length mANP (with 40 amino acids) was synthesized in a core facility for use in developing the mANP radioimmunoassay and for the experiments with isolated hearts.

RADIOIMMUNOASSAY

Radioimmunoassays were developed for both mANP (with the use of the polyclonal antibody described above) and for wild-type ANP (with the use of commercially available antibody [Phoenix Pharmaceuticals]). Technical details are provided in the Supplementary Appendix. Both antibodies were labeled with iodine-125.

The binding specificity of anti-mANP antibody was demonstrated by first generating standard curves with varying amounts of synthetic mANP and then measuring samples from the subjects at several dilutions to assess cross-reactivity. Standard curves were generated for calculating the ANP and mANP concentrations, in picograms per milliliter, in samples from both family members and control subjects. The range of the standard curve was 2 to 500 pg per milliliter for both ANP and mANP. All peptide measurements were made in duplicate, and values were reported as the average of the two. The normal range for ANP on the basis of this assay was determined to be 14 to 36 pg per milliliter among 100 samples.

A commercially available immunoradiometric assay was used to measure B-type natriuretic peptide (BNP) concentrations (Shionogi) (see the Supplementary Appendix). A calibration curve (range, 0 to 2000 pg per milliliter) was constructed from standard BNP solutions to estimate BNP concentrations in the samples. The normal range for BNP with the use of this assay was determined to be 8 to 16 pg per milliliter among 100 samples.

ISOLATED-HEART MODEL

The institutional animal care and use committee at the University of Iowa approved all the procedures for studies in animals. Atrial monophasic action potentials and effective refractory periods were measured in isolated, perfused hearts obtained from male rats. Each set of measurements was performed in separate isolated hearts. Details of the preparation of the isolated hearts are provided in the Supplementary Appendix.

After establishment of a stable perfused-heart preparation, the posterior atria were cut away to

expose the left and right atrial endocardium, and the atrioventricular node was mechanically crushed, resulting in atrioventricular dissociation. The right atrium was paced with a Bloom Electrophysiology Stimulator (Fischer Medical Technologies) at a cycle length of 150 msec and a pulse width of 0.1-msec with the use of a bipolar platinum-tipped electrode (NuMed), and the right ventricle was paced at a cycle length of 500 msec. A monophasic action potential (MAP) probe (Harvard Apparatus) was maintained in a single position on the anterior left atrial endocardium. Amplified signals (IsoDam, World Precision Instruments) were digitally acquired at 2 kHz (USB-6210 and LabVIEW 8.2, National Instruments). The MAP duration was measured at 90% repolarization on atrial beats without far-field ventricular interference. The effective refractory period was measured by delivering extrastimuli through the pacing catheter at decremental coupling intervals after at least 8 beats of a drive train with a 150-msec cycle length. The effective refractory period was defined as the longest extrastimulus coupling interval that did not result in a propagated response, as measured by the left atrial MAP probe. In separate heart preparations, the perfusion buffer was supplemented after baseline data acquisition with 100 nM of either ANP or mANP to assess the effect of both the wild-type and mutant hormones on MAP duration and the effective refractory period.

STATISTICAL ANALYSIS

We used a two-sided t-test for the comparison of data, assuming equal variance. Data are expressed as means (\pm SE); P values below 0.05 were considered to indicate statistical significance.

RESULTS

CASE SUBJECTS

We collected clinical data for 16 members of the family (Fig. 1 and Table 1). Eleven affected family members had received a diagnosis of familial atrial fibrillation at a mean age of 40 years; of these subjects, three had received the diagnosis during pregnancy. Transition from paroxysmal to chronic atrial fibrillation (in three of the subjects) or to arrest of atrial activation (in four of the subjects) suggested progressive electrical remodeling. Five subjects presented with tachycardia-induced cardiomyopathy, which improved or resolved with effective pharmacologic rate control.

Subsequent echocardiography ruled out cardiac hypertrophy and contractile dysfunction but showed dilatation of the left atrial chamber in seven subjects and of the left ventricular chamber in four subjects.

NPPA MUTATION

Genomewide linkage analyses ruled out known loci for atrial fibrillation and identified peak two-point lod scores at marker *DIS2667*, ranging from 2.32 (in the analysis of only affected subjects) to 3.56 (in the analysis of all subjects, assuming 100% mutation penetrance) at a recombination frequency of 0%. Fine mapping identified a disease-associated haplotype on chromosome 1p36-p35, a region spanning 24 Mb that was inherited by all affected subjects (peak multipoint lod scores, 2.66 for affected subjects only and 3.90 for all subjects, assuming 100% mutation penetrance) (Fig. 1). A recombination event within this interval in a 38-year-old asymptomatic man (Subject III-9), if it was assumed that he did not inherit the disease-associated mutation, further narrowed the critical region to 11 Mb.

We selected *NPPA* as a candidate gene because of its localization in the mapped interval, its expression in the atria of the heart, and its established role in cardiovascular physiology.^{11,12} We excluded seven other genes, including two — solute carrier family 9, member 1 (*SLC9A1*) and chloride intracellular channel 4 (*CLIC4*) — that have direct roles in ion regulation. Genomic DNA sequencing of *NPPA* identified a two-base-pair deletion (c.456-457delAA) in exon 3 that causes a frameshift, which abolishes the stop codon and extends the reading frame. Translation of the mutant gene would generate a fusion protein comprising the normal mature peptide containing 28 amino acids plus an anomalous carboxyl terminus of 12 residues (for details, see the Supplementary Appendix). Each of the 11 clinically affected family members was heterozygous for the mutation; the mutation was absent in the other 5 family members and in 560 control subjects.

MUTANT PEPTIDE

Radioimmunoassay showed that the mutant peptide was present in the plasma of heterozygotes in concentrations that were higher by a factor of 5 to 10 than the concentrations of wild-type ANP (Fig. 2). Anti-ANP antibody was found to be specific for wild-type ANP, since the aberrant carboxyl tail of mANP apparently prevented binding

Table 1. Phenotypic Data for Members of a Family with Autosomal Dominant Atrial Fibrillation.*

Pedigree No.	Years of Age		Heart Rhythm	Hypertension Requiring Treatment	Tachycardia-Induced Cardiomyopathy	Echocardiographic Measurements†				
	At Diagnosis	At Time of Study				LVH	LAE	LVDD	LVSD	LVEF‡
II-2	58	69	PAF, SB, JR	No	Yes	None	Moderate	54 (48)	36 (32)	60
II-4	42	67	PAF, SB, JR	Yes	No	None	None	49 (50)	28 (34)	65
II-5	51	66	PAF, SB, JR	Yes	Yes	None	Severe	66 (53)	40 (36)	50
III-1	43	44	PAF	Yes	No	None	None	Normal	Normal	60
III-2	45	41§	Chronic AF	Yes	Yes	None	None	54 (56)	34 (38)	65
III-3	38	43	PAF	No	No	None	Moderate	50 (54)	35 (36)	60
III-4	—	35	NSR	No	No	None	None	58 (56)	35 (40)	65
III-5	—	36	NSR	No	No	None	None	44 (56)	26 (40)	60
III-6	30	45	PAF	No	NA	NA	NA	NA	NA	NA
III-7	—	44	NSR¶	No	No	NA	NA	NA	NA	NA
III-8	37	38	PAF, SB, JR	No	Yes	None	Mild	64 (57)	40 (38)	55
III-9	—	38	NSR	No	No	NA	NA	NA	NA	NA
III-10	28	38	Chronic AF	No	Yes	None	Mild	56 (57)	39 (38)	50
III-11	—	38	NSR	No	No	None	None	40 (56)	28 (39)	60
III-12	35	36	Chronic AF	No	No	None	Mild to moderate	54 (53)	38 (36)	55
III-13	34	34	PAF	No	No	None	Mild	46 (53)	30 (36)	60

* AF denotes atrial fibrillation, JR junctional escape rhythm, LAE left atrial enlargement, LVDD left ventricular diastolic dimension, LVSD left ventricular systolic dimension, LVEF left ventricular ejection fraction, LVH left ventricular hypertrophy, NA not available, NSR normal sinus rhythm, PAF paroxysmal atrial fibrillation, SB sinus bradycardia, and ULN upper limit of the normal range for that patient in millimeters. Dashes denote not applicable.

† Echocardiographic measurements in affected subjects were performed during effective ventricular rate control when atrial fibrillation was present.

‡ The normal value is 50% or more.

§ Echocardiography was performed before the onset of atrial fibrillation.

¶ Normal sinus rhythm was not documented but was reported in the subject's medical history.

|| Subject III-8 had a ventricular pacemaker.

of this antibody. BNP levels were normal, which was consistent with an absence of overt ventricular disease.¹² To determine the integrative electrophysiological effects of circulating mANP on the heart, an isolated whole-heart model was studied. As compared with wild-type ANP, mANP caused significant shortening of the MAP duration and the effective refractory period (Fig. 3).

DISCUSSION

ANP is a circulating hormone that, through stimulation of the intracellular second messenger cGMP, plays a primary physiological role in the regulation of intravascular blood volume and vascular tone through natriuresis, diuresis, and vasodilatation.¹² Through cGMP signaling, ANP also modulates currents of sodium, calcium, and potassium channels in cardiac myocytes.¹³⁻¹⁵ Moreover, in atria of intact human hearts, ANP has been shown to shorten atrial conduction time and the effective refractory period, which provides a potential electrophysiological substrate for arrhythmia.¹⁶ ANP has also been shown to cause dose-dependent, autonomically mediated shortening of the atrial MAP duration and the effective refractory period in dogs.¹⁷ Thus, the suggestion that a mutation in *NPPA* could be responsible for the development of atrial fibrillation is consistent with some of the known aspects of ANP physiology.

To elucidate the mechanism by which a mutation in *NPPA* could lead to familial atrial fibrillation, we first showed that the mutation we identified results in the production of a mutant protein product. The concentration of circulating mANP was several times as high as that of wild-type ANP. One possible explanation for this difference in concentration is that mANP may have a prolonged half-life. Indeed, natriuretic peptides with a longer carboxyl terminus have increased resistance to degradation by neutral endopeptidase 24.11.¹⁸ In particular, *Dendroaspis augusticeps* natriuretic peptide (DNP), a unique natriuretic peptide isolated from snake venom, has a carboxyl-terminal extension of 15 amino acids and increased cGMP-stimulating potency.^{18,19} The phenotype that we observed in family members could thus be explained by high levels of circulating mANP with ANP-like activity. Such a mechanism would be consistent with previous experimental studies showing electrophysiological derangements on exposure of atrial myocytes to patho-

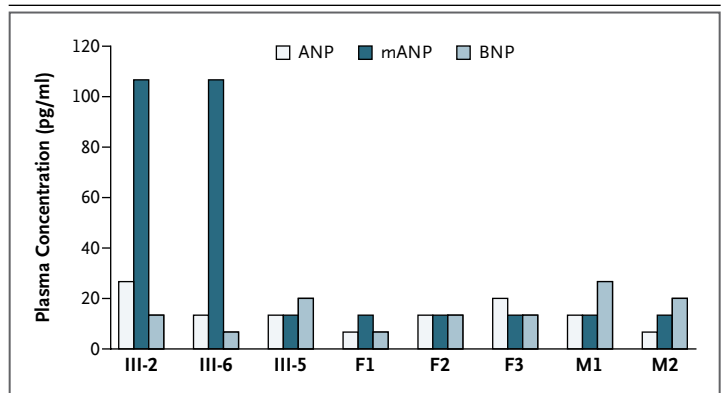
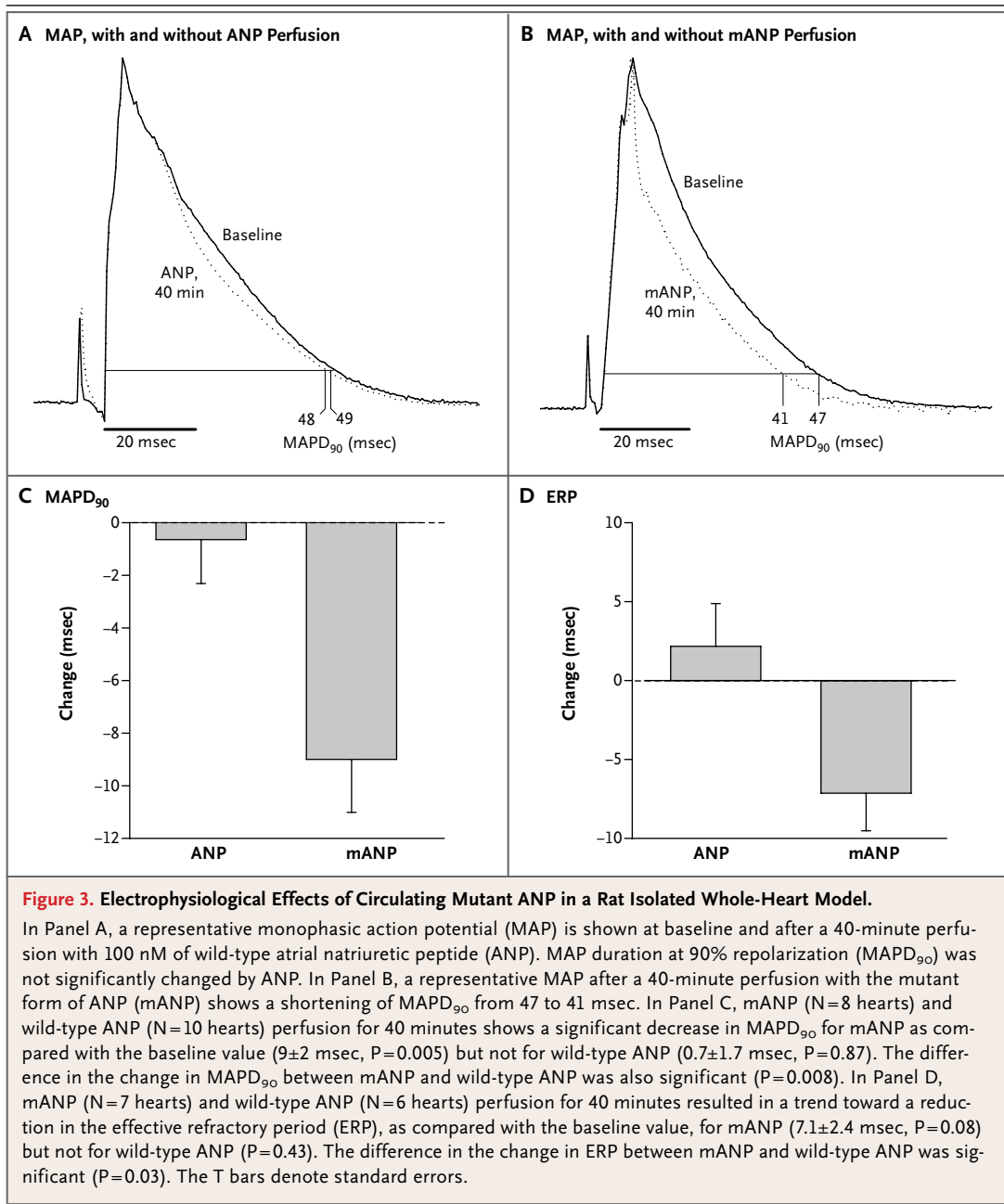


Figure 2. Radioimmunoassay Analysis Showing the Presence of Mutant ANP in Plasma from Heterozygotes for the *NPPA* Mutation.

A radioimmunoassay with polyclonal antibodies against wild-type atrial natriuretic peptide (ANP), mutant ANP (mANP), and B-type natriuretic peptide (BNP) shows levels of circulating mANP that are 5 to 10 times higher than the levels of ANP in two family members with the *NPPA* mutation (in Subject III-2 during chronic atrial fibrillation and in Subject III-6 during normal sinus rhythm). In the affected subjects, plasma ANP and BNP levels are normal. Low-level cross-reactivity of the polyclonal anti-mANP antibody was observed in samples from unaffected subject (Subject III-5) and in five control subjects (three female [F] and two male [M]).

physiological doses of ANP.¹⁴⁻¹⁷ Moreover, the mild structural remodeling, despite effective ventricular rate control, in several affected members of the family we studied is consistent with a proapoptotic effect on myocytes observed with excessive ANP-cGMP signaling.²⁰ However, regardless of potential dose-related and structural remodeling effects, our isolated (denervated) heart model showed a direct effect of mANP, but not wild-type ANP, on atrial electrophysiology. Although an additional novel function of the mANP fusion protein cannot be ruled out, the 12-residue carboxyl terminus has no strong sequence homology with known proteins. Atrial fibrillation developed in affected subjects over a period of several decades, as observed in patients with primary defects in ion channels and gap junctions,⁷⁻¹⁰ which suggests insidious but progressive electrical remodeling that conferred susceptibility to atrial arrhythmia.

The linkage analysis we performed has some limitations. Several subjects in the family who were classified as “unaffected” had not yet reached the mean age at which atrial fibrillation was diagnosed in other family members. If the analysis is based only on the family members who were known to be affected, the lod score was 2.66, which is below the threshold of 3.0 commonly accepted to confirm linkage. However, the fact that



the mutation in the gene encoding ANP segregates with known disease and the demonstration that mANP has electrophysiological effects that could confer a predisposition to atrial fibrillation strongly suggest that we have correctly identified the causative mutation.

In families with atrial fibrillation, investigators have identified mutations in ion channels that are predicted to either shorten or lengthen the duration of cardiac action potentials.²¹ Our

findings uncover a novel molecular genetic basis for abnormal repolarization and electrical instability in the cardiac atria and suggest the ANP-cGMP signaling pathway as a potential therapeutic target.^{22,23}

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Dr. Burnett reports serving on an advisory board at Nile Therapeutics and receiving lecture fees from Scios. No other potential conflict of interest relevant to this article was reported.

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