PLoS GENETICS

Neuropeptide Y Gene Polymorphisms Confer Risk of Early-Onset Atherosclerosis

Svati H. Shah[1,2]*, Neil J. Freedman[1], Lisheng Zhang[1], David R. Crosslin[2], David H. Stone[3], Carol Haynes[2], Jessica Johnson[2], Sarah Nelson[2], Liyong Wang[4], Jessica J. Connelly[2], Michael Muehlbauer[5], Geoffrey S. Ginsburg[1,6], David C. Crossman[7], Christopher J. H. Jones[8], Jeffery Vance[4], Michael H. Sketch, Jr.[1], Christopher B. Granger[1], Christopher B. Newgard[5], Simon G. Gregory[2], Pascal J. Goldschmidt-Clermont[4], William E. Kraus[1], Elizabeth R. Hauser[2]

1 Department of Medicine, Duke University Medical Center, Durham, North Carolina, United States of America, 2 Center for Human Genetics, Duke University Medical Center, Durham, North Carolina, United States of America, 3 Department of Surgery, Duke University, Durham, North Carolina, United States of America, 4 Miller School of Medicine, University of Miami, Miami, Florida, United States of America, 5 Sarah W. Stedman Center for Nutrition and Metabolism at Duke, Durham, North Carolina, United States of America, 6 Duke Institute for Genome Sciences and Policy, Durham, North Carolina, United States of America, 7 University of Sheffield School of Medicine, Sheffield, United Kingdom, 8 The Wales Heart Research Institute, Cardiff, United Kingdom

Abstract

Neuropeptide Y (*NPY*) is a strong candidate gene for coronary artery disease (CAD). We have previously identified genetic linkage to familial CAD in the genomic region of *NPY*. We performed follow-up genetic, biostatistical, and functional analysis of *NPY* in early-onset CAD. In familial CAD (GENECARD, N = 420 families), we found increased microsatellite linkage to chromosome 7p14 (OSA LOD = 4.2, p = 0.004) in 97 earliest age-of-onset families. Tagged *NPY* SNPs demonstrated linkage to CAD of a 6-SNP block (LOD = 1.58–2.72), family-based association of this block with CAD (p = 0.02), and stronger linkage to CAD in the earliest age-of-onset families. Association of this 6-SNP block with CAD was validated in: (a) 556 non-familial early-onset CAD cases and 256 controls (OR 1.46–1.65, p = 0.01–0.05), showing stronger association in youngest cases (OR 1.84–2.20, p = 0.0004–0.09); and (b) GENECARD probands versus non-familial controls (OR 1.79–2.06, p = 0.003–0.02). A promoter SNP (rs16147) within this 6-SNP block was associated with higher plasma NPY levels (p = 0.04). To assess a causal role of *NPY* in atherosclerosis, we applied the NPY1-receptor–antagonist BIBP-3226 adventitially to endothelium-denuded carotid arteries of apolipoprotein E-deficient mice; treatment reduced atherosclerotic neointimal area by 50% (p = 0.03). Thus, *NPY* variants associate with atherosclerosis in two independent datasets (with strong age-of-onset effects) and show allele-specific expression with NPY levels, while NPY receptor antagonism reduces atherosclerosis in mice. We conclude that *NPY* contributes to atherosclerosis pathogenesis.

Introduction

The prevalence of early-onset cardiovascular disease in Americans (under 40 years of age) is approximately 10–15% [1] and the hereditary nature of coronary artery disease (CAD) is well-established [2]. The relative risk of developing CAD in a first degree relative is 3.8 to 12.1, with higher risk correlating with earlier age-of-onset [3]. Recent successes suggest that CAD genes can be identified through comprehensive genetic and functional studies [4–6]. However, the genetic architecture of CAD remains complex and poorly understood.

To identify genetic risk factors in early-onset CAD, we implemented a strategy combining the strengths of family-based studies with validation by case-control association, in conjunction with careful consideration of phenotype and functional data. In our own GENECARD linkage study of early-onset CAD, we have found five genomic regions of linkage with multipoint linkage odds

(LOD) scores >1.0 [7]. The neuropeptide Y gene *(NPY)* is located adjacent to the peak microsatellite marker in the 7p14 peak. Because of its proximity to the peak marker, and because NPY has been implicated in disorders of vascular smooth muscle cell proliferation [8,9], we sought to examine *NPY* further as a candidate gene for early-onset CAD.

NPY is the most abundant peptide in the heart and brain, and is produced by sympathetic neurons, endothelial cells [10], and platelets [11]. NPY has diverse functions including roles in sympathetic nerve stimulation through co-release with norepinephrine; immune function [12]; regulation of food consumption [9]; and modulation of heart rate, vasoconstriction, coronary blood flow and ventricular function [13]. These cardiovascular functions are primarily mediated through the NPY1 receptor [12,14,15]. In injured rat carotid arteries, non-atherosclerotic neointimal hyperplasia is aggravated by local delivery of NPY, and ameliorated by treatment with NPY1 receptor antagonist [8,9]. In

Author Summary

Early-onset coronary artery disease (CAD) has a very strong genetic component as evidenced by the heritable nature of this disease. However, little is known about the actual genes underlying disease risk. Neuropeptide Y (NPY) is an abundant protein in humans that has been implicated in cardiovascular disease pathophysiology, but comprehensive evaluation of the gene coding for this protein has never been pursued in cardiovascular disease. Therefore, using gene-wide evaluation of variants within the *NPY* gene in a family-based as well as a non-familial study, we have shown that a cluster of six related *NPY* genetic variants is associated with early-onset CAD risk. We then show that one of these variants, which resides within the promoter region of this gene, is associated with higher NPY levels. Finally, to further support the functional role of this gene in CAD, we find that antagonism of the primary receptor of this gene results in marked attenuation of atherosclerosis in a mouse model. In conclusion, these findings demonstrate the role of the *NPY* gene in cardiovascular disease risk and add important additional information about the genetic architecture of this complex disease.

humans, NPY levels predict cardiovascular complications in end-stage renal disease [16], and NPY is implicated in congestive heart failure [17]. An *NPY* variant rare in most populations has been associated in Scandinavian populations with hyperlipidemia and carotid atherosclerosis [18,19], CAD in type 1 diabetics [20], and MI in hypertensive patients [21]; however, the effects of this variant on NPY expression remain obscure.

To date there have been no systematic studies of the role of the *NPY* gene, or of the functional consequences of genetic variation at this locus, in cardiovascular disease pathogenesis. In response to the results of the genome-wide linkage analyses, the phenotypic correlations, and the strong but limited prior published work, we proposed to test the hypothesis that *NPY* variants affect atherosclerosis through effects on NPY plasma levels. We pursued a comprehensive gene-wide approach to correlating *NPY* variants with CAD and plasma NPY levels in humans, and tested the effects of NPY1 receptor blockade on atherosclerosis in mice.

Results

Table 1 presents baseline characteristics of the three datasets: GENECARD (N = 946 affected, 37 unaffected individuals); CATHGEN (N = 556 cases, 256 controls); and GENECARD probands versus CATHGEN controls (N = 221 cases, 256 controls). Despite GENECARD families being selected on early age-of-onset, genetic heterogeneity manifest as differences in age-of-onset could still be present, as observed in the discovery of the *BRCA1* breast cancer gene [22]. Hence, we performed ordered subset analysis (OSA) to understand the effect of age-of-onset on linkage to CAD. We found increased linkage on the chromosome 7p14 peak in a subset of 97 families with the youngest age-of-onset (overall LOD = 1.04; subset LOD = 4.22; p = 0.004 for increase, Figure 1). The mean age-of-onset in these families was 37.8 years, and they had significantly higher mean total- and LDL-cholesterol and were more often male, compared with affected members of the remaining 323 families. No other genomic regions showed a correlation between linkage and age-of-onset. The *NPY* gene resides within this linkage peak and is a strong biological candidate. As a result we aimed to evaluate *NPY* polymorphisms, NPY levels, and age-of-onset of CAD, along with evaluating the role of *NPY* in a mouse model system.

Table 1. Baseline characteristics.

Variable	GENECARD Affected (N = 946)	GENECARD Probands (N = 221)	CATHGEN Cases (N = 556)	CATHGEN Very-Young Cases (N = 74)	CATHGEN Controls (N = 256)
Age	51.5 (7.1)	50.8 (6.6)	51.1 (7.9)	43.4 (8.1)	69.3 (6.5)
Age-of-onset	43.7 (5.8)	43.8 (5.7)	46.2 (6.4)	34.7 (3.3)	N/A
Sex (% male)	68.0%	68.0%	79.7%	83.8%	38.7%
Race					
% Caucasian	91.9%	83.1%	71.9%	68.9%	77.4%
% African- American	2.3%	7.4%	20.5%	21.9%	16.7%
Dyslipidemia	82.4%	83.7%	72.5%	81.2%	41.8%
Lipids					
TC	205.6 (57.4)	216.3 (59.0)	194.2 (55.8)	201.1 (71.6)	192.5 (50.3)
TG	221.6 (166.5)	224.7 (149.5)	225.7 (263.2)	281.2 (391.8)	163.4 (140.2)
HDL	38.2 (10.7)	35.5 (9.6)	39.8 (11.8)	38.5 (9.6)	51.7 (17.7)
LDL	117.9 (45.9)	131.6 (53.0)	113.5 (43.0)	115.2 (46.3)	106.3 (34.7)
Hypertension	55.2%	60.4%	68.5%	66.2%	65.2%
Diabetes	20.9%	25.6%	31.7%	27.0%	16.4%
BMI (SD)	29.7 (5.7)	30.6 (6.3)	30.8 (6.5)	31.9 (6.5)	28.8 (7.4)
Smoking	33.2%	26.5%	68.7%	75.7%	38.3%
History of MI	63.1%	61.7%	52.2%	52.7%	0%
Family history CAD	100%	100%	54.1%	70.3%	22.3%

*Continuous variables presented as mean (SD).
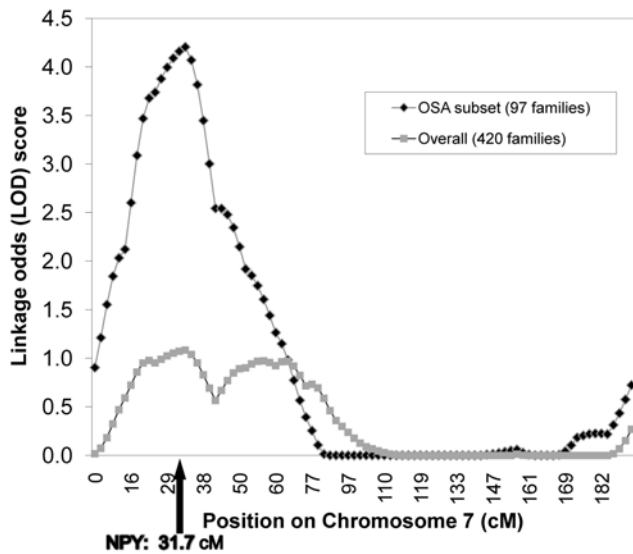doi:10.1371/journal.pgen.1000318.t001

Figure 1. Ordered subset analysis (OSA) using age-of-onset in GENECARD, chromosome 7. We constructed a CAD linkage map using the GENECARD microsatellite genome screen on chromosome 7. Results are depicted for all 420 families (grey), and for the subset generated by OSA (black), demonstrating increased LOD at the peak microsatellite from 1.04 (420 families) to 4.22 (97 lowest age-of-onset families, mean 37.8 years), p = 0.004 for increase in LOD.
doi:10.1371/journal.pgen.1000318.g001

NPY Variants Are Associated with Early-Onset CAD

Twenty-four SNPs were genotyped and were in Hardy-Weinberg equilibrium. Rs5571 was monomorphic and not analyzed further. Consistent with microsatellite results, nine *NPY* SNPs had LOD>1.0 in GENECARD, with higher LODs for several SNPs in the subset of 97 very-young-age-of-onset OSA families. Six of these linked SNPs also showed family-based association with CAD by PDT in GENECARD (Table 2). These SNPs are in varying degrees of LD (Figure 2). Association with CAD was validated in the non-familial dataset CATHGEN for five SNPs within the 6-SNP block; the sixth SNP (rs16147) demonstrated borderline significance (Table 3). CATHGEN results also validated our GENECARD findings for age-of-onset effects, with stronger association in the youngest age-of-onset CAD cases (<38 years of age, threshold defined by the GENECARD OSA subset). Although the CATHGEN sample size decreased using this age-of-onset threshold, SNPs within the 6-SNP block remained significantly associated to CAD (Table 3), with odds ratios higher than those obtained with the full dataset, and with several SNPs showing greater significance than the overall cohort (e.g., rs16119, allelic OR 2.20, p = 0.0004). These very-young-age-of-onset cases had a higher prevalence of family history of CAD (p = 0.003) and a trend for higher prevalence of dyslipidemia (p = 0.07).

All six key *NPY* SNPs were also associated with early-onset CAD in the comparison of GENECARD probands with CATHGEN controls (Table 4). Although we had adjusted for race in our regression models, to further address the potential for population stratification we performed analyses stratified by race. In these analyses, the association between NPY SNPs and early-onset CAD remained consistent and often more significant in self-reported Caucasians (Table S1), suggesting that the results are not confounded by population stratification from race. However, there was no association between *NPY* SNPs and CAD in non-Caucasians, most likely due to the lower power to detect such a

difference given the small sample size (N = 162 CATHGEN non-Caucasian CAD cases, 72 non-Caucasian controls). Haplotype analysis in the CATHGEN dataset showed association of several two-SNP haplotypes with CAD, recapitulating the LD structure of the individual SNPs, but not providing additional information. The haplotype most strongly associated was composed of rs5574 and rs16474 (p = 0.005).

Because rs16147 is a promoter SNP with established effects on expression of the gene [23–25], and all other key *NPY* SNPs are in LD with rs16147, we will focus the remainder of our results primarily on rs16147. However, as expected, findings were consistent across all six SNPs. As with the other five SNPs, there was consistency of the CAD-associated allele for rs16147 (G allele) and of its allele frequency across all datasets (CATHGEN cases 0.49; very-young CATHGEN cases 0.55; GENECARD probands 0.55; CATHGEN controls 0.43) (Table S2).

To test the possibility that associations between *NPY* SNPs with CAD are mediated through traditional CAD risk factors, we performed multivariable regression in our case-control datasets, using hypertension, dyslipidemia, diabetes, BMI, and smoking as covariates with *NPY* SNP genotype. In this analysis, the association of rs16147 with CAD remained significant in our comparison of GENECARD probands with CATHGEN controls (genotype OR 1.60 [95% CI 1.11–2.31], p = 0.01; allele OR 2.00 [1.11–3.59], p = 0.03), suggesting that *NPY* genotype contributes to CAD risk independently of traditional risk factors, at least in family-based cohorts. In the CATHGEN cohort, however, adjusting for risk factors attenuated association for rs16147 (genotype OR 1.15 [0.87–1.51], p = 0.34; allele OR 1.24 [0.82–1.88], p = 0.32), but left the association of rs16120 with CAD intact (allele model, OR 1.54 [1.01–2.35], p = 0.04). Race-stratified analyses in Caucasians also showed attenuation of association between *NPY* SNPs and early-onset CAD, although three of the SNPs remained significantly associated (Table S1).

To understand which CAD risk factor(s) most mediate association of *NPY* genotype with CAD, we performed a forward stepwise logistic regression, first fitting a model with genotype and then adding each risk factor. The greatest attenuation of association was caused by dyslipidemia, with minimal additional attenuation by other variables. Table S3 lists risk factors significant in the multivariable model for the SNP remaining significant after adjustment (rs16120), demonstrating the independent strength of effect of genotype, as well as those for intermediate risk factors, in a full fitted model. Neither BMI nor cholesterol levels varied significantly by *NPY* genotype (data not shown). Thus, it appears that *NPY* SNPs may contribute to atherosclerosis risk either independently of traditional risk factors, especially in high-risk families, or via mechanisms that may relate to dyslipidemia in non-familial CAD.

NPY Risk Alleles Are Associated with a Younger Age-of-Onset

An alternate way to examine the relationship between quantitative covariates and genetic variants is comparing means of traits by genotype (measured genotype analysis). We performed this analysis in CATHGEN to elucidate further the influence of *NPY* variants on age-of-onset. We found that the risk allele for rs16147 is indeed associated with a younger age-of-onset (mean age-of-onset: 45.8 (SD 6.5) in subjects with 1 or 2 copies of risk allele versus 47.3 (5.9) in subjects with 0 copies, p = 0.03). These results support our GENECARD findings and suggest *NPY* may affect the course of atherogenesis such that atherosclerosis manifests earlier in life.

Table 2. Linkage and family-based association of *NPY* SNPs with CAD (GENECARD).

SNP	Overall Two-Point LOD All Families (N = 420)		LOD OSA Very Young AOO Families (N = 97)		PDT	Geno-PDT
	Dom	Rec	Dom	Rec	P-value	P-value
RS7800861	0.25	0.09	0.43	0.35	0.49	0.50
RS16148	0.38	0.25	0.45	0.31	0.10	0.25
RS16147	**1.88**	**1.39**	**2.10**	**1.16**	**0.05**	**0.03**
RS16143	0.20	0.05	0.47	0.18	0.69	0.50
RS16478	0.16	0.17	0.27	0.21	0.41	0.29
RS16142	0.24	0.08	0.19	0.11	0.41	0.29
RS16141	**1.62**	**1.15**	**1.92**	**1.19**	0.12	0.17
RS16140	0.01	0.01	0.27	0.19	0.62	0.67
RS16139	0.03	0.01	0.03	0.02	1.00	1.00
RS5572	0.89	0.46	0.24	0.31	1.00	1.00
RS9785023	**1.87**	**1.17**	**2.13**	**1.03**	**0.04**	**0.05**
RS16138	0.19	0.06	0.41	0.20	1.00	0.39
RS1468271	0.09	0.26	0.16	0.68	0.78	0.78
RS5574	**2.13**	**1.26**	**2.73**	**1.37**	**0.02**	**0.05**
RS16132	0.40	0.39	**1.07**	0.92	0.17	0.17
RS16131	**1.42**	0.86	**1.09**	0.68	0.08	0.08
RS16475	0.10	0.29	0.70	0.76	0.16	0.16
RS16126	0	0	0	0	1.00	1.00
RS16474	**2.72**	**2.26**	**1.72**	**1.13**	**0.04**	**0.02**
RS16473	**1.91**	**1.62**	**1.56**	0.94	0.08	0.18
RS16120	**1.63**	**1.33**	**1.32**	0.73	**0.03**	**0.04**
RS16119	**1.58**	**1.14**	**1.62**	0.85	**0.04**	0.08
RS17374047	0.07	0.28	0.18	0.16	0.74	0.57

PDT: Pedigree-Disequilibrium-Test.
SNPs with PDT p-value≤0.05 in bold. SNPs listed in order of genomic location (3′→5′).
Dom: dominant; *Rec:* recessive.
doi:10.1371/journal.pgen.1000318.t002

Allele-Specific Effects on NPY Levels

To examine allele-specific effects, we assessed the relationship of *NPY* variants with peripheral NPY levels in 220 subjects randomly selected from the CATHGEN case/control dataset. We found that rs16147 was associated with higher NPY levels for the minor (risk, G) allele compared with the major (A) allele (46.3 vs. 41.0 pmol/L, p = 0.04, Figure 3). Concordantly, NPY levels were higher in CAD cases compared with controls (46.8 vs. 41.1 pmol/L, p = 0.02).

Antagonism of NPY Reduces Atherosclerosis

To determine whether NPY-evoked signaling contributes to atherosclerosis, we attenuated the vascular effects of NPY [26] with an NPY1 receptor antagonist that does not cross the blood-brain barrier: BIBP 3226 (BIBP) [8,26,27], which has been shown to reduce non-atherosclerotic neointimal hyperplasia in rats [8,26]. In order to accelerate typical atherosclerosis in a focal manner, we employed carotid endothelial denudation in $apoe^{-/-}$ mice, as reported by other groups [28,29]. With this approach, we could apply BIBP just focally to the carotid artery in a peri-arterial Pluronic gel. Atherosclerosis developed typically in this carotid model, with abundant evidence of macrophage foam cells, SMC-like cells constituting fibrous caps of complex intimal lesions, and extracellular cholesteryl ester (Figure 4A–4C). Peri-carotid appli-

cation of Pluronic gel by itself had no effect on extent of atherosclerosis (data not shown), and BIBP in the gel did not engender cell toxicity, as judged by apoptosis: cleaved caspase-3 levels were $70\pm20\%$ higher in control than in BIBP-treated carotids (p<0.02, Figure 4D–4E). While control specimens demonstrated greater apoptosis than BIBP-treated specimens, they also demonstrated 1.7 ± 0.3-fold greater cell proliferation, as judged by immunofluorescence for proliferating cell nuclear antigen (PCNA, Figure 4F, 4G). However, while BIBP did not affect the prevalence of macrophages, SMCs or collagen in atherosclerotic plaques, it reduced total plaque cell number, collagen content and plaque area by 56% (p<0.03, Figure 5). Thus, it appears that NPY, through the NPY1 receptor and perhaps other NPY receptors, contributes to atherogenesis.

Discussion

Our data provide the first evidence that *NPY* gene variants associate with CAD in humans, particularly those with early-onset CAD, and that NPY contributes to atherosclerosis in mice through its NPY1 receptor. Our results supporting the role of *NPY* in CAD pathogenesis emerge from multiple lines of evidence, including linkage, family-based and case-control association studies in multiple cohorts, as well as allele-specific differential NPY levels.
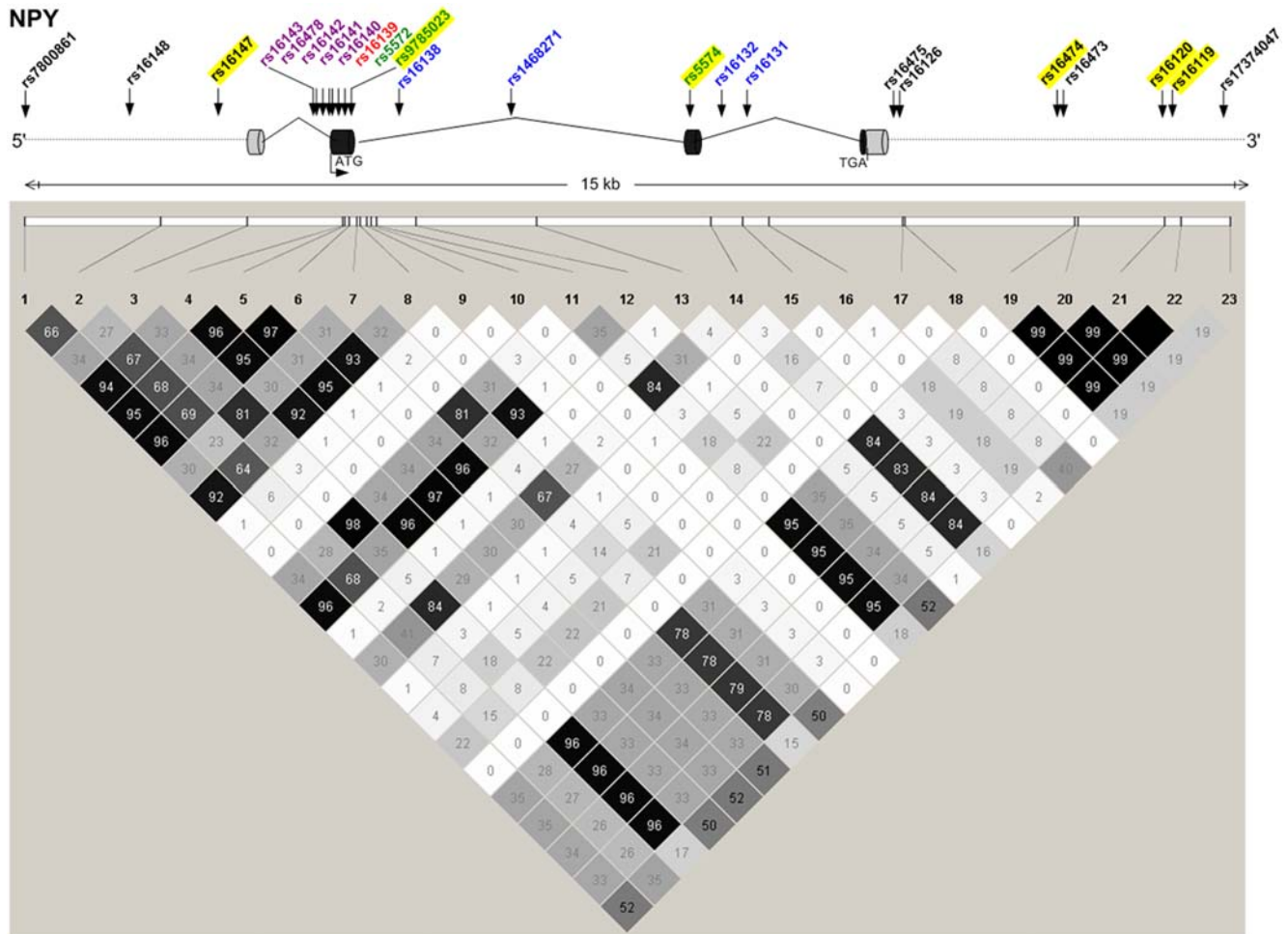
Figure 2. *NPY* structure, genotyped SNPs, and LD between SNPs. *NPY* gene structure is displayed diagrammatically (top), with exons symbolized as cylinders, and sites of translation initiation (ATG) and termination (TGA) indicated. Genotyped SNPs are noted with arrows and color-coded: coding-nonsynonymous (red); coding-synonymous (green); intronic (blue); mRNA-untranslated region (lavender); 5′ and 3′ gene-flanking region (black). The bottom panel constitutes a LD graphic with R^2 values ($n \times 10^2$) between SNPs; darker squares signify higher degrees of LD. SNPs genotyped as part of this study are highlighted in yellow.
doi:10.1371/journal.pgen.1000318.g002

Because we observed increased linkage and association between *NPY* SNPs and CAD in the very youngest age-of-onset cases, NPY may make a particularly appealing therapeutic target for CAD prevention in families with early-onset disease. Together with our mouse data, our results support the following scenario by which *NPY* variants promote atherosclerosis: presence of the rs16147 risk

Table 3. Association of *NPY* SNPs with early-onset CAD in CATHGEN, adjusted for race and sex.

SNP	CATHGEN Cases				CATHGEN Very-young AOO Cases			
	Genotype		Allele		Genotype		Allele	
	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
RS16147	1.25 (0.97–1.62)	0.08	1.33 (0.91–1.94)	0.15	1.55 (1.01–2.38)	0.05	1.84 (0.91–3.72)	0.09
RS9785023	1.27 (0.99–1.63)	0.06	1.46 (1.00–2.12)	0.05	1.59 (1.04–2.43)	0.03	1.98 (0.99–3.99)	0.06
RS5574	1.29 (1.01–1.66)	0.04	1.48 (1.04–2.12)	0.03	1.51 (0.99–2.30)	0.06	2.01 (1.03–3.92)	0.04
RS16474	1.32 (1.03–1.70)	0.03	1.57 (1.08–2.29)	0.02	1.62 (1.06–2.47)	0.03	1.93 (0.96–3.89)	0.07
RS16120	1.34 (1.05–1.73)	0.02	1.65 (1.13–2.40)	0.01	1.57 (1.03–2.40)	0.04	2.07 (1.01–4.23)	0.05
RS16119	1.32 (1.02–1.70)	0.03	1.57 (1.07–2.31)	0.02	1.59 (1.07–2.35)	0.02	2.20 (1.43–3.38)	0.0004

AOO: age of onset; OR: Odds Ratio; 95% CI: 95% Confidence Interval; p: p-value. All case groups compared with CATHGEN controls.
doi:10.1371/journal.pgen.1000318.t003

Table 4. Association of NPY SNPs with early-onset CAD, GENECARD probands compared with CATHGEN controls, adjusted for race and sex.

SNP	GENECARD Probands			
	Genotype		Allele	
	OR (95% CI)	p-value	OR (95%CI)	p-value
RS16147	1.55 (1.15–2.09)	0.004	1.79 (1.12–2.88)	0.016
RS9785023	1.54 (1.15–2.06)	0.004	1.88 (1.18–3.00)	0.008
RS5574	1.57 (1.17–2.11)	0.003	1.89 (1.21–2.96)	0.005
RS16474	1.63 (1.21–2.19)	0.001	2.06 (1.28–3.31)	0.003
RS16120	1.58 (1.18–2.12)	0.002	2.02 (1.25–3.24)	0.004
RS16119	1.56 (1.15–2.11)	0.004	1.98 (1.21–3.24)	0.007

OR: Odds Ratio; 95% CI: 95% Confidence Interval.
doi:10.1371/journal.pgen.1000318.t004

allele leads to increased plasma NPY levels, which, acting on NPY1 (and perhaps other) receptors, promotes arterial smooth muscle cell proliferation [30], thereby promoting atherogenesis.

To our knowledge, ours is the first study to take a gene-wide approach to *NPY* in cardiovascular disease. Furthermore, the *NPY* variant rs16147 has never been reported for any cardiovascular phenotypes. Because rs16147 is not in LD with upstream SNPs, it appears that the LD block tagged by rs16147 does not extend beyond the gene (www.hapmap.org). This inference is supported by our own data: the upstream rs7800861 is not in LD with rs16147 or other key SNPs.

The allele-specific effects of the *NPY* promoter SNP rs16147 (A/G) on *NPY* expression have been documented in three previous
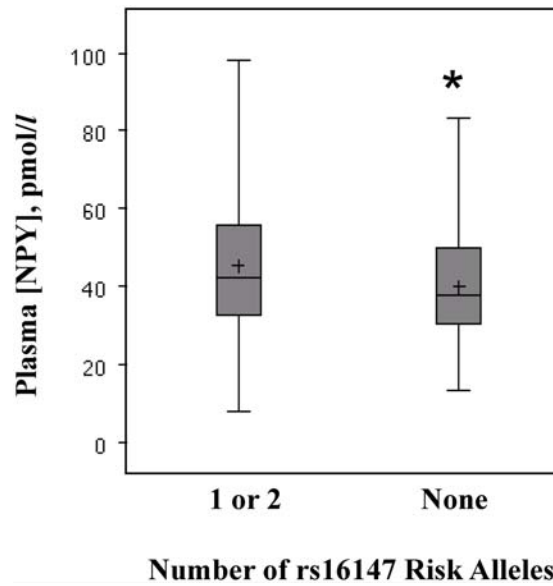

Figure 3. Rs16147 is associated with higher plasma NPY levels. NPY levels were measured in fasting plasma (N = 220). Box-and-whisker plots are presented displaying mean (+), median, and 25–75th interquartile range (shaded boxes), according to number of risk alleles at rs16147. *P = 0.04 for comparison of 1 or 2 vs. 0 risk alleles.
doi:10.1371/journal.pgen.1000318.g003

studies [23–25]. In accord with our work, two of these studies have observed that the rs16147 G allele (reverse strand C allele) increases *NPY* expression [23,24]. Furthermore, a putative SP1 transcription factor binding site within the rs16147 stretch of *NPY* sequence is lost with the rs16147 A allele [24]; consequently, we
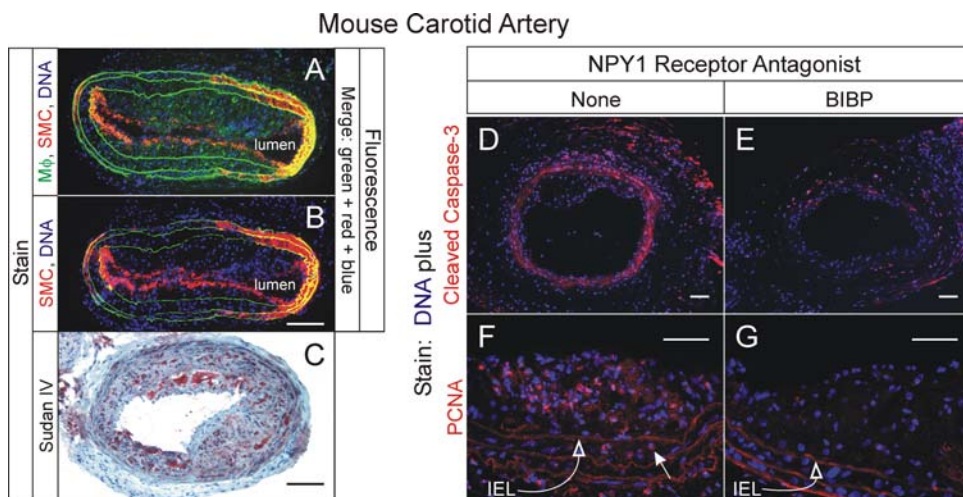

Figure 4. Mouse carotid atherogenesis: cellular effects of a NPY Y1 receptor antagonist. Immediately after wire-mediated endothelial denudation, carotid arteries of *apoe*$^{-/-}$ mice were encased in Pluronic gel lacking ("None," "control") or containing the NPY1 receptor-selective antagonist BIBP 3226 ("BIBP"). Carotid arteries were harvested 6 wk (A–C) or 2 wk (D–G) postoperatively, and processed as described in Methods. A, B, Typical atherosclerosis in a representative control-treated carotid artery. Frozen sections were stained for DNA and (immunofluorescently) with IgG specific for SMC α-actin and either the macrophage marker Mac3 (A), or an isotype negative control rat IgG (B). Green autofluorescence of the elastic laminae can be seen in both A and B. Alternatively, frozen sections were stained for cholesteryl ester with Sudan IV and hematoxylin counterstain (C). Scale bar = 100 μm. D, E, Carotid sections were stained with IgG for cleaved caspase-3 (apoptosis marker) and counterstained for DNA; specimens stained with non-immune rabbit IgG yielded no red immunofluorescence (not shown). Scale bar = 50 μm. F, G, Carotid sections were stained with IgG for proliferating cell nuclear antigen (PCNA), counterstained for DNA, and oriented with the lumen side upward. The closed arrow designates a single PCNA-positive nucleus in the tunica media, representative of >20 such nuclei in the field shown; specimens stained with non-immune rabbit IgG (not shown) yielded only elastic lamina autofluorescence, like that observed in panels F and G. IEL, internal elastic lamina, indicated by the open arrows. Scale bar = 50 μm. All images are representative of ≥4 independent specimens stained with each modality.
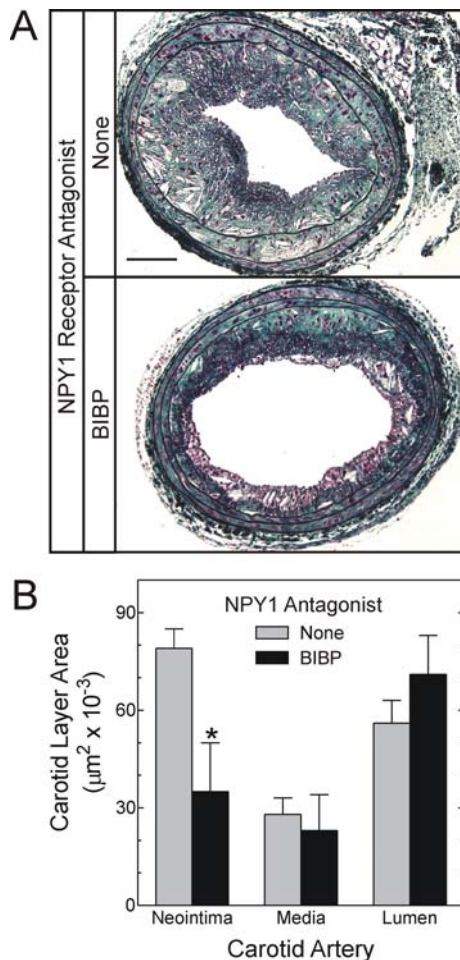doi:10.1371/journal.pgen.1000318.g004

Figure 5. Reduction of atherosclerosis by antagonism of the NPY Y1 receptor. Mice treated identically to those in Figure 4 underwent carotid harvest 6 weeks after endothelial denudation, as described in Methods. A, Carotid sections from mice subjected to the indicated treatment were stained with a modified connective tissue stain (green = collagen, black = elastin, red = cytoplasm) [39]. Sections shown are representative of 3 obtained from each of 5 mice in each treatment group. Scale bar = 100 μm. B, Computerized morphometry was used to quantitate the indicated carotid artery dimension, as described in Methods; the means±S.E. of 5 independent carotid arteries from each treatment group are shown. Compared with control: p<0.03.
doi:10.1371/journal.pgen.1000318.g005

would expect the A allele to demonstrate lower *NPY* expression. In contrast, Zhou et al recently studied NPY expression in post-mortem brain and virally transformed lymphoblastoid cells and found that the rs16147 A allele results in higher, rather than lower expression of NPY [25]. Zhou et al. also found that haplotypes reporting on the rs16147 A allele result in higher NPY plasma levels [25]. However, it is important to note that Zhou et al. collected plasma from patients under resting conditions, whereas we collected plasma from patients under arguably stressful conditions, immediately before they underwent coronary angiography. Furthermore, differences in cell and tissue types examined in these studies may underlie the discordant results obtained for NPY expression. Given previous studies showing that NPY levels are higher in cardiovascular disease [16,31] (findings that were corroborated in our study), and the strong concordance of our results showing a higher frequency of the rs16147 G allele in CAD cases, it follows that the G allele would result in increased NPY

expression, as observed in the previous NPY studies [23,24] and in our study. Taken together, all of these studies nevertheless imply a functional role for rs16147.

One prior linkage scan has implicated chromosome 7p in atherogenesis, showing modest linkage to carotid plaque (LOD 0.60–0.78, p = 0.03–0.05) [32]. A recently published study has also shown linkage on chromosome 7p15 (LOD 3.3) to pulse pressure, a measure of central arterial stiffness and a predictor of cardiovascular mortality [33]. None of the genomewide association studies (GWAS) of atherosclerosis have published association with variants on chromosome 7p. However, we reviewed individual SNPs through the Framingham SHARe GWAS database (dbGAP, http://www.ncbi.nlm.nih.gov/projects/gap) and found association of several flanking *NPY* SNPs with cardiovascular phenotypes (Text S1, p = 0.04–0.0005). These results provide further support for a role for *NPY* in human atherosclerosis.

Although previously associated with atherosclerosis primarily in Scandinavian subjects [18–20], the *NPY* SNP rs16139 was not associated with CAD in our sample. Our inability to replicate association for rs16139 may have resulted from the low minor allele frequency of rs16139 in our cohorts (similar to other non-Scandinavian populations [34]), and hence the small power of our studies for rs16139. Alternatively, the relationship between rs16139 and atherosclerosis in Scandinavians, which is as yet mechanistically obscure, may not pertain to our genetically more diverse cohorts. Concomitantly, we note that rs16147 is very common, and despite a modest relative risk conferred, this variant may have a higher population attributable risk in genetically diverse cohorts. In fact, 21% of our CATHGEN subjects are homozygous for the rs16147 risk allele. This prevalence is similar in magnitude to that observed with the recently identified chromosome 9p21 CAD susceptibility variant discovered through GWAS [5].

We cannot exclude the possibility that our CATHGEN data are affected by population stratification; however we adjusted for race and CATHGEN subjects were recruited from only one site. Furthermore, race-stratified analyses showed consistent, and often more significant, association between NPY SNPs and CAD in Caucasians, though our analyses were underpowered to assess such relationships in non-Caucasians. Importantly, there is no evidence of population stratification in previous GENECARD analyses [7] and, unlike the non-familial case-control regression analyses which are sensitive to population stratification, the family-based association analyses are robust to population stratification.

The allele frequencies of the *NPY* SNPs were remarkably similar in very-young-age-of-onset CATHGEN cases and GENECARD probands. Consequently, these independent cohorts appear to be genetically as well as clinically similar, in that they demonstrate a strong genetic component to their risk profiles. These very young individuals represent a prime target for prevention. In identifying the association between *NPY* variants and atherosclerosis, our results highlight the importance of pursuing a comprehensive gene-wide SNP survey. The replication of our GENECARD findings in our CATHGEN validation dataset is very consistent, not just for the *NPY* gene itself but also for the association of the individual associated alleles of each SNP, suggesting it is extremely unlikely that results are due to chance alone. It remains to be determined whether association between *NPY* and CAD is influenced by traditional CAD risk factors, particularly dyslipidemia, and whether a particular constellation of risk factors along with *NPY* SNPs is related to the very early-onset CAD associations observed in both GENECARD and CATHGEN. A more

complete understanding of the relationship between *NPY*, CAD risk factors, and CAD will require additional studies.

In our study, the correlation between SNPs in the form of LD makes a Bonferroni correction for multiple comparisons too conservative. Using a method that corrects for LD [35] resulted in 11 minimally redundant SNPs (corrected p<0.0047). Thus, one SNP for our CATHGEN very-young-age-of-onset group (rs16119, p = 0.0004) and most SNPs for the GENECARD proband comparison group would remain significant. More importantly, we have used additional statistical and functional studies to demonstrate that association of *NPY* SNPs with CAD does not result from chance alone: the convergence of evidence from linkage and association, as well as replication of the same SNP/CAD association in independent cohorts. The possibility that NPY contributes to atherosclerosis is supported by the correlation of plasma NPY levels with *NPY* SNP risk alleles, and the inhibition of murine atherosclerosis with an antagonist of the NPY1 receptor, which mediates most cardiovascular effects of NPY [8].

In summary, *NPY* is a strong candidate gene for early-onset CAD. *NPY* SNPs may help refine CAD risk estimates and target therapies for young members of families with CAD.

Methods

Study Sample and Phenotype Description

The GENECARD study enrolled 920 families with early-onset CAD (age-of-onset before 51 years for men, 56 years for women) to perform affected-sibling-pair linkage [7]. GENECARD families were recruited based on the presence of at least two siblings with early-onset CAD defined by: stress testing with ischemia; myocardial infarction (MI); coronary revascularization; or angiography with ≥50% stenosis in one major vessel. Unaffected family members had no clinical evidence of CAD and age >55 years for men (>60 years for women). The 420 families from the initial linkage scan [7] are included in this report, including a limited number of unaffected family members (N = 37).

An independent non-familial validation cohort was selected from the CATHGEN biorepository, consisting of subjects recruited sequentially through the cardiac catheterization laboratories at Duke University Medical Center (Durham, NC). Fasting whole blood and plasma samples were obtained from the femoral artery during cardiac catheterization and frozen prior to use. CAD cases were defined as CAD-index≥32 (at least one vessel with ≥95% stenosis) with age-of-onset <56 years. CADi is a numerical summary of angiographic data directly related to outcome [36]. Age-of-onset was defined as age at first MI, coronary revascularization, or catheterization meeting CADi threshold. Controls were defined as age-at-catheterization >60 years; CAD-index≤23, no history of cardiovascular disease, and no clinically significant CAD. Dyslipidemia was defined as a previous diagnosis and/or treatment of hypercholesterolemia (yes/no), confirmed by review of medical records. We also constructed a third case-control group comprising GENECARD probands included in the original linkage scan from United States sites (N = 221) and CATHGEN controls. Institutional Review Boards approved all study protocols. Informed consent was obtained.

Genotyping and Plasma NPY Levels

Tagged NPY SNPs were identified using the SNPSelector program, which employs a linkage disequilibrium (LD) tagging algorithm that prioritizes functional SNPs [37], and were then genotyped using either Taqman or Illumina BeadArray (www.illumina.com). The 7900HT Taqman SNP genotyping system incorporates a standard PCR-based, dual fluor, allelic discrimina-

tion assay with a dual laser scanner. Assays were purchased through Applied Biosystems (www.appliedbiosystems.com). QC samples, composed of 12 reference controls, were included in each quadrant of the plate. Illumina BeadStation genotyping was performed using the 500G system. All SNPs examined were successfully genotyped for ≥95% of the individuals in the study. Error rate estimates for SNPs meeting QC benchmarks were <0.2%.

Plasma NPY levels were measured by an NPY-specific radioimmunoassay (Alpco Diagnostics, Salem NH) on 220 CATHGEN subjects, randomly selected (regardless of case/control status) from all subjects included in the genotyping studies. NPY levels were measured in fasting unextracted plasma collected at time of cardiac catheterization (prior to administration of supplementary anticoagulants if given), which was subsequently frozen and stored at −80°C, and analyzed in a single RIA run. Cross-reaction of the NPY RIA assay as reported by the company is: human NPY 100%; human PYY <2.0%; human pancreatic polypeptide (PP) <1.0%; NPY 1–21 <0.1%; NPY 20–36 <0.4%. The assay was characterized as a mean recovery of 82% (range 75–88%) for NPY-spiked plasma. Precision was characterized by an intra-assay CV of 2.6–3.9% in our samples, which were run in one batch. NPY levels were approximately normally distributed.

Mouse Atherosclerosis Studies

All animal experiments complied with institutional guidelines. Gender-matched apolipoprotein E-deficient (*apoe*$^{-/-}$) C57BL/6 mice were used, at the age of 10±2 weeks. To accelerate atherosclerosis, we performed endothelial denudation of the left common carotid artery with a 0.36-mm flexible angioplasty guidewire (Johnson and Johnson), inserted via the external carotid artery as described [28,38]. After ligating the external carotid artery, we encased the common carotid in 150 μl of 22.5% (w/v) Pluronic gel, containing either 1% (v/v) water that lacked (control) or contained the NPY1 receptor antagonist BIBP 3226 [27] (21 μmol/L, [final]; Sigma-RBI, Inc.). The skin was closed after the Pluronic gelled (virtually instantaneously). Postoperatively, mice were fed a Western diet (Harlan Teklad #88137) ad libitum for two-six weeks (until arterial harvest), and there were no differences in weight between mice treated with vehicle or BIBP 3226. BIBP 3226 does not affect blood pressure in rodents [26]. As we previously described [39], arteries were perfusion/fixed and embedded in paraffin, or embedded in OCT and frozen. Five-μm sections were stained with a modified connective tissue stain (paraffin sections), Sudan IV (frozen sections, for cholesteryl ester), or immunostained (frozen sections) as previously described [38,39]: for macrophages (FITC-conjugated rat IgG$_1$κ anti-mouse Mac3 or negative control FITC-conjugated rat IgG$_1$κ (Pharmingen, Inc.)); smooth muscle cells (SMCs) (Cy3-conjugated 1A4 IgG targeting SMC α-actin (Sigma)); for apoptotic cells (monoclonal rabbit IgG targeting cleaved (activated) caspase-3 (Cell Signaling, Inc.)); or for proliferating cell nuclear antigen (Santa Cruz Biotechnology, Inc). All sections were digitally photographed with fixed light settings for all immunofluorescence specimens within a single staining batch, to facilitate quantitation [39]. Atherosclerosis, cellular (or protein antigen) prevalence, and PCNA-positive nuclear prevalence [38] were quantified with Scion Image (www.scioncorp.com) or by manual counting by an observer blinded to specimen identity [39], from three cross sections obtained from the distal, middle, and proximal portions of each specimen, as described [39]. Data from these locations were averaged for each carotid specimen.

Statistical Analysis

Ordered subset analysis (OSA) examines the impact of covariates by defining homogeneous subsets of families for linkage

[40], and has been successfully used for gene mapping in complex diseases [41–44]. Similar methods were used to identify the *BRCA1* breast cancer susceptibility gene, where evidence for linkage was only present in earlier-onset families [22,45]. Subsets of families with earlier ages-of-onset may help evaluate genetic heterogeneity and define subgroups with a stronger genetic effect. *A priori* specification of the age-of-onset threshold is unnecessary, as OSA uses maximal linkage evidence to define subsets. OSA was conducted using nonparametric multipoint family-specific LODs from microsatellite genotypes from the original screen as input [7], with age-of-onset as a covariate. A permutation procedure provides empirical p-values for the significance of the increase in the maximum-subset-LOD from the overall LOD. To assess SNP linkage, two-point LODs were calculated using Merlin [46]. The Pedigree Disequilibrium Test (PDT) and Genotype-PDT were used for family-based association. PDT, an extension of the transmission-disequilibrium-test (TDT), allows incorporation of extended pedigrees and is valid even with population substructure [47]. Power calculations (QUANTO:http://hydra.usc.edu/) showed power $\geq$0.80 to detect effect sizes of $\geq$2.45 for the lowest allele frequency SNP (0.02, rs16139), and effect sizes $\geq$1.35 for the highest allele frequency SNP (0.49, rs16147).

In CATHGEN, association was assessed using logistic regression models adjusting for race and sex; and for race, sex, hypertension, diabetes, dyslipidemia, smoking and body-mass-index (BMI) (multivariable model). Measured genotype analysis using generalized linear models was performed to compare differences in means of quantitative traits (NPY levels, age-of-onset) by *NPY* genotype. Baseline differences were assessed using a chi-square or *t*-test. The Graphical Overview of Linkage Disequilibrium (GOLD) program [48] was used to assess LD. Haplotype analysis used HaploStats 1.1.0 (Mayo Clinic, Rochester, MN). We report p-values uncorrected for multiple comparisons, but also present results in the context of correction for LD between SNPs [35]. The extent of atherosclerosis was compared between control and NPY1 receptor antagonist groups in mice with one-way ANOVA and Tukey's post-hoc test for multiple comparisons. SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis.

Supporting Information

Table S1 Race-stratified analyses: association of NPY SNPs with early-onset CAD in CATHGEN Caucasians.
Found at: doi:10.1371/journal.pgen.1000318.s001 (0.04 MB DOC)

Table S2 Minor allele frequencies and replication of associated allele for six *NPY* SNPs associated with CAD.
Found at: doi:10.1371/journal.pgen.1000318.s002 (0.03 MB DOC)

Table S3 CAD risk factors in multivariable regression model for rs16120, CATHGEN Cases vs. Controls.
Found at: doi:10.1371/journal.pgen.1000318.s003 (0.03 MB DOC)

Text S1 Generalizability of *NPY* Genetic Variant Association with Cardiovascular Phenotypes: Results from Framingham SHARe database.
Found at: doi:10.1371/journal.pgen.1000318.s004 (0.03 MB DOC)

Acknowledgements

Author Contributions

Conceived and designed the experiments: SHS NJF LZ DHS LW JJC GSG DCC CJHJ JV CBG SGG PJGC WEK ERH. Performed the experiments: SHS NJF LZ DRC DHS CH JJ SN LW MM CBN. Analyzed the data: SHS NJF LZ DRC CH JJ SN LW JJC MM SGG ERH. Contributed reagents/materials/analysis tools: SHS NJF MM DCC MHSJ. Wrote the paper: SHS NJF DRC DHS CH SN LW JJC GSG CBG CBN PJGC WEK ERH.

References

1. American Heart Association (2006) Heart and Stroke Statistical Update. http://www.americanheart.org/statistics.
2. Marenberg ME, Risch N, Berkman LF, Floderus B, De Faire U (1994) Genetic susceptibility to death from coronary heart disease in a study of twins. N Engl J Med 330: 1041–1046.
3. Rissanen AM (1979) Familial occurrence of coronary artery diease: effect of age at diagnosis. Am J Cardiol 44: 60–66.
4. Connelly JJ, Wang T, Cox JE, Haynes C, Wang L, et al. (2006) GATA2 Is Associated with Familial Early-Onset Coronary Artery Disease. PLoS Genet 2.
5. Helgadottir A, Thorleifsson G, Manolescu A, Gretarsdottir S, Blondal T, et al. (2007) A common variant on chromosome 9p21 affects the risk of myocardial infarction. Science 316: 1491–1493.
6. Wang L, Hauser ER, Shah SH, Pericak-Vance MA, Haynes C, et al. (2007) Peakwide mapping on chromosome 3q13 identifies the kalirin gene as a novel candidate gene for coronary artery disease. Am J Hum Genet 80: 650–663.
7. Hauser ER, Crossman DC, Granger CB, Haines JL, Jones CJ, et al. (2004) A genomewide scan for early-onset coronary artery disease in 438 families: the GENECARD Study. Am J Hum Genet 75: 436–447.
8. Li L, Lee EW, Ji H, Zukowska Z (2003) Neuropeptide Y-induced acceleration of postangioplasty occlusion of rat carotid artery. Arterioscler Thromb Vasc Biol 23: 1204–1210.
9. Lin S, Boey D, Herzog H (2004) NPY and Y receptors: lessons from transgenic and knockout models. Neuropeptides 38: 189–200.
10. Zukowska-Grojec Z, Karwatowska-Prokopczuk E, Rose W, Rone J, Movafagh S, et al. (1998) Neuropeptide Y: a novel angiogenic factor from the sympathetic nerves and endothelium. Circ Res 83: 187–195.
11. Myers AK, Farhat MY, Vaz CA, Keiser HR, Zukowska-Grojec Z (1988) Release of immunoreactive-neuropeptide by rat platelets. Biochem Biophys Res Commun 155: 118–122.
12. Wheway J, Mackay CR, Newton RA, Sainsbury A, Boey D, et al. (2005) A fundamental bimodal role for neuropeptide Y1 receptor in the immune system. J Exp Med 202: 1527–1538.
13. Pedrazzini T, Brunner HR, Waeber B (1993) Neuropeptide Y and cardiovascular regulation. Curr Opin Nephrol Hypertens 2: 106–113.
14. Pedrazzini T, Seydoux J, Kunstner P, Aubert JF, Grouzmann E, et al. (1998) Cardiovascular response, feeding behavior and locomotor activity in mice lacking the NPY Y1 receptor. Nat Med 4: 722–726.
15. Pedrazzini T (2004) Importance of NPY Y1 receptor-mediated pathways: assessment using NPY Y1 receptor knockouts. Neuropeptides 38: 267–275.
16. Zoccali C, Mallamaci F, Tripepi G, Benedetto FA, Parlongo S, et al. (2003) Prospective study of neuropeptide y as an adverse cardiovascular risk factor in end-stage renal disease. J Am Soc Nephrol 14: 2611–2617.
17. Feng QP, Hedner T, Andersson B, Lundberg JM, Waagstein F (1994) Cardiac neuropeptide Y and noradrenaline balance in patients with congestive heart failure. Br Heart J 71: 261–267.
18. Karvonen MK, Pesonen U, Koulu M, Niskanen L, Laakso M, et al. (1998) Association of a leucine(7)-to-proline(7) polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels. Nat Med 4: 1434–1437.
19. Karvonen MK, Valkonen VP, Lakka TA, Salonen R, Koulu M, et al. (2001) Leucine7 to proline7 polymorphism in the prepronueuropeptide Y is associated with the progression of carotid atherosclerosis, blood pressure and serum lipids in Finnish men. Atherosclerosis 159: 145–151.
20. Pettersson-Fernholm K, Karvonen MK, Kallio J, Forsblom CM, Koulu M, et al. (2004) Leucine 7 to proline 7 polymorphism in the prepronueuropeptide Y is associated with proteinuria, coronary heart disease, and glycemic control in type 1 diabetic patients. Diabetes Care 27: 503–509.
21. Wallerstedt SM, Skrtic S, Eriksson AL, Ohlsson C, Hedner T (2004) Association analysis of the polymorphism T1128C in the signal peptide of neuropeptide Y in a Swedish hypertensive population. J Hypertens 22: 1277–1281.
22. Hall JM, Lee MK, Newman B, Morrow JE, Anderson LA, et al. (1990) Linkage of early-onset familial breast cancer to chromosome 17q21. Science 250: 1684–1689.

23. Buckland PR, Hoogendoorn B, Guy CA, Coleman SL, Smith SK, et al. (2004) A high proportion of polymorphisms in the promoters of brain expressed genes influences transcriptional activity. Biochim Biophys Acta 1690: 238–249.
24. Itokawa M, Arai M, Kato S, Ogata Y, Furukawa A, et al. (2003) Association between a novel polymorphism in the promoter region of the neuropeptide Y gene and schizophrenia in humans. Neurosci Lett 347: 202–204.
25. Zhou Z, Zhu G, Hariri AR, Enoch MA, Scott D, et al. (2008) Genetic variation in human NPY expression affects stress response and emotion. Nature 452: 997–1001.
26. Li L, Jonsson-Rylander AC, Abe K, Zukowska Z (2005) Chronic stress induces rapid occlusion of angioplasty-injured rat carotid artery by activating neuropeptide Y and its Y1 receptors. Arterioscler Thromb Vasc Biol 25: 2075–2080.
27. Doods HN, Wienen W, Entzeroth M, Rudolf K, Eberlein W, et al. (1995) Pharmacological characterization of the selective nonpeptide neuropeptide Y Y1 receptor antagonist BIBP 3226. J Pharmacol Exp Ther 275: 136–142.
28. von Hundelshausen P, Weber KS, Huo Y, Proudfoot AE, Nelson PJ, et al. (2001) RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. Circulation 103: 1772–1777.
29. Zernecke A, Schober A, Bot I, von HP, Liehn EA, et al. (2005) SDF-1alpha/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. Circ Res 96: 784–791.
30. Zukowska-Grojec Z, Pruszczyk P, Colton C, Yao J, Shen GH, et al. (1993) Mitogenic effect of neuropeptide Y in rat vascular smooth muscle cells. Peptides 14: 263–268.
31. Morris MJ, Cox HS, Lambert GW, Kaye DM, Jennings GL, et al. (1997) Region-specific neuropeptide Y overflows at rest and during sympathetic activation in humans. Hypertension 29: 137–143.
32. Pankow JS, Heiss G, Evans GW, Sholinsky P, Province MA, et al. (2004) Familial aggregation and genome-wide linkage analysis of carotid artery plaque: the NHLBI family heart study. Hum Hered 57: 80–89.
33. Franceschini N, MacCluer JW, Rose KM, Rutherford S, Cole SA, et al. (2008) Genome-wide linkage analysis of pulse pressure in American Indians: the Strong Heart Study. Am J Hypertens 21: 194–199.
34. Jia C, Liu Z, Liu T, Ning Y (2005) The T1128C polymorphism of neuropeptide Y gene in a chinese population. Arch Med Res 36: 175–177.
35. Nyholt DR (2004) A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am J Hum Genet 74: 765–769.
36. Smith LR, Harrell FE jr, Rankin JS, Califf RM, Pryor DB, et al. (1991) Determinants of early versus late cardiac death in patients undergoing coronary artery bypass graft surgery. Circulation 84: III245–253.
37. Xu H, Gregory SG, Hauser ER, Stenger JE, Pericak-Vance MA, et al. (2005) SNPselector: a web tool for selecting SNPs for genetic association studies. BioInformatics 21: 4181–4186.
38. Kim J, Zhang L, Peppel K, Wu JH, Zidar DA, et al. (2008) Beta-arrestins regulate atherosclerosis and neointimal hyperplasia by controlling smooth muscle cell proliferation and migration. Circ Res 103: 70–79.
39. Zhang L, Peppel K, Sivashanmugam P, Orman ES, Brian L, et al. (2007) Expression of tumor necrosis factor receptor-1 in arterial wall cells promotes atherosclerosis. Arterioscler Thromb Vasc Biol 27: 1087–1094.
40. Hauser ER, Watanabe RM, Duren WL, Bass MP, Langefeld CD, et al. (2004) Ordered subset analysis in genetic linkage mapping of complex traits. Genet Epidemiol 27: 53–63.
41. Allingham RR, Wiggs JL, Hauser ER, Hauser MA, Graham FL, et al. (2002) Ordered Subset Analysis in Primary Open-Angle Glaucoma (POAG) Evidence for Linkage to Chromosomes 14 and 15. 52th Annual Meeting of the American Society of Human Genetics, October 15–19 71: 453.
42. Bowden DW, Rudock M, Ziegler J, Lehtinen AB, Xu J, et al. (2006) Coincident linkage of type 2 diabetes, metabolic syndrome, and measures of cardiovascular disease in a genome scan of the diabetes heart study. Diabetes 55: 1985–1994.
43. Samani NJ, Burton P, Mangino M, Ball SG, Balmforth AJ, et al. (2005) A genomewide linkage study of 1,933 families affected by premature coronary artery disease: The British Heart Foundation (BHF) Family Heart Study. Am J Hum Genet 77: 1011–1020.
44. Woodroffe A, Krafchak CM, Fuse N, Lichter PR, Moroi SE, et al. (2006) Ordered subset analysis supports a glaucoma locus at GLC1I on chromosome 15 in families with earlier adult age at diagnosis. Exp Eye Res 82: 1068–1074.
45. Hall JM, Friedman L, Guenther C, Lee MK, Weber JL, et al. (1992) Closing in on a breast cancer gene on chromosome 17q. Am J Hum Genet 50: 1235–1242.
46. Abecasis GR, Cherny SS, Cookson WO, Cardon LR (2002) Merlin–rapid analysis of dense genetic maps using sparse gene flow trees. Nat Genet 30: 97–101.
47. Martin ER, Monks SA, Warren LL, Kaplan NL (2000) A test for linkage and association in general pedigrees: the pedigree disequilibrium test. Am J Hum Genet 67: 146–154.
48. Abecasis GR, Cookson WO (2000) GOLD–graphical overview of linkage disequilibrium. BioInformatics 16: 182–183.