

Original Investigation

Plasma Lipids, Genetic Variants Near *APOA1*, and the Risk of Infantile Hypertrophic Pyloric Stenosis

Bjarke Feenstra, PhD; Frank Geller, MSc; Lisbeth Carstensen, PhD; Paul A. Romitti, PhD; Izabella Baranowska Körberg, PhD; Bruce Bedell, MSc; Camilla Krogh, MD, PhD; Ruzong Fan, PhD; Anna Svenningsson, MD, PhD; Michele Caggana, ScD; Agneta Nordenskjöld, MD, PhD; James L. Mills, MD; Jeffrey C. Murray, MD; Mads Melbye, MD, DrMedSci

IMPORTANCE Infantile hypertrophic pyloric stenosis (IHPS) is a serious condition in which hypertrophy of the pyloric sphincter muscle layer leads to gastric outlet obstruction. Infantile hypertrophic pyloric stenosis shows strong familial aggregation and heritability, but knowledge about specific genetic risk variants is limited.

OBJECTIVES To search the genome comprehensively for genetic associations with IHPS and validate findings in 3 independent sample sets.


DESIGN, SETTING, AND PARTICIPANTS During stage 1, we used reference data from the 1000 Genomes Project for imputation into a genome-wide data set of 1001 Danish surgery-confirmed samples (cases diagnosed 1987-2008) and 2371 disease-free controls. In stage 2, the 5 most significantly associated loci were tested in independent case-control sample sets from Denmark (cases diagnosed 1983-2010), Sweden (cases diagnosed 1958-2011), and the United States (cases diagnosed 1998-2005), with a total of 1663 cases and 2315 controls.

MAIN OUTCOMES AND MEASURES Association of genetic variation with the presence of infantile hypertrophic pyloric stenosis.

RESULTS We found a new genome-wide significant locus for IHPS at chromosome 11q23.3. The single-nucleotide polymorphism (SNP) with the lowest *P* value at the locus, rs12721025 (odds ratio [OR], 1.59; 95% CI, 1.38-1.83; *P* = 1.9×10^{-10}), is located 301 bases downstream of the apolipoprotein A-I (*APOA1*) gene and is correlated (r^2 between 0.46 and 0.80) with SNPs previously found to be associated with levels of circulating cholesterol. For these SNPs, the cholesterol-lowering allele consistently was associated with increased risk of IHPS.

CONCLUSIONS AND RELEVANCE This study identified a new genome-wide significant locus for IHPS. Characteristics of this locus suggest the possibility of an inverse relationship between levels of circulating cholesterol in neonates and IHPS risk, which warrants further investigation.

JAMA. 2013;310(7):714-721. doi:10.1001/jama.2013.242978

 Supplemental content at jama.com

Author Affiliations: Department of Epidemiology Research, Statens Serum Institut, Copenhagen, Denmark (Feenstra, Geller, Carstensen, Krogh, Melbye); Department of Epidemiology, University of Iowa, Iowa City (Romitti); Department of Women's and Children's Health and Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden (Körberg, Svenningsson, Nordenskjöld); Department of Pediatrics, University of Iowa, Iowa City (Bedell, Murray); Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland (Fan, Mills); Wadsworth Center, New York State Department of Health, Albany (Caggana).

Corresponding Author: Bjarke Feenstra, PhD, Department of Epidemiology Research, Statens Serum Institut, Artillerivej 5, 2300 Copenhagen S, Denmark (fee@ssi.dk).

Infantile hypertrophic pyloric stenosis (IHPS) is the leading cause of gastrointestinal obstruction in the first months of life, with an incidence of 1 to 3 per 1000 live births in Western countries.^{1,2} It affects 4 to 5 times as many boys as girls³ and typically presents 2 to 8 weeks after birth⁴ with projectile vomiting, weight loss, and dehydration.

Although IHPS is a clinically well-defined entity, the etiology of the condition is complex and remains unclear. A genetic predisposition is well established; IHPS aggregates strongly in families and has an estimated heritability of more than 80%.² However, environmental factors, such as

erythromycin exposure⁵ and feeding practice,^{6,7} have also been implicated in IHPS etiology. Moreover, sharp changes in incidence seen in several countries over the last decades⁸ underline the importance of modifiable environmental exposures.

Cases of IHPS sometimes occur as part of a syndrome of known genetic etiology.⁹ For example, Smith-Lemli-Opitz syndrome is an autosomal recessive congenital disorder caused by mutations in the 7-dehydrocholesterol reductase (*DHCR7* [NCBI Entrez Gene 1717]) gene.¹⁰ Affected individuals are unable to complete the final step in cholesterol biosynthesis, caus-

ing a wide range of metabolic and developmental abnormalities, including IHPS in 10% to 15% of cases.¹⁰

Less is known about the genetic background of isolated IHPS. Several association studies have focused on genes involved in gastric contractility.¹¹⁻¹³ However, these studies were relatively small and produced conflicting results. We recently conducted a genome-wide association study (GWAS) of IHPS and identified 3 susceptibility loci near the muscleblind-like splicing regulator 1 gene (*MBNL1* [NCBI Entrez Gene 4154]) and the NK2 homeobox 5 gene (*NKX2-5* [NCBI Entrez Gene 1482]).¹⁴ Still these variants only explain a small fraction of the variance in disease liability.

The present study followed a 2-stage approach to identify novel genetic variants for IHPS. In the first (discovery) stage, we used a hypothesis-free approach to identify variants associated with IHPS. In the second stage, we carried forward the most promising variants for validation in 3 independent case-control sample sets. Finally, we also investigated the biological relevance of novel genetic loci through follow-up experiments based on prospectively collected plasma samples from cases and controls.

Methods

Participants

Eligible IHPS cases for the discovery sample were defined as singleton children of Danish ancestry who in their first year of life had a surgery code for pyloromyotomy in the Danish National Patient Registry and did not have any additional major malformations. Eligible controls for the discovery sample were nonaffected Danish singleton children who did not have any major malformations. In addition, we excluded severe pregnancy complications from both cases and controls. All Danish samples were drawn from the Danish National Biobank; cases were sampled from dried blood spot samples and controls were sampled from dried blood spots or buffy coats (see the eAppendix in the Supplement for details).

For the validation stage, we used IHPS case and unaffected control samples from 3 different countries. For the validation sample from Denmark, we used the same case and control definitions as for the discovery sample. The US sample was obtained from archived, residual newborn blood spots of participants of mostly non-Hispanic white descent delivered by New York State residents between 1998-2005. Cases were identified from the population-based New York Congenital Malformations Registry. Controls were a random sample of all New York State live births delivered during the same time period and frequency-matched by birth year and race/ethnicity to cases. The Swedish validation cases were identified as patients with IHPS who had undergone pyloromyotomy at pediatric surgery clinics. Available Swedish controls included healthy middle-aged anonymous blood donors, infants born in 2006, and unaffected relatives of IHPS cases. Swedish cases provided samples from whole blood; controls provided samples from whole blood or placenta.

The results of the genetic study suggested a possible relationship between low plasma lipid levels and risk of IHPS.

To investigate this hypothesis, we set up a follow-up study comparing lipid levels in cases and controls. The dried blood spot samples used for genotyping were not suitable for lipid measurements. Instead, we used plasma from prospectively collected umbilical cord blood samples from the Danish National Birth Cohort (DNBC).¹⁵ We included all DNBC children who met our IHPS case definition and had adequate amounts of plasma available as cases. To increase statistical power, we sampled 4 controls for each case. Controls were matched to cases on sex and gestational age at birth and then selected randomly among the DNBC children who were already included as controls in the discovery sample of the genetic study.

The study was approved by the scientific ethics committee for the capital city region (Copenhagen) and the Danish Data Protection Agency for the Danish sample. The scientific ethics committee also granted exemption from obtaining informed consent from Danish participants because the study was based on biobank material. The ethics committee at Karolinska Institutet approved the study for the Swedish sample and informed consent was obtained from all Swedish participants. The New York State Department of Health institutional review board approved the study and did not require informed consent from the US participants because the samples were deidentified. The study was also approved by the National Institutes of Health Office of Human Subjects Research Protections for the US sample.

Genotyping

For the discovery phase, samples were genotyped using the Illumina Human 660W-Quad version 1.0 Bead Array. After quality control, 529 128 SNPs remained available for association and imputation analyses. The Danish validation samples were genotyped at deCODE Genetics using the Centaurus platform (Nanogen) or TaqMan assays (Applied Biosystems). The US samples were genotyped at LGC Genomics using KASP assays, and the Swedish samples were genotyped at Karolinska Institutet using TaqMan assays. (See the eAppendix in the Supplement for a detailed description of sampling, genotyping, and quality control.)

Plasma Measurements

For the plasma samples, aliquots of 40 μ L were prepared and diluted 1:1 with phosphate-buffered saline, and spectrophotometric measurements were done using the Roche Cobas c 111 Analyzer, yielding measurements of circulating low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), and total cholesterol, as well as triglycerides. All measurements were conducted at the University of Iowa.

Statistical Analysis

We imputed unobserved genotypes using phased haplotypes from the integrated phase 1 release of the 1000 Genomes Project.¹⁶ (See the eAppendix in the Supplement for imputation details.) We used logistic regression to test for differences in allele dosages between cases and controls under an additive genetic model. We carried out combined analysis of the discovery and validation data using the inverse variance

Table 1. Study Sample Characteristics

	Stage 1 Samples: Discovery		Stage 2 Samples: Validation					
	Denmark		Denmark		United States		Sweden	
	Cases (n = 1001) ^a	Controls (n = 2371)	Cases (n = 796)	Controls (n = 879)	Cases (n = 738) ^a	Controls (n = 697)	Cases (n = 129) ^a	Controls (n = 742)
Boys, No. (%)	826 (83)	1276 (54)	666 (84)	412 (47)	619 (84)	584 (84)	110 (85)	395 (54) ^b
Year of birth, range	1987-2008	1986-2008	1983-2010	1998-2003	1998-2005	1998-2005	1958-2011	NA ^c
Year of birth, mean (SD)	1996 (6)	2001 (3)	1990 (7)	2000 (1)	2001 (2)	2001 (2)	1998 (6)	NA ^c
Maternal age, mean (SD), y	28 (5)	29 (5)	28 (5)	24 (2)	28 (6)	30 (6)	28 (5) ^d	NA
Season of birth, No. (%)								
Winter	247 (25)	562 (24)	170 (21)	192 (22)	165 (22)	193 (28)	34 (26)	
Spring	246 (25)	566 (24)	184 (23)	207 (24)	196 (27)	178 (26)	26 (20)	
Summer	280 (28)	635 (27)	242 (30)	249 (28)	194 (26)	170 (24)	30 (23)	NA
Autumn	228 (23)	608 (26)	200 (25)	231 (26)	183 (25)	156 (22)	39 (30)	
Age at diagnosis, mean (SD), d	37 (18) ^e		39 (22)		NA		41 (19) ^f	
Sample types (No.)	DBSS (1001)	DBSS (961); buffy coat (1410)	DBSS (796)	DBSS (879)	DBSS (738)	DBSS (697)	Whole blood (129)	Whole blood (380); placenta (362)

Abbreviations: DBSS, dried blood spot samples; ICD-8, *International Statistical Classification of Diseases, Eighth Revision*; ICD-10, *International Statistical Classification of Diseases, Tenth Revision*; NA, data not available.

^a Cases were defined in the following ways in each location: in Denmark, by surgery codes for pyloromyotomy (ICD-8 codes 41840, 41841, and 44100 up through December 1995; ICD-10 codes KJDH60 and KJDH61 from January 1, 1996) in the Danish National Patient Register; in the United States, by the British Pediatric Association code for pyloric stenosis (750.510) in the New York State Congenital Malformations Registry; and in Sweden, by pyloromyotomy registered in pediatric surgery clinic records. Refer to the "Methods" section for information about the genotyping platforms.

^b Sex data were missing for 14 of the Swedish controls. The percentage is based on the 728 participants with available sex data.

^c Among the Swedish controls, 362 were infants born in 2006; the remaining 380 were middle-aged anonymous blood donors for whom year of birth was not available.

^d Based on 96 cases with available maternal age.

^e Based on 996 cases with available age at diagnosis.

^f Based on 101 cases with available age at diagnosis.

method, applying genomic control¹⁷ to the discovery stage results. We estimated heterogeneity between studies using the I^2 statistic.¹⁸ We also assessed the robustness of the meta-analysis results by using hierarchical logistic regression as an alternative approach for combined analysis of discovery and replication data. We conducted the genetic analyses overall and stratified by sex and also tested for sex \times genotype interaction.¹⁹ We conditioned on the top SNP at associated loci to explore possible allelic heterogeneity. Family-based association testing was performed using an extended sib transmission disequilibrium test.²⁰

We evaluated the association between lipid levels in umbilical cord blood and the risk of IHPS by odds ratios (ORs) estimated in a conditional logistic regression using R version 2.15.3. The analysis took into account matching by sex and gestational age at birth using strata and was adjusted for gestational age. P values were obtained using likelihood ratio tests. We tested for possible nonlinear effects by adding a quadratic term to the model and conducted additional analyses based on lipid-level quartiles to allow for nonlinear association.

We used SHAPEIT,²¹ IMPUTE2,²² and SNPTEST²³ software for imputation and association testing. METAL²⁴ and R were used for meta-analysis, and we used PLINK²⁰ for family-based association testing. We used a genome-wide significance threshold of $P < 5 \times 10^{-8}$ in the combined analyses of discovery and validation stage results. The lipid measurement study used a significance threshold of $P < .05$. All statistical tests were 2-sided.

Results

Table 1 shows sample characteristics of the participants contributing to the 2 stages of the genetic study. In the discovery stage, we analyzed the association between the disease and 9 737 928 imputed genetic variants in 1001 cases and 2371 controls. Genomic inflation factors were 1.05 in the complete discovery data and 1.02 and 1.03 in the data restricted to boys and girls, respectively. Imputed SNPs at 4 loci showed P values less than 1×10^{-7} and were selected for further study. These included 2 novel loci on chromosomes 11q23.3 and 19p13.2, as well as 2 already confirmed loci on chromosomes 3q25.1 and 5q35.2. The third known locus on chromosome 3q25.2 was also selected for completeness. The chromosomal regions harboring the 5 selected loci were reimputed with the original IMPUTE2 algorithm (ie, without prephasing) for increased accuracy, and association tests were repeated for these regions. To confirm the associations at these loci, we genotyped a total of 7 SNPs in validation samples from Denmark, Sweden, and the United States with a total of 1663 cases and 2315 controls. One novel locus (11q23.3) was validated with genome-wide significance (Table 2), the 3 known loci (3q25.1, 3q25.2, and 5q35.2) were confirmed, and 1 locus (19p13.2) could not be validated (eTable 1 in the Supplement). Results based on hierarchical logistic regression were very similar (eTable 2 in the Supplement). Figure 1 displays forest plots for the 4 genome-wide significant loci.

The variant rs12721025 yielded the lowest *P* value (OR, 1.59; 95% CI, 1.38-1.83; *P* = 1.9×10^{-10}) at the 11q23.3 locus. This SNP is located 301 bases downstream of the apolipoprotein A-I gene (*APOA1* [NCBI Entrez Gene 335]) with additional apolipoprotein genes *APOC3* (NCBI Entrez Gene 345), *APOA4* (NCBI Entrez Gene 337), and *APOA5* (NCBI Entrez Gene 116519) within 50 kb (kilobase) centromeric (Figure 2). A region of strong linkage disequilibrium extends several hundred kb to the telomeric side. Here the variant rs77349713, which is intronic in

the salt-inducible kinase 3 gene (*SIK3* [NCBI Entrez Gene 23387]), was also associated with genome-wide significance (OR, 1.53; 95% CI, 1.33-1.75; *P* = 1.2×10^{-9}).

We explored the possible functional effect of the 11q23.3 associations by considering all 222 genotyped or imputed variants (SNPs and indels) at the locus with *P* < 1×10^{-6} . These were all correlated with rs12721025 (*r*² between 0.46 and 0.91), and we found no evidence for allelic heterogeneity (all of these SNPs had *P* > .01 when conditioning on rs12721025). Most of the SNPs

Table 2. Discovery Stage, Validation Stage, and Combined Results for the Novel 11q23.3 Locus Associated With IHPS

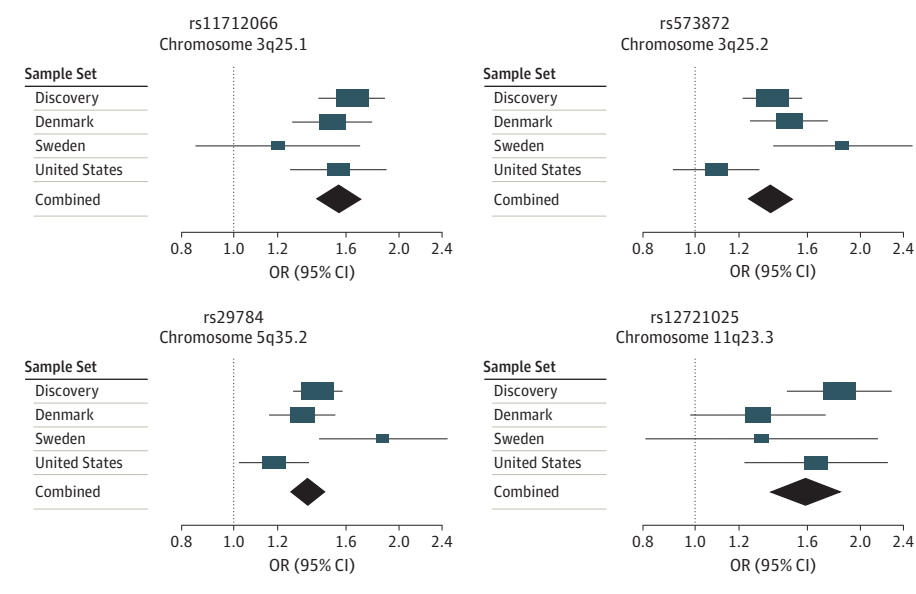
SNP	Position, bp	Alleles		Sample Set	Effect Allele Frequency		No.		OR (95% CI)	<i>P</i> Value	<i>I</i> ² (95% CI) ^a	<i>P</i> Value for Het ^b
		Eff	Alt		Cases	Controls	Cases	Controls				
rs12721025	116706047	A	G	Discovery	0.0911	0.0569	1001	2371	1.83 (1.47-2.28)	1.0×10^{-7}		
				Denmark	0.0741	0.0582	789	876	1.30 (0.98-1.70)	.06		
				Sweden	0.0820	0.0634	128	733	1.32 (0.81-2.16)	.27		
				United States	0.0860	0.0538	738	697	1.66 (1.23-2.33)	7.4×10^{-4}		
				Combined			2656	4677	1.59 (1.38-1.83)	1.9×10^{-10}	31 (0-75)	.23
rs77349713	116765476	C	T	Discovery	0.0896	0.0533	1001	2371	1.76 (1.43-2.16)	1.1×10^{-7}		
				Denmark	0.0713	0.0554	793	876	1.31 (0.99-1.73)	.06		
				Sweden	0.0930	0.0760	129	730	1.25 (0.78-1.98)	.35		
				United States	0.0867	0.0603	738	697	1.48 (1.11-1.97)	6.7×10^{-3}		
				Combined			2661	4674	1.53 (1.33-1.75)	1.2×10^{-9}	21 (0-90)	.28
rs150758276	117085874	C	G	Discovery	0.0392	0.0188	1001	2371	2.94 (2.00-4.31)	4.0×10^{-8}		
				Denmark	0.0269	0.0201	781	872	1.35 (0.86-2.12)	.19		
				Sweden	0.0310	0.0236	129	741	1.32 (0.61-2.89)	.48		
				United States	0.0373	0.0244	738	697	1.55 (1.00-2.39)	.05		
				Combined			2649	4681	1.86 (1.48-2.35)	1.4×10^{-7}	66 (50-88)	.03

Abbreviations: Alt, alternative allele; Eff, effect allele; Het, heterogeneity; IHPS, infantile hypertrophic pyloric stenosis; OR, odds ratio; SNP, single-nucleotide polymorphism.

^a Heterogeneity estimate.

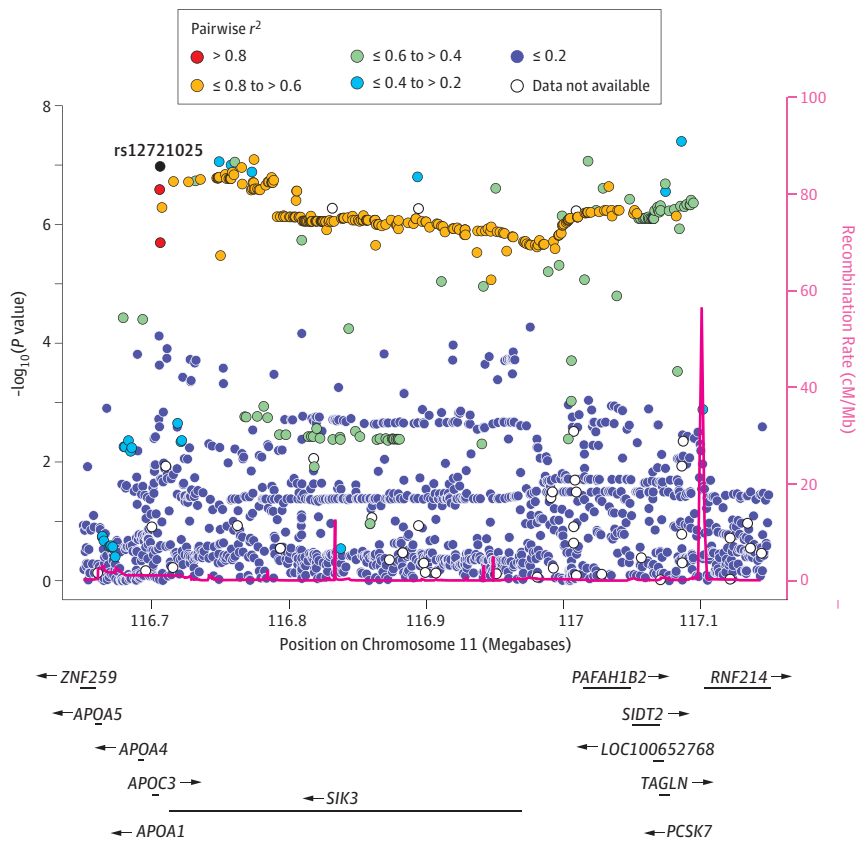
^b Cochran Q test of heterogeneity.

Figure 1. Forest Plot



Discovery stage, validation stage, and combined results for the most significant single-nucleotide polymorphism at each of the 4 genome-wide significant infantile hypertrophic pyloric stenosis loci. The size of each box is proportional to the inverse variance of the effect estimate (which is correlated with the number of participants) in each sample set.

Figure 2. Regional Association Plot Showing Imputed SNP Results in the Discovery Genome-wide Association Study for the Novel IHPS Locus on Chromosome 11q23.3



Single-nucleotide polymorphisms (SNPs) are plotted by chromosomal location (x-axis) and association with infantile hypertrophic pyloric stenosis (IHPS) ($-\log_{10} P$ value; left y-axis). The colors reflect the linkage disequilibrium of each SNP with rs12721025 (based on pairwise r^2 values from the 1000 Genomes Project). Estimated recombination rates (from HapMap) are plotted in pink (right y-axis) to reflect the local LD structure. Genes are indicated in the lower panel of the plot. The figure was generated using LocusZoom (<http://csg.sph.umich.edu/locuszoom/>).

were intronic in the genes *APOA1*, *SIK3*, *PFAH1B2* (NCBI Entrez Gene 5049), *SIDT2* (NCBI Entrez Gene 51092), *TAGLN* (NCBI Entrez Gene 6876), or *PCSK7* (NCBI Entrez Gene 9159) (see eTable 3 in the Supplement). Two SNPs were in exons, but both were synonymous. Fifty of 222 SNPs were previously found to be associated with levels of total cholesterol, HDL cholesterol, or both,²⁵ with P values down to 5.7×10^{-7} .²⁶ For all of these SNPs, the cholesterol-lowering allele consistently was associated with increased risk of IHPS (eTable 4 in the Supplement). A search of the GWAS catalog²⁷ did not reveal any associations to other phenotypes, and no associations to gene expression were found in a search of the expression quantitative trait loci (eQTL) browser.²⁸ However, chromatin immunoprecipitation sequencing (ChIP-seq) data from the ENCODE Consortium (explored using the UCSC genome browser, NCBI build 37²⁹) showed that small islands of histone modification involving monomethylation and trimethylation of histone H3 on lysine 4 (H3K4me1 and H3K4me3) directly cover rs12721025.

The functional characteristics of the 11q23.3 locus suggest the hypothesis that low levels of circulating lipids in newborns are associated with increased risk of IHPS. We addressed this hypothesis by measuring plasma levels of total, LDL, and HDL cholesterol as well as triglycerides in prospectively collected umbilical cord blood from a set of 46 IHPS cases and 189 controls of Danish ancestry, most of

which were also in the discovery sample (see eTable 5 in the Supplement for sample characteristics). The subgroup had 96% male cases and 94% male controls due to the matching, whereas in the initial GWAS there were 83% and 54% male cases and controls, respectively. For the cholesterol study, 70% of the cases were born in summer or winter compared with 53% in the GWAS group, and the mean (SD) age at diagnosis was lower for cases in the cholesterol study, 34 (14) days compared with 37 (18) days in the GWAS group. The eFigure in the Supplement summarizes the distribution of the 4 biomarkers in cases and controls.

Table 3 shows levels of total cholesterol levels in umbilical cord blood plasma for cases and controls overall and divided into quartiles. The mean total cholesterol levels for 46 cases and 189 matching controls were 65.2 mg/dL (95% CI, 58.7-71.8) and 75.2 mg/dL (95% CI, 72.0-78.5), respectively. (To convert cholesterol to mmol/L, multiply by 0.0259.) The risk of IHPS was inversely and significantly associated with total cholesterol level with an OR of 0.77 per 10 mg/dL (95% CI, 0.64-0.92; $P = .005$). An omnibus test of differences between quartiles was also significant ($P = .02$). Results for LDL and HDL cholesterol and triglycerides are shown in eTable 6 in the Supplement. For HDL cholesterol and triglycerides, adding a quadratic term to the analyses indicated nonlinear effects ($P = .05$ and $P = .004$, respectively). For both of these biomarkers, there were significant differences between quartiles

Table 3. Total Cholesterol Levels in Umbilical Cord Blood and Sample Characteristics for IHPS Cases and Controls

	Cases (n=46)	Controls (n=189)	OR (95% CI)	P Value
Total cholesterol, mean (95% CI), mg/dL	65.2 (58.7-71.8)	75.2 (72.0-78.5)		
Boys, No. (%)	44 (96)	177 (94)		
Year of birth, range	1998-2003	1997-2003		
Maternal age, mean (SD), y	29 (5)	30 (4)		
Gestational age, mean (SD), wk	40.1 (1.7)	40.0 (1.2)		
Season of birth, No. (%)				
Winter	17 (37)	44 (23)		
Spring	5 (11)	44 (23)		
Summer	15 (33)	45 (24)		
Autumn	9 (20)	56 (30)		
OR per 10-mg/dL increase in total cholesterol			0.77 (0.64-0.92)	.005 ^a
Test for nonlinearity				.07 ^b
Total cholesterol by quartile, mean (95% CI), mg/dL				
Quartile 1 (≤58 mg/dL)	48.2 (44.9-51.5)	51.0 (49.3-52.7)	1.00 [Reference]	
No. of participants	20	46		
Quartile 2 (58-70 mg/dL)	64.2 (62.1-66.3)	65.3 (64.3-66.3)	0.54 (0.23-1.30)	
No. of participants	10	48		
Quartile 3 (70-84 mg/dL)	76.5 (74.4-78.7)	78.3 (77.3-79.4)	0.51 (0.21-1.21)	
No. of participants	11	45		
Quartile 4 (>84 mg/dL)	110.4 (81.6-139.2)	104.3 (98.5-110.1)	0.21 (0.07-0.63)	
No. of participants	5	50		
Test for no difference in ORs between quartiles				.02 ^c

Abbreviations: IHPS, infantile hypertrophic pyloric stenosis; OR, odds ratio.

SI conversion factor: To convert total cholesterol to mmol/L, multiply by 0.0259.

^a Test for no change in risk per 10-mg/dL increase using conditional logistic regression.

^b Test for nonlinearity using conditional logistic regression and comparing a model including cholesterol as a quadratic term with a model only including cholesterol as a linear term.

^c Omnibus test for no difference in ORs between quartiles using conditional logistic regression. The null hypothesis assumes that the ORs are equal for all 4 quartiles.

($P = .04$ and $P = .01$, respectively, in the omnibus test) and quartile 3 had the lowest ORs.

To explore the role of other lipid-related variants on IHPS risk, we identified 247 SNPs representing 134 regions reported in the GWAS catalog to be associated with lipid levels. Only 1 region on chromosome 19p13.2 was associated with IHPS below a Bonferroni-adjusted threshold of $P < 3.8 \times 10^{-4}$ in the discovery cohort. For all SNPs in this region, the cholesterol-lowering allele conferred increased risk of IHPS. This region was represented by rs2228671, a synonymous SNP in the LDL receptor gene (*LDLR* NCBI Entrez Gene 3949). The association was not seen in the validation cohorts (eTable 1 in the Supplement).

A subset of 94 familial cases and 93 unaffected relatives in 37 pedigrees was excluded from the Swedish case-control analyses. These data were instead analyzed using family-based association testing. This gave very limited statistical power, particularly for the rarer SNPs, and results did not reach statistical significance (eTable 7 in the Supplement). All genome-wide significant SNPs showed the same direction of effects in the two sexes, and there was no interaction between sex and genotype (eTables 8 and 9 in the Supplement).

Discussion

This study identified a novel genome-wide significant locus for IHPS on chromosome 11q23.3 in a region harboring the apolipoprotein (*APOA1/C3/A4/A5*) gene cluster and also con-

firmed 3 previously reported loci. The most significant SNP at the new locus, rs12721025, is located immediately downstream of *APOA1* and is covered by several different histone modification regions. Given that the intronic variant rs77349713 in *SIK3* also reached genome-wide significance and that a region of linkage disequilibrium covers several additional genes (*PAFAH1B2*, *SIDT2*, *TAGLN*, and *PCSK7*), we cannot rule out that other genes in the region could play a role in the etiology of IHPS.

APOA1 encodes apolipoprotein A-I, which is the major protein component of HDL cholesterol in plasma. Furthermore, rs12721025 is correlated with SNPs previously found to be associated with levels of circulating cholesterol. For these SNPs, the cholesterol-lowering allele consistently conferred increased risk of IHPS. These findings suggest the hypothesis that low levels of plasma cholesterol in newborns are associated with increased risk of IHPS. We addressed this hypothesis experimentally using prospectively collected umbilical cord blood samples and found lower cholesterol levels at birth in infants who went on to develop IHPS compared with matched controls who did not develop the disease.

Infantile hypertrophic pyloric stenosis is a prominent clinical feature in many reports of Smith-Lemli-Opitz syndrome, an inborn defect of cholesterol biosynthesis in the gene *DHCR7* associated with low cholesterol levels in infants at birth. In one large case series, 6 of 49 cases with proven *DHCR7* mutations had IHPS.³⁰ Also, a large epidemiological study found that Smith-Lemli-Opitz syndrome is 150 times more prevalent in

IHPS cases than in the general population.³ A study of 10 patients with isolated IHPS and 8 controls found no cholesterol metabolism anomalies but did find that plasma cholesterol levels were lower in cases compared with controls.³¹ However, the study was too small for firm statistical conclusions and appears not to have been followed up.

A number of previous findings would also be consistent with low lipid levels representing an important risk factor for IHPS. First, the protective effect of female sex could at least partly be due to higher cholesterol levels, because it is well-known that levels of LDL cholesterol and HDL cholesterol are on average higher in newborn girls compared with boys.³² Second, the IHPS risk associated with bottle-feeding^{6,7} could in part be caused by insufficient lipid levels because bottle-feeding is known to be associated with lower total and LDL cholesterol levels in infancy.³³ Finally, the decrease in IHPS incidence observed in the 1990s in several countries^{8,34-37} coincided temporally with increasing percentages of mothers breast-feeding their infants,³⁸ suggesting that better nutritional status in infants may have prevented IHPS from developing in a fraction of potential cases.

Other previously reported lipid-related variants were not significantly associated with IHPS. However, different loci regulate different aspects of lipid metabolism at different stages over a lifetime, and most lipid genetics studies have used adult participants. Further functional study is clearly needed to illuminate the biological mechanisms underlying our findings. One approach that might be revealing would focus on the essential role of cholesterol in nervous system development³⁹⁻⁴¹ given the deficiencies in enteric innervation seen in pyloric sphincter muscle tissue from patients with IHPS.⁴²⁻⁴⁴

The previously identified IHPS loci point to candidate genes involved in alternative splicing, cardiac muscle development, and embryonic gut development,¹⁴ and further investigation is needed of the interplay between these loci and the genetic and biochemical findings reported here.

Strengths of our study include the use of samples from 3 different populations with a total of more than 2600 cases and 4600 controls. Furthermore, by leveraging 1000 Genomes Project data, we were able to impute and analyze almost 10 million genetic variants in the discovery stage. Also, we were able to perform a follow-up study based on prospectively collected samples that investigated the potential biological relevance of the new IHPS locus.

The study also has limitations. First, our data permit no assertions about putative causal SNPs at the associated loci, something that would require further fine-mapping studies, eg, through targeted sequencing of affected individuals in families.⁴⁵ Furthermore, it is important to emphasize that our results do not establish a causal link between cholesterol levels and IHPS. If some of the risk is mediated through cholesterol, further study is required to assess the relative importance of other genetic and environmental risk factors. Finally, the lipid measurement study was limited by small numbers of cases and also only covered the time right at birth, ie, several weeks before the condition typically developed in the cases.

In conclusion, we identified a novel genetic locus that associates with IHPS at genome-wide significance. Characteristics of this locus suggest the possibility of an inverse relationship between levels of circulating cholesterol in neonates and IHPS risk. Further investigation is required to illuminate the functional significance of the association identified here.

ARTICLE INFORMATION

Author Contributions: Dr Feenstra had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Dr Feenstra and Mr Geller contributed equally to the study.

Study concept and design: Feenstra, Geller, Melbye.

Acquisition of data: Feenstra, Geller, Romitti, Körberg, Bedell, Krogh, Svenningsson, Caggana, Nordenskjöld, Mills, Murray.

Analysis and interpretation of data: Feenstra, Geller, Carstensen, Romitti, Körberg, Fan, Murray, Melbye.

Drafting of the manuscript: Feenstra, Geller, Melbye.

Critical revision of the manuscript for important intellectual content: Feenstra, Geller, Carstensen, Romitti, Körberg, Bedell, Krogh, Fan, Svenningsson, Caggana, Nordenskjöld, Mills, Murray, Melbye.

Statistical analysis: Feenstra, Geller, Carstensen, Fan.

Obtained funding: Feenstra, Geller, Romitti, Nordenskjöld, Mills, Murray, Melbye.

Study supervision: Feenstra, Melbye.

Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Dr Feenstra, Mr Geller, and Dr Melbye reported being listed on the priority patent application filed by Statens Serum Institut at the Danish Patent and Trademark Office on the use of genetic profiling to identify newborns at risk of IHPS, which contains

subject matter drawn from the work also published here. No other conflicts were reported.

Funding/Support: The study was supported by grants from the Lundbeck Foundation (R34-A3931), the Novo Nordisk Foundation, the Danish Medical Research Council (271-06-0628), the Swedish Research Council, the Centers for Disease Control and Prevention (5U01DD000492), and the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. The GWAS data for the control samples were generated for our study of preterm birth within the GENEVA consortium with funding provided through the National Institutes of Health Genes, Environment, and Health Initiative (U01HG004423). Dr Feenstra is supported by an Oak Foundation Fellowship.

Role of the Sponsor: The funding agency had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Previous Presentation: We have submitted an abstract based on results from this study to the American Society of Human Genetics meeting; October 22-26, 2013.

Additional Contributions: We thank the participants and their families, as well as the staff involved in recruiting and managing the study groups for their contributions to this study. The

participants received no compensation for their contribution; the staff did not receive compensation besides their salaries.

REFERENCES

- Mitchell LE, Risch N. The genetics of infantile hypertrophic pyloric stenosis: a reanalysis. *Am J Dis Child.* 1993;147(11):1203-1211.
- Krogh C, Fischer TK, Skotte L, et al. Familial aggregation and heritability of pyloric stenosis. *JAMA.* 2010;303(23):2393-2399.
- Schechter R, Torfs CP, Bateson TF. The epidemiology of infantile hypertrophic pyloric stenosis. *Paediatr Perinat Epidemiol.* 1997;11(4):407-427.
- Ranells JD, Carver JD, Kirby RS. Infantile hypertrophic pyloric stenosis: epidemiology, genetics, and clinical update. *Adv Pediatr.* 2011;58(1):195-206.
- Honein MA, Paulozzi LJ, Himelright IM, et al. Infantile hypertrophic pyloric stenosis after pertussis prophylaxis with erythromycin: a case review and cohort study. *Lancet.* 1999;354(9196):2101-2105.
- Pisacane A, de Luca U, Criscuolo L, et al. Breast feeding and hypertrophic pyloric stenosis: population based case-control study. *BMJ.* 1996;312(7033):745-746.

7. Krogh C, Biggar RJ, Fischer TK, Lindholm M, Wohlfahrt J, Melbye M. Bottle-feeding and the risk of pyloric stenosis. *Pediatrics*. 2012;130(4):e943-e949.
8. Pedersen RN, Garne E, Loane M, Korsholm L, Husby S; EUROCAT Working Group. Infantile hypertrophic pyloric stenosis: a comparative study of incidence and other epidemiological characteristics in seven European regions. *J Matern Fetal Neonatal Med*. 2008;21(9):599-604.
9. Peeters B, Benninga MA, Hennekam RC. Infantile hypertrophic pyloric stenosis: genetics and syndromes. *Nat Rev Gastroenterol Hepatol*. 2012;9(11):646-660.
10. Kelley RI, Hennekam RC. The Smith-Lemli-Opitz syndrome. *J Med Genet*. 2000;37(5):321-335.
11. Svenningsson A, Lagerstedt K, Omrani MD, Nordenskjöld A. Absence of motilin gene mutations in infantile hypertrophic pyloric stenosis. *J Pediatr Surg*. 2008;43(3):443-446.
12. Lagerstedt-Robinson K, Svenningsson A, Nordenskjöld A. No association between a promoter NOS1 polymorphism (rs41279104) and infantile hypertrophic pyloric stenosis. *J Hum Genet*. 2009;54(12):706-708.
13. Saur D, Vanderwinden JM, Seidler B, Schmid RM, De Laet MH, Allescher HD. Single-nucleotide promoter polymorphism alters transcription of neuronal nitric oxide synthase exon 1c in infantile hypertrophic pyloric stenosis. *Proc Natl Acad Sci U S A*. 2004;101(6):1662-1667.
14. Feenstra B, Geller F, Krogh C, et al. Common variants near MBNL1 and NKX2-5 are associated with infantile hypertrophic pyloric stenosis. *Nat Genet*. 2012;44(3):334-337.
15. Olsen J, Melbye M, Olsen SF, et al. The Danish National Birth Cohort: its background, structure and aim. *Scand J Public Health*. 2001;29(4):300-307.
16. Abecasis GR, Altshuler D, Auton A, et al; 1000 Genomes Project Consortium. A map of human genome variation from population-scale sequencing. *Nature*. 2010;467(7319):1061-1073.
17. Devlin B, Roeder K. Genomic control for association studies. *Biometrics*. 1999;55(4):997-1004.
18. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med*. 2002;21(11):1539-1558.
19. Magi R, Lindgren CM, Morris AP. Meta-analysis of sex-specific genome-wide association studies. *Genet Epidemiol*. 2010;34(8):846-853.
20. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet*. 2007;81(3):559-575.
21. Delaneau O, Marchini J, Zagury JF. A linear complexity phasing method for thousands of genomes. *Nat Methods*. 2012;9(2):179-181.
22. Howie BN, Donnelly P, Marchini J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *PLoS Genet*. 2009;5(6):e1000529.
23. Marchini J, Howie B. Genotype imputation for genome-wide association studies. *Nat Rev Genet*. 2010;11(7):499-511.
24. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics*. 2010;26(17):2190-2191.
25. Teslovich TM, Musunuru K, Smith AV, et al. Biological, clinical and population relevance of 95 loci for blood lipids. *Nature*. 2010;466(7307):707-713.
26. WikiGWA: an open platform for collecting and using genome-wide association results. <http://www.wikigwa.org/>. Accessed July 26, 2013.
27. Catalog of published genome-wide association studies. National Human Genome Research Institute. <http://www.genome.gov/gwastudies>. Accessed July 26, 2013.
28. eQTL resources at the Pritchard lab. <http://eqtl.uchicago.edu>. Accessed July 26, 2013.
29. UCSC genome browser. <http://www.genome.ucsc.edu/cgi-bin/hgGateway>. Accessed July 26, 2013.
30. Ryan AK, Bartlett K, Clayton P, et al. Smith-Lemli-Opitz syndrome: a variable clinical and biochemical phenotype. *J Med Genet*. 1998;35(7):558-565.
31. Hennekam RC, Waterham HR, Wanders RJ, Aronson DC. No cholesterol metabolism anomalies detectable in infants with hypertrophic pyloric stenosis. *Am J Med Genet*. 2001;99(3):256-257.
32. Carlson LA, Hardell LI. Sex differences in serum lipids and lipoproteins at birth. *Eur J Clin Invest*. 1977;7(2):133-135.
33. Owen CG, Whincup PH, Odoki K, Gilg JA, Cook DG. Infant feeding and blood cholesterol: a study in adolescents and a systematic review. *Pediatrics*. 2002;110(3):597-608.
34. Nielsen JP, Haahr P, Haahr J. Infantile hypertrophic pyloric stenosis: decreasing incidence. *Dan Med Bull*. 2000;47(3):223-225.
35. Persson S, Ekblom A, Granath F, Nordenskjöld A. Parallel incidences of sudden infant death syndrome and infantile hypertrophic pyloric stenosis: a common cause? *Pediatrics*. 2001;108(4):70.
36. Sommerfield T, Chalmers J, Youngson G, Heeley C, Fleming M, Thomson G. The changing epidemiology of infantile hypertrophic pyloric stenosis in Scotland. *Arch Dis Child*. 2008;93(12):1007-1011.
37. de Laffolie J, Tural S, Heckmann M, Zimmer KP, Schier F. Decline in infantile hypertrophic pyloric stenosis in Germany in 2000-2008. *Pediatrics*. 2012;129(4):e901-e906.
38. Yngve A, Sjöström M. Breastfeeding in countries of the European Union and EFTA: current and proposed recommendations, rationale, prevalence, duration and trends. *Public Health Nutr*. 2001;4(2B):631-645.
39. Mauch DH, Nägler K, Schumacher S, et al. CNS synaptogenesis promoted by glia-derived cholesterol. *Science*. 2001;294(5545):1354-1357.
40. Fan QW, Yu W, Gong JS, et al. Cholesterol-dependent modulation of dendrite outgrowth and microtubule stability in cultured neurons. *J Neurochem*. 2002;80(1):178-190.
41. Fünfschilling U, Jockusch WJ, Sivakumar N, et al. Critical time window of neuronal cholesterol synthesis during neurite outgrowth. *J Neurosci*. 2012;32(22):7632-7645.
42. Langer JC, Berezin I, Daniel EE. Hypertrophic pyloric stenosis: ultrastructural abnormalities of enteric nerves and the interstitial cells of Cajal. *J Pediatr Surg*. 1995;30(11):1535-1543.
43. Vanderwinden JM, Liu H, De Laet MH, Vanderhaeghen JJ. Study of the interstitial cells of Cajal in infantile hypertrophic pyloric stenosis. *Gastroenterology*. 1996;111(2):279-288.
44. Piotrowska AP, Solari V, Puri P. Distribution of heme oxygenase-2 in nerves and interstitial cells of Cajal in the normal pylorus and in infantile hypertrophic pyloric stenosis. *Arch Pathol Lab Med*. 2003;127(9):1182-1186.
45. Cirulli ET, Goldstein DB. Uncovering the roles of rare variants in common disease through whole-genome sequencing. *Nat Rev Genet*. 2010;11(6):415-425.