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## Brief Genetics Report

# Variation at the Insulin Gene VNTR (Variable Number Tandem Repeat) Polymorphism and Early Growth Studies in a Large Finnish Birth Cohort

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Variation at the insulin gene (*INS*-)VNTR (variable number of tandem repeats) minisatellite polymorphism has been reported to be associated with both early growth and adult metabolic phenotypes. However, the samples studied have been small and the relationship between *INS*-VNTR variation and parameters of early growth inconsistent, with four previous studies producing conflicting results. We have studied the relationship between *INS*-VNTR class (measured by genotyping the nearby *-23HphI* variant with which it is in tight linkage disequilibrium) and early growth in 5,646 members of the Northern Finnish Birth Cohort of 1966. Comparing class III homozygotes with other genotypes using multivariate linear regression analysis, we found no significant associations with any early growth measure (birth weight, birth length, ponderal index, and head circumference at 1 year), even after stratifying subjects by growth trajectory during infancy and/or birth order. For example, among infants with limited postnatal growth realignment ( $n = 2,470$ ), class III/III infants were no heavier at birth (difference [ $\pm$ SE] in the means [fully adjusted],  $58 \pm 51$  g;  $P = 0.26$ ) than class I/- infants. No significant associations were detected following reanalysis with an additive model (for example, for birth weight,  $\beta = 20$  g [95% CI  $-3$  to  $44$ ],  $P = 0.09$ ). Studies of this large population-based cohort have failed to generate convincing evidence that *INS*-VNTR variation influences early growth. *Diabetes* 53:2126–2131, 2004

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ALSPAC, Avon Longitudinal Study of Parents and Children; ARMS, amplification refractory mutation system; GDM, gestational diabetes mellitus; NFBC66, Northern Finland Birth Cohort of 1966; VNTR, variable number of tandem repeats.

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There are two main explanations for the widely observed relationship between restricted early growth and increased susceptibility to type 2 diabetes. One mechanism, encapsulated in the thrifty-phenotype hypothesis, links poor intrauterine nutrition to permanent metabolic changes (“programming”) that predispose to subsequent diabetes (1). Amply supported by studies in animal models (2), evidence that this mechanism is important in humans is less convincing (3,4). A complementary explanation attributes these associations to variation in genes with effects on both early growth and metabolic phenotypes (5). Evidence that paternal diabetes is associated with lower offspring birth weight (6,7) supports such a genetic explanation, and analyses within families segregating rare variants (e.g., in glucokinase) provide proof of principle that genes influencing insulin secretion (and/or action) can have pleiotropic effects on early growth (5). Such rare variants cannot, however, explain the observed population associations.

In this context, several groups have sought to establish the role of insulin gene polymorphisms with respect to early growth. Variation at the insulin gene VNTR (variable number of tandem repeats) minisatellite has been implicated in susceptibility to type 2 diabetes (8,9), polycystic ovarian syndrome (10), and obesity (11). The importance of insulin as a major growth factor in early life, and evidence that the VNTR has a direct effect on insulin (and *IGF2*) transcription (12,13), provides strong grounds for suspecting that *INS*-VNTR variation also influences early growth.

In the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort ( $n = 1,049$ ), Dunger et al. (14) found that VNTR class III homozygote infants had larger head circumference at birth than children of other genotypes. In infants displaying limited postnatal growth realignment (“nonchangers”), in whom birth size may more closely reflect fetal genotype, these class III associations extended to greater birth weight and length. However, there are some difficulties with these data (15). First, the direction of the association is contrary to expectation, given the consensus view that VNTR class III alleles reduce pancre-

atic insulin gene transcription (12,13) and increase risk of adult metabolic phenotypes (8–10). Second, a study of 418 offspring of Pima origin (16) reported an association between class III alleles and birth weight in the opposite direction, whereas in a recent study (17) of 1,184 infants from the U.K., no significant associations with birth weight were observed. Most recently, a study (18) of 452 additional subjects from the ALSPAC cohort confirmed the class III association with greater head size but failed to corroborate the association with birth weight.

Since much of the inconsistency that has troubled complex trait association studies has resulted from the interpretation of findings from inadequately sized samples (19), we studied the relationship between the VNTR genotype and early growth phenotypes (birth weight, birth length, ponderal index, placental weight, and head circumference at 1 year) in 5,753 subjects from the Northern Finland Birth Cohort of 1966 (NFBC66). In this sample, as in other longitudinal cohorts (20), birth weight variation is significantly associated with adult metabolic traits (U.S., M.-R.J., unpublished observations). Of these subjects, 5,646 were successfully genotyped for the  $-23HphI$  variant, a close to perfect proxy for VNTR class in non-African populations (21). Overall, 68.3% (3,859 subjects) were homozygous for the A allele, 29.2% (1,646) were heterozygotes, and 2.5% (141) T allele homozygotes. The frequency of the  $-23HphI$  T allele (equivalent to VNTR class III) is, as previously noted (22,23), lower in Finns than in other European populations (17% in NFBC66). Genotype frequencies did not deviate significantly from Hardy-Weinberg equilibrium. Characteristics of the typed subjects are provided in Table 1.

Analyses of birth weight are displayed in Table 2. The primary analysis of all typed subjects found no significant relationship between VNTR genotype and birth weight under a recessive model that compared class III homozygotes with all other genotypes ( $P = 0.26$ ). Under an additive model, each additional class III allele was associated with a mean birth weight difference of 20 g (95% CI  $-3$  to 44), but this, too, did not reach significance ( $P = 0.09$ ). In the ALSPAC cohort (14), VNTR class effects on early growth parameters were most evident in infants with limited postnatal growth realignment; we were unable to corroborate these findings (nonchangers: recessive model,  $P = 0.26$ ; additive model,  $P = 0.53$ ). The only subanalysis yielding nominally significant differences in birth weight was obtained after stratification for birth order. In first-born children ( $n = 1,770$ ), class III homozygotes were  $21 \pm 74$  g (mean  $\pm$  SE) heavier than heterozygotes and  $81 \pm 72$  g heavier than class I homozygotes. After adjustment for sex, gestational age, and pertinent maternal factors (including maternal BMI and smoking), these differences were significant under the additive ( $P = 0.011$ ) but not the recessive ( $P = 0.40$ ) model. Although first-born individuals are overrepresented among those babies showing positive growth realignment during infancy (28.4% of first born versus only 20.2% of later born), there was no significant relationship between genotype and birth weight in the “change-up” group (recessive model,  $P = 0.34$ ; additive model,  $P = 0.08$ ).

Selected analyses for other early growth phenotypes are provided in Tables 3 and 4. No significant associations

were found for birth length, ponderal index at birth, or head circumference at 1 year, although the additive analysis for ponderal index attained nominal significance ( $P = 0.045$ , two sided). Stratification of the cohort by early growth trajectory, birth order, and/or sex failed to reveal any associations (data not shown). There was some suggestion (Table 4) that III/III homozygotes had smaller placentas, a difference that attained nominal significance ( $P = 0.024$  under the recessive analysis) in the nonchangers. However, in the absence of corroboration from other analyses, interpretation of this finding (and others that reach nominal significance) needs to take account of the large number of different tests performed. There were no significant associations between offspring *INS*-VNTR genotype and the maternal variables listed in Table 1 (data not shown).

This study of a large Finnish birth cohort, therefore, does not substantiate the relationship between VNTR class and early growth reported in a smaller, though exquisitely characterized, cohort of children born in the U.K. one-quarter of a century later (14) or that seen (in the opposite direction) in a study of Pima Indian children (16). It is worth pointing out that the main analysis of birth weight in the Finnish cohort (Table 2) could be interpreted as being consistent with a very modest effect in the direction identified in the ALSPAC study (14), given that the additive analysis reaches nominal significance ( $P < 0.05$ ) on a one-sided test. However, in the absence of corroboration on the recessive model and failure to detect any augmentation of the effect size following stratification by postnatal growth trajectory, we do not support such an interpretation.

What are the possible explanations for this apparent discrepancy? Genotyping error in the current study seems unlikely given the duplicate genotyping and documented low error rate. Neither is the answer likely to lie in ethnic differences in VNTR subclass composition, presence (or absence) of nearby modifying variants, or variable local linkage disequilibrium relationships because these are known to be broadly similar in all non-African populations (21). Although the comparatively low class III allele frequency in this northern Finnish population reduces the power to detect effects restricted to class III homozygotes, the increased overall sample size should more than compensate (the number of class III homozygotes is twice that in the ALSPAC study). The fact that our population-based sample cannot detect (and allow for) parent-of-origin effects implicated in VNTR effects on both early growth (16) and subsequent phenotypes (9,11) represents an intrinsic limitation of the current study, but, again, cannot explain the failure to detect the VNTR association observed in the similarly constrained ALSPAC sample (14).

According to the fetal insulin hypothesis, diabetes-susceptibility alleles are expected to reduce fetal size through compromised insulin secretion or action; however, where the mother also carries susceptibility alleles, and is therefore predisposed to gestational diabetes mellitus (GDM), such associations might be obscured by consequent fetal macrosomia (5). No systematic information on blood glucose levels during pregnancy or GDM status is available for the NFBC66 mothers because at the time of cohort recruitment, GDM was not widely recognized and diagnostic criteria not established. However,

TABLE 1  
Characteristics of the study population

Variable or adjustment factor	Males		Females	
	Number for whom information is available	Mean $\pm$ SD or percentage	Number for whom information is available	Mean $\pm$ SD or percentage
Early growth variables				
Birth weight (g)	2,735	3,555 $\pm$ 539	2,911	3,442 $\pm$ 501
Birth length (cm)	2,714	50.7 $\pm$ 2.2	2,885	49.9 $\pm$ 2.1
Ponderal index at birth (100 $\times$ g/cm <sup>3</sup> )	2,714	2.72 $\pm$ 0.24	2,885	2.75 $\pm$ 0.26
Placental weight (g)	2,374	658 $\pm$ 149	2,525	644 $\pm$ 140
Head circumference at 1 year (cm)	2,447	47.3 $\pm$ 1.4	2,644	46.3 $\pm$ 1.4
Stratification variables				
Birth order	2,730	—	2,904	—
First born	—	31.8	—	31.1
Later born	—	68.2	—	68.9
Growth realignment in first year	2,303	—	2,485	—
Nonchanger	—	51.3	—	51.9
Change up	—	23.1	—	22.3
Change down	—	25.6	—	25.8
Variables adjusted for				
Gestational age at delivery (weeks)	2,649	40.0 $\pm$ 1.9	2,804	40.1 $\pm$ 1.9
Parity	2,730	2.94 $\pm$ 2.28	2,904	3.00 $\pm$ 2.27
Mother's height (cm)	2,596	160 $\pm$ 5	2,777	160 $\pm$ 5
Mother's BMI (kg/m <sup>2</sup> )	2,484	23.2 $\pm$ 3.2	2,675	23.2 $\pm$ 3.3
Mother working during pregnancy	2,682	38.1	2,857	37.7
Mother's age (years)	2,735	—	2,911	—
<20	—	8.6	—	9.3
20–34	—	73.0	—	71.6
$\geq$ 35	—	18.4	—	18.1
Mother smoking at 2 months' gestation	2,680	—	2,839	—
None	—	85.9	—	86.5
1–10 cigarettes/day	—	12.1	—	11.1
>10 cigarettes/day	—	2.0	—	2.4
Socioeconomic status	2,715	—	2,892	—
Higher professional	—	28.4	—	28.5
Lower professional	—	17.3	—	16.0
Skilled worker	—	33.3	—	33.9
Unskilled worker	—	21.0	—	21.6
Farmer (farm size <8 ha)	—	11.6	—	11.7
Farmer (farm size $\geq$ 8 ha)	—	9.6	—	10.2
Desirability of pregnancy	2,682	—	2,839	—
Pregnancy wanted	—	65.1	—	63.3
Pregnancy wanted later	—	23.3	—	24.7
Pregnancy unwanted	—	11.6	—	12.0
Maternal frame of mind during pregnancy	2,690	—	2,840	—
As usual	—	87.2	—	86.6
Depressed	—	11.1	—	11.4
Very depressed	—	1.7	—	2.0

undiagnosed GDM is unlikely to explain the failure to detect class III associations with increased birth size. The ALSPAC data indicate that, in the case of the *INS*-VNTR, the diabetes-associated (class III) allele leads to increased, not decreased, birth size (14,18). In this situation, GDM (presumably associated with maternal class III) would exacerbate, not obscure, the class III association with birth size. In addition, no VNTR associations were revealed when we excluded offspring with negative postnatal growth realignment on the basis that most offspring born following pregnancies complicated by GDM-associated macrosomia would be "change-downers." Four of the NFBC66 mothers had a preexisting diagnosis of diabetes; their exclusion from the analyses had no impact on the findings.

Two possible explanations remain. The first attributes the discrepant findings to biological differences between the various study samples (e.g., environmental exposures, antenatal management, secular trends) and/or to study design-related issues (ascertainment schemes, accuracy, and choice of measures of early growth) that have an effect on the power to detect VNTR association effects. In particular, it is important to note that we did not have data on head circumference at birth, the phenotype most strongly associated with the VNTR genotype in the ALSPAC cohort (14,18). Nonetheless, the persistence of the VNTR association with head circumference from birth to 7 years of age in the ALSPAC study (18) suggests that this is not a complete answer. The second explanation is that certain of the analyses in the smaller sets have been

TABLE 2  
Analyses of birth weight in the NFBC66 by *INS*-VNTR genotype, stratified by growth realignment and birth order

Stratification	<i>n</i>	III/III individuals	Mean of III/III individuals*	Between-mean differences (III/III vs. I/III)*	Between-mean differences (III/III vs. I/I)*	<i>P</i> (recessive model)†	β (95% CI) (additive model)‡	<i>P</i> (additive model)‡
None	5,646	141 (2.5)	3,523 ± 44	25 ± 46	27 ± 45	0.26	20 (−3 to 44)	0.09
Nonchanger	2,470	58 (2.3)	3,453 ± 58	−30 ± 60	−11 ± 59	0.26	9 (−20 to 38)	0.53
Change up	1,086	21 (1.9)	3,265 ± 100	35 ± 104	86 ± 102	0.34	44 (−5 to 93)	0.08
Change down	1,232	32 (2.6)	3,916 ± 88	96 ± 92	70 ± 90	0.24	16 (−31 to 63)	0.51
Firstborn	1,771	44 (2.5)	3,414 ± 71	21 ± 74	81 ± 72	0.40	51 (12–90)	0.01
Later born	3,863	97 (2.5)	3,572 ± 54	31 ± 56	0 ± 55	0.32	7 (−22 to 37)	0.62
Male	2,735	81 (3.0)	3,569 ± 60	32 ± 63	6 ± 61	0.36	20 (−15 to 54)	0.26
Female	2,911	60 (2.1)	3,461 ± 65	4 ± 67	24 ± 64	0.47	22 (−12 to 55)	0.20

Data are means ± SE or *n* (%), unless noted otherwise. \*Mean and differences between the means for the three genotypes are unadjusted; †*P* for the recessive model (III/III homozygotes versus all other genotypes) is adjusted for sex, gestational age, and maternal background factors (including BMI and smoking); ‡β is the regression coefficient for the change in birth weight associated with each unit increase in the number of class III alleles as obtained under an additive model on full adjustment, shown together with 95% CIs and the associated *P* value. For all comparisons, a positive value implies that birth weight is higher in individuals having more class III alleles.

subject to type 1 error and effect-size inflation (“the winner’s curse”) (19), which have led to an overestimation of the evidence that VNTR class and early growth are truly associated. The available data do not allow us to distinguish between these alternatives, which are, in any event, not mutually exclusive.

Preliminary analyses of the 31-year data from the NFBC66 cohort have failed to find any clear evidence of a relationship between VNTR class variation and adult metabolic phenotypes (A.J.B., M.-R.J., M.I.M., unpublished observations). Therefore, while we can conclude that studies of this large population-based cohort have failed to generate convincing evidence that insulin gene VNTR class variation influences early growth, these studies of the insulin gene do not allow us to discriminate between genetic and environmental explanations for the observed

associations between early growth and adult metabolic phenotypes.

#### RESEARCH DESIGN AND METHODS

The NFBC66 originally ascertained 96% of all women in the northernmost two provinces of Finland with expected dates of delivery during 1966 (12,058 live births) (24). Extensive data were collected on parental environment, pregnancy progress, and outcome. Several early growth phenotypes were captured using standardized methods including birth weight, birth length, and placental weight. Ponderal index was calculated as the ratio of birth weight to birth length cubed. Follow-up data were collected at 12 months’ age, including weight and head circumference (in 84.9 and 90.2%, respectively). At 31 years of age, all individuals still living in northern Finland or the Helsinki area (*n* = 8,463) were recontacted and invited for clinical examination (response rate 71%) and DNA sampling (5,753 samples available). The subset with DNA is representative of the original cohort in terms of birth and early growth parameters and the major environmental and social factors known to influence these characteristics.

TABLE 3  
Analyses of other early growth parameters in the NFBC66 by *INS*-VNTR genotype: stratification by sex

Early growth variable/ stratification	<i>n</i>	III/III individuals	Mean of III/III individuals*	Between-mean differences (III/III vs. I/III)*	Between-mean differences (III/III vs. I/I)*	<i>P</i> (recessive model)†	β (95% CI) (additive model)‡	<i>P</i> (additive model)‡
<b>Birth length (cm)</b>								
None	5,599	139 (2.5)	50.3 ± 0.2	0 ± 0.2	0 ± 0.2	0.91	0 (−0.1 to 0.1)	0.63
Male	2,714	80 (2.9)	50.6 ± 0.2	−0.1 ± 0.3	−0.2 ± 0.3	0.78	0 (−0.2 to 0.1)	0.87
Female	2,885	59 (2.0)	49.9 ± 0.3	−0.1 ± 0.3	0 ± 0.3	0.57	0.1 (−0.1 to 0.2)	0.37
<b>Ponderal index (100 × g/cm<sup>3</sup>)</b>								
None	5,599	139 (2.5)	2.76 ± 0.02	0.02 ± 0.02	0.03 ± 0.02	0.10	0.01 (0–0.03)	0.05§
Male	2,714	80 (2.9)	2.75 ± 0.03	0.04 ± 0.03	0.04 ± 0.03	0.06	0.02 (0–0.04)	0.10
Female	2,885	59 (2.0)	2.77 ± 0.03	0.01 ± 0.04	0.02 ± 0.03	0.69	0.01 (−0.01 to 0.03)	0.24
<b>Head circumference at 1 year (cm)</b>								
None	5,091	121 (2.4)	46.9 ± 0.1	0 ± 0.1	0.2 ± 0.1	0.24	0 (−0.1 to 0.1)	0.65
Male	2,447	75 (3.1)	47.3 ± 0.2	0 ± 0.2	0 ± 0.2	0.5	0 (−0.1 to 0.1)	0.78
Female	2,644	46 (1.7)	46.2 ± 0.2	−0.1 ± 0.2	0 ± 0.2	0.26	0 (−0.1 to 0.1)	0.67

Data are means ± SE or *n* (%), unless noted otherwise. \*Mean and differences between the means for the three genotypes are unadjusted; †*P* value for the recessive model (III/III homozygotes versus all other genotypes) is adjusted for sex, gestational age, and maternal background factors (including BMI and smoking); ‡β is the regression coefficient for the change in the parameter of interest associated with each unit increase in the number of class III alleles as obtained under an additive model on full adjustment, shown with 95% CIs and the associated *P* value. §*P* = 0.045. For all comparisons, a positive value implies that the parameter of interest is greater in individuals carrying more class III alleles.

TABLE 4  
Analyses of placental weight in the NFBC66 according to *INS*-VNTR genotype

Stratification	<i>n</i>	III/III individuals	Mean of III/III individuals*	Between-mean differences (III/III vs. I/III)*	Between-mean differences (III/III vs. I/I)*	<i>P</i> (recessive model)†	β (95% CI) (additive model)‡	<i>P</i> (additive model)‡
None	4,899	119 (2.4)	639 ± 13	-16 ± 14	-10 ± 13	0.71	4 (-5 to 12)	0.38
Male	2,374	70 (2.9)	648 ± 18	-9 ± 18	-10 ± 18	0.94	1 (-11 to 13)	0.87
Female	2,525	49 (1.9)	626 ± 20	-26 ± 21	-16 ± 20	0.60	7 (-5 to 19)	0.26
Nonchangers								
Total	2,137	47 (2.2)	606 ± 19	-39 ± 20	-32 ± 19	0.02	-2 (-13 to 9)	0.74
Male	1,017	26 (2.6)	608 ± 26	-47 ± 27	-37 ± 27	0.13	3 (-13 to 19)	0.69
Female	1,120	21 (1.9)	604 ± 28	-32 ± 29	-29 ± 28	0.15	-5 (-21 to 11)	0.54

Data are means ± SE or *n* (%), unless noted otherwise. \*Mean and differences between the means for the three genotypes are unadjusted; †*P* value for the recessive model (III/III homozygotes versus all other genotypes) is adjusted for sex, gestational age, and maternal background factors (including BMI and smoking); ‡β is the regression coefficient for the change in placental weight associated with each unit increase in the number of class III alleles as obtained under an additive model on full adjustment, shown together with 95% CIs and the associated *P* value. For all comparisons, a positive value implies that the parameter of interest is greater in individuals carrying more class III alleles.

**Genotyping.** The -23*Hph*I variant (rs689) was typed as a surrogate for VNTR class because in Finns, as in other non-African populations, these are in tight linkage disequilibrium (21). Genotyping was performed in duplicate, using both a PCR-restriction fragment-length polymorphism and a mass-spectrometry assay. The amplicon for the former was generated using oligonucleotides 5'-AGCAGGTCTGTTCCAAGG and 5'-CTTGGGTGTGTAGAAGAAGC and included an obligate restriction site; following restriction, products were separated on a 7.5% acrylamide gel. The mass-spectrometry assay used an amplicon generated with primers 5'-ACGTTGGATGTCCACAGGGCCATGGCAGAAG and 5'-ACGTTGGATGTGGCCTTCAGCCTGCCTCAG. Following dNTP removal with shrimp alkaline phosphatase, the sequencing oligo (5'-CAGAAGGACAGTGATCTGGG) was extended using thermosequencing, and the products were resin captured, arrayed, and analyzed in a Bruker Biflex III mass spectrometer according to the manufacturer's protocol (Sequenom, San Diego, CA). Discrepant calls were resolved using a four-primer amplification refractory mutation system (ARMS)-PCR assay designed to use the flanking oligos, 5'-CTCAGCCCTCCAGGACAGGCTGCATCAGA and 5'-AGAGCTTCCAACAGGTGTGAGCCGCACA, and allele-specific oligos, 5'-TCAGCCTGCCTCAGCCCTGCCTGACA (class I) and 5'-GGCCATGGCAGAAGGACAGTGATCTGCGA (class III). Amplification products were separated on a 5% acrylamide gel. In addition, class III homozygote genotypes were reconfirmed (with no discrepancies detected) by ARMS-PCR, direct sequencing, and/or Pyrosequencing. A final round of ARMS-PCR retyping of 384 samples identified only one genotype discrepancy compared with the assigned genotype. Thus, we estimate our overall error rate as <0.1%. Additional details on assay design and quality control data are available from the authors.

**Statistical analysis.** The relationship between the -23*Hph*I genotype and phenotypes of interest was examined by linear multivariate regression modeling using the SAS (version 8.2) and SPSS (version 11.5 for Windows) programs. We considered two different genotype models. Given previous findings (14), the first ("recessive") model compared TT (class III) homozygotes against all other genotypes. The second ("additive") model assumed a linear relationship between the number of T alleles and the trait of interest. Analyses were optionally adjusted for potential confounding and explanatory variables, with four such adjustments considered (none; sex alone; sex and gestational age; and sex, gestational age, and maternal background factors, including maternal BMI and height, depression, employment, smoking during pregnancy, and parental socioeconomic status). The full list of variables included in these adjustments is shown in Table 1. Unless otherwise stated, all *P* values reported are those for the fully adjusted analyses. Analyses were repeated after stratification by postnatal growth realignment (14) and birth order. The former divided subjects into "nonchangers," "change-downers," and "change-uppers" based on comparison of sex- and gestational age-adjusted SD scores for weight at birth and 1 year (the latter available for 4,574 individuals). The boundaries for each stratum were set at a change in SD score of ±0.67 (14). Stratification by parity considered first borns and those from second or subsequent pregnancies ("later borns"). Additional adjustment for actual age at the 12-month visit had no impact on the findings (data not shown).

**Power.** Our power to detect an effect size of 25% of an SD (~130 g for birth weight) with a significant *P* value of 0.05 was >80% (given that the at-risk genotype was present in only 2.5% of the sample). The additive analysis had

equivalent power to detect a difference in the parameter of interest of <10% of an SD.

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