

Common *STAT3* Variants Are Not Associated With Obesity or Insulin Resistance in Female Twins

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Abstract

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In animal models, *STAT3* action in the hypothalamus and liver appears essential for normal body weight and glucose homeostasis in response to insulin. We hypothesized that variation in the *STAT3* gene may be associated with body fat and/or insulin resistance in the general population. Five tagging SNPs spanning the *STAT3* gene, *rs8074524*, *rs2293152*, *rs2306580*, *rs6503695*, and *rs7211777* were genotyped in 2776 white female twins (mean age, 47.4 ± 12.5 yrs) from the St Thomas' United Kingdom Adult Twin Registry (Twins UK). Minor allele frequencies were as follows: *rs8074524* (0.19), *rs2293152* (0.37), *rs2306580* (0.06), *rs6503695* (0.35), and *rs7211777* (0.34). The minor allele of *rs2293152* was associated with higher homeostasis model assessment index of insulin resistance ($p = 0.013$) in the full cohort and confirmed in sib-transmission/disequi-

librium test (TDT): ($p = 0.015$; $n = 60$). However, there were no associations with fasting serum insulin or glucose or with obesity variables. Although defective *STAT3* action results in obesity and insulin resistance in animal models, we failed to establish any indicative associations with common SNPs in this human study.

Key words: fat distribution, genetic susceptibility, insulin resistance, signal transduction

Signal transducers and activators of transcription (*STAT*)¹ are a family of latent cytoplasmic transcription factors that are produced in many cell types. They share a DNA-binding domain, an Src homology 2 domain, and a putative Src homology 3 domain and are activated by tyrosine phosphorylation and dimerization in response to a wide variety of extracellular ligands, such as cytokines and growth factors (1). It is well known that *STAT3* is involved in the regulation of energy homeostasis through the adipose tissue cytokine leptin (2). Mice with a neural-specific disruption of the *Stat3* gene are hyperphagic, obese, diabetic, infertile, and hyperleptinemic, suggesting a leptin-resistant condition (3). Other pathways that converge and function through *STAT3* may have similar effects. Mice lacking *Stat3* specifically in the liver exhibit insulin resistance and glucose intolerance when fed a high-fat diet, and restoration of hepatic *STAT3* expression corrects the abnormalities (4). It has recently been shown that intracerebroventricular insulin infusion stimulates tyrosine phosphorylation of *STAT3* in the liver and that inhibition of hepatic glucose production and expression of gluconeogenic genes induced by insulin is impaired in mice with liver-specific *STAT3* deficiency (5).

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¹ Nonstandard abbreviations: *STAT*, signal transducers and activators of transcription; tSNP, tagging single nucleotide polymorphism; LD, linkage disequilibrium; Twins UK, St Thomas' United Kingdom Adult Twin Registry; MAF, minor allele frequency; MZ, monozygotic; DZ, dizygotic; HOMA, homeostasis model assessment; TDT, transmission/disequilibrium test; SiM, insulin sensitivity measure.

Table 1. General characteristics of subjects

Variable	n	Mean ± SD
Age (yrs)	2776*	47.4 ± 12.5
Postmenopausal (%)	2453	47.7
Obesity-related variables		
Leptin (ng/mL)	2776	16.5 ± 12.0
BMI (kg/m ²)	2759	24.7 ± 4.4
Weight (kg)	2760	65.3 ± 11.8
Waist (cm)	2708	78.3 ± 10.2
Total fat (kg)	2719	23.4 ± 8.8
Total fat (%)	2678	35.6 ± 8.0
Central fat (kg)	2696	1.33 ± 0.73
Central fat (%)	2696	31.2 ± 11.5
Insulin sensitivity†		
Fasting glucose (mM)	2317	4.49 ± 0.55
Fasting insulin (μU/mL)	1984	6.96 ± 5.96
HOMA	1791	1.37 ± 1.31
2-hour glucose (mM)	738	5.18 ± 1.10
2-hour insulin (μU/mL)	738	34.2 ± 25.4
SiM	738	88.5 ± 68.8

SD, standard deviation; HOMA, homeostasis model assessment; SiM, insulin sensitivity measure; MZ, monozygotic; DZ, dizygotic; SNP, single nucleotide polymorphism. Type 2 diabetes, diagnosed patients or subjects with fasting glucose >7.8 mmol/L or 2-hours glucose >11.1 mM.

* Number of subjects (846 MZ, 1930 DZ) with leptin data and genotype data on at least 1 SNP.

† Non-fasting subjects, patients with either type 1 or type 2 diabetes, and patients on any anti-diabetic drugs were excluded.

STAT3 action in the mouse hypothalamus and liver, therefore, appears essential for normal body weight and glucose homeostasis in response to the actions of both leptin and insulin. We hypothesized that variants in the *STAT3* gene may influence body fat mass and insulin sensitivity in humans. In this first gene-wide association study of *STAT3* variation in relation to body fat and insulin action, we have used tagging single nucleotide polymorphisms (tSNPs) to test associations with phenotypes in a large sample of white female twins ($n = 2771$; mean age, 47.4 ± 12.5 yrs). tSNPs effectively capture information of most common variants by taking into account patterns of linkage disequilibrium (LD) across the gene (6).

Characteristics of subjects in the St Thomas' United Kingdom Adult Twin Registry (Twins UK) study sample are shown in Table 1. The 25 common [minor allele frequency (MAF) >0.05] SNPs in the selected 83,170 bp region, including the 75,171 bp *STAT3* gene, are situated in

two blocks in strong LD ($r^2 > 0.8$). SNP *rs2292152* sited between the blocks is not in LD with any other common SNP in the gene (Figure 1). Five tSNPs were selected: *rs8074524*, *rs2293152*, *rs2306580*, *rs6503695*, and *rs7211777*, all of which are in non-coding regions. Table 2 shows the genotype and allele frequencies of the tSNPs in the Twins UK cohort, based on one monozygotic (MZ) and both dizygotic (DZ) twins genotyped for each pair.

We found no associations between any of the five tSNPs and general or central obesity scores, so individual variables were not tested further. The only association for a 2 *df* overall genotypic test (i.e., a codominant model) showing borderline nominal significance ($p = 0.052$) was between the intronic SNP *rs2293152* and the homeostasis model assessment (HOMA) index of insulin resistance. We performed follow-up analyses to determine the best model for all insulin resistance-related phenotypes and found a recessive association between *rs2293152* and HOMA in subjects with available data ($n = 1791$) (Table 3). Subjects homozygous for the minor allele had significantly higher HOMA ($p = 0.013$) in comparison to subjects homozygous or heterozygous for the major allele, explaining 0.34% of variance. Association with HOMA was confirmed in sib-transmission/disequilibrium test (TDT) ($p = 0.015$; $n = 60$), which is based on dizygous twins drawn from the full cohort who are discordant for *rs2293152* genotype, suggesting that population stratification did not account for the association in the full cohort. However, we found no significant association between *rs2293152* and fasting insulin and glucose levels used in calculation of the HOMA score. No tSNPs were associated with insulin sensitivity measure (SiM) based on 738 subjects. Haplotype analyses based on *rs7211777* and *rs6503695* were also tested; however, no significant effects were observed for any phenotype.

The findings are generalizable, as few differences were found between Twins UK subjects and singletons in the U.K. female population, other than that MZ twins had a slightly lower weight and a smaller variance for weight than DZ twins and singletons (7). The main strengths of our study lie in the large number of subjects with measures of regional fat distribution and insulin resistance and comprehensive coverage of variation in this 75 kb gene using tagging SNPs. The test of association with obesity variables involving 1930 DZ (865 pairs) and 846 MZ (413 pairs) twins provided power in excess of 80% (and $\alpha = 0.05$) to detect a locus effect of 0.5%. Although there were fewer subjects than this available for insulin resistance measures, the current study had 80% ($\alpha = 0.05$) power to detect a locus effect explaining 0.65% of the variance in HOMA index ($n = 1791$) and 1.5% of the variance in SiM ($n = 738$). The apparent association of *rs2293152* with HOMA in the full cohort accounted for 0.34% of variance; however, the sample of 738 was underpowered to detect an associa-

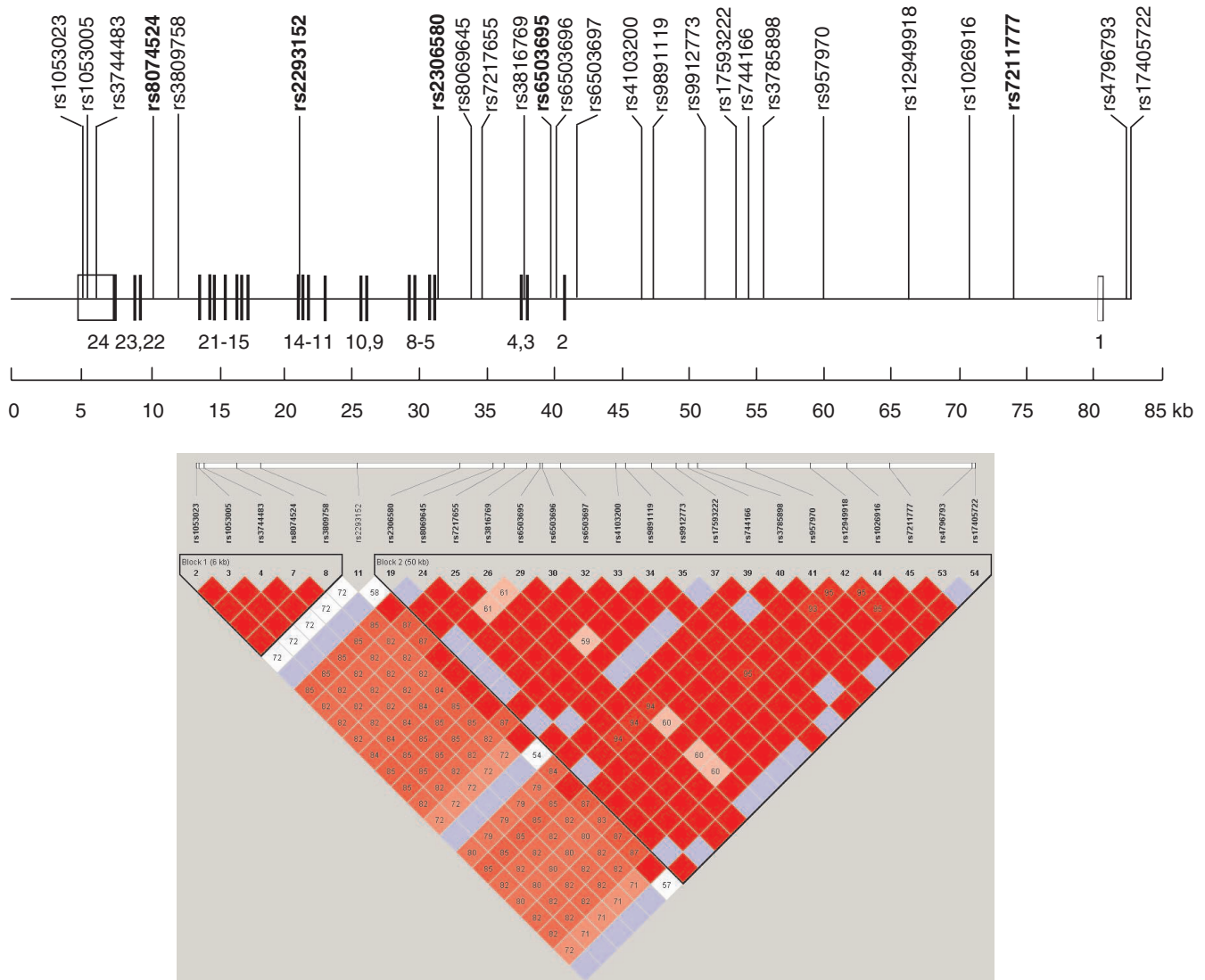


Figure 1: Linkage disequilibrium plot of *STAT3* region HapMap SNPs. On gene map, numbers refer to exons, with coding exons as solid boxes and untranslated exons as open boxes. tSNPs are shown in bold type. Note reverse strand orientation. Genotype data for SNPs of MAF >0.05 in the region Chr17:40835507–40918677 in 90 Utah residents with European ancestry were downloaded from <http://www.hapmap.org> (Phase II HapMap Release, January 2006). The pairwise LD plot (r^2) was obtained using the integral Tagger program. SNP *rs2293152* lies between two blocks and does not tag any other common SNPs in this gene.

tion of comparable strength with SiM. The *rs2293152* genotyping success rate was the lowest at 87%, but the association with HOMA cannot be dismissed as spurious on the grounds of sample size, as 2059 genotypes still represent a powerful study. Also, the effect of reduced sample size on power was in some way offset by the relatively high minor allele frequency (0.37).

Inoue et al. (4) originally showed that *Stat3* $-/-$ mice develop insulin resistance associated with increased glucose production and more recently that insulin acting in the hypothalamus down-regulates gluconeogenic gene expression in the liver by stimulating IL-6 signaling via *STAT3*

(5). Gorogawa et al. (8) have found insulin secretion to be impaired in pancreatic β cell-specific *Stat3* $-/-$ mice. We found a significant increase in HOMA in *STAT3* *rs2293152* rare homozygotes compared with the rest. However, fasting glucose concentration, which reflects basal hepatic glucose production, and fasting insulin, which reflects β cell competence, showed no significant changes. It seems, therefore, that the *STAT3* variant has minimal effect on gluconeogenesis or insulin secretion, and the modest association with HOMA may not be genuine. We would not expect profound effects on phenotype matching those of animal knockout models as a result of SNP variation, potentially resulting in

Table 2. STAT3 SNP genotype and allele distributions

HapMap no.	rs no.	Genotype			Total*	Minor allele frequency (95% CI)	H-W test†		MAF in HapMap
		11	12	22			χ^2	<i>p</i>	
7	rs8074524	1377	650	81	2108	19.3 (18.1 to 20.5)	0.15	NS	15.8
11	rs2293152	797	995	267	2059	37.1 (35.7–38.6)	1.21	NS	40.4
19	rs2306580	1939	260	9	2208	6.3 (5.6–7.1)	0.21	NS	11.2
29	rs6503695	915	1050	240	2205	34.7 (33.3–36.1)	0.78	NS	34.8
45	rs7211777	979	1010	249	2238	33.7 (32.3–35.1)	1.82	NS	35.8

STAT, signal transducers and activators of transcription; SNP, single nucleotide polymorphism; CI, confidence interval; H-W, Hardy-Weinberg; MAF, minor allele frequency; NS, not significant; MZ, monozygotic; DZ, dizygotic.

* Includes one individual from each MZ pair and both individuals from each of the DZ pairs.

† Tested in one of each MZ pair, one of each DZ pair, and all singleton DZ twins.

small changes in the level of STAT3 gene expression or minor changes in protein structure associated with carriage of a particular allele. However, the expectation would be for a trend toward a comparable effect, which might become significant only in a much larger study sample.

The absence of any association with obesity variables is interesting in view of a recent discovery that mice deficient in interleukin 18 (IL18), a cytokine that signals via STAT3, are hyperphagic, obese, and insulin resistant (9). Hepatic insulin resistance in the *IL18* $-/-$ mice involved an enhanced expression of genes associated with gluconeogenesis in the liver, which resulted from defective phosphorylation of STAT3. Resistance seemed to be secondary to increased food intake and obesity (specifically accumulation of body fat) because the effect emerged only after 6 months.

In tagging only common SNPs, we excluded the possibility of discovering any substantial effect associated with low frequency SNPs (MAFs <0.05), which could be functional. However, evidence from animal models suggests that defective STAT3 activity remains a strong candidate in the development of obesity, with insulin resistance emerging as a secondary effect.

Research Methods and Procedures

Study Design

The Twins UK Registry comprises unselected, white, mostly female volunteers ascertained from the general population through national media campaigns in the U.K. (10). The study sample comprised 2776 subjects (423 MZ pairs, 940 DZ pairs, and 50 singletons) with available leptin data. No difference was observed in the distributions of age and menopausal status between subjects with and without leptin data (*n* = 362). The number of individuals in the study cohort with data on other phenotypic variables is shown in

Table 1. Means and ranges of quantitative phenotypes in Twins UK are similar to an age-matched sample of the U.K. female population (7). Informed consent was obtained from all participants before they entered the studies, which were approved by the local research ethics committee.

Zygosity, Body Composition, and Biochemical Analyses

Zygosity was determined by standardized questionnaire and confirmed by DNA fingerprinting. Height was measured to the nearest 0.5 cm using a wall-mounted stadiometer. Weight (light clothing only) was measured to the nearest 0.1 kg using digital scales. BMI was used as a measure of general adiposity and calculated as weight divided by height squared (kg/m^2). Waist circumference (cm) was measured at the level midway between the lower rib margin and the iliac crest. Body composition was measured by dual emission X-ray absorptiometry (Hologic QDR-2000, Vertec, Waltham, MA). Serum leptin concentration was determined after an overnight fast using a radioimmunoassay (Linco Research, St. Louis, MO). Fasting insulin was measured by immunoassay (Abbott Laboratories Ltd., Maidenhead, U.K.), and glucose was measured on an Ektachem 700 multichannel analyser using an enzymatic colorimetric slide assay (Johnson and Johnson Clinical Diagnostic Systems, Amersham, U.K.). A random sub-sample of 738 subjects underwent an oral glucose tolerance test in which glucose and insulin levels were measured before and 2 hrs after a 75-g oral glucose load.

Selection of tSNPs

The STAT3 gene spans over 75 kb and contains 24 exons. Twenty-five polymorphic SNPs with MAF >0.05 in an 83,170 bp region including the STAT3 gene are listed on the HapMap database (HapMap Data Release #20/Phase II Jan 2006; available at <http://www.hapmap.org>) (Table 1, available online at the Obesity website, www.obesityresearch.org). Geno-

Table 3. Nominally significant associations of STAT3 rs2293152 with insulin sensitivity phenotypes

	GEE				p	Explained variance	No. of pairs	sTDT		p	
	Number (11 + 12/22)	Mean (SD)		11 + 12				22	Mean (SD)		
		11 + 12	22						11 + 12		22
HOMA	1361/193	1.35 (1.29)	1.57 (1.63)	1.36 (0.67)	0.013	0.34%	60	1.13 (0.67)	1.36 (0.96)	0.015	
Fasting glucose (mM)	1762/254	4.48 (0.55)	4.55 (0.59)	4.52 (0.54)	NS	—	79	4.43 (0.42)	4.52 (0.54)	NS	
Fasting insulin (μ U/mL)	1508/210	6.91 (5.89)	7.57 (7.42)	7.13 (5.25)	NS	—	69	6.26 (4.30)	7.13 (5.25)	0.048	
SiM	554/76	88.2 (69.0)	92.0 (71.9)	116.9 (96.0)	NS	—	22	100.9 (46.7)	116.9 (96.0)	NS	

STAT, signal transducers and activators of transcription; GEE, generalized estimating equation; sTDT, sib-transmission/disequilibrium test; SD, standard deviation; HOMA, homeostasis model assessment; SiM, insulin sensitivity measure; NS, not significant.

types of 90 Centre d'Etude du Polymorphisme Humain: European parent-offspring trio subjects were downloaded from HapMap into Haploview (available at: <http://www.broad.mit.edu/mpg/haploview/>). The integral Tagger program (available at: <http://www.broad.mit.edu/tagger>) was then used to select five tSNPs that capture common variants (MAF >5%) at a minimum r^2 of 0.8, using the aggressive multimarker tagging mode. This provides not only individual tSNPs but also multimarker combinations (haplotypes composed of 2 or 3 markers), which capture additional variants. As shown in Supplementary Table 2 (available online at the *Obesity* website, www.obesityresearch.org), 9 of the 25 common variants are tagged by the haplotypes constructed by rs7211777 and rs6503695.

Genotyping in Cohorts

The tSNPs were genotyped by Pyrosequencing (Biotage, Uppsala, Sweden). Genotyping accuracy as assessed by inclusion of duplicates (50 pairs of MZ twins) in the arrays was approximately 98%, and negative controls (water blanks) were included on each plate. Genotyping success rate for each tSNP varied between 87.0% and 94.8%. Primers and polymerase chain reaction conditions for tSNP genotyping in the full cohort are given in ESM Table 2.

Statistical Analyses

Hardy-Weinberg equilibrium for genotype was tested by a χ^2 test, with 1 *df* in one twin of each pair chosen at random to prevent inflated significance. Phenotypes significantly ($p < 0.05$) deviating from normal were log-transformed to obtain normal distributions before analysis. Factor analysis was used to combine strongly correlated indices of obesity into two measures: one for general obesity (serum leptin, BMI, weight, total fat mass, and percentage total fat) and one for central obesity (waist circumference, central fat mass, and percentage central fat). Insulin-resistance measures HOMA and SiM, described previously (11), were available for subsets of subjects with leptin data. Preliminary association analyses were performed using STATA 8 (StataCorp LP., College Station, TX). To reduce the likelihood of generating false-positive associations through multiple testing, single variables characterizing obesity were analyzed only if initial tests with the general and central obesity scores yielded a positive association for at least one of these combined variables. This strategy was also used for the two indices of insulin sensitivity. Association analyses were performed using Generalized Estimating Equations (12), which allow for the relatedness between twins and yield unbiased standard errors and *p* values. To limit the number of models tested for individual SNP association analyses, we first performed a 2-*df* overall test of genotypic association. Only in the presence of a significant association were additive, dominant, and recessive

models (all 1-*df*) further tested to find the best mode of inheritance. To control for population stratification, DZ twin pairs discordant for genotype were also used in sib-TDT association analysis to confirm the results of regular association tests, as described elsewhere (11). Age and menopausal status were included as covariates in the models. The program developed by Purcell et al. (13) was used to calculate the genetic power of the study. Assuming a sibling correlation of 0.3, a sample of 840 DZ pairs is adequate to detect a locus effect of 0.5% with 80% power (and $\alpha = 0.05$). The current study of 940 DZ pairs with an additional 423 MZ pairs and 50 singletons provided even greater power.

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