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A Nonsense Polymorphism (R392X) in TLR5 Protects from Obesity but Predisposes to Diabetes

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The *TLR5* gene encodes an innate immunity receptor. Mice lacking *Tlr5* (T5KO) develop insulin resistance and increased adiposity. Owing to the segregation of a dominant nonsense polymorphism (R392X, rs5744168), a portion of humans lack *TLR5* function. We investigated whether the nonsense polymorphism influences obesity and susceptibility to type 2 diabetes (T2D). R392X was genotyped in two cohorts from Saudi Arabia, a region where obesity and type 2 diabetes (T2D) are highly prevalent. The nonsense allele was found to protect from obesity ($p_{combined}$ = 0.0062; odds ratio, 0.51) and to associate with lower body mass index (BMI) ($p_{combined}$ = 0.0061); this allele also correlated with a reduced production of proinflammatory cytokines. A significant interaction was noted between rs5744168 and sex in affecting BMI ($p_{interaction}$ = 0.006), and stratification by gender revealed that the association is driven by females ($p_{combined}$ = 0.0016 and 0.0006 for obesity and BMI, respectively). The nonsense polymorphism also associated with BMI in nonobese women. After correction for BMI, the 392X allele was found to represent a risk factor for T2D with a sex-specific effect ($p_{interaction}$ = 0.023) mediated by females (p = 0.021; odds ratio, 2.60). Fasting plasma glucose levels in nondiabetic individuals were also higher in women carrying the nonsense allele (p = 0.012). Thus, in contrast to T5KO mice, loss of human *TLR5* function protects from weight gain, but in analogy to the animal model, the nonsense allele predisposes to T2D. These effects are apparently sex-specific. Data in this study reinforce the hypothesis that metabolic diseases, including T2D, are associated with immune dysregulation. *The Journal of Immunology*, **2013, 190: 3716–3720.**

The presence of a close link between metabolism and innate immunity has emerged in recent years. Thus, chronic subclinical inflammation has been associated with obesity, and inflammatory mediators were shown to have a role in promoting insulin resistance and type 2 diabetes (T2D) (1). TLRs are molecules of the innate immune system playing a fundamental role in pathogen recognition and activation of innate immune responses.

Studies in mouse models genetically deficient in *Tlr2*, *Tlr4*, or *Tlr5* have indicated that these animals are differentially susceptible to obesity and to the development of insulin resistance compared with their wild-type littermates. Specifically, 1) *Tlr4* knockout mice fed a high fat diet are protected from obesity and insulin resistance (2); 2) animals lacking *Tlr2* show either higher or lower adiposity/insulin resistance depending on the experimental conditions, suggesting that additional factors, possibly related to microbiome composition, interact with the genetic defect (3, 4); and 3) mice lacking *Tlr5* (T5KO), a receptor highly expressed in the gut mucosa, exhibit hyperphagia and develop hyperlipidemia, insulin resistance, and increased adiposity (5).

In humans, nonsynonymous variants in *TLR4* were associated with T2D and higher body fat, although these findings are not unequivocal (6, 7). Evidence linking natural variation in *TLR2* or *TLR5* with T2D or obesity is still lacking. In human African and Eurasian populations a nonsense polymorphism in *TLR5* (rs5744168, R392X) is found at low frequency (minor allele frequency of <12%) (8). The variant exerting a dominant effect was associated with increased susceptibility to Legionnaires' disease and decreased production of IL-6 (9). In this study we investigated a possible role for R392X in modulating susceptibility to obesity and T2D in subjects from Saudi Arabia, a region with a high prevalence of both conditions (10).

Materials and Methods

Patients and controls

Two independent cohorts of Saudis from the Biomarker Screening in the Riyadh Project were enrolled (Table I). Diagnosis of T2D was based on the World Health Organization proposed cut-off, that is, fasting plasma glucose ≥7.0 mmol/l or 126 mg/dl. Subjects with medical complications (coronary artery disease, nephropathy, and end-stage renal disease or liver disease) were excluded. Anthropometry included measurement of height (to the nearest 0.5 cm) and weight (to the nearest 0.1 kg); BMI was calculated as kilograms per square meter. According to the World Health Organization criteria, individuals were classified as obese when their BMI was >30 kg/m^2.

Written consent was obtained and ethical approval was granted by the Ethics Committee of the College of Science Research Center, King Saud University, Riyadh, Kingdom of Saudi Arabia.

To perform functional analyses, 250 healthy European individuals were genotyped for rs5744168. PBMCs were obtained from 12 heterozygous

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Abbreviations used in this article: BMI, body mass index; CI, confidence interval; OR, odds ratio; T2D, type 2 diabetes.

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Table I. Subject characteristics in the two cohorts

	Study Cohort		Replication Cohort	
Subject Characteristic	Obese	Nonobese	Obese	Nonobese
n	450	462	235	234
Females (%)	238 (53)	246 (53)	143 (61)	73 (31)
Age ± SD, y	49.46 ± 11.87	42.49 ± 17.64	47.34 ± 11.47	42.80 ± 16.92
BMI ± SD, kg/m^2	33.91 ± 4.06	23.94 ± 0.68	36.47 ± 4.25	22.26 ± 2.37
T2D (%)	292 (65)	158 (34)	83 (35)	79 (34)

subjects (carrying one nonsense allele; 4 females and 8 males) and for 24 GG homozygotes (carrying two functional *TLR5* genes; 12 females and 12 males) we identified; subjects homozygous for the nonsense allele were not analyzed owing to their rarity and to the dominant nature of the stop codon variant. Written consent was obtained from these additional individuals.

Genotyping and statistical analysis

rs5744168 was genotyped by allelic discrimination real-time PCR using a predesigned TaqMan probe assay (Applied Biosystems, Foster City, CA). Reactions were performed using TaqMan Genotyping Master Mix in an ABI 9700 analyzer (Applied Biosystems). Genotyping rate was >0.97 in all cohorts. Genetic association was investigated by multiple linear or logistic regression (as appropriate) using the rs5744168 genotype as the independent predictor variable. A dominant model was used in the regressions with sex and age as covariates; BMI was added as a covariate when addressing the association between T2D or fasting glucose levels and rs5744168; and T2D was accounted for when addressing the effect of R392X on obesity and BMI. An interaction term was included in the linear/logistic models to test for the interaction between R392X and sex or BMI. Allelic counts are provided in Supplemental Tables I and II.

Functional analysis

Ten milliliters of whole blood was collected in Vacutainer tubes containing EDTA (Becton Dickinson, Rutherford, NJ). PBMCs were separated on lymphocyte separation medium (Organon Teknika, Durham, NC, USA) and washed twice in PBS. Viable leukocytes were determined using a Scepter hand-held automated cell counter (Millipore, Bedford, MA). Freshly isolated PBMCs (2.5 × 10^5) were seeded in 96- well plates in triplicate and incubated for 24 h with medium alone, recombinant endotoxin-free flagellin from *Salmonella typhimurium* (40 ng/ml; InvivoGen, San Diego, CA), or LPS (10 ng/ml). Production of IL-6, TNF-α, and IL-1β was evaluated in the supernatants of the cultured PBMCs using commercial ELISA kits (R&D Systems, Minneapolis, MN) and following the procedures suggested by the manufacturer. Cytokine concentration was calculated from a standard curve of the corresponding recombinant human cytokine. Statistical analyses were performed using SPSS version 11. Differences between the groups were assessed using nonparametric analyses (Mann–Whitney *U* test). All *p* values are two-tailed.

Results

The TLR5 nonsense polymorphism is poorly tagged in genome-wide platforms

The frequency of the *TLR5* nonsense polymorphism (rs5744168, A/G) is low in Africa (3%), Europe (11%), and Asia (1%) (8), and the variant has not been included in the HapMap Project. Analysis of the 1000 Genomes Project Pilot 1 data using the SNAP utility (http://www.broadinstitute.org/mpg/snap) indicated that rs5744168 is in tight linkage disequilibrium with few variants in Europeans that were not included in commercial genotyping arrays. In Africans one single variant present in genotyping platforms (rs1100886) shows limited linkage disequilibrium for rs5744168 ($r^2 = 0.83$), whereas no data are available for Asians. R392X is thus not efficiently tagged in common genome-wide association studies.

Protection from obesity in females is conferred by the nonsense allele

We analyzed R392X in a study population of 450 obese subjects (cases) and 462 nonobese controls (Table I). The frequency of the nonsense allele was 0.039 in the whole sample. A significant deviation of rs5744168 from Hardy–Weinberg equilibrium was observed in cases ($p = 0.008$) with an excess of homozygotes; this deviation was not present in controls ($p = 0.23$). Logistic regression indicated that the nonsense allele protects from obesity ($p = 0.037$; OR, 0.55; 95% confidence interval [CI], 0.32–0.97) (Table II). In this cohort, a significant association of the nonsense allele with lower BMI was also detected ($p = 0.049$) (Table II).

Table II. Association analysis of rs5744168 with obesity and BMI

	Study Cohort		Replication Cohort		Combined	
Trait	*p* Value[a]	OR (95% CI)[b]	*p* Value	OR (95% CI)	*p* Value	OR (95% CI)
Obesity						
All	**0.037**	0.55 (0.32–0.97)	**0.010**	0.27 (0.10–0.73)	**0.0062**	0.51 (0.32–0.83)
Males	0.652	0.83 (0.38–1.83)	0.147	0.29 (0.055–1.54)	0.405	0.75 (0.39–1.47)
Females	**0.014**	0.37 (0.17–0.82)	**0.019**	0.13 (0.022–0.71)	**0.0016**	0.33 (0.17–0.66)
	p Value	BETA[c]	*p* Value	BETA	*p* Value	BETA
BMI						
All	**0.049**	−1.42	**0.003**	−4.72	**0.006**	−2.01
Males	0.749	0.30	0.203	−2.65	0.954	−0.05
Females	**0.008**	−3.06	**0.003**	−6.77	**0.0006**	−3.84
BMI (excluding obese)						
All	NS	NS	NS	NS	0.256	−0.55
Males	NS	NS	NS	NS	0.586	0.38
Females	NS	NS	NS	NS	**0.037**	−1.43

[a]The *p* values were calculated using logistic or linear regression (as appropriate) using a dominant model; nominally significant *p* values are in boldface type.
[b]OR and 95% CI refer to the nonsense allele.
[c]Regression coefficient.

Table III. Association analysis of rs5744168 with T2D and fasting plasma glucose levels

Trait	*p* Value[a]	OR (95% CI)[b]
T2D		
All	0.282	1.35 (0.78–2.34)
Males	0.328	0.68 (0.31–1.48)
Females	**0.021**	2.60 (1.16–5.82)
	p Value	BETA[c]
Plasma glucose level		
All (non-T2D)	**0.044**	0.39
Males (non-T2D)	0.515	0.18
Females (non-T2D)	**0.012**	0.64

[a]The *p* values were calculated using logistic or linear regression (as appropriate) using a dominant model; nominally significant *p* values are in boldface type.
[b]OR and 95% CI refer to the nonsense allele.
[c]Regression coefficient.

Stratification by sex revealed that the association with both obesity and BMI was driven by females (Table II).

These findings were verified in a replication cohort of 235 obese and 234 nonobese controls of the same ethnicity. These subjects are part of a larger cohort to be enrolled for a study on T2D susceptibility, aiming at recruiting both high- and low- risk (based on BMI) subjects, and thus the proportion of T2D cases is similar in obese and nonobese individuals. Both groups complied to Hardy–Weinberg equilibrium, and the frequency of the nonsense allele amounted to 0.030. Again, association with obesity ($p = 0.010$) was observed in the whole cohort and was driven by females ($p = 0.019$; OR, 0.13; 95% CI, 0.022–0.71) (Table II). The effect of R392X on BMI in women was replicated in this cohort ($p = 0.003$) (Table II).

Combination of the two independent cohorts confirmed that the nonsense allele protects from obesity ($p = 0.0062$), and that the effect is specific for females ($p = 0.0016$; OR, 0.33; 95% CI, 0.17–0.66). As expected, the association between lower BMI and R392X was observed in women but not in males in the combined cohort (Table II). In line with these results, a significant interaction in the whole cohort was detected between allelic status (dominant model) at rs5744168 and sex in the BMI association ($p_{interaction} = 0.006$), whereas the interaction *p* value did not reach full significance ($p_{interaction} = 0.090$) in the association for obesity. Furthermore, the nonsense polymorphism was associated with BMI in nonobese females, providing further evidence for the role of this variant in protecting from weight gain (Table II).

The nonsense allele is associated with diabetes predisposition and higher fasting glycemia in females

We next evaluated the role of R392X in predisposing to T2D; toward this aim, all subjects were analyzed by fitting a logistic regression including BMI as covariates. No effect was observed in the whole sample, but a significant interaction between rs5744168 and sex was observed ($p_{interaction} = 0.023$). Stratification for gender revealed a predisposing role of the nonsense allele in females ($p = 0.021$; OR, 2.60) (Table III). Fasting plasma glucose levels were measured for the 884 nondiabetic individuals in the study (control subjects from the study and replication cohorts). Linear regression controlling for BMI and age showed a significant association between the nonsense allele and fasting glucose levels in females but not in males (Table III). The interaction *p* value was not significant for this association ($p_{interaction} = 0.11$), possibly because of lack of power (only nondiabetic individuals were used for this analysis, with a consequent reduction in sample size and frequency of the nonsense allele).

The nonsense allele correlates with a lower production of proinflammatory cytokines

Finally, we verified whether the presence of R392X would modulate the production of proinflammatory cytokines. Toward this end, we stimulated in vitro PBMCs of 12 heterozygous subjects (carrying one nonsense allele) and 24 GG homozygotes (carrying two functional *TLR5* genes) with flagellin (TLR5 agonist) or LPS and evaluated IL-1β, IL-6, and TNF-α in culture supernatants. Results showed that whereas IL-1β, IL-6, and TNF-α production was comparable in LPS-stimulated cells, the production of all three cytokines following flagellin treatment was reduced in R392X heterozygous compared with homozygous individuals; these differences reached statistical significance for IL-1β and TNF-α ($p < 0.05$) (Fig. 1). No sex-specific effect was detected.

Discussion

A dominant nonsense allele makes a portion of humans deficient in *TLR5* function. Evidence indicating that T5KO mice develop in-

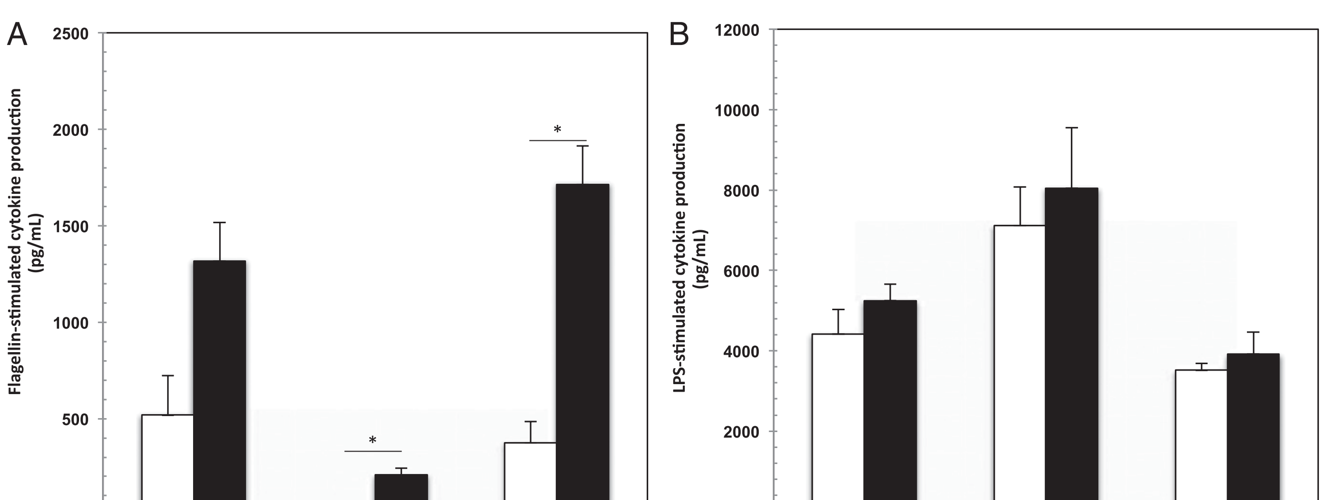

FIGURE 1. IL-6, TNF-α, and IL-1β production by PBMCs of healthy individuals who were genotyped for rs5744168. Results obtained with PBMCs of 12 AG heterozygous (carrying one nonsense allele) (open bars) and 24 GG homozygous (carrying two functional TLR5 genes) (filled bars) individuals are presented. (**A**) Results obtained upon stimulation of PMBCs with flagellin are shown. (**B**) Illustrates data in LPS-stimulated cells; background was subtracted. Mean values ± SE and statistical significance (*$p < 0.05$) are presented.

creased adiposity and insulin resistance, as well as the established role of chronic inflammation in the pathogenesis of obesity and T2D, makes the nonsense polymorphism a very good candidate for contributing to these conditions in humans. Most recent knowledge on the genetic susceptibility to obesity/overweight and T2D derives from genome-wide association studies; our analysis nevertheless indicated that R392X is likely to be poorly tagged in most large-scale studies. Thus, we analyzed the role of this variant among Saudis, a population affected by a high prevalence of both obesity and T2D (10). In contrast to the observations in knockout mice, results indicated that in two independent cohorts the *TLR5* nonsense allele is associated with protection from obesity and lower BMI. Stratification by sex revealed that the effect is driven by females, and exclusion of obese subjects from the analysis still resulted in a significant association with BMI in women.

Obesity is a risk factors for the development of T2D, although genetic susceptibility is thought to play a stronger role in nonobesity-related T2D (11). Thus, we analyzed the effect of R392X on diabetes susceptibility by taking BMI into account. Again, a significant association was detected in females, indicating that lack of *TLR5* activity results in a higher risk to develop T2D. A confirmation of this finding was obtained by analysis of fasting plasma glucose levels in nondiabetic individuals, which were higher in females carrying the nonsense allele. Notably, in T5KO mice insulin resistance is not dependent on increased adiposity, as mice undergoing caloric restriction are lean but underresponsive to exogenous insulin (5). In these animals, no sex-specific effect was reported. Several quantitative traits (including fat deposition) are nevertheless sexually dimorphic in humans and/or show sex-specific heritablity linked to the autosomes (12), thus separating the sexes or modeling for gender-based differences has been suggested in association studies (12). In fact, an interaction between gender and genetic factors has been described for other genes involved in T2D (13–16). The reasons underlying these sex-specific associations, including the one we describe for *TLR5*, remain to be elucidated and might include a role for sex hormones, epistatic effects with X-linked variants, or differences in dietary habits and lifestyle between the sexes that interact with the genetic status.

TLR activation provokes the translocation of NF-κB to the nucleus and the transcription of inflammatory mediators such as IL-1, IL-6, and TNF-α (17), resulting in the activation of the immune system and triggering of inflammatory responses. The *TLR5* gene product, in particular, recognizes bacterial flagellin (18) and is expressed in myelomonocytic cells, gut epithelial cells (19, 20), and small intestine dendritic cells residing in the lamina propria (21). Recent data obtained on Crohn's disease showed that these patients have increased immune responses to certain Ags of the microbiota. Such immune responses are originated by ligation of TLR5 by flagellin sequences from the *Clostridium* phylogenetic cluster XIVa, an important component of the intestinal microbiota. The *TLR5* nonsense allele was previously shown to be associated with markedly decreased IL-6 production in response to flagellin (9), and subjects with the *TLR5* nonsense allele produce significantly lower levels of proinflammatory cytokines (TNF-α and IL-1β) (22). These results were confirmed by analyses performed on an additional group of European healthy individuals who were genotyped for rs5744168. Thus, the production of proinflammatory cytokines was reduced upon stimulation with flagellin, but not with LPS, in subjects that were heterozygous for rs5744168. It is therefore tempting to speculate that the nonsense polymorphism in *TLR5* that is more frequent in nonobese Arabs results in protection from obesity and lower BMI as a consequence of a reduced ability of the flagellin/TLR5 interaction to induce cytokine production and chronic immune activation. In fact, proinflammatory mediators such as IL-6 and TNF have an established role in the pathogenesis of obesity and metabolic dysfunction (23).

In line with the central role of *Tlr5* in gut immune homeostasis, T5KO mice differ from their wild-type littermates in terms of intestinal microbiome composition (5, 24). This effect is thought to mediate both the metabolic phenotype of these animals and the development of spontaneous colitis in a proportion of them (5, 24). Thus, modification of the gut microbiota might also occur in humans lacking *TLR5* function and might at least partially explain the results we describe in this study. Indeed, components of the gut microbiota confer the ability to extract calories from otherwise indigestible common polysaccharides in the diet, thus affecting energy harvest from food (reviewed in Ref. 25); consistently, human obese individuals show alterations in the gut microbiota composition at the phylum level and in terms of encoded metabolic pathways (26). Also, the human gut microbiome is modified by gender (27), dietary habits (28), and possibly other lifestyle patterns, suggesting a very complex interplay between genetic and environmental effects.

Another interesting possibility is that the association we observed with T2D is accounted for by lack of *TLR5* function in Langherans islets. Indeed, the expression of TLR5 (but not of other TLRs) and of its cytoplasmic adaptor, MyD88, is upregulated in rat islet cells following glucose challenge (29). In these same cells, flagellin stimulation decreases glucose-induced insulin secretion and determines the production of both proinflammatory molecules and heat-shock chaperones (29). Because islet cells are particularly vulnerable to damage during active secretion, possibly as a result of endoplasmic reticulum stress (30), TLR5 might function as a defense during infection by downregulating insulin release, ultimately contributing to β cell homeostasis.

Further studies will be required to gain insight into the mechanisms underlying the association between the *TLR5* nonsense allele and metabolic traits. In particular, it will be extremely interesting to verify whether lack of *TLR5* function in humans alters the gut microbiome composition, as observed in mice, and whether this depends on gender or other factors. Indeed, as we noted above, sex-specific effects are relatively common in metabolic traits. Unfortunately, the reasons for these gender differences remain elusive, mainly because they are difficult to model using in vitro experiments. In fact, we detected no sex-specific effect on cytokine production following PBMC stimulation with flagellin, although this might result from the limited sample size.

In conclusion, to our knowledge this is the first case-control study to investigate the association of a common *TLR5* nonsense polymorphism with obesity/BMI and T2D; data in this study reinforce the hypothesis that metabolic diseases are associated with immune dysregulation.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Material for:

A nonsense polymorphism (R392X) in Toll-like Receptor 5 protects from obesity but predisposes to diabetes

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Supplemental Table 1. Allelic counts of rs5744168 for obese and non-obese subjects

	Study Cohort		Replication Cohort	
	Obese	Non obese	Obese	Non obese
All (A/G)	29/859	41/841	7/459	21/433
Males (A/G)	16/400	18/396	2/180	13/295
Females (A/G)	13/459	23/445	5/279	8/138

Supplemental Table 2. Allelic counts of rs5744168 for T2D and non-T2D subjects.

	T2DM	**No T2DM**
All (A/G)	48/1114	50/1478
Males (A/G)	23/589	26/682
Females (A/G)	25/525	24/796