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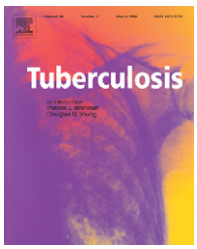

Vitamin D receptor gene polymorphisms and susceptibility *M. tuberculosis* in Native Paraguayans

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Summary
Tuberculosis (TB) is a significant health problem for most of the world's populations, and prevalence among indigenous groups is typically higher than among their nonindigenous neighbors. Native South Americans experience high rates of TB, but while research in several other world populations indicates that susceptibility is multifactorial, polygenic, and population-specific, little work has been undertaken to investigate factors involved in Native American susceptibility. We conducted a family-based association study to examine immunologically relevant polymorphisms of a candidate gene, the vitamin D receptor, in conjunction with three measures of TB status in two Native Paraguayan populations, the Aché and the Avá. This is the first large-scale genetic analysis of Native South Americans to examine susceptibility to both infection and disease following exposure to *M. tuberculosis*. These two types of susceptibility reflect differences in innate and acquired immunity that have proven difficult to elucidate in other populations. Our results indicate that among the Aché, the *Fok*I *F* allele protects individuals from infection, while the *Taq*I *t* allele protects against active disease but not infection. In particular, *FF* homozygotes are 17 times more likely to test positive for exposure to TB, but no more likely to have ever been diagnosed with active TB. *TT* individuals are 42 times less likely to mount a delayed-type hypersensitivity response, and the *T* allele was significantly more likely to have been transmitted to offspring who have been diagnosed with active TB. This ongoing research is of vital importance to indigenous groups of the Americas, because if there is a population-specific component to TB susceptibility, it will likely prove most effective to incorporate this into future treatment and prevention strategies.

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Introduction

Tuberculosis (TB) is a leading cause of death worldwide,[1] and differences in susceptibility to it have been noted for decades.[1–3] Because susceptibility to TB is multifactorial, polygenic, and displays population heterogeneity,[4–11] a broad array of human populations must be examined to elucidate commonalities and differences that may ultimately help communities and governments develop effective prevention and treatment strategies.

Few susceptibility studies have focused on Native American populations, despite extremely high TB prevalence. The Aché of Paraguay, for example, experience prevalence of infection greater than 30%, annual incidence of active pulmonary TB of 7%, and lifetime prevalence of active TB greater than 30%.[12] A complex interplay between host genetics, and environmental and cultural factors likely contribute to these high rates. The Aché present a unique opportunity to analyze the effects of these factors separately, because the entire extant population shares similar socio-economic, nutrition, and exposure conditions, and further, genealogical information has been extensively documented.[13] Here, vitamin D receptor (VDR) polymorphisms are examined in conjunction with the presence of pulmonary TB disease outcomes in the Aché and a neighboring group, the Avá.

The VDR is involved in a wide range of biological functions, including mediation of vitamin D_3 interactions with the immune system.[14–17] Among Gujarati Asians living in the UK, a dose-response effect of vitamin D_3 serum levels and risk of TB is seen,[18] and two VDR gene polymorphisms,[19] in combination with low serum vitamin D_3, were strongly associated with pulmonary TB. The first, a *Taq*I RFLP in exon 9 (allele *t*), increases stability of the mRNA, and was also associated with pulmonary TB in native Gambian populations.[8] There, the *tt* genotype was underrepresented in pulmonary TB patients. In India,[20] the *tt* genotype was associated with the less severe tuberculoid form of leprosy (another mycobacterial disease), while the *TT* genotype was associated with lepromatous leprosy. These data imply[21] an influence over the host's T-helper immune response, with *tt* assisting a shift to Th_1.

The second polymorphism, a *FokI* restriction site in exon 2, increases the length of the protein.[19] The *F* isoform has moderately higher transcriptional activity than the *f* variant.[22,23] Among UK Gujarati Asians, the *ff* genotype was associated with increased TB susceptibility, particularly extrapulmonary TB.[18] In a predominantly Native Peruvian community, individuals homozygous for *F* were significantly more likely to have positive treatment outcome than *Ff* or *ff* individuals.[1] Individuals of the *Tt* genotype were also more likely to have positive, and more rapid, treatment outcome than *TT* patients.

In south Indians, the *TaqI,* and *FokI* variants, as well as two others, a *Bsm*I and an *Apa*I RFLP both in intron 8, were examined in a case-control study involving patients with spinal TB.[24] The *BsmI Bb* genotype was found to be elevated in patients, as was the *FF.* Further, individuals with the *BbAATtFF* genotype were 7.2 times more likely to have spinal TB, and the *batF* haplotype was increased in spinal TB patients, while the *BAtf* haplotype was significantly increased in controls. In a companion study, the *FF* genotype was associated with active pulmonary TB among males,[25] while in females,[26,27] the *tt* genotype was associated with spinal TB. Although VDR data appear to conflict across populations, this may be explained by the multiple roles of vitamin D_3. Selvaraj et al.[24] suggest that higher utilization of vitamin D_3 for those with the *FF* genotype may result in lowered serum vitamin D_3, increasing risk for spinal TB.

Given the associations of the VDR *Taq*I and *Fok*I variants with TB in other populations, as well as the high prevalence of TB among Native South Americans,[12] we examined the *Apa*I, *Taq*I and *Fok*I polymorphisms in the Aché and Avá of Paraguay in relation to TB status. Among the Aché, we used a family-based analysis to test for allelic assocaition between the variants and three different TB outcomes. Among the Avá, we were able to examine a small number of individuals in conjunction with exposure to TB only.

Methods

Population

The Aché are an indigenous population from eastern Paraguay. Until the 1970s, when they came into permanent contact with outsiders, Aché lived in small mobile hunting and gathering bands of 15–70 individuals. Currently there are approximately 1000 Aché living in several agricultural settlements. Extensive historical, demographic, social, and medical data have been compiled for the Aché, including a complete genealogy for nearly the entire population.[13]

The Avá are also an indigenous population of eastern Paraguay, part of an ethnic group known as the Chiripá, a subgroup of peoples known as Guaraní. Although the Ava have been in contact with peoples of European descent for four centuries, they have maintained a distinct ethnic identity and social organization. They live in villages of 100–800 individuals and rely on horticulture for their subsistence. In contrast to the Aché, the Avá are a large group of more than 7000 living in many communities throughout eastern Paraguay.

Samples

This study was conducted with approval of the Human Research Review Committee, the University of New Mexico (HRRC 00–273 and HRRC 00-473). During several field sessions over a period of three years, 273 buccal swabs and 98 blood samples were collected from Aché and 69 buccal swabs were collected from Avá. Individuals were recruited during large community meetings without regard to TB infection or disease status, and include both sexes and all ages. Pedigree construction was performed using the Cyrillic 2.1 software package (FamilyGenetix Ltd.).

Aché individuals for whom family information was available were placed in a single large, multigenerational pedigree that included some family members for whom there are no DNA samples, although disease status was known for a few of these. There was no family information for 40 sampled individuals. Five hundred and six individuals were represented in the pedigree, comprising 208 nuclear families. Genotypes of all individuals for whom parental genotypes were available corresponded as expected—there

was no apparent nonpaternity observed in the pedigree. The pedigree contains both affected and unaffected parents, and discordant sibships. For one of the statistical tests of association (the pedigree disequilibrium test—PDT), the large Aché pedigree was subdivided into 20 smaller pedigrees (termed, in this paper, "lineages") related no more closely than at the great-grandparent level. This was done because the PDT relies on the assumption of asymptotic normality (in the number of pedigrees) to assess significance.

Determination of TB status

Infection and disease status were determined in 1992 and 1998 for 184 Aché in this sample.[12] Infection was determined with purified protein derivative (PPD) skin tests; individuals with a wheal size of 5 mm or greater were considered positive. This lower cut-off value for the test reflects the documented tendency of Aché to be unresponsive to PPD.[12] Here, individuals are considered to have positive PPD status if they were positive in either 1992 or 1998. Disease status was determined using clinical data (e.g., clinical examination, radiology and bacteriology).[12] Anyone who had TB in either year was considered positive for disease.

All buccal swab samples were tested three times for the presence of *M. tuberculosis* DNA. The target, *IS*6110, is diagnostic of the *M. tuberculosis* complex, and has been approved for testing of other types of body fluids for TB.[28] PCR status was assigned based on number of tests positive out of 3 (either 0, 1, 2 or all 3). Each of the four possible PCR statuses was analyzed separately, as test sensitivity and specificity are still being determined. However, a companion study underway with nonhuman primates of known disease status suggests that samples with even a single positive test should be considered positive for TB.

Laboratory methods

Amplification

DNA was extracted using a standard phenol-chloroform procedure.[29] All fragments were amplified using a standard PCR protocol comprising 1 U Amplitaq Biosystems Taq Gold and 1 × buffer, 3–4 mM $MgCl_2$, 0.4 μM each primer, and 0.6 nM dNTPs, for a total 30 μl PCR volume. For all buccal swab samples, a target 123 base-pair fragment of the repetitive element IS*6110* from *M. tuberculosis* complex organisms,[30] was amplified following Eisenach et al.[31] To avoid contamination from previous amplifications, all PCR reactions were set up in a separate laboratory in which no previous DNA work has ever been performed. DNA was then added to the reaction tubes in the main lab as a final step.

A 372 bp segment containing the *TaqI* and *Apa*I polymorphisms was amplified using the forward primer 5′-CACAGATGTGAAGGCTGGTG-3′ and the reverse primer 5′-AGGCGGTCCTGGATGG-3′ and an annealing temperature of 56° with a touchdown procedure for a total of 30 cycles. The 268-bp segment of exon 2 containing the *FokI* polymorphism was amplified following Ref.[22]

Sequencing and genotyping

To determine the presence of private polymorphisms among the Aché and Avá, a subset of samples were sequenced. Samples from 41 Aché were selected as follows: both unaffected and affected individuals from small "family" groupings with two parents and at least one affected child, and every "lineage" in the single large Aché pedigree was represented by at least one sampled individual. Six Ava individuals were chosen from the box of samples at random for sequencing as well. Sequencing was performed by the Arizona State University Core DNA Lab on an ABI 377 sequencer and analyzed using the SeqManII 5.03 Sequence Analysis Software (DNASTAR Inc.). All sequencing reactions were performed in both directions. Samples that were not sequenced were genotyped using the restriction enzymes *TaqI* and *FokI* (New England Biosystems) following manufacturer's specifications.

Analytical methods

Two different types of analyses were conducted in order to examine the data, and we emphasize that these are not independent. Rather, each is a different way of analyzing limited data on a small, but important, sample. First, odds ratios were calculated using EpiInfo version 3.3.2 to determine if there was any association between TB status and genotype using data from the Aché and the Avá. Then, in the Aché individuals for whom genealogical information was available, two types of family-based tests for linkage and allelic association were used to determine if particular alleles are associated with TB. Association analyses based on the transmission-disequilibrium test (TDT) were implemented because of appropriateness to the type of data: family-based sample, various configurations of parents and offspring and/or discordant sibships, and candidate markers identified from linkage studies in other populations. The classic TDT[32] tests for linkage by comparing transmission and nontransmission of marker alleles to affected offspring. If the marker and disease locus are unlinked, the probability of marker allele transmission from heterozygous parents should be 0.5. The original TDT requires nuclear family groups with both parents and a single affected child, but extensions and variations have been formulated.[33] One problem with larger pedigrees is that most variations of the TDT, while valid tests of linkage, are not valid tests of allelic association when families are related—even in the absence of allelic association in the population, genotypes of related individuals are correlated if there is linkage,[34] and the various sibships or nuclear families in the pedigree thus cannot be treated as independent.

The PDT[34,35] uses the nuclear family triads (two parents and an affected child) and/or discordant sibships as the independent units. A measure of linkage disequilibrium (LD) is calculated for each of these within each pedigree, and then LD is the average of those quantities. Two types of PDT were calculated in this study: the avePDT, which averages over all informative units (giving equal weight to all families) and the sumPDT, which sums over all informative units (giving more weight to pedigrees with more informative units). Under the assumption of high disease prevalence (30%), the PDT is more powerful than other types of TDTs,[35]

particularly relevant for this study, in which at least 30% of Aché are infected with *M. tuberculosis*.

Because the PDT software employs the pedigree as the unit of analysis, the single large Aché pedigree was divided into 20 smaller pedigrees as described above. Within each sub-pedigree, PDT calculates the statistics based on informative families, which are defined in this study as either nuclear families with at least one affected member and at least one parent heterozygous at the marker of interest, or discordant sibships with at least one affected and one unaffected sibling with different marker genotypes.

The family-based association test (FBAT) was also implemented. The genotype distribution observed in affected offspring is compared to the expected distribution under the null hypothesis of no linkage and no association, or of no association in the presence of linkage.[36,37] Offspring genotypes are treated as random variables conditioned on all phenotypes and parental genotypes, which avoids confounding due to genetic model specification, admixture, or population stratification.[36,38,39] Although there is also a haplotype-based variation of the FBAT (HBAT), there was an insufficient number of informative families with each haplotype for the statistic to be calculated.

Results

Genotypes

Forty-one Aché and six Avá samples were sequenced at the exon 2 fragment containing the *Fok*I polymorphism and at the intron 8/exon 9 fragment containing the *Taq*I and *Apa*I polymorphisms. No further variation was found in the exon 2 fragment in either population, and all samples were then typed for presence/absence of the restriction site using the *Fok*I enzyme. In all cases for which there were both sequencing and restriction digest results, these were identical. Table 1 presents the distribution of *Fok*I genotypes among Aché and Avá.

In intron 8 and exon 9, both Aché and Avá show variation. Both populations are polymorphic at the *Apa*I site in intron 8 and at the *Taq*I site in exon 9. Among Aché, there is also a polymorphism at nucleotide 1237 not previously noted in the literature. All remaining Aché samples were thus sequenced at the intron 8/exon 9 fragment. Table 1 presents the distribution of intron 8/exon 9 polymorphisms determined by direct sequencing for all Aché and for six Avá. Remaining Avá samples were genotyped at *Taq*I using the restriction digest.

At *Taq*1 there is a significant difference in genotype frequencies between Aché and Avá ($\chi^2 = 12.128$; df $= 2$; $p = 0.002$). There are more Aché heterozygotes than expected by chance, while there are more Avá *TT* homozygotes. Correspondingly, calculations of heterozygosity[40] reflect this: among Aché $H = 0.43$, compared to Avá, with a much lower H ($= 0.03$). Among Aché, in addition to the *Apa*I G to T transversion of intron 8, there is also a G to C transversion at the same nucleotide. Both variants obliterate an *Apa*I site, although only the former is the *Apa*I polymorphism previously noted. Presence of a G is termed the *A* allele, while presence of the T is the *a* allele. Here, presence of a C at this site will be termed *a*′. As can be seen in Table 1, 93% of the Aché are heterozygous for *a*; and in fact, 98% of this sample has at least one copy of *a*. All of the six Avá sequenced at the *Apa*I site had the *aa* genotype.

Table 1 Distribution of genotypes among Aché and Avá.

	Aché	Avá
*Fok*I		
ff	1 (<1%)	4 (6%)
Ff	87 (33%)	11 (16%)
FF	176 (67%)	52 (78%)
Total	264	67
*Taq*I		
tt	18 (7%)	1 (2%)
Tt	116 (48%)	18 (30%)
TT	108 (45%)	42 (68%)
Total	242	61
*Apa*I		
aa	219 (93%)	6 (100%)
aa′	2 (1%)	0
Aa	10 (4%)	0
AA	5 (2%)	0
Total	236	6
nt. 1237		
CC	172 (73%)	6 (100%)
CG	38 (16%)	0
GG	26 (11%)	0
Total	236	6

At nucleotide 1237 of intron 8, there is a C to G transversion among Aché at an allele frequency of $G = 0.19$. This variant was not noted among the six Ava sequenced at intron 8/exon 9. Frequencies of the intron 8 polymorphism in Aché are presented in Table 1, and in Table 2, the genotype frequencies for all polymorphisms examined in this study are presented according to TB status measure.

Using the FBAT software, LD was measured for all four pairs of markers in Aché based on the allele frequencies using two measures. Using D, a score of zero indicates linkage equilibrium between two markers (and thus scores different from zero indicate LD). The standardized measure of LD |D′| was also calculated; here, a score of 1 indicates perfect LD. The pairwise LD (|D′|) matrix is presented in Table 3. Intron 8 markers *Apa*I and nt.1237, as expected given their proximity, are closely linked (|D′| indicates perfect LD).

Association tests

FBAT test statistics for PPD status are presented in Table 4. Among Aché, the *f* allele is significantly more often transmitted from heterozygous parents to PPD positive offspring, with a *Z*-score of 2.641 and $p = 0.008$. Significant associations were not found between any marker and previous disease status, or for any measure of PCR status.

Table 5 shows that for disease status, there is a marginally statistically significant association between *Taq*I using the avePDT and disease: the *T* allele was more likely to be transmitted to an affected child, or to be present in an

Table 2 Distribution of genotypes at *Fok*I and *Taq*I Among Aché and Avá by TB status.

	Aché		Aché		Aché		Avá	
	PPD +	PPD −	Disease +	Disease −	PCR +	PCR −	PCR +	PCR −
ff	0	1	0	1	0	1	1	3
Ff	25	36	19	42	5	69	2	9
FF	42	75	35	81	19	44	10	40
Total	67	112	54	124	24	114	13	52
	179		178		138		65	
tt	4	5	4	5	2	16	0	1
Tt	41	46	28	58	11	101	5	13
TT	22	59	22	59	10	99	7	34
Total	67	110	54	122	23	216	12	48
	177		176		239		60	

Table 3 Pairwise linkage disequilibrium (|D′|) matrix for Aché markers.

	*Apa*I	nt. 1237	*Taq*I
nt. 1237	−0.003(1.00)		
*Taq*I	−0.001(0.006)	−0.039(0.41)	−0.002(0.03)
*Fok*I	0.0000(0.06)	0.011(0.41)	−0.002(0.03)

affected sib. No significant associations were found for PPD or PCR status.

Odds ratios calculated to compare genotype vs. TB status are summarized in Tables 6 and 7 for Aché and Avá subjects, respectively. Disease and PPD status were available for 178 Aché samples genotyped at *Fok*I. Individuals of any *Fok*I genotype were equally likely to have been diagnosed with active disease or to have had a positive PPD test. PCR status was available for 138 Aché and 64 Avá. Aché with at least one copy of *f* were17 times (OR = 0.17; 95% confidence interval: 0.05, 0.52; $p<0.001$) less likely than *FF* homozygotes to have three PCRs positive for TB, indicating that they were significantly more likely to have *M. tuberculosis* DNA in their mouths.

Disease and PPD status were available for 176 Aché, and PCR status was available for 240 Aché and 60 Avá, for whom *Taq*1 genotype was determined. Because *tt* was associated with resistance to TB in other populations, odds ratios were calculated using the *tt* genotype as the resistant group. For Aché PPD status, individuals with the *TT* genotype were 42 times (OR = 0.42; 95% confidence interval: 0.21, 0.83; $p = 0.007$) less likely than those with at least one copy of *t* to respond to PPD (excluding *tt* homozygotes changes only the upper bound of the confidence interval to 0.84 and $p = 0.008$). No association was found between active disease or PCR status and *Taq*I genotype, but among the Aché and Avá a similar (nonsignificant) trend is seen for *TT* homozygotes to be less likely to have three positive PCRs.

Disease and PPD status were also available for 163 Ache for whom intron 8 was typed, and PCR status was available for 117 of them. At nt. 1237, no associations were found using FBAT, PDT or the odds ratios. Variation at the *Apa*I site was sufficiently minimal (see Table 1) that it was not possible to perform any tests for association there.

Discussion

No associations were found between VDR polymorphisms and Avá TB status. At the *Taq*I site, no significant differences were noted by any measure of PCR positivity, but the *T* allele was more likely to have been transmitted to Aché offspring who had positive active disease. Individuals with the *TT* genotype, however, are significantly less likely to have a positive PPD than those with a copy of *t*. The PCR results indicate that Aché of all *Taq*I genotypes were equally likely to be exposed to *M. tuberculosis*. Further, it is known that all Aché are exposed to TB, but many are PPD negative, even those vaccinated with BCG.[12] Thus, positive response to PPD may be a sign of better health, as individuals with a *t* are displaying an appropriate cell-mediated immune response, and thus the association of *t* with positive PPD is neither contradictory with data from other populations, nor with the association of the *T* allele and positive disease status.

At the *Fok*1 site, Aché with at least one copy of allele *f* are significantly less likely than *FF* homozygotes to have three positive PCRS, but no more likely to have been diagnosed with active disease or to respond to PPD. Further, heterozygous parents were significantly more likely to have passed a copy of the *f* allele to PPD positive offspring. Thus, there is a trend for *FF* individuals to harbor *M. tuberculosis* DNA in their mouths, but they are no more likely to have active disease: perhaps the *F* allele protects against infection (assessed by PPD status) and thus disease, while the *f* allele does not. While the two types of analyses (odds ratios and FBATs) are not independent, these results suggest that this area will be important for future research.

There is a high likelihood that frequencies of alleles conferring susceptibility or resistance to TB were severely altered among Aché during the period of contact.[12] Approximately 37% of the Aché population died of respiratory diseases within weeks or months of exposure to nonAché during the early 1970s.[13] While these diseases were probably not TB, it seems reasonable that

Table 4 FBAT test statistics calculated for each marker: Aché PPD status.

Marker	Allele	#Fam	S	*E*(S)	Var(S)	*Z*	*p*
1237	*C*	14	0.571	2.516	3.092	−1.106	0.268584
1237	*G*	14	7.713	5.767	3.092	1.106	0.268584
*Taq*I	*T*	30	−0.721	2.006	4.137	−1.341	0.180046
*Taq*I	*t*	30	4.001	1.274	4.137	1.341	0.180046
*Fok*I	*F*	21	0.489	4.588	2.408	−2.641	0.008260
*Fok*I	*f*	21	5.724	1.626	2.408	2.641	0.008260

Fam: number of informative families, *S*: test statistic, *E*(*S*): expected test statistic, Var(*S*): variance of *S*, *Z*: standard normal variable. *p*: probability

Table 5 PDT test statistics calculated for Aché active disease status.

Marker	#Fam	#Tr	#DSP	sumPDT		avePDT	
				ChiSq	*p*	ChiSq	*p*
1237	8	5	25	0.576	0.4477	0.382	0.5367
*Apa*I	8	7	27	1.256	0.2623	0.273	0.6015
*Taq*I	8	6	26	1.286	0.2568	0.302	0.5824
*Fok*I	6	6	22	2.273	0.1317	2.951	0.0858

#Fam: number of pedigrees with informative families, #Tr: number of triads, #DSP: number of discordant sib pairs.

Table 6 Summary of odds ratios by genotype: Aché.

	ff/*Ff*†	*FF*	*TT*	*Tt*	*tt*
3 PCRs	0.17**	1.00	0.81	0.87	1.00
PPD	1.21	1.00	0.42*	1.00†	1.00†
Active disease	1.02	1.00	0.47	0.60	1.00

*$p<0.01$
**$p<0.001$
†*ff*/*Ff* or *Tt*/*tt* combined due to sample size.

Table 7 Summary of odds ratios by genotype: Avá.

	ff/*Ff**	*FF*	*TT*	*Tt*/*tt**
3 PCRs	1.00	1.00	0.58	1.00

No tests were statistically significant at $p<0.1$
**ff*/*Ff* or *Tt*/*tt* combined due to sample size.

susceptibility to TB may share common immune and genetic components with susceptibility to other infectious respiratory diseases. By 1977, the worst of the TB epidemic was over, and prompt medical intervention since then has minimized the number of Aché deaths.[12] An assessment of Aché and Avá in light of Hardy–Weinberg proportions indicates that at the *Fok*I site, there are significant differences between expected and observed genotype frequencies in both populations. Among Avá there is an excess of the *FF* genotype, and a deficit of heterozygotes and *ff* homozygotes ($0.02<p<0.025$). Among Aché, there is an excess of heterozygotes, and a deficit of both homozygotes ($0.02<p<0.025$). The frequency of the *ff* genotype is the lowest observed in the world thus far (Table 8), even compared to Native Peruvians, and Avá and Gujarati Asians have the next lowest frequencies. While it is tempting to speculate that this may reflect selection among Aché, it is equally possible that it is an artifact of population history.

This is the first large-scale genetic study of Native South Americans to examine susceptibility to both infection and disease following exposure to *M. tuberculosis*. These two types of susceptibility reflect differences in innate and

Table 8 Frequency of VDR genotypes in various populations.

Population	*Fok*I			*Taq*I		
	FF	*Ff*	*ff*	*TT*	*Tt*	*tt*
Italian males[41]	0.370	0.500	0.130			
French[42]				0.345	0.51	0.145
British females[43]	0.357	0.481	0.162			
US females, mostly white[44]				0.437	0.437	0.126
US, mostly white[45]	0.360	0.494	0.146	0.364	0.460	0.176
African (Gambian)[8]				0.480	0.43	0.090
Gujarati[18]	0.609	0.338	0.053	0.406	0.502	0.092
Indian[25]				0.420	0.430	0.150
Indian[20]				0.431	0.446	0.123
Korean females[46]	0.393	0.437	0.170			
Chinese[47]				0.952	0.048	0.00
Han Chinese[48]				0.904	0.096	0.00
Taiwanese females[49]	0.423	0.337	0.239			
Taiwanese females[50]				0.899	0.100	0.00
Japanese[51]				0.74	0.26	0.00
Japanese[52]				0.657	0.313	0.030
Peruvian[11]	0.083	0.349	0.568	0.873	0.124	0.003
Aché	0.670	0.330	0.004	0.441	0.513	0.046
Avá	0.776	0.164	0.060	0.689	0.311	0.00

acquired immunity. Among the Aché, both polymorphisms are associated with protection against infection (PPD status), while the *Fok*1 variant is associated with exposure (PCR status), and the *Taq*1 variant is associated with active disease. Unfortunately, the Avá sample size was too small to detect any significant associations. It is interesting, however, that Aché are more heterozygous at the *Taq*1 site than Avá. Although the Aché population is small, this could reflect population subdivision[13] in the recent past or it could be the result of balancing selection. Both explanations are plausible: population substructure is well documented, as are the multiple effects that the VDR exerts on crucial biological functions.

Based on our data, we hypothesize that individuals with at least one copy of the *Taq*1 *t* allele are better able to mount an appropriate cell-mediated immune response to *M. tuberculosis* exposure. We also hypothesize that the *Fok*1 *F* allele protects against infection (assessed by PPD status) and thus disease, while the *f* allele does not, and thus the extremely low frequency of the *ff* genotype could reflect selection. Further work is underway to test these hypotheses with genetic, cultural, environmental, nutritional and historical data.

This ongoing research is important, because if there is a population-specific component to TB susceptibility, it may prove effective to incorporate this into treatment and prevention strategies. The Aché may be representative of other long-isolated forest hunter-forager groups in the spectrum of pathogens that they typically encountered during their history and even today. Assuming that immune functions were programmed by pathogens that exerted a selective effect on the population over time, and given that an individual's own pathogen-encounter history influences future immune responses, Aché immune genetics may be very similar to those of other Native South American forest communities. As a recently contacted group, the Ache provide a means of examining the complex and interacting effects of changing subsistence systems, settlement/mobility patterns, socio-economic status, environmental stressors, and introduced diseases on a population.

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