

# Loss of Function of Transient Receptor Potential Vanilloid 1 (TRPV1) Genetic Variant Is Associated with Lower Risk of Active Childhood Asthma\*

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Transient receptor potential cation channels of the vanilloid subfamily (TRPV) participate in the generation of  $\text{Ca}^{2+}$  signals at different locations of the respiratory system, thereby controlling its correct functioning. TRPV1 expression and activity appear to be altered under pathophysiological conditions such as chronic cough and airway hypersensitivity, whereas TRPV4 single nucleotide polymorphisms (SNP) are associated with chronic obstructive pulmonary disease. However, to date, there is no information about the genetic impact of either TRPV1 or TRPV4 on asthma pathophysiology. We now report on the association of two functional SNPs, *TRPV1-I585V* and *TRPV4-P19S*, with childhood asthma. Both SNPs were genotyped in a population of 470 controls without respiratory symptoms and 301 asthmatics. Although none of the SNPs modified the risk of suffering from asthma, carriers of the *TRPV1-I585V* genetic variant showed a lower risk of current wheezing (odds ratio = 0.51;  $p = 0.01$ ), a characteristic of active asthma, or cough (odds ratio = 0.57;  $p = 0.02$ ). Functional analysis of *TRPV1-I585V*, using the  $\text{Ca}^{2+}$ -sensitive dye fura-2 to measure intracellular  $[\text{Ca}^{2+}]$  concentrations, revealed a decreased channel activity in response to two typical TRPV1 stimuli, heat and capsaicin. On the other hand, *TRPV4-P19S*, despite its loss-of-channel function, showed no significant association with asthma or the presence of wheezing. Our data suggest that

genetically determined level of TRPV1 activity is relevant for asthma pathophysiology.

TRPV1<sup>2</sup> and TRPV4 are nonselective cation channels, members of the vanilloid subfamily of transient receptor potential cation channels (1). TRPV1 is expressed primarily on nociceptive neurons and can be activated by capsaicin, noxious heat, and protons (2). The widely distributed TRPV4 cationic channel participates in the transduction of mechanical and/or osmotic stimuli in different tissues (3, 4). TRPV4 channels also respond to heat, acidic pH, and endogenous arachidonic acid metabolites (5, 6).

Both channels are expressed in airway sensory nerves (TRPV1) (7) and epithelial (8, 9) and smooth muscle cells (TRPV4) (10). As integrators of different physical and chemical stimuli, they participate in the generation of  $\text{Ca}^{2+}$  signals (11, 12) that contribute to airway defense mechanisms such as cough (13, 14) and mucociliary clearance (9), but TRPV1 and TRPV4 also show a pathophysiological downside. TRPV1 activity has been related to different aspects of chronic respiratory disease such as neurogenic inflammation (15, 16), irritant-induced chronic cough (13, 14), and airway hypersensitivity (17). TRPV4 activation disrupts alveolar barrier in animal models (18) and has been associated with chronic obstructive pulmonary disease in humans (19). Together, these evidences make *TRPV1* and *TRPV4* interesting candidate genes for asthma. To understand their implication in asthma pathophysiology, *i.e.* whether the etiology or the pathological symptoms are associated with a gain or loss of channel function, we have only considered two nonsynonymous variants that may alter channel activity.

## EXPERIMENTAL PROCEDURES

**Population Characteristics**—Data for this analysis were obtained from the Childhood Respiratory Health Study (CRHS). The study was conducted in two phases (I and II). Phase I had a population-based cross-sectional design and was carried out in the cities of Barcelona and Sabadell (Spain). Parents of 12,382 children aged 7–8 and registered in all existing primary schools (public and private) were invited to participate. For a total of 10,821 children, a parent self-administered questionnaire was completed (response rate 87.4%). The questionnaire was based on the International Study on Asthma and Allergies in Childhood (ISAAC) (20).

Phase II had a two-stage population-based case-control study design. From Phase I completed questionnaires, children were classified in different groups according to their answers to respiratory symptom questions. Parents of all children with asthma diagnosed by a doctor (cases) and a random sample of children free of symptoms (controls) were invited to complete a self-administered questionnaire including questions on respiratory symptoms, drugs prescribed for asthma/wheeze, rhini-

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<sup>2</sup> The abbreviations used are: TRPV, transient receptor potential cation channels of the vanilloid subfamily; OR, odds ratio; CI, confidence interval.

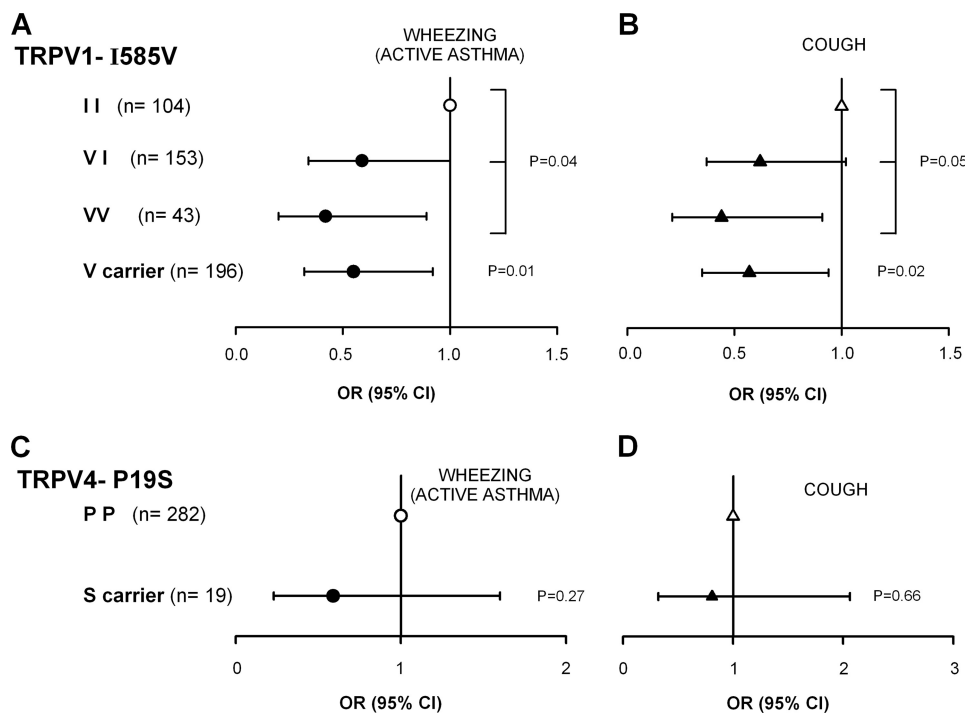


FIGURE 1. Risk of wheezing and cough by TRPV1 and TRPV4 genotype in asthmatics. A–D, crude odds ratio (95% CI) of wheezing (A and C) and cough (B and D) as a function of TRPV1-I585V (A and B) and TRPV4-P19S (C and D) by multivariate logistic regression analysis. Adjusted ORs did not vary significantly from the crude ones. For TRPV1, values are: II,  $n = 104$  (76 of whom presented wheezing and 69 cough); VI,  $n = 153$  (94 of whom presented wheezing and 84 cough); VV,  $n = 43$  (23 of whom presented wheezing and 20 cough); V, carrier,  $n = 196$  (117 of whom presented wheezing and 79 cough). For TRPV4, values are: PP,  $n = 282$  (184 of whom presented wheezing and 163 cough); S carrier,  $n = 19$  (10 of whom presented wheezing and 10 cough). II, Ile-Ile; VI, Val-Ile; VV, Val-Val; V carrier, Val carrier; PP, Pro-Pro; S carrier, Ser carrier.

tis, eczema, and use of health care services. A total number of 2070 completed questionnaires for asthma cases and 1014 for controls were obtained with a response rate of 79.2 and 78.7%, respectively. For the purpose of the present study, a total of 811 children were included for the genetic analysis. Of the total number of genotyped children, those with asthma diagnosed by a doctor and from whom information about wheeze in the last 12 months (194 children presented wheeze in the last 12 months, while 107 children with diagnosed asthma did not present wheeze in the last twelve months) and control children free of symptoms ( $n = 470$ ) were included in the association study of clinical phenotype with TRPV1 and TRPV4 genotypes. Ethical approval was obtained from the IMIM-Universitat Pompeu Fabra-Hospital del Mar Ethics Committee, and written consent was obtained from each participant.

**SNP Detection**—TRPV1 and TRPV4 single nucleotide polymorphisms (SNPs) (rs8065080 and rs3742030, respectively) were genotyped using SNPLex (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, DNA was fragmented at 99 °C for 10 min, and 150 ng of fragmented DNA were dispensed in a 384 microplate and followed by phosphorylation, oligonucleotide ligation, exonuclease cleanup, PCR, and hybridization steps using liquid-handling robots. Finally, the specifically bound fluorescent probes were eluted and analyzed on an Applied Biosystems 3730xl DNA analyzer. Genotypes were analyzed with GeneMapper 3.5 software.

**Plasmids and Cell Transfection**—Human TRPV1-Ile-585 construct was generated by PCR from the original pCAGGSM2-IRES-

GFP-R1R2TrpV1 vector encoding TRPV1-Val-585 variant (a gift from B. Nilius, Leuven, Belgium). Site-directed mutagenesis primers were 5'-CCCGAACAAGAAGACGATGTAGACAAACATGAAACGGC-3' and 5'-GCCGTTTCATGTTTGTCTACATCGTCTTCTTGTTCGGG-3'. Mutant fragments were analyzed by sequencing with ABiPrism 3.1 BigDye kit and subcloned into the original vector using SbfI restriction sites flanking the mutated region. Subcloned constructs were sequenced for final verification.

HeLa cells were transiently transfected with ExGen500 (Fermentas MBI) following the manufacturer's instructions. Cells were transfected with pIRES-GFP-TRPV1-human (1  $\mu$ g) cDNA or pEGFPN1 (1  $\mu$ g) as described previously (21).

**Measurement of Intracellular  $[Ca^{2+}]$** —Cytosolic  $Ca^{2+}$  signal was determined at room temperature in cells loaded with 4.5  $\mu$ M fura-2 AM (20 min) as described previously (4). Cytosolic  $[Ca^{2+}]$  increases are presented as the ratio of emitted fluo-

rescence (510 nm) after excitation at 340 and 380 nm, relative to the ratio measured prior to cell stimulation (fura-2 ratio 340/380). All experiments were carried out at room temperature, except for the short periods of stimulation with solutions at 50 °C. Cells were bathed in an isotonic solution containing (in mM): 140 NaCl, 5 KCl, 1.2  $CaCl_2$ , 0.5  $MgCl_2$ , 5 glucose, 10 HEPES (300 mosmol/liter, pH 7.4, with Tris). All chemicals were obtained from Sigma.

**Statistical Analysis**—An exact test was used to evaluate Hardy-Weinberg equilibrium for individual SNP genotypes in all individuals (22). To test the hypothesis of association between genetic polymorphisms and cases with asthma, multivariate logistic regression models were used. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated assuming homozygosity for the more common allele among controls as the reference class. Analyses were also adjusted for sex, force expiratory volume in 1 s, passive smoking, treatment, family history of asthma, and older siblings. All analyses were performed by using the SNPAssoc R package (23).

Functional TRPV1 data were expressed as means  $\pm$  S.E. of  $n$  number of cells analyzed. Statistical analysis was performed with one-way analysis of variance using the OriginPro software. Tukey's tests were used for post hoc comparison of means. The criterion for a significant difference was a final value of  $p < 0.05$ .

## RESULTS AND DISCUSSION

**Population-based Genetic Association Studies**—An SNP corresponding to an isoleucine-to-valine mutation at position 585

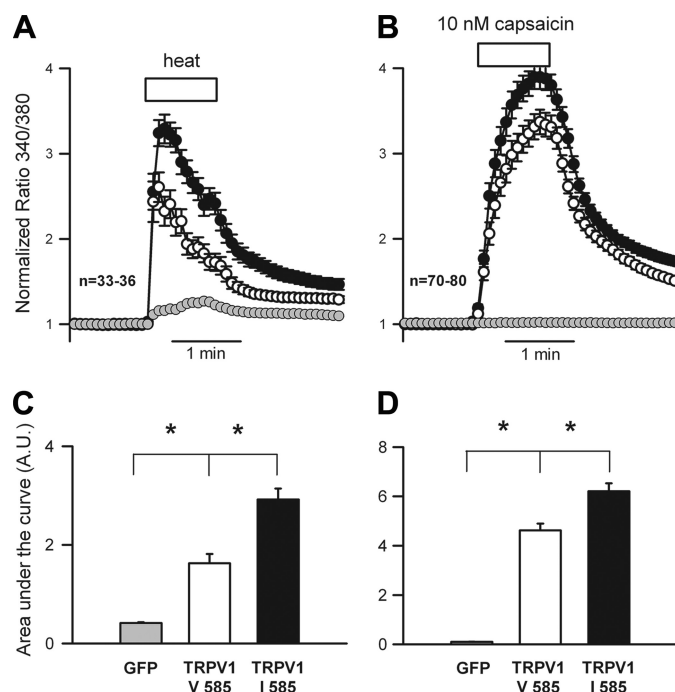
## REPORT: TRPV1 Genetic Variant and Asthma

of the TRPV1 protein (rs8065080; *TRPV1-I585V*) (24) and an SNP generating a proline-to-serine mutation at amino acid 19 of the TRPV4 protein (rs3742030; *TRPV4-P19S*) (25) were evaluated for their association with asthma. The most frequent alleles, *TRPV1-Ile-585* (61% of the population) and *TRPV4-Pro-19* (97% of the population), were used as the reference alleles. We first tested for association between diagnosed asthma and the presence of *TRPV1-I585V* and *TRPV4-P19S* SNPs by multivariate logistic regression analysis. Asthma was not associated with *TRPV1-I585V* allele neither in homozygous (OR, 0.96, 0.62–1.49 95% CI,  $n = 122$ ) nor in heterozygous children (OR, 1.17, 0.86–1.60 95% CI,  $n = 392$ ) when compared with noncarriers ( $n = 297$ ;  $p = 0.4$ ). Similarly, the OR of *TRPV4-P19S* carriers (1.39, 0.75–2.58;  $n = 43$ ; homozygous and heterozygous were pulled together due to the low number of the former) was not different ( $p = 0.29$ ) from noncarriers ( $n = 770$ ). Next, within asthmatics, we evaluated the association of these SNPs with the presence of wheezing or cough within the last year. The magnitude and direction of the association (OR < 1 implies a decrease risk) were consistent with a progressive protective effect of the *TRPV1-Val-585* variant against the presence of wheezing (Fig. 1A) or cough (Fig. 1B) among asthmatics. By contrast, *TRPV4-P19S* showed no significant association with either current wheeze or current cough (Fig. 1, C and D). We also tested whether *TRPV1-Val-585* was associated with other outcomes among asthmatics including lung function (force expiratory volume in 1 s and force vital capacity), atopy (Phadiatop test), and bronchial responsiveness (hypertonic saline serum) but found no association (results not shown).

**Functional Analysis of TRPV1-I585V Variant**—To evaluate the functional consequences of the genetic variants, we expressed TRPV1-Val-585 and TRPV1-Ile-585 in HeLa cells. TRPV1 channel activation by typical stimuli such as heat (>43 °C) and capsaicin, the main ingredient in hot chili peppers and a potent tussive agent, triggers  $\text{Ca}^{2+}$  entry into the cells. Thereby, we assessed TRPV1 channel activity using fluorescence microscopy imaging techniques in cells loaded with the calcium sensor fura-2. No significant differences in basal calcium levels were detected among the transfected cells (results not shown). However, cells transfected with TRPV1-Ile-585 responded to heat (50 °C; Fig. 2A) and capsaicin (10 nM; Fig. 2B) with significantly higher increases in intracellular  $[\text{Ca}^{2+}]$  than those transfected with TRPV1-Val-585. A quantitative analysis of the  $\text{Ca}^{2+}$  signal, calculating the mean area under the curve as an indicator of the magnitude of the  $\text{Ca}^{2+}$  signal, is shown in Fig. 2, C and D.

The impact of TRPV4-P19S mutation on TRPV4 channel activity has already been evaluated. TRPV4-P19S induces a loss of channel function in response to mild hypotonic stimuli and has been recently associated with hyponatremia (25).

Our combined functional and population-based genetic epidemiological studies provide evidence for the involvement of TRPV1, but not TRPV4, in symptoms typically associated to asthma: wheezing and cough. We also acknowledge that given the low frequency of *TRPV4-P19S*, the statistical power to detect its real impact on asthma pathophysiology is also low. TRPV1 is generally viewed as a molecular integrator of nociceptive stimuli and inflammatory reactions. In the lung,  $\text{Ca}^{2+}$  plays



**FIGURE 2. Expression and functional analysis of TRPV1-Val-585 and TRPV1-Ile-585 in HeLa cells.** A, changes in cytosolic  $[\text{Ca}^{2+}]$  (normalized fura-2 ratio) evoked by heating the bathing solution to 50 °C (for the time indicated) in cells transfected with control GFP (●), TRPV1-Val-585 (○), and TRPV1-Ile-585 (●). B, average of areas under the curves derived from the experiment in A. C, changes in cytosolic  $[\text{Ca}^{2+}]$  generated by exposing the cells to 10 nM capsaicin. A.U., arbitrary units. D, average of areas under the curves derived from the experiment in C. Data are expressed as the mean  $\pm$  S.E. ( $n$  values shown in the figure). \*,  $p < 0.05$  one-way analysis of variance.

a crucial role in the activation of almost all cells. In fact, TRPV1-mediated  $\text{Ca}^{2+}$  entry in response to irritants and endogenous activators triggers pulmonary chemoreflexes leading to bronchoconstriction, mucus hypersecretion, airway irritation, and cough (17). It also appears to be responsible for the release of neuropeptides that favor the inflammatory response (17). Besides, sensitivity to TRPV1 activators is elevated in both animal models of asthma (15) and human patients (26). Consistent with all these previous reports, we observed that the *TRPV1-Val-585* variant that decreases the channel response to different agonists is associated with lower risk of asthmatics to present wheezing and cough. Although we could not show any evidence that *TRPV1-I585V* was associated with asthma or with other asthma outcomes, our data suggest that this genetic variant can be implicated in the clinical heterogeneity of childhood asthma and eventually in the remission of asthma.

The 20–30% loss of channel function shown by TRPV1-Val-585 (depending on stimuli) is associated with a modest but significant lower risk of active asthma. Although the structural explanation for the increased activity of TRPV1-Ile-585 is not known at present, it is worth noting that TRPV4-V620L, a similar mutation that when aligned appears two amino acids away from position 585 of the TRPV1, shows a 4-fold increase channel activity and that it is linked to skeletal dysplasia (27). It will be interesting to evaluate whether other TRPV1 genetic variants with stronger effect on channel activity show higher impact on the disease.

In summary, our data provide the first genetic evidence for the involvement of the TRPV1 nonselective cation channel in human disease and provide support to recent reports pointing toward the important role of intracellular Ca<sup>2+</sup> dysregulation in asthma pathophysiology (28).

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