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# Immunological Characteristics and Two Novel Mutations in TACI in a Cohort of 28 Pediatric Patients with Common Variable Immunodeficiency

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**Abstract** Common variable immunodeficiency (CVID) is a heterogeneous syndrome characterized by impaired immunoglobulin production. Mutations in the gene encoding TACI (*TNFRSF13B*) were previously found to be associated with CVID. Previous studies have identified a variety of sequence variants in TACI where A181E and C104R were the most common, with variable frequencies in different ethnic populations. So far, no mutations were identified in the recently reported "TACI highly conserved" (THC) cytoplasmic domain, important for the induction of class switch recombination. Our study evaluated immunological and clinical data on a cohort of 28 Argentinean pediatric CVID patients and allowed the identification of two novel mutations in *TNFRSF13B*, including one, S231R, affecting the highly conserved THC domain. In contrast, none of the patients presented with A181E and C104R mutations.

**Keywords** Common variable immunodeficiency · TACI · mutations · pediatric · immunoglobulin

## Introduction

Common variable immunodeficiency (CVID) (OMIM#240500) is a heterogeneous syndrome diagnosed on the basis of an impaired ability to produce specific antibodies after vaccination or antigen exposure, markedly reduced serum levels of IgG, IgA, and frequently IgM and exclusion of other defined causes of antibody deficiency (e.g. Hyper IgM syndromes, X-linked lymphoproliferative disorder). CVID has an estimated prevalence from 1 in 25,000 to 1 in 66,000 and is the most prevalent human primary immunodeficiency requiring medical attention. Most cases of CVID are sporadic, but at least 10% are familial. CVID is characterized by chronic/recurrent sinopulmonary infections, and the clinical course is complicated by systemic immunopathology including gastrointestinal, lymphoproliferative, autoimmune, and granulomatous diseases [1–5].

Mutations in T cell costimulators such as ICOS (MIM 604558) [6], or B cell receptors such as TNFRSF13B (TACI; MIM 604907) [7, 8], CD19 (MIM 107265) [9], TNFRSF13C (BAFFR; MIM 606269) [10], CD81 (MIM 613496) [11], or CD20 (MIM 112210) [12] are found to be associated with CVID.

The tumor necrosis factor receptor (TNFR) superfamily member, TACI, is expressed on B cells and binds two ligands, B cell activating factor (BAFF, TNFSF13B) and A proliferation-inducing ligand (APRIL, TNFSF13). BAFF and APRIL are specific immune mediators that trigger IgG and IgA class switch recombination (CSR) in B cells by engaging the receptor TACI. Recently, it has been shown that BAFF and APRIL elicited CSR by inducing recruitment of the adaptor MyD88, to a highly conserved cytoplasmic domain of TACI (THC) different from the Toll-interleukin 1 (IL-1) receptor (TIR) domain of Toll-like receptors (TLRs) [13]. This interaction triggered CSR via the DNA editing enzyme AID by activating transcription factor NF-κB. TACI-induced CSR was impaired in mice and humans cells lacking MyD88 or the kinase IRAK4, which indicates that MyD88 controls a

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B cell-intrinsic, TACI-dependent pathway for immunoglobulin diversification.

Previous studies carried out mainly in adults with CVID have identified a variety of sequence variants in *TNFRSF13B*, where A181E and C104R were the most common, with variable frequencies in different ethnic populations [14]. Actually, only these two variants were evaluated in a recent report on pediatric CVID population [15]. Both variants were also seen in a heterozygous state in healthy individuals, albeit at lower frequencies. So far, no mutations were identified in the highly conserved THC domain of TACI.

In this work, we evaluated the presence of *TNFRSF13B* mutations in 28 Argentinean patients with pediatric presentation of CVID. This study not only showed the absence of the most frequent variants of this gene in our population but also identified two novel mutations, one of them affecting the highly conserved segment of the THC domain.

## Methods

### Patient and Control Cohort

The study included 28 patients with hypogammaglobulinemia from 28 unrelated families. All were diagnosed with probable CVID according to the European Society for Immunodeficiencies (ESID) criteria [16].

In patients presenting with normal or with increased IgM levels *CD40 ligand*, *CD40*, *AID*, *and UNG* genes were also analyzed.

Considering the immunophenotype of the B cells, we classify the patient in $smB^+$ (switched memory B cells>2%) and $smB^-$ (switched memory B cells<2%) subgroups according to the classifications reported by EUROclass [17].

Informed written consent was obtained from each patient or parental guardian prior to participation, in accordance with the Declaration of Helsinki. The research protocol was approved by the internal ethics review board of the Hospital de Pediatria Juan P. Garrahan.

A total of 60 age-matched healthy donors were included in this study. These participants provided consent under a separate ethics protocol for healthy donors.

### Flow Cytometric Analysis of Peripheral Blood Lymphocytes

Peripheral blood samples from patients and their relatives were analyzed by standard three color flow cytometry for the quantification of B cell subpopulations. Staining was performed using the following mAbs at optimal concentrations: anti-CD27-PE (BD), anti-CD19-PC5 (Beckman Coulter, IOTest), anti-IgM-FITC (BD Pharmingen), anti-IgD-FITC (BD Pharmingen), anti-IgG-PE (BD Pharmingen). Samples acquisition was performed on a FACSort Cytometer (BD), and data were analyzed with Cell Quest software (BD). B cell sub-populations percentages ($CD27^-IgD^+$, $CD27^+IgD^+$, $CD27^+IgD^-$, $IgM^+$, and $IgG^+$) were performed by analyses of the $CD19^+$ lymphocyte gate. Pediatric reference values for B cell subpopulations, obtained in our laboratory, were used for comparing the CVID group.

For TACI detection, peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation over a Ficoll-Hypaque Plus (Amersham) gradient. PBMC aliquots were stained for four colour flow cytometry with anti-TACI-PE (R&D Systems), anti-BAFF-R-FITC (R&D Systems) anti-CD19-PerCP-Cy5.5 (BD Biosciences) and anti-CD27-APC (BD Biosciences). At least $1\times10^5$ cells were acquired on a FACS Canto II cytometer, analyzed by FACSDiva software and the percentages of BAFF-R, TACI, and CD27 expression were analyzed on $CD19^+$ lymphocytes.

### Sequence Analysis of *TNFRSF13B*

We analyzed all five coding exons of *TNFRSF13B* in the 28 patients and in the 60 healthy controls by direct sequencing with primers and conditions as previously described [10]. Sequence analysis was performed with DNA Sequencing Analysis software (PE Applied Biosystems) on ABI 3130 (Applied Biosystems).

### Total RNA Preparation, cDNA Synthesis, and RT-PCR

Total RNA was extracted from peripheral blood mononuclear cells, and cDNA was prepared using a first-strand cDNA synthesis kit (Amersham Biosciences, UK) according to the manufacturer's instructions. PCR amplification was performed using primers and conditions as previously described [18].

### Bioinformatics Analysis of TACI Amino Acid Substitutions

Polymorphism phenotyping (PolyPhen) [19] is a bioinformatics method that predicts the possible impact of an amino acid substitution on the structure and function of a human protein by using physical and comparative considerations. The result is given as a score resulting for the protein change as “putative benign” (0.00–1.50), “possibly damaging” (1.51 to 2.00), and “probably damaging” (>2.01).

### Statistical Analysis

The statistical analysis of results was performed with two-tailed Student's *t* tests using the PRISM software (GraphPad

Software Inc.). Other statistical analyses were performed using Fisher exact test with SPSS 15.0 for Windows.

## Results

Clinical and immunological data of our cohort of patients are summarized in Table I. Among the 28 patients, 27 cases were sporadic and one was familial (P12), his father presenting with hypogammaglobulinemia. The male:female ratio was 1:1. The mean age at the beginning of symptoms was 5.7 years (range 1–14 years), while the mean age at diagnosis was 11.1 years (range 4–16.1 years). Transient hypogammaglobulinemia of infancy was excluded in the patients with clinical symptoms younger than 4 years old. As mentioned by others [20], we also observed a delay of approximately 6 years (5.4 years) in the diagnosis. The European cohort [17] showed a delay in diagnosis that averaged 8 years (median, 4 years).

All of the patients presented with more than 2% of $CD19^+$ B cells in total blood lymphocytes, allowing a correct evaluation of B cell subpopulations and excluding other genetic defects such as X-linked agammaglobulinemia. The percentage of total B cells was greater than 2 standard deviation below the mean value for age only in 3 out of 28 (Table I). Peripheral B cell subset analysis was available for 25 patients (Table I, Patients Part B). Percentages of $CD27^-IgD^+$ naïve cells were increased in 76% of CVID patients (19/25) compared to healthy controls. Correspondingly, the proportion of $CD27^+IgD^+$ non-switched memory B cells was decreased in 76% of patients (19/25) (Table I), being comparable to other reported CVID pediatric population [15]. Whereas, the percentages of $CD27^+IgD^-$ switched memory B cells were decreased in 24 of the 25 patients.

The difference in percentages of naïve, switched memory, and non-switched memory B cells were found to be significant ($p<0.0001$, $p<0.0001$, and $p<0.001$, respectively), when the CVID patient group was compared to an age-matched healthy control population (Table I).

Immunoglobulin levels were recorded at diagnosis: IgG was markedly decreased as expected (at least 2 standard deviations, SDs, below the mean for age), and IgA was very low in most cases (Table I). Serum titers of IgM showed variation between patients, as previously observed in European patients [17], being even normal or increased in five patients of our cohort (P2, P15, P19, P22, and P28). These patients were evaluated for mutations in *CD40L*, *CD40*, *AICD*, and *UNG* genes, and no mutations were found.

The most frequent clinical manifestation was respiratory tract infection. Indeed, 43% (12 out of 28) of patients presented upper respiratory tract infections, namely sinusitis and otitis media, and as much as 79% of patients showed recurrent lower respiratory tract infections.

The clinical findings not related to infections are summarized in Table I. Autoimmunity was mainly hematologic such as thrombocytopenia or hemolytic anemia. Granulomatous disease was proven by biopsy in patients P19 and P26 who presented with this complication. All patients manifesting either autoimmunity, granulomatous disease, or splenomegaly belonged to the EUROClass classification $smB^-$ (switched memory B cells below 2% of total B cells); nevertheless, no significant association between non-infectious complications and the percentage of switched memory B cells was found, by using Fisher exact test for $2\times2$ contingency tables.

Along with the immunological evaluation, we carried out *TNFRSF13B* gene analysis that allowed the identification of two individuals (7%) carrying different heterozygous mutations, P5 and P28 (Q57H and S231R respectively, Fig. 1a, b). The single nucleotide polymorphisms (SNPs) T27T (rs8072293), P97P (rs35062843), V220A (rs56063729), P251L (rs34562254) and S277S (rs11078355) were found, in the present cohort of patients and controls, at comparable frequencies to the previously published frequencies data in CVID and healthy individuals (Fig. 1c) [10, 14, 18, 21, 22]. Interestingly, the C104R and A181E mutations, are typically described as the most frequent variants in TACI-associated CVID [10, 21, 22] but were not found in our patient cohort. Further, the W40R despite having been encountered in a patient with TACI-associated CVID [22], appears to be a polymorphism as it was found in our control subjects, and also in the SNPdatabase (dbSNP) (rs72553874).

The two different *TNFRSF13B* mutations observed in our cohort, Q57H and S231R, have not been previously described. These amino acid substitutions were neither reported as SNPs in the NCBI dbSNP database nor found in our healthy control group (Fig. 1c).

We carried out bioinformatics analysis of the novel missense mutations, since these tools may be useful in predicting the effects of amino acid substitutions. PolyPhen [19], a bioinformatics method that evaluates the possible impact of a missense change on the structure and function of a human protein by using physical and comparative considerations, predicted that both Q57H and S231R are "possibly damaging" of altering local protein structures (score 1.664 and 1.764, respectively) (Fig. 2a). Another method, Sorting Intolerant from Tolerant [23], did not reveal useful information by indicating a low confidence in this prediction (data not shown), probably due to the limited number of comparative sequences represented at the novel mutation positions. Indeed, the lack of TACI highly homologous proteins with resolved structures certainly restricts the estimation of the impact of an amino acid

**Table I** Clinical and immunological characteristics of Argentinean patients with pediatric presentation of CVID

| | No | Age at diagnosis (years) | Sex [a] | First clinical manifestation (years) | Igg (Mg/Dl) | Iga (Mg/Dl) | Igm (Mg/Dl) | %B cell[b] | Switched memory B cells[c] | Non-switched memory B cells[d] | Naive B cells[e] | Euroclass[f] | Non-infectious complications[g] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patients part A | 1 | 5.3 | M | 5.0 | 150 | 49 | 21 | 6% | ND | ND | ND | ND | None |
| | 2 | 11.0 | F | 2.0 | 473 | <7 | 171 | 15% | ND | ND | ND | ND | Splenomegaly |
| | 3 | 12.5 | M | 11.9 | 222 | <7 | 16 | 21% | ND | ND | ND | ND | None |
| Patients part B | 4 | 4.0 | F | 3.0 | 322 | 16 | 24 | 17% | Norm (10%) | ↓ (7%) | Norm (79%) | $smB^+$ | None |
| | 5 | 5.4 | M | 4.2 | 360 | 19 | 10 | 6% | ↓ (5%) | ↓ (5%) | Norm (81%) | $smB^+$ | None |
| | 6 | 5.8 | M | 4.0 | 6 | <7 | 13 | 13% | ↓ (0%) | ↓ (3%) | ↑ (97%) | $smB^-$ | None |
| | 7 | 6.8 | M | 1.5 | 296 | <7 | 13 | 14% | ↓ (3%) | Norm (9%) | ↑ (85%) | $smB^+$ | None |
| | 8 | 7.2 | M | 6.0 | 472 | <7 | 21 | 15% | ↓ (1%) | ↓ (3%) | ↑ (95%) | $smB^-$ | None |
| | 9 | 8.8 | F | 8.0 | 288 | <7 | 24 | 31% | ↓ (3%) | ↑ (19%) | Norm (76%) | $smB^+$ | None |
| | 10 | 9.0 | F | 6.0 | 123 | 20 | 21 | 13% | ↓ (0%) | ↓ (6%) | ↑ (89%) | $smB^-$ | None |
| | 11 | 10.0 | M | 5.0 | 61 | <7 | 10 | 3% | ↓ (2%) | ↓ (2%) | ↑ (95%) | $smB^-$ | None |
| | 12 | 11.0 | M | 7.0 | 439 | 13 | 32 | 5% | ↓ (2%) | Norm (9%) | ↑ (88%) | $smB^-$ | None |
| | 13 | 11.0 | M | 8.0 | 74 | <7 | 27 | 19% | ↓ (0%) | ↓ (2%) | ↑ (96%) | $smB^-$ | Autoimmunity (TID) |
| | 14 | 11.0 | F | 6.0 | 484 | <7 | 36 | 20% | ↓ (0%) | ↓ (1%) | ↑ (97%) | $smB^-$ | None |
| | 15 | 11.2 | F | 2.0 | 262 | <7 | 58 | 26% | ↓ (0%) | ↓ (2%) | ↑ (97%) | $smB^-$ | None |
| | 16 | 12.0 | M | 3.0 | 450 | 18 | 20 | 24% | ↓ (1%) | ↓ (5%) | ↑ (93%) | $smB^-$ | Autoimmunity (AIT) |
| | 17 | 12.0 | M | 1.0 | 220 | <7 | 18 | 7% | ↓ (0%) | ↓ (7%) | ↑ (91%) | $smB^-$ | None |
| | 18 | 13.0 | F | 11.0 | 333 | <7 | 15 | 20% | ↓ (5%) | Norm (14%) | Norm (77%) | $smB^+$ | None |
| | 19 | 13.0 | F | 6.0 | 321 | 16 | 78 | 8% | ↓ (1%) | ↓ (5%) | ↑ (86%) | $smB^-$ | Granulomatous disease |
| | 20 | 13.1 | F | 7.3 | 376 | 25 | 33 | 3% | ↓ (1%) | ↓ (2%) | ↑ (91%) | $smB^-$ | Autoimmunity (AIT) and splenomegaly |
| | 21 | 14.0 | M | 3.0 | 336 | <7 | 15 | 5.6% | ↓ (1%) | ↓ (5%) | ↑ (93%) | $smB^-$ | None |
| | 22 | 14.0 | F | 1.0 | 524 | <7 | 40 | 12% | ↓ (3%) | ↓ (7%) | ↑ (88%) | $smB^+$ | None |
| | 23 | 14.1 | M | 5.0 | 301 | <7 | 25 | 13% | ↓ (3%) | Norm (10%) | ↑ (85%) | $smB^+$ | None |
| | 24 | 14.9 | F | 4.0 | 426 | 8 | 35 | 7% | ↓ (2%) | ↓ (0%) | ↑ (91%) | $smB^-$ | Autoimmunity (AIT) |
| | 25 | 15.0 | F | 8.0 | 36 | <7 | 10 | 7% | ↓ (0%) | ↓ (2%) | ↑ (97%) | $smB^-$ | Splenomegaly |
| | 26 | 15.3 | M | 12.0 | 147 | <7 | 28 | 25% | ↓ (0%) | ↓ (2%) | ↑ (96%) | $smB^-$ | Granulomatous disease and splenomegaly |
| | 27 | 15.6 | F | 14.0 | 106 | <7 | 20 | 11% | ↓ (0%) | ↓ (2%) | ↑ (98%) | $smB^-$ | Splenomegaly |
| | 28 | 16.1 | F | 4.9 | 33 | <7 | 129 | 16% | ↓ (2%) | Norm (15%) | Norm (80%) | $SMB^-$ | Autoimmunity (AIT, AIHA) and splenomegaly |

**Table I** (continued)

| Healthy donors | Flow cytometry Range of age (years) | *N* | % B cell (b) | Switched memory B cells (c) | Non-switched memory B cells (d) | Naive B cells (e) |
|---|---|---|---|---|---|---|
| | 2.4–15.8 | 60 | (14±4)% | (14±7)% | (11±4)% | (74±10)% |

[a] *M* male, *F* female

[b] %B cells: as percentage of lymphocyte gate

[c] Switched memory B cells as percentage of CD19+ gate=CD27+IgD−

[d] Non-switched memory B cells as percentage of CD19+ gate=CD27+IgD+

[e] Naive B cells as percentage of CD19+ gate=CD27-IgD+

[c, d, e] *ND* not done, ↓ decreased number, ↑ increased number, *Norm* within normal range, compared to the healthy control cohort

[f] smB+ means <2% class switched memory B cells; smB− means ≤2% class switched memory B cells

[g] *TID* type I diabetes; *AIT* autoimmune thrombocytopenia; *AIHA* autoimmune hemolytic anemia

replacement on protein three-dimensional structure and function, by using sequence conservation methods.

We evaluated the effect of the nucleotide changes at the mRNA level in terms of expression and splicing fidelity. In both cases, the mutated alleles were present in mRNA analysis (Fig. 2b), without apparent splice effect.

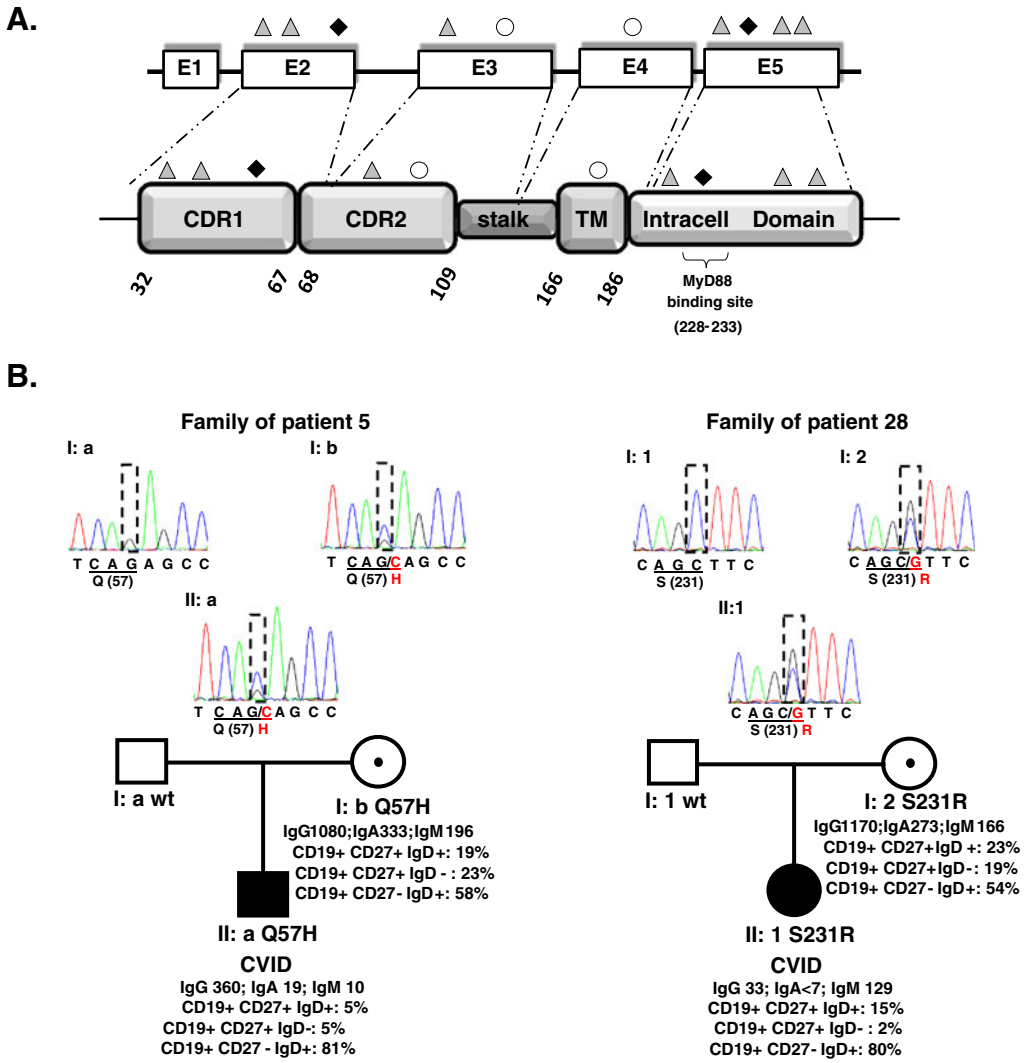
In both patients with *TNFRSF13B* novel mutations, we also analyzed TACI expression on peripheral B cells. By using a monoclonal antibody recognizing the TACI extracellular domain, we readily detected surface expression of this protein on B cells from patients and healthy controls (Fig. 2c). Although in patient 5, the expression of TACI in $CD19^+$ B cells was reproducibly decreased, this probably reflects the diminished percentage of total memory B cells observed in this patient ($CD27^+IgD^+$ 5% and $CD27^+IgD^-$ 5%, both measured as percentages of $CD19^+$ gate) with respect to our control group (Table I). In this sense, taking into account that TACI is mainly expressed in resting peripheral blood B cells $CD27^+$ cells (Fig. 2c) [24], it is not unexpected that the mean fluorescence intensity of TACI remains with the expected range for P5 (Fig. 2c).

Similar to patient 28, in cells carrying S231R substitution generated in vitro by using site-directed mutagenesis, TACI expression was not impaired either [13]. We also noticed in patient CVID-S231R a relative expansion of $TACI^+CD27^-IgD^+$ B cells (Fig. 2c). In both patients, surface expression on B cells of BAFF-R, another member of the TNFR superfamily proteins, was comparable to healthy control cohort (Fig. 2c).

Among the first-degree unaffected relatives of patients P5 and P28, both healthy mothers carry S231R and Q57H heterozygous mutations, respectively (see immunological parameters in Fig. 1b), supporting the incomplete penetrance for heterozygous mutations in *TNFRSF13B* previously shown [22]. Likewise, between both patients carrying these new mutations a lack of correlation was found with clinical as well as immunological parameters. Indeed, while patient CVID-S231R presented less than 2% switched memory B cells and autoimmunity and splenomegaly, patient CVID-Q57H belonged to the $smB^+$ subgroup without these non-infectious complications.

## Discussion

Our study evaluated immunological and clinical data on a cohort of 28 Argentinean pediatric CVID patients and allowed the identification of two novel mutations in *TNFRSF13B*, including one, S231R, affecting the "TACI highly conserved" (THC) cytoplasmic domain, important for the induction of CSR [13]. Patient carrying S231R mutation constitutes the first CVID case associated with a substitution affecting the




C.

| Mutations | T27T △ | W40R△ | Q57H◆ | P97P△ | C104R○ | A181E○ | V220A△ | S231R◆ | P251L△ | S277S△ | IVS4 -60t>c | IVS3 +25a>c |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patients MAF % | 64,3 | 0,0 | 1,8 | 8,9 | 0,0 | 0,0 | 3,6 | 1,8 | 5,4 | 53,6 | 21,4 | 32,1 |
| Controls MAF % | 81,3 | 1,3 | 0,0 | 6,3 | 1,3 | 0,0 | 3,8 | 0,0 | 11,3 | 38,8 | 14,3 | 21,4 |

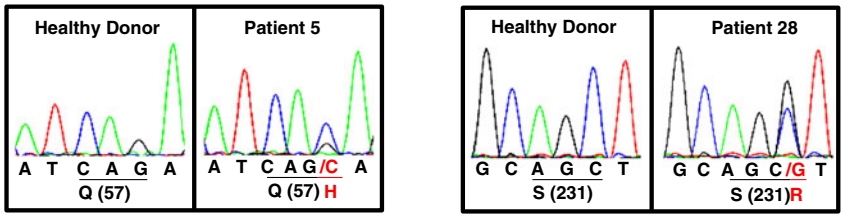
**Fig. 1** Sequence analysis of *TNFRSF13B* in Argentinean patients with pediatric presentation of CVID. **a** Schematic representation of TACI gene (*up*) and protein (*down*), *gray triangles* = polymorphism; *white circles*=C104R and A181E; *black diamonds* = novel mutations; *E* = exon; *CRD* = cysteine rich domain; *TM* = trans-membrane domain **b** DNA sequence of *TNFRSF13B* depicted by DNA electropherograms and pedigrees of two families with novel mutations in TACI. Symbols: *circles* indicate female; *squares*, male; *black filled symbols*, CVID patient; *white filled symbols*, healthy family members. Below the pedigrees are indicated the B cell phenotype and the immunoglobulin levels (in milligrams per deciliter) for all carriers of mutations. **c** Frequency of TACI mutations in CVID patients and in healthy donors (range of age: 2.4–15.8 years; *N*=60). *MAF* = Minor allele frequency. *Gray triangles* = polymorphism; *white circles*= C104R and A181E; *black diamonds* = novel mutations

THC cytoplasmic domain, which has previously shown to decrease TACI-induced NF-κB activation [13]. This patient presented at 5 years of age with thrombocytopenic purpura, then, she developed recurrent upper respiratory tract infections (sinusitis) as well as recurrent pneumonia and, later on splenomegaly and autoimmune hemolytic anemia.

**A.**

| Patient | Gene Symbol (name, Refseq) | Nucleotide change | Protein change | Polyphen score | Predicted SNP effect |
|---|---|---|---|---|---|
| 5 | TNFRSF13B (TACI, NM_01245 2.2) | c.171G>C | Q57H | 1.664 | Possibly damaging |
| 28 | | c.693C>G | S231R | 1.764 | Possibly damaging |

**B.**




**C.**

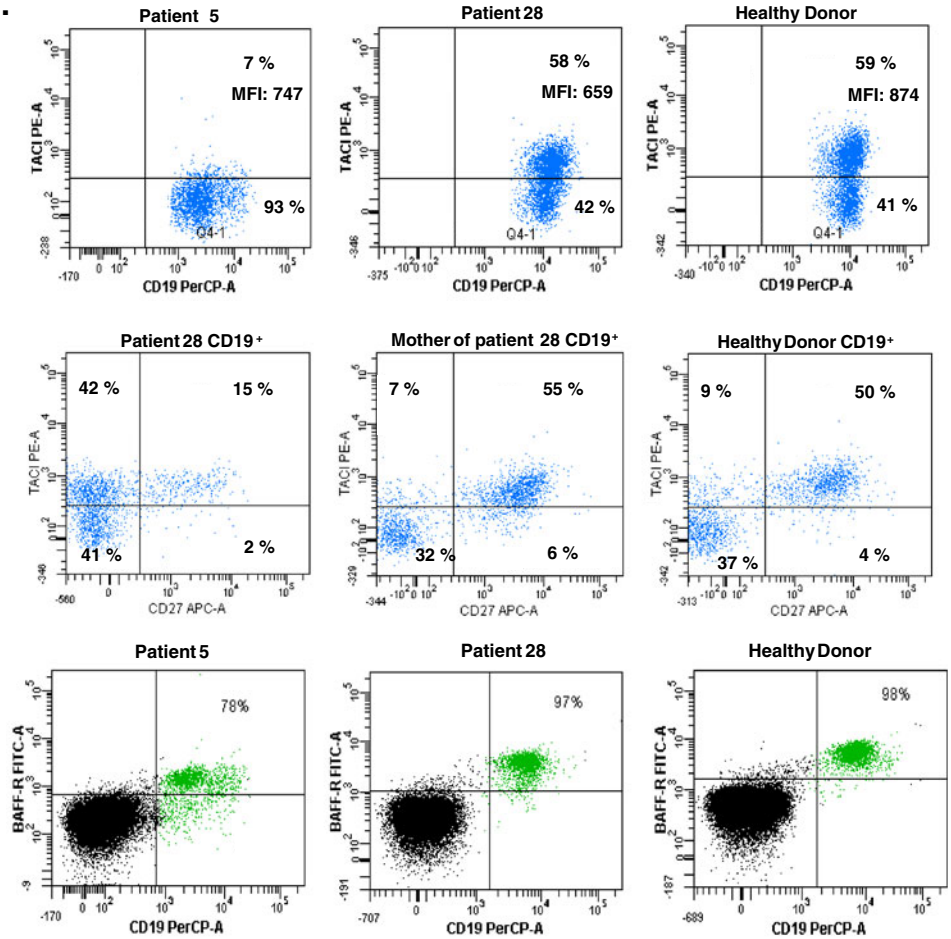



**Fig. 2** Analysis of novel mutations of TACI. **a** Bioinformatics analysis of novel TACI amino-acid substitutions by using polymorphism phenotyping (Polyphen). **b** Sequence analysis of mRNA in patients with novel mutations. **c** Flow cytometric analysis of TACI expression on the surface of B cells in patients with novel mutations and healthy donors (*up*); co-expression of TACI with CD27 in B cells of patient CVID-S231R, her mother and a healthy donor (*middle*); expression of BAFF-R on the surface of B cells (in *green*) in patients with novel TACI mutations and in healthy donors (*down*). *MFI* Mean fluorescence intensity

The immunological abnormalities and clinical symptoms found in our group of patients are comparable to those found in a recent report of other pediatric CVID population and also in adult populations, with respiratory tract infections as the most frequent clinical manifestation [15, 17].

B cell phenotypes revealed a decrease of non-switched memory B cells percentage (19/25) and an increase of naïve B cells (19/25). Additionally, we observed a marked decrease in switched memory B cells with a higher incidence of patients belonging to the $smB^-$ subgroup (18/

25) when compared with adult CVID populations [17]. This finding was also reported in a pediatric CVID study [15] and could show that pediatric patients have a more severe defect in B cell differentiation than adults.

In the last few years, genetic defects have been described in a very low number of CVID patients such as biallelic and monoallelic mutations in *TNFRSF13B*. In previous studies, the proportion of CVID patients carrying at least one *TNFRSF13B* mutation was estimated at 10% [10, 14, 18, 21, 22]. Most of the molecular alterations described so far in TACI were mainly observed in adults and the most frequent mutations were C104R and A181E. Actually, the latter are the only ones that were assessed in a recent report on a CVID pediatric population [15]. In this work, we evaluated *TNFRSF13B* gene in an early onset population of CVID patients and two novel mutations were identified (7%), but none of the patients presented with C104R or A181E changes, indicating the need to evaluate the entire gene for other mutations.

Among the novel alterations, S231R becomes relevant considering a recent report showing that TACI engagement promotes recruitment of MyD88 to a conserved cytoplasmic motif of the receptor for inducing CSR. This MyD88-binding site spans amino acids 228–239 in TACI, and it has been shown a decrease of MyD88 binding, as well as an attenuation of its signaling in cells carrying S231R substitution, generated by using site-directed mutagenesis [13]. These authors conclude that MyD88 controls a previously unknown B cell-intrinsic, TACI-dependent, TIR-independent pathway for Ig gene diversification and this, in a specific manner, as the lack of MyD88 did not hamper the IgM secretion. Interestingly, in our CVID-S231R patient, elevated IgM serum levels was found at the time of diagnosis, as also reported for some TACI-deficient CVID patients [9, 10]. In this regard, it is noteworthy that while a significant deficiency in non-switched memory B cells was presented by our cohort, CVID-S231R patient showed a preserved percentage of these cells.

The other not previously described mutation, Q57H, is located in the exon 2 of *TNFRSF13B* gene in a sequence encoding the extracellular cysteine rich domain 1 (CRD1). TACI is expressed as two splice variants containing either one or two CRDs, of which only CRD2 seems to be functionally relevant [25]. However, we cannot formally rule out a harmful effect of Q57H mutation on TACI signaling, since CRD1 could play another, as yet undefined role. Additional CRDs can have roles in providing stability, regulation [26], or in forming further contacts to the ligand to optimize affinity and specificity. Although, a significant reduction of protein expression on cell surface was detected in this patient, it could be related to a reduced subset of $CD27^+$ B cells. In addition, despite a "possibly damaging" effect over protein structure obtained by the PolyPhen bioinformatics analysis, the limited homology of TACI with other TNFR superfamily members and the fact that only the three-dimensional structure of CRD2 was solved, highlight the importance of resolving the structure of the CRD1 domain of TACI.

These novel *TNFRSF13B* mutations could be considered rare events and therefore the lack of any correlation with clinical or immunological parameters is not surprising. For example, patient carrying S231R showed less than 2% of switched memory B cells, while the CVID-Q57H belonged to the $smB^+$ subgroup. Regarding non-switched memory B cells, CVID-S231R presented a relative increase, whereas in CVID-Q57H, this subset appeared decreased. It remains to prove whether the interference caused by S231R mutation for binding to MyD88 is the basis of this specific switched memory B cells deficiency in P28. Otherwise, although both patients showed recurrent respiratory tract infections, only patient CVID-S231R presented non-infectious complications such as autoimmunity and splenomegaly.

Screening of the unaffected family members of the patients revealed that in both cases healthy mothers carried the mutation. Incomplete penetrance and familial segregation were previously observed for heterozygous *TNFRSF13B* sequence variants, and it has been suggested that heterozygous mutations increase the risk, without being sufficient to cause CVID [22]. Because TACI assembles as a trimer or higher-order oligomer, TACI mutants with disrupted function might potentially exert a dominant-negative (DN) effect on signaling by WT TACI in heterozygotes. Thus, the ratio of the number of expressed proteins with or without mutation could determine the possibility to form an active tripartite molecule. Indeed, most missense mutations affecting the intracellular domain of *TNFRSF6*, other member of the TNFR superfamily, are known to exert a DN effect accounting for the onset of autoimmune lymphoproliferative syndrome (ALPS). However, recently, Fried et al. [27] suggested that the mutations that impaired TACI function did not exert a DN effect but could cause B-cell dysfunction in heterozygotes because of haploinsufficiency. In this case, we can speculate that a second event could be necessary for disease expression, as observed in ALPS patients carrying an inherited as well as a somatic mutation in *TNFRSF6* [28]. Furthermore, in our current results, a normal surface expression of TACI was shown on peripheral B cells in the patient carrying S231R mutation and in her mother, but preliminary functional studies indicated that this variant in heterozygous state impairs the induction of CSR assessed on B cells from the patient and from her mother (Almejun MB, unpublished data). Further studies are needed to unravel the additional genetic and/or environmental factors that act in concert with a heterozygous genetic alteration on *TNFRSF13B* to give rise to the development of CVID.

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