Genetic Profile of Inborn Errors of Immunity Using Whole Exome Sequencing in Individuals With BCG Localized Adverse Events

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Abstract

Purpose: In *Mycobacterium tuberculosis* endemic regions, BCG vaccine is administered early after birth to confer protection against severe form of tuberculosis disease. Previous reports suggest that BCG adverse events, even localized ones (BCGitis), can be the first manifestation of immunodeficiency. We investigated children with a history of BCGitis who needed drug treatment looking for possibly pathogenic variants in inborn errors of immunity genes (IEI-genes).

Methods: Forty-four probands were evaluated. The exome sequences obtained by Next-Generation Sequencing were filtered for variants in the 344 IEI-genes described by the International Union of Immunological Societies (IUIS) and classified according to the recommendations of the American College of Medical Genetics. The identified candidate variants were validated by Sanger sequencing.

Results: Out of the 44 probands, 36 were sporadic cases and 8 were familial cases. Thirty-one in 44 (70.5%) presented immunoallergic or other infectious clinical conditions besides BCGitis; 19 in 44 (43.2%) presented variants classified as pathogenic or likely pathogenic in 17 different IEI-genes, of which 35.3% were genes related to defects in intrinsic and innate immunity, including Mendelian Susceptibility to Mycobacterial Disease (MSMD) genes (*IRF8*, *IFNGR1*, *JAK1*, *STAT1*, *TLR3* and *TBK1*). Remaining genes were distributed in another five IUIS classifications groups (*CARD14*, *CFH*, *CHD7*, *FOXN1*, *NFAT5*, *NLRP3*, *NOD2*, *PMS2*, *STAT3*, *TNFRSF13B* and *TNFSF12*).

Conclusion: The high prevalence of pathogenic or likely pathogenic variants found in IEI-genes may be associated with BCGitis, which should be considered a sign of an inborn error of immunity.

Introduction

It is estimated that one quarter of individuals in the world are infected with *Mycobacterium tuberculosis*, but only 5–10% become symptomatic [1]. A variety of factors are associated with development of tuberculosis disease, amongst which young age is included [2,3]. In *M. tuberculosis* endemic regions, BCG vaccine is administered early after birth and it is known to confer protection against severe forms of tuberculosis disease [4].

BCG is a live attenuated vaccine that can lead to adverse events (BCG-AE) which might be classified either as disseminated or localized [5]. Whereas disseminated BCG-AE (*BCGosis*) are indisputably associated with an underlying immunodeficiency [6-8], localized BCG-AE (*BCGitis*) are generally less severe and are often attributed to vaccine administration errors in Brazil [9].

Retrospective studies with patients diagnosed with Inborn Immunity Errors (IEI) - previously denominated as Primary Immunodeficiency Disease (PID) - have shown that localized BCG-AE also occurred in these patients [6, 10-13]. However, the real frequency of individuals with IEI who develop localized BCG-AE as a first manifestation of the immunological impairment is unclear.

Whole exome sequencing (WES) has proven to be an effective tool to detect novel IEI-causing genes in patients with syndromes of unknown etiology [14, 15]. WES combined with selective analysis of IEI-associated genes has been effective in identifying 17 disease-causing variants in 30% of patients diagnosed with severe Common Variable Immunodeficiency (CVID) phenotypes [16]. The NGS WES analysis of 278 families with immunodeficiency from 22 countries focused on 475 IEI known or candidate genes and achieved a probable molecular diagnosis in 110 (40%) unrelated patients [17].

Using a similar approach, we investigated patients with BCG localized adverse events and some close family members looking for pathogenic variants in genes known to cause IEIs.

Methods

Patient Selection

This study performed a molecular assessment of children who presented with BCGitis after BCG vaccination. They were followed up at the Reference Center for Special Immunobiologicals and at the Immunology Clinic of the Department of Pediatrics at the Universidade Federal de São Paulo/UNIFESP, in São Paulo, Brazil, from 2009 to 2018. The Brazilian Ministry of Health criteria for BCGitis diagnosis were used in this study [9]. Children who had criteria indicating the use of a specific drug treatment (isoniazid - INH) and whose legal guardians accepted to participate in the study were included in the study and were named probands. For familial cases, when available, other affected family members were included. HIV infection was as exclusion criteria.

Patients were assessed for advanced immunological tests, such as flow cytometry immunophenotyping of T cells (CD3+), CD4+ T cells (CD3+CD4+) and CD8+ T cells (CD3+CD8+), B cells (CD3-CD19+) and NK cells (CD3-CD56+CD16+). Phagocytic function assessment was performed using dihydrorhodamine test. Evaluation of the IFNγ-IL-12/IL-23 axis defects was performed after specific stimulation of whole blood with BCG, BCG and IL-12p70 and BCG and IFNγ, with subsequent dosage of IL-12p70 and IFNγ in culture supernatant by the X-MAP technology [18]. Lymphocyte and monocyte expression of the IFNγ receptor chain (CD119) *ex vivo* and of the IL-12 receptor chain b1 (CD212) after 72h stimulation with phytohemagglutinin were performed by flow cytometry. When necessary, complement assays were performed and serum immunoglobulins were assessed.

Data Analysis

Whole Exome Sequencing (WES) was performed on Ion Torrent™ plataform according to manufactorer instructions (Supplemental methods).

Exome sequences determined by NGS of all cohort subjects were filtered for variants in the 344 genes associated with 354 Inborn Errors of Immunity (IEI-Genes) described by the Primary Immunodeficiency Diseases Committee Report of International Union of Immunological Societies - IUIS [19]. Considering

relationship between related genes to Mendelian Susceptibility to Mycobacterial Disease genes (MSMD-Genes) to BCGitis, three newly described genes in the IUIS update [20] were added to our analysis (Table S1). Further filtering steps were applied as specified on supplemental methods.

Candidate variants were validated by Sanger sequencing and then submitted to familial segregation.

To assess the importance of IEI-related genes on patients presenting with BCG adverse event, an enrichment analysis was performed using as control population WES data from 1,562 unrelated samples from the Baylor Hopkins Center for Mendelian Genomics, searching for rare single nucleotide variants (SNV) on IEI-related genes. A contingency table containing the number of individuals presenting these qualified SNVs was accomplished for each gene found in the BCG cohort samples and in the control samples. The *p* value was determined by Fisher's exact test and it was subsequentially was corrected by Benjamini and Hochberg method.

For variant interpretation, the American College of Medical Genetics and the Association for Molecular Pathology (ACMG/AMP) guidelines [21] were used. Final classification was obtained using VarSome platform [22].

Probands who presented variants in IEI-Genes classified as Pathogenic or Likely Pathogenic were categorized according to clinical outcome in respect to the development of other symptoms besides BCGitis: Only BGCitis group, with probands who did not present other symptoms besides BCGitis; Mild or Transient Infectious Conditions group, with probands who presented mild or transient infectious conditions besides BCGitis; and Immunoallergic Conditions group, with probands with immunoallergic conditions besides BCGitis.

Ethics

This study was approved by the Ethics Committee of Universidade Federal de São Paulo (protocol number 842,006 and 1,641,734). All participants or legal guardians signed the consent forms.

Results

The 44 children (probands) with BCGitis from non-consanguineous Brazilian families included 36 probands without family history of other BCGitis (sporadic cases) and 8 probands with familial BCGitis (Figure 1, Table S2).

In four familial cases it was possible to analyze additional affected individuals (multiplex). In another four familial cases, only proband was analyzed (simplex). Among the probands simplex families, variants in IEI-Genes classified as Pathogenic or Likely Pathogenic were found in two probands (Family 7 and Family 13). Among the multiplex families, identified variants did not explain the family aggregation of BCGitis in three of them (Family 2, Family 37 and Family 44) and these families will be subject to further analysis. The proband of Family 2 showed a variant in MSMD-Gene classified as Likely Pathogenic (Figure 1).

Among all probands, 70.5% (31/44) presented mild or transient infectious conditions or immunoallergic conditions besides BCGitis. Table 1 summarizes probands characteristics with Pathogenic or Likely Pathogenic variants by Group of clinical phenotypes.

Table 1
Characteristics of probands with Pathogenic and Likely Pathogenic variants by Group of clinical phenotypes of the probands

Parameters	Only BCGitis	BCGitis *plus* Mild or Transient Infections	BCGitis *plus* Immunoallergic Conditions
Number of probands	6	3	10
Male (%)	3 (50)	3(100)	6 (60)
Median age at BCGitis diagnosis in days (range)	57 (30-75)	132 (29-251)	92 (8-184)
Median BCGitis treatment in days (range)	142 (0-181)	178(150-249)	96 (32-212)
Age at last clinical assessment in years (range)	11 (9-11)	7(3-10)	9 (8-12)
Leukopenia (%)	1 (33)	0	5(50)
Persistent Limphopenia (%)	0	0	1 (10)
Alteration in IL12-IFNg axis (%)	3 (50)	1 (33)	8 (80)
Family history of Tuberculosis (%)	0	0	4 (40)
Hospitalization (%)	0	1 (33)	7 (70)
Number of Genes with variants P or LP	6	3	11
Number of Variants P or LP	6	4	14
P=Pathogenic; LP=Likely Pathogenic			

In the whole cohort, Pathogenic or Likely Pathogenic variants in IEI-Genes were found in 19/44 (43.2%) of probands. Tables 2 to 4 shows Pathogenic or Likely Pathogenic variants in IEI-Genes, including parental origin of inherited alleles by groups of probands according to clinical phenotype.

Table 2

Relevant clinical, familial and laboratory characteristics of probands with clinical phenotype "Only BCGitis" with genetic variants classified as Pathogenic or Likely Pathogenic

Prob	BCG-AE	Relevant caracteristics	Blood cells	IL12-IFNg axis	GENE[a]	Variant	ClinVar/ HGMD	Genot/ (inherit)	Enrichment analysis (p value)	Variant Classification [b] (ACMG Criteria)[c]
10_1	Injection site abscess and Suppurative lymphadenitis	Recurrent lymphatic edema in the right arm after BCG	normal	Low IFNg cytokine	*CFH*	1:196659285C>T c.C1252T p.P418S	- / -	het (mother)	0.02740	Likely Pathogenic (PS4, PM2, PP2)
16_1	Regional enlarged lymph node > 3cm	None	normal	normal	*NFAT5*	16:69681120A>G c.A161G p.Q54R	- / -	het (father)	0.02740	Likely Pathogenic (PS4, PM2)
18_1	Suppurative lymphadenitis	None	normal	Low IFNg and IL-12 cytokines/ absent receptor IFNg in monocytes	*TLR3*	4:186998119G>A c.G346A p.D116N	- / -	het (father)	0.02740	Likely Pathogenic (PS4, PM2, PP3)
29_1	Suppurative lymphadenitis	Exeresis of lymph node was required	normal	normal	*TBK1*	12:64873821G>T c.G731T p.G244V	- / DM	het (mother)	0.02740	Pathogenic (PS4, PM1, PM2, PP2, PP3)
2_1	Regional enlarged lymph node > 3cm	Maternal cousin had BCGits	transient neutropenia	normal	*STAT1*	2:191873713T>C c.A255G p.I85M	- / -	het (father)	0.02740	Likely Pathogenic (PS4, PM2, PP2)
23_1	Suppurative lymphadenitis	Secondary bacterial infection Sister had BCGitis	normal	Low IFNg and IL-12 cytokines	*NOD2*	16:50733638G>A c.G232A p.A78T	- / -	het (father)	0.00073	Likely Pathogenic (PS4, PM2)

Prob.= Proband; BCG-AE = Type of BCG adverse event; ([a]) = Human Genome Reference GRCh37/hg19. VUS = Variant of Uncertain Significance; DM=disease-causing mutation; Genot./(inherit) = Genotype/(Inheritance); het = heterozygosis; comp-het = compound heterozygosis; IFN = Interferon; IL-12 = Interleukin-12; N/A = not available; HGMD = Human Gene Mutation Database; ([b]) = using VarSome platform ; ACMG = American College of Medical Genetics; ([c]) Richards et al., 20

Table 3

Relevant clinical, familial and laboratory characteristics of probands with clinical phenotype "Mild or Transient Infections" with genetic variants classified a Pathogenic or Likely Pathogenic

Prob	BCG-AE	Relevant caracteristics	Blood cells	IL12-IFNg axis	GENE[a]	Variant	ClinVar/ HGMD	Genot (inherit)	Enrichment analysis (p value)	Variant Classifica[b] (ACMG Criteria)[c]
3_1	Ulcer >1 cm	Urinary tract infection, recurrent otitis	transient neutropenia	Low IFNg cytokine	*TNFSF12*	17:7452627C>A c.C157A p.Q53K	- / -	het (mother)	0.02740	Likely Pathogeni (PS4, PP3
57_1	Suppurative Lymphadenitis	Recurrent pneumonia with hospitalization, urinary tract infection, sinusitis, normal CH100 and C2; normal Igs	N/A	N/A	*CFH*	1:196711052G>C c.G3004C p.G1002R	VUS / -	het (father)	0.02740	Likely Pathogeni (PS4, PM2 PP2)
68_1	Suppurative Lymphadenitis	BCGitis with secondary bacterial infection with hospitalization	transient low CD8 T and NK cells	normal receptors/ cytokines N/A	*PMS2*	7:6035238G>T c.C830A. p.T277K	VUS / -	comp-het/ (father)	0.02740	VUS (PS4, PM2
					PMS2	7:6042238G>A c.C383T p.S128L	VUS / -	comp-het/ (no mother - no father)	0.02740	Pathogeni (PS2, PS4 PM2, PP3

Prob.= Proband; BCG-AE = Type of BCG adverse event; ([a]) = Human Genome Reference GRCh37/hg19. VUS = Variant of Uncertain Significance; DM=disease-causing mutation; Genot./(inherit) = Genotype/(Inheritance); het = heterozygosis; comp-het = compound heterozygosis; IFN = Interferon; IL-12 = Interleukin-12 N/A = not available; HGMD = Human Gene Mutation Database; ([b]) = using VarSome platform ; ACMG = American College of Medical Genetics; ([c]) Richards et 2015.

Table 4
Relevant clinical, familial and laboratory characteristics of probands with clinical phenotype "Immunoallergic conditions" with genetic variants classified as Pathogenic or Likely Pathogenic

Prob	BCG-AE	Relevant caracteristics	Blood cells	IL12-IFNg axis	GENE[a]	Variant	ClinVar/ HGMD	Genot/ (inherit)	Enrichment analysis (p value)	Variant Classification [b] (ACMG Criteria)[c]
6_1	Suppurative Lymphadenitis	Asthma with hospitalization; recurrent stomatitits	transient low B cells	Low IFNg and IL-12 cytokines	*CHD7*	8:61655415T>C c.T1424C p.M475T	- / -	het (no father, no mother)	0.02740	Pathogenic (PS2, PS4, PM2, PP2)
11_1	Suppurative Lymphadenitis	Neonatal sepsis and asseptic meningitis, recurrent pneumonia with hospitalization, sinusitis, rinitis; normal Igs, high IgE (501UI/mL). Maternal uncle and aunt had TB	low CD4 T and transient low B cells	Low IFNg cytokine	*FOXN1*	17:26862145T>A C.T1556A p.L519Q	VUS / DM	het (father)	0.02740	Likely Pathogenic (PS4, PM2, PP3)
14_1	Injection site abscess and Suppurative lymphadenitis	Asthma, recurrent pneumonia with pleural effusion and hospitalization, recurrent otitits, atopic dermatitits, lactose and gluten intolerance; elevated IgE (3.416 UI/mL)	normal	normal	*CHD7*	8:61764578G>A c.G5666A p.G1889D	- / -	het (father)	0.02740	Likely Pathogenic (PS4, PM2, PP2)
					STAT3	17:40498688G>A c.C172T P.H58Y	- / DM	het (no father, no mother)	0.02740	Pathogenic (PS2, PS4, PM2, PP2, PP3)
20_1	Suppurative Lymphadenitis	Asthma, recurrent pneumonia, recurrent otitis. Paternal great uncle had TB and paternal cousins had recurrent pneumonia	normal	Low IL-12 cytokine	*NOD2*	16:50744718G>A c.G896A p.G299D	- / -	het (father)	0.02740	Likely Pathogenic (PS4, PM1, PM2, PP3)

Table 4 *(continuation)* – Relevant clinical, familial and laboratory characteristics of probands with clinical phenotype "Immunoallergic conditions" with genetic variants classified as Pathogenic or Likely Pathogenic

Prob	BCG-AE	Relevant caracteristics	Blood cells	IL12-IFNg axis	GENE[a]	Variant	ClinVar/ HGMD	Genot/ (inherit)	Enrichment analysis (p value)	Variant Class[b] (ACM Criteri
21_1	Suppurative Lymphadenitis	Rhinitis. Maternal grandmother, aunt and cousin had TB	normal	normal	*TNFRSF13B*	17:16852187A>G c.T310C p.C104R	VUS / DM	het (father)	0.42819	Patho (PS1, PM1, PP2, PP5)
					STAT1	2:191873774T>C c.A188G p.Q63R	- / -	het (father)	0.02740	Likely Patho (PS4, PP2,
					NLRP3	1:247588562T>A c.T1817A p.L606Q	- / -	het (father)	0.02740	Likely Patho (PS4, PP2)
27_1	Injection site abscess	Asthma, recurrent pneumonia with hospitalization. Grandfather had TB	normal	Low IL-12 cytokine	*IFNGR1*	6:137527386A>G c.T260C p.I87T	Pathogenic /DM	het (father)	0.02740	Patho (PS4, PP3, PP5)
32_1	Suppurative lymphadenitis and secondary bacterial infection	Asthma, recurrent severe pneumonia with hospitalizations	transient low NK cells	Low IL-12 cytokine	*CHD7*	8:61654602G>T c.G611T p.G204V	- / -	het (no mother/ (father N/A)	0.02740	Path (PS4, PM6,
50_1	Suppurative Lymphadenitis	Sepsis, asthma, recurrent pneumonia with hospitalization, otitis and oral moniliasis. Brother had recurrent pneumonia	transient low total CD4 T, naive CD4 T and B cells	Low IFNg and IL-12 cytokines	*JAK1*	1:65316585T>G c.A1657C p.N553H	- / -	het (no mother, no brother)	0.02740	Likely Patho (PS2, PM2,

Table 4 *(continuation)* – Relevant clinical, familial and laboratory characteristics of probands with clinical phenotype "Immunoallergic conditions" with genetic variants classified as Pathogenic or Likely Pathogenic

Prob	BCG-AE	Relevant caracteristics	Blood cells	IL12-IFNg axis	GENE[a]	Variant	ClinVar/ HGMD	Genot/ (inherit)	Enrichment analysis (p value)	Variant Classificati[b] (ACMG Criteria)[c]
7_1	Injection site abscess	17p13 Microdeletion Syndrome, asthma, recurrent pneumonia, pleural effusion with hospitalizations, recurrent stomatitis. Father had BCGits	transient low CD8 T cells	Low IFNg and IL-12 cytokines	*IRF8*	16:85946749G>A c.G460A p.D154N	- / -	het (mother)	0.02740	Likely Pathogenic (PS4, PM2)
					NLRP3	1:247587762G>C c.G1017C p.K339N	- / -	het (father)	0.02740	Likely Pathogenic (PS4, PM1, PM2 PP2)
13_1	Suppurative Lymphadenitis	Rhinitis and sinusitis/ eosinofilia. Maternal grandmother had BCGitis	normal	Low IL-12 cytokine	*CARD14*	17:78181965G>C c.G2836C p.E946Q	- / -	het (mother)	0.02740	Likely Pathogenic (PS4, PM2, PP3)

Prob.= Proband; BCG-AE = Type of BCG adverse event; ([a]) = Human Genome Reference GRCh37/hg19. VUS = Variant of Uncertain Significance; DM=disease-causing mutation; Genot./(inherit) = Genotype/(Inheritance); het = heterozygosis; comp-het = compound heterozygosis; IFN = Interferon; IL-12 = Interleukin-12 N/A = not available; HGMD = Human Gene Mutation Database; ([b]) = using VarSome platform ; ACMG = American College of Medical Genetics; ([c]) Richards et al., 2015.

Six probands with Pathogenic or Likely Pathogenic variants in IEI-Genes presented clinical phenotype "Only BCGitis" (Table 2). Among them, four had suppurative lymphadenitis, one associated with injection site abscess and one associated with secondary bacterial infection. Two other probands had regional enlarged lymph node > 3cm. Fifty percent (3/6) presented altered IL12p70-IFNγ axis: one with low IFNγ, two with low IFNγ and low IL-12 and one with low IFNγ and IFNγ receptor not detected. Six variants classified as Pathogenic or Likely Pathogenic in six different IEI-Genes were identified in WES of those six probands. Among these IEI-genes, 50% (3/6) are related to defects in intrinsic and innate immunity (*STAT1*, *TBK1* and *TLR3*). The remaining three genes are related to autoinflammatory disorders (*NOD2*), complement deficiencies (*CFH*) and immune deregulation diseases (*NFAT5*).

Three probands with Pathogenic or Likely Pathogenic variants in IEI-Genes presented clinical phenotype "Mild or Transient Symptoms" (Table 3). Two had suppurative lymphadenitis (SL) and one had an ulcer >1 cm (UL). In this group, only 33.1% (1/3) of probands presented alteration in IL12p70-IFNγ axis (low IFNγ) and 66.7% (2/3) probands had transient cytopenia (one neutropenia and one low CD8 T and low NK cells). Three variants classified as Pathogenic or Likely Pathogenic in three different IEI-Genes were identified in WES of these three probands. One of these variants is compound heterozygosis. These genes are IEI-Gene related to combined immunodeficiencies with associated or syndromic features (*PMS2*), IEI-Gene related to predominantly antibody deficiencies (*TNFSF12*) and IEI-Gene related to complement deficiencies (*CFH*).

The largest clinical group of this cohort with detectable Pathogenic or Likely Pathogenic variants in IEI-Genes was composed of 10 probands with clinical phenotype "Immunoallergic conditions" (Table 4). Among them, eight had suppurative lymphadenitis (SL), one associated with injection site abscess (ISA) and another one, associated with secondary bacterial infection (BSI). Two other probands had injection site abscess (ISA). Seven out of 10 (70%) required at least one hospital admission due to asthma or recurrent pneumonia and 8/10 (80%) probands had an altered IL12p70-IFNγ axis (seven with low IL-12, three with also low IFNγ, and one with low IFNγ). Cytopenia was identified in 6/10 (60%) of these probands, of which 5/6 (83.3%) were transient cytopenia (neutropenia, total lymphopenia, low CD4 T cells, low naive CD4 T cells, low B cells or low NK cells), and 1/6 (16.7%) had persistent low CD4 T cells. Four out of 10 (40%) probands reported tuberculosis cases in their families and another two probands were familial simplex cases of BCGitis.

WES identified 14 variants classified as Pathogenic or Likely Pathogenic in 11 IEI-Genes in these 10 probands. Three in 10 (30%) had variants classified as Pathogenic or Likely Pathogenic in two or more genes each. Four of the 11 IEI-Genes (36.4%) were related to defects in intrinsic and innate immunity (*IFNGR1, IRF8, JAK1* and *STAT1*), all of them in MSMD-genes. Another 3/11(27.3%) were IEI-genes related to autoinflammatory disorders (*CARD14, NLRP12 and NOD2*). Another 3/11 (27.3%) were IEI-Genes related to combined immunodeficiencies with associated or syndromic features (*CHD7, FOXN1, STAT3*). One of the 11(9.0%) genes was an IEI-Genes related to predominantly antibody deficiencies (*TNFRSF13B*).

Figure 2 shows IEI-Genes variants classified as Pathogenic or Likely Pathogenic by group of clinical phenotypes of the probands, as well as the number of probands with variants in each IEI-Gene.

Parental origin of inherited alleles is also shown in Tables 2 to 4. *De novo* variants classified as Pathogenic or Likely Pathogenic were found in 3 IEI-genes: *CHD7*, *STAT3* and one allele of *PMS2*. One variant in *JAK1* was also assumed to be *de novo*, as it was novel-LOF and it was not found in the WES of the mother and brother (father's sample unavailable for testing). Three other novel-LOF variants were found, two in *CHD7* and one in *STAT1*.

All four novel-LOF variants classified as Pathogenic or Likely Pathogenic were found in probands with "Immunoallergic conditions" clinical phenotype.

Discussion

Twenty-four Pathogenic or Likely Pathogenic variants were identified in 17 IEI-genes from 43.2% children of a Brazilian cohort of BCGitis. A significant percentage (70.5%) of patients with BCGitis developed other clinical findings such as immunoallergic conditions or recurrent infections that required hospitalization and had altered immunological tests.

Timely diagnosis of IEI significantly improves the patient´s outcome. However, as clinical phenotypes of IEI are very heterogeneous, a high level of suspicion is necessary for the diagnosis [23].

Many new IEIs have been described recently. Although in constant expansion, the understanding of clinical phenotypes and the exact molecular mechanisms of all IEIs are still limited [20]. Moreover, the percentage of IEIs diagnoses based on BCGitis is still uncertain [24].

Severe BCG-AE are usually associated with genetic defects or allelic variants associated with IEI affecting innate or adaptive immunity [25]. Although *BCGosis* is more frequent in patients with severe clinical conditions such as severe combined immunodeficiency (SCID), localized BCG-AE have been described in these patients, with incidence rates ranging from 16.6% [12] to one third of patients investigated [26].

In this study, different *in silic*o predictors and a search software, aggregator and impact analysis tool for human genetic variation - VarSome [22] - were used, in observance of ACMG criteria for variant pathogenicity classification.

As expected, there was a predominance of IEI-genes related to Defects in Intrinsic and Innate Immunity among variants classified as Pathogenic or Likely pathogenic: they represented 35.3% (6/17) of IEI-genes with identified variants. Among them, the most frequent were MSMD-genes, with 4/17 (23.5%) classified as Pathogenic or Likely Pathogenic variants.

MSMD is a group of rare innate immunity errors characterized by individual selective susceptibility to clinical disease caused by weakly virulent mycobacterial species, such as BCG strains and environmental mycobacteria, in healthy patients with normal resistance to other microorganisms, in the absence of immunological abnormalities evident in routine evaluation [27-29].

In countries where BCG is administered early in life, BCG infections are often the first sign of MSMD disease [28, 30, 31], with severity in patients with MSMD varying from localized infections to widespread and life-threatening infections [29, 32].

The incomplete penetrance in MSMD genes suggests the presence of modifying factors explaining different susceptibility of individuals with the same variant but with variable clinical outcomes [30]. Allelic heterogeneity can also interfere, as different defects of the same gene can result in different disorders [28, 33].

Among variants previously described in the literature and found in this study, one of them occurred in a MSMD-gene: a heterozygous variant in the *IFNGR1* gene (Proband 27_1). This variant has been previously identified by Jouanguy *et al.* [34] as a partial IFNGR1 deficiency in one child with curable BCG infection and his sibling with latent tuberculosis. This variant was also reported by Remiszewski *et al.* [35] who identified a 20-year-old female with disseminated *Mycobacterium avium* disease involving bones, lungs and brain. She was completely healthy until this illness and had been vaccinated with BCG in infancy without complications. Functional analysis of this variant was performed by van de Wetering *et al.* [36] and confirmed that the severely reduced function of the I87T mutant receptor can lead to partial IFNGR1 deficiency.

IFNGR1 deficiency caused by variants in *IFNGR1* gene that are either autosomal recessive or autosomal dominant have a high degree of allelic, cellular and clinical phenotype heterogeneity [37-39]. Most recessive IFNGR1 deficiencies result in complete loss of cellular responsiveness to IFNγ due to mutations that preclude the expression of IFNGR1 on the cell surface.

Another group of IFNGR1 deficiencies is due to missense mutations which result in normal expression of IFNGR1 at the cell surface, however, the resulting receptors show either diminished or hindered binding of IFNγ [36]. This is compatible with the alterated IL12p70-IFNγ axis test of proband 27_1.

Siblings reported by Jouanguy *et al.* [34] had also some degree of atopy, as observed with proband 27_1. Studies have shown that genetic variants in these IFN-pathway genes may have some degree of susceptibility to a range of common, chronic human diseases, which have an inflammatory component with high IgE levels and clinical phenotypes of asthma, atopic dermatitis and eczema herpeticum [40-42].

Variants associated to Autoinflammatory Disorders and classified as Pathogenic or Likely Pathogenic in IEI-Genes were found in 3/17 (17.6%) of identified IEI-genes of this cohort. Interestingly, these variants were observed especially within probands from the group that displayed immunoallergic conditions.

In autoinflammatory disorders, IEI are usually caused by hyperfunction of the immune system, with frequent manifestations of recurrent inflammatory episodes [43]. Since the disease mechanism of various inflammatory disorders is related to increased production of proinflammatory cytokine IL1β and possible deviation from Th1 response to Th17, a less effective containment of BCG replication [44] could justify the BCG-AE presented by these patients.

No proband in the cohort presented a SCID phenotype and no variant classified as Pathogenic or Likely Pathogenic in IEI-Genes related to Immunodeficiencies affecting cellular and humoral immunity was identified. Although BCGitis is reported in SCID patients, BCG dissemination is more likely to occur [6].

Three variants classified as Pathogenic or Likely Pathogenic among IEI-genes related to Combined immunodeficiencies with associated or syndromic features were identified in *CHD7* gene. All patients had a phenotype of severe immunoallergic conditions with recurrent infections (Table 4). Two of the *CHD7* variants are *novel* (probands 14_1 and 32_1) and the other one is a *de novo* variant (proband 6_1).

Heterozygous pathogenic variants in *CHD7* are the most frequent cause of CHARGE Syndrome [45-47] and pathogenic *CHD7* variant is considered as a major criterion for the diagnosis of CHARGE syndrome (Hale *et al*., 2016). All three probands with variants in *CHD7* will be investigated in depth and will be described in another article.

Proband 14_1 had another variant classified as pathogenic among IEI-genes related to Combined immunodeficiencies with associated or syndromic features: that was a *de novo* variant in *STAT3*. *STAT3* acts as a central transcription factor downstream of multiple cytokine and growth factor receptors and thus regulates antimicrobial responses and cell survival [48]. *STAT3* mutations can cause autosomal dominate hyper-IgE syndrome (AD-HIES), characterized by elevated IgE levels, persistent eczema, repeated skin abscesses, recurrent pneumonia with abscess and pneumatocele formation, candida infections, peculiar face and skeletal and connective tissue abnormalities [49, 50]. This phenotype is compatible with the relevant clinical characteristics presented by proband 14_1, including high IgE levels.

Two of the 17 (11.8%) Pathogenic or Likely Pathogenic variants were related to Predominantly Antibody Deficiencies IEI-genes (*TNFSF12* and *TNFRSF13B*). Studies suggest antibodies can also provide protection against intracellular pathogens such as micobacteria by targeting innate immune antimicrobial activity via Fc receptor-mediated opsonization and phagocytosis [51-53], which are found in all innate immune cells [54]. Lu *et al*. [53] demonstrated that different antibody profiles appear to correlate with different stages of TB disease (active or latent) and may be able to lead to cell cytotoxicity mediated by NK cells, phagolysosomal maturation, inflammasome activation or intervene in other defense mechanisms.

The same can occur to humoral immune response to vaccination with BCG [55, 56]. In this way, IEI that affect production of antibodies or B cell function may result in inefficiency to contain BCG replication, leading to an adverse event.

One proband from this cohort had a chromosome 17p13 Microdeletion Syndrome (proband 7_1). To date, no IEI-gene was identified in this region of chromosome 17 [57-60]. However, there are two reports associating of 17p13 microdeletion to thymic hypoplasia suggesting partial DiGeorge's Anomaly [61] and one case associated with T cell lymphopenia [62]. However, no monogenic disorder was identified in these two studies.

Among the family cases, many had relevant clinical conditions in addition to the BCG-AE, however, the genetic variants found did not meet the criteria adopted in this study for analysis, particularly an explanation for the familial aggregation of BCGitis.

Variants in IEI-genes classified as Pathogenic or Likely Pathogenic were found in all groups of clinical phenotypes analyzed (Figure 2). That is in line with the substantial phenotypic and clinical heterogeneity observed and described within groups of patients with variants in the same gene and even between individuals from same pedigree [63].

Diagnostic sequencing and genetic testing have the drawback that the effect of a variant on function cannot be inferred from sequencing alone, with a large proportion of variants classified as variants of uncertain significance (VUS) persisting in bioinformatic databases [64].

In this study, many cases with relevant clinical findings presented VUS variants or even no variants in IEI-Genes were found at all. Ewans *et al.* [65] observed that even if a disease-causing gene is not identified in a first analysis, or the variants found are initially classified as VUS, a future reanalysis increases molecular diagnoses. So, re-analyses will be carried out in due course and other groups of genes will be investigated in the probands of this cohort.

In this study we identified several cases in which the proband inherited a candidate causative variant from an unaffected parent. This observation led us to hypothesize a possible incomplete penetrance for those cases. Al Dhaheri *et al.* [66] raise four explanations for this sort of observation: a) a misclassification of a parent presenting a mild phenotype instead of being unaffected; b) an environmental effect requirement for this specific genotype; c) a bialelic variant requirement in which the second hit was lost due to technical limitation; and d) an oligogenic disease, in which the patient inherited the second failed gene from the other parent and, due to a technical limitation, it could not be identified.

Likewise, it is important to remember that the identification of associations between an allele and an observed clinical outcome does not necessarily mean that the allele itself conveys a functional difference [36].

The overall diagnostic yield of genetic variants identified by WES in cohorts of different pathologies varies in the literature from 5% [67], 25,2% [68] to 40 - 50% [69, 70], even considering patients with immunological impairment [16, 17, 71]. From a genetic point of view, these yield variations can be explained, for example, by possible repeated expansion variants, somatic variants and deep intron variants with indeterminate splicing effects [70].

To identify which variants found in a WES might have functional or neutral changes remains challenging. Indeed, even using a standardized approach, a consensus classification is not achieved in 100% of cases even among experts [72]. This study does not intend to offer a definitive genetic diagnosis, but sought to demonstrate a possible relationship that a localized BCG-AE may indicate an IEI, emphasizing the importance of adequate follow-up and investigation of these patients.

To consider that children with localized BCG-AE may have some genetic-molecular disorder linked to an immunological defect is essential to define early intervention and prevent clinical complications and unfavorable outcome usually observed in children with undiagnosed primary immunodeficiencies timely.

As far as we know, this is the first study to assess exome of patients who had BCGitis using a panel of IEI-related genes, with many plausible variants identified, suggesting that BCGitis may be signaling an inborn error of immunity.

Declarations

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Conflicts of interest/competing interests

The authors declare no conflicts of Interest/competing interests

Availability of data and material

The raw data supporting the conclusions of this article can be made available by the authors under request.

Code availability

Not applicable

Authors' contributions

SAMGM, RPM and MIMP designed the study, performed data analysis and wrote the manuscript.

RFS, MMM, CPG, PV, ARM, PNA and EA performed data analysis and helped with manuscript preparation.

JBP, CSA and ACO discussed results and helped with manuscript preparation.

TNFM and LYW helped with collection of study samples and clinical information and with manuscript preparation.

All authors commented and approved the final version of the manuscript.

Ethics approval

This study was approved by the Ethics Committee of Universidade Federal de São Paulo (842,006 and 1,641,734).

Consent to participate/ Consent for publication

All participants or legal guardians signed the consent forms and provided consent for publication of data.

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Statement of interest

none to declare

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Figures

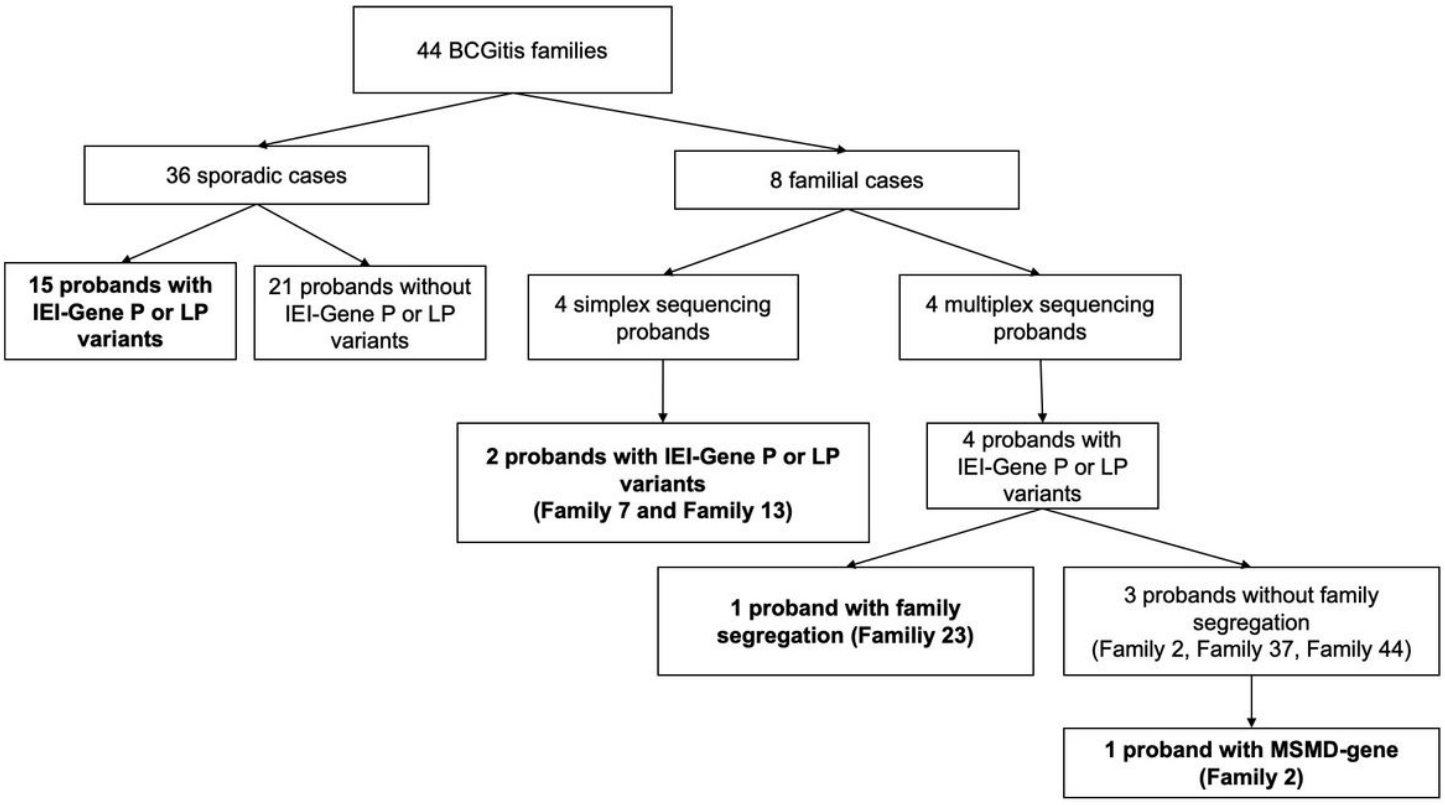

Figure 1

Description of study cohort. IEI-Genes: Genes related to Inborn Errors of Immunity. *P: Pathogenic variant. LP: Likely Pathogenic variant. MSMD-Gene: Genes related to Mendelian susceptibility to mycobacterial diseases.*

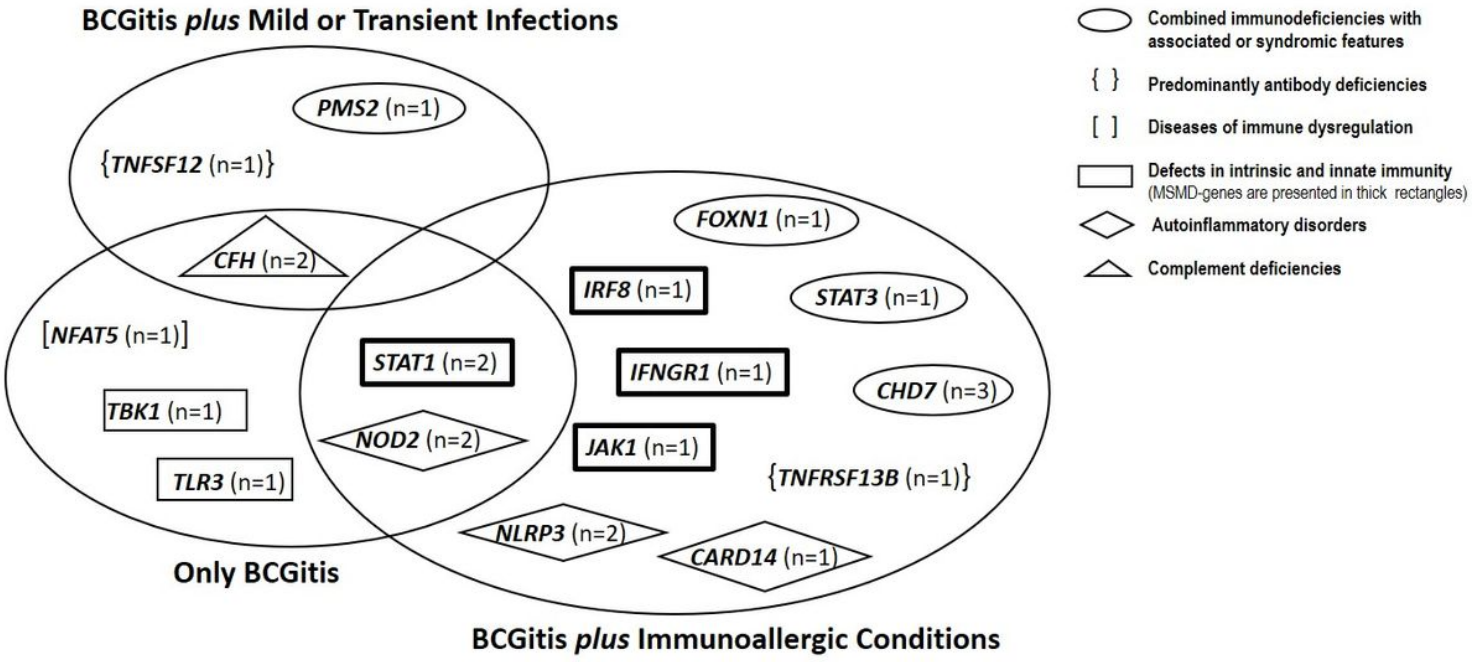

Figure 2

Genes related to Inborn Errors of Immunity in which variants classified as Pathogenic or Likely Pathogenic were identified, by Group of clinical phenotypes of the probands. *Number of probands with variants in each gene is shown in parentheses. IEI-genes Classification according IUIS 2020 (Tangye et al., 2020).*

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplemTable1.docx
- SupplemTable2.docx