Title page

Genetic screening of male patients with primary hypogammaglobulinemia can guide diagnosis and clinical management

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Abstract

The precise diagnosis of an immunodeficiency is sometimes difficult to assess, especially due to the large spectrum of phenotypic variation reported among patients. Common variable immunodeficiency disorders (CVID) do not have, for a large part, an identified genetic cause. The identification of a causal genetic mutation is important to confirm, or in some cases correct, the diagnosis. We screened >150 male patients with hypogammaglobulinemia for mutations in three genes involved in pediatric X-linked primary immunoglobulin deficiency: *CD40LG*, *SH2D1A* and *BTK*. The *SH2D1A* screening allowed to reclassify two individuals with an initial CVID presentation as XLP after mutations identification. All these mutations were associated with a lack of protein expression. In addition, 4 patients with a primary diagnosis of CVID and one with a primary IgG subclass deficiency were requalified as XLA after identifying *BTK* mutations. Interestingly, two out of these 5 patients carried a damaging coding *BTK* mutation associated with a lower, but detectable, BTK expression in monocytes, suggesting that a dysfunctional protein explains the disease phenotype in these patients. In conclusion, our results advocate to include *SH2D1A* and *BTK* in newly developed targeted NGS genetic testing, to contribute to providing the most appropriate medical treatment and genetic counselling.

Keywords

Immunodeficiency, BTK, SAP, CD40L, CVID

1 **Abbreviations**

2 CVID: Common variable immunodeficiency disorders

3 EBV: Epstein-Barr virus

4 HC: healthy controls

5 HIGM: X-linked hyperIgM syndrome

6 Ig: immunoglobulin

7 IgAD: IgA deficiency

8 MFI: mean fluorescent intensity

9 NGS: next generation sequencing

10 PE: Phycoerythrin

11 SAP: SLAM-associated protein

12 XLA: X-linked agammaglobulinemia

13 XLP: X-linked lymphoproliferative disease

14

1 **Introduction**

2 Common variable immunodeficiency disorders (CVID) are characterized by a defect in
3 immunoglobulin (Ig) production. Most patients present recurrent infections of the respiratory
4 and gastrointestinal tracts and, less frequently, lymphoid proliferation or autoimmune diseases
5 [1]. The prevalence of CVID has been estimated at 1/30,000 in the European population, and
6 this disorder is the second most frequent immunodeficiency of adulthood, after IgA deficiency
7 (IgAD). About 20% of patients have at least one relative affected by IgAD or CVID, suggesting
8 a genetic origin in such cases. Genetic abnormalities in 24 genes involving different immune
9 pathways have been demonstrated in less than 10% of CVID patients to date [2]. Some genetic
10 variants, such as the ones in *TNFRSF13B* [3], do not seem sufficient to cause CVID phenotype
11 while others, such as the ones in *CD19* [2,4], present a full penetrance toward Ig deficiency.

12 The clinical presentation of patients with CVID is wide and made by exclusion of other
13 possible causes [5], which can sometimes lead to a diagnosis reclassification after identification
14 of genetic mutations or occurrence of new biological evidence or symptoms. Few CVID
15 patients were reclassified to XLA (X-linked agammaglobulinemia) when a mutation in the *BTK*
16 gene was found (OMIM: *300300) [6–16]. XLA is characterized by a failure during B-cell
17 development [17,18], and is usually diagnosed before the age of 18 months, after the loss of
18 maternal antibody protection [19], most of patients present an absence of B-cells and
19 immunoglobulin. Similarly, occasional reports of mutations in *SH2D1A* were showed in CVID
20 patients [20–24]. Mutations in *SH2D1A*, encoding SAP (SLAM-associated protein; OMIM:
21 *300490), are found in patients with XLP (X-linked lymphoproliferative disease) [25,26],
22 which is characterized by an extreme susceptibility to Epstein-Barr virus (EBV) infections,
23 resulting in near-fatal infectious mononucleosis, acquired hypogammaglobulinemia, malignant
24 lymphoma or a combination of these conditions [27,28].

1 In order to identify disease-causing mutations and their potential to support a diagnosis,
2 we explored a large cohort of French hypogammaglobulinemic patients (see figure S1) showing
3 a wide range of primary diagnosis (Table 1). We therefore systematically screened this
4 population for mutations in the *SH2D1A* and *BTK* genes, as well as for mutations in *CD40LG*
5 (OMIM: *300386), a gene responsible for the hyperIgM syndrome (HIGM1) that presents
6 numerous similarities with CVID [29,30].
7

Materials and methods

Patients

Investigated patients came from the DEFI cohort, a French cohort of patients with primary hypogammaglobulinemia [1,5]. All patients gave informed consent for DNA sequencing, and the study was approved by the local institutional review board, in accordance with the Helsinki declaration. Initial diagnoses are reported in table 1.

Genes

The *CD40LG* gene, located in Xq26.3, encodes for the CD40 ligand. After PCR amplification of the five exons and their flanking intronic regions, the PCR products were directly sequenced in 150 male patients. We identified 3 SNPs (rs11575982, rs3092923 and rs148594123) with a frequency equivalent to the matching 1000 Genomes population (<http://www.internationalgenome.org/1000-genomes-browsers/>) [31,32].

The *SH2D1A* gene is located in Xq25 and encodes for SAP. After PCR amplification of the four exons and their flanking intronic regions, we directly sequenced the PCR products in a total of 230 male subjects. We identified 2 SNPs (rs200198093 and rs72610640) with a frequency equivalent to the matching 1000 Genomes population.

The *BTK* gene is located in Xq22.1. After PCR amplification of the 19 exons and their flanking intronic regions, we directly sequenced the PCR products in a total of 181 male patients from the DEFI cohort.

Molecular analysis

Genomic DNA was extracted from buffy coats. All coding exons and intronic flanking regions of *CD40LG*, *SH2D1A* and *BTK* were amplified (primers available upon request) and sequenced with the Big Dye Terminator kit (Applied Biosystems) on an ABI3730 DNA sequencer.

1 Total RNA was extracted from PHA T-cell blast and EBV-transformed B-cells with
2 TRIzol (Invitrogen). The mRNA was reverse-transcribed with oligo-dT primers and
3 SuperScriptIII reverse transcriptase (Invitrogen), to obtain cDNA.

4 Cloning of the amplified cDNA

5 The PCR products obtained after RNA extraction and cDNA amplification were
6 inserted into the TOPO® TA cloning vector (Invitrogen) and sequenced.

7 Flow cytometry analysis of BTK expression

8 Intracellular BTK staining was performed on thawed PBMC with the Fix and Perm®
9 kit (Caltag), used according to the manufacturer's instructions. PBMC were first stained with
10 FITC-conjugated anti-CD14 (IgG2a; Immunotech), PerCP-Cyanine5.5-conjugated anti-CD45
11 (IgG1; BD Biosciences) and APC-conjugated anti-CD19 (IgG1; BD Biosciences) for 15
12 minutes at room temperature. The cells were fixed, washed and permeabilized and Fc receptors
13 were blocked by incubation with human serum for 15 minutes at room temperature. PBMC
14 were stained by incubation with Phycoerythrin (PE)-labeled anti-BTK antibody (IgG2a; BD
15 Biosciences) or with an irrelevant Ab (CD10, IgG2a; BD Biosciences) for 15 minutes at room
16 temperature. The stained cells were analyzed with a BD FACS Canto™ flow cytometer (BD
17 Biosciences). The results are expressed by the difference (Δ) of monocytes (CD45+CD14+)
18 mean fluorescence intensity (MFI) between anti-BTK and irrelevant Ab. Fifteen male healthy
19 controls (HC) presented a median Δ MFI of 107 [79-173].

20 Flow cytometry analysis of CD40L expression

21 PHA T-cell blasts were stained by incubation with PerCP-conjugated anti-CD3 (SK7,
22 BD Biosciences) and PE-conjugated anti-CD40L (555700, BD Biosciences) antibodies for 30

1 minutes at 4°C. The stained cells were analyzed in a FACSCalibur flow cytometer (BD
2 Biosciences).

3 SAP Western blotting

4 Lysates of $5 \cdot 10^6$ PHA T-cell blasts were separated by SDS-PAGE and immunoblotted
5 with a polyclonal rabbit anti-SAP antibody (2778, Cell Signaling Technology) and a polyclonal
6 goat anti-actin antibody (1616, Santa Cruz).

7

Results

CD40LG mutation screening

Four patients with a primary HIGM diagnosis were included in our cohort for genetic screening. Of those, only 3 presented a mutation in *CD40LG*: 228_231delAAGA, 409+1G>A and 410-2A>G (Figure 1A). These mutations were associated with an absence of CD40L cell surface detection on activated T-cell (see table 2). The fourth patient with an HIGM diagnosis may carry a mutation in another HIGM-causing gene (e.g. *AICDA*, *CD40*) [33,34] and as such should be screened for it. We did not identify any *CD40LG* mutation in any other patient of the cohort.

SH2D1A mutation screening

No patient included in our cohort were initially diagnosed with XLP. After identifying a first patient initially diagnosed with CVID but carrying a *SH2D1A* mutation (patient S1) in the first 150 patients, we expanded our *SH2D1A* screening to 80 additional individuals and identified a second CVID patient with a *SH2D1A* mutation (patient S2, table 3 and figure 1B).

Patient S1 was mutated in intron 2: 201+3A>G. We identified and sequenced two splicing mRNA variants in activated T-cells by RT-PCR: a first variant where exon 2 were spliced out (cDNA fragment of 323bp) and a second variant where exon 2 and a portion of exon 3 were spliced out (cDNA fragment of 268bp). We did not detect SAP protein expression in activated T-cells protein extracts using immunoblot technique (Figure 2). Patient S2 carried a E67G coding mutation in exon 2 that was associated with an absence of SAP protein on the immunoblot performed with activated T-cells protein extract (Figure 2).

The clinical presentation of of these 2 patients was as followed. Patient S1 first diagnosis was pediatric CVID at eight years of age after detection of hypogammaglobulinemia during bacterial pneumonia. He also suffered from severe infectious mononucleosis at six years of age.

The patient was treated with gammaglobulins, administered intramuscularly for the first 10 years, and then intravenously. Patient S1 had 4% circulating B-cells including 87% naive (CD27-IgM+), 3% switched memory (CD27+IgM-) and 7% CD27+IgM+ B-cells (Table 3). He presented a family history suggestive of X-linked disease, indeed, his older brother died with a refractory digestive EBV-driven B large cell lymphoma in the course of an otherwise unremarkable CVID, without previous severe EBV primo-infection. Patient S2 was diagnosed with CVID at the age of 40 based on the detection of profound hypogammaglobulinemia after severe bacterial pneumonia without familial history of X-linked disease. He denied suffering severe EBV primary infection, despite chronic positive plasma circulating EBV viral load. The patient had 4% circulating B-cells including 19% naive (CD27-IgM+), 57% switched memory (CD27+IgM-) and 15% CD27+IgM+ B-cells (Table 3).

BTK mutation screening

In our cohort, 7% of patients presented with an initial diagnosis of XLA (Table 1). As our genetic screening revealed, *BTK* mutations was found only in patients presenting a very low B-cell count (<1%, see table 4), we included 31 additional hypogammaglobulinemic patients with B-cell counts below 1%. We detected a *BTK* mutation in 17 out of 20 patients with a primary XLA diagnosis (B1-B17, table 4, Figure 1C). Despite a clinical/immunological phenotype consistent with the diagnosis of XLA, we could not confirm genetically the diagnosis in 3 patients (B18-B20, table 4). These 3 patients may carry a mutation in another gene causing agammaglobulinemia (e.g. *BLNK*, *PI3KR1*) [35,36] and as such should be screened for it. Strikingly, we found a *BTK* mutation in 5 patients (B21-B25) with a primary diagnosis of CVID or IgG subclass deficiency (Table 4, boxes in Figure 1C). Overall, we described 20 different mutations in 22 patients, including 5 novel mutations while the others were previously reported in the BTK database (<https://databases.lovd.nl/shared/genes/BTK>) [37].

We observed a phenotypic variability in patients carrying *BTK* mutations despite showing all less than 1% B-cells; indeed, while a majority of patients presented their first symptoms at early age in infancy some were described at later age (up to 18 years old for B22, see table 4). In addition, of all the *BTK* mutated patients only 3 had an IgG level above 4g/L and all with a primary diagnosis of CVID or IgG subclass deficiency (B21, B22 and B25; see table 4). We hypothesized that this variability could be explained by the impact of each mutation on the *BTK* protein function and expression. Considering the absence or very low levels of B-cells in XLA patients, we carried out intracellular FACS assay to measure *BTK* expression in monocytes. We measured the delta between *BTK* mean fluorescent intensity (MFI) and the irrelevant antibody MFI (i.e. Δ MFI) in 15 healthy controls and observed a median Δ MFI of 107 [79-173] (Figure 3A). We then obtained data for patients harboring *BTK* mutations (17 different mutations out of 21, see table 4 and Figure 3). As expected, *BTK* protein was undetectable in all 13 patients' monocytes with a primary diagnosis of XLA, as illustrated with patient B14 on Figure 3A. When considering the 3 patients with a primary diagnosis of XLA but without identified *BTK* mutation, we detected a lower Δ MFI in 2 patients (B18 and B19, Figure 3B) and a Δ MFI in the normal range for the B20 patient (Figure 3B).

Finally, we measured *BTK* expression in 4 out of 5 patients carrying a *BTK* mutation but without a primary diagnosis of XLA, as we could not measure *BTK* expression for patient B24. However, this patient carried the same M630V mutation as for the XLA B5 patient, so we can only extrapolate that he might also present an undetectable *BTK* expression (Table 4). *BTK* expression was undetectable in patients B22 and B25 monocytes, who were primarily diagnosed with CVID and IgG subclass deficiency, respectively (Table 4, Figure 3C). Patients B21 and B23 showed a decreased but detectable *BTK* expression in monocytes (Figure 3C), in normal range for B21 (Δ MFI=80) and just below for B23 (Δ MFI=69). R28H mutation is described in multiple studies as responsible for the XLA phenotype (Table 4), moreover, it is

1 reported as pathogenic in the ClinVar database
2 (<https://www.ncbi.nlm.nih.gov/clinvar/variation/11348/>). These suggest strongly that R28H is
3 responsible of patient B21 phenotype. G164D coding mutation is predicted by the CADD
4 bioinformatics tool [38] to be damaging for the protein function (score: 29.1). This mutation is
5 not described in populations databases such as the 1000 Genomes project [31]. These data are
6 not sufficient to describe G164D as responsible of the disease, additional family testing would
7 permit to settle this down. Altogether, these results suggest an alternative pathophysiological
8 mechanism to the absence of detectable BTK protein: lower level of BTK may indicate a
9 dysfunction of the BTK signaling pathway that could consequently alter the development of B-
10 cells and promote hypogammaglobulinemia.

11

Discussion

We sequenced three genes (*CD40LG*, *SH2D1A*, *BTK*) located on the X chromosome in male patients with primary humoral deficiencies from the French national DEFI cohort (Figure S1). Deleterious mutations in these genes are responsible of profound defects in immunoglobulin production, are usually diagnosed in children and associated with specific phenotypes. We intentionally did not restrict patients' selection to any particular clinical or biological phenotypes as we intended to identify mutations outside of a commonly recognized clinical presentation. We demonstrated that the systematic screening of these genes is useful to confirm and/or reclassify immune defects diagnosis in adults with humoral primary immunodeficiency.

CD40LG genetic screening of 150 male patients did not reveal any mutation outside of the classical clinical presentation of HIGM, indicating that the *CD40LG* mutations are responsible for a very specific clinical and immunological phenotype that cannot be confused with CVID or any other defects. One patient with a HIGM phenotype did not exhibit any mutation in *CD40LG*; to further characterize this patient's disease, additional genetic analyses should be performed for autosomal recessive HIGM genes such as *AICDA*, *CD40* or *UNG* [33,34].

Here, we characterized 2 patients primarily diagnosed as CVID but presenting a *SH2D1A* mutation with an absence of SAP detection by immunoblot. XLP main feature is an extreme susceptibility to EBV infection, and one of these 2 patients presented a severe infectious mononucleosis at six years of age. However, the second one claimed he did not suffer any severe EBV primary infection during his childhood and had no familial history of X-linked disease. This indicates that, despite the low percentage of diagnosed patients (2 out of 145 if we consider only CVID), the *SH2D1A* mutation knowledge is important in all male patients diagnosed with CVID, whether or not they have a medical or familial history consistent with

1 severe EBV infection. Indeed, the XLP diagnosis significantly modifies the therapeutic and
2 follow-up options of the patients.

3 The systematic sequencing of *BTK* in 181 male patients confirmed the suspected XLA
4 diagnosis in 17/20 patients. Moreover, we identified 5 patients with a primary diagnosis of
5 CVID or IgG subclass deficiency carrying a mutation in the *BTK* gene. This misdiagnosis could
6 have several explanations: persistence of low levels but detectable IgG, IgA or IgM; insufficient
7 genealogic investigation to detect familial X-linked disease; onset of disease at adulthood;
8 absence of serious pulmonary complications. It emphasizes that all male patients with a
9 diagnosis of primary humoral immunodeficiency and with very low levels of circulating B-cells
10 (<1%), regardless of age at diagnosis, residual Ig concentration or familial history, should be
11 screened for *BTK* defects, either by FACS detection in monocytes or by direct sequencing of
12 the coding region.

13 The 3 patients referred to us with a diagnosis of XLA and in whom we could not identify
14 *BTK* mutations are of great interest. B18 and B19 patients do display clinical features consistent
15 with XLA, including an absence of circulating B-cells. BTK expression in monocytes, despite
16 being detectable, was lower in these patients compared to controls. B20 also shows an absence
17 of circulating B-cells but with a normal level of BTK expression and a family history of
18 autosomal recessive disease. Notwithstanding no mutations were found in these individuals,
19 including after screening the 5' and 3'UTR regulatory regions; at this stage, we cannot exclude
20 the possibility that the weak or undetectable BTK expression may be the result of a mutation in
21 another *BTK* regulatory region not covered by the sequencing method we implemented.
22 Alternatively, other intracellular proteins in the B-cell lineage may be absent or defective in
23 these patients, accounting for the weak translation of *BTK* and the low level of BTK activity in
24 B-cell precursors. These patients may carry a mutation in genes responsible of autosomal
25 recessive agammaglobulinemia, and especially B20 considering his family history. Beyond

1 these previously identified genes, an agnostic screening using whole-exome or whole-genome
2 next-generation sequencing (NGS) might also reveal novel disease-causing genes.

3 With the rising of NGS technologies, the possibility of genetic screening has increased.
4 Our results advocate to include *SH2D1A* and *BTK* genes in newly developed targeted NGS
5 panel genetic testing for all primary immunodeficiency diagnosis including CVID or related
6 hypogammaglobulinemia. This is supported by the fact that such NGS panel are already in
7 place, they include *SH2D1A* and *BTK* but also other genes responsible of HIGM, XLP or XLA
8 diseases (e.g. *CD40*, *PIK3R1*, etc.). This will further help characterize monogenic forms of
9 CVID, which still count for less than 10% of patients [2], from polygenic forms of CVID. To
10 achieve deciphering the pathophysiology and help improve treatments of these complex forms
11 of CVID, and in addition with NGS, efforts should be combined and integrated from different
12 omics approaches such as epigenomics, proteomics and metabolomics [39,40].

13 In conclusion, our systematic genetic screening in a large cohort of adults with primary
14 humoral immunodeficiency highlights several key features of CVID and
15 hypogammaglobulinemia genetics: (1) There is a good correlation between *CD40LG* genotype
16 and phenotype; (2) a *SH2D1A* mutation can occur in a patient with atypical clinical features;
17 (3) *BTK* genotype do not always correlates with cellular phenotype (*BTK* expression in
18 monocytes), immunological phenotype (Ig concentrations at the time of immunodeficiency
19 diagnosis) and clinical phenotype. A knowledge of the genetic status of each patient is therefore
20 crucial for a firm diagnosis, as it leads to the most appropriate medical treatment and genetic
21 counselling.

22

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8

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1 Tables

2 **Table 1:** Diagnosis provided by the clinical investigators before the genetic screening of *CD40LG*, *SH2D1A* and *BTK*.

	<i>CD40LG</i> <i>n=150</i>	<i>SH2D1A</i> <i>n=230</i>	<i>BTK</i> <i>n=181</i>
<i>HIGM</i>	4	4	4
<i>XLP</i>	0	0	0
<i>XLA</i>	11	14	20
<i>CVID</i>	109	145	120
<i>IgAD</i>	0	3	0
<i>IgG-SCL</i>	9	15	13
<i>Good syndrome</i>	4	9	8
<i>Other</i>	13	40	16

3

4 HIGM: X-linked hyperIgM syndrome. XLP: X-linked lymphoproliferative disease. XLA: X-linked agammaglobulinemia. CVID: Common

5 variable immunodeficiency disorders. IgAD: IgA deficiency. IgG-SCL: IgG subclass deficiency.

6

1 **Table 2:** *CD40LG* mutations and clinical features of the patients

2

3

<i>Patient ID</i>	<i>Age at first symptoms (years)</i>	<i>B-cells (%)</i>	<i>IgG (g/L)</i>	<i>IgA (g/L)</i>	<i>IgM (g/L)</i>	<i>Family history</i>	<i>Opportunistic infections</i>	<i>Initial diagnosis</i>	<i>Mutation</i>	<i>Previously described</i>	<i>FACS expression</i>
<i>C1</i>	4	9	1.3	0	7.5	no	yes	HIGM	228-231delAA GA	no	none
<i>C2</i>	0	7	3.3	0.4	1.2	no	yes	HIGM	409+1G>A	yes [41]	none
<i>C3</i>	1	7	0.1	0	0.9	no	yes	HIGM	410-2A>G	no	none

1 **Table 3:** *SH2D1A* mutations and clinical features of the patients

2

<i>Patient ID</i>	<i>Age at first symptoms (years)</i>	<i>B-cells (%)</i>	<i>IgG (g/L)</i>	<i>IgA (g/L)</i>	<i>IgM (g/L)</i>	<i>Family history</i>	<i>Initial diagnosis</i>	<i>Mutation</i>	<i>Previously described</i>	<i>WB expression</i>
<i>S1</i>	3	4	1.8	0.4	0.4	yes	CVID	201+3A>G	yes [42]	none
<i>S2</i>	6	4	1.2	0	0.2	no	CVID	E67G	yes [43]	none

3 See figure 2 for Western blot.

4

1 **Table 4:** *BTK* mutations and clinical features of the patients

<i>Patient ID</i>	<i>Age at first symptoms (years)</i>	<i>B-cells (%)</i>	<i>IgG (g/L)</i>	<i>IgA (g/L)</i>	<i>IgM (g/L)</i>	<i>Family history</i>	<i>Bronchiectasis</i>	<i>Initial diagnosis</i>	<i>Mutation</i>	<i>Previously described *</i>	<i>FACS expression **</i>
<i>B1</i>	0	0	0.5	0.3	0.6	yes	yes	XLA	T117P	yes [44,45]	ND
<i>B2</i>	1	0	NA	0.1	0.1	no	no	XLA	R332L,H333L	no	none
<i>B3</i>	0	0	0	0	0	no	no	XLA	C502R	yes [46]	none
<i>B4</i>	3	0.1	0	0	0	no	no	XLA	K577N	yes [46]	none
<i>B5</i>	1	0	ND	0	0	yes	no	XLA	M630V	yes [46]	none
<i>B6</i>	0	0	1.2	0	0	yes	yes	XLA	F644L	yes (unpublished***)	ND
<i>B7</i>	0	0	0.7	0	0.4	yes	yes	XLA	R525X	yes [47]	ND
<i>B8</i>	2	0	2.0	0	0.4	no	yes	XLA	100delG	no	none
<i>B9</i>	0	0	0.8	0.2	0.3	yes	yes	XLA	1953delT	yes [47]	none

<i>B10</i>	4	0	ND	0.1	0	no	yes	XLA	g.2275_13114del10838insCC C	no	none
<i>B11</i>	4	0	ND	0	0.1	yes	no	XLA	174_175insT	yes [45]	none
<i>B12</i>	1	0	3.7	0.1	0.1	no	yes	XLA	215_216insA	yes [48]	ND
<i>B13</i>	1	0	1.5	0	0	yes	no	XLA	1349+5G>A	yes [49]	none
<i>B14</i>	0	0	ND	0	0	yes	no	XLA	1566+1G>T	yes [45]	none
<i>B15</i>	0	0	0.8	0	0	no	no	XLA	1567-12delTTTG	yes [50]	none
<i>B16</i>	0	0	0	0	0	yes	yes	XLA	1750+5G>A	yes [51]	none
<i>B17</i>	0	0.1	0	0	0.2	no	yes	XLA	1750+5G>A	yes [51]	none
<i>B18</i>	22	0	0	0	0.1	yes (XL)	yes	XLA	No mutation	-	low
<i>B19</i>	14	0.4	0	0	0.1	yes	no	XLA	No mutation	-	low

<i>B20</i>	0	0	1	0	0	yes (AR)	yes	XLA	No mutation	-	yes
<i>B21</i>	5	0.3	4.4	1.6	0.4	no	no	CVID	R28H	yes [50,52]	yes
<i>B22</i>	18	0.1	4.4	1.8	0.1	no	no	CVID	F114V	no	none
<i>B23</i>	15	0.1	2.1	0	0.3	no	yes	CVID	G164D	no	low
<i>B24</i>	0	0	0	0	0	yes	yes	CVID	M630V	yes [46]	ND
<i>B25</i>	0	0.1	4.2	1.2	0.4	no	yes	IgG s/class	1350-2A>G	yes [53]	none

1

2 *Only the oldest description is cited, see the btkbase for a complete list: <https://databases.lovd.nl/shared/genes/BTK>.

3 **See figure 3 for FACS expression.

4 ***See in btkbase.

5 Patients B1 to B17 had clinical and biological features consistent with XLA and an identified BTK mutation. Patients B16 and B17, as well as,
6 B5 and B24 do not belong to the same family. Patients B18 to B20 had an initial diagnosis of XLA based on phenotypic data, no mutation in
7 *BTK* were found. Newly described mutations are in bold. CVID or IgG subclass deficiency patients' mutations are boxed. Patients B21 to B25
8 had a primary diagnosis of humoral deficiency, 4 CVID and 1 IgG subclass deficiency. XL: X-linked; AR: autosomic recessive.

9

Figure Legends

Figure 1: (A) Schematic diagram of the *CD40LG* coding region, with 5 coding exons, 783 nucleotides (nt) and 261 amino acids (AA). The 3 patients' mutations are represented. TM: transmembrane domain. Dotted lines are the trimer interface. (B) Schematic diagram of the coding region of *SH2D1A*, with 4 exons, 387 nt and 128 AA. SH2: Src homology 2 domain. The 2 patients' mutations are represented. (C) Schematic diagram of the coding region of *BTK*, with 18 coding exons, 1980 nt and 659 AA. Newly described mutations are in bold. CVID or IgG subclass deficiency patients' mutations are boxed. PH: pleckstrin homology. ZF: zinc finger. SH3: Src homology 3 domain. SH2: Src homology 2 domain. TK: tyrosine kinase.

Figure 2: Immunoblot of SAP in PHA T-cell blasts protein extraction. Actin is represented as a control of protein expression. HC: healthy control.

Figure 3: BTK expression in gated monocytes (CD45+, CD14+). (A) healthy control (HC) and a representative XLA patient (B14, absence of BTK detection). We measured the delta between BTK mean fluorescent intensity (MFI) and the irrelevant antibody MFI (i.e. Δ MFI), the 15 HC showed a median of Δ MFI = 107 [79-173]. (B) Patients with XLA as primary diagnosis but without identified *BTK* mutations (WT: wild type). B18 and B19 patients showed reduced BTK expression but not B20. (C) Patients with CVID or IgG subclass deficiency as primary diagnosis but with *BTK* mutations. B21 and B23 patients showed reduced BTK expression; B20 show no BTK expression. Ab: Antibody.

Fig1 A

Figure 1

Fig2

Figure 2

Fig3

1

2 Figure 3

Table S1: Diagnosis provided by the clinical investigators before the genetic screening of *CD40LG*, *SH2D1A* and *BTK*.

	<i>CD40LG</i> <i>n=150</i>	<i>SH2D1A</i> <i>n=230</i>	<i>BTK</i> <i>n=181</i>
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<i>IgAD</i>	0	3	0
<i>IgG-SCI</i>	9	15	13
<i>Thymoma</i>	4	9	8
<i>Other</i>	13	40	16

HIGM: X-linked hyperIgM syndrome. XLP: X-linked lymphoproliferative disease. XLA: X-linked agammaglobulinemia. CVID: Common variable immunodeficiency disorders. IgAD: IgA deficiency. IgG-SCI: IgG subclass deficiency.

FigS1