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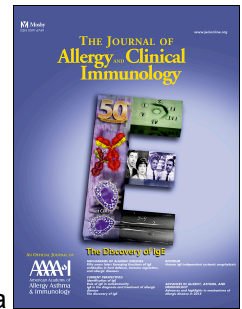
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Non-infectious complications in pediatric-onset CVID patients correlated with defects in somatic hypermutation
but not in class switch recombination

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19 **Abstract**

20 **Background:** Common variable immunodeficiency is a heterogeneous syndrome characterized by impaired
21 immunoglobulin production and usually presents with a normal quantity of peripheral B cells. Most attempts
22 aiming to classify these patients have mainly been focused on T cell phenotypes or B cell phenotypes and their
23 ability to produce protective antibodies, but it is still a major challenge to find a suitable classification that
24 includes the clinical and immunological heterogeneity of these patients.

25 **Objective:** In this study, we evaluated the late stages of B cell differentiation in a heterogeneous population of
26 pediatric-onset CVID patients in order to clinically correlate and assess their ability to perform somatic
27 hypermutation and/or class switch recombination.

28 **Methods:** We performed a previously reported assay, IgκREHMA, to evaluate the in vivo somatic
29 hypermutation status. We amplified switch regions from genomic DNA to investigate the quality of the double
30 strand break repairs in class switch recombination process in vivo. We also tested the ability to generate
31 immunoglobulin germline and circle transcripts and to upregulate activation-induced cytidine deaminase gene
32 through in vitro T-dependent and T-independent stimuli.

33 **Results:** Our results showed that patients could be classified in two groups according to their degree of
34 alterations in somatic hypermutation. This stratification showed a significant association between patients of
35 Group A -severe alteration- and the presence of non-infectious complications. Additionally, sixty percent of
36 patients presented with an increased microhomology usage at switched regions. In vitro activation revealed that
37 CVID patients behaved heterogeneously in terms of responsiveness to T-dependent stimuli.

38 **Conclusions:** The correlation between non-infectious complications and somatic hypermutation could be an
39 important tool for physicians to further characterize patients with CVID. This categorization would help to
40 improve elucidation of the complex mechanisms involved in B cell differentiation pathways.

41

42 **Clinical Implications:** Stratification of our CVID cohort according to their capacity of performing somatic
43 hypermutation highlighted an inverse relationship with non-infectious clinical complications.

44 **Capsule Summary:** Categorization of CVID patients could be useful to hypothesize the molecular defects
45 underlying B cell phenotype as well as on disturbed B cell differentiation pathways in these patients.

46

47 **Abbreviations**

48 **Ab** antibody

49 **AICDA** activation induced cytidine deaminase

50 **CSR** class switch recombination

51 **CT** circle transcripts

52 **CVID** common variable immunodeficiency

53 **DSB** double strand breaks

54 **GLT** germline

55 **Ig** immunoglobulin

56 **NHEJ** non-homologous end joining

57 **SHM** somatic hypermutation

58 **PBMC** peripheral blood mononuclear cells

59 **TD** T-dependent

60 **TI** T-independent

61

62 **Running head**

63 SHM and CSR analysis in pediatric CVID patients

64 **Key words**

65 Common Variable Immunodeficiency; SHM; CSR; hypermutation; pediatric; switch

66

67 Introduction

68 The hypogammaglobulinemia of at least two immunoglobulin isotypes characterizes the heterogeneous group of
 69 the primary immunodeficiency disorder known as common variable immunodeficiency (CVID)
 70 (OMIM#240500) [1-5]. These patients are particularly susceptible to recurrent infections of the respiratory tract
 71 as well as gastrointestinal system and manifest non-infectious complications such as autoimmunity,
 72 gastrointestinal disorders, and lymphoproliferation [1, 5].

73 Despite the rarity of this condition, CVID is the most common immunodeficiency of clinical significance whose
 74 main immunological defect, the failure of immunoglobulin production, is usually associated with a normal
 75 peripheral B cells count [1, 5]. Genetic defects associated with survival, differentiation and/or immune
 76 interactions of B cells have been proposed as being responsible for the pathophysiology of the disease. Several
 77 attempts aiming to classify CVID patients have mainly focused on T cell phenotypes or B cell phenotypes and
 78 their ability to produce protective antibodies (Ab) [2, 6, 7], but it is still a major challenge to find a suitable
 79 classification that includes the clinical and immunological heterogeneity of these patients and their variable
 80 response to treatment. The plasma level of IgG has poor predictive value in CVID since patients with slightly
 81 decreased levels of IgG can be highly susceptible to infections and benefit from replacement with intravenous
 82 gammaglobulin therapy [8]. Nevertheless, the molecular mechanisms underlying the maturation of the Ab
 83 response triggered by natural infections and vaccines have been poorly investigated in these patients.

84 During somatic hypermutation (SHM), point mutations are introduced into the V region of immunoglobulin (Ig)
 85 genes (both IgH as IgL), increasing Ab affinity for the antigen, and this process is essential for the generation of
 86 long-lived plasma and memory B cells [9-11]. Failure in the affinity-maturation process of all isotypes of Abs
 87 could underlie a qualitative humoral immunodeficiency. In previous reports, impaired SHM was associated with
 88 a high frequency of severe respiratory tract infections in a cohort of mainly adult CVID patients [12]. In
 89 addition, Ab-response maturation also requires class switch recombination (CSR) process in which C μ region is
 90 replaced by a downstream Ig CH gene, resulting in a change from IgM to IgG, IgA or IgE, a mechanism
 91 involving S regions located upstream of each CH except for C δ [13].

92 Both processes SHM and CSR are dependent on activation-induced cytidine deaminase (AID) function, an
 93 enzyme expressed in antigen-activated B cells, which generates high affinity antibodies and antibody CSR,
 94 respectively [14]. To achieve CSR, AID generates double strand breaks (DSBs) mainly repaired through non-
 95 homologous end joining (NHEJ) mechanism [15] by using little or no sequence homology. The NHEJ machinery

requires a large number of factors (Ku70/Ku80, DNAPKcs, DNA ligase IV, Artemis Cernunos, etc) [16-20], and defects in this mechanism lead to alternative pathways (such as the use of microhomology) in attempt to repair DSBs at the S junctions, as shown in patients with primary immunodeficiencies involving defects in NHEJ genes [21, 22].

In this study, we evaluated the capacity to perform SHM and CSR ex vivo and in vitro in a population of pediatric-onset CVID patients, whose immunological and clinical data were previously reported [23] allowing us to propose three different subsets of CVID patients.

Methods

Patients and controls.

This study included twenty-five unrelated CVID patients (12 males), with a mean age of onset of symptoms of 5.6 years (range 1 – 14 years) and a mean age of diagnosis of 11.3 years (range 4 - 16.1 years). Mutations in *CD40 ligand*, *CD40*, *AID*, *UNG*, *PMS2*, *DCLRE1C* and *DNA LIG4* genes were excluded in patients presenting with normal or with increased IgM levels. Patients with decreased peripheral B cells were also excluded from the study. We considered patients as belonging to the smB+ (switched memory B cells >2%) or smB- (switched memory B cells <2%) subgroups depending on their B cell immunophenotype, according to the EUROclass CVID classification [6]. These patients were previously reported ([23] Patients Part B) and the main clinical and immunological data were summarized in Table I and Table II. Informed written consent was obtained from the patient or parental guardian prior to participation in accordance with the Declaration of Helsinki.

Twenty-two healthy donors matched for age, gender (11 males) and ethnic background were included in the study and provided written consent under a separate ethics protocol for healthy donors.

Five cord blood samples were included as negative control in the IgkREHMA assay, donated after informed consent was obtained from the delivering women.

The research protocol was approved by the internal ethics review board of the Hospital Garrahan.

Total RNA Preparation, cDNA Synthesis

Total RNA was extracted from peripheral blood mononuclear cells (PBMC) using TRIzol reagent (Invitrogen), and cDNA was prepared using a first-strand cDNA synthesis kit (Amersham Biosciences, UK) according to the manufacturer's instructions.

RNA from memory and naïve B cell subset were obtained by using RNeasy Micro Kit (Qiagen), while cDNA was prepared with a SuperScript™ III (Invitrogen) according to the manufacturer's instructions.

Human cell isolation

PBMC were isolated by density centrifugation over a Ficoll-Hypaque Plus (Amersham) gradient. B cells from 7 patients and 4 healthy donors were purified from PBMC by negative selection using the MACS B cell isolation kit II (Miltenyi Biotec). Memory and naïve B cells subsets were purified from the total B cells obtained using FACSaria cell sorter (BD Biosciences) with CD27-FITC, IgD-PE, CD19- PE-Cy7 (all from eBioscience).

All fractions were obtained with a purity of >95%.

Cell cultures and reagents

Cultures of PBMC or naïve B cells from COVID patients or healthy donors were performed in complete RPMI medium supplemented with 10% (v/v) FBS (GIBCO). In the T-dependent stimulation, cells were incubated with CD40L (PeproTech), 500 ng/ml and IL-4 (Schering-Plough), 200 U/ml. CD40 was cross-linked with 1 µg/ml mouse 89 mAb (Schering-Plough). Mouse IgG1 mAb with irrelevant binding activity (Santa Cruz Biotechnology) was used as control. In the T-independent stimulation, cells were incubated with phosphorothioate-modified 5'-tcgtcgtttgtcgtttgtcgtt-3' oligodeoxynucleotide-2006 (Operon Technologies), 5 µg/ml and IL-10 (Peprotech), 50 ng/ml.

RT-PCR and quantitative RT-PCR

Quantification of human germline $I\gamma 1$ -C $\gamma 1$ or $I\alpha$ -C α transcripts, switched $I\gamma 1$ - $I\gamma 2$ -C μ or $I\alpha$ -C μ circle transcripts, AICDA transcript and GAPDH, was performed from total RNA extraction in 2 or 4-day culture with TRIzol (Invitrogen). cDNA was generated by reverse transcription with Superscript II RT (Invitrogen). PCR primers and conditions used for standard or quantitative RT-PCR analysis were done as reported [24-26]. Results of qRT-PCR were normalized to ACTB mRNA and were presented as relative expression (RE) compared with that of B cells incubated with control antibody (Control).

Characterization of SHM

The IgkREHMA assay was performed as previously described [12].

Characterization of switch recombination junctions

Genomic DNA was purified from peripheral blood cells from patients and healthy donors using standard methods. Amplification of S μ -S α from in vivo switched cells was performed using a previously described nested PCR assay [21]. The PCR-amplified switch fragments were gel purified (GE Healthcare), cloned into a pGEM-T vector (Invitrogen), and sequence analysis was performed with DNA Sequencing Analysis software (PE Applied Biosystems) on ABI 3130 (Applied Biosystems). The CSR junctions were determined by aligning the switched fragment sequences with the S μ (X54713) and S $\alpha 1$ (L191219) or S $\alpha 2$ (AF030305). Analysis of microhomology usage and mutation pattern at the CSR junctions was performed as described previously [21, 27].

Statistical analysis

158 The statistical analysis of results was performed with Student's two tailed *t* tests using the PRISM software,
159 Spearman two tail test or one way ANOVA followed by Dunnett's multiple comparison post test. The
160 frequencies of non-infectious complications among the groups were compared using the *Chi*-square test
161 (GraphPad Software Inc.). Other statistical analyses were performed using *Chi*-square test with SPSS 15.0 for
162 Windows.
163

Results

B-cells of CVID patients carry significantly diminished SHM

To evaluate the SHM levels in peripheral cells from our patients we performed an assay developed by Andersen et al [12]. This assay is based on cleavage by a restriction enzyme of a hotspot in the rearranged V κ A27 transcripts (Ig κ REHMA, restriction enzyme hot-spot mutation assay). Briefly, somatic mutations in the restriction sites of the most commonly used light chain gene (κ A27) abrogate cutting with a specific enzyme. The variability observed in our healthy control group remained within the levels previously reported (Figure 1A and 1B, [12]) which did not seem to be modified by donor age above 2 years old.

The comparison of our CVID patient group with the healthy donor group revealed a significant difference in mutations of the V κ A27 gene (Figure 1A and 1B) (Student's two tailed *t* test, $p < 0.0001$). At the same time a heterogeneous behavior in undergoing SHM could be observed in the pediatric CVID population (Figure 1A and 1B) and this heterogeneity correlates with the percentage of switched memory B cells (Figure 1C) (Spearman $r = 0.68$, $p = 0.0002$) but not with total memory B cells nor non-switched memory B cells (data not shown).

Almost all of the patients (24/25) showed decreased values of SHM (Table I). Based on these results a relevant cut-off could optimize the distinction of patients with clinical complication and/or reduced switched memory B cell. Thus, we established two subgroups of patients with altered SHM: Group A with less than -3 standard deviation (SD) respect to the healthy control group ($< 18\%$ of mutated fraction of V κ A27) and Group B with values ranging between -2 and -3 SD compared with the healthy control group ($\geq 18\%$ and $< 28\%$ of the mutated fraction) (Table I). Only one patient (P25) had values above the reference interval of our healthy control group.

We can see that 88% of the patients in group A (14/16) had a low number of switched memory B cells ($< 2\%$) belonging to the so called smB- group of EUROclass (see Materials) who also showed clinical complications or more severe presentation of the disease, i.e. 6/9 patients with autoimmunity, splenomegaly or granulomas fell into this group (Table I). On the other hand, 7 of the 8 patients in the group B presented CD27+ B cells (total memory B cells) above 5% of peripheral B lymphocytes, and 6 of them remained thus far, free of non-infectious complications (Table I). Interestingly, we found a significant association between patients of Group A and the presence of non-infectious complications with respect to patients of Group B ($p < 0.05$, *Chi-square* test).

In spite of this strong correlation between SHM, B cell immunophenotype and clinical presentation, Patient 25 had reference values of mutated fraction of V κ A27, developed autoimmunity and presented switched memory B cells below 2% (Figure 1B and Table I). An important issue is whether the values of hot-spot mutations obtained

from PBMC are comparable with those from particular B cell subsets, i.e. memory B cells. For this purpose, we FACS-sorted CD27⁺ B cells and performed IgkREHMA analysis from 7 patients (P8, P11; P12, P17, P18, P20 and P23) presenting with more than 2 % of switched-memory B cells, more than 10 % of total memory B cells, and also from 4 healthy donors. The analysis showed that these patients, who initially showed reduced levels of mutated fractions of V κ A27, continued so on memory B cells (Figure 1D). Even though the mutation rate could be higher in the switched memory B-cell population in respect to non-switched memory B-cells, even patients with a comparable switched memory B-cells to healthy donors (P20 and P23) showed a marked decrease of SHM frequency (Figure 1D). Overall, SHM frequencies ranged below the reference interval in CVID patients even when the analysis was performed specifically in memory B cells.

Studies of CSR

CVID patients demonstrate increased microhomology usage

To investigate the quality of the DSB repairs in in vivo CSR process, we amplified S μ -S α regions from genomic DNA of patients and controls by using a previously described nested PCR [21]. Despite the low levels of serum IgA in our patients, S μ -S α fragments could be amplified, even if with decreased intensity than in healthy donors (data not shown). Therefore, to assess the nature of switched junctions, we cloned and sequenced 194 S μ -S α fragments from 20 CVID pediatric patients (see Table II) and 53 fragments from 10 healthy controls (Figure 2). All of the switched fragment sequences were unique and thus represented independent CSR events. Figure 2A shows some of the more representative aligned sequences from patients and controls.

Globally, we observed a clear decrease in the proportion of blunt junctions (0 bp of homology) in our CVID cohort in comparison to controls. Indeed, a significant increase of sequences presenting 7 to 9 bp microhomology usage was observed in patients even with more than 10 bp homology on the junction repair compared to healthy donors ($p < 0.001$ and $p < 0.01$ respectively *Chi-square* test, Figure 2B).

Overall, our results showed a significant increase in the extent of donor-acceptor homology on S μ -S α joints in CVID patients with respect to healthy controls with a mean perfect sequence homology of 6.9 ± 5.3 bp in CVID patients vs 2.3 ± 3.5 bp in healthy controls ($P < 0.0001$ Student's two tailed *t* test) (Figure 2B and C). The increase in microhomology usage was found in 12 of the 20 evaluated patients (60 %) (Table II) and most of the S μ -S α joints from these patients were flanked by imperfect repeats (see the dashed line boxes in Figure 2A). Eleven of the 12 patients displaying abnormal microhomology usage had less than 2% of switched memory B cells (Table II). The remaining eight patients had no statistical differences with respect to the control group and

four of them presented with more than 10% of total memory B cells (CD19⁺ CD27⁺) (Table II). In spite of this increased microhomology usage, no significant association with clinical non-infectious complications was observed between *abnormal* and *normal* microhomology usage subgroups of patients ($p=0.26$, *Chi-square* test).

Altogether, we found a significant correlation between the media of microhomology usage in each patient and the proportion of switched memory B cells (Figure 2D) (Spearman $r = -0.72$, $p=0.0003$), this significant difference was not noted when compared with IgG or IgA serum levels before substitution with intravenous gammaglobulin (data not shown). These results indicate a CSR deficiency in an important group of patients likely associated with a defective repair of switch junctions.

Analysis of T-dependent (TD) and T-independent (TI) stimulation further characterize patient subgroups

In an attempt to localize the precise step of the CSR defect, we tested the ability to generate germline (GLT) (IH-CH), circle (CT) (IH-C μ) transcripts and to upregulate AICDA in PBMC before and after being activated in vitro with TD (CD40L and IL-4) and TI (CpG and IL-10) stimuli in 15 patients (P1, P2, P3, P4, P6, P8, P9, P11, P12, P15, P17, P18, P23, P24 and P25) and 5 healthy donors.

Healthy donors' PBMC showed an increase in the expression of GLT γ 1 (I γ 1-C γ 1), CT γ 1 (I γ -C μ) (Figure 3B) along with an upregulation of AICDA measured by RT-PCR and qRT-PCR (Figure 3A and B) after TD stimuli (similar results were observed by TI stimulation, data not shown). By contrast, none of the CVID patients showed GLT γ 1, CT γ 1 and AICDA transcripts upregulation by TI stimuli, and they behaved heterogeneously in terms of responsiveness to TD stimuli (Figure 3A and B).

To limit possible effects on transcript induction due to the presence of memory B cells, naïve B cells obtained from 6 patients with more than 10% of total memory B cells (P8, P11, P12, P17, P18 and P23) and from 4 healthy donors were activated in vitro as previously described (Figure 3C and D). We observed similar results through TI stimulation in naïve B cells with respect to that observed in PBMC. However, through TD stimuli patient 23 and patient 18 showed less upregulation of AICDA along with a lack of expression of GLT α and CT α transcripts (Figure 3D), as well as with GLT γ 1 and CT γ 1 expression (data not shown).

Taking these results into account, three subgroups were delineated: subgroup 1, lacking response to TD stimulation (P1, P2, P3, P4, P6, P9, P11, P15 and P25); subgroup 2, no expressing GLTs but retaining AICDA activation (P17, P18 and P23); and subgroup 3, showing a similar behavior to the healthy controls (P8, P12, and P24).

Discussion

In the recent years, the analysis of several Ig deficiencies have made it possible to describe molecular mechanisms underlying CSR and SHM pathways, which are key elements in the maturation of Abs responses [28]. However, functional defects in the ability to perform CSR may or may not be associated with a defect in SHM [22, 29, 30]. Among the genetic alterations found causing defects on CSR and SHM are those causing HIGM syndromes, such as deficiencies of *CD40* and *CD40L* [31, 32], *AICDA* [33] or *UNG* genes [34], which helped to understand the essential role of these molecules in B cells for both mechanisms. Thus the study of the ability of B cells to perform SHM and CSR in primary immunodeficiencies could help to identify new proteins involved either in one or in both pathways [7, 35-40].

In the present study by evaluating the qualitative aspects of the Abs produced by patients with CVID we described SHM and/or CSR defects in our cohort of pediatric patients. Indeed, even if in our cohort as reported [6, 7] low or absent switched memory B cells have mainly been associated with secondary complications, this does not seem consistently related to the ability to trigger CSR. Thus, intrinsic CSR events remained unaffected in both; patients presenting (P12 and P18) or lacking (P24) switched memory B cells and non-infectious clinical complications. Likewise, by pointing out specific impairments in Ab production we can attempt to categorize CVID patients in order to guide the search for new genetic targets and correlate treatment based on the clinical course of each category..

The results obtained by analyzing SHM pathway showed that to a greater or lesser extent, almost all CVID patients had an altered SHM of the antibody light chain making it possible to group them into two categories: 1) severe defect in SHM (Group A in IgκREHMA assay); and 2) moderate defect in SHM (Group B in IgκREHMA assay) (Table I). This assay allows the analysis of the frequency of mutated Igk transcripts in a CDR mutation hotspot. It is important to note that mutations in this hotspot seem to be positively selected [41] and the levels of mutated transcripts correlate highly with percentage of memory B cells [12]. Indeed, in our cohort, heterogeneity of SHM correlated with the percentage of switched memory B cells. This correlation could indicate that the light chain mutation levels might relate on the absence of memory subpopulations, however as previously observed in Andersen et al [12], we also found a reduced proportion of mutated fraction on memory B cells sorted from CVID patients. Furthermore, even patients with similar fraction of switched memory B-cells as healthy donors (P20 and P23), showed a marked decrease of SHM frequency (Figure 1D). Thus, since a low number of memory B cells cannot fully explain the results of IgκREHMA assay in our CVID patient cohort, an intrinsic defect in the SHM machinery should not be ruled out at least in some of them.

By analyzing the clinical aspect of patients in each group of altered SHM we observed that 6/9 patients in Group A (severe defect in SHM) presented with autoimmunity, splenomegaly or granulomas (Table I). On the other hand, 6 of the 8 patients in Group B remained thus far, free of non-infectious complications (Table I). As an important clinical tool, we found a significant association between patients of Group A and the presence of non-infectious complications with respect to patients of Group B ($p < 0.05$, Chi-square test). This could potentially make clinicians more aware of non-infectious complications that are more likely to arise in a particular group of patients with CVID and not exhaust valuable resources in monitoring patients who are less likely to develop non-infectious complications.

To further characterize CVID B cells analysis of unique switch junctions representing independent CSR events lead us to distinguish two groups of patients: those with a significant increase in the extent of donor-acceptor homology (microhomology) at the $S\mu$ - $S\alpha$ regions and those patients with switch junctional sequences statistically comparable to healthy donors. (Table II) In spite of this increased microhomology usage, no significant association with clinical non-infectious complications were observed between *abnormal* and *normal* microhomology usage subgroups of patients ($p = 0.26$, Chi-square test).

The mean of microhomology usage for each patient correlated with the percentage of switched memory B cells (Figure 2D), suggesting that those patients triggering lower but measurable CSR in vivo have less extent of donor-acceptor homology on $S\mu$ - $S\alpha$ joints. By contrast, we could not find any correlation between microhomology usage and Ig serum levels, which seems to be in accordance with the poor predictive value of plasma level of IgG in CVID, since even patients with slightly decreased levels of IgG can be highly susceptible to infections [8].

In our attempt to stratify CVID patients according to SHM or CSR analysis no difference in infection control became evident. As previously reported, the most frequent clinical manifestation in this CVID cohort was respiratory tract infection with 43% of them presenting upper respiratory tract infections, namely sinusitis and otitis media, and as many as 79% of patients presenting with recurrent lower respiratory tract infections [23].

Almost all of the patients in Group A according to SHM assay performance presented with failures in CSR evaluated mechanisms (P4, P6, P7, P9, P10, P11, P13, P14, P15 and P16).

The presence of microhomology and imperfect repeats are reminiscent of defects in the NHEJ repair pathway [35, 42, 43]. It is important to note that in our CVID cohort no patient presented clinical or immunological phenotypes reminiscent to those found in patients with known DNA-repair defects. Nevertheless, normal

expression of other DNA repair proteins involved in CSR [42, 44, 45] or in repairing AID-induced lesions [38, 46] could not be excluded. Similarly, we cannot rule out a defect in a yet uncharacterized molecule involved in NHEJ (S regions in CSR) and/or MMR (V regions in SHM) or in an alternative pathway of DNA repair involved in both late differentiation processes of B cells. It has been described that patients with mutations in *PMS2* gene with impaired S region-repair have an altered pattern of SHM [43]. This defect, however, has been excluded by *PMS2* sequencing in our CVID patient cohort. The improved DNA-sequencing technologies, i.e. next generation sequencing, should enable us in the near future to elucidate the possible gene defects underlying the observed changes.

In addition, a defect in proteins activated in response to DNA damage and suppressed in B cells via Bcl-6 should also be considered [47]. Although to date no animal model can support this hypothesis, the identification of genes that are directly repressed by Bcl-6 has strengthened the idea of essential and specialized functions of this protein in the germinal center (GC) formation [48, 49]. Bcl-6 appears to be crucial for GC B cells undergoing the high rates of proliferation required for CSR and SHM. In fact, it has been shown that Bcl-6 suppresses the responses of cell cycle arrest and apoptosis, either by suppression of p53 [50] or p21 [47]. In this sense it would also be interesting to evaluate the FOXO1/AKT/PI3K pathway, which is involved in regulation of apoptosis, cell cycle arrest, differentiation and longevity of B cells [51, 52].

On the other hand, patients of Group B with moderated defect in SHM but correctly repairing S regions (P17, P18, P23 and P24), a defect in an AICDA cofactor exclusively involved in the activation or repair of the V regions could be considered. The existence of such cofactor of AICDA has been previously postulated but still no protein playing that role was identified [53]. However, although P24 was able to activate CSR molecular events in vitro via TD stimuli, P17, P18 and P23 only conserved AICDA activation (Figure 3). Abnormalities involving a first step of CSR, namely GLTs activation, could underlie this condition.

Finally, patients 21 and 25 presented with failure in CSR with a mildly defective SHM evoking the phenotype caused by mutations in the AICDA C-terminal region [54, 55]. A cofactor acting at AICDA C-terminal region, specifically required for the development of CSR and dispensable for SHM, has been also suggested but not yet identified [53, 56]. As an example, defects in 14-3-3 adapter proteins involved in AICDA recruitment to S regions could be evaluated in these patients [57], particularly in Patient 21 with an immunological phenotype reminiscent to defects in genes known to be involved in CSR (i.e. hyper IgM syndromes). However, abnormalities in *AICDA*, *UNG*, *CD40*, *CD40L* and *PMS2* were ruled out through sequence analysis.

Furthermore, a normal expression of ICOS co-stimulatory molecule was seen by flow cytometry, ruling out this deficiency previously described in some CVID patients [58, 59].

Altogether molecular definition of CSR and/or SHM defects in CVID patients can guide future investigations aiming to further elucidate the complex mechanisms involved in B cell differentiation pathways [29, 60]. Moreover, clinicians could consider assessments of SHM in CVID patients in view of the observed correlation between severe impairment and non-infectious complications in pediatric patients with CVID.

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Figure legends:

Figure 1: A. IgκREHMA assay, fragment length analysis by capillary electrophoresis of cut VκA27 PCR products. Healthy donor (HD); cord blood (CB) sample, patient 3 (P3) and patient 16 (P16). The peaks of 106 and 109 bp represent the quantity of VκA27 fragments cleaved in one of the 2 hot-spot Fnu4HI restriction sites, respectively. The peaks of 244 bp represent the quantity of VκA27 fragments cleaved in the signal peptide Fnu4HI site, but not cleaved in the hot spot as a consequence of one or 2 mutations eliminating the Fnu4HI sites.

B. SHM values obtained through IgκREHMA assay as a percentage of the mutated fraction in CVID patients (patients) and healthy donors (controls). Comparison of mean and SD for Student's two tailed *t* test *** $p < 0.0001$.

C. Correlation between the percentage of mutated fraction and the proportion of switched memory B cells in CVID patients.

D. Mutated fractions of purified CD19⁺ CD27⁺ B cells from 7 CVID patients (P) and 4 healthy donors (HD). The numbers at the top of the bars represent the proportion of switched memory B-cells.

Figure 2: A. Selected sequence of Sμ-Sα junctions from patients and controls. The recombination junctional sequences are aligned with Sμ (above) and Sα or Sα2 (below) reference sequences. Microhomology (perfectly matched sequence homology) is indicated by a box (solid lines). Imperfect repeat was determined by identifying the longest overlap region near the switch junction by allowing one mismatch on either side of the breakpoints (dashed line box). The Sμ and Sα breakpoints for each switch fragment are indicated by ▲ and ▼, respectively, and their positions in the reference sequences are indicated above or below the arrowheads. The number of base pairs involved in microhomology and imperfect repeats for each joint is shown in the bottom of each sequence.

B. The Sμ-Sα switch junctions from CVID patients were compared with those from healthy donors. Statistical analysis was performed using the *Chi*-square test, ** $p < 0.01$; *** $p < 0.001$.

C. Pie charts demonstrating the microhomology usage at Sμ-Sα junctions in patients and controls. The proportion of switch junctions with a given size of perfectly matched sequence homology is indicated by the size of the slices.

D. Correlation between the media of microhomology usage and the proportion of switched memory B cells.

Figure 3: A. qRT-PCR analysis of AICDA in patients and controls cells cultured for 2 or 4 days with anti-CD40 and IL-4 or CpG and IL-10, respectively. Bars represent the mean $\pm$ SEM of three independent experiments. Data was analyzed by the $2^{-\Delta\Delta Ct}$ method using ACTB as a reference gene, considering the relative expression (RE) of AICDA mRNA from the control culture as 1. Statistical analysis was performed using one way ANOVA followed by Dunnett's multiple comparison post test $\Psi = *** p < 0.001$; $\eta = * p < 0.05$. HD: Healthy Donors, P= Patients.

B. Expression of AICDA, Iγ-Cγ1 (GLTγ1), Iγ1-Cμ (CTγ1) and GAPDH (charge control) transcripts by RT-PCR in PBMC from patients and control before (-) and after (+) stimulation with CD40L and IL-4. P:

524 Patients, C: Control. **C.** qRT-PCR analysis of AICDA in purified naïve B cells from 6 CVID patients and 4
525 healthy donors cultured for 2 or 4 days with anti-CD40 and IL-4 or CpG and IL-10, respectively. Data was
526 analyzed as described above. **D.** Expression of AICDA, I α -C α (GLT α), I α -C μ (CT α) and GAPDH (charge
527 control) transcripts by RT-PCR in purified naïve B cells from 6 CVID patients and 1 healthy donor before (-)
528 and after (+) stimulation with CD40L and IL-4. P: Patients, C: Control.

Table I

Table I SHM measured by IgκREHMA and their correlation with clinical and immunological data										
	Nº (a)	IgκREHMA: % SHM (b)	Switched memory B-cells (c)	Non switched memory B-cells (d)	EUROclass (e)	Noninfectious complications (f)	Age at the time of the sample (years)	Ig serum levels (g)		
								IgG	IgA	IgM
GROUP A PATIENTS	1 (27)	2,8	↓ (0%)	↓ (2%)	smB-	SPM	15,8	115	<7	18
	2 (6)	3,3	↓ (0%)	↓ (3%)	smB-		8,2	12	<7	10
	3 (13)	3,5	↓ (0%)	↓ (2%)	smB-	AI (TID)	15,0	62	<7	33
	4 (15)	3,8	↓ (0%)	↓ (2%)	smB-		12,9	220	<7	49
	5 (17)	4,0	↓ (0%)	↓ (7%)	smB-		13,5	193	<7	15
	6 (25)	5,8	↓ (0%)	↓ (2%)	smB-	SPM	15,7	42	<7	10
	7 (10)	8,6	↓ (0%)	↓ (6%)	smB-		10,7	135	24	26
	8 (28)	8,7	↓ (2%)	Norm (15%)	smB-	AI (ITP/AIHA) and SPM	17,3	29	<7	145
	9 (21)	10,7	↓ (1%)	↓ (5%)	smB-		15,1	352	<7	17
	10 (26)	10,8	↓ (0%)	↓ (2%)	smB-	GR and SPM	15,8	162	<7	32
	11 (7)	11,2	↓ (3%)	Norm (9%)	smB+		7,8	302	<7	13
	12 (9)	11,7	↓ (3%)	↑ (19%)	smB+		9,7	293	<7	28
	13 (20)	12,2	↓ (1%)	↓ (2%)	smB-	AI (ITP) and SPM	13,4	391	22	30
	14 (8)	12,6	↓ (1%)	↓ (3%)	smB-		7,2	456	<7	19
	15 (14)	13,1	↓ (0%)	↓ (1%)	smB-		12,7	495	<7	38
	16 (11)	14,8	↓ (2%)	↓ (2%)	smB-		10,9	75	<7	12
GROUP B PATIENTS	17 (22)	18,5	↓ (3%)	↓ (7%)	smB+		15,0	536	<7	43
	18 (18)	19,1	↓ (5%)	Norm (14%)	smB+		13,0	342	<7	18
	19 (23)	19,2	↓ (3%)	Norm (10%)	smB+		14,9	293	<7	25
	20 (5)	19,7	↓ (5%)	↓ (5%)	smB+		5,9	372	21	9
	21 (19)	20,6	↓ (1%)	↓ (5%)	smB-	GR	13,4	315	19	83
	22 (12)	22,1	↓ (2%)	Norm (9%)	smB-		11,1	452	12	36
	23 (4)	25,6	Norm (10%)	↓ (7%)	smB+		4,0	312	15	28
	24 (24)	26,0	↓ (2%)	↓ (0%)	smB-	AI (ITP)	14,9	435	11	33
	25 (16)	50,5	↓ (1%)	↓ (5%)	smB-	AI (ITP)	12,5	462	20	23
HEALTHY DONORS	N	IgκREHMA: mean % SHM (b)	Mean age at the time of the sample (years)							
	22	45,9 ± 9,0 (range 30,3 - 63,1)	10,5 (range 2,2 - 17,4)							
PATIENTS	25	14,5 ± 10,3 (range 2,8 - 50,5)	12,3 (range 4,0 - 17,3)							

(a) In parenthesis patient number in previous report [20]

(b) % SHM: % of mutated fraction

(c) switched memory B cells = CD19+CD27+IgD-

(d) non switched memory B cells = CD19+CD27+IgD+

(e) smB+ means < 2% of switched memory B cells; smB- means ≤2% switched memory B-cells

(f) AI: Autoimmunity; GR: Granulomas; SPM: Splenomegaly. ITP: Idiopathic thrombocytopenic purpura; AIHA: Autoimmune hemolytic anemia; TID: Type I Diabetes

(g) Immunoglobulin serum levels at the time of the sample (mg/dl)

(c) y (d) ↓= decreased number, ↑= increased number, Norm= within normal range

Table II S μ -S α junctions microhomology analysis and their correlation with clinical and immunological data

Nº	S μ -S α Perfectly matched short homology (a)	Nº of fragment S μ -S α analyzed	Switched memory B-cells (b)	Non switched memory B-cells (c)	EUROclass (d)	Noninfectious complications (e)
6	12,50 $\pm$ 2,65 *	11	↓ (0%)	↓ (2%)	smB-	SPM
13	10,70 $\pm$ 6,38 *	10	↓ (1%)	↓ (2%)	smB-	AI (ITP) and SPM
4	9,71 $\pm$ 7,67 *	8	↓ (0%)	↓ (2%)	smB-	
7	9,17 $\pm$ 5,34 *	9	↓ (0%)	↓ (6%)	smB-	
10	8,93 $\pm$ 6,33 *	10	↓ (0%)	↓ (2%)	smB-	GR and SPM
25	8,75 $\pm$ 6,41 *	8	↓ (1%)	↓ (5%)	smB-	AI (ITP)
15	8,33 $\pm$ 6,75 *	10	↓ (0%)	↓ (1%)	smB-	
14	7,57 $\pm$ 5,08 *	10	↓ (1%)	↓ (3%)	smB-	
16	7,57 $\pm$ 2,15 *	8	↓ (2%)	↓ (2%)	smB-	
11	7,56 $\pm$ 3,68 *	9	↓ (3%)	Norm (9%)	smB+	
9	7,10 $\pm$ 4,48 *	11	↓ (1%)	↓ (5%)	smB-	
21	7,00 $\pm$ 3,92 *	8	↓ (1%)	↓ (5%)	smB-	GR
2	6,60 $\pm$ 5,46 ns	9	↓ (0%)	↓ (3%)	smB-	
3	6,43 $\pm$ 1,99 ns	9	↓ (0%)	↓ (2%)	smB-	AI (TID)
1	6,22 $\pm$ 4,38 ns	9	↓ (0%)	↓ (2%)	smB-	SPM
24	4,89 $\pm$ 4,70 ns	10	↓ (2%)	↓ (0%)	smB-	AI (ITP)
17	4,71 $\pm$ 5,28 ns	8	↓ (3%)	↓ (7%)	smB+	
8	4,09 $\pm$ 4,37 ns	21	↓ (2%)	Norm (15%)	smB-	AI (ITP/AIHA) and SPM
18	3,92 $\pm$ 5,53 ns	9	↓ (5%)	Norm (14%)	smB+	
23	1,50 $\pm$ 1,76 ns	7	Norm (10%)	↓ (7%)	smB+	
CONTROLS	2,28 $\pm$ 3,52	53				
PATIENTS	6,95 $\pm$ 5,32 †	194				

(a) Mean and SD from patients and controls. *p<0.0025 by two tailed Student's *t* test with Bonferroni correction for multiple comparisons (the same was obtained by applying one way ANOVA followed by Dunnett's multiple comparison post test, using the control treatment as statistical control in Dunnett's comparisons), ns: not significant. †p<0.005 by two tailed Student's *t* test.

(b) switched memory B cells = CD19+CD27+IgD-

(c) non switched memory B cells = CD19+CD27+IgD+

(d) smB+ means < 2% of switched memory B cells; smB- means $\leq$ 2% switched memory B-cells

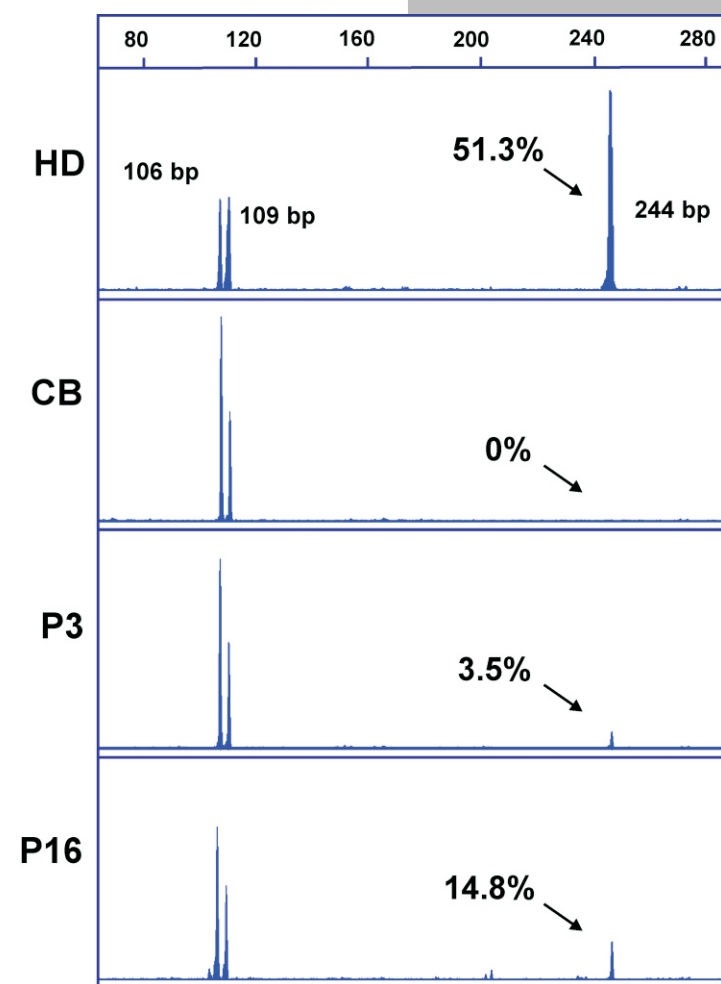
(e) AI: Autoimmunity; GR: Granulomas; SPM: Splenomegaly. ITP: Idiopathic thrombocytopenic purpura; AIHA: Autoimmune hemolytic anemia; TID: Type I Diabetes

(b) y (c) ↓= decreased number, ↑= increased number, Norm= within normal range

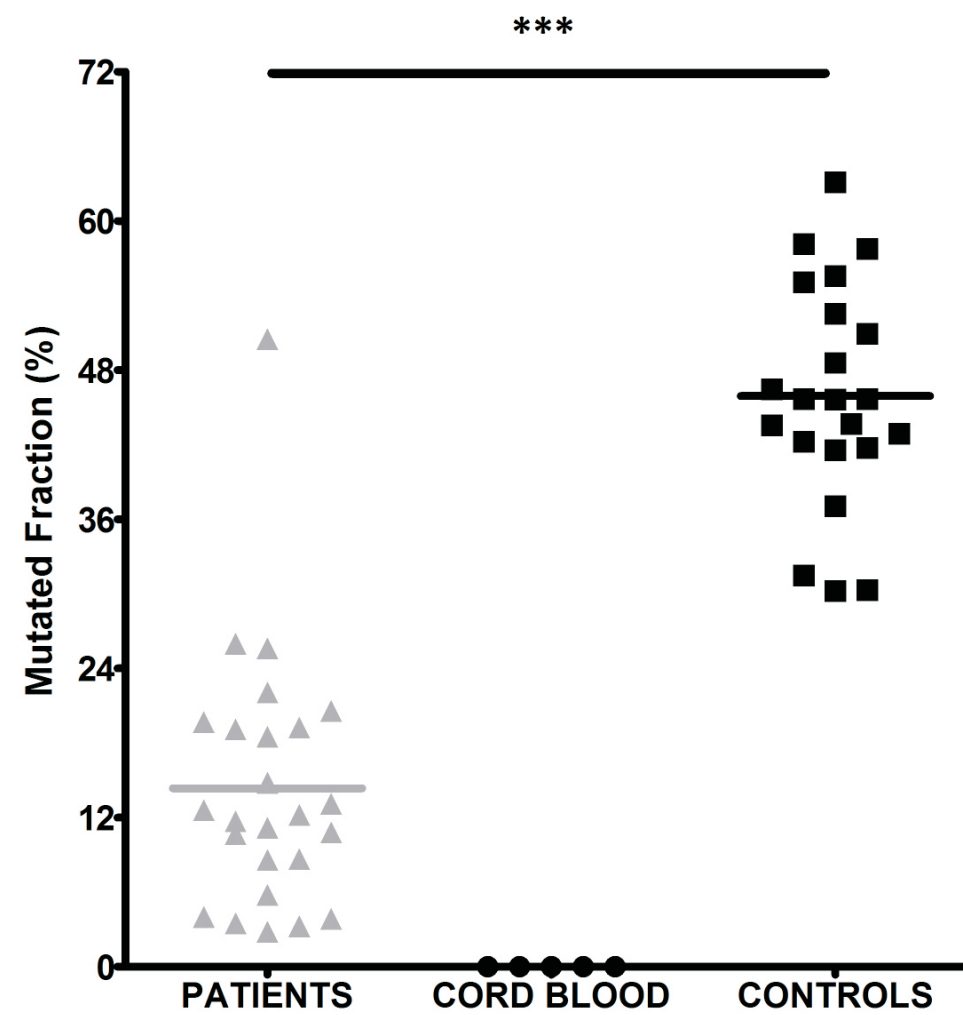
Figure No. 1
Figure 1

ACCEPTED MANUSCRIPT

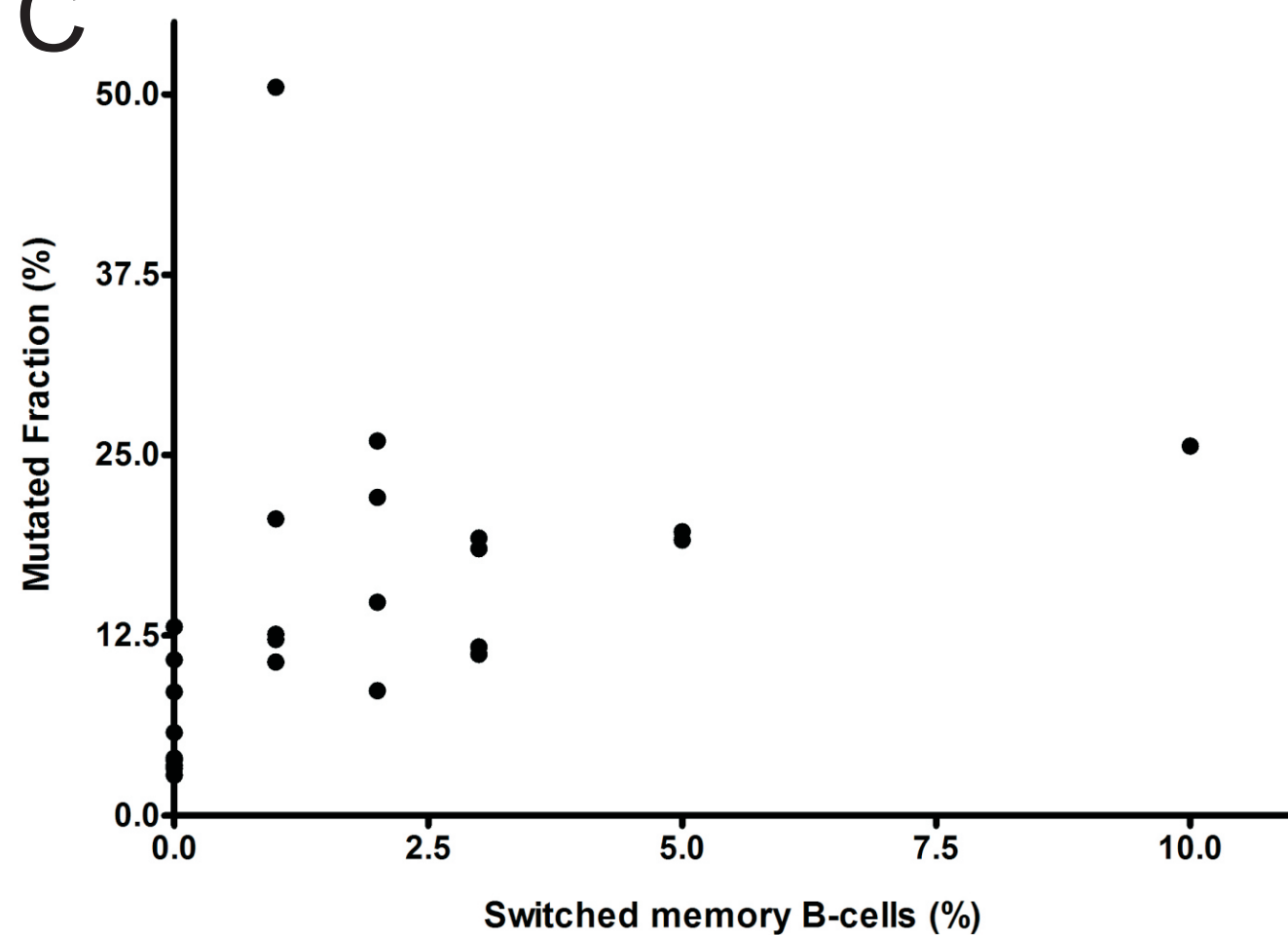
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B



C



D

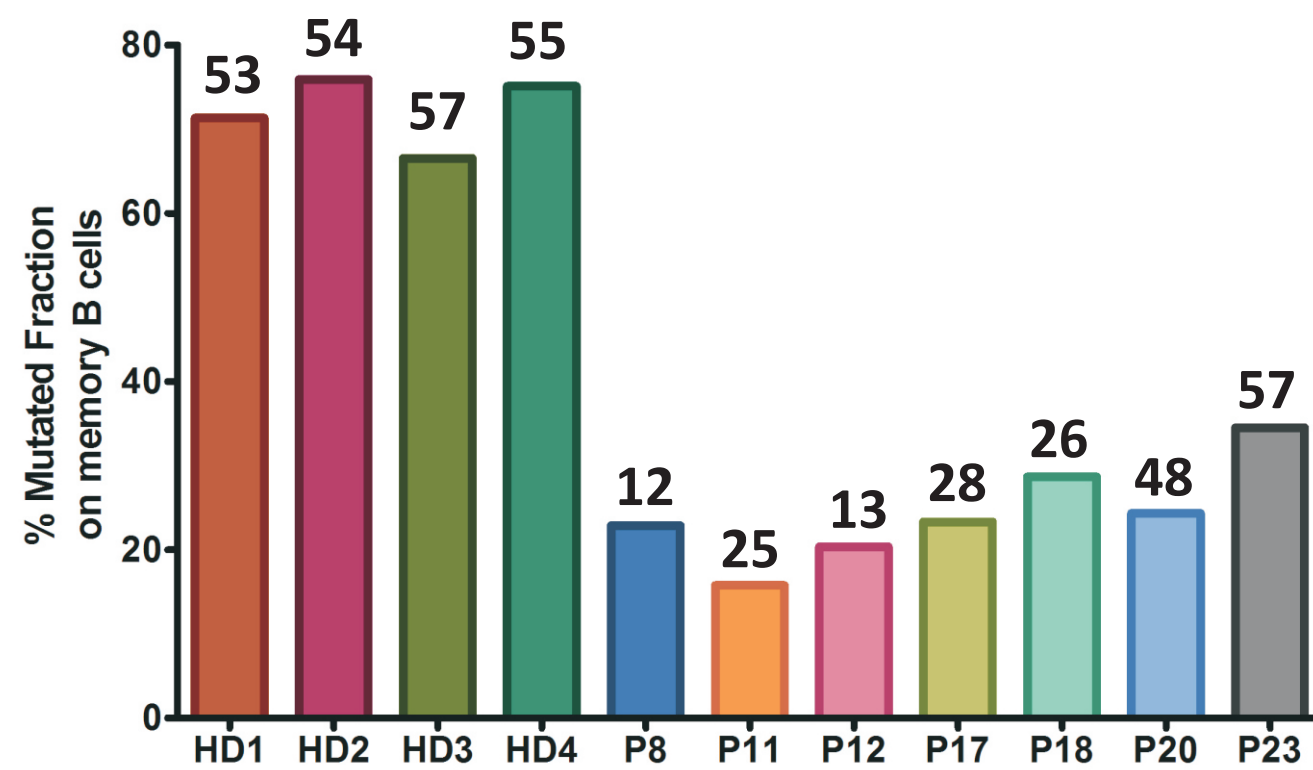
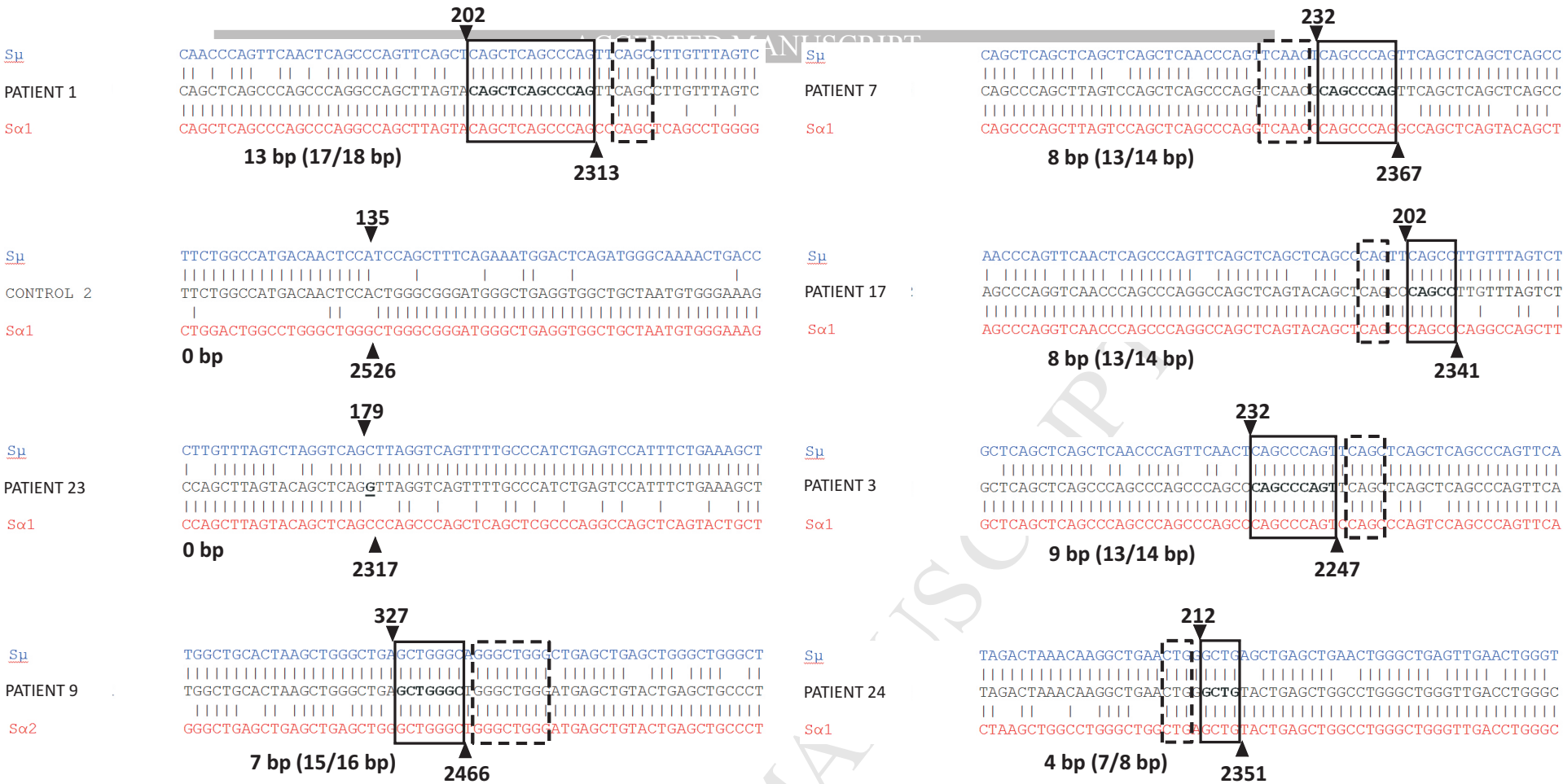


Figure 2

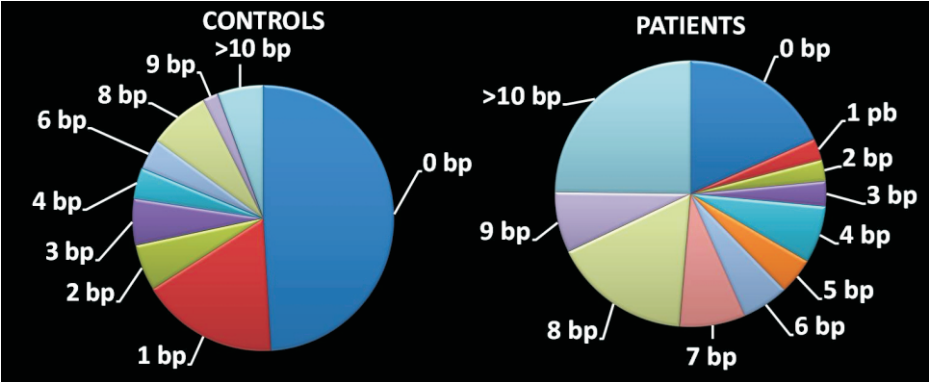
A



B

Characterization of Sμ-Sα junctions							
	Perfectly matched short homology						Total number of S fragments
	0 bp		1-3 bp	4-6 bp	7-9 bp	>10 bp	
	No insertions	1 bp insertions					
Controls	16 (30,2%)	10 (18,9%)	15 (28,3%)	4 (7,6%)	5 (9,4%)	3 (5.6%)	53
Patients	16 (8,2%)*	23 (11.9%)	15 (7,7%)*	35 (18,0%)	55 (28,4%)*	50 (25,8%)*	194

C



D

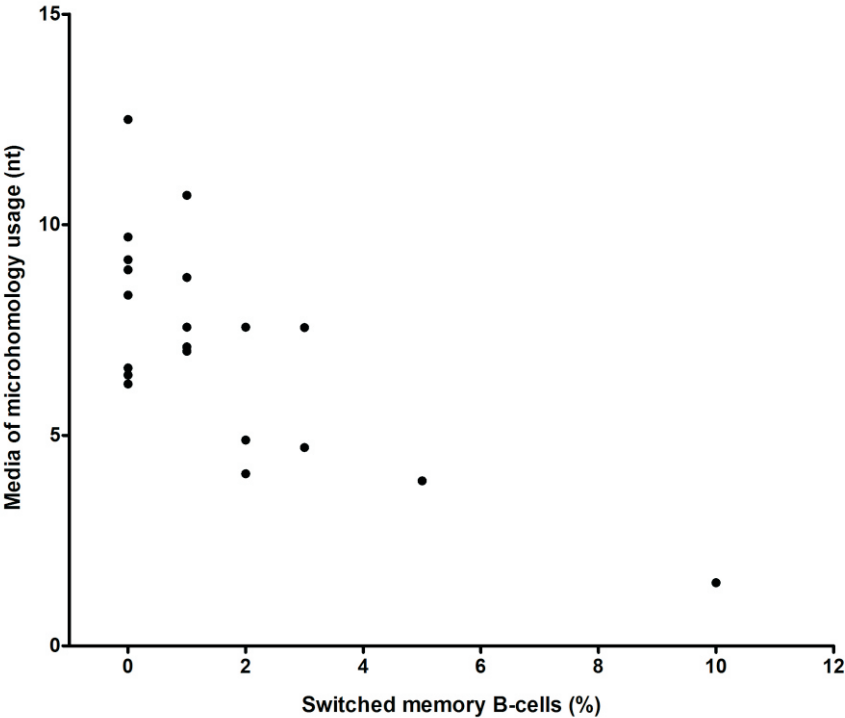
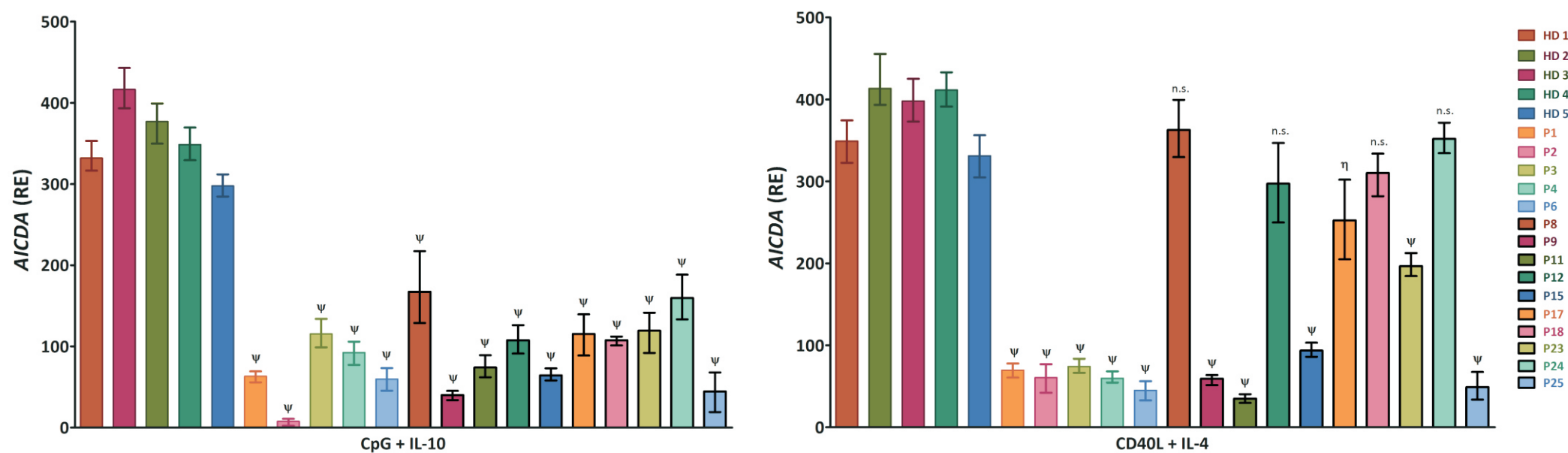
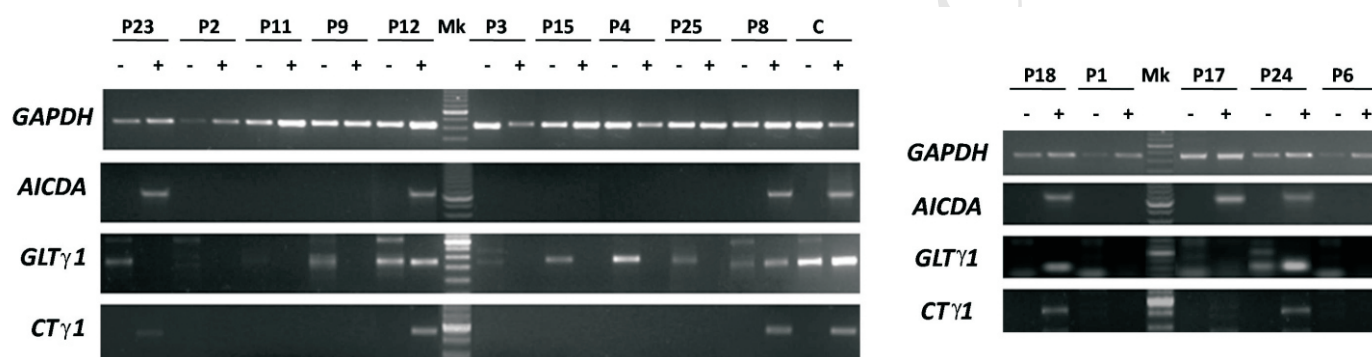


Figure 3

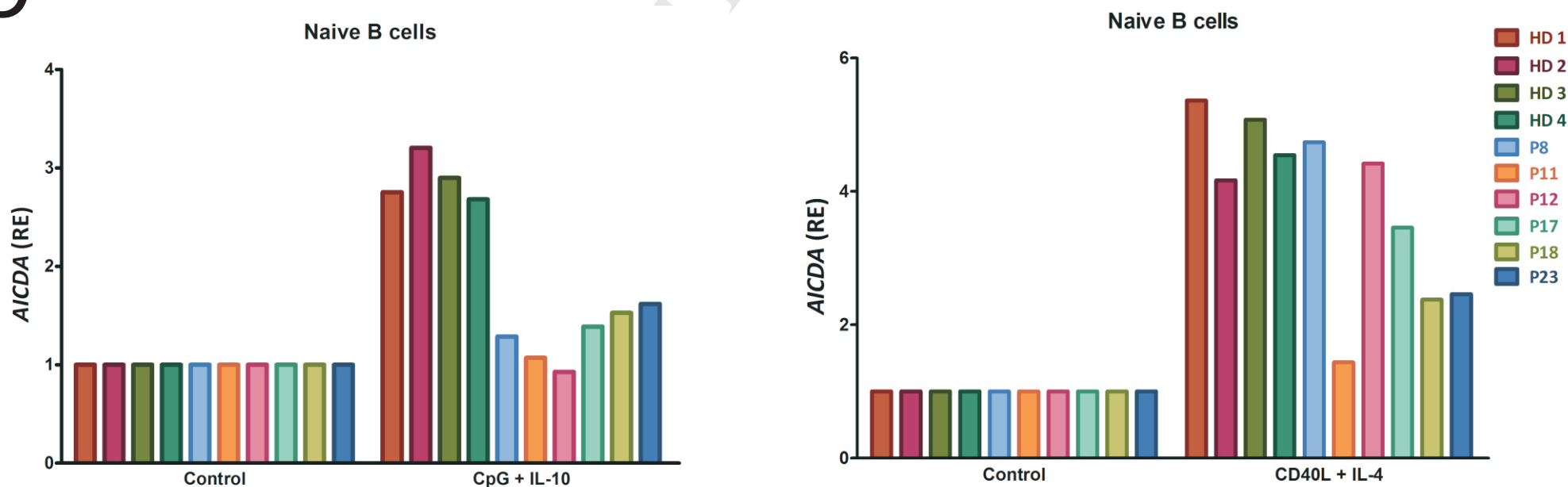
A



B



C



D

