Clinical, Immunological and Molecular Variability of RAG Deficiency: a Retrospective Analysis of 22 RAG Patients

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Clinical, immunological and molecular variability of RAG deficiency: a retrospective analysis of 22 RAG patients

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48 ABSTRACT

49 **Purpose:** RAG deficiency is associated with a variety of clinical phenotypes. We described clinical, immunological and
50 molecular characterization within a cohort of 22 RAG patients focused on the possible correlation between clinical and
51 genetic data.

52 **Methods:** Immunological and genetic features were investigated by Multiparametric Flow Cytometry and by Sanger or
53 Next generation sequencing (NGS) respectively.

54 **Results:** Patients represented a broad spectrum of RAG deficiencies: SCID n=8, OS n=6, LS/AS n=4 and CID n=4. Four
55 novel mutation in *RAG1* gene and one in *RAG2* were reported.

56 The primary symptom at presentation were infections (81.8%). Infections and autoimmunity occurred together in the
57 majority of cases (63.6%). Fifteen out of 22 (68.2%) patients presented autoimmune/hyperinflammatory manifestations.
58 Four patients experienced severe autoimmune cytopenia refractory to different lines of therapy.

59 Total lymphocytes count was reduced or almost lacking in SCID group. CD4 cells count was higher in OS patients. B
60 lymphocytes were variably detected in AS and CID groups. Eighteen patients underwent HSCT permitting definitive
61 control of autoimmune/hyperinflammatory manifestations in twelve of them (80%).

62 **Conclusion:** RAG deficiency still represents a challenge in the tracing of effective management and follow-up, notably
63 considering the inability to predict the disease course in atypical cases. Immune dysregulation manifestations are common
64 features often refractory to conventional medical management. Severe and early autoimmune refractory cytopenia is
65 frequent and could be the first symptom of onset. Prompt recognition of RAG deficiency in patients with early onset of
66 autoimmune/hyperinflammatory manifestations could contribute to the choice of a timely and specific treatment
67 preventing the onset of other complications.

68
69 **Keywords:** RAG deficiency, RAG1/RAG2, hypomorphic mutation, CID phenotypes, cytopenia

71 Abbreviation

72 TCR, T-Cell Receptor
73 SCID, Severe Combined Immunodeficiency
74 OS, Omenn Syndrome
75 AS, Atypical SCID
76 LS Leaky/SCID
77 CID/G/A, Combined immunodeficiency with diffuse granulomatous disease and/or autoimmunity
78 EBV, Epstein-Barr virus
79 CVID, Common Variable Immunodeficiency
80 ICL, Idiopathic CD4+ T cell lymphopenia
81 NGS Next Generation Sequencing
82 RTE, Recent Thymic Emigrants
83 IUIS, Union of Immunological Societies
84 PIDTC, Primary Immune Deficiency Treatment Consortium
85 CMV, Cytomegalovirus
86 IVIG, Intravenous Immunoglobulin
87 ITP, Immune thrombocytopenia
88 AIHA, Autoimmune Hemolytic Anemia
89 HSCT, Hematopoietic Stem Cell Transplantation
90 MAV, Myeloablative Conditioning
91 MMF, Mycophenolate Mofetil
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98 INTRODUCTION

99 Systematic rearrangement of antigen receptor genes via V(D)J recombination is essential for maturation of progenitor
100 lymphocytes, genesis of immunoglobulin and T cell receptor (TCR) and production of a broad repertoire of antigen-
101 specific T and B cells [1-6]. Complete RAGs deficiency has been known to cause severe combined immunodeficiency
102 (SCID) phenotype with lack of T and B cells (T-B-NK+ SCID) [7], life-threatening infections and failure to thrive in
103 early infancy. Today, RAGs diseases are associated to an expanding broad spectrum of phenotypes ranging from SCID,
104 Omenn syndrome (OS) [8-13], 'leaky' or 'atypical' SCID, (LS/AS) whose peculiarities reside in varying numbers of
105 oligoclonal T and B cells, and in some cases a predominance of $\gamma\delta$ + T cells ($\gamma\delta$ AS) and autoimmune cytopenias [14].
106 Hypomorphic mutations causing a residual RAG protein function leading to a delayed onset and diagnosis, characterized
107 by diffuse granulomatous disease and/or autoimmunity (CID-G/A) and susceptibility to severe Herpesviridae infections
108 (in particular EBV) [15-17]. Common variable immunodeficiency (CVID), idiopathic CD4+ T cell lymphopenia (ICL)
109 [18], IgA deficiency and hyper-IgM syndrome have been also reported [19-21].
110 Although infections are the predominant presenting features in RAG deficiency patients, autoimmune manifestations
111 including cytopenias, autoimmune hepatitis, myopathy, and nephrotic syndrome [17, 22-23] should be considered
112 associated manifestations.
113 This autoimmunity has been linked not only to checkpoint breaks in both T and B cell tolerance but also to intestinal
114 microbiota which may play an additional role in sustaining autoimmune pathology [24].
115 Herein, we report the clinical, immunological phenotype and molecular characterization of 22 RAG patients referred to
116 our Center presenting with a broad spectrum of symptoms including autoimmune and/or hyperinflammatory
117 manifestations.

118

119

120 METHODS

121 Patients

122 A total of 22 patients from 18 different families were assessed between 2009 and 2020. Patients were classified into four
123 groups (SCID, OS, LS/AS, CID) based on clinical presentation, immunological data, on the criteria published by the
124 Primary Immune Deficiency Treatment Consortium (PIDTC) [25], ESID 2019 [26] and other groups [24,27,28].

125

126 - **SCID:** at least one of: invasive bacterial, viral or fungal/opportunistic infection; persistent diarrhoea and failure to thrive;
127 affected family member AND manifestation in the first year of life AND two of 4 T cell criteria fulfilled: absence or very
128 low number of T cells (CD3 T cells < 300/microliter), reduced naïve CD4 and/or CD8 T cells; elevated g/d T cells; no or
129 very low T cell function by response to mitogen or TCR stimulation; AND T cells maternal engraftment excluded AND
130 HIV excluded

131 - **OS:** Desquamating erythroderma in the first year of life AND one of the following: lymphoproliferation, hepatomegaly
132 splenomegaly; failure to thrive; chronic diarrhea; recurrent pneumonia AND eosinophilia or elevated IgE AND T-cell
133 deficiency (detectable CD3 T cells, ≥ 300 /microliter, low naïve cells, reduced proliferation, oligoclonality) AND maternal
134 engraftment excluded AND HIV excluded

135 - **LS/AS:** Reduced number of CD3 T cells (for age up to 2 years < 1000/microliter; for > 2 years up to 4 years <
136 800/microliter, for > 4 years < 600/microliter) AND Absence of maternal engraftment AND < 30% of lower limit of
137 normal T cell function (as measured by response to PHA) AND Presence of activated, oligoclonal, and autologous T cells

not associated with typical features of OS. Expansion of $\gamma\delta$ T cells upon cytomegalovirus (CMV) infection or Epstein-Barr virus (EBV)-driven lymphoproliferative disease and autoimmune cytopenias could be associated.

- **CID:** at least one of: severe infection (especially VZV, CMV, EBV, HPV, and molluscum); one manifestation of immune dysregulation (autoimmunity, IBD, severe eczema, lymphoproliferation, granuloma); malignancy; affected family member AND 2 of 4 T cell criteria fulfilled: reduced CD3 or CD4 or CD8 T cells (using age-related reference values); reduced naïve CD4 and/or CD8 T cells; elevated g/d T cells; reduced proliferation to mitogen or TCR stimulation.

The clinical characterization is summarized in Table 1. Two patients have been enrolled in the pCID study (DRKS00000497).

Multiparametric Flow Cytometric Analysis

All flow cytometric analysis were performed on ethylenediamine tetraacetic acid (EDTA) blood samples within 24 h of venipuncture. After red blood cells lysis with ammonium chloride the lymphocytes were incubated with the appropriate antibody cocktail for 30 min at 4°C, washed with PBS and suspended in PBS. At least 50,000 events in the lymphocyte live gate were acquired for each sample. Samples were acquired on FACSCANTO II (BD Biosciences, San Diego, CA, USA) and analyzed with FlowJo software (Tree Star Inc, version 8.8.6, Ashland, Ore).

Genetic Analysis

Ion Torrent Gene Target Library Preparation and NGS Sequencing were performed according to manufacturer's instructions (Thermo Fisher Scientific)

Next generation Sequencing (Ion Torrent) and Sequencing: genomic DNA isolated from peripheral blood of patients using standard protocols (QIAamp DNA Blood kit by QIAGEN GmbH, Hilden, Germany). PCR reactions were carried out using GoTaq DNA polymerase by standard methods (Promega, Madison, WI). Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 3130 and 310 automated sequencers (Applied Biosystems).

Sanger sequencing for all mutations and parents' carrier status were performed.

Ion Torrent bioinformatics analysis

Mapping and variants calling were performed using the ion Torrent suite software v3.6. Sequencing reads were aligned against the USC hg19 reference genome using the program distributed within the Torrent mapping Alignment Program (TMAP) map4 algorithm (Thermo Fisher; <https://github.com/iontorrent/TS>). The aligned reads were processed for variant calling by using the Torrent Suite Variant Caller TVC program; variants found in Variant Calling Format (VCF) file were annotated using ANNOVAR. The called variants with minimum coverage of 20X, standard Mapping Quality, and Base Phred Quality were examined on Integrative Genome Viewer (IGV) and BIOMART. After applying filtering criteria, all nonsense, frameshift, and canonical splice site variants were evaluated to determine their potential pathogenicity.

Statistical Analysis

Data were analyzed with Graph-Pad Prism, version 6.2 (Graph Pad Software, la Jolla, CA). $p < 0.05$ was considered significant.

RESULTS

1.1 Clinical, Immunological, and Genetic Phenotypes of RAG-deficient cohort

178 We report clinical, immunological and molecular characterization within a cohort of 22 RAG patients diagnosed between
179 2009 and 2020.

180 The cohort characteristics are described in Table 1. The age at presentation ranged from birth to 6 years (72 months), with
181 a median of 16.2 months whereas the median age of genetic diagnosis was 23.5 months (from birth to 11 years) with a
182 diagnostic delay of 7.3 months.

183 In particular, the mean age of first symptom was 4.2 months (± 2.5 s.d.) and genetic diagnosis was 7.1 months (± 3.06 s.d.)
184 for SCID; 2.6 months (± 1.5 s.d.) and 3.4 months (± 1.8 s.d.) for OS; 13.2 months (± 7.9 s.d.) and 16.8 months (± 9.0 s.d.)
185 for LS/AS; 4.7 years (± 42 s.d.) and 6.7 years (± 58.9 s.d.) for CID (figure 1B). Male patients were slightly predominant
186 (54.2%).

187 The majority of patients (8 cases, 36.4%) were affected by SCID presenting with T-B-NK+ phenotype except for two
188 patients with T+B-NK+ phenotype in which maternal T engraftment was observed, followed by OS (6 cases, 27.2%),
189 LS/AS (4 cases, 18.2%) and CID (4 cases, 18.2%) (Fig. 1a). *RAG1* and *RAG2* mutations were present in 19 (86.4%) and
190 3 (13.6 %) patients respectively (Table 2). Clinical manifestations are summarized in Table 1.

191 Consanguinity was documented in 9 out of 20 families (45%). Noteworthy, there was no significant difference between
192 patients with SCID and OS when comparing age at first clinical symptoms and at diagnosis (Fig. 1b). The median age of
193 the first symptom as well as of the genetic diagnosis, was markedly lower in SCID and OS than in LS/AS and CID patients
194 ($p < 0.05$) (Fig. 1b). The symptom at presentation was infection ($n=18$; 81.8%) except for OS where dermatitis represents
195 the onset manifestation in all cases. On the other hand, infections and autoimmunity occurred together in the majority of
196 cases ($n=14$; 63.6%). Three SCID and one OS early diagnosed and promptly transplanted did not present any severe
197 manifestation (Fig. 1c). Chronic viremia was common among the cohort whereas respiratory and gastrointestinal
198 infections were prevalent in SCID patients (Fig. 1d). Interestingly, 15 out of 22 (68.2%) patients presented autoimmune
199 and/or hyperinflammatory manifestations (Fig. 1c). The most frequent was dermatitis occurring in 11 patients (50%).
200 Lymphoproliferation and hepatosplenomegaly were present in 5 patients (22.7%) (Suppl. Fig. 1A). Nephropathy occurred
201 in 2 patients whereas one patient developed Miller Fisher syndrome (Fig. 1d, and Table 1).

202 Notably, four patients, three CID and one OS (PID-12, PID-13, PID-20, PID-10) with autoimmune/hyperinflammatory
203 complications experienced a severe autoimmune cytopenia (AIC) (Fig. 2a, b) with a median age at onset of cytopenia of
204 3.2 years.

205 All patients received intravenous immunoglobulins (IVIG) and steroids as first-line therapy with limited response.
206 No complete remission was observed in any patients despite second-line therapies with Rituximab (PID12 and PID20)
207 (or Mycophenolate mofetil (MMF) (PID-20).

208 Increased IgE levels were observed on the OS and CID groups and IgA and IgM resulted very low or undetectable in
209 SCID and OS patients. AS and CID patients showed normal to hypergammaglobulinemia values. Serum IgG at onset
210 resulted highly variable, for SCID and OS, probably reflecting maternal trans-placental transfer (Fig. 3a).

211 Total lymphocyte counts were particularly reduced or almost lacking in SCID group as expected, whereas CD4 cell
212 count was higher in OS patients. Expansion of T-cell receptor $\gamma\delta$ was documented in two patients (PID-3 and PID-11).
213 Despite circulating T cells were present, the proportion of naïve T cells was reduced in CID patients (data not shown).
214 Interestingly, B lymphocytes were variably detected in AS and CID groups (Fig. 3b). NK cells were present in all groups
215 (Fig. 3b).

216 *RAG1* mutations were detected in nineteen patients, while *RAG2* mutations in three of them. Four novel mutations in
217 *RAG1* gene and two in *RAG2* were reported. All mutations are summarized in Table 2. In total eighteen patients received
218 HSCT (Fig. 3c).

219 Among fifteen patients with autoimmune/hyperinflammatory manifestations, HSCT was required for definitive
220 management in twelve patients (80%). PID-13 and PID-18 died for multiorgan failure before HSCT, while PID-12 died
221 of infectious complications after transplant (invasive aspergillosis already present at time of HSCT). Patients with immune
222 dysregulation underwent HSCT at an older age compared to those without immune dysregulation (median 12.7 vs. 7.4
223 years) partially due to the diagnostic delay (Fig. 2d). Two patients resolved treatment- refractory AIC thanks to HSCT.
224 Eighteen patients (81.8%) are alive at the time of this study. Thirteen patients experienced post-HSCT complications
225 including herpetic infections, and low grade (i.e., grade I-II graft-versus-host disease)
226

227 1.2 Particular cases

228 Of note, three patients need further more in depth description because of the peculiarities of clinical phenotype. In details,
229 PID-11 with an homozygous frameshift mutation was characterized by the persistent CMV viremia, *P. aeruginosa* sepsis
230 and mild BCGitis at the age of seven months. In addition, he developed Miller Fisher's Syndrome (Fig. 4a, b).
231 Immunological evaluation revealed marked CD4-penia, expansion of $\gamma\delta$ + T-cells and memory B cells with
232 hypergammaglobulinemia and ITP. In order to control the expansion of B autoreactive clones, Rituximab therapy and
233 plasmapheresis were used allowing a complete depletion of B cells and progressive improvement of neurological and
234 respiratory function. Patient received HLA-haploidentical transplant from the father with full engraftment. Four months
235 post-transplantation severe AIHA associated to a CMV reactivation was observed and BAFF plasma level resulted
236 increased (12.000 pg/ml) as previously described in patients with RAG mutations and autoimmunity [29-31]. Antiviral
237 drugs, high-dose IVIg, Rituximab, plasmapheresis and multiple blood transfusions were required to control autoimmunity
238 and achieve a complete remission.

239 PID-3 carrying the homozygous p.R841W *RAG1* mutation was characterized by long lasting diarrhea, erythroderma,
240 candidiasis and tubulo-interstitial nephritis with infiltrate of lymphocytes, histiocytes, plasmacells and eosinophils. He
241 showed T+B-NK+ phenotype due to maternal T engraftment. Immunological evaluation revealed CD4-, CD8-penia and
242 $\gamma\delta$ + T-cells expansion.

243 Lastly, PID-14, previously reported [32], presented a disease onset at six years of age characterized by relapsing nasal
244 polyposis, severe agammaglobulinemia and absence of B cell suggesting initially a humoral defect. His past history was
245 notable for recurrent middle-ear infections, chickenpox and mild persistent EBV viremia. Bone marrow examination
246 showed a marked decrease of B cell progenitors, with an incomplete arrest at pro-B cell stage and few pre-B and mature
247 B cells. No sign of myelodysplasia was found. Later in the follow-up a marked reduction of CD4+/CD45RA+/CD31+
248 recent thymic emigrants (RTE) were detected over-time, along with a corresponding increase in the proportion of memory
249 T cells. Since T lymphocytes pool deteriorate with age, we reassessed his clinical diagnosis that changed from
250 agammaglobulinemia to CID. This patient, who did not receive HSCT, is currently alive and well on IVIGs at the age of
251 16 years
252

253 DISCUSSION

254 We report 22 RAG deficient patients, documenting an increase in the number of RAG diagnosis following the application
255 of NGS from a median of 1.5 up to 2.6 per year. In addition, we observed a median delay of 7.33 months between clinical
256 onset and genetic diagnosis in the mild clinical presentation associated to hypomorphic RAG mutations. The increased
257 frequency observed is consistent with recent reports including newborn screening that confirm *RAG1/2* as second most
258 prevalent genes associated with SCID and the most common genes associated with leaky SCID/CID [33]. *RAG1*
259 mutations have been reported more frequently than *RAG2* [34] as occurred in our cohort.

260 Differently from other studies focused on populations with high rate of consanguinity [34,35], many patients enrolled in
261 this work had non-consanguineous parents (59%), suggesting a highly mutation rate in these genes as well as a large
262 occurrence of heterozygous carriers.

263 Intriguingly, although individuals with *RAG* heterozygous missense mutations do not seems associated to typical *RAG*
264 phenotype, we observed three patients presenting as CID and autoimmune cytopenia carrying only a single missense
265 heterozygous mutation in *RAG* genes (data not shown) suggesting that other factors, not yet fully understood, could
266 influence this genetic background.

267 Two patients defined as AS and CID (PID-3 and PID-13), carrying the same homozygous mutations (p.R841W), showed
268 distinct phenotypes, with severe autoimmune manifestations: the second one, despite having received a more timely
269 diagnosis, died before being able to undergo HSCT; similarly, two siblings (PID-12 and PID-16) with compound
270 heterozygosity (p.R405G and p.R624H) were diagnosed in different ages. The older sister diagnosed at 3 years of life
271 died of invasive aspergillosis already present at time of HSCT. The younger sister thanks to a prenatal diagnosis
272 underwent to HSCT at 3 months of life showing a different clinical outcome. This underlines the importance of other
273 genetic or environmental factors on disease course and how a prompt HSCT may be crucial.

274 Furthermore, three compound heterozygous *RAG1* deficient patients, classified as SCID (PID-16), CID (PID-12, PID-
275 14) and OS (PID-7), carried the same p.R624H mutation in combination with the p.R405G, p.Y728H and p.R561H
276 respectively. The p.Y728H showed a markedly decreased but detectable recombinase activity in line with his milder
277 phenotype [33]. Nevertheless, other factors such a chronic and uncontrolled infections or unknown genetic characteristics
278 could influence genotype-phenotype correlation besides the level of recombinase activity. Time to diagnosis is also very
279 different ranging from 2 months (PID7) to 3 and 6 years (PID12 and PID14) revealing a very different behavior under
280 the same common p.R624H mutation. Of note the two sisters PID-12 and PID-16 received two different diagnoses at the
281 birth (CID and SCID respectively).

282 In addition, chronic, uncontrolled Herpesviridae viremia was frequent among CID group. Usually, CID patients develop
283 more severe manifestations (as autoimmunity) often triggered by viral infections that closely preceded the onset of
284 autoimmunity as in our patients PID-11 [36].

285 Nonetheless, the presence of residual B cells in CID patients makes them prone to EBV infection with higher risk EBV-
286 driven lymphoproliferation suggesting the need of a closer viral and radiological monitoring [36,37].

287 Considering the high frequency of hypomorphic mutations and milder phenotype, a longitudinal re-evaluation of patients
288 lacking a molecular diagnosis is recommended, as in our PID-14 who was initially classified as humoral defect and only
289 later reconsidered as CID.

290 Thus, *RAG* deficiency should be suspected also in patients where B cell defect is predominant and in those patients
291 without typical sign of SCID or OS as immunodeficiency associated to immune dysregulation-

292 Our data, in line with other reports [22], showed that severe immune dysregulation manifestations are a common feature
293 of *RAG* deficiency often refractory to conventional medical management.

294 In particular, severe and early autoimmune refractory cytopenia found in other cohort and others [22], seems to be very
295 frequent in *RAG* deficiency also as the first symptom of onset. The early recognition of these patients in patients with
296 early onset or severe autoimmune cytopenia could contribute to the choice of a prompt specific treatment and prevent the
297 onset of others complications [38,39].

298 Literature reports show that HSCT using an HLA-identical donor demonstrates excellent overall survival greater than
299 75% [40,41]; moreover, innovative transplant approaches have showed encouraging results in case where a HLA-matched
300 donor is not available [42,43]. Immunosuppressive or immunomodulatory drugs should be strongly considered to control

immunodysregulation in patients with a milder phenotype (CID or AS) and delayed presentation as well as patients affected by CVID or agammaglobulinemia in the absence of HLA-identical donor.

CONCLUSIONS

NGS has greatly accelerated the diagnosis of all RAGs cases preventing the worsening of disease thanks to a better and early treatment. On the other hand, it still presents a challenge in the tracing of effective management and follow-up, considering the inability to predict the disease course in atypical cases. This study describes the immunological, clinical and molecular characteristics of 22 patients with RAG deficiency highlighting the heterogeneity of manifestations associated with this condition in particular of patients with hypomorphic mutations and milder phenotypes. In this regard, extending RAG analysis to a cohort of older pediatric and adult patients affected by undefined CVID and/or immune dysregulation [44], may help to expand the knowledge of natural history of *RAG* deficiency.

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Code availability: not applicable

Author Contribution

CC, DA, BR, interpreted the results and wrote the manuscript

CC, GMU, SDC, MC, MGD, MD, GDM performed molecular and functional experiments and developed gene sequencing analysis.

CC, GMU, GDM created gene clusters to filter variants and integrated clinical and bioinformatics analysis of data retrieved by genetic platforms

DA, BR, LP, NC, MA, FG, GP, PM, FL, PP, PR, AF, and CaC provided or referred clinical samples and patient's clinical data.

CC, DA, BR, GDM, CaC designed the research, participate to the study design and data interpretation.

FL, PP, PR, AF, GDM and CaC made substantial contributions to revising the manuscript.

All authors have critically revised and approved the manuscript

Ethics approval

This retrospective study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Ethical Committee of the Children's Hospital Bambino Gesù in Rome approved this study. Informed consent was obtained from patient's parents/legal guardians.

Consent to participate: Informed consent was obtained from patient's parents or legal guardians.

Consent for publication: Informed consent for publication was obtained from patient's parents or legal guardians.

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Table 1 Clinical features of RAG patients. SCID, Severe Combined Immunodeficiency; OS, Omenn Syndrome; AS, Atypical SCID; LS, Leaky/SCID; CID, Combined immunodeficiency, EBV, Epstein-Barr virus; CMV, Cytomegalovirus; HHV-6; Human herpesvirus 6; RSV, Respiratory syncytial virus; URI, Upper respiratory infections; LRI, Lower respiratory infections; AIHA, Autoimmune hemolytic anemia.

ID	AGE AT PRESENTATION	GENDER	ADMITTING CLINICAL DIAGNOSIS	FINAL CLINICAL DIAGNOSIS	GENETIC DIAGNOSIS	CHRONIC VIREMIA	OPPORTUNISTIC/ RECURRENT INFECTIONS	IMMUNE DYSREGULATION AUTOIMMUNITY MALIGNANCIES	OTHER	HSCT
PID-1	5 MONTHS	M		AS SCID	RAG2	ADENOVIRUS			WIDESPREAD DERMATITIS, LYMPHOADENOPATY	YES
PID-2	5 MONTHS	F	FEVER, VOMITING	SCID	RAG2	ADENOVIRUS	FEVER		APHTHOUS GINGIVOSTOMATITIS	YES
PID-3	2 YEARS	M		AS SCID	RAG1	ADENOVIRUS, R HINOVIRUS	LONG-LASTING DIARRHOEA, CANDIDIASIS	TUBULOINTERSTITIAL NEPHRITIS WITH INFILTRATE OF LYMPHOCYTES, HISTIOCY TES, PLASMACELLS AND EOSINOPHILS	DERMATITIS	YES
PID-4	2 MONTHS	M	FEVER	SCID (T-B-NK+)	RAG1	CMV ADENOVIRUS	CMV RETINITIS, RSV, BRONCHIOLITIS, PNEUMONIA			YES
PID-5	6 MONTHS	F	RESPIRATORY DISTRESS	SCID (T-B-NK+)	RAG1					YES
PID-6	4 MONTHS	M	DIARRHOEA, FEVER	SCID	RAG1		LONG-LASTING ROTAVIRUS, ADENOVIRUS DIARRHOEA, PNEUMONIA			YES

PID-7	2 MONTHS	M	HYPOGAMMAGLOBULINEMIA	OS	RAG1	CMV			GENERALIZED ERYTHRODERMA; SEVERE DERMATITIS	YES
PID-8	3 MONTHS	F	DERMATITIS AND INFECTIONS	SCID	RAG1		OTITIS (PSEUDOMONAS AERUGINOSA), URINARY INFECTION, BRONCHIOLITIS		DERMATITIS, GROWTH RETARDATION, DUST ALLERGY	YES
PID-9	2 MONTHS	F	OS	OS	RAG1		PNEUMONIA, STAPHYLOCOCCAL SEPSIS		SEVERE DERMATITIS, GROWTH RETARDATION	YES
PID-10	BIRTH	M	OS	OS	RAG1		PNEUMOCYSTOSIS, LRI (FLAVOBACTERIUM MENINGOSEPTICUM)	TUBULE INTERSTITIAL NEPHRITIS WITH LYMPHO-MONOCYTE INFILTRATE, THROMBOCYTOPENIA	HERPETIC KERATITIS, DERMATITIS	YES
PID-11	11 MONTHS	M	MILLER-FISHER (CMV- RELATED)	AS	RAG1	CMV, PNEUMONIA	P.AERUGINOSA PNEUMONIA, SEPSIS, BCGite	MILLER-FISHER SYNDROME (CMV-RELATED), PITYRADICULONEURITIS WITH CRANIAL NERVE INVOLVEMENT		YES
PID-12	3 YEARS	F	LRI	CID	RAG1	HHV-6, CMV, EBV	URI, LRI	AIHA, NEUTROPENIA		YES
PID-13	1 YEAR	M	RESPIRATORY INFECTIONN, THROMBOCYTOPENIA	CID	RAG1	CMV, EBV	HAEMOPHILUS INFLUENZAE, BOCAVIRUS RESPIRATORY INFECTION, LONG-LASTING ROTAVIRUS DIARRHOEA	THROMBOCYTOPENIA, AIHA	ACUTE RESPIRATORY FAILURE SEVERE HEPATOSPLENOMEGALY WITH LIVER FAILURE, DERMATITIS	NO
PID-14	6 YEARS	M	AGAMMAGLOBULINEMIA	CID	RAG1	EBV	URI, OTITIS, SEVERE CHICKEN POX		NASAL POLYPOSIS; CHRONIC BRONCOPNEUMOPATHY	NO

PID-15	1,8 YEARS	F	URI, OTITIS, VOMITING, DIARRHOEA	SCID	RAG1	CMV, ADENOVIRUS	RESPIRATORY INFECTION, OTITIS, LONG-LASTING DIARRHOEA		CHRONIC HEPATOPATHY	YES
PID-16	BIRTH	F	PRENATAL DIAGNOSIS	SCID (T-B-NK+)	RAG1					YES
PID-17	5 MONTHS	M	DERMATITIS	OS (T-B-NK+)	RAG2		LRI, STAPHYLOCOCCUS SEPSIS, ENTEROBACTER CLOACAE URINARY TRACT INFECTIONS	ALOPECIA	EXUDATIVE DERMATITIS, , EPATOSPLENOMEGALY, STAPHYLOCOCCUS SKIN INFECTION	YES
PID-18	3 MONTHS	M	PURULENT OTITIS	OS	RAG1		PURULENT OTITIS, NECROTIZING FASCIITIS, CEREBRAL ABSCESS, SEPSIS		DERMATITIS	NO
PID-19	7 MONTHS	F		SCID	RAG1					YES
PID-20	3 YEARS	F		CID	RAG1	HHV6	PNEUMONIA; LRI	VITILIGO, AI THYROIDITIS AIHA	ECZEMA, EPATOSPLENOMEGALY	NO
PID-21	5 DAYS	F		OS	RAG1				SEVERE DERMATITIS	YES
PID-22	5 MONTHS	M	DIARRHOEA, BRONCHITIS	SCID (with maternal engraftment)	RAG1	HHV6	NOROVIRUS LONG- LASTING DIARRHOEA, PNEUMONIA, INFECTIOUS GASTROENTERITIS, HHV6 DNAemia		ECZEMA, EPATOSPLENOMEGALY, PAPULAR RASH	YES

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Table 2 Molecular Characterization of RAG patients. SCID, Severe Combined Immunodeficiency; OS, Omenn Syndrome; AS, Atypical SCID; LS, Leaky/SCID; CID, Combined immunodeficiency; dbSNP, Single Nucleotide Polymorphism Database; In red novel not described mutations. * values from reference 45.

ID	Disease	Gene	Mutation		dbSNP and references	Zygosity	Inheritance	OMIM	Protein Domain	Recombinase activity allele a (approximately)	Recombinase activity allele b (approximately)
PID-1	AS SCID	RAG2	a) c.685C>T; p.R229W		rs765298019	Homozygous	Unknown	OMIM *179616	a) core region	10.5 ± 0.5*	
PID-2	SCID	RAG2	a) c.1A>G; p.M1V	b) c.1403_1406del ATCT	Cifaldi C. et al.; rs786205616	Compound heterozygous	Familial	OMIM *179616	a) core region	n.d.	0% deduced
PID-3	AS SCID	RAG1	a) c.2521C>T; p.R841W		rs104894287	Homozygous	Familial	OMIM *179615	a) Zn binding domain (catalytic core)	10.0 ± 0.5*	
PID-4	SCID	RAG1	a1) c.1681C>T; p.R561C a2) c.1815G>C; p.M605I	b) c.2780T>C; p.F927S	rs104894285; Dobbs et al.	Compound heterozygous	Familial	OMIM *179615	a) pre RNaseH (catalytic core); b) catalytic RNAaseH (catalytic core)	n.d.; n.d.	n.d.
PID-5	SCID	RAG1	a) c.1221G>A; p.R410Q	b) c.1863delG; p.A622QfsTer9	rs199474684; Cifaldi et al.	Compound heterozygous	Unknown	OMIM *179615	a) NBD (catalytic core)	0%	0% deduced
PID-6	SCID	RAG1	a. c.985delC; p.329fs		n.d.	Homozygous	n.d.	OMIM *179615		0% deduced	
PID-7	OS	RAG1	a) c.1682G>A; p.R561H	b) c.1871G>A; p.R624H	rs104894284; rs199474680	Compound heterozygous	Familial	OMIM *179615	a) pre RNaseH (catalytic core); b) catalytic RNAaseH (catalytic core)	2.0 ± 0.6*	0.0 ± 0.4*
PID-8	SCID	RAG1	a) c.1228C>T; p.R410W	b) c.2780T>C; p.F927S	rs199474684; Cifaldi et al.	Compound heterozygous	Familial	OMIM *179615	a) NBD (catalytic core) b) Zn binding domain (catalytic core)	0.0 ± 0.0*	n.d.
PID-9	OS	RAG1	a) c.351delT	b) c.1577T>G; p.L526A	Cifaldi et al.	Compound heterozygous	n.d.	OMIM *179615	a) pre RNaseH (catalytic core);	0% deduced	n.d.
PID-10	OS	RAG1	a) c.1870C>T; p.R624C	b) c.2521C>T; p.R841W	rs199474688; rs104894287	Compound heterozygous	Familial	OMIM *179615	a) catalytic RNAaseH (catalytic core) b) Zn binding domain (catalytic core)	n.d.	10.0 ± 0.5*
PID-11	AS	RAG1	a) c.256_257del; p.K86VfsTer33		rs772962160	Homozygous	Familial	OMIM *179615		2.7 ± 0.3*	

PID-12	CID	RAG1	a) c.1870C>T; p.R624H	b) c.1213A>G p.R405G	rs199474680; Cifaldi et al.	Compound heterozygous	Familial	OMIM *179615	a) catalytic RNAaseH (catalytic core) b) NBD (catalytic core)	0.0 ± 0.4*	Recombinase activity ongoing
PID-13	CID	RAG1	a) c.2521C>T; p.R841W		rs104894287	Homozygous	Familial	OMIM *179615	a) Zn binding domain (catalytic core)	10.0 ± 0.5*	
PID-14	CID	RAG1	a) c.1871G>A; p.R624H	b) c.2182T>C; p.Y728H	rs199474680; Cifaldi et al.	Compound heterozygous	Familial	OMIM *179615	a) catalytic RNAaseH (catalytic core) b) Zn binding domain (catalytic core)	0.0 ± 0.4*	25.7% (REF)
PID-15	SCID	RAG1	a) c.1767C>G p.Y589X		rs991089005	Homozygous	n.d.	OMIM *179615		0% deduced	
PID-16	SCID	RAG1	a) c.1870C>T; p.R624H	b) c.1213A>G p.R405G	rs199474680; Cifaldi et al.	Compound heterozygous	Familial	OMIM *179615	a) catalytic RNAaseH (catalytic core) b) NBD (catalytic core)	0.0 ± 0.4*	n.d.
PID-17	OS	RAG2	a) c.281A>G p.H94R	b) c.1090_1093delAACA		Compound heterozygous	n.d.	OMIM *179616	a) N-term	nd	n.d.
PID-18	OS	RAG1	a) c.519delT p.E174Sfs*26		rs1241698978 Hanna IJspeert, 2014J Allergy Clin Immunol	Homozygous	n.d.	OMIM *179615		0.5 ± 0.2*	
PID-19	SCID	RAG1	a)c.1361 T>A p. L454Q		rs199474677	Homozygous	n.d.	OMIM *179615	a) NBD (catalytic core)	5.4 ± 0.7*	
PID-20	CID	RAG1	a)c.2095C>T p.R699W	b) c.2408A>G; p:N803D	Lee et al; n.d.	Compound heterozygous	n.d.		a) catalytic RNAaseH (catalytic core) b) Zn binding domain (catalytic core)	19.3 ± 1.8*	n.d.
PID-21	OS	RAG1	a) c.1870C>T; p.R624C	b) c.2521C>T; p.R841W	rs199474688; rs104894287	Compound heterozygous	Familial	OMIM *179615	a) catalytic RNAaseH (catalytic core) b) Zn binding domain (catalytic core)	n.d.	10.0 ± 0.5*
PID-22	SCID	RAG1	a) c. 1210 C>T; p.R404W			Homozygous			a) NBD (catalytic core)	n.d.	

* values from [45] Predicting the Occurrence of Variants in RAG1 and RAG2. Dylan Lawless, Journal of Clinical Immunology (2019) 39:688–701. Bold: novel not described mutations. SCID = Severe Combined Immunodeficiency; OS = Omenn syndrome; AS= Atypical SCID CID-G/A= combined immunodeficiency with granuloma/autoimmunity; CVID= Common Variable Immunodeficiency;

504 **FIGURES LEGEND**
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506 **Fig. 1 Panel a** RAG cohort. Clinical diagnosis of RAG patients for the four main categories (n=22). **Panel b** Difference
507 in timing for first symptom of immunodeficiency and genetic diagnosis of four RAG groups. Symbols represent individual
508 patients and red bars representing median. **Panel c** Infections and autoimmunity in RAG cohort (number of total cases).
509 **Panel d** Infections and autoimmune manifestations in RAG cohort distributed among the four groups (frequency as %
510 total cases).

511 **Fig. 2 Panel a** Occurrence of autoimmune cytopenia (AIC) in RAG cohort (n=4/22). **Panel b** Severity of autoimmune
512 cytopenias by cell nadir during disease flare (symbols representing individual patients, mean with SD. Dashed line normal
513 range from Bambino Gesù Children's Hospital. Autoimmune manifestation (AI); Autoimmune cytopenia (AIC); Absolute
514 neutrophil count (ANC); Immune thrombocytopenia (ITP); autoimmune hemolytic anemia (AIHA); platelet (PLT).

515 **Fig. 2 Panel a** Immunoglobulin titers of each group. **Panel b** Immunophenotype of RAG cohort. CD3+, CD4+, CD8+ T
516 cell, CD19+ B cells and NK cells count among groups **Panel c** Occurrence of HSCT in our RAG cohort. For each
517 category, the total number of patients is indicated. **Panel d** Occurrence of HSCT in patients with (AI+ n=12) or without
518 (AI- n=6) autoimmunity (frequency as % annotated total cases, n=18) and age of HSCT.

519 **Fig. 4 Panel a** Abdominal CT scan and ultrasound showed hepatosplenomegaly in two CID patients.
520 **Panel b** Magnetic resonance images showed marked thickening, impregnation of the roots of the cauda (left) and cranial
521 nerves (right) reflecting a clinical picture of Miller Fisher's Syndrome characterized by severe axial hypotonus,
522 ophthalmoparesis, ataxia, generalized areflexia, progressive paralysis of cranial nerves.
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Figures

Figure 1

Panel a RAG cohort. Clinical diagnosis of RAG patients for the four main categories (n=22). Panel b Difference in timing for first symptom of immunodeficiency and genetic diagnosis of four RAG groups. Symbols represent individual patients and red bars representing median. Panel c Infections and autoimmunity in RAG cohort (number of total cases). Panel d Infections and autoimmune manifestations in RAG cohort distributed among the four groups (frequency as % total cases).

Figure 2

Panel a Occurrence of autoimmune cytopenia (AIC) in RAG cohort (n=4/22). Panel b Severity of autoimmune cytopenias by cell nadir during disease flare (symbols representing individual patients, mean with SD. Dashed line normal range from Bambino Gesù Children's Hospital. Autoimmune manifestation (AI); Autoimmune cytopenia (AIC); Absolute neutrophil count (ANC); Immune thrombocytopenia (ITP); autoimmune hemolytic anemia (AIHA); platelet (PLT).

Figure 3

Panel a Immunoglobulin titers of each group. Panel b Immunophenotype of RAG cohort. CD3+, CD4+, CD8+ T cell, CD19+ B cells and NK cells count among groups Panel c Occurrence of HSCT in our RAG cohort. For each category, the total number of patients is indicated. Panel d Occurrence of HSCT in patients with (AI+ n=12) or without (AI- n=6) autoimmunity (frequency as % annotated total cases, n=18) and age of HSCT.

Figure 4

Panel a Abdominal CT scan and ultrasound showed hepatosplenomegaly in two CID patients. Panel b Magnetic resonance images showed marked thickening, impregnation of the roots of the cauda (left) and cranial nerves (right) reflecting a clinical picture of Miller Fisher's Syndrome characterized by severe axial hypotonus, ophthalmoparesis, ataxia, generalized areflexia, progressive paralysis of cranial nerves.