ADVANCES IN PRIMARY IMMUNODEFICIENCY IN CENTRAL-EASTERN EUROPE

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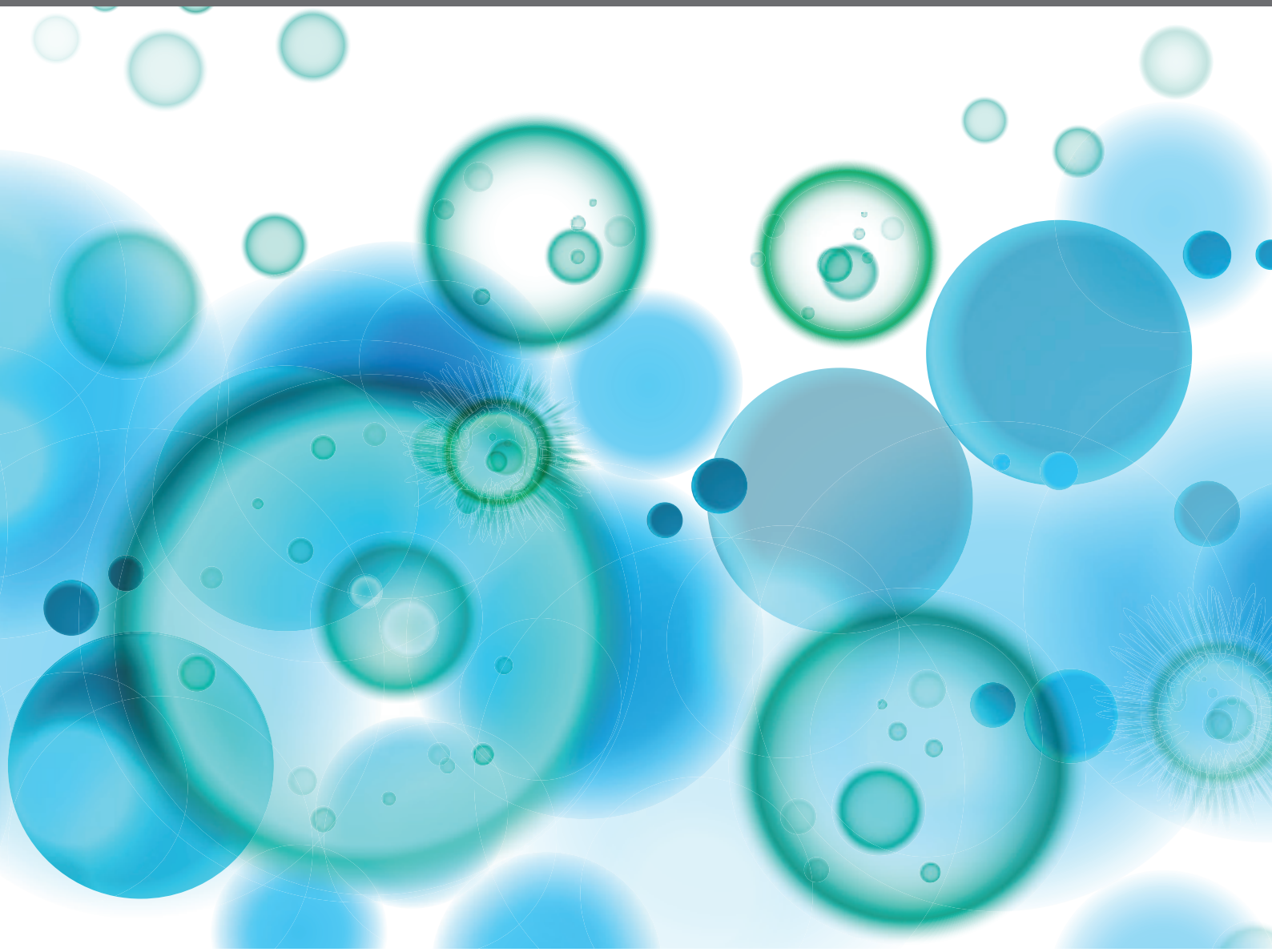

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ADVANCES IN PRIMARY IMMUNODEFICIENCY IN CENTRAL-EASTERN EUROPE

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EDITORIAL
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Editorial: Advances in Primary Immunodeficiency in Central-Eastern Europe

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Keywords: primary immunodeficiencies, antibody deficiencies, severe combined immunodeficiency, J Project, Nijmegen Breakage Syndrome, awareness, treatment, education

Editorial on the Research Topic

Advances in Primary Immunodeficiency in Central-Eastern Europe

INTRODUCTION

Primary immunodeficiencies (PIDs), since 2019 International Union of Immunological Societies (IUIS) Expert Committee updated classification designated as Inborn Errors of Immunity (IEI) are genetically inherited, heterogeneous disorders affecting at least two million people over the world. Although remarkable improvements in diagnosis and treatment have been made, they remain underestimated. The initial report done under the auspices of the World Health Organization in 1970 identified 16 distinct primary immunodeficiencies. Over the years following this report, tremendous progresses in the field of recognition has been made. This was possible due to great energy and enthusiasm from scientists and doctors, as well as new diagnostic and therapeutic tools. Next-generation sequencing techniques lead to an increased number of recognized disorders. According to the 2019 report of the IUIS, 416 distinct IEI with 430 different gene have been defined (1, 2). This progress was done over the past decades mainly in Western Europe, the US, Japan, and Australia (1–4).

DEVELOPMENT OF PID CARE IN EASTERN AND CENTRAL EUROPE (ECE)

Until the late 80's and early '90s, ECE countries remained isolated with limited access to the newest scientific achievements, diagnostic tools, and therapeutic methods. Only personal connections and direct collaboration with clinical and research centers in Western Europe and the US made some progress possible in that region. It was depressing to see registry data of the European Society for Immunodeficiencies in 2002 showing that most Eastern European countries had reported fewer than 10 patients with PIDs disorders. These data suggested that PIDs may have been not only underreported but underdiagnosed in Eastern and also in Central Europe. A lot of efforts were made

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to overcome the large gap between ECE and Western Europe in terms of PID diagnostics, including molecular tests, treatment, and education. These efforts, over the past two decades, resulted in remarkable progress of clinical care, laboratory diagnosis, and awareness in the field of PID in the whole area of ECE.

THE IMPACT OF THE J PROJECT

One of the most important initiatives was the J Project, started in the early 2000s, with the goal to increase awareness, facilitate diagnosis including genetic tests, and improved therapy according to the latest knowledge in the area of ECE region (4–8). Over the past 17 years this project has extended to 32 countries mostly in ECE and partly in Asia and promoted close to 300 education meetings in the PID field for physicians, laboratory workers and patient advocates. The number of J Project meetings has exceeded 40 per year recently. As a result, the number of diagnosed patients reached tens of thousands and an increasing proportion of them are receiving therapy, primarily immunoglobulin substitution and hematopoietic stem cell transplantation. The J Project continues to spread conceptually to countries and areas where PID patient care is negligible or missing like Uzbekistan (2018), North Cyprus (2019), Far East Russia (2019), Kyrgyzstan (2020), and Tajikistan (2020). In addition to improved clinical PID care, more and more clinical research papers focusing on PID are published in national and international journals (8).

THE AIM OF THIS RESEARCH

The main aim of this Research Topic was to expose the successful efforts of single immunological centers and countries as well as the effects of scientific collaboration within J Project groups and ECE region, and Western Europe and/or the US in the field of primary immunodeficiencies. Our colleagues from ECE countries were invited by the Editors of Frontiers in Immunology to submit original research articles, commentary, opinion and reviews resulting from mentioned collaboration and their own experience covering the molecular defects of PIDs, diagnostics achievements, clinical characteristics of different PIDs, region-specific PIDs, current treatment of different PIDs with immunoglobulin replacement therapy (IgRT), hematopoietic stem cell transplantation (HSCT), biological treatment in autoinflammatory diseases as well as collaboration within J Project Group. After rigorous reviews, 11 articles from ECE reflecting new diagnostic tools and their influence on recognition of IEI, country-related registries, analysis of clinical course were selected for publications.

THE SUBJECT OF PAPERS PUBLISHED IN THIS ISSUE

Two papers about TREC and KREC implementation for severe combined immunodeficiency (SCID) and other severe PIDs were included. The first one assessed accuracy of TREC and KREC pilot study in children aged 0-18 years old with suspicion of primary immunodeficiency, indicating its role beyond newborn screening programs. The next one reported the first 14 months of transborder collaboration in the field of newborn screening pilot study. As proved earlier newborn screening for SCID and other severe IEI let introduce proper treatment procedures such as HSCT and IgRT before the first symptoms and complication occurred. The analysis of detailed flow cytometry and evaluation of peripheral T lymphocytes maturation in one of the papers revealed existence of senescent and exhausted T cell population in Nijmegen Breakage Syndrome (NBS) patients. The observed significant aberration in peripheral T cell maturation in NBS individuals makes probable hypothesis of its role in increased susceptibility to malignancies, which, however needs further investigations. Selected papers described genetic causes of different inborn errors of immunity. The joint work of several authors from ECE proposed *RAG1* p.K86Vfs*33 as a founder variant originating from the Vistula watershed and present in all Slavs. Novel mutations were found respectively in: *CDC42* in patient with syndromic immunodeficiency, autoinflammation, hemophagocytic lymphohistiocytosis and malignancy and *STAT1* GOF in a mother and child with recurrent, severe aphthous stomatitis and mucosal ulcers. Differentially methylated *PIK3AP1* and *SPON2* were observed in patients with Periodic Fever, Aphthous Stomatitis, Pharyngitis, and Adenitis (PFAPA), indicating a potential role in PFAPA etiology, but requiring further investigations. The increased awareness of PID led to better recognition, including shorter delay in adults with common variable immunodeficiency as well as better understanding of PID- related interstitial lung diseases in children and national registry, as shown in the collection of Research Topic.

CONCLUSION

It needs to be emphasized that these papers document a substantial improvement of PID awareness and research in the ECE, especially over the past decade. The Editors hope that this Special Issue of Frontiers in Immunology helps readers to learn more about the remarkable development in the ECE region on PID-related specific diseases, their molecular background, novel mutations in different phenotypes. It should also stimulate further research and cooperation within the J Project in ECE countries and elsewhere in this rapidly developing field of molecular medicine. Finally we are grateful to the Editors of Frontiers in Immunology for their invitation to put together this Research Topic.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Expanding TREC and KREC Utility in Primary Immunodeficiency Diseases Diagnosis

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Primary immunodeficiency diseases (PID) area heterogeneous group of disorders caused by genetic defects of the immune system, which manifest clinically as recurrent infections, autoimmune diseases or malignancies. Early detection of PID remains a challenge, particularly in older children with milder and less specific symptoms. This study aimed to assess TREC and KREC diagnostic ability in PID. Data from children assessed by clinical immunologists at Speransky Children's Hospital, Moscow, Russia with suspected immunodeficiencies were analyzed between May 2013 and August 2016. Peripheral blood samples were sent for TREC/KREC, flow cytometry (CD3, CD4, CD8 and CD19), IgA and IgG analysis. A total of 434 children [189 healthy, 97 with group I and II PID (combined T and B cell immunodeficiencies & well-defined syndromes with immunodeficiency) and 148 group III PID (predominantly antibody deficiencies)] were included. Area under the curve (AUC) for TREC in PID groups I and II diagnosis reached 0.82 (CI = 0.75–0.90), with best model providing sensitivity of 65% and specificity of 92%. Neither TREC, nor KREC had added value in PID group III diagnosis. In this study, the predictive value of TREC and KREC in PID diagnosis was examined. We found that the TREC had some diagnostic utility for groups I and II PID. Possibly, addition of TREC measurements to existing clinical diagnostic algorithms may improve their predictive value. Further investigations on a larger cohort are needed to evaluate TREC/KREC abilities to be used as diagnostic tools on a wider scale.

Keywords: TREC, KREC, primary immunodeficiency diseases, PID, primary immunodeficiency diseases diagnosis

HIGHLIGHTS

- ***What is already known about this topic?***
  Assessment of TREC levels is actively used in the screening of severe combined immunodeficiency disorders (SCID).
- ***What does this article add to our knowledge?***
  This study shows that TREC may have a place not just in SCID screening but in the diagnosis of PID.
- ***How does this study impact current management guidelines?***
  Evidence suggests that TREC may be a good addition to already existing diagnostic methods in groups I and II PID diagnosis. It may be particularly useful in less affluent environments with lack of access to flow cytometry.

INTRODUCTION

Primary immunodeficiency diseases (PID) are a heterogeneous group of disorders caused by genetic defects of the immune system, which manifest clinically as recurrent infections, autoimmune diseases or malignancies. The most severe forms of PID, severe combined immune deficiency (SCID) is intensively studied and has been found to be associated with fatal consequences in the first 2 years of life (1, 2).

Most forms of SCID can be detected by measuring the levels of T-cell recombination excision circles (TREC) in dried blood spots using real-time polymerase chain reaction (PCR) (3), while kappa-deleting recombination excision circles (KREC) are used to screen for agammaglobulinemia (4). TREC is a by-product of the T-cell receptor gene recombination, and KREC is a by-product of the B-cell receptor recombination. Low levels of these molecules in T- and B-cells in peripheral blood were shown to be associated with T- and/or B-cell lymphopenia (4). The best possible outcome for patients with SCID can be achieved by timely hematopoietic stem cell transplantation or gene therapy before the development of infectious complications (5, 6), while early diagnosis is associated with a significant increase in treatment effectiveness (5). In patients with agammaglobunemia, the best outcome is achieved by initiating replacement therapy using intravenous or subcutaneous immunoglobulins.

PID influence child health-related quality of life, limiting physical, emotional, social and school functioning (7). Therefore, early detection of not only SCID but all PID patients is vital to improve the chances of appropriate management, in order to significantly reduce potential complications and improve life quality (6, 8).

At present, early detection of PID remains a challenge. This is particularly true in older children and in adults, potentially due to milder and less specific symptoms, a low level of awareness of PID amongst clinicians, as well as unavailability of necessary diagnostic devices such as flow cytometry in hospital laboratories (9, 10). In some countries flow cytometry is not readily available and TREC/KREC analysis may represent a feasible alternative. It is therefore imperative to not only allow diagnosis of patients in low-resource facilities but also develop more cost-effective alternatives. Many studies showed that dried blood spots are robust (11) and useful as a potential alternative sample source for clinical purposes, epidemiological studies, and biobanking (12).

Flow cytometry is a more commonly used but more expensive diagnostic technique for PID detection, when compared with PCR (13). It requires a significant amount of training in highly specialized tertiary centers and therefore cannot be used as a screening tool.

Potential applications of TREC/KREC analysis were highlighted in the reviews by van Zelm and co-authors (14, 15). These included support therapy monitoring, patient classification and newborn screening for PID. Apart from apparent clinical benefits, assessment of B- and T- cell neogenesis in PID patients following stem cell transplantation (16) and KREC assessment in patients presenting with abnormalities in B-cell subsets to explain B-cell compartment aberrancies (17) may improve current state of knowledge.

This pilot study aims to assess diagnostic accuracy of TREC and KREC in children from 0 to 18 years of age with suspected PID.

METHODS

Study Setting, Eligibility Criteria, and Ethics

In this prospective study, we recruited all children referred by primary care physicians (polyclinic pediatricians) to a tertiary level center (Moscow City Pediatric Hospital #9 named after Speransky, Moscow, Russia) with suspected immunodeficiencies and assessed by board-certified clinical immunologists between May 2013 and August 2016. The investigations and sample collection were conducted following ethical approval by the Speransky Children's Hospital Ethics Committee. Parental written consent was obtained for all participants as part of routine procedure at Speransky Children's Hospital. Parents/guardians were informed of the procedures in lay terms. The study design has been described in detail elsewhere (18).

Outcome Definition

The primary outcome of interest in this study was PID. We considered that a child has a PID if he or she had PID diagnosed by a physician. The diagnosis of different groups of PID was based on IUIS Phenotypic Classification for Primary Immunodeficiencies (19): group I was defined as immunodeficiencies affecting cellular and humoral immunity; group II corresponded to combined immunodeficiencies with associated or syndromic features, group III was defined as predominantly antibody deficiencies.

Sample Analysis

Peripheral blood samples were taken by venipuncture during morning hours, aliquoted and sent for complete blood count, flow cytometry, immunoglobulin (IgA, IgG), and TREC/KREC

Abbreviations: AUC, Area under receiver operating characteristic curve; CD, cluster of differentiation; CVID, Common Variable Immunodeficiency; DNA, Deoxyribonucleic acid; ICD-10, International Classification of Diseases, 10th Revision; Ig, immunoglobulin; IUIS, International Union of Immunological Societies; KREC, kappa-deleting element recombination circle; PCR, polymerase chain reaction; PID, Primary immunodeficiency diseases; ROC, Receiver Operating Curve; SCID, Severe Combined Immune Deficiency; TREC, T-cell recombination excision circles.

analysis. All blood samples were EDTA-anticoagulated and analyzed on the day of collection to avoid cellular death. Immunoglobulin levels were measured in blood serum.

Sample analysis was performed as described elsewhere (18). In brief, three-four color flow cytometric immunophenotyping with directly labeled monoclonal antibodies was used to determine the following immune cell subsets: CD3-CD19+, CD3-CD(16 + 56)+, CD3+CD4+, and CD3+CD8+ following manufacturer's protocol. Further analysis was performed with a FACS Canto II flow cytometer using FACSDiva v7.0 software (Becton Dickinson). The total leucocyte count and differential was measured with Advia 2120i hematology analyzer (Siemens). The absolute size of each lymphocyte subpopulation was calculated by multiplying the relative size of the lymphocyte subpopulation by the absolute lymphocyte count. Immunoglobulin levels were assessed using a biochemical analyzer Architect C8000 (Abbott, USA, Abbott kits) in accordance with manufacturers' protocol. TREC and KREC assays were performed using real-time PCR with fluorescent hybridization probes and reagents for TREC/KREC assays: T&B PCR kit (ABV-test, Russia) (20), in whole blood. The TREC/KREC levels were assayed in whole blood samples as described previously (16, 18, 20), In brief, DNA was extracted from 100 μl EDTA anticoagulated whole blood by using RIBO-prep nucleic acid extraction kit (Amplisense®, Russia). The Real-time qPCR was performed using CFX 96 Real-Time PCR System (Bio Rad, USA). Amplification of ALB was used to assess correct sampling and quality of DNA extraction, as well as to determine TREC and KREC levels. The number of TREC/KREC copies was calculated per 10^5 white blood cells, accounting for the quantity of ALB using the formula: [The number of TREC/KREC copies/the number of ALB copies] × 200,000. The normal/cutoff levels of TRECs and KRECs of 1,000 copies/10^5 cells were used.

Statistical Analysis

Shapiro-Wilk test was used to assess whether analyzed variables were normally distributed. Since the null hypothesis was not rejected, Spearmen correlation coefficient was used to assess the strength of the correlation between the variables. Sensitivity, specificity and their 95% confidence intervals were computed with stratified bootstrap replicates (21). Area under Receiver Operating Characteristic (ROC)—curve (AUC) calculation was followed by 95% confidence interval as suggested by DeLong et al. (22). The diagnostic accuracy measures used were: sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). TREC/KREC accuracy in diagnosing group I/II and group III PIDs were assessed. The ROC-analysis was performed separately for PID groups I/II and

TABLE 1 | Characteristics of study participants.

PID Group	Total number of patients	Clinical diagnosis (number of patients)	Age	Gender	
				Male	Female
Group I ("Combined PID") Immunodeficiencies affecting cellular and humoral immunity	17	D81 Combined immunodeficiencies (17)	0–12 months 1–6 years 6–12 years 12–18 years	9 2 1 0	5 0 0 0
Group II ("Syndromic PID") CID with associated or syndromic features	80	D82 Immunodeficiency associated with other major defects (13) D82.1 Di George syndrome (39) D82.4 Hyperimmunoglobulin E syndrome (5) D84.8 Other specified immunodeficiencies (15) G11.3 (8)	0–12 months 1–6 years 6–12 years 12–18 years	7 26 10 4	9 17 6 3
Group III ("Antibody PID") Predominantly antibody deficiencies	148	D80.0 Immunodeficiency with predominantly antibody defects (4) D80.1 Non-familial hypogammaglobulinaemia (47) D80.2 Selective deficiency of immunoglobulin A (34) D80.3 Selective deficiency of immunoglobulin G (24) D80.4 Selective deficiency of immunoglobulin M (1) D80.5 Immunodeficiency with increased immunoglobulin M (4) D83 Common variable immunodeficiency (34)	0–12 months 1–6 years 6–12 years 12–18 years	6 21 28 38	2 17 21 13
Control group (Healthy children)	226	No clinical diagnosis of PID	0–12 months 1–6 years 6–12 years 12–18 years	9 41 36 25	5 48 33 29

All codes and diagnoses are in accordance with international classification of diseases, 10th revision (ICD-10).

PID group III. The performance characteristics of all lymphocyte subpopulations as well as combinations of TREC and KREC were evaluated and compared in terms of (a) the sensitivity (proportion detected of those with PID) at a fixed specificity (proportion of controls correctly detected not to have PID) and (b) AUC.

Due to TREC levels decreasing with age, every TREC measurement was divided by the corresponding TREC reference interval for the patient's age group prior to analysis.

Results were considered statistically significant if p-value was smaller than 0.05. All calculations were done using R package version 3.4.1.

RESULTS

Study Population

The data was extracted from clinical notes and the laboratory database of Speransky Children's Hospital. Out of 3,055 patients requiring flow cytometry within the given period of time, due to financial restrictions (those eligible for flow cytometry to be covered by compulsory health insurance in accordance to local regulations. Regulations did not change throughout the study period.), a total of 839 samples were analyzed using flow cytometry and TREC assay and 931 samples were analyzed using flow cytometry and KREC assay. Data on confirmed clinical diagnosis was available from 471 participants. All data points required for TREC/KREC diagnostic properties assessment were available from 434 children and were included in the statistical analysis. Out of 434 children with a doctor's confirmed diagnosis, 189 were immunologically healthy, 97 were group I and II PID patients and 148 were group III PID patients. The following conditions were diagnosed in each subcategory in accordance to International Classification of Diseases, 10th revision (ICD-10) and classified following IUIS Phenotypic Classification for Primary Immunodeficiencies (19): group I (combined immunodeficiencies), group II (immunodeficiency associated with other major defects, ataxia telangiectasia and other specified immunodeficiencies), group III (immunodeficiency

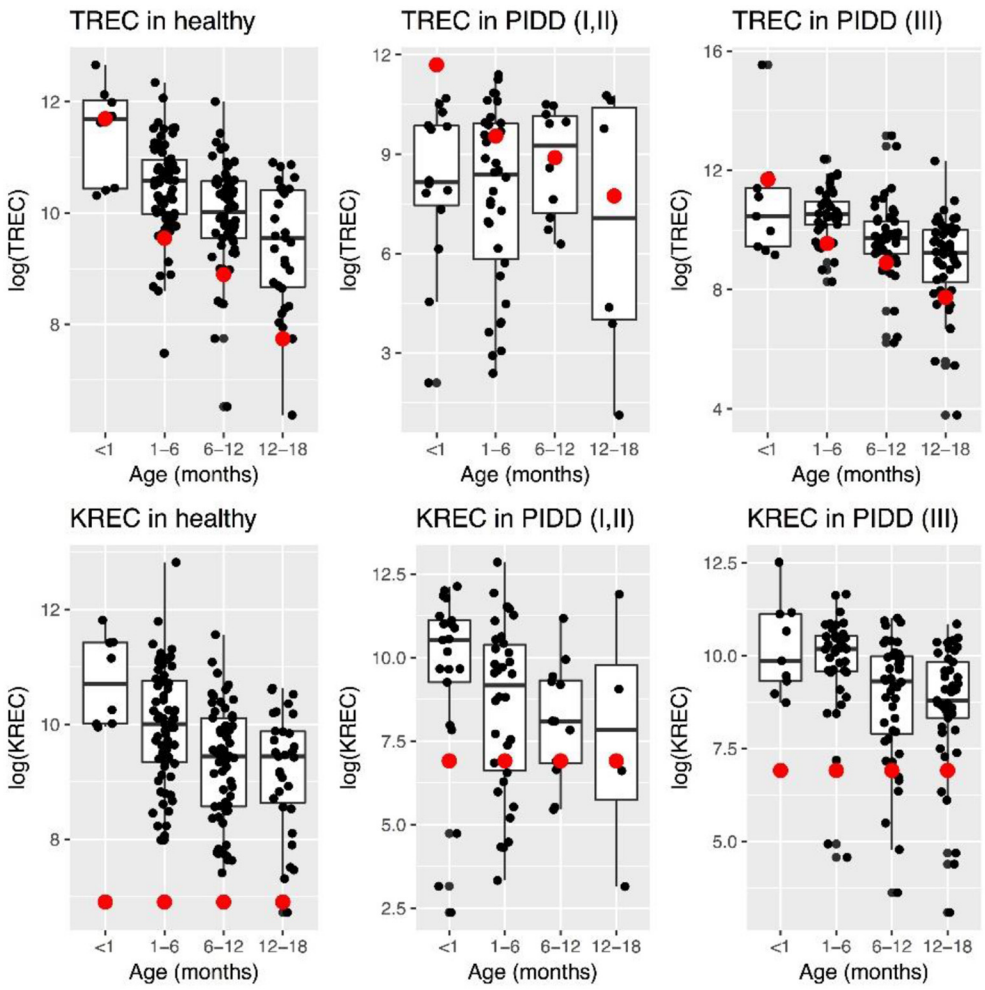

FIGURE 1 | Patterns of change in TREC and KREC log-transformed (natural logarithm) levels in healthy individuals and PID patients at different age. Red dot represents minimal normal level for a given age group.

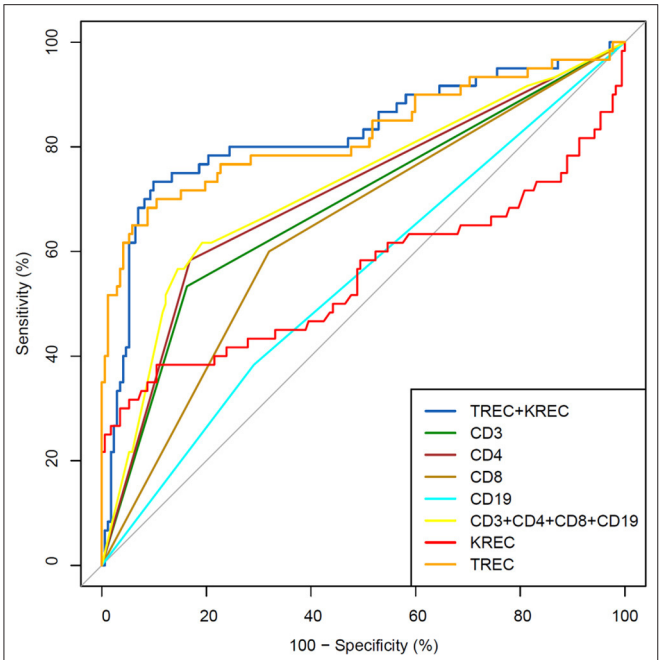

FIGURE 2 | Receiver operating characteristic (ROC) curves for each of lymphocyte subpopulations (CD3, CD4, CD8, and CD19) individually; combined diagnostic ability of all lymphocyte subpopulations and diagnostic ability of TREC and KREC combination in "Combined PID" and "Syndromic PID" diagnosis. Healthy individuals ($n = 172$); children diagnosed with "Combined PID" and "Syndromic PID" ($n = 60$). AUC for TREC and a combination of TREC and KREC = 0.82 (95% CI = 0.75–0.90).

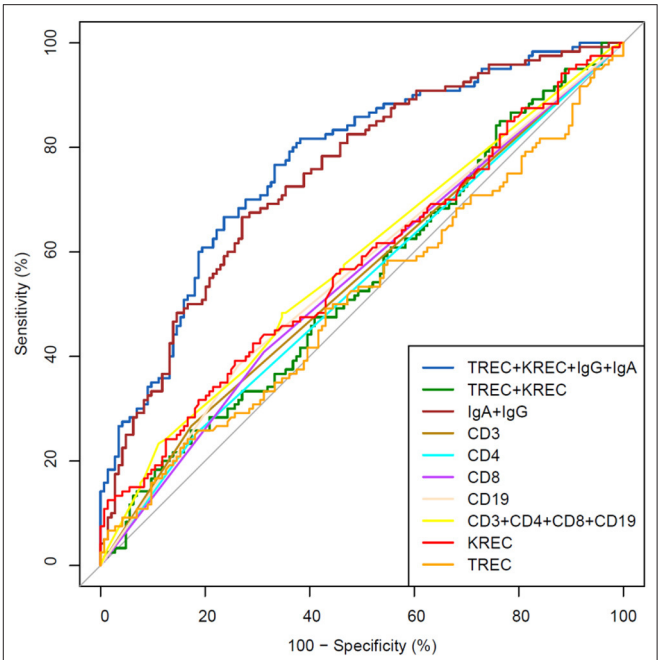

FIGURE 3 | Receiver operating characteristic (ROC) curves for each of lymphocyte subpopulations (CD3, CD4, CD8, and CD19) individually; combined diagnostic ability of all lymphocyte subpopulations; IgA and IgG combined; TREC and KREC combined and diagnostic ability of TREC, KREC, IgA, and IgG combination in "Antibody PID" diagnosis. Healthy individuals ($n = 144$); children diagnosed with "Antibody PID" ($n = 120$). IgA, IgG, TREC, and KREC AUC = 0.77 (95% CI = 0.71–0.82).

with predominantly antibody defects and common variable immunodeficiency). For the sake of the readers' convenience we will use the following terminology in the following sections of the manuscript: "Combined PID" for group I, "Syndromic PID" for group II and "Antibody PID" for group III. The diagnoses were reached using clinical signs and immune phenotype. Genetic testing was not available at the recruitment site.

All the samples were analyzed using flow cytometry, turbidimetry, and TREC/KREC assays and were included into the primary analysis of this study. Demographic data of the participants is presented in **Table 1**.

Descriptive Results of Flow Cytometry and TREC/KREC Testing

Levels of TREC decreased with age in healthy children, while this was less evident in "Combined PID" and "Syndromic PID." Overall TREC levels were lower in "Combined PID" and "Syndromic PID" compared to healthy individuals (**Figure 1**).

TREC/KREC Diagnostic Accuracy

The area under the curve for lymphocyte subpopulations (CD 3, 4, 8, and 19), immunoglobulins (IgA, IgG) and TREC/KREC levels were assessed. Separate analyses were undertaken for "Combined PID" and "Syndromic PID" (**Figure 2**) and "Antibody PID" (**Figure 3**). The same analysis was performed to assess ability of TREC/KREC to differentiate between "Combined PID," "Syndromic PID," and "Antibody PID."

TABLE 2 | Diagnostic accuracy measures for different cutoff points of the predicted probabilities for TREC in "Combined PID" and "Syndromic PID" diagnosis.

Cutoff point (probability)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	Youden index
0.05	28	92	97	13	9.5
0.1	29	90	93	21	14.2
0.15	32	90	90	32	22
0.2	33	89	85	41	25.7
0.25	37	88	78	54	31.8
0.3	46	90	78	67	45.7
0.35	59	89	72	83	54.3
0.4	**75**	**88**	**65**	**92**	**57.4**

Optimum cut-off point based on maximum value of the J index is presented in bold.

AUC for TREC and a combination of TREC and KREC for "Combined PID" and "Syndromic PID" diagnosis was 0.82 (95% CI = 0.75–0.90). As KREC did not add value to the model's predictive capacity, the data for TREC is presented (**Table 2**). The cutoff point of a probability of 0.4 showed the best diagnostic accuracy with regards to the sensitivity and specificity (65 and 92%), $J = 57.4$.

A combination of IgA, IgG, TREC and KREC demonstrated the best AUC 0.77 (95% CI = 0.71–0.82) for "Antibody PID" diagnosis but neither TREC or KREC nor combination of two

TABLE 3 | Diagnostic accuracy measures for different cutoff points of the predicted probabilities for a combination of TREC and KREC in "Antibody PID" diagnosis.

Cutoff point (probability)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	Youden index
0.35	46	75	98	4	2.5
0.4	46	64	93	11	3.6
0.45	47	56	61	42	2.5
0.5	**57**	**56**	**14**	**91**	**5.2**
0.55	42	54	4	95	0
0.6	50	55	2	99	0.3

Optimum cut-off point based on maximum value of the J index is presented in bold.

yielded good diagnostic ability (AUC 0.54, 95% CI = 0.47–0.61), **Table 3**. TREC and KREC combination did not demonstrate any added value to immunoglobulin level detection.

We then performed analysis to study ability of TREC/KREC to differentiate between "Combined PID," "Syndromic PID," and "Antibody PID." As KREC did not add value to the predictive model, the predictive model based on TREC levels is presented (**Figure 4**). The AUC for TREC in "Combined PID" and "Syndromic PID" diagnosis was 0.79 (95% CI = 0.70–0.87). A cutoff point of a probability of 0.4 showed the best diagnostic accuracy with regards to the sensitivity and specificity (66 and 84%; (**Table 4**), $J = 50.2$.

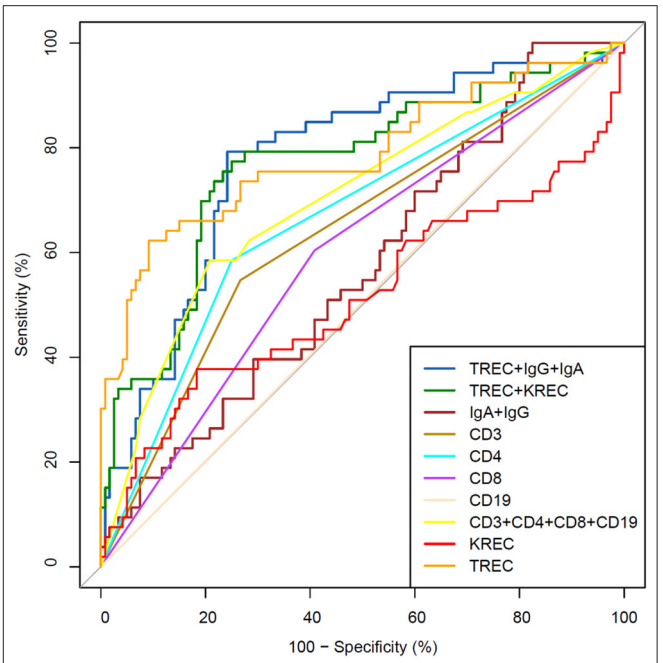

FIGURE 4 | Receiver operating characteristic (ROC) curves for each of lymphocyte subpopulations (CD3, CD4, CD8, and CD19) individually; combined diagnostic ability of all lymphocyte subpopulations; IgA and IgG combined; TREC and KREC combined and diagnostic ability of TREC, KREC, IgA, and IgG combination in differentiating between "Combined PID" and "Syndromic PID" and "Antibody PID." "Combined PID" and "Syndromic PID" individuals ($n = 53$); children diagnosed with "Antibody PID" ($n = 120$). TREC AUC = 0.79 (95% CI = 0.70–0.87).

TABLE 4 | Differential diagnosis for "combined PID" and "syndromic PID" and "antibody PID." Accuracy measures for different cutoff points of the predicted probabilities for TREC.

Cutoff point (probability)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	Youden index
0.05	31	80	96	7	2.9
0.1	33	89	96	13	9.5
0.15	34	88	94	18	12.6
0.2	36	89	93	28	20
0.25	38	88	89	35	23.7
0.3	40	84	79	47	25.9
0.35	51	86	76	68	43.8
0.4	**65**	**85**	**66**	**84**	**50.2**

Optimum cut-off point based on maximum value of the J index is presented in bold.

DISCUSSION

In this study, we tested the accuracy of TREC and KREC in PID diagnosis. The models showed decent utility of TREC in the diagnosis of "Combined PID" and "Syndromic PID." To our knowledge, this is the first attempt to use TREC/KREC not in SCID screening but in PID diagnostics.

PID is a large group of disorders encompassing more than 400 conditions affecting development and/or functioning of the immune system (23). Flow cytometry is a sensitive and important tool in evaluating the immune system function and in PID diagnosis (24). However, it is expensive, not easily available in developing countries and requires appropriate training and equipment. TREC and KREC may represent cheaper alternatives and/or add value to PID diagnosis and screening. Low cost methodology can be used in small laboratories and rural settings, where complex and expensive tools are unavailable, to provide access to primary PID evaluation.

Prior studies have noted the need in screening tool for early SCID diagnosis, to reduce the risk of infections and organ damage (6, 25). Early diagnosis is particularly important as lack of early treatment is associated with severe complications and increased mortality rates (26). TREC is a common screening approach used for early SCID detection around the globe, providing a good combination of sensitivity and specificity (27, 28) with high cost-effectiveness (29). KREC's role in early screening is still debatable but some data suggest that it may add value in certain cases (30). While TREC's indispensability in SCID screening is obvious, very little can be found in the literature on the question of TREC/KREC use in other PID diagnosis or their use as a screening tool (31–33). Previous attempts to this end have predominantly focused on Common Variable Immunodeficiency (CVID).

We hypothesized, that TREC and KREC may be adopted as a surrogate method of PID diagnosis. Our models demonstrated good AUCs indicating the potential of TREC to be used as an additional tool in PID diagnosis. Our data show that when TREC is used for differentiation between "Combined PID" and "Syndromic PID" patients and healthy individuals, the cut-off point probability of 0.4 provides high specificity (92%) with

acceptable sensitivity (65%), further supporting our hypothesis that use of TREC may have its place in PID diagnosis. TREC may serve as an addition to existing tests and may be used as a prerequisite to flow cytometry. We did not use any clinical questionnaires in this study but expect that laboratory findings combined with additional clinical data using standardized instruments may facilitate development of a stronger diagnostic model, improving utility.

TREC and KREC whether individually or combined, did not show good diagnostic ability in diagnosing "Antibody PID." However, this result was expected, with immunoglobulin measurement having the primary role in aiding the diagnosis. Our further analysis highlights potential for TREC level assessment in discrimination between "Combined PID," "Syndromic PID," and "Antibody PID." When a cut-off of 0.4-point probability was used, TREC reached specificity of 84%. At present, the wide variety and lack of specificity of PID clinical manifestations, do not allow physicians to determine the exact area of immune defect during initial clinical examination. Thus, the extensiveness of the primary laboratory examination in a patient with suspected PID is often determined by subjective clinical criteria, based on physician expertise. Laboratory tests normally used vary from immunoglobulin level assessment to assessment of a wide range of lymphocyte subpopulations. There are no predefined universal guidelines on how detailed the laboratory analysis should be, while selection of diagnostic tests depends on the given clinical settings and clinical immunologist. Normally, if initial tests reveal deviations in humoral immunity the next logical step is cellular immunity assessment to exclude combined immunodeficiency and diagnose "Antibody PID." Our data suggests that TREC appears to be a useful additional tool to aid in the differentiation between combined immunodeficiency and antibody deficiencies, particularly in the settings with limited access to flow cytometry. The qualitative method of TREC/KREC assessment is easy and can be applied equally well to both whole blood and Guthrie cards, as DNA extraction can be performed on either of these samples.

A few diagnostic algorithms/approaches to children with suspected PID were proposed. Among them excellent algorithm from Dutch immunologist Esther DeVries', based on a pattern recognition approach and decision trees (34), and the Jeffrey Modell Foundation's 4 steps (35). We do not propose changes to existing clinical approaches in routine PID diagnosis and fully acknowledge that TREC/KREC should not be used as a replacement of flow cytometry. Addition of TREC/KREC measurements to aforementioned clinical approaches, nevertheless, may improve predictive capacity of the tools, which is worth further investigation.

The main limitation of this study is related to the use of ICD-10 classification for PID diagnosis. The same ICD-10 code may sometimes include heterogenous group of immunodeficiencies. Genetic testing would be a preferable option; however, this was not available for most of the patients due to economical restrictions. Another limitation is the lack of Guthrie card use in our study with all samples analyzed using whole blood, which does not allow for a result extrapolation. No difference is expected, however, between DNA extraction from the whole blood sample and dried blood spot. Recruitment of patients of three PID groups only, may also be considered a limitation, even though it is unlikely to have influenced the outcomes of this study. We acknowledge that participants defined as "immunologically healthy" in this study can be considered as "healthy" to a high degree of certainty, but no information on naive cells and memory cells were collected. It is clear, however, that "immunologically healthy" participants in this study do not belong to Group I ("Combined PID"). Our study would also have benefited from assessment of TREC/KREC diagnostic accuracy in patients with a particular subtype of PID [e.g., X-linked agammaglobulinemia (XLA)], but it was not possible due to restricted number of patients. This should be addressed in future research.

In conclusion, we found evidence that TREC may have a place in aiding PID diagnosis. The models showed decent diagnostic accuracy measures for TREC in diagnosing "Combined PID" and "Syndromic PID". Further investigations in a larger cohort in combination with addition of genetic diagnoses and/or questionnaires focused on clinical symptoms are needed to improve diagnostic performance and to further evaluate TREC potential on a wider scale. It is too premature to draw definitive conclusions, but with a few diagnostic algorithms available (e.g., Esther DeVries' and Jeffrey Modell Foundation's 4 steps), addition of TREC to such algorithms may allow for an improved predictive ability. We would like to stress that TREC-based PID diagnosis may be particularly important in the recourse-limited countries and further research will benefit children in these settings.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Speransky Children's Hospital Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

IK, MF, AP, AK, and DM conceived and designed the experiments and study analysis. MG and ND performed the experiments. IK, NZ, SZ, and AS collected, extracted, and sorted the data. OB, RM, and AZ analyzed the data. AE reviewed additional available evidence on the matter. IK, PH, and DM wrote the manuscript.

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Conflict of Interest: MG, MF, and IK are board members for ABV-test. MG, MF, AP, and IK has a patent with ABV-test. DM has given paid lectures for Merck Sharp & Dohme (MSD) and Bayer. DM also is a member of ILSI Europe: Immune Competence Across Lifespan: Impact of Nutrition on Immune Competence and its Consequences Later in Life expert group.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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A Novel *CDC42* Mutation in an 11-Year Old Child Manifesting as Syndromic Immunodeficiency, Autoinflammation, Hemophagocytic Lymphohistiocytosis, and Malignancy: A Case Report

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Background: The *CDC42* (*Cell Division Cycle 42*) gene product, CDC42, is a member of the family of small Rho GTPases, which are implicated in a broad spectrum of physiological functions in cell cycle regulation, including establishing and controlling of the cell actin cytoskeleton, vesicle trafficking, cell polarity, proliferation, motility and migration, transcription activation, reactive oxygen species production, and tumorigenesis. The *CDC42* gene mutations are associated with distinct clinical phenotypes characterized by neurodevelopmental, growth, hematological, and immunological disturbances.

Case presentation: We report the case of an 11-year-old boy with syndromic features, immunodeficiency, and autoinflammation who developed hemophagocytic lymphohistiocytosis and malignant lymphoproliferation. In this patient, a novel heterozygous p.Cys81Tyr mutation in the *CDC42* gene was found by whole exome sequencing.

Conclusions: The Cdc42 molecule plays a pivotal role in cell cycle regulation and a wide array of tissue-specific functions, and its deregulation may result in a broad spectrum of molecular and cellular dysfunctions, making patients with *CDC42* gene mutations susceptible to infections, immune dysregulation, and malignancy. In the patient studied, a syndromic phenotype with facial dysmorphism, neurodevelopmental delay, immunodeficiency, autoinflammation, and hemophagocytic lymphohistiocytosis shares common features with Takenouchi–Kosaki syndrome and with C-terminal variants in *CDC42.* It is important to emphasize that Hodgkin's lymphoma is described for the first time in the medical literature in a pediatric patient with the novel p.Cys81Tyr mutation in the *CDC42* gene. Further studies are required to delineate precisely the *CDC42* genotype–phenotype correlations.

Keywords: Cdc42, immunodeficiency, hemophagocitic lymphohistiocytosis, malignancy, gene mutation

BACKGROUND

The *CDC42* (*Cell Division Cycle 42*) gene product, CDC42, is a member of the family of Rho GTPases (small G proteins of the Rho-family), which belongs to the Ras superfamily of small GTPases. The Rho-family GTPases have a broad spectrum of physiological functions in cell cycle regulation, including establishing and controlling of the cell actin cytoskeleton, vesicle trafficking, cell polarity, cell proliferation, motility and migration, transcription activation, reactive oxygen species production, and tumorigenesis (1). The signaling and the regulatory function of CDC42 are based on its tightly regulated cycle of activation with GTP binding and an inactive state with GTP hydrolysis, and intricate interactions with multiple proteins that impact on cell functions during its active state (2). It has been shown that CDC42 plays a number of physiologically pivotal, tissue-specific roles in the cardiovascular, genitourinary, respiratory, nervous, and immune systems, and its dysfunction is implicated as a background for syndromic immunodeficiency and immune dysregulation in patients reported so far (3–9). This is the first report of a pediatric patient with syndromic immunodeficiency, autoinflammation, hemophagocytic lymphohistiocytosis, and malignant lymphoproliferation in whom a novel, heterozygous p.Cys81Tyr mutation in the *CDC42* gene was found.

CASE PRESENTATION

We report the case of a 9-year-old boy who was referred to the pneumonology, allergology, and clinical immunology unit of the Poznan Pediatric University Hospital because of pneumonia, bilateral otitis media, and vesicular dermatitis.

Since the age of 2 years, he suffered from recurrent respiratory tract infections and required multiple hospitalizations because of recurrent bronchitis and pneumonia, maxillary sinusitis, otitis media, purulent dermatitis with *Pseudomonas aeruginosa* and *Staphylococcus aureus* infection, and severe varicella complicated by pneumonia, sinusitis, and gastrointestinal infection. He completed a full course of vaccinations, including BCG (Bacille Calmette–Guerin) and MMR (measles–mumps–rubella) vaccines without adverse effects following immunization (AEFI). The family history was complicated by multiple sclerosis in the patient's father.

He presented with neurodevelopmental delay and dysmorphic features with oblique palpebral fissures and eyebrows, retrognathia, low set small auricles with thick helices, and clinodactyly of the V fingers. The erythematous papulovesicular rash was present on the skin of the face, in the perioral region, and in the retroauricular area. In the nasopharynx and in the oral cavity, inflammatory lesions were observed. The most striking symptom was lymphadenopathy with numerous bilaterally enlarged cervical and submandibular lymph nodes. During hospitalization, he required antibiotic therapy, bilateral paracenthesis with tympanostomy, and drainage of maxillary sinuses. Laboratory evaluation revealed an antibody production defect and a memory B cell deficiency. Therefore, replacement therapy with intravenous immunoglobulin (IVIg) was initiated, and further genetic testing was recommended. The patient received three IVIg transfusions in monthly intervals, but afterward the parents decided to discontinue the therapy, and the boy was lost to follow-up.

At the age of 11 years, the boy was referred again to our clinic because of recurrent fevers, accompanied by vomiting, abdominal pain, cervical lymphadenopathy, and splenomegaly. The episodes of fever started 5 months before the hospitalization; they were not associated with any signs and symptoms of infection, they would reach 39.5 degrees, and did not respond to treatment with antibiotics. At that time, no other family members were ill, the boy had no contact with any toxic substances or infections, and he did not travel to the Mediterranean or exotic regions.

The laboratory tests showed pancytopenia, lymphopenia and neutropenia, high inflammatory markers, hypoalbuminemia, IgG and IgM hypoimmunoglobulinemia, hyperferritinemia, hypertriglyceridemia, hypertransaminasemia, and positive EBV-DNA (93,400 copies/ml) in the peripheral blood. Further laboratory findings comprised a markedly elevated (7,255 U/ml) serum concentration of the soluble interleukin 2 receptor (sIL-2R, sCD25) and a decreased intracellular expression of perforin (CD107a) on NK cells and increased on CD8+ T cells. Concomitantly, neither in the bone marrow nor in the lymph node were signs of hemophagocytosis found. Hence, seven of the eight diagnostic criteria (four clinical and three immunological criteria) of hemophagocytic lymphohistiocytosis (HLH) were fulfilled (10) (data displayed in **Table 1**).

With the concern of severe respiratory infection (**Figure 1**) and sepsis in an immunocompromised patient, initial empiric pharmacotherapy was based on broad spectrum antibiotics meropenem and vancomycin, *Pneumocystis jiroveci* prophylaxis with cotrimoxazole, and antiviral and antimycotic medications acyclovir and fluconazole. The chemo-immunotherapy for HLH was initiated with methylprednisolone pulse therapy, etoposide, and cyclosporine. The boy also required supplemental transfusions of albumins, immunoglobulins, prothrombin complex, and red blood cell preparations. The initial response to the therapy was satisfactory with an improvement in the patient's general state, a resolution of fevers, and a decrease in the serum inflammatory markers. Subsequently, however, the boy's state deteriorated, febrile episodes returned, and exacerbation of the supraclavicular and abdominal lymphadenopathy was observed (**Figure 2**). Based on complex diagnostic procedures including histopathology, immunology, and magnetic resonance imaging (MRI), the diagnosis of IV stage nodular sclerosis (NS) Hodgkin's lymphoma was established. The boy underwent successful chemotherapy based on the Euronet Pediatric Hodgkin's Lymphoma protocol (EuroNet-PHL-C1) including OEPA (vincristine, etoposide, prednisone, and doxorubicin) followed by 18-fluorodeoxyglucose-positron emission tomography-computed tomography (FDG-PET-CT) reevaluation. Further considerations and decisions regarding therapeutic options, including bone marrow transplantation, are ongoing.

GENETIC ANALYSIS

In the search for the genetic cause of HLH, syndromic immune deficiency, autoinflammation, and recurrent fevers, whole exome sequencing (WES) of DNA extracted from peripheral blood

TABLE 1 | Results of laboratory investigations in the patient studied aged 11 years.

Test	Results
Immunology	• Pancytopenia WBC 1.68 × 10^3, HGB 8.9 G/L, RBC 3,10 × 10^6, PLT 39 × 10^3 • Hypoimmunoglobulinemia IgG 479 mg/dl, IgM 11 mg/dl, IgA < 5 mg/dl • Peripheral blood lymphocyte immunophenotyping Lymphocytes CD45+/SSC low: 38% (1,125/mcl) low T CD3+ 81.0% (930 cc), low Th CD4+ 17.0% (195/mcl), high Tc CD8+ 59.0% (677/mcl) markedly decreased CD4+/CD8+ ratio 0.29, very low B cells CD19+ 6% (69/mcl) NK CD3–CD45+CD16+CD56+ 9.0% (103/mcl), activated CD3+HLA-DR+ 58% Low Th naïve CD4+CD45RA+ 10.0% (20/mcl), Th memory CD4+CD45RO+ 90.0% (176/mcl) low CD4+CD45RA+/CD4+CD45RO+ ratio 0.11 Low Recent thymic emigrants CD4+CD31+CD45RA+ 9.0% (18/mcl) Low Th Naïve CD4+CD27+CD45RO– 14.7% (29/mcl) Th Central memory CD4+CD27+CD45RO+ 72.8% high (142/mcl) low Low Th Effector memory CD4+CD27-CD45RO+ 10.6% (21/mcl) Low Th Terminally differentiated memory CD4+CD27-CD45RO– 1.8% (4/mcl) Th Regulatory CD4+CD127-CD25+ 6.7% (13/mcl) T follicular helper CD4+CD45RO+CD185+ 35.1% (62/mcl) Markedly decreased Tc Naïve CD8+CD27+CD197+ 10.2% (69/mcl) Tc Central memory CD8+CD27+CD45RO+ 32.9% (223/mcl) high Tc Effector memory CD8+CD27–CD197 × CD45RO+ 53.7% (364/mcl) CD107a decreased intracellular expression on NK cells, increased on CD8+ T cells
Microbiology	• CMV-DNA positive • EBV-DNA I. 12,900 copies/ml–II. 93,400 copies/ml • VZV, Enterovirus, Adenovirus, Parechovirus, HBV, HCV, HIV, hPVB19, HSV1, HSV2, HHV6, HHV7 RT-PCR in peripheral blood negative • Influenza virus A, AH1N1, Influenza virus B, Coronavirus NL63, 229E, OC43, HKU1, Parainfluenzavirus 1,2,3,4, Metapneumovirus A,B, Bocavirus, RSV A,B, Rhinovirus, Adenovirus, Enterovirus, Parechovirus, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Streptococcus pneumoniae*, *Staphylococcus aureus* RT-PCR in nasopharyngeal aspirate negative • *Yersinia enterocolitica* IgM, IgG, IgA negative • *Bartonella henselae* DNA negative • *Toxoplasma gondii* DNA negative • Galactomannan (Aspergillus antigen) negative • *Pneumocystis jiroveci* DNA negative • Quantiferon TB Gold negative • Blood, throat, urine cultures negative
Inflammatory markers	• CRP 22.52 mg/dl • PCT 3.90 ng/ml • Ferritin 3,649.4 ng/ml, TG 365.0 mg/dl, Fibrinogen 504 mg/dl • sIL-2R (sCD25) 7,255 U/ml
Neoplastic markers	• Beta-HCG <2.39 mIU/ml • AFP 1.5 ng/ml • LDH 124 IU/L
Histopathology	• Supraclavicular (Virchow's) lymph node: classical Hodgkin's lymphoma, nodular sclerosis • Immunohistochemistry: large atypical lymphoma cells positive for CD30, CD15, PAX-5, MUM.1, LMP/EBV, EBI-3, granzyme B, EMA; negative for ALK-1, CD43, CD3, CD20

was performed. For the enrichment SureSelectXT Human All Exon v7 (Agilent) was used and sequencing was performed on the Illumina Platform HiSeq 1500. Bioinformatics analysis was performed as previously described with the modification that the Hg38 version of the human genome reference sequence was used for alignment (11). The details of the WES results and variant filtering are given in the **Supplementary Material**.

We prioritized a heterozygous missense variant in the *CDC42* gene (NM_044472.3 c.242G>A, p.Cys81Tyr, Hg38:1:022086502-G>A, LRG_1326t2:c.[242G>A];[242=]). The variant has not been reported previously; it was not found in the GnomAD database (https://gnomad.broadinstitute.org) nor in an in-house database of >1,000 Polish exomes. The variant was predicted to be pathogenic by Mutation Taster, Mutation Assessor, FATHMM-MKL, FATHMM-XF, LRT, DEOGEN2, EIGEN, EIGEN PC, SIFT, SIFT4G, PROVEAN, MVP, REVEL, PrimateAI, MetaSVM, and MetaLR, whereas FATHMM suggested that it was "tolerated" (https://varsome.com). The CADD score (28,1) indicated pathogenicity. Notably, an alternative variant at the same amino acid position (chr1:22086502 G>T, Cys81Phe) has been classified as "Likely Pathogenic" in association with the Takenouchi–Kosaki syndrome by ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). The fragment of the *CDC42* gene encompassing the c.242G>A variant was PCR-amplified and analyzed with the method of deep amplicon sequencing (ADS) in the proband and his parents. The presence of the variant was confirmed in the proband and excluded in both parents, indicating that the variant occurred *de novo*.

According to the ACMG recommendations (12), the variant was classified as "Likely Pathogenic." This was based on the following criteria: the variant resulted from a *de novo* mutation in a patient with no family history (Pathogenic Strong, PS2);

the variant not found in GnomAD data (Pathogenic Moderate, PM2); an alternative variant at the same amino acid position classified as Likely Pathogenic by ClinVar (Pathogenic Moderate, PM5); multiple lines of computational evidence supporting a deleterious effect (Pathogenic Supporting, PP3).

DISCUSSION

The broad spectrum of heterogeneous clinical manifestations in disturbed CDC42 molecule regulation results from the wide variety of cellular pathways with CDC42 activity. In a mammalian model, it has been shown that CDC42 plays a fundamental role in cell biology and is implicated in a wide array of physiologically pivotal, tissue-specific activities, such as organogenesis, cellular specification, migration, and functional maturation in the cardiovascular system, the pancreas, the kidney, the lung, salivary and mammary glands, the central nervous system (2, 13), the skin (2), the bones (2), the sensory organ of the inner ear (1, 14), and the photoreceptors (15). An important clinicopathological issue is the role of CDC42 GTPase in hematopoiesis and in immune system homeostasis. CDC42 controls the multilineage development of blood progenitors and their egress from the bone marrow to the periphery, influences the tight balance between myelopoiesis and erythropoiesis (16, 17), and contributes to the immune system regulation by coordinating survival, proliferation, migration, receptor expression, activation signal transduction, and cytokine secretion in T (18–20) and B cells (21). Dysregulated CDC42-dependent actin dynamics may, therefore, impede multiple stages of B cell development and affinity maturation, resulting in an aberrant germinal center response and immunodeficiency, autoimmunity, and lymphoproliferation (22).

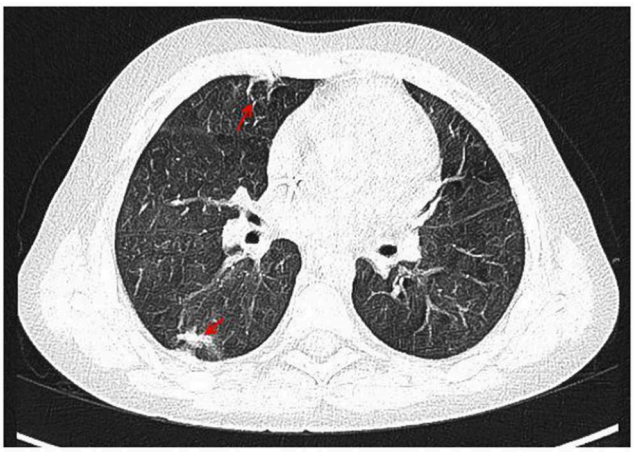

FIGURE 1 | CT of the chest with contrast medium, arterial phase, coronal view. Irregular, thickened peribronchium with narrowing of the lumen of segmental and subsegmental bronchi, fibrosis (marked with arrows) with compensatory dilation of the bronchial lumen (bronchiectasis), signs of bronchiolitis in PS10 and LS 10, irregular nodules in LS9 and LS10, a trace of fluid in the pericardium, enlarged right upper paratracheal, below carina, paraaortic, perivascular lymph nodes.

In the patient studied, in whom a novel, heterozygous *de novo* p.Cys81Tyr mutation in the *CDC42* gene was identified for the first time, a marked complexity of the clinical phenotype is observable, characterized by syndromic features, neurodevelopmental delay, immunodeficiency, autoinflammation, hemophagocytic lymphohistiocytosis, and malignant lymphoproliferation. The clinical phenotype of patients with a *CDC42* gene mutation and the triad of recognizable symptoms, such as macrothrombocytopenia, developmental delay, and distinctive facial features, has been initially described by Takenouchi and Kosaki in 2015 (3). It is worth noting that the initially reported patient with the Takenouchi–Kosaki syndrome and the p.Tyr64Cys mutation did not present with the diverseness of clinical manifestations

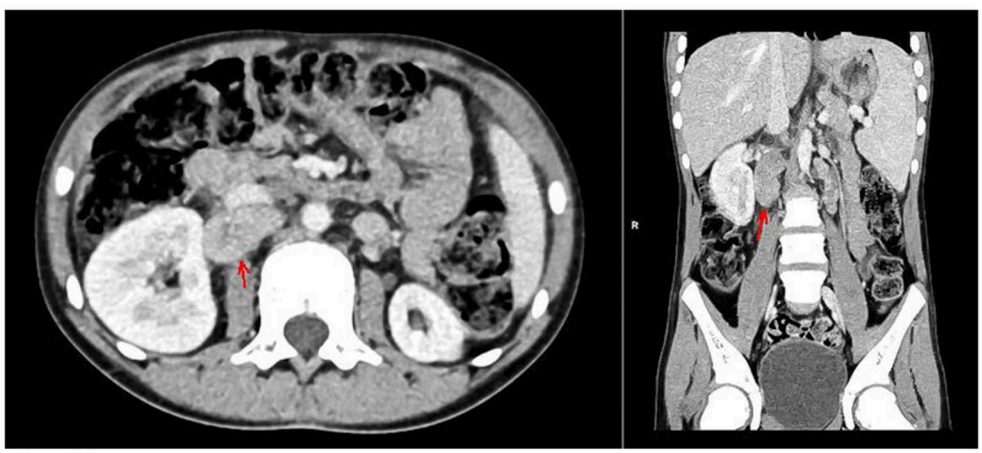

FIGURE 2 | CT of the abdomen with contrast medium, arterial phase, axial, and coronal views. Hypodense foci in the II and VIII segments of the liver, enlarged spleen, *ca.* 14 cm long with nonhomogenous attenuation, hypoplastic left kidney, lymphadenopathy of lymph nodes in the regions of the head of the pancreas, portal vein, right renal hilum, inferior vena cava (marked with arrows), and retroperitoneal, iliac and aortic lymph nodes, forming a mass 8.5 cm long.

observable in our patient with the p.Cys81Tyr variant since immunodeficiency, systemic autoinflammatory disease, HLH, and lymphoma were not present in the original case. Further evidence supporting the notion that the *CDC42* gene mutation causes a syndromic form of thrombocytopenia has also been provided by Takenouchi et al. (4) and Motokawa et al. (5) in their reports of the next two patients, sharing the same p.Tyr64Cys variant (6) and overlapping phenotypes including facial dysmorphism, psychomotor developmental delay, lymphedema of the lower extremities, camptodactyly, sensorineural hearing loss, and immunodeficiency. Further evidence for the intricate genotype–phenotype relationship and the heterogeneity of the clinical features correlating with mutations affecting the *CDC42* gene was provided by Martinelli et al. (7). The authors reported 15 patients, divided in three groups according to different disease-causing *CDC42* mutations, namely, group I—p.Tyr64Cys, p.Arg66Gly, p.Arg68Gln, group II—p.Cys81Phe, p.Ser83Pro, p.Ala159Val, and group III—p.Ile21Thr, p.Tyr23Cys, pGlu171Lys. The various mutations are related to an unusually broad spectrum of anomalies with predominant growth retardation and developmental disorders with an intellectual disability, while macrothrombocytopenia was noted foremostly in group I. In patients with the p.Cys81Phe variant, affecting the same amino acid as in the novel p.Cys81Tyr here reported, severe autoinflammatory disease, lymphohistiocytic hemophagocytosis, and lymphoproliferation were not present. In contrast to patients with the classical Takenouchi–Kosaki syndrome and similarly to our patient, systemic autoinflammatory disease and development of HLH were predominating manifestations in four patients with three distinct C-terminal *de novo* variants (p.C188Y, p.R186C, p.*192C*24) in *CDC42* reported by Gernez et al. (8). Likewise, four patients with disturbed hematopoiesis, rash, autoinflammation, and HLH, reported by Lam et al. (9), were sharing the same C-terminal p.R186C variant in the *CDC42* gene. The two latter studies point to the unique effect of the C-terminal mutations in *CDC42*, resulting in neonatal-onset deregulation of the inflammatory response and the development of secondary HLH. While our patient did not present with symptoms of a significant autoinflammatory disease and HLH in his early childhood, it may be assumed that HLH might be secondary in the setting of autoinflammation and malignant transformation.

Since CDC42 plays a pivotal role in a tight regulation of a plethora of complex cell functions, its deregulation resulting in impaired cell proliferation, migration, and transcription programming, was shown to be oncogenic. Overexpression of CDC42 was reported in several cancers, including non-small cell lung cancer, colorectal adenocarcinoma, melanoma, breast cancer, and testicular cancer, supporting the role of CDC42 in the promotion of tumorigenesis as an oncogene (23). The modulation of transcription factors, such as signal transducer and activator of transcription 3 (STAT3) and nuclear factor κB (NFκB), which, in turn, regulate cancer cell growth and survival, as well as alter cancer cell metabolism (24), is a further mechanism by which the activation of CDC42 contributes to malignant cell transformation. Numerous, somatic genetic alterations affecting CDC42 were described in human lymphomas (25). While the *CDC42* mutation may play a role in the genesis of the lymphoma, it may be assumed that hematopoietic stem cell transplantation would be a curative strategy for the patient. However, whether the development of Hodgkin's lymphoma in the reported patient may be mechanistically connected with the novel p.Cys81Tyr variant in the *CDC42* requires further investigations.

CONCLUDING REMARKS

We describe a novel heterozygous p.Cys81Tyr mutation in the *CDC42* gene, which encodes a small GTPase of the Rho subfamily, playing a pivotal role in cell cycle regulation. In the patient studied, diverse clinical manifestations compose a syndromic phenotype with facial dysmorphism, neurodevelopmental delay, immunodeficiency, autoinflammation, hemophagocytic lymphohistiocytosis, and malignant lymphoproliferation, sharing common features with the Takenouchi–Kosaki syndrome and patients with the C-terminal *CDC42* mutations. It is worth noting that Hodgkin's lymphoma is described here for the first time in a patient with a mutation in *CDC42*. It may be therefore assumed that different disease-causing *CDC42* mutations have a different impact on the GTPase structure, activity, and binding to effectors, and finally, on the heterogeneity of clinical manifestations in affected patients, leading to new challenges in the syndrome's recognition and delineation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the parent for the publication of this case report.

AUTHOR CONTRIBUTIONS

AS-P was responsible for the conception and design of the study, collection and interpretation of clinical data, and drafted the manuscript. RP was responsible for genetic analysis and interpretation of data and critically revised the manuscript. MP and EB participated in the clinical evaluation of the case and critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Facilitated Subcutaneous Immunoglobulin Replacement Therapy in Clinical Practice: A Two Center, Long-Term Retrospective Observation in Adults With Primary Immunodeficiencies

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Facilitated subcutaneous immunoglobulin (fSCIG) replacement therapy is the latest method of IgG administration; however, real-life data are limited. We retrospectively analyzed the everyday experience of fSCIG administration, particularly, the method used to switch from intravenous immunoglobulin (IVIG) or subcutaneous immunoglobulin (SCIG) to fSCIG and the dosing modifications required. Of the 39 adult patients with primary immunodeficiency (PID) who received fSCIG, 34 remained on the therapy at the end of the study. The median observation time was 18 (range, 3–24) months. Two patients were IgG-treatment-naïve; 23 had previously received IVIG and 14 had received SCIG. In 25 cases, a non-ramp-up dosing mode was used to switch to fSCIG (including two half-monthly doses given biweekly in 14 cases, and full monthly doses given in 11 cases), a ramp-up mode was used in six cases; other methods were used in eight cases. The median IgG trough level at baseline was 7.9 g/L ($n = 38$), 7.9 g/L ($n = 32$) at Month 6, 9.0 g/L ($n = 30$) at Month 12, 8.6 g/L ($n = 22$) at Month 18, and 9.0 g/L ($n = 11$) at Month 24. No serious bacterial infections or hospitalizations due to PID complications occurred. At the end of the study, 24 patients (71%) received fSCIG every 4 weeks, six (18%) received fSCIG every 3 weeks, and four (12%) received fSCIG biweekly. In conclusion, our study provides real-life evidence of clinical efficacy of personalized fSCIG treatment when switching from prior immunoglobulin replacement using various switching modes and dosing frequencies.

Keywords: common variable immunodeficiency, hypogammaglobulinemia, fSCIG, personalized approach, pregnancy, primary immunodeficiency, replacement immunoglobulin therapy, real-life data

INTRODUCTION

Immunoglobulin G (IgG) replacement therapy is the most essential pharmacological intervention in patients with humoral primary immunodeficiency (PID). The substitution of antibodies significantly reduces mortality as it prevents patients from many serious health conditions, most commonly, recurrent bacterial infections (1). Due to the primary character of the immunity defect, the treatment must be systematically conducted throughout ones' lifetime, either intravenously (IVIG) or subcutaneously (SCIG). Two methods of SCIG application are currently available, termed conventional (either using an infusion pump or without, termed rapid-push SCIG) and facilitated (fSCIG), which is aided by the initial administration of human recombinant hyaluronidase in the same needle as IgG.

Home SCIG is safe, clinically-effective, cost-effective, and often preferred by patients and medical staff (2, 3). However, conventional SCIG can be burdensome due to the high frequency of infusions required (i.e., weekly dosing) (4). Weekly dosing is required in conventional SCIG, as only a limited volume of IgG can be infused into the subcutaneous tissue. Meanwhile, the fSCIG method allows a larger volume of IgG to be administered, and therefore, only requires dosing every 4 weeks, analogous to IVIG (5). This may reduce the burden of SCIG treatment, as well as improve patients' quality of life and their adherence to treatment. Therefore, the fSCIG method can fulfill patients' expectations; i.e., it involves home-based 4-weekly infusions that can be self-administrated with shorter administration and fewer needle sticks (4).

The extensions of pivotal clinical studies have shown fSCIG is an effective and safe option both for adults and children with PID (6, 7). However, data from real-life experience, especially regarding practical aspects of switching patients to fSCIG, remain limited. Indeed, most data has been derived from case reports (8–12), with only one single-site, real-life study published in 2014 (13). Unfortunately, this real-life study was limited to only 14 patients, with a short follow-up (8 months), and a fixed dosing schedule (every 3 weeks) (13). Moreover, in terms of switching from other types of IgG replacement to fSCIG, available data is limited to the ramp-up dosing mode.

In this long-term, retrospective, open observational study, we aimed to report the everyday experience of fSCIG treatment in adult patients with PID. In particular, we examined the reasons for switching, the mode of switching, and the modification of dosing (according to patients' expectations of the planned 4-weekly application and the shared-decision making model) in a long-term follow-up in routine clinical practice.

MATERIALS AND METHODS

This was a retrospective analysis of routinely-collected, real-life data obtained from the medical documentation of PID patients receiving fSCIG treatment. The patient database was closed on June 30, 2019. The study started on January 24, 2017, which was the day of the first administration of fSCIG therapy.

Patients eligible to participate in the study were: adults (aged $\geq$18 years), with humoral PID diagnosed according to the European Society for Immunodeficiencies (ESID) criteria (14), who were receiving fSCIG substitution treatment covered by the Drug Program no B.62 financed by the National Health Fund in Poland (15). Patients were treated in two Immunology Centers specialized in PID therapy located in Warsaw, Poland. In both centers, all modes of IgG administration (IVIG, SCIG, and fSCIG) and all licensed immunoglobulin products were available. After completing an educational period, patients continued self-treatment in a home-based manner and were controlled every 3 months.

We collected demographic data and data on the everyday experience of fSCIG administration. This included the reasons for switching from IVIG or SCIG to fSCIG, as well as the practical way the method of administration and dose was changed according to the patients' expectations in the context of the 4-weekly application and the infusion characteristics (i.e., number of administration sites, flow characteristics, and volume of the administered drug). As the shared-decision model of care is applied in the two treatment centers (16), it was possible to report the reasons for changing the therapy. During routine control visits, the professional medical staff (physicians and nurses) assessed treatment effectiveness in terms of clinical response, treatment compliance, and patient's subjective evaluation (i.e., patient satisfaction, burden of the treatment). Based on the assessment and presentation of the available alternative methods of therapy, the decision regarding the next course of treatment was made in the cooperation with the patient.

The ramp-up mode of switching is described in the registration clinical trial protocol (6) and recommended in the HyQvia Summary of Product Characteristics (17) as follows: 25% of the dose infused at Week 1, 50% at Week 2, 75% at Week 4, and 100% at Week 7.

The fSCIG monthly dose was calculated based on the individual's demand over the past 3 months, independent of the previous mode of administration, and then corrected according to the patient's needs (18). The aim of introducing fSCIG therapy is to obtain the individual IgG trough level that provides optimal protection from infectious diseases. Lucas et al. showed a wide range of IgG trough levels and drug doses may be used to control infections (19). Here we aimed to achieve a trough IgG level of $>$5.0 g/L (20) that optimally reduced infection rates in the particular patient and was preferably within the reference range of the local laboratory (i.e., 7.0–12.0 g/L). In assessing patient's needs, we also took into account their subjective overall well-being and symptoms related to wear-off effect and fatigue (21).

To assess efficacy, we determined IgG trough levels (drawn just before the next infusion) and the proportion of patients with serious bacterial infections or hospitalizations due to PID complications. A serious bacterial infection was defined as bacterial pneumonia, bacteremia/septicemia, osteomyelitis/septic arthritis, bacterial meningitis, and visceral abscess (22). One patient with hyperglobulinemia at baseline, was excluded from IgG trough level analysis.

In this study, mainly descriptive statistical methods were used. For continuous variables, we calculated the median and range; and for categorical variables, we determined the frequency counts and percentages. The significance of changes in the IgG trough

levels was assessed using the Wilcoxon signed-rank test. The significance of differences between infused volumes of IgG in patients receiving different dosing intervals was assessed using the multiple comparisons (two-tailed) Kruskal-Wallis test. All computations were done using Microsoft Excel software and TIBCO Software Inc. Statistica ver. 13.

The Bioethics Committee of the Military Institute of Medicine in Warsaw approved the study (Approval No. 55/WIM/2019). All patients gave written consent for the treatment and inclusion of their medical and treatment history records within this study.

RESULTS

At the time the database was closed, there were 39 adult patients with PID that had received fSCIG replacement therapy (median observation time of 18 months; range, 3–24 months). The first patient started fSCIG in January 2017. Prior to fSCIG treatment, two patients were naïve to any immunoglobulin replacement therapy (IgRT). Among those that had received prior IgRT, 23 received IVIG, five self-administered SCIG, and nine received both IVIG and SCIG. The median time of prior IgRT (both IVIG and SCIG) for the 37 patients was 38 (range, 1–252) months. The detailed characteristics of the included patients are presented in (**Table 1**).

The median number of fSCIG infusions per patient was 26 ($N = 34$; range, 6–59). Due to different treatment durations, data were available for 32 patients in Month 6 of fSCIG therapy, 30 patients in Month 12, and 22 patients in Month 18. Eleven patients received fSCIG for 24 months. Overall 894 infusions were analyzed in this study. At the time the database was closed, 34 patients were still on fSCIG therapy. Five patients discontinued fSCIG for the following reasons: two male patients returned to 20% conventional SCIG due to side effects after the first fSCIG application; one woman with unspecified hypogammaglobulinemia switched to rapid-push application of 20% L-proline-stabilized IgG on the second educational visit at her request (she had problems programming the pump and preferred to apply smaller volumes of the drug at one site), and; two women were non-compliant (i.e., they gave up any type of IgRT and were lost from follow up; one in the 12th week of observation, and the other in week 36th). One patient with hyperglobulinemia at baseline, was excluded from IgG trough level analysis.

TABLE 1 | Patient characteristics.

Characteristic	All study participants ($N = 39$)*
Age, median (range), years	35 (18–68)
Sex	
Female	21 (54%)
Male	18 (46%)
Type of primary immunodeficiency	
Common variable immunodeficiency	27 (71%)
Unspecified hypogammaglobulinemia	5 (13%)
X-linked or recessive agammaglobulinemia	3 (8%)
Antibody deficiency due to congenital defect	2 (5%)
Specific antibody deficiency with	1 (3%)
bronchiectasis	1 (3%)
Hyperglobulinemia with infections	
Disease duration, median (range), years	10 (1–41)
Place of living	25 (64%)
Big city	11 (28%)
Small city	3 (8%)
Village	
Education level	18 (46%)
High	14 (36%)
Secondary	7 (18%)
Basic	
Professional activity	24 (62%)
Professionally active (working/studying)	15 (38%)
Disability benefit	
IgG replacement therapy before fSCIG	23 (59%)
Only IVIG	9 (23%)
IVIG>SCIG	5 (13%)
Only SCIG	2 (5%)
IgG naïve	

All data are presented as n (%), unless otherwise indicated.
fSCIG, facilitated subcutaneous immunoglobulin; IVIG, intravenous immunoglobulin; SCIG, subcutaneous immunoglobulin.

Reasons for Switching to fSCIG

The reasons for switching to fSCIG therapy were patient preference (28 patients, 72%), medical reasons (four patients, 10%), or both (seven patients, 18%). Medical reasons included: headaches or flu-like syndromes after IVIG (four cases), difficult or lack of venous access (five cases), severe common variable immune deficiency (CVID) enteropathy combined with IVIG insufficiency (one case), lack of adherence to conventional SCIG (one case).

Treatment Scheme and Dosing

The following methods of introducing fSCIG therapy were used: non-ramp-up dosing in 25 patients (i.e., two initial half-monthly doses biweekly in 14 patients, and full monthly dosing in 11 patients), ramp-up dosing in six patients, and other methods that were modified according to patient's preferences and center accessibility in eight patients.

At the time the database closed, 34 patients were continuing fSCIG treatment: 24 patients (71%) received fSCIG every 4 weeks, six patients (18%) every 3 weeks and, four patients (12%) every 2 weeks. The main reasons for changing from monthly to more frequent dosing were as follows: wear-off effect (six cases), large dose and local swelling that persisted longer than 3 days (three cases), pregnancy (two cases), low trough IgG level despite the high dose of 800 mg/kg/4 weeks (one case).

According to diagnosis, patients who needed biweekly dosing were: one patient with hypogammaglobulinemia and myopathy, one patient with combined immunodeficiency and five patients with complicated CVID (i.e., three cases with polyclonal lymphadenopathy, three cases with autoimmune disorders, and two cases with bronchiectasis). Please note that more than one condition could occur in one patient.

The median initial fSCIG dose was 480 mg/kg/4 weeks ($N = 33$; range, 260–800 mg/kg/4 weeks). The median fSCIG

dose was 510 mg/kg/4 weeks ($N = 32$; range, 260–800 mg/kg/4 weeks) at Month 6, 560 mg/kg/4 weeks ($N = 30$; range, 260–800 mg/kg/4 weeks) at Month 12, 505 mg/kg/4 weeks ($N = 22$; range 350–770 mg/kg/4 weeks) at Month 18, and 470 mg/kg/4 weeks ($N = 11$; range, 370–590 mg/kg/4 weeks) at Month 24.

Infusion Characteristics

In the majority of patients ($n = 32$, 94%), one application site was used, while two patients used two application sites. The median of volume infused per site was 300 ml ($N = 33$; range 150–500 ml). No statistically significant changes were found between the median infused volumes of the drug in the groups defined by different dosing intervals (i.e., every 2, 3, or 4 weeks; $p = 0.317$; **Supplementary Figure 1**). Three patients used syringe pumps (CRONO S-PID 100 or Syringe Pump T34L) and 31 used infusion pumps (Bodyguard 323 Color) with the median infusion speed of 300 ml/h ($N = 33$; range, 250–300 ml/h).

Clinical Efficacy and IgG Trough Level

There were no cases of serious bacterial infection or hospitalization due to a PID complication in our study.

The IgG trough levels and therapeutic IgG doses are summarized in (**Table 2**). Data for a 24-month observation period were available for 11 patients. There was a systematic growth in the median IgG trough level starting from 8.01 g/L at baseline and reaching 9.0 g/L at Month 24 (**Figure 1A**). A statistically significant difference was found between median IgG through levels between baseline and Month 12 ($p = 0.024$) in the whole study population (**Supplementary Figure 2**).

Among the group of patients converting from SCIG to fSCIG ($n = 11$), higher fSCIG doses were required to maintain the same IgG trough levels (**Figure 1B**). Meanwhile, patients previously treated with IVIG ($n = 21$) required comparable doses of fSCIG to maintain their IgG trough levels (**Figure 1C**). Either ramping-up the therapy dose (**Figure 1D**), or using a non-ramp-up method (**Figure 1E**), could maintain clinical efficacy.

The trough IgG levels and doses of six patients previously treated with IVIG and then SCIG, who then switched to fSCIG were analyzed separately. These patients required doses analogous to their prior IVIG therapy, and not their immediately-preceding SCIG dose, to maintain clinical efficacy when switching to fSCIG (**Figure 2**).

Tolerability

No serious adverse drug reactions were observed in our study. There was one type of local side effect (edema at the infusion site) observed in all patients and lasting for a median time of 24 ($N = 34$; range, 12–72) h. The duration of the swelling was independent of the patient's body mass index.

In addition, general side effects were observed in seven patients; among them, two decided to discontinue fSCIG. The first patient (who was previously on IVIG without any side effects) complained of low-grade fever, chills, fatigue, and edema with redness close to the site of infusion for 72 h after the first fSCIG application, after which the patient switched to 20% conventional SCIG (which was well-tolerated without side effects). The second patient (who was previously on IVIG and then 16% SCIG) reported chills, low grade fever, anxiety, a prickling sensation and palm rash after the first infusion of 12.5 g fSCIG; however, the symptoms disappeared spontaneously within 12 h. Nonetheless, after the first fSCIG infusion, the patient decided to switch to 20% conventional SCIG (which was well-tolerated long-term, without side effects). Another three patients complained of low-grade fever, chills, and fatigue appearing 24 h after the fSCIG application. All three patients had received IVIG or SCIG prior, without any side effects. In two patients, the symptoms disappeared within 3 months, and in one male patient, a sub-febrile condition persisted until the last follow-up visit at 24 months.

DISCUSSION

Long-term observations from clinical trials have proven the effectiveness and safety of fSCIG both in pediatric and adult PID patients (6, 7). The switch method from IVIG to fSCIG proposed in the clinical setting was a ramp-up model, with a final frequency of injections every 3–4 weeks. Although clinical data has confirmed the efficacy and safety of fSCIG, here we focus on real-life observations, which provide useful information on

TABLE 2 | Trough IgG levels and dosing (both presented as median).

	24 months of observation $n = 11$		SCIG to fSCIG $n = 11$		IVIG to fSCIG $n = 21$		Ramp-up $n = 5$		Non-Ramp-up $n = 28$	
	IgG g/L	Dosing mg/kg/4 weeks	IgG g/L	Dosing mg/kg/4 weeks	IgG g/L	Dosing mg/kg/4 weeks	IgG g/L	Dosing mg/kg/4 weeks	IgG g/L	Dosing mg/kg/4 weeks
Baseline	8.01	430	8.07	420	7.8	470	7.8	550	8.0	455
Month 3	7.12	460	8.06	460	7.12	480	8.7	550	7.0	470
Month 6	7.21	490	8.79	560	7.21	490	8.9	530	7.5	490
Month 12	9.0	590	9.53	570	8.0	480	9.8	470	8.7	565
Month 18	8.63	500	Nd*	Nd*	Nd*	Nd*	Nd*	Nd*	Nd*	Nd*
Month 24	9.0	470	Nd*	Nd*	Nd*	Nd*	Nd*	Nd*	Nd*	Nd*

Nd, no data.

fSCIG, facilitated subcutaneous immunoglobulin; IVIG, intravenous immunoglobulin; SCIG, subcutaneous immunoglobulin.

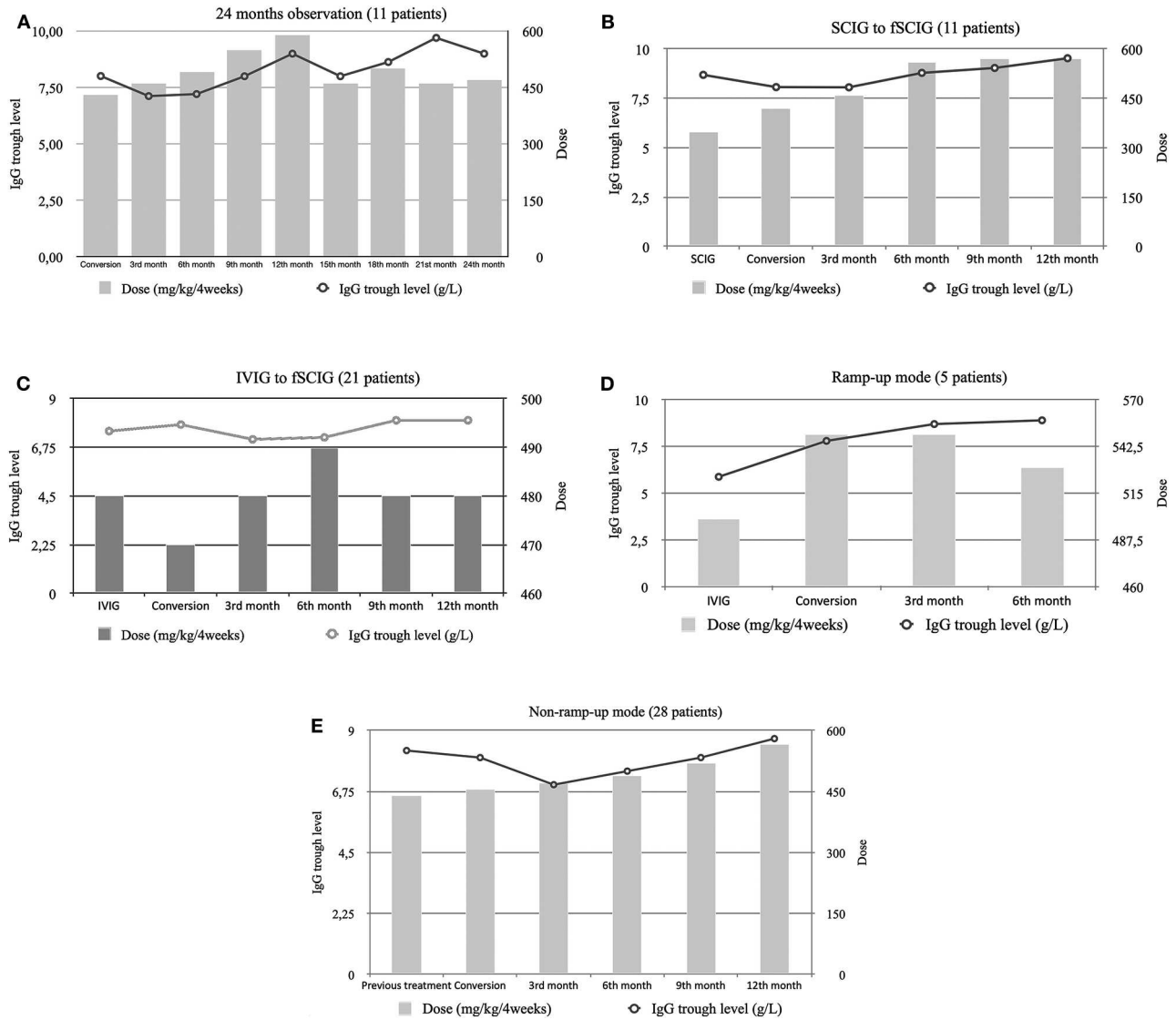

FIGURE 1 | Median trough immunoglobulin G (IgG) levels and median dose per 4 weeks of immunoglobulin therapy in: **(A)** patients that completed the 24-month follow-up, **(B)** patients that switched from subcutaneous immunoglobulin (SCIG) to fSCIG, **(C)** patients that switched from intravenous immunoglobulin (IVIG) to fSCIG, **(D)** patients that switched in a ramp-up mode, and **(E)** patients that switched in a non-ramp-up mode.

heterogenous populations and real-life treatment patterns, and may reveal rare adverse events (23).

Indeed, only one other real-life observation focusing on the practical aspects of everyday fSCIG experience has been conducted (13), albeit with some limitations (i.e., small sample size, short follow-up, and a fixed dosing schedule), which were improved in the methodology of our study. Ponsford et al. (13) reported identified two types of patients choosing fSCIG over IVIG or SCIG: those with clinical problems on current treatment and those looking for convenience and flexibility (13). However, we found patient preference was the main reason for switching to fSCIG.

In the study by Ponsford et al. (13), patients expressed positive opinions regarding fSCIG, especially a lower disease burden. There was also a reduction in the frequency of injections required for fSCIG compared to SCIG, and similar to our results, good tolerability of the infusion speed was reported. Moreover, all 14 patients in the study by Ponsford et al. (13) experienced temporary side effects at the injection site during infusion. We also found patients reported swelling lasting up to 72 h post-infusion.

Our study presents real-life data from a Polish setting. Currently in Poland, all IgG application modes (IVIG, conventional SCIG, and fSCIG) are available and are reimbursed by the National Health Fund (15). The previous report describing a Polish cohort of 77 adult patients with CVID treated in four centers was published in 2017 (3). Over the follow-up period, over 70% of patients (55/74) changed the therapy mode,

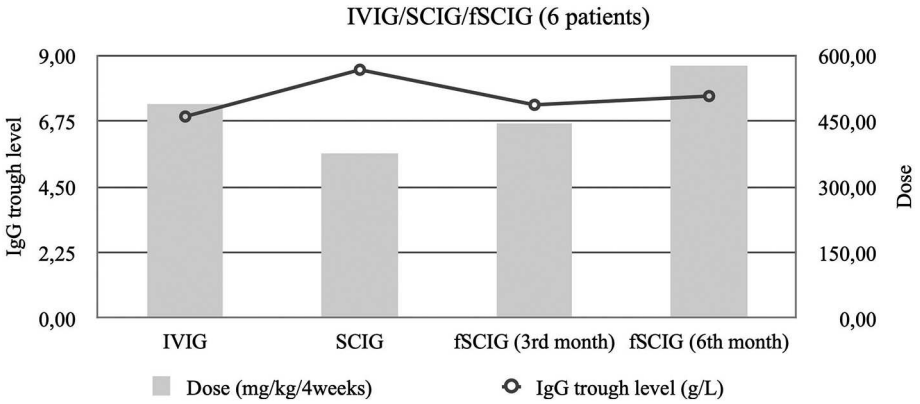

FIGURE 2 | Median trough immunoglobulin G (IgG) levels and median immunoglobulin dose per 4 weeks among six patients that started of facilitated subcutaneous immunoglobulin (fSCIG) treatment after previous immunoglobulin (SCIG) and intravenous immunoglobulin (IVIG).

mainly from IVIG to SCIG or fSCIG, either directly (IVIG to fSCIG) or indirectly (IVIG to SCIG and then to fSCIG), which was determined mainly by the availability of treatment methods at that time, not the patient's preference itself. The method of switching from IVIG to fSCIG seems preferable for patients previously treated with IVIG every 4 weeks. Indeed, 23 patients in our study refused a conventional SCIG treatment because of the need to administer the drug every 1/2 weeks.

Real-world evidence of fSCIG treatment is limited to case reports of PID (8–11), which are mainly complicated by other medical conditions and require fSCIG introduction due to medical needs. Indeed, in our study, such medical needs included: problems with venous access, adverse events on previous treatment, severe PID complications combined with previous treatment insufficiency, and lack of adherence to previous treatment.

Carne et al. (8) presented the longest (60 months) case of continuous home fSCIG therapy, covering 240 infusions. The patient started on the SCIG method, but trough IgG levels could not be maintained despite high IgG doses (>1 g/kg/month). A change to SCIG facilitated by ovine hyaluronidase was introduced (as at the time, no licensed product containing human recombinant hyaluronidase was available), which normalized IgG trough levels (8). Pedini et al. (9) reported four cases of patients with CVID and accompanying cytopenia, including idiopathic thrombocytopenic purpura (ITP; $n = 3$) and autoimmune hemolytic anemia (AIHA; $n = 1$), who were treated with fSCIG. All patients achieved stable remissions from cytopenia and anemia, and the patient with AIHA was able to switch to a minimal prednisone dose (9).

Danieli et al. (10) reported five cases of patients with PID with different comorbidities who were successfully treated with fSCIG. To achieve satisfactory IgG trough levels and reduce infection rates, a dosing regimen of every 2–3 weeks was used. Similarly, in our study, we found patients with a complex case of PID required more frequent dosing of every 2/3 weeks. Despite this, we found the most common reason for increasing the dose frequency was a wear-off effect, which is often a problem among patients treated with IVIG (24). However, as prior pharmacokinetic analyses indicate fSCIG has a similar pharmacokinetic profile to IVIG (25), such a wear-off effect will likely occur with fSCIG therapy. Therefore, although dosing for fSCIG is designed to be at 3 to 4-weeks intervals (17), clinical practice indicates more frequent dosing every 2 weeks may be needed to achieve the desired effects with good tolerability in certain patients.

Another paper by Wiesik-Szewczyk et al. (11) presents the first case of a successful switch from IVIG to fSCIG during the third trimester of the first pregnancy in a CVID patient. The treatment was effective and well-tolerated by the mother, and provided the baby with sufficient IgG levels (11). In our study, there were two other pregnancies, including a second pregnancy of the patient described before. Both pregnant patients remained on fSCIG therapy and preferred to switch from monthly to biweekly dosing. From these early observations, fSCIG seems to be a good treatment option for pregnant patients with PID.

Although fSCIG therapy was generally well-tolerated in our study and others (8–11, 13), serious conditions may occur, such as necrosis (12). However, more real-life data on fSCIG are needed with larger patient groups, long follow-ups, and different settings, not only to assess its efficacy and safety, but also patient opinions, convenience, and quality of life.

To conclude, here we report the first Polish observational study on the practical experience of fSCIG administration. The major limitation of our study was its retrospective character and a potential record bias. Nonetheless, based on the results from our study, which included a large number of patients and a long follow-up, we conclude fSCIG is safe and efficient for IgG treatment in a real-life setting. Finally, we proved fSCIG efficacy was maintained because of the personalized approach: using different modes of switching (ramp-up, non-ramp-up, and other) and individualized modification of dosing (every 2, 3, or 4 weeks) based on our long-term follow-up data on the shared-decision model of care.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bioethics Committee of the Military Institute of Medicine in Warsaw (Approval No. 55/WIM/2019). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EW-S and DS conceived the idea for the study, contributed to the design of the research, involved in data collection, and coordinated the project. EW-S, DS, LP, and KJ-R analyzed the data. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00981/full#supplementary-material

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Shorter Diagnostic Delay in Polish Adult Patients With Common Variable Immunodeficiency and Symptom Onset After 1999

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Common variable immunodeficiency (CVID) is the most clinically significant primary antibody immunodeficiency recognized in adulthood. Previously published data have shown an average diagnostic delay of 10 years for Polish adult patients with CVID. In the current study, we aimed to analyze the current diagnostic delay of adult patients with CVID in Poland. To this end, we identified patients from four immunological centers specialized in the care of adult patients with primary immunodeficiencies (PID). Demographic and clinical data of patients were collected using an internet database. We identified 103 adult patients (F:M 44.7%:55.3%) in Poland with CVID. The median age at onset of symptoms was 24 (0–66), 33 (4–70) at diagnosis, and 37 (18–73) years at the time of analysis. The median diagnostic delay for the entire study population was 6 (0–57) years. However, this delay was higher in patients with symptom onset before the year 2000 than after the year 1999 [15 (0–57) vs. 3 (0–19) years; $p < 0.001$]. Comparing patients (median $\leq$ 6 years, $N = 53$) with short diagnostic delay (SDD) and those (median $>$ 6 years, $N = 50$) with long diagnostic delay (LDD), the LDD group had a statistically significant higher incidence of infections of the lower respiratory tract before diagnosis (90.0 vs. 71.70%). During the entire observation period, cytopenias (44.00 vs. 22.64%), granulomatous lesions (28.00 vs. 11.32%), and solid tumors (14.00 vs. 1.89%) were significantly more frequent in the LDD group. In conclusion, we found a significant reduction in the median diagnostic delay in Polish CVID patients with disease onset in the last two decades.

Keywords: primary antibody deficiency, hypogammaglobulinemia, common variable immunodeficiency, diagnostic delay, adults, epidemiology

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INTRODUCTION

Primary immunodeficiencies (PIDs) are rare diseases. Because of their innate nature, they are diagnosed mainly in childhood (1). More than half of PID cases are associated with a defect in antibody production or function (2). In this group, the most common symptomatic deficiency is common variable immunodeficiency (CVID) (1, 3). CVID is a heterogeneous group of disorders characterized by recurrent upper and lower respiratory tract infections, which occur in more than 85% of patients (4). Besides, up to 70% of patients have at least one non-infectious manifestation, such as autoimmunization, granulomatous lesions, unexplained polyclonal lymphoproliferation, enteropathy, or malignancy (5–7).

Epidemiological data indicate that CVID has two peaks of onset. The first peak occurs in childhood, and the second peak occurs in the third or fourth decade of life. However, symptoms of CVID can start at any time of life, even in elderly patients (8). In Europe, 60% of CVID diagnosis occurs in adults (**Table 1**).

Due to low awareness among physicians regarding PID in adults, the onset of symptoms in adulthood, and a heterogeneous clinical picture of CVID, it may take up to several years to establish a proper diagnosis (14). The analysis of nearly 3,000 CVID cases showed a relationship between diagnosis delay and a higher risk of death [1.04 (1.02, 1.06), $p = 0.0003$], and organ complications (13). Aghamohammadi et al. demonstrated that the delay in diagnosis correlated significantly with the severity of the infection and the number of hospitalizations in children with primary antibody deficiencies, including CVID (15). Diagnostic delay of CVID generates high socioeconomic costs. According to Sadeghi et al., a diagnosis of CVID in a single patient can save US$ 6500 annually (16).

Similar to other rare diseases, data on CVID epidemiology are derived mainly from registries. In the last decade, several papers have been published, analyzing data from the ESID register (8, 13) or national registers (1, 3, 9, 10, 12). According to these studies, the diagnostic delay ranges between 3 and 9 years (**Table 1**). The period between the onset of first symptoms and CVID diagnosis is reportedly significantly shortened after 2000 in Spain (8) and the United Kingdom (3). In several other countries, there has been a tendency to shorten the delay of diagnosis, but the differences have not reached statistical significance (1, 8).

In Poland, we have very limited knowledge regarding CVID epidemiology. Considering the estimated prevalence of 1:25,000–1:50,000 and the population of Poland, which is about 38.386 million (17), there should be about 760–1,500 patients with CVID in this country. According to available data, 78 new cases were identified in 2014 (including 49 in children, 29 in adults) (18), and the median diagnostic delay in one of the pediatric centers (Kraków, 32 patients) was 1.8 years (8). According to data published in 2018, in a group of 77 adult Polish CVID patients, the mean diagnosis delay was 10.13 ± 10.53 years (19).

This study aimed to determine the length of the diagnostic delay of CVID in a group of Polish adult patients and compare groups of patients with short (SDD) and long diagnostic delay (LDD).

MATERIALS AND METHODS

Study Population

Data of CVID patients were collected from May 24, 2017, to December 31, 2019, using an internet database. The database did not contain personal data, and the patients were identified by code numbers. Only the attending physician of a particular patient could link the code number and patient's data. Entries older than 12 months were updated every year.

The study group consisted of patients treated under the Polish Ministry of Health's drug programs B.62 and B.78. A drug program is defined as follows: "guaranteed compensation, including therapies with innovative, expensive active substances, which are not financed by other guaranteed benefits. The treatment is carried out in selected disease entities and includes a strictly defined group of patients" (20). Within the aforementioned drug programs, immunoglobulin replacement therapy and monitoring are reimbursed for patients with primary humoral immunodeficiencies. Patients were treated at four immunological centers specializing in the care of adult patients with primary immunodeficiencies (Department of Allergology, Clinical Immunology and Internal Diseases, Ludwik Rydygier Collegium Medicum in Bydgoszcz Nicolaus Copernicus University in Torun, Bydgoszcz; Department of Internal Medicine, Connective Tissue Diseases and Geriatrics, Medical University of Gdansk, Gdansk; Outpatient Clinic for the Immunological and Hypercoagulable Diseases, The University Hospital in Krakow, Cracow; and Department of Internal Medicine, Pneumonology, Allergology and Clinical Immunology, Central Clinical Hospital of the Ministry of National Defense, Military Institute of Medicine, Warsaw). All patients met the Registry Working Definitions of the European Society for Immunodeficiencies (ESID) for CVID (21).

Of note, the most important epidemiological and clinical data are available as a Data Sheet, in the **Supplementary Materials**.

Data Collection

We collected data on the age of onset of the first symptoms, age at the time of CVID diagnosis, immunoglobulin (Ig) levels at the time of diagnosis, and type of infections before diagnosis. We also recorded the most important organ complications and co-morbidities associated with CVID from the time of the first symptoms until the data were entered in the database or updated. The year in which the first symptoms occurred was considered as the year in which the frequency of infection increased, a severe infection requiring hospitalization or intravenous antibiotic treatment, or the year in which symptoms of autoimmunity, polyclonal lymphoproliferation, or malignancy occurred. The age of onset of the first symptoms and that at the time of diagnosis was calculated as the difference in years between the year of birth of the patient and the year in which the event occurred. The diagnostic delay was calculated as the difference of full years between the years of onset of symptoms and diagnosis.

Due to the median delay in diagnosis for all patients (6 years), the cohort was divided into the following groups: SDD (median delay ≤ 6 years; $N = 53$) and LDD (median delay > 6 years; $N = 50$). The groups were compared in terms of age of first symptoms,

TABLE 1 | Summary of most relevant CVID epidemiological studies in selected countries.

Country	Reported period (years)	Number of patients	Age at time of analysis	Age at onset	Age at diagnosis	Diagnostic delay	% of patients diagnosed as adults	References
Denmark	1994–2013	179	50.1± 17.0	29 (IQR; 3–87)	40 (IQR; 29–56) min 4; max 87	7 (IQR; 3–17)	–	Westh et al. (9)
Germany	2012–2017	728	40 (3–88)	–	Max 79	Mean: 7.35 median: 3	65%	El-Helou et al. (1)
Italy	1985–2015	75	50.08 ± 15.81; Median: 49	32 [17.82]*	40 [16.01]*	7 (IQR; 3–13)	–	Graziano et al. (10)
Poland	2017	77	39.19 ± 13.61	22.16 ± 14.32	32.29 ± 14.94	10.13 ± 10.53	76.6%	Wiesik-Szewczyk et al. (11)
Switzerland	2008–2014	98	–	–	–	Median: 5.95	87.5%	Marschall et al. (12)
United Kingdom	(2008**) 2012–2017	1,404	–	–	–	4 (IQR; 1–10) 4 (0–69)	–	Shillitoe et al. (3)
Europe (23 countries)	2004–2014	2,700	–	18 (0–81) 22.4 ± 19.0	31 (4–89)	4 (0–69) 8.8 ± 11.4	69.5%	Odnoletkova et al. (13)
Europe (16 countries)	2004–2012	2,212	–	–	–	4.1 (IQR; 1–11.8)	86.7%	Gathmann et al. (8)

If not otherwise indicated, data are presented as median (minimum-maximum) or median (interquartile range—IQR) or mean ± SD.
**Median [SD].*
***United Kingdom Primary Immunodeficiency (UKPID) registry exists from 2008.*

age of diagnosis, as well as IgG, IgA, and IgM levels at the time of diagnosis, the incidence of infection in the period before diagnosis, and incidence of complications and co-morbidities throughout the observation period.

Statistical Analysis

The normality of the observed values was tested using the Shapiro-Wilk test. For the continuous variables, mean and standard deviation were calculated if they followed a normal distribution; for non-normal distributions, the median (minimum to maximum) was used. Continuous variables were analyzed using Student's T, Mann-Whitney-U, and Kruskal–Wallis tests. Categorical variables were analyzed using the Chi-square test. For all data analyses, differences were considered statistically significant when $p < 0.05$. The statistical analysis was performed using the STATISTICA software (TIBCO Software Inc. Palo Alto, CA, USA), version 13.

Ethics Statement

The study was approved by the Ethics Committee of the Military Institute of Medicine, Warsaw (7/WIM/2020). All patients provided written consent to the collection and analysis of their demographic and medical data.

RESULTS

Characterization of CVID Patient Population

This study consisted of 103 adult patients, including 46 women (44.7%) and 57 men (55.3%) with CVID. At the time of data analysis, their median age was 37 (18–73) years.

The first symptoms of the disease commonly appeared from 0 to 14 years (39 patients; 37.9%) and 25–39 years of age (38 patients; 36.9%). Additionally, for the age of diagnosis, a bimodal distribution was observed. CVID was diagnosed in the highest percentage of patients at 10–19 years (21 patients; 20.4%) and 30–39 years of age (32 patients; 31.1%) (**Figure 1A**).

The median age at onset of symptoms was 24 (0–66) years. The first symptoms occurred in 44 patients (42.7%) before 18 years of age. In 41 patients (39.8%), disease onset occurred before the year 2000. In the decades following 1980, the mean age of patients at the onset of symptoms increased from 8.11 ± 5.97 to 29.7 ± 14.1 years (**Table 2**).

The median age at the time of CVID diagnosis was 33 years (4–70). In subsequent decades, the diagnosis was established in increasingly older patients. The age of diagnosis at specified intervals is presented in **Table 2**. Childhood (<18 years of age) diagnosis was established in 23 patients (22.3%). In 74 patients (71.8%), CVID was diagnosed between 2010 and 2019 (**Table 2**). In 7 cases (6.8%), including five patients under 18 years of age, the diagnosis was established in the same calendar year in which the first symptoms occurred.

The median diagnostic delay of the study group was 6 (0–57) years (mean 9.91 ± 10.3 years). In men, the median delay was 9.0 (0–39) years and 5.0 (0–57) years in women. These differences were not statistically significant ($p = 0.191$). The mean diagnostic delay was 41.5 ± 10.8 years in patients whose first symptoms occurred between 1950 and 1979. In subsequent decades, this delay systematically decreased, and from 2010 to 2019, it was 2.24 ± 1.77 years (**Table 2**). The median delay in patients with first symptoms before 2000 was 15 (0–57) years and 3 (0–19)

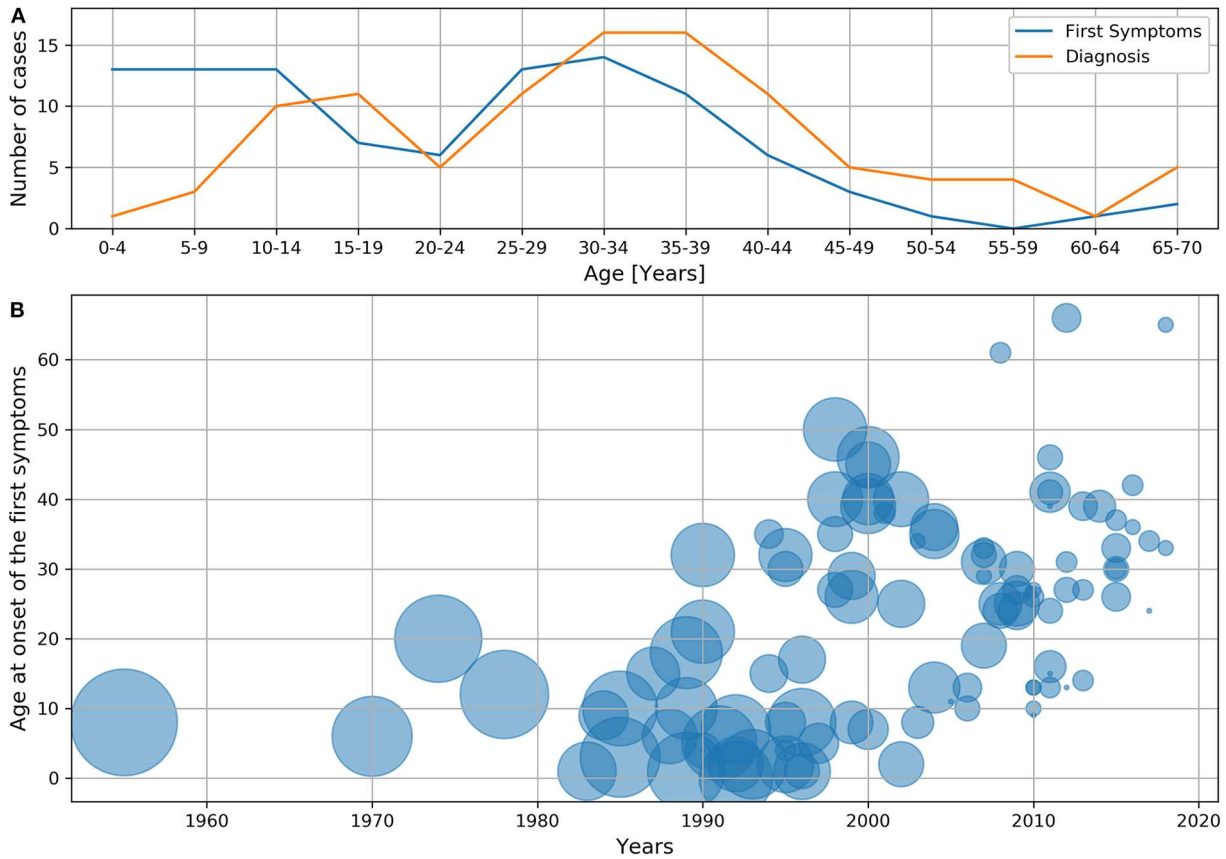

FIGURE 1 | Age of first symptoms or diagnosis and diagnostic delay: **(A)** Age of first symptoms and age of diagnosis. **(B)** Diagnostic delay depending on the age and year at which the first symptoms occurred. The diameter of the circle corresponds to the delay expressed in years, and the center indicates the age and year at which the first symptoms occurred.

TABLE 2 | Mean delay of CVID diagnosis and mean age of patients in subsequent decades, depending on the age of first symptoms and the age of diagnosis.

Years	First symptoms			Diagnosis		
	N	Diagnostic delay	Age	N	Diagnostic delay	Age
1950–1979	4 (3.88%)	41.5 ± 10.8	11.5 ± 6.19	–	–	–
1980–1989	9 (8.74%)	21.4 ± 7.58	8.11 ± 5.97	–	–	–
1990–1999	28 (27.18%)	13.1 ± 7.85	16.1 ± 14.7	5 (4.85%)	4.80 ± 4.60	16.0 ± 14.4
2000–2009	29 (28.16%)	7.66 ± 4.97	27.7 ±1 3.5	24 (23.30%)	8.96 ± 7.29	25.1 ± 11.2
2010–2019	33 (32.04%)	2.24 ± 1.77	29.7 ± 14.1	74 (71.84%)	10.6 ± 11.3	36.3 ± 14.9
p value*	–	<0.001	<0.001	–	0.535	<0.001

The values are presented as number (%) or mean ± SD.
**Kruskal-Wallis test.*

years after 1999 ($p < 0.001$). Further, we observed a reduction in diagnostic delay, even if the first symptoms occurred in elderly patients (**Figure 1B**), which was the most prominent after the year 2000.

In the decades following 1990, the mean delay assessed at the time of diagnosis increased from 4.80 ± 4.60 to 10.6 ± 11.3 years. The differences in subsequent analyzed periods were not statistically significant (**Table 2**).

Comparison of Patients With SDD and LDD

There were statistically significant differences between groups of patients with SDD and LDD (**Table 3**) in the age of appearance of first symptoms [27.0 (1–66) vs. 15.0 (0–50) $p = 0.004$], the age at which the diagnosis was established [31.0 (4–70) vs. 34.5 (12–70) $p = 0.04$], and the age at the time of analysis [35.0 (18–73) vs. 41.0 (19–72) $p = 0.036$]. In both groups, at the time of diagnosis, IgG, IgA, and IgM levels were comparable. Infections

TABLE 3 | Comparison of patients with short and long diagnostic delay.

	Short Diagnostic Delay Median ≤ 6 years	Long Diagnostic Delay Median > 6 years	*p*
N (%)	53 (51.46%)	50 (48.54%)	–
Women: men N (%)	28:25 (52.8%:47.2%)	18:32 (36.0%:64.0%)	0.086
Age at the first symptoms [years]	27.0 (1–66)	15.0 (0–50)	**0.004**
Age at the time of diagnosis [years]	31.0 (4–70)	34.5 (12–70)	**0.040**
Age at the time of analysis [years]	35.0 (18–73)	41.0 (19–72)	**0.036**
IgG at the time of diagnosis [mg/dl]	138.0 (0–543)	204.0 (0–640)	0.870
IgM at the time of diagnosis [mg/dl]	15.0 (0–93)	10.5 (0–903)	0.638
IgA at the time of diagnosis [mg/dl]	5.0 (0–67.5)	6.0 (0–53)	0.509
INFECTIONS BEFORE CVID DIAGNOSIS *N* (%)			
Upper respiratory tract (except sinusitis)	50 (94.34%)	49 (98.0%)	0.336
Nose and paranasal sinuses	48 (90.57%)	47 (94.0%)	0.515
Lower respiratory tract	38 (71.70%)	45 (90.0%)	**0.019**
Middle ear	40 (75.47%)	35 (70.0%)	0.532
Gastrointestinal tract	12 (22.64%)	11 (22.00%)	0.938
Urinary tract	7 (13.21%)	8 (16.00%)	0.688
Skin and subcutaneous tissue	9 (17.00%)	5 (10.00%)	0.301
Generalized infection/sepsis	6 (11.00%)	11 (22.00%)	0.154
CVID COMPLICATIONS AND CO-MORBIDITIES *N* (%)			
Any autoimmunization	22 (41.51%)	29 (58.00%)	0.094
Cytopenia	12 (22.64%)	22 (44.00%)	**0.021**
Thrombocytopenia	9 (16.98%)	13 (26.00%)	0.264
Enteropathy	7 (13.2%)	3 (6.0%)	0.217
Bronchiectasis	13 (25.49%)	8 (16.00%)	0.240
Polyclonal lymphoproliferation (Lymphadenopathy, GLILD, etc.)	16 (30.19%)	18 (36.00%)	0.531
Granulomatous lesions	6 (11.32%)	14 (28.00%)	**0.032**
Splenomegaly	4 (7.55%)	8 (16.00%)	0.181
Malignancy total	3 (5.66%)	9 (18.00%)	**0.049**
Lymphoma	2 (3,77%)	2 (4.00%)	0.953
Solid tumors	1 (1.89%)	7 (14.00%)	**0.022**

Bolded p-values indicate statistical significance ($p < 0.05$).

of the lower respiratory tract occurred in a significantly higher percentage of patients in the LDD group than in the SDD group (90.0 vs. 71.70%; $p = 0.019$). There were no significant differences in other infections between SSD and LDD groups.

Cytopenias (44.00 vs. 22.64% $p = 0.021$), granulomatous lesions (28.00 vs. 11.32% $p = 0.032$), and malignancies (18.00 vs. 5.66% $p = 0.049$), including solid tumors (14.0 vs. 1.89% $p = 0.022$), were significantly more frequent in the LDD group than in the SDD group (**Table 3**). Autoimmunization, thrombocytopenia (as the most frequent cytopenia), polyclonal lymphoproliferation, splenomegaly, and lymphoma were more frequent in the LDD group than in the SDD group, although these differences were not statistically significant. Further, in the SDD group, bronchiectasis, and enteropathy were more frequent. However, these differences were not significant.

DISCUSSION

According to data published after 2010, the median delay in CVID diagnosis (**Table 1**) ranges from 3 years in Germany (1) to 7 years in Italy (10) and Denmark (9). In the 23 European countries analyzed together, the median diagnostic delay is 4 (0–69) years, and the mean is 8.8 ± 11.4 years (13).

In our group, the median delay was 6 years. However, for patients whose first symptoms appeared between 2010 and 2019, the mean delay was shortened to slightly over 2 years. According to the 2014 ESID registry, a significant shortening of the median delay was achieved only in Spain (9.0 vs. 4.6 years) (8). Furthermore, the United Kingdom demonstrated a statistically significant but weak correlation for a decrease in diagnostic delay over time from 2012 to 2017 (3).

At present, in Poland, CVID diagnosis is more rapid than that before 2000, even in elderly patients. However, compared to other European countries, a lower percentage of patients whose diagnosis was established in the year in which the first symptoms occurred remains (6.8 vs. 16.0%) (13).

In the group of Polish patients, we observed an increase in the mean diagnostic delay, assessed at the time of diagnosis, over the last three decades (**Table 2**). This finding may result from patients who had undiagnosed CVID symptoms for several years. A similar phenomenon occurred in Europe, in which the mean delay assessed at the time of diagnosis before and in 1980 was 7.4 years, and in and after 2000 was 8.8 years (13).

Most CVID cases were identified in Poland after 1999, which is higher than in other European countries (95.15 vs. 69.1%) (13). This striking difference was possibly due to efforts by the Polish Ministry of Health, which provided reimbursements of immunoglobulin treatment for patients with primary immunodeficiency in 2015 as part of its drug programs (11). Additionally, after year 2000 new Polish centers for adult PID patients were established that improved accessibility for clinical immunologist consultations.

Comparing the group of patients with SDD and LDD, statistically significant differences were found between the age of first symptoms and the age at which CVID was diagnosed. Patients from the SDD group were older at the time of onset, while they were younger at the time of diagnosis compared to patients from the LDD group. This could be due to at least three reasons. First, at the time of analysis, there were no patients under 18 years old in the study group, which increased the median age at the onset of the first symptoms and decreased the median age at which the diagnosis was established. Second, in patients who were older at the time of data analysis, the first symptoms could have occurred in childhood, which lowered the age median when the

first symptoms occurred in the LDD group. Third, before 2000, the delay in diagnosis was considerably longer than in recent years, which resulted in patients waiting longer to be diagnosed, even if symptoms occurred at a young age.

A statistically significant difference in the percentage of patients who had infections before CVID diagnosis was found exclusively in the case of lower respiratory tract infection, which was higher in the LDD group. This finding could be due to the delay in the initiation of IgG substitution.

Many studies have highlighted the occurrence of numerous complications and co-morbidities during the course of CVID (6, 8, 13, 22, 23). In this study cytopenias, granulomatous lesions, solid tumors, and neoplastic diseases were more frequent in the LDD group. The more frequent occurrence of the above-mentioned non-infectious complications in the LDD group could be a consequence of several phenomena. Due to low awareness among physicians of PID in adults, in patients who do not present with recurrent infections, diagnostics focus may divert from CVID. For instance, granulomatous lesions may occasionally be misdiagnosed as sarcoidosis (24). Moreover, the analysis of 21 patients with CVID and idiopathic thrombocytopenic purpura showed that only 19% of patients were diagnosed with immunodeficiency before the diagnosis of ITP (25). The more frequent occurrence of cancers, in the LDD group may be associated with the patients' older age and longer disease duration. In addition, Kiaee et al. performed a meta-analysis showing that CVID patients diagnosed with malignancy were older at the time of diagnosis, relative to patients without malignancy (5).

Recurrent lung infections are a recognized risk factor for bronchiectasis (26). Although the percentage of patients with lower respiratory tract infections was higher in the LDD group examined here, the percentage of patients with bronchiectasis in the LDD group was lower than that in the SDD group. This discrepancy may have resulted from the lack of discrimination between patients affected by chronic conditions from those with sporadic lower respiratory tract infections. Nevertheless, other cofactors, such as very low IgA or IgM level, or low neonatal Fc receptor expression, reportedly contribute to bronchiectasis (26). It can be assumed that the occurrence of bronchiectasis may have directed and accelerated the diagnosis toward CVID.

In the available literature, data on the relationship between delayed diagnosis and the occurrence of individual complications are unclear. Odnoletkova et al. reported in a group of 2700 patients with CVID that the diagnostic delay is associated with a higher risk of death, bronchiectasis, solid tumors, and enteropathy (13). In contrast, Razi et al., in a meta-analysis of 8,535 patients, did not show a correlation between delayed diagnosis and the occurrence of bronchiectasis. The incidence of bronchiectasis in the group of patients with 3 years or longer delay compared to the group with a shorter delay did not show a statistical difference (37.4 vs. 25.8%) (23). Further, in a group of 40 patients, researchers found a correlation between diagnosis delay and bronchiectasis ($r = 0.323$, $p = 0.042$), but did not confirm the correlation between chronic diarrhea and diagnostic delay (27).

Undoubtedly, further research on the relationship between the delayed diagnosis and occurrence of complications is necessary for a larger patient population.

Our study had a few limitations. Only adult patients were included in the analysis, which may have resulted in the overestimation of some indicators, such as the age of first symptoms or that of diagnosis. Further, four clinical, immunological centers participated in the study. Therefore, the analysis consisted of only a segment of Polish patients with CVID. Additionally, the relatively small number of patients made it difficult to analyze the incidence of rare complications statistically. Finally, for patients whose first symptoms occurred a long time ago (even in the 1950s), we had incomplete medical documentation, especially from the initial period of the disease. In a few cases, the type of infection before establishing a diagnosis was based on the oral records of the patients.

CONCLUSION

To the best of our knowledge, this is the first study on the delay in CVID diagnosis in the largest group of Polish patients. Notably, in recent years, the median time of delay in CVID diagnosis in Poland has significantly shortened and reached values comparable to that of other European countries. Presently, even an adult patient whose first symptoms occur at a late age can be diagnosed more quickly. However, further efforts are needed to assess the epidemiological and clinical landscape of patients with CVID and other primary immunodeficiencies (PID). To this end, we plan to establish the Polish Register of Primary Immunodeficiency Deficiencies in Adults (POLPIDA) that will facilitate a better, more comprehensive understanding of the needs of Polish patients for the diagnosis and therapy of PID and especially CVID. We believe that our continued effort will help reduce the incidence and severity of clinical complications.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Military Institute of Medicine, Warsaw, Poland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MZ and EW-S designed the study and wrote the first draft of the manuscript. This text was produced with an equal contribution of both authors. MZ, EW-S, AM-B, and KN-B collected data

and performed managed the literature searches. MZ performed the statistical analyzes. AM-B, KN-B, ZZ, and KJ-R performed a critical revision of the manuscript for intellectual content. All authors have read and agreed to the published version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00982/full#supplementary-material

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CASE REPORT
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Recurrent, Severe Aphthous Stomatitis and Mucosal Ulcers as Primary Manifestations of a Novel *STAT1* Gain-of-Function Mutation

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Chronic mucocutaneous candidiasis (CMC) characterized by persistent and recurrent Candida infection of the skin, nails, and the mucosa membranes has been proposed as the major infectious phenotype in patients with gain-of-function mutation of signal transducer and activator of transcription 1 (STAT1) 1. However, viral infections caused mostly by herpesviruses, and a broad range of autoimmune disorders may also be part of the clinical phenotype. We report here on a 31 years old female patient suffering from severe mucosal aphthous mucositis and ulcers and recurrent herpes simplex for decades. We found a previously unknown heterozygous sequence variant in *STAT1* (c.1219C>G; L407V) affecting the DNA-binding domain of the protein in the patient and her 4 years old daughter. We found this mutation gain-of-function (GOF) by using immunoblot and luciferase assays. We detected low proportion of IL-17A-producing CD4+ T cell lymphocytes by using intracellular staining and flow cytometry. Candida-induced secretion of IL-17A and IL-22 by mononuclear cells from the patient was markedly decreased compared to controls. These data suggest that the novel mutant allele may result in impaired differentiation of CD4+ T cells to CD4+/IL-17+ cells. The clinical phenotype of the disease in this patient was unique as it was dominated primarily by severe aphthous stomatitis and ulcerative esophagitis and only partly by typical CMC resulting in diagnostic delay. We suggest that patients with severe recurrent aphthous stomatitis and esophagitis should be evaluated for *STAT1* GOF mutation. Based on the broad clinical spectrum of the disease, we also suggest that CMC and CMC disease may not be an appropriate term to define clinically *STAT1* GOF mutation.

Keywords: mucosal ulcers, STAT1, gain-of-function mutation, IL-17-mediated immunity, mucocutaneous candidiasis

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INTRODUCTION

Impaired interleukin (IL)-17 mediated T cell immunity has been described to associate with chronic mucocutaneous candidiasis (CMC) (1–3). The most common genetic cause of CMC is thought to be gain-of-function (GOF) mutation in signal transducer and activator of transcription 1 (*STAT1*) affecting predominantly the coiled-coil domain (CCD) and less frequently the DNA-binding domain (DBD) (4–6). Importantly, bacterial and viral disease are also common in patients with *STAT1*-GOF occurring in 74 and 38%, respectively, and viral infections, herpesviruses in particular, preferably cause diseases on the surface barrier (6, 7).

Candida species, especially *C. albicans* reside on body surfaces of healthy individuals as innocent commensals. This symptomless commensalism, also referred to as colonization, may proceed to symptomatic candidiasis especially in patients with HIV infection and AIDS, and in those who have genetically impaired CD4+CD17+ T cell immunity (7). Mucosal candidiasis is commonly seen in patients with aphthous stomatitis and mucosal ulcer, but it is challenging to define if epithelial damage is caused primarily by Candida or, alternatively, fungal superinfection is a consequence of impaired barrier structure and function (8, 9).

We report here on a female patient who has been suffering primarily from severe, recurrent and persistent aphthous stomatitis since infancy. It was only at age 25 when genetic analysis was performed and revealed a novel *STAT1* GOF mutation (c.1219C>G; L407V). An impaired CD4+IL17+ T cell differentiation and function was also found. The patient was diagnosed with autoimmune mucositis and CMC was not considered as a primary disease-causing entity leading to remarkable delay in molecular diagnosis. We propose that patients with unexplained chronic aphthous stomatitis and esophagitis may have *STAT1* GOF mutation. We also suggest that *STAT1* GOF mutation is a more accurate disease term than CMC because of the broad and heterogeneous clinical manifestations including non-fungal infections, autoimmune disease, endocrinopathies, and rarely, cerebral aneurisms (6).

CASE REPORTS

Patient 1

This 31-year-old Hungarian female patient was born at term with 2,380 g birth weight and 47 cm length. During the time Hungary was polluted from air by the radiation originated from Chernobyl in May 1986 her mother spent a full day outdoor and developed an undiagnosed disease with fatigue, dizziness and diarrhea lasting for a week. Otherwise the pregnancy was uneventful, and the patient was born with no complication. The patient's father developed bladder cancer at age 41 which was treated successfully by surgery and chemotherapy. The umbilical cord of the patient detached 13 days after birth and local infection of the stub by Candida was diagnosed and treated successfully with local agents. She was immunized with Bacille-Calmette-Guérin vaccine at 3 days after birth and had seropurulent discharge from the site of injection at 6 and 9 months of age, respectively, for a few days. She received other childhood immunizations including diphtheria, pertussis, tetanus, poliomyelitis (Sabin vaccine), and one shot of measles-mumps-rubella vaccine without complication. Remarkably, she developed fever of 39–40 °C after each shot. At 7 months of age she developed aphthous stomatitis which recurred monthly at the beginning and more frequently later on but oral candidiasis was not visible. The second episode of aphthous stomatitis at age 3 required hospitalization and this time oral candidiasis was also diagnosed and she was treated with local nystatin and metronidazole. Pharyngeal and stool cultures yielded *C. albicans*. Sedimentation rate was 9 mm/h, platelet number, 122.5 Giga/L, serum iron and zinc levels were 5.5 and 7.43 μM, respectively. Tuberculin skin test evoked an 8 × 8 mm erythematous nodule, HIV serology and direct and indirect Coombs tests were negative. T cell number evaluated with rosetta test was 55% of lymphocytes (normal, 75–80%), T cell proliferation assay showed 35% blasts (normal, 40–45%), serum IgM, 1,15 g/L, IgA, 1,12 g/L, IgG, 12,3 g/L), complement hemolytic activity and granulocyte respiratory burst activation (nitroblue tetrazolium reduction and superoxide anion generation) were normal. She was discharged with the clinical diagnosis of undefined "Immunodeficiency." At age 5 she developed chickenpox, received hyper immune globulin and oral acyclovir and developed only about 20 vesicles and a mild course of the disease. Later on, however, she developed herpes simplex each year for 4–5 years, usually in August, and from age 24 she had recurrent herpes simplex every other year for 3 times mostly in the back and once in the lower abdomen with lesions of 8–10 cm size. At age 7 she was admitted to hospital with high fever and cough. WBC was 19,3 Giga/L, sedimentation rate, 98 mm/h, platelet number, 224 Giga/L, hemoglobin, 122 g/L, serum IgM, A, and G were 0.87, 1.14, and 20.8 g/L, respectively. Chest X-ray showed signs of bacterial pneumonia and did not suggest fungal etiology. Culture of urine, stool, throat scrub, nasal scrub, sputum and stool did not yield bacterial or candida pathogen. Serology and sputum culture for Mycoplasma, Chlamydia, Legionella, and *P. jiroveci* gave negative results. Treatment with various combinations of vancomycin, ceftazidime, brulamycin, clindamycin, and monobactam antibiotics and diflucan, the latter given because of the prolonged antimicrobial treatment, resulted in recovery from the severe, lower respiratory tract infection. At 13 years of age she met a bicycle accident and developed multiple fractures in her left tibia and fibula. Even after normal bone healing her left lower extremity remained 2 cm shorter than the right one. Since the first hospital admission she was scheduled for yearly immunology checkup and a decrease in the ratio of CD3+ and CD4+ T lymphocytes and occasionally an increase in the B lymphocytes was detected. Autoimmune and allergy serology tests gave negative results, only ASCA IgA and IgG was somewhat elevated (26–67 U/l) over the years. During her school age she was no more susceptible to viral respiratory or gastrointestinal infections than her schoolmates. She was somewhat even more resistant to community infections including flu compared to her schoolmates. Remarkably, however, when she had infection occasionally, she always had high fever and when she received antibiotic treatment, she always developed oral stomatitis. Despite the lack or recurrent infections

her weight percentile remained below 3 and she had microcytic anemia all over the time. Since her school age she has had persistent and recurrent eczema in the crook of the arm, the ham, the neck and the eyelids. At 17 years of age she was hospitalized for high fever, bilateral cervical lymphadenopathy, severe stomatitis with deep ulcers and difficulty eating and swallowing. Epstein-Barr virus serology revealed past infection with negative serum anti-VCA IgM and positive anti-EBV nuclear antigen. Adenovirus and cytomegalovirus serology gave negative results. Elevated liver enzymes were first detected at age 18 and remained slightly elevated afterwards. At age 18 she developed gastroenteritis and high fever which was attributed to Campylobacter species infection. At the age 21 chest X-ray was performed because of cough and rales on both sides and revealed patchy alveolar infiltrations around both hilus and fine interstitial infiltrations in both lower lobes. Inactive, 5–6 mm-sized, homogenic infiltrations were found in the upper lobes. Amoxicillin-clavulanic acid, clarithromycin and methylprednisolone treatment resulted in partial relief of cough, and the pneumonia finally resolved for levofloxacin and amoxiclav. Bronchoscopy and mycobacterial culture gave negative results. Calcification of the previously found upper lobar lesions was detected by X-ray. A host of autoimmune and allergy serology tests gave negative results and the etiology of the lung disease remained undefined. *Campylobacter coli* infection was diagnosed again at age 22. Between 19 and 25 years of age when she was a medical student, oropharyngeal and esophageal ulcers developed more and more frequently and at age 25 she did not become symptomless and had daily fever without defined infectious disease. During this period, she underwent esophago-gastro-duodenoscopy 9 times and colono-ileoscopy twice. These examinations revealed severe ulcerous lesions all over the length of the esophagus once with Candida patches, scars and narrowing at several locations. In the stomach and duodenum lymphatic stasis was detected. Colono-ileoscopy ruled out inflammatory bowel disease; however, histology of the esophagus at the peak of the symptoms complied with the manifestation of the Crohn's disease in the upper gastrointestinal tract, and no histology could detect any sign of fungal infection. At age 25 she underwent surgery for gluteal abscess and treated with amoxiclav and ciprofloxacin empirically.

We first saw the patient at age 25 during the episode what the patient defined as the most severe attack of oropharyngeal ulceration (**Figure 1**). Her weight was 37 kg and her height was 167 cm. Several deep ulcers of 5–14 mm in the buccal mucosa were seen and the patient could only swallow liquid. The facial skin was covered with papulopustulosus lesions of rosacea mostly in the nasolabial and maxillary areas. Chest CT scan revealed miliary lesions in the left apical and posterior segments. Blood culture yielded alfa-hemolytic Streptococci and buccal swab culture was positive for *S. aureus*. Bronchofiberoscopy showed atrophic, fragile bronchial mucosa but mucosal swab culture gave negative result. Coccidioides serology on admission and 8 months later revealed "TP" antibodies in the serum. CMC was thought and targeted *STAT1* sequencing was performed which revealed a novel sequence variant affecting the DBD. Functional analysis suggested GOF mutation (see Results). Parenteral

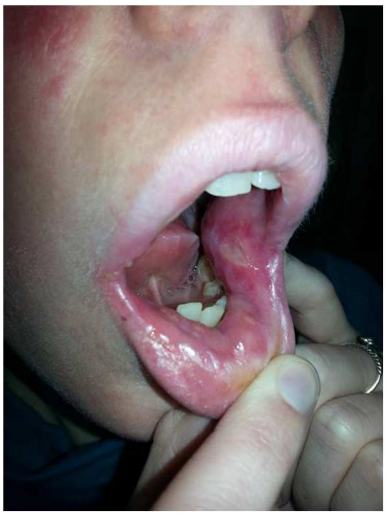

FIGURE 1 | Painful, deep ulcer in the left buccal mucosa at age 26.

acyclovir, clindamycin and fluconazole treatment was started, but her condition did not improve. Her treatment was switched to a combination of Caspofungin and low dose (30 mg/day) prednisone with local agents including nystatin, amphotericin B, xanthoflavin, lidocaine, and ascorbic acid was applied. Her condition improved in a week and full oral food intake and calorization could be started. The patient's general condition and appetite improved, and she started to gain weight. After she became free of ulcer, fluconazole maintenance therapy at a dose of 150 mg/week and prednisone at a 5 mg maintenance dose were continued for a few weeks. She was without therapy and remained stable for 1 year. At 27 year of age she became pregnant and in the second semester she developed upper respiratory tract disease and took amoxiclav for 7 days which precipitated stomatitis and ulcers. Prednisone at 10 mg dose was effective for the ulcers but in the third semester soor appeared on the oral mucosa and *C. glabrata* was detected from the vaginal scrub. At this age she experienced onychomycosis in her right thumb which proved to be recalcitrant to local therapy. She started again fluconazole at a dose of 2 × 150 mg/week Her mucositis was well controlled by treatment with local antifungals and 10 mg, and later on 5 mg per day prednisolone. Otherwise the pregnancy was uneventful, and in January 2016, she gave birth to a girl of 2,740 g and 47 cm (Patient 2, see below). She went off medications and for more than a year was well. At 29 years of age she again developed oropharyngeal and esophageal ulcers that was treated with steroids and fluconazole which resulted in rapid improvement. Over the past years she has had transient and mild oral aphtha and esophagitis responsive to short courses of prednisolone (maximal 30 mg per day) and fluconazole (maximal 2 × 150 mg weekly). She has been on 5 mg per day prednisolone and 150 mg per week fluconazole maintenance therapy.

Patient 2

The baby of Patient 1 was breastfed for 6 months and she has had only mild gluteal and oral candidiasis after

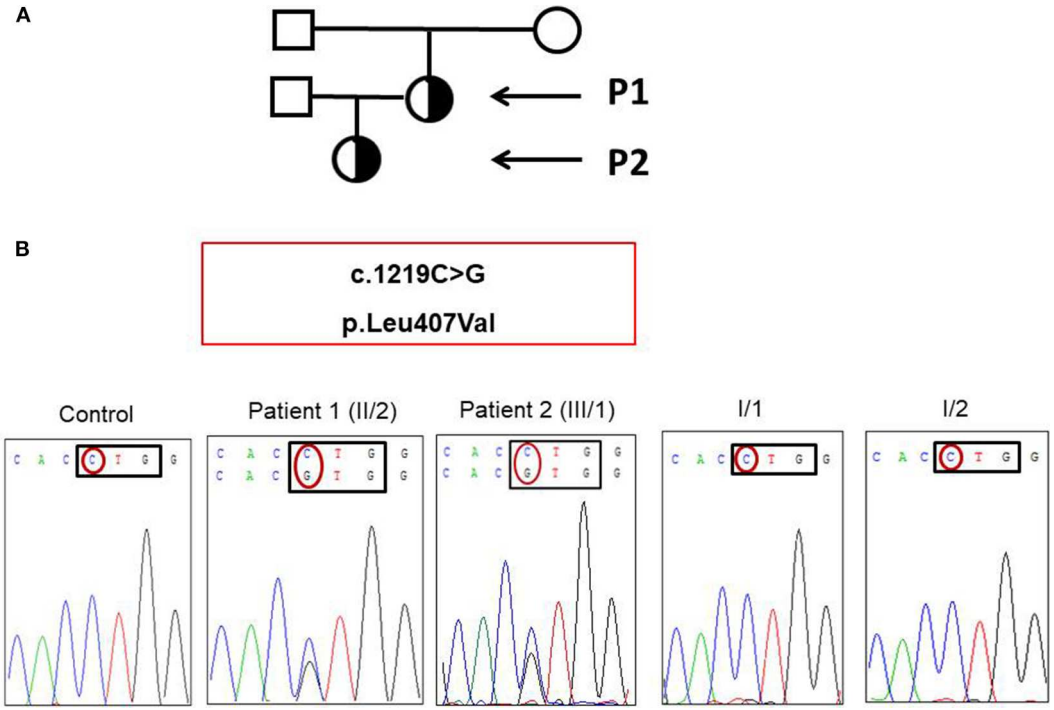

FIGURE 2 | (A) Pedigree of patient 1 and patient 2 (black arrows). **(B)** Novel heterozygous mutation affecting the DNA binding domain of *STAT1* was detected in the patient and her daughter.

birth (**Figure 2A**). She received routine immunization, and in addition, varicella, meningococcus C and tick encephalitis vaccines without complication. She had fluconazole responsive oral candidiasis treated for 2 months at age 1 year. She started kindergarten at 1½ year of age, and she had not developed Candida infection again but had 16 consecutive episodes of purulent otitis media and treated with several antibiotics over the past year. She has also had recurrent upper respiratory tract viral infections and underwent adenectomy at 2½ years of age. Despite the recurrent infections her growth and development has been appropriate for age. Genetic analysis revealed the same *STAT1* GOF mutation as found in her mother (See Results).

METHODS

Genetic Analyses

Genomic DNA from the patient and her relatives were isolated with the Gen Elute Blood Genomic DNA kit (Sigma-Aldrich, St Louis, Missouri, USA). Mutations were analyzed by amplifying exons and flanking intronic regions of *STAT1* by PCR. The PCR primers and sequencing primers are available on request. Amplicons were sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA). And targeted regions were analyzed by an ABI 3,130 capillary sequencer (Applied Biosystems). Sequence variants were determined compare to reference sequence, GenBank accession no. ENST00000361099 of the STAT1 cDNA to identify the position of mutations. Mutations are nominated according to Dunnen and Antonarakis (9). The pathogenicity of missense variant identified was investigated by *in silico* analysis using SIFT and PolyPhen2 software. According to the PolyPhen2 score the amino acid substitution is predicted to be "probably damaging" if the score is >0.908. According to the SIFT score the amino acid substitution is predicted to be "deleterious" if the score is <0.05.

Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare Bio-Science AB, Uppsala, Sweden) and resuspended in DMEM (Sigma-Aldrich Inc., St Louis, Missouri, USA) supplemented with 10% heat-inactivated FBS (Gibco) and 1% Pen/Strep (Sigma- Aldrich). Adherent monocytes were removed by incubation for 3–4 h at 37°C.

Western Blot

Generation of EBV-transformed lymphoblasts was carried out as previously described (10). EBV-B cells were stimulated by incubation with IFN-γ (PeproTech, London, UK, Eu). We assessed dephosphorylation by stimulation with IFN-γ and then incubation with staurosporine (Sigma-Aldrich) and nuclear proteins were extracted with Protein Fractionation Kit (Thermo Scientific, Rockford, IL, USA) and subjected to immunoblot analysis. We used antibodies against phosphorylated STAT1 (pY701, BD, San Jose, CA, USA), STAT1 (C-24, Santa Cruz, Dallas, Texas, USA), and lamin B1 (Santa Cruz, Dallas, Texas, USA).

Generation of IL-17-Producing T Cells

Non-adherent blood cells were cultured in anti-CD3 antibody-coated plates (Miltenyi Biotec, Bergisch Gladbach, Germany, Eu) in the presence of interleukin (IL)-23, IL-6, IL-1β, and transforming growth factor (TGF)-β1 (Pepro Tech). After 2 days, cells were restimulated with IL-2 (PeproTech), together with IL-1β, IL-23, IL-6, and TGF-β1. After 5 days, the cells were stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and ionomycin (IMC; Sigma-Aldrich) in the presence of GolgiPlug (Sigma-Aldrich) for flow cytometry analysis and for ELISA without GolgiPlug. The detailed protocol was described earlier (10).

Flow Cytometry

For flow cytometry analysis, the cells were incubated for the surface labeling with allophycocianin (APC)-conjugated anti-hCD4 IgG_1 monoclonal antibody (mAb) (BD, San Jose, CA, USA) and Peridinin chlorophyll (PerCP)-conjugated anti-hCD3 IgG_1 monoclonal antibody (BD). After fixation the cells were stained with phycoerythrin (PE)-conjugated mouse anti-human IL-17A IgG_1 mAb (R&D Systems, Minneapolis, MN, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-human IL-22 IgG_1 mAb (R&D Systems) antibodies. Cells were analyzed with an Accuri C6 flow cytometer (BD).

Elisa

C. albicans (ATCC 10231) was maintained and heat-inactivated as described earlier (10). Candida-induced secretion of IL-17A and IL-22 was determined by ELISA. The supernatants were harvested after stimulation of cells with PMA and ionomycin and after *in vitro C. albicans* stimulation. Human IL-17 Quantikine ELISA Kit and Human IL-22 Quantikine ELISA Kit (R&D Systems) were used according to the manufacturer's instructions.

Luciferase Reporter Assay

STAT1 mutations were generated by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit, Stratagene, La Jolla, CA, USA). We transfected the STAT1-deficient U3C fibrosarcoma cells with 100 ng/well reporter plasmids (Cignal GAS Reporter Assay, SA Biosciencies) and plasmids carrying the c.1219C>G (L407V), c.821G>A (R274Q), c.2117T>C (L706S), and wild type allele of *STAT1* or a mock vector in the presence of Lipofectamine LTX (Invitrogen). The transfected cells were then stimulated with IFN-γ (10 and 1,000 U/ml) and analyzed by Dual Luciferase assay system (Promega).

RESULTS

Mutational Analysis of *STAT1*

We found a novel heterozygous c.1219C>G mutation of *STAT1* located in exon 14 of the patient and her daughter (**Figure 2B**). This mutation was not found in the samples of the mother and father. The parents have had no recurrent and persistent mucosal disease. *In silico* predictions (SIFT and PolyPhen2) of the novel *STAT1* variant showed that this mutation was predicted to be "deleterious" and "probably damaging" with a score of 0.00 (SIFT), score of 0.999 (HumDiv, PolyPhen2; sensitivity: 0.14; specificity: 0.99), and score of 0.980 (HumVar, PolyPhen2; sensitivity: 0.57; specificity: 0.94), respectively. This mutation was not found in 50 healthy controls by using bidirectional DNA sequencing.

Gain-of-Phosphorylation of Mutant STAT1

We prepared EBV-B lymphoblasts from the patient and a healthy control. After incubation with IFN-γ we detected increased STAT1 phosphorylation in nuclear extracts of patient's EBV-B cells compared to that of control cells (**Figure 3**). We determined the dephosphorylation of STAT1 after stimulation with tyrosine kinase inhibitor staurosporine, and we found that dephosphorylation was impaired in the patient carrying the mutant allele (**Figure 3**). These data suggested GOF and loss-of-dephosphorylation of STAT1 due to the c.1219C>G mutation.

The New c.1219C>G Mutation of *STAT1* Results in GOF for γ-Activated Factor (GAF)-Dependent Cellular Responses

Plasmids with the new mutant alleles were created by site-directed mutagenesis and the position of the mutation was confirmed by Sanger sequencing. U3C cells, that lack of endogenous STAT1 were transfected with the new c.1219C>G (L407V) allele, a CMC-causing c.821G>A (R274Q) allele serving as positive control, an MSMD-causing c.2117T>C (L706S) allele serving as negative control, and the wild type allele of *STAT1*. The luciferase activity of the reporter gene was measured under the control of the γ-activated sequence (GAS) promoter. After 1,000 IU/ml IFN-γ stimulation two times stronger luciferase activity was measured in U3C cells transfected with the novel L407V allele and GOF R274Q allele than in cells with the wild type allele. These data suggested that the new allele is GOF for GAF-dependent cellular responses to IFN-γ (**Figure 4**).

Impaired Differentiation of CD4+ T Cells Into IL-17+ Cells in the Patient

We determined the percentage of CD3+/IL-17+ and CD3+/IL-22+ and CD4+/IL-17+ and CD3+/IL-22+ T cells after incubation with PMA and IMC as described. Cells from the patient with heterozygous L407V allele showed markedly decreased proportion of IL-17+ and IL-22+ T cells among CD3+ and CD4+ cells compared to healthy controls (**Figure S1**).

Secretion of IL-17A and IL-22 Cytokines

Cytokine concentrations were detected in the supernatant of PBMCs after stimulation with heat-killed Candida and from the supernatant of IL-17+ T cells after stimulation with PMA and IMC. PBMCs and T cells from the patient secreted decreased amounts of IL-17A and negligible amount of IL-22 compared to controls (**Figure S2**).

DISCUSSION

The unique feature of patient 1 was the chronic damage of mucous membranes of the oropharynx and esophagus without visible and detectable candidiasis most of the time. This report highlights the remarkable lack of awareness on

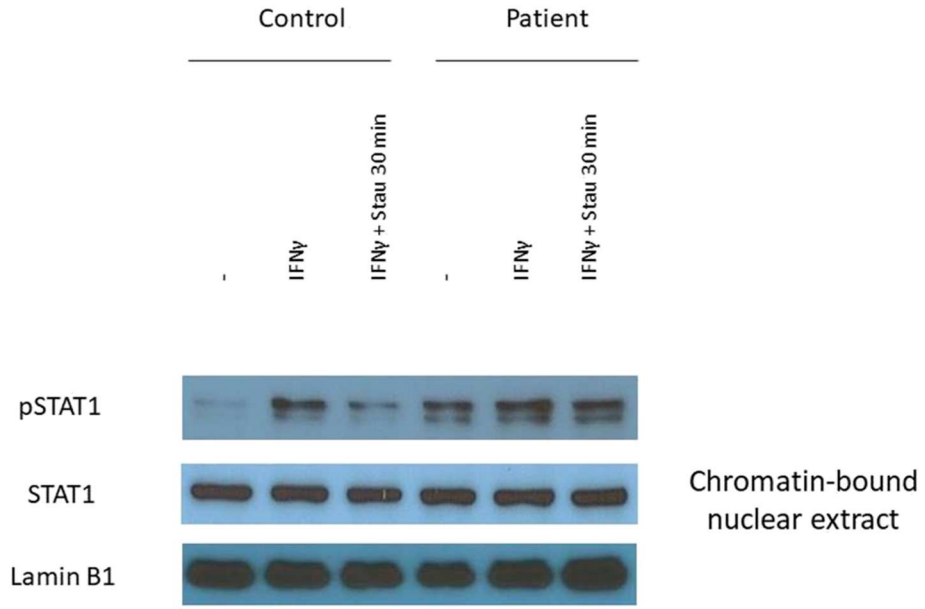

FIGURE 3 | The L407V amino acid change in STAT1 results in gain-of-phosphorylation. The expression of pSTAT1 and STAT1 were assessed by immunoblot. EBV-B lymphocytes were stimulated with IFN-γ for 30 min. To determine the inhibition of dephosphorylation we added tyrosine inhibitor staurosporine into the cell suspension for 30 min.

PID in the medical community and emphasizes the ongoing importance of physician education campaigns like the J Project (11, 12). Clinicians should be more aware of the heterogenous phenotype of *STAT1* GOF mutation. In patient 1 the "misleading" manifestation of the disease was the massive involvement of oropharyngeal mucosa and recurrent and persistent aphthous stomatitis and esophageal ulcers for decades. Aphthous stomatitis could be a complication of CMC. It is possible that once Candida causes damage to the mucous membranes additional host defenses like evasion of phagocytes and serum factors may control the overgrowth of fungi. In patient 1 several mucosal biopsies were performed, and the samples from the esophagus were negative for fungal infection, but positive for EBV genomic markers by PCR suggesting further the critical role of herpes viruses in the pathology of mucositis (7). *STAT1* GOF mutation may result in massive inflammation at mucosal surfaces resulting in aphthous stomatitis and ulcers which may be independent of candida mucositis; moreover, they might be manifestations of Crohn's disease in the upper gastrointestinal tract, as an autoimmune complication of STAT1 GOF mutation. The fact that the mucosal ulcers are responsive to prednisone suggest a role of immune activation in the pathogenesis. From the clinical point of view, we believe that mutational analysis of *STAT1* and enumeration and characterization of Th17 cells is warranted in patients with recurrent and progressive oral and esophageal mucositis without solid clinical and immunology diagnosis.

Meyer et al. (13) proposed that the single L407V amino acid change, did not affect the subcellular resting distribution of STAT1 or nuclear clearance after interferon-γ stimulation. It was suggested that changing from leucine to isoleucine or valine may not affect the hydrophobic nature of the side chains

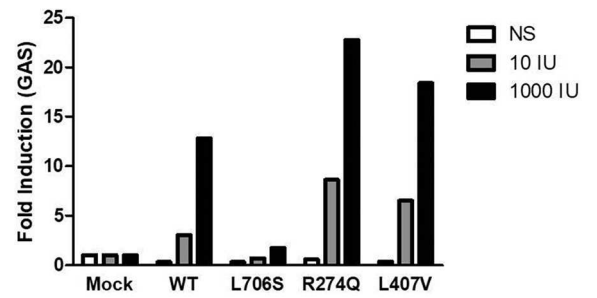

FIGURE 4 | Fold induction of γ-activated sequence (GAS)-dependent reporter gene transcription activity was assayed after stimulation with 10 IU/ml or 1,000 IU/ml IFN-γ. The R274Q allele used as positive control, the L706S allele as negative control, and the L407V as a new mutant allele were transfected to U3C cells. The data represent 2 independent experiments performed in triplicate.

(13). In contrast, we found impaired development of IL-17+ and IL-22+ T cells associated with the higher susceptibility to Candida infections in patient 1. These findings were in concert with the low amount of IL-17+ and IL-22+ T cells and small concentrations of IL-17A and IL-22 cytokines released by Candida-exposed mononuclear blood cells. Our data suggest that this novel mutation which may not interfere with transport mechanisms such as import-export of STAT1 is still pathogenic. Higher activity of the luciferase reporter gene under the control of the GAS promoter analyzed in U3C cells transfected with L407V allele after IFN-γ stimulation compared to those transfected with the wild type or MSMD-causing alleles confirmed this hypothesis.

In conclusion, the new mutant allele is GOF and adds to the list of *STAT1* GOF mutations (14). The unique, but not exceptional clinical phenotype in this patient remains to be elusive. Heterozygous *STAT1* GOF patients show an unexpectedly broad clinical phenotype (6, 14). Environmental factors and effects of modifying genes involved in phenotypic manifestation of *STAT1* L407V mutation may have a role.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University of Debrecen. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. ME: diagnostic workup, evaluation of data, and writing the paper. EJ: design of diagnostic setup and follow the patient. BS and BT: performing laboratory experiments. ZB-C: designed the experiments, follow up and treatment the patient, and writing the paper. LM: study design, treatment coordination, differential diagnostic workup, treatment the patient, writing the paper, and coordinating research.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00967/full#supplementary-material

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The Clinical and Genetic Spectrum of 82 Patients With *RAG* Deficiency Including a c.256_257delAA Founder Variant in Slavic Countries

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Background: Variants in recombination-activating genes (*RAG*) are common genetic causes of autosomal recessive forms of combined immunodeficiencies (CID) ranging from severe combined immunodeficiency (SCID), Omenn syndrome (OS), leaky SCID, and CID with granulomas and/or autoimmunity (CID-G/AI), and even milder presentation with antibody deficiency.

Objective: We aim to estimate the incidence, clinical presentation, genetic variability, and treatment outcome with geographic distribution of patients with the *RAG* defects in populations inhabiting South, West, and East Slavic countries.

Methods: Demographic, clinical, and laboratory data were collected from *RAG*-deficient patients of Slavic origin via chart review, retrospectively. Recombinase activity was determined *in vitro* by flow cytometry-based assay.

Results: Based on the clinical and immunologic phenotype, our cohort of 82 patients from 68 families represented a wide spectrum of *RAG* deficiencies, including SCID ($n = 20$), OS ($n = 37$), and LS/CID ($n = 25$) phenotypes. Sixty-seven (81.7%) patients carried *RAG1* and 15 patients (18.3%) carried *RAG2* biallelic variants. We estimate that the minimal annual incidence of *RAG* deficiency in Slavic countries varies between 1 in 180,000 and 1 in 300,000 live births, and it may vary secondary to health care disparities in these regions. In our cohort, 70% ($n = 47$) of patients with *RAG1* variants carried p.K86Vfs*33 (c.256_257delAA) allele, either in homozygous ($n = 18$, 27%) or in compound heterozygous ($n = 29$, 43%) form. The majority (77%) of patients with homozygous *RAG1* p.K86Vfs*33 variant originated from Vistula watershed area in Central and Eastern Poland, and compound heterozygote cases were distributed among all Slavic countries except Bulgaria. Clinical and immunological presentation of homozygous *RAG1* p.K86Vfs*33 cases was highly diverse (SCID, OS, and AS/CID) suggestive of strong influence of additional genetic and/or epigenetic factors in shaping the final phenotype.

Conclusion: We propose that *RAG1* p.K86Vfs*33 is a founder variant originating from the Vistula watershed region in Poland, which may explain a high proportion of homozygous cases from Central and Eastern Poland and the presence of the variant in all Slavs. Our studies in this cohort of *RAG1* founder variants confirm that clinical and immunological phenotypes only partially depend on the underlying genetic defect. As access to HSCT is improving among RAG-deficient patients in Eastern Europe, we anticipate improvements in survival.

Keywords: *RAG1, RAG2*, primary immunodeficiency, geographic distribution, incidence, Slavic children

INTRODUCTION

Recombination-activating gene 1 (*RAG1*) and 2 (*RAG2)* encode lymphoid-specific proteins that are expressed during the early stages of T-cell and B-cell development and initiate the process of V(D)J recombination by introducing DNA double-strand breaks (DSBs) for recognizing millions of possible antigens (1). Genotype–phenotype correlation is strong, as null variants of *RAG1* and *RAG2* genes result in the T- B- severe combined immune deficiency (SCID) phenotype, whereas hypomorphic *RAG* variants have been associated with distinct clinical entities including Omenn syndrome

(OS) and combined immunodeficiency with granuloma and/or autoimmunity (CID/G-AI) with herpesvirus infections and lymphoproliferation (2–5) and with selective polysaccharides antibody deficiency (6). Furthermore, in the era of widespread next-generation sequencing, RAG deficiency is being identified among adults with variants of antibody deficiencies with a frequency of 1 in 500 patients (7).

The *RAG1* and *RAG2* genes are polymorphic. Described clinical phenotypes are associated with a variety of variants including non-sense, frameshift, in-frame deletion or insertion, and missense variants of the *RAG1* and *RAG2* genes that affect various domains of the proteins (1). Among numerous *RAG* variants, some of them were observed in a Jewish population with a high rate of consanguineous marriages (8). In our previous report of 11 OS patients from the East Slavic regions, we described the high rate of c.256_257delAA (p.K86Vfs*33) in the *RAG1* gene ($n = 4$, 50%) (9). This variant was also observed in Polish and Serbian patients with OS and SCID phenotypes (10–13), which suggests a founder effect.

Currently, there is no published systematic evaluation of Slavic patients with *RAG* deficiency. In this report, we aim to describe the genetic landscape of *RAG* deficiency by estimating the incidence, genetic diversity, clinical and immunological presentation, and survival rate of a large cohort of Slavic patients. In addition, we focus on *RAG1* p.K86Vfs*33 as a candidate founder variant among Slavic patients by studying the geographic distribution of allele and genotype frequencies of *RAG1* p.K86Vfs*33 mutation among patients in major Slavic populations.

MATERIALS AND METHODS

Patients and Kindreds

Patients with pathogenic *RAG* variants were recruited retrospectively for this study through extensive collaboration with clinical immunologists who collected the data of national primary immunodeficiency (PID) registries from *East Slavs* (Russia, Belarus, and Ukraine), *West Slavs* (Poland, Czech Republic, and Slovakia), and *South Slavs* (Serbia, Slovenia, Montenegro, Croatia, and Bulgaria). The patients were divided into ethnic groups (East, West, and South Slavs) according to their country of origin.

Ethics Statement

Informed consent forms were signed by the parents as requested and approved by the institutional review boards of various institutions involved. The protocol of study was approved by the institutional review board of Belarusian Research Center for Pediatric Oncology, Hematology, and Immunology (IRB0012-2015).

Study Design

A detailed questionnaire was completed by the physicians including demographic data (gender, country, place of birth, and year of birth), variants, and clinical data (age at disease manifestation, age at diagnosis, clinical and immunologic phenotype, and outcome).

Assignment to Phenotypic Subgroups

Corresponding clinicians from each country assigned patients to one of the following subgroups—SCID, OS, atypical SCID (AS), or combined immunodeficiency (CID)—on the basis of clinical and immunologic phenotype, and age at manifestation.

The Primary Immunodeficiency Treatment Consortium (PIDTC) diagnostic subdivides SCID into three categories: typical SCID, atypical SCID, and OS, based on total T-cell count, lymphocyte proliferation, presence of maternal T cells, characteristic phenotypic features, and gene defects. CID was classified by late and mild clinical presentation according to published criteria (4, 14). Roifman et al. distinguished CID from SCID based on a total CD3+ T-cell count of >500/μl (15). The 2019 ESID criteria for diagnosis of CID requires a symptomatic patient (infections, immune dysregulation) or history of affected family members with immune phenotype of two of the four parameters (low CD3 or CD4 or CD8 T cells, low naïve CD4 and/or CD8 T cells, elevated γδ T cells, and reduced proliferation to mitogen or TCR stimulation). This ESID 2019 criterion does not discuss underlying genetic defects for CID patients (16). The IUIS 2020 classification does list genetic defects for CID but fails to include *RAG* deficiency in this category (17).

To estimate the number of newborns with *RAG* variant in each country, the information about affected siblings in families with *RAG* variants was collected through hospital or personal records of affected families.

Mapping of Variants

Mapping was performed by the *ArcGIS 10.5* program on the map of Central–Eastern Europe and Russia.

RAG1/RAG2 Sequencing

Gene sequencing was performed using standard techniques (panel-based, Sanger) in local laboratories in Europe. The reference DNA and protein sequences for *RAG1* are from NIH RefSeq NM_000448.2 and NP_000439.1 and those for *RAG*2 are from NM_001243785.1 and NP_001230714.1, respectively.

Measurement of Recombination Activity

For single-allele mutation, assay is based on a v-Abl *Rag1*/*Rag2*$^{-/-}$ pro-B cell line containing a single pMX-INV integrated cassette (18).

TRECs and KRECs

TRECs and KRECs quantity was measured by RQ-PCR using plasmid standards, and ALB was taken as an internal control according to manufacturer instructions.

Statistical Analysis

Allele frequencies were compared by chi-square test or Fisher's exact tests. Kaplan–Meier curve was computed for survival post-hematopoietic stem cell transplant (HSCT). $p < 0.05$ was considered significant. Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Population Demographics

We retrospectively collected and studied 82 patients with RAG deficiency from 67 families in 12 countries, born in the period of 1992–2018. Thirty-five of 82 (43%) patients were reported previously (9–13, 19–23). Distribution was even between males ($n = 40$, 49%) and females ($n = 42$, 51%). The median age at diagnosis of *RAG* was 1.3 years (range, 1 day to 24 years).

At the time of manuscript submission (March 2020), 43 of the 82 patients died (52%, 8 out of 43 after HSCT). All patients alive received HSCT (ranging from 1 to 18 years) except three females with LS/CID presentation from Poland (42_f), Bulgaria (54_f), and Czech Republic (55_f) who are alive without transplantation at the ages of 3, 6, and 25 (**Supplementary Tables E1** and **E2** in this article's Online Repository).

The largest group of patients ($n = 40$, 48.8%) was West Slavs from Poland ($n = 29$, $n = 1$ from Lithuania, Polish origin), Czech Republic ($n = 8$) and Slovakia ($n = 2$). The second largest group was East Slavs ($n = 30$, 36.5%) from Russia ($n = 24$), Ukraine ($n = 4$), and Belarus ($n = 2$). The smallest group was South Slavs ($n = 12$, 14.6%) from Serbia ($n = 6$), Slovenia ($n = 3$), Croatia ($n = 1$), Montenegro ($n = 1$), and Bulgaria ($n = 1$).

Clinical, Immunological, and Genetic Phenotypes

All patients have genetically confirmed pathogenic *RAG1 or RAG2* variants. *RAG1* variants were detected in 67 patients in 54 families (82%) and *RAG2* variants were detected in 15 patients from 13 families (18%). Nineteen children (23%) out of 16 families and 8 children (10%) out of 7 families were homozygotes while 47 patients (59%) out of 39 families and 8 patients (10%) out of 6 families were compound heterozygotes for *RAG1* and *RAG2* variants, respectively (**Supplementary Tables E1** and **E3**).

Clinical phenotypes, as determined by attending clinician following immunological and clinical guidelines, included SCID ($n = 20$), OS ($n = 37$), AS ($n = 21$), and CID ($n = 4$) with highly mixed immune phenotypes (different numbers of T cells in all groups and domination of B+ phenotype in AS group); however, *in vitro* relative recombinase activity showed good correlation between genotype and clinical cathegories. (**Figure 1C**, and **Supplementary Tables E1** and **E3** in this article's Online Repository). Four (10%) out of 41 vaccinated SCID patients with RAG1/2 variants developed BCGosis infection, and 12 (3%) BCGitis were noted. As expected, the AS group had increase frequency of autoimmune cytopenias and history of herpesvirus infections (**Figure 1A,B** and **Supplementary Table E2**).

Patients With RAG1 Variants

Patients with RAG1 variants are described in **Supplementary Tables E1** and **E2**. Most patients presented with OS (46%) (31 children from 26 families) followed by AS (28%) (19 patients from 16 families), SCID (19%) (13 patients from 11 families), and CID (6%) (4 patients from 4 families).

Patients diagnosed as having OS and SCID were characterized by classical clinical and immunologic phenotype (**Supplementary Table E1**). Their clinical phenotype allowed for diagnosis before the age of 1 year. On the other hand, the median age at the time of diagnosis in AS and CID was 4 years (± 6.02 years) and ranged from 5 months to 24 years (**Supplementary Tables E1** and **E2**).

In the AS group, infectious complications were notable for high prevalence of CMV infection ($n = 9$, 42%) in both localized ($n = 2$, retinitis) and systemic forms ($n = 7$). Among patients who received BCG vaccination, only a fraction (4 of 18, 22%) developed BCG infections (five with BCGitis and one with BCGosis) (**Supplementary Table E2** and **Figure 1A**).

Autoimmune findings in the AS and CID group were observed in 12/19 and 4/4 of patients (**Supplementary Table E2**), with autoimmune cytopenia being most prevalent in AS ($n = 11/19$, 58%) including autoimmune hemolytic anemia ($n = 8$), isolated immune thrombocytopenia (ITP) ($n = 2$), and multiple lineage cytopenia ($n = 2$). Autoimmune colitis was diagnosed in 9% (2/19) of AS patients with autoimmunity. In the CID group, one patient had skin granuloma, one developed vasculitis, and one had vitiligo (**Supplementary Table E2** and **Figure 1B**).

Immunological phenotype of the AS group is summarized in **Supplementary Table E1**. Curiously, high fraction of the patients had preserved fraction of B cells (>65% in 13 of 19 patients) at the time of diagnosis. However, 2 of 7 (28%) patients had complete absence of KRECs. This suggests abnormal B cell maturation even in the setting of preserved fraction of total B cells. Four patients presented with normal IgG level, and one had IgA deficiency at the age of 4 years that progressed to agammaglobulinemia with age.

In this study, we additionally determined the recombination activity of patient's RAG1/RAG2 mutant proteins. Of the *RAG* variants, 38 variants were unique, including frameshift ($n = 7$), non-sense ($n = 4$), and missense ($n = 27$), and 23 *RAG1* mutations were new in our cohort, and for all of them, recombination activity was determined (**Supplementary Tables E1** and **E2**). We had the possibility to demonstrate that mutations associated with SCID and OS had lower residual activity than mutations detected in patients with less severe clinical presentations (AS and CID) (**Figure 1C**). Nevertheless, genotype–phenotype correlation was less evident in our cohort; we have not found significant differences between OS/SCID and AI/CID in clinical and laboratory markers. Among the patients with homozygous *RAG1* p.K86Vfs*33 (c.256_257delAA) variant ($n = 18$), the clinical and immunological presentation was highly diverse, 4 patients presented as OS, 1 as SCID, and 13 as AS (**Supplementary Tables E1** and **E2**); these findings were supported by **Supplementary Table E1** Pasic et al. (12). This is suggestive of the strong influence of other genetic and/or epigenetic factors in shaping the final clinical and immunological phenotype.

RAG1 Variants

Homozygous *RAG1* variants were detected in 18 families, but consanguinity was identified only in 4 (22%) [3 families from Poland (2a_m, 2b_m; 39_f; 41a_m, 41b_m) and 1 from Serbia (44a_m, 44b_f, 44c_m)].

The most frequent genetic *RAG1* anomaly among patients of all groups and in 10 out of 11 countries was *RAG1* p.K86Vfs*33 (c.256_257delAA) (**Supplementary Table E1**). This

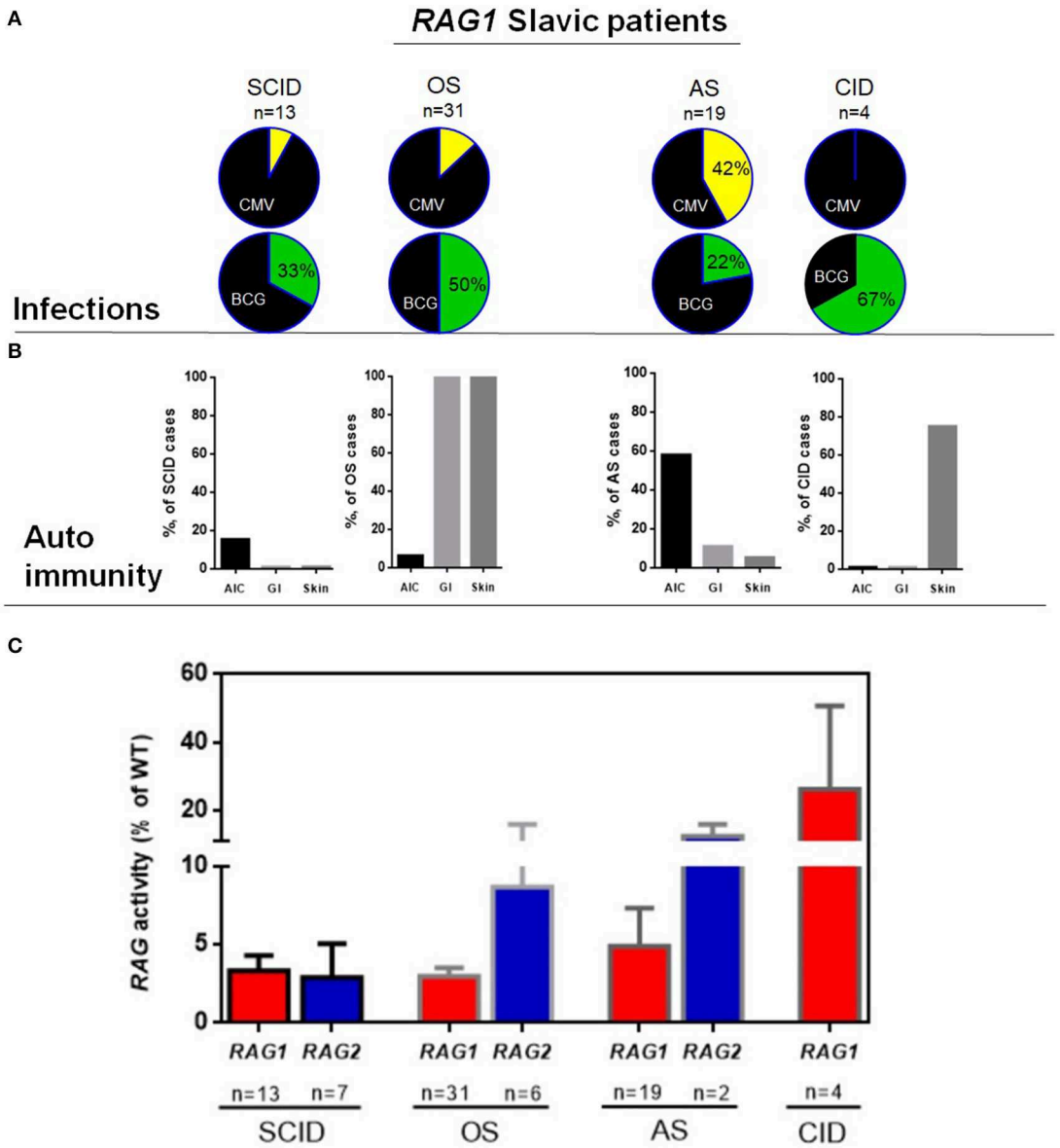

FIGURE 1 | Characteristics of RAG Slavic cohort. **(A)** Infectious complications in *RAG1* patients, upper row CMV in four groups, lower row BCG complications among patients, who received BCG vaccination. **(B)** Frequency of autoimmunity in 4 groups of *RAG1* deficiency. **(C)** Recombinase activity of *RAG1/RAG2* variants for the 82 patients divided into four major clinical presentations for *RAG1* and three clinical presentations for *RAG2*.

deletion occurred in one or both alleles in 22 out of 30 patients with OS, in 7 out of 13 SCID patients, and in 16 out of 19 patients with AI and 2/4 CID phenotype. Because of the enrichment of the p.K86Vfs*33 variant among all cohorts, we calculated the frequency of this allele among East, West, and South Slavic patients (**Figures 2A–F**) and their families (data not shown). Nine families had two or three children with immunodeficiency, and therefore, we conducted a separate analysis of families and analyzed frequency between countries counting one case per family.

Patients of South and West Slavic origin demonstrated the highest frequency of c.256_257delAA allele (**Figures 2C,E**). We analyzed the relative frequency of this predominant allele among Slavic patients and families. The differences in the allele frequency of c.256_257delAA frequency among the patients ($\chi^2 = 8.4$; $p = 0.015$) and the families ($\chi^2 = 22.8$; $p < 0.0001$) of the East/West/South Slavs proved to be statistically significant. In pairwise comparisons, the West Slavic population had a higher relative frequency of this allele than the East Slavic (families $p = 0.0006$; patients $p = 0.006$), but it did not differ from the South Slavic population (families $p = 0.14$; patients $p = 0.81$).

Besides the analysis of different Slavic cohorts, we calculated the contribution of each country to the overall frequency of *RAG1* c.256_257delAA allele and showed the highest occurrence in Poland (**Figure 2D**). *RAG1* c.256_257delAA allele (p.K86Vfs*33) was present in over 50% of *RAG1*-deficient patients from Serbia,

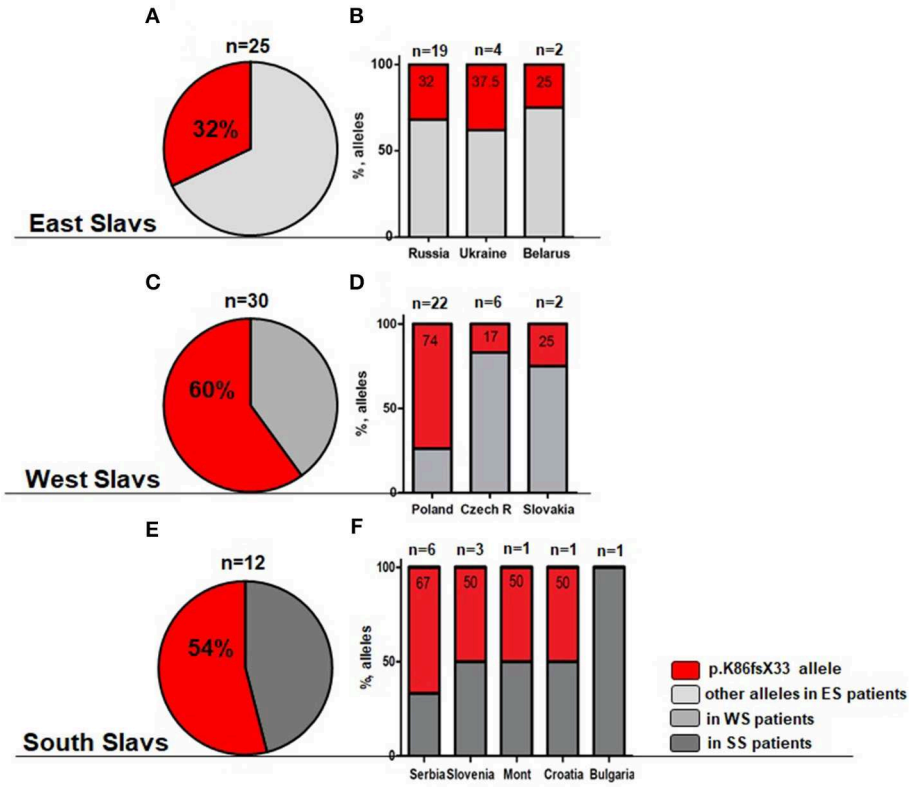

FIGURE 2 | Overall frequency of c.256_257delAA allele (p.K86Vfs*33) among all cohort of *RAG1* patients. **(A,B)** Light gray—East Slavs (ES); **(C,D)** Gray—West Slavs (WS); **(E,F)** Dark gray—South Slavs (SS); **(B,D,F)** Percentage of p.K86Vfs*33 allele in studied countries. In Montenegro and Croatia, only one patient was studied.

Slovenia, Montenegro, and Croatia, but extrapolating this finding to the whole South Slavic population is premature because only one patient was from Croatia and Montenegro (**Figure 2F**).

Among East and South Slavs, we observed only two families (one from Ukraine and one from Serbia) where p.K86Vfs*33 was detected in a homozygous constitution, but consanguinity was proved only in the Serbian patients. The Polish cohort of families showed 12 homozygous genotypes among 18 *RAG1* positive families, with only three cases of proven consanguinity.

Due to the high frequency of the *RAG1* p.K86Vfs*33 variant in Poland and homozygous genotypes, we studied its geographical distribution in West, East, and South Slavic populations. Our findings are illustrated on the map of East Europe and Russian Federation based on the patient's place of origin (one patient from each family) (**Figure 3**).

We studied the distribution of *RAG1* p.K86Vfs*33 variant in 11 out of 13 Slavic ethnic groups and it was widespread and found in 10 out of 11 countries except Bulgaria (**Figure 3**). The occurrence of p.K86Vfs*33 was observed from Ljubljana (Slovenia) in the West to Vladivostok in the East and from Moscow in the North to Cetinje (Montenegro) in the South (**Figures 3**, **4**). One patient who originated from Slovenia was born in Austria (Graz), but after delivery, he was treated in the homeland of Slovenia (**Figure 3**).

The geographic center of the presumed origin of the *RAG1* p.K86Vfs*33 variant corresponds to the Vistula watershed area of Central and Eastern region in Poland in which the variant was the most frequent (**Figure 3B**) and suggestive of shared origin for the p.K86Vfs*33 alleles in all Slavic populations.

Due to a small number of cases, gradual decrease in the prevalence of p.K86Vfs*33 among East and South Slavs could not be determined, but it is clearly shown that heterozygous families are spread among other Slavic countries from the Polish area of homozygosity (**Figure 3**).

The broadest area of East Slavs in Russia is located mainly in the European part of the Russian Federation (25). In our cohort of patients from Russia, the majority (11/19) is concentrated on the territory west of the Ural Mountains, but 8 patients from 5 families were born in Siberia and the Russian Far East, and all inherited our studied variant (**Figure 4**). This finding does not contradict our assumption that p.K86Vfs*33 has a Slavic origin. According to the map of the resettlement of peoples in Russia, most Russians live in the central part or in the south and northwest of Russia as well as in the Ural region. To the east of the Ural Mountains, Russians live predominantly near the southern border of Russia, which is shown in **Figure 3** and in **Figure 4** as a light gray area on the white map of the Russian Federation.

Patients with RAG2 Variants

Patients with RAG2 variants were detected only in three countries among West (Poland and Czech Republic) and East Slavs (Russia) (**Supplementary Table E3**). Among 15 patients from 13 families, 7/15 presented as SCID, 6/15 presented as OS,

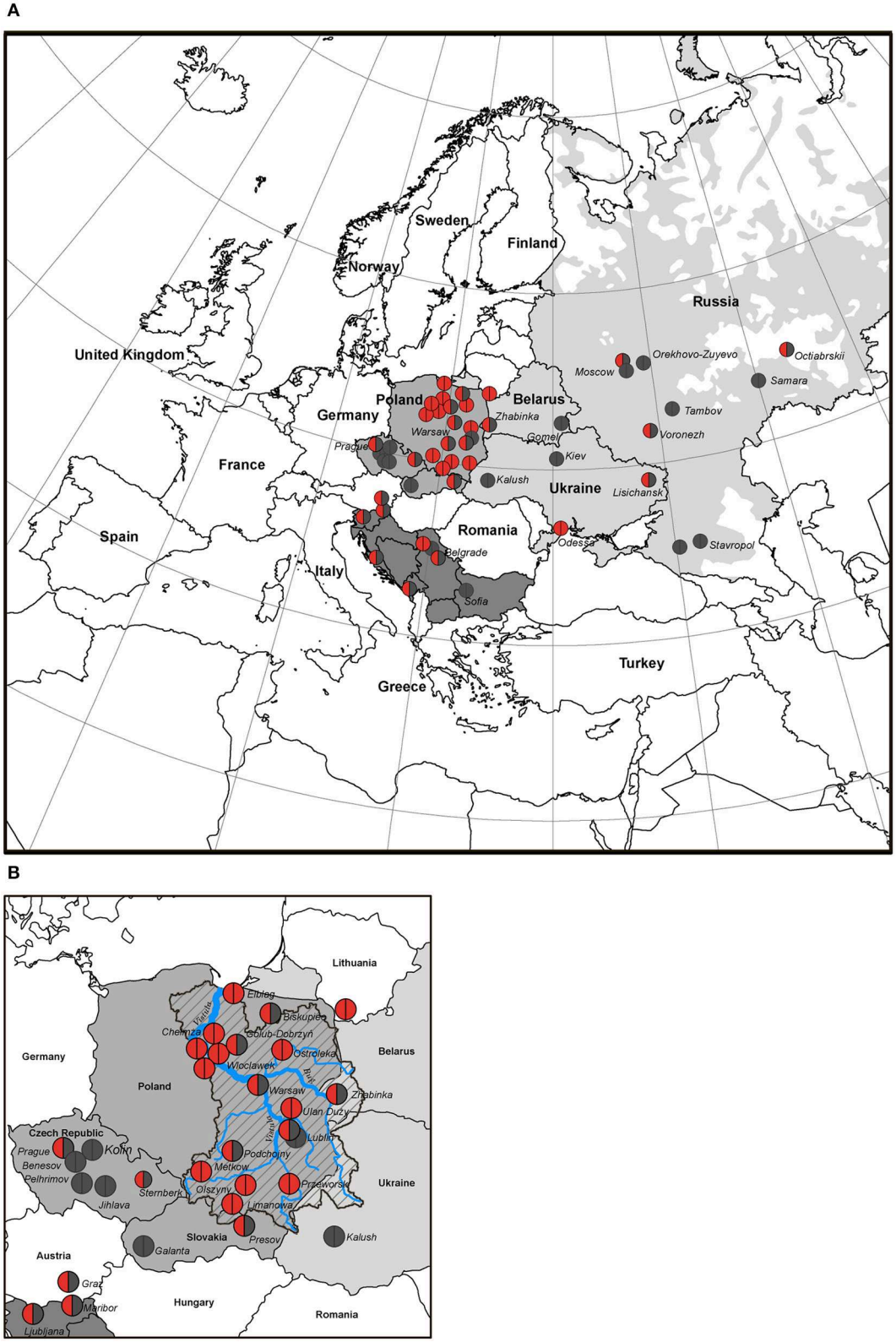

FIGURE 3 | Distribution map of families with *RAG1* p.K86Vfs*33 variant in Slavic countries (50 of 55 families). **(A)** The birthplace of the patients is indicated by the location of the circles; homozygous p.K86Vfs*33 variant is represented by red circles; heterozygous p.K86Vfs*33 variant is half red/half gray, and other variants are gray. **(B)** Map of the Western Slavs; the Vistula River basin is overlaid on the map of Poland. The blue line is the Vistula River, a zone marked by an oblique black line—the Vistula River basin, the geographic area coincides with the region of the largest concentration of families where patients with p.K86Vfs*33 homozygous variants were born.

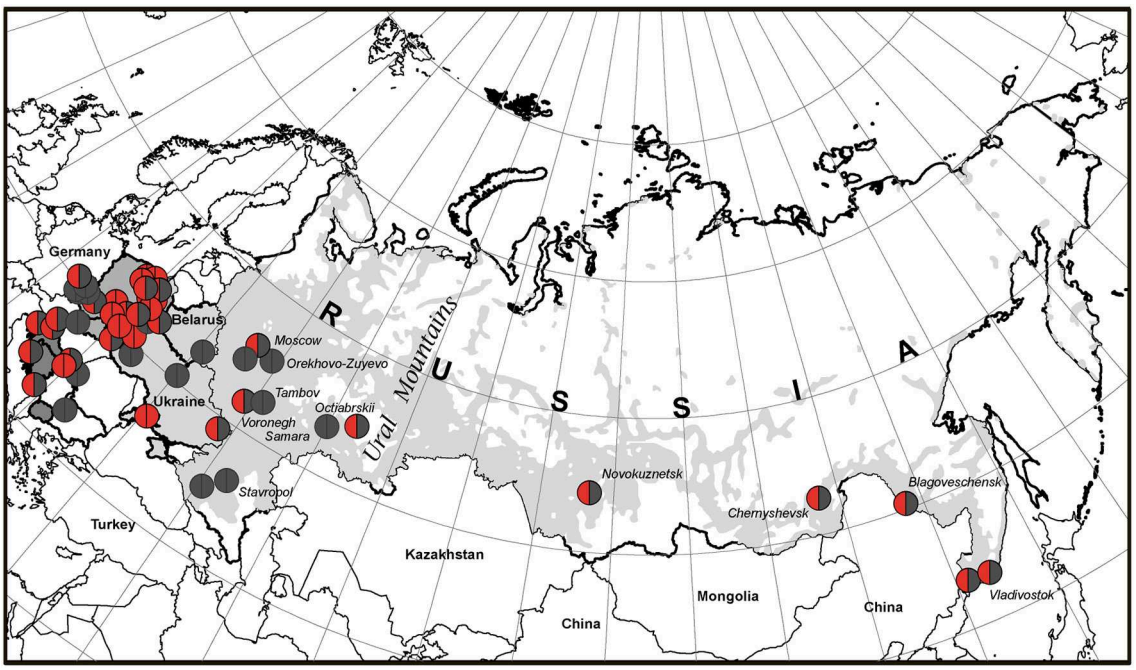

FIGURE 4 | The geographic picture of the p.K86Vfs*33 distribution among East Slavs. Light gray zone on the map of Russia is the territory of predominant Russian settlement (24).

and 2/15 presented as AS. Two Polish females manifested as atypical SCID/CID and one had a sister with SCID (8a_ f). In one Russian family, one male presented as OS (3b_ m) and one presented as SCID (3a_ m).

Females with late manifestation of *RAG2* variant presented with encephalitis, HSV, bronchitis, pneumonia, neutropenia and pneumonia (8b_f), local BCGitis, and failure to thrive (12_f).

Interestingly, the obtained data showed that 53% (8/15) of patients had homozygous variants in the *RAG2* gene, but consanguinity was established only in one family history (1_m). Despite the marked diversity of the genotypes, there are some repeated variants, p.Y434H in Russian, p.R229Q in Polish and Czech children (in homo and in heterozygous state), and p.W453R only among Polish patients. However, an obvious dominating region among variants was not established due to a small number of the studied cases.

The *RAG2* variants in Slavic cohort showed a wide range of recombination activity (**Supplementary Table E3**). Mutations associated with severe combined immune deficiency and OS had lower activity than those detected in patients with atypical SCID presentations (**Supplementary Table E3** and **Figure 1D**). Our data support genotype–phenotype correlation in *RAG2* deficiency in Slavic children (18).

The Average Annual Incidence of Slavic Patients With *RAG* Deficiency and Survival

We examined the incidence of all types of *RAG* deficiency together (*RAG1/RAG2* genotype; OS/SCID/AS/CID phenotype) in each country during different periods. The average incidence of *RAG* deficiency was calculated based on the number of all diagnosed patients and their confirmed siblings and the number of live births in studied countries during the period from the year of the first registered patient in the country to the last one (**Table 1**). The average 10-year incidence was estimated by the number of all patients of Slavic origin diagnosed since 2008 to 2017 in each country (**Table 1**, last column).

The obtained data showed that frequency of diagnosed *RAG* deficiency ranged from 1 per 75,000 in Slovenia to one per 1,100,000–1,600,000 live births in Russia and Ukraine.

A wide range in frequency among Slavic countries suggest underdiagnosis of atypical late-onset cases. We compared the two largest *RAG* cohorts from Russia ($n = 24$) and Poland ($n = 30$). In further analysis, we graphically illustrated the number of diagnosed patients in the two countries (the age at diagnosis was added to the year of birth) and showed that Russia achieved great success in identification of *RAG* patients in the last 8 years (**Figure 5A**, lower). Exponential growth (**Figure 5A**, upper) was observed; 19 out of 24 *RAG* patients were diagnosed from 2011 to 2017, and their geography covered the entire country (**Figure 4**). Poland, as the largest West Slav country, uses advanced immunologic diagnostics services and one to three patients have been confirmed approximately each year since 1999 (**Figure 5B**).

Despite an increased awareness for SCID and OS variants and improved access for HSCT in most Slavic countries, the mortality rate of *RAG* patients is still high in Eastern Europe. We studied relative mortality rate among OS/SCID/CID groups of patients with *RAG* deficiency (**Figure 5C**—ES, **Figure 5D**—WS, and **Figure 5E**—SS). Neither comparison of transplanted patients in two groups (WS and ES) as biggest cohorts revealed

TABLE 1 | Average annual incidence of *RAG* deficiency in Slavic countries.

No	Country	Number of patients with RAG	Number of families	Whole period	Sum of newborns for the period	The average annual incidence frequency	Number of patients in 10 year period*	Sum of newborns for the 10-years period	The average 10-years incidence*
EAST SLAVS									
1	Russia	24	18	2002–2017	26 557 598	1:1 106 566	18	17 553 840	1:975 213
2	Ukraine	4	4	2004–2013	4 834 300	1:1 208 575	3	4 795 200	1:1 598 400
3	Belarus	2	2	1998–2009	1 145 223	1:572 611	1	1 127 193	-
WEST SLAVS									
4	Poland	28	23	1999–2017	7 201 693	1:257 203	11	3 904 235	1:354 930
5	Czech Republic	8	8	1993–2016	2 395 156	1:299 395	6	1 126 990	**1:187 832**
6	Slovakia	2	2	2007–2015	515 219	1:257 609	2	572 776	1:286 388
SOUTH SLAVS									
7	Serbia	6	3	1992–2011	1 529 226	1:254 871	1	671 049	-
8	Slovenia	3	3	2003–2013	223 206	**1:74 402**	1	212 986	-
9	Montenegro	1	1	1995	9 492	-	0	76 785	-
10	Croatia	1	1	2010	43 361	-	1	411 277	-
11	Bulgaria	1	1	2013	66 578	-	1	715 571	-

*Bold values indicate the highest average frequency of RAG deficiency. *10-year period is 2008–2017.*

any statistically significant difference in survival (**Figure 5F**). Additionally, we compared the results of HSCT before and after 2011, due to changing the protocols in last years and we were able to show that survival of East Slavic patients became much better after 2011 and reached the same percentage as in West Slavs (**Figures 5G,H**). We suggest that poor outcome in HSCT before 2011 (three out of five died after HSCT) in East Slavs was due to delayed diagnosis in AS/CID patients and associated complications (chronic infections and immune dysregulation) (**Supplementary Figure E1C**). Patients with OS were transplanted with a high rate of survival in ES/WS/SS (**Supplementary Figure E1B**) and with SCID in ES/WS countries (**Supplementary Figure E1D**).

DISCUSSION

Several cohort studies with international collaborators are published, and a recent review of literature enumerate the number of published cases with *RAG* deficiency over 400 (2, 4, 7–9, 13, 14, 23). Among the population with high rate of consanguity, founder variants for *RAG* genes were proposed among the Amish and in the Middle East (26–28).

In this study, we present the largest geographically defined cohort of patients with *RAG* deficiency. Our patients are predominantly of Slavic origin from 11 European countries retrospectively collected in a 27-year period (1992–2018). In our current and previous study of patients with *RAG1* p.K86Vfs*33 (c.256_257delAA), we confirmed that different clinical phenotypes can been found in patients with identical variants even within the same family (9, 13). Family studies for homozygous *RAG1* p.S480G and *RAG2* p.M459L were also reported in consanguineous marriages of Arabic descent (29, 30). Furthermore, similar non-familiar findings were reported with patients with *RAG1* c.519delT (p.E174SfsX27) (13). Among all these reports, our study with 18 homozygous patients *RAG1* p.K86Vfs*33 (c.256_257delAA) is the largest in the literature within a geographically linked population.

The data from California's SCID newborn screening identified *RAG1/2* variants in 28.6% of SCID/OS cases, with an incidence of about 1:250,000 (31). In populations with domination of consanguineous marriages, *RAG* deficiency was diagnosed in 48% (21/44) of Turkish (32) and 51% (19/37) of Iranian SCID patients (33). The incidence of *RAG* deficiency is difficult to establish, due to the highly variable phenotype, late age of manifestation of hypomorphic variants, and different level of diagnostics capacities in different countries (5). Recently, it was identified that RAG deficiency among adults with a variety of antibody abnormalities occurs with a frequency of 1 in 500 patients with any variant of antibody deficiency (7). That is much higher than the expected number of *RAG1/2* SCID or OS cases. Based on our data reported in this study, we determined that the minimal incidence of *RAG* deficiency varied from 1:189,000 in the Czech Republic to 1:1,200,000 in Ukraine; the average was 1 per 190,000–300,000 live newborns.

It is still unclear if all *RAG* patients with AS/CID phenotype (with residual recombination activity) can be detected by newborn screening (NBS) (14). Retrospective testing of NBS card showed absence of TRECs in case reports (34). Current observation showed that KREC level can support and underlying *RAG* deficiency is relevant and further justifies the consideration of KREC in universal NBS for SCID and AS/CID. Our retrospective data suggest that most of the patients with late-onset forms of *RAG* deficiency (CID) are likely missed, but the level of discovery of SCID/OS patients is reasonable in most Slavic countries. There is a great need for early identification of these patients before infectious and autoimmune complications occur. The availability of next-generation sequencing and the

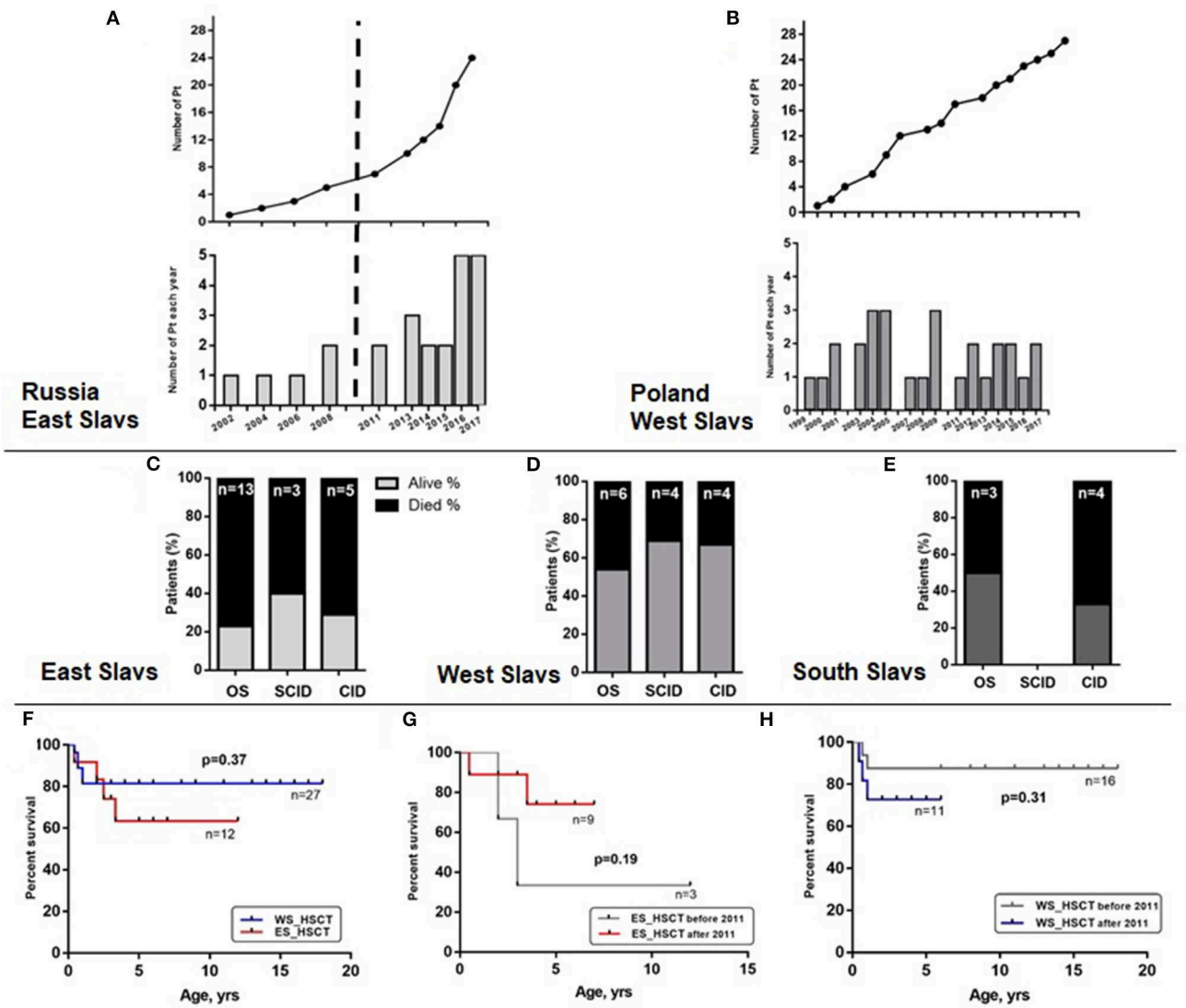

FIGURE 5 | Identification and survival of Slavic patients. Number of patients diagnosed in Russia **(A)** and Poland **(B)**. Percentage of dead patients (black region) among clinical groups of patients with *RAG* variants and East **(C)**, West **(D)**, or South Slavic **(E)** origin. Overall survival displayed as Kaplan–Meier survival curve of WS and ES patient groups with HSCT **(F)**. Overall survival curve of ES **(G)** and WS **(H)** patient transplanted before and after 2011.

access to HSCT service make it possible to improve the outcome for this group.

In the present study, 27% of patients were classified by attending physicians as atypical SCID. This phenotype was first described by Schuetz C. et al. in 2008, which they named "atypical/leaky SCID." During the last 10 years, there is growing awareness of these patients (2, 4, 7, 9, 13, 19, 20, 22, 30). Distinct clinical features include inflammatory and/or autoimmune complications. Autoimmune cytopenias (AIC) were the most frequent in our cohort, found in 58% (11/19 *RAG1* patients with AS phenotype). In the general population, ITP is the most common AIC. This is comparable to published data (23, 35). Regarding immunological features, although one third of the RAG1 patients had absence of very low B cell count, over two thirds of the cases had presence of low to normal B cells and immunoglobulins that progressive decline with age. Of note, many of our patients did not have full immune evaluation.

Slavs are the largest ethno-linguistic group in Europe (25, 36). In 10 out of 11 countries, the majority of patients carried p.K86Vfs*33 in RAG1 in homo or heterozygous state. Based on our data reported in this study, we established the geographic center of the origin of this specific RAG1 allele; it corresponds to the Vistula watershed area in Central and Eastern Poland (**Figure 3B**). Since SCID (Bernatowska E. unpublished data) and *RAG2* patients are evenly diagnosed in Poland, the lower frequency of absence *RAG1* variant p.K86Vfs*33 in the Western part of Poland may be likely related to distance from the

geographic center of the founder variant or due to lower medical care in this region. Together, these data suggest that it is most likely that the founder *RAG1* variant p.K86Vfs*33 is of a Slavic origin.

Location of the Slavic homeland prior to their great expansion in the fifth to sixth centuries is one of the key questions of European history. Although it is assumed that prehistorically the original habitat of Slavs was Asia, from which they migrated in the third or second millennium BC to populate parts of Eastern Europe, a debate concerning the European homeland of Slavs seems to remain unsolved. Different theories concerning the Slavs' geographic origin based on archaeological, anthropological, and/or linguistic data have been formulated. One places the cradle of Slavs in the watershed of the Vistula and Oder rivers (present-day Poland), and the other locates it in the watershed of the middle Dnieper (present-day Ukraine) (32). The obtained data strengthen the hypothesis that the Slavs could originate from the watershed of the Vistula due to the high number of families with homozygous variant from that region (**Figure 3B**). However, at present, it is impossible to deny that the variant could be found in the watershed of the middle Dnieper due to low number of cases and low incidence of *RAG* diagnostics in the Ukraine. Additionally, the only homozygous variant among East Slavs was found in the Ukraine (Odessa), where the parents denied their consanguinity. Collectively, these data indicate that p.K86Vfs*33 could be established as a "Slavic" *RAG1* variant or a founder variant.

Population migrations in Europe have led to the distribution of ethnic groups and cultures, and consequently to genetic mixing. Migrations, together with other factors, have also determined the prevalence of genetic variants among other populations (25, 36). In the GnomAD database, this deletion was found in heterozygous state in six people of European population (non-Finnish) of 282,486 individuals [https://gnomad.broadinstitute.org/variant/11-36595109-TAA-T]. Reviewing published cases, we established that patients with homo- and heterozygous p.K86Vfs*33 *RAG1* variants were detected in European cohorts (2, 13, 37–40). A recent Turkey study reported one patient with homozygous p.K86Vfs*33 in *RAG1* among the cohort of 44 SCID patients (32) and a SCID infant with EBV-positive B-cell lymphoma of the liver with homozygous p.K86Vfs*33 was diagnosed in Austria (40). It should be noted that in all these previous studies, the ethnic origin of the patients was not documented.

Our study highlight that a founder RAG1 variant is frequently present in Eastern Europe, especially in the watershed area of the Vistula. As cases accumulate in a specific region in Poland, screening of carriers of pathogenic RAG variants may be of high importance and could be offered in case of parental interest to reduce the incidence of RAG deficiency in this population. Our study underscores the importance of region of birth and ethnic background in genetic diagnosis. Further studies are needed to explore the carrier frequency of p.K86Vfs*33 in Central and Eastern Poland, as well in other Slavic countries.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Informed consent forms were signed by the parents as requested and approved by the institutional review boards of various institutions involved. The protocol of study was approved by the institutional review board of Belarusian Research Center for Pediatric Oncology, Hematology and Immunology (IRB0012-2015). Written, informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SS designed the research, collected data, interpreted, analyzed the results and wrote the manuscript. DV prepared the maps. YY and TK studied RAG activity. MS-P, YR, BW-K, ND-L, BM, OP, PS, TF, TM, RF, AS, MS, TA, GM, PC, KK, SKo, TJ, KD, AG, MP, EN, SKa, KB-P, BB, ER, TV, AP, HG, MD, IM, AB, LC, LK, MG, JR, AM, EP, CG, and AL-P provided patient's information. JW, AS, TF, LN, OA, NK, and MB guided the writing of the manuscript. All authors have read and approved the contents of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00900/full#supplementary-material

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T Lymphocytes in Patients With Nijmegen Breakage Syndrome Demonstrate Features of Exhaustion and Senescence in Flow Cytometric Evaluation of Maturation Pathway

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Patients with Nijmegen Breakage Syndrome (NBS) suffer from recurrent infections due to humoral and cellular immune deficiency. Despite low number of T lymphocytes and their maturation defect, the clinical manifestations of cell-mediated deficiency are not as severe as in case of patients with other types of combined immune deficiencies and similar T cell lymphopenia. In this study, multicolor flow cytometry was used for evaluation of peripheral T lymphocyte maturation according to the currently known differentiation pathway, in 46 patients with genetically confirmed NBS and 46 sex and age-matched controls. Evaluation of differential expression of CD27, CD31, CD45RA, CD95, and CD197 revealed existence of cell subsets so far not described in NBS patients. Although recent thymic emigrants and naïve T lymphocyte cell populations were significantly lower, the generation of antigen-primed T cells was similar or even greater in NBS patients than in healthy controls. Moreover, the senescent and exhausted T cell populations defined by expression of CD57, KLRG1, and PD1 were more numerous than in healthy people. Although this hypothesis needs further investigations, such properties might be related to an increased susceptibility to malignancy and milder clinical course than expected in view of T cell lymphopenia in patients with NBS.

Keywords: Nijmegen Breakage Syndrome, T lymphocyte maturation, flow cytometry, primary immune deficiency, immune senescence, immune exhaustion

INTRODUCTION

Nijmegen Breakage Syndrome (NBS) (MIM #251260) is a rare autosomal recessive disease belonging to a group of chromosomal instability disorders. The disease is caused by mutations in *NBN* gene (MIM #602667) encoding nibrin. The defect leads to defective response to DNA double strand break repair occurring both physiologically and in response to ionizing radiation and radical-producing agents (1–6). The principal clinical manifestations of the syndrome include progressive microcephaly, dysmorphic facial features, mild growth retardation, mild-to-moderate intellectual disability, and an increased predisposition to malignancies (7–10).

Due to humoral and cellular immune deficiency (11–14) patients with NBS suffer from recurrent infections. Low concentration of serum immunoglobulins and/or inadequate specific antibody response (13), are caused by general B cell lymphopenia (11, 15–17) and/or lower frequency of switched memory B-cells (18). Severe impairment in T-cell dependent antigen response and features of defective cellular immunity have been attributed to T cell lymphopenia and defective T lymphocyte maturation (13, 18, 19).

This prospective study was initiated in attempt to describe peripheral T lymphocyte maturation profile in patients with NBS according to the currently known differentiation pathway (20).

PATIENTS AND METHODS

Peripheral EDTA-K2 anticoagulated blood samples were collected between November 2016 and December 2018 from 46 patients with common Slavic 657del5 mutation in nibrin (21), and from 46 healthy subjects, with the same female-to-male ratio as in the study group. Detailed clinical data were collected at the time of patient's (or healthy control's) visit in the outpatient department. None of the patients was treated for malignancy or demonstrated other features of lymphoproliferation at their enrollment into the study. In case of previous malignancy, the interval between initiation of the study and the end of treatment associated with remission was at least 2 years. All healthy controls have been sex and age-matched and met additional requirement of smallest possible deviation from the patient's age. They were also free from infections and have not been vaccinated recently.

Distribution of basic lymphocyte populations, including T, B, NK, as well as T helper and cytotoxic lymphocytes, were determined by flow cytometry using the lyse-no-wash approach and Multitest six-color cocktails of antibodies with Trucount tubes, to determine absolute cell counts of respective cell populations (Becton Dickinson, cat. no. 644611) (**Table 1**). Antibody manufacturer's instructions were followed during the staining procedure. At least 15,000 events were acquired to BD FACSCanto II flow cytometer, with lymphocyte gate based on CD45 expression and side scatter characteristics. Lyse-no-wash settings for the FACS Canto Clinical software were used without any custom modification. Briefly, 50 μl aliquots of blood were incubated with optimally titered antibodies for 15 min in room temperature. The incubation was followed by erythrocyte lysis using 0.45 ml of BD FACSLysing Solution (Becton Dickinson, cat. no. 349202) diluted according to the manufacturer's instructions. Definition of basic lymphocyte subsets, i.e., T, B, NK, CD4, and CD8 T lymphocytes was performed according to standard procedures (22). Absolute numbers of individual cell subsets were calculated based on proportion of the respective cell subpopulation and absolute lymphocyte count (22).

Peripheral T lymphocyte maturation profile was analyzed according to the currently known differentiation pathway, using six-color cocktails of mouse fluorochrome-associated monoclonal antibodies specific for human receptors and differential expression of CD27, CD31, CD45RA, CCR7 (CD197), and CD95 (23) (details on monoclonal antibodies are presented in **Table 1**). Co-expression of CD31 and CD45RA was used to identify recent thymic emigrants among $CD4^+$ lymphocytes and a population of naïve T $CD8^+$ lymphocytes including also recently emigrating cells from the thymus. All remaining cell subsets have been identified both within $CD4^+$ and $CD8^+$ T lymphocyte populations. Naïve population (TN) has been identified as $CD27^+CD45RA^+CD197^+$, memory T lymphocytes with stem cell-like properties (TSCM) as $CD27^+CD45RA^+CD95^+$, central memory (TCM) as $CD27^+CD45RA^-CD197^+$, effector memory (TEM) as $CD27^+CD45RA^-CD197^-$, terminal effector memory expressing RA (TEMRA), as $CD27^-CD45RA^+CD95^+$, including low- (L-TEMRA, $CD27^+CD45RA^+CD197^-$ and high-differentiated H-TEMRA, $CD27^-CD45RA^+CD197^-$), and late effector memory/terminally differentiated subsets (TD), as $CD27^-CD45RA^-CD197$. We also analyzed features of immunosenescence manifested by expression of CD57 and KLRG1 (24), as well as features of exhaustion associated with expression of PD1 (CD279) (25, 26). Briefly, 100 μl of peripheral blood samples were incubated with an adequate amount of antibodies for 15 min in darkness in room temperature. The sample was then lyzed with BD FACSLysing solution (Becton Dickinson), washed twice with wash buffer (PBS+0.1% sodium azide), and after suspending in wash buffer—acquired into appropriately calibrated BD FACS Canto II cytometer and analyzed with BD Facs Diva v.7 software. Gating strategy applied throughout the study is presented on **Figure 1**. The same approach was applied for both $CD4^+$ and $CD8^+$ T lymphocytes. Patient data were compared with results obtained in healthy controls collected during the study, and the differences were analyzed with Mann-Whitney and Fischer's exact tests.

The study was approved by the Bioethical Committee at the Children's Memorial Health Institute, Warsaw (Poland) and carried according to Helsinki Declaration. Written consent for participation was obtained from all patients older than 16 years, and parents or legal guardians in case of patients younger than 16 years.

RESULTS

The study group included 46 NBS patients aged 0.6–38.7 years (median 12.1 year), with female to male ratio 27:19, and 46 healthy sex-matched controls at similar age (0.6–39.8 year, median 11.9, p = NS).

Patients with NBS demonstrated significantly lower proportion and absolute lymphocyte count in comparison to healthy controls (27.2 vs. 46.8%, $p < 0.01$, and 1,330 vs. 2,676 cells/μl, $p < 0.01$). T lymphocytes composed significantly lower

Abbreviations: NBS, Nijmegen Breakage Syndrome; RTE, recent thymic emigrants; TN, naïve T lymphocytes; TSCM, memory T lymphocytes with stem-cell like properties; TCM, central memory T lymphocytes; TEM, effector memory T lymphocytes; TEMRA, revertant terminal effector memory T lymphocytes expressing CD45RA; L-TEMRA, low differentiated revertant terminal effector memory T lymphocytes expressing CD45RA; H-TEMRA, high differentiated revertant terminal effector memory T lymphocytes expressing CD45RA; TD, terminally differentiated T lymphocytes.

TABLE 1 | Six-color antibody panels for composition of the T cell compartment analysis.

Tube	FITC	PE	PerCP	APC	APC/Cy7	PE/Cy7
Trucount	CD3 (SK7)[a]	CD16/CD56 (B73.1)[a]/(NCAM16.2)[a]	CD45 (2D1)[a]	CD19 (SJ25C1)[a]	CD8 (SK1)[a]	CD4 (SK3)[a]
1	CD45RO (UCHL1)[b]	CD31 (WM59)[b]	CD3 (SK7)[a]	CD4 (SK3)[a]	CD8 (SK1)[a]	CD45RA (HI100)[b]
2	CD27 (L128)[a]	CD197 (150503)[b]	CD3 (SK7)[a]	CD4 (SK3)[a]	CD8 (SK1)[a]	CD45RA (HI100)[b]
3	CD27 (L128)[a]	CD8 (SK1)[a]	CD3 (SK7)[a]	CD95 (DX2)[a]	CD4 (SK3)[a]	CD45RA (HI100)[b]
4	KLRG1 (SA231A2)[c]	CD8 (SK1)[a]	CD3 (SK7)[a]	CD57 (HCD57)[c]	CD4 (SK3)[a]	CD279 (EH12.1)[b]

Clones are shown between brackets, manufactures by characters.
[a]*BD Biosciences, San Jose, CA, USA.*
[b]*Pharmingen, San Diego, CA, USA.*
[c]*Biolegend, San Diego, CA, USA.*

proportion and absolute count in NBS patients than in healthy controls (52.7 vs. 72.9%, $p < 0.01$, and 667 vs. 1,949 cells/μl, $p < 0.01$). Similar observation was made for $CD4^+$ (25.9 vs. 43.1%, $p < 0.01$, and 322 vs. 1,074 cells/μl, $p < 0.01$) and CD8+ T lymphocyte subsets (16.7 vs. 22.8%, $p < 0.01$, and 234 vs. 612 cells/μl, $p < 0.01$), as well as B lymphocytes (8.7 vs. 14.1%, $p < 0.01$, and 103 vs. 359 cells/μl, $p < 0.01$). In contrast, NK composed a significantly greater population in NBS patients than in healthy controls (32.3 vs. 9.7%, $p < 0.01$, and 388 vs. 236 cells/μl, $p < 0.01$; **Figure 2**).

We found several aberrancies in the T lymphocyte maturation profile in NBS patients in comparison to healthy controls, both in terms of relative and absolute counts of individual cell populations. Populations of lymphocytes identified as $CD31^+CD45RA^+$ and $CD27^+CD45RA^+CD197^+$, were significantly less numerous in NBS patients than in healthy controls, both within the $CD4^+$ (median 6.6 vs. 48.9%, 25 vs. 534 cells/μl $p < 0.01$, and 11.6 vs. 67.6%, 46 vs. 732 cells/μl, $p < 0.01$, respectively) and $CD8^+$ cell subset (median 32.7 vs. 59.4%, 73 vs. 400 cells/μl, $p < 0.01$, and 13.7 vs. 48.6%, 25 vs. 302 cells/μl, $p < 0.01$, respectively; **Figures 3**, **4**). Despite poor generation of naïve cells, we identified lack of differences in proportions of TSCM, considered to be the youngest antigen-primed T cell population, between NBS patients and healthy controls (median 2.1 vs. 1.8% $CD4^+$, $p =$ NS and 6.8 vs. 6.6% $CD8^+$, $p =$ NS), although significant differences in absolute counts of these cells were still detected both within $CD4^+$ (8 vs. 18 cells/μl, $p < 0.01$), and CD8+ subset (12 vs. 42 cells/μl, $p < 0.01$). TCM composed significantly greater proportion of lymphocytes in NBS patients than in healthy controls (28.6 vs. 16.8% T $CD4^+$, $p < 0.01$ and 4.3 vs. 2.8% T $CD8^+$, $p < 0.01$), but significant differences in absolute counts were found only within the $CD4^+$ cell subset ($CD4^+$ 86 vs. 184 cells/μl, $p < 0.01$; $CD8^+$ 13 vs. 16 cells/μl, $p =$ NS). TEM composed a significantly greater population of T $CD4^+$ in NBS patients (median 24.4 vs. 10.5%, $p < 0.01$, and 83 vs. 109 cells/μl, $p < 0.01$), with a similar population within T $CD8^+$ lymphocytes (median 18.9 vs. 17.1%, $p =$ NS), however, still significantly lower absolute number of cells from this population (46 vs. 99 cells/μl, $p < 0.01$). Low differentiated revertant $CD45RA^+$ T lymphocytes composed similar populations within $CD4^+$ and $CD8^+$ cells in NBS patients in relation to healthy controls (median 1.7 vs. 1.8%, $p =$ NS, and 9.1 vs. 13.9%, $p =$ NS, respectively). On the other hand, more differentiated stages of TEMRA, i.e., H-TEMRA composed significantly higher proportions of T cells in NBS patients than in healthy controls (median 1.2 vs. 0.4% T $CD4^+$, $p < 0.01$, and 20.6 vs. 6.3% T $CD8^+$, $p < 0.01$). The same observation was made for TEMRA identified as $CD27^-CD45RA^+CD95^+$ (median 0.7 vs. 0.1% T $CD4^+$, $p < 0.01$, and 6.8 vs. 3.2% T $CD8^+$, $p < 0.05$). In terms of absolute counts L-TEMRA were significantly less numerous within both $CD4^+$ and $CD8^+$ populations (7 vs. 24 cells/μl, $p < 0.01$, and 24 vs. 81 cells/μl, $p < 0.01$, respectively), but no significant differences were found either for H-TEMRA (5 vs. 4 cells/μl, $p =$ NS and 40 vs. 39 cells/μl, $p =$ NS, respectively) or total TEMRA identified as CD27-CD45RA+CD95+ (2 vs. 1 cells/μl, $p =$ NS and 16 vs. 19 cell/μl, $p =$ NS, respectively). The most differentiated stage of $CD27^-CD45RA^-CD197^-$ T lymphocytes composed patients significantly greater proportion of both $CD4^+$ and $CD8^+$ cell subsets in NBS patients in comparison to healthy controls (median 13.0 vs. 3.2% T $CD4^+$, $p < 0.01$, and 7.4 vs. 3.5% T $CD8^+$, $p < 0.01$, respectively), but statistical difference in absolute count was found only in case of CD4+ cell subset (44 vs. 30 cells/μl, $p < 0.01$), with similar counts of TD cells within CD8+ subset (22 vs. 15 cells/μl, $p =$ NS; **Figures 3**, **4**).

Significant differences between NBS patients and healthy controls were found in expression of senescence markers CD57 and KLRG1, especially in terms of proportion of cells expressing the studied cell markers. CD57 was present on median 5.9 vs. 0.5% T $CD4^+$ ($p < 0.01$) and 25.2 vs. 9.6% T $CD8^+$cells ($p < 0.01$), with significant differences in absolute counts of $CD57^+$ $CD4^+$ cells (17 vs. 4 cells/μl, $p < 0.01$), but not within the $CD8^+$ cell population (52 vs. 50 cells/μl, $p=$ NS). Similar observation was made for KLRG1, which was detected on median 46.0 vs. 7.2% T $CD4^+$ lymphocytes ($p < 0.01$) (120 vs. 54 cells/μl, $p < 0.01$) and 88.0 vs. 44.4% T $CD8^+$ lymphocytes ($p < 0.01$). No statistically significant difference in absolute count of $KLRG1^+$ $CD8^+$ lymphocytes was found (163 vs. 177 cells/μl, $p =$ NS; **Figure 5**).

PD1 as a marker of exhaustion was detected on a significantly higher proportion of both $CD4^+$ and $CD8^+$ lymphocytes in NBS patients than in healthy controls, with median $CD4^+PD1^+$ lymphocytes composing 10.7 vs. 2.6% ($p < 0.01$) and $CD8^+PD1^+$ composing 7.3 vs. 3.3% ($p < 0.01$). However, statistical differences in absolute counts between the study cohort and healthy controls were found only within $CD4^+$ population (33

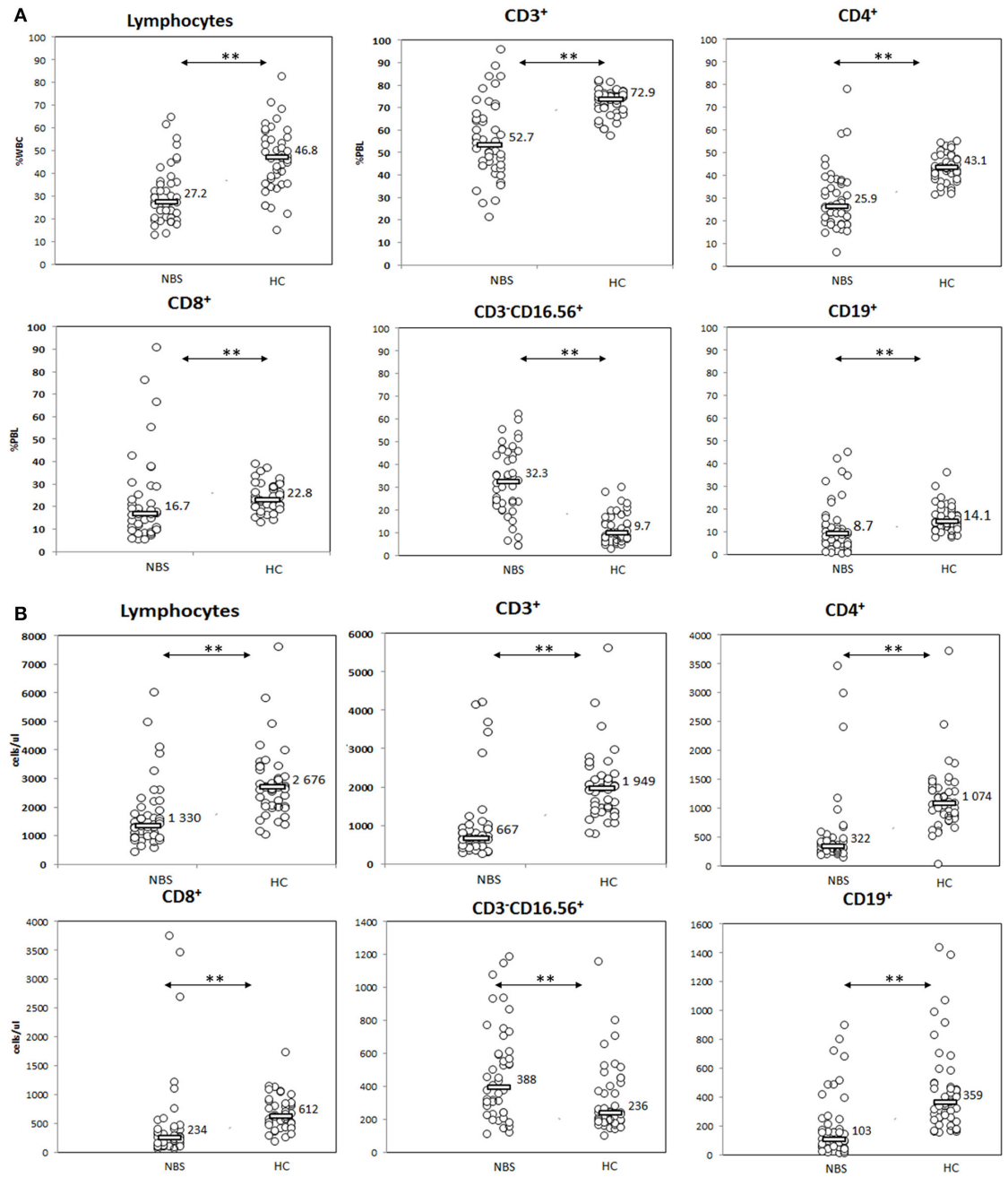

FIGURE 1 | Evaluation of basic lymphocyte subsets. Patients with NBS demonstrated significantly smaller general population of lymphocytes, with disturbed distribution of T ($CD3^+$), T $CD4^+$ ($CD3^+CD4^+$), T $CD8^+$ ($CD3^+CD8^+$), NK ($CD3^-CD16^+CD56^+$), and B ($CD19^+$) lymphocyte subsets in comparison to healthy controls. Individual results in respective study cohorts are presented as circles. Median values are presented as bars with numerical values. Statistical significance: ** <0.01. **(A)** Relative counts and **(B)** absolute counts.

vs. 29 cells/μl, $p = 0.0198$), but not within $CD8^+$ cells (16 vs. 23 cells/μl, $p =$ NS; **Figure 5**).

DISCUSSION

This study for the first time presents a detailed analysis of T lymphocytes and their subpopulations that reflect the development of T cells in periphery in a large cohort of patients with Nijmegen breakage syndrome. Thus, our observations extend and supplement earlier information on T cell development in this rare, but still underestimated disease.

For many years CD45 isoforms were used to differentiate between naive ($CD45RA^+CD45RO^-$) and memory ($CD45RA^-CD45RO^+$) T lymphocytes (27, 28). Along with

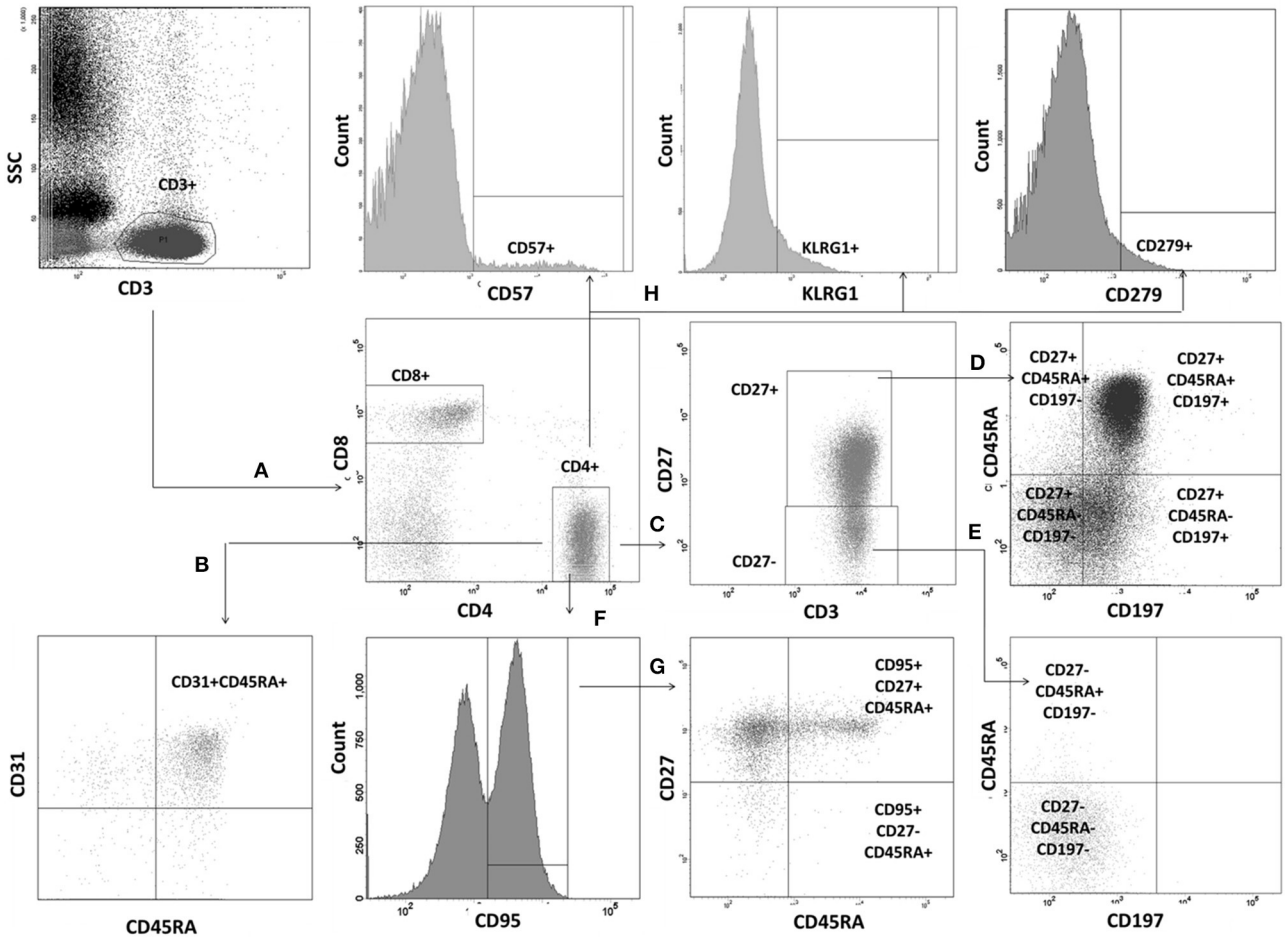

FIGURE 2 | Gating strategy for evaluation of T lymphocyte maturation process. Lymphocyte subsets were identified by differential expression of CD27, CD31, CD45RA, CD95, and CD197. T lymphocytes were gated based on CD3 vs. side scatter characteristics. Identification of individual populations is presented for T helper cells. Identical strategy was used for T $CD8^+$ cell subsets. **(A)** T helper and T suppressor cells were identified within T cell gate as $CD3^+CD4^+$ and $CD3^+CD8^+$, respectively. **(B)** RTE $CD31^+CD45RA^+$ were identified within the $CD3^+CD4^+$ gate. **(C)** Two additional gates: $CD27^+$ and $CD27^-$ were set. **(D)** Within $CD27^+$ gate TN have been defined as $CD27^+CD45RA^+CD197^+$, TCM as $CD27^+CD45RA^-CD197^+$, TEM as $CD27^+CD45RA^-CD197^-$, and low-differentiated effector RA^+ L-TEMRA as $CD27^+CD45RA^+CD197^-$). **(E)** High differentiated effector RA+ (H-TEMRA $CD27^-CD45RA^+CD197^-$) and terminally differentiated (TD, $CD27^-CD45RA^-CD197^-$) have been identified within the $CD27^-$ gate. **(F)** CD95 gate was drawn within the T $CD4^+$ gate. **(G)** Two populations were identified within $CD95^+$ gate: TSCM ($CD27^+CD45RA^+CD95^+$), and effector RA^+ (TEMRA, $CD27^-CD45RA^+CD95^+$). **(H)** Identification of cells with positive expression of CD57, KLRG1, and PD1 on the whole T $CD4^+$ population.

the development of more refined methods of cell analysis, it was found that other surface markers, such as CD197 corresponding to chemokine receptor CCR7 (29–31), CD27 (32–34), CD31 (35), and CD95 (36, 37), used in combination with CD45RA offered significantly more detailed view into the T lymphocyte maturation process (20). New combinations of markers allowed unique identification of recent thymic emigrants among T $CD4^+$ cells ($CD31^+CD45RA^+$) (35, 38). In contrast to $CD4^+$, the population of $CD31^+CD45RA^+CD8^+$ T lymphocytes includes also a naïve subset (39, 40). Several newly identified populations, such as $CD27^-CD45RA^+CD197^+CD95^+$ (currently known as TEMRA) or $CD27^+CD45RA^+$ $CD95^+$ (currently known as TSCM), have been initially identified among T $CD8^+$ cells and only later among T $CD4^+$ lymphocytes (29, 41). The newly identified cell subsets were also found to demonstrate varying effector capabilities (42–44) and significant differences in response depending on stimulating antigen (45–47).

Nibrin is known to participate in T cell development (48, 49) by affecting thymic output (50) and the V(D)J rearrangement process (6, 51, 52) resulting in increased proportions in T lymphocytes expressing TCRγδ receptor (19) and shift toward increased proportions of cells in mature development stages (14, 19, 53). The purpose of this prospective study was to provide more detailed description of the T lymphocyte maturation process in patients with Nijmegen Breakage Syndrome than available from previous reports (19, 53). We also searched for differences in the process between healthy subjects and patients

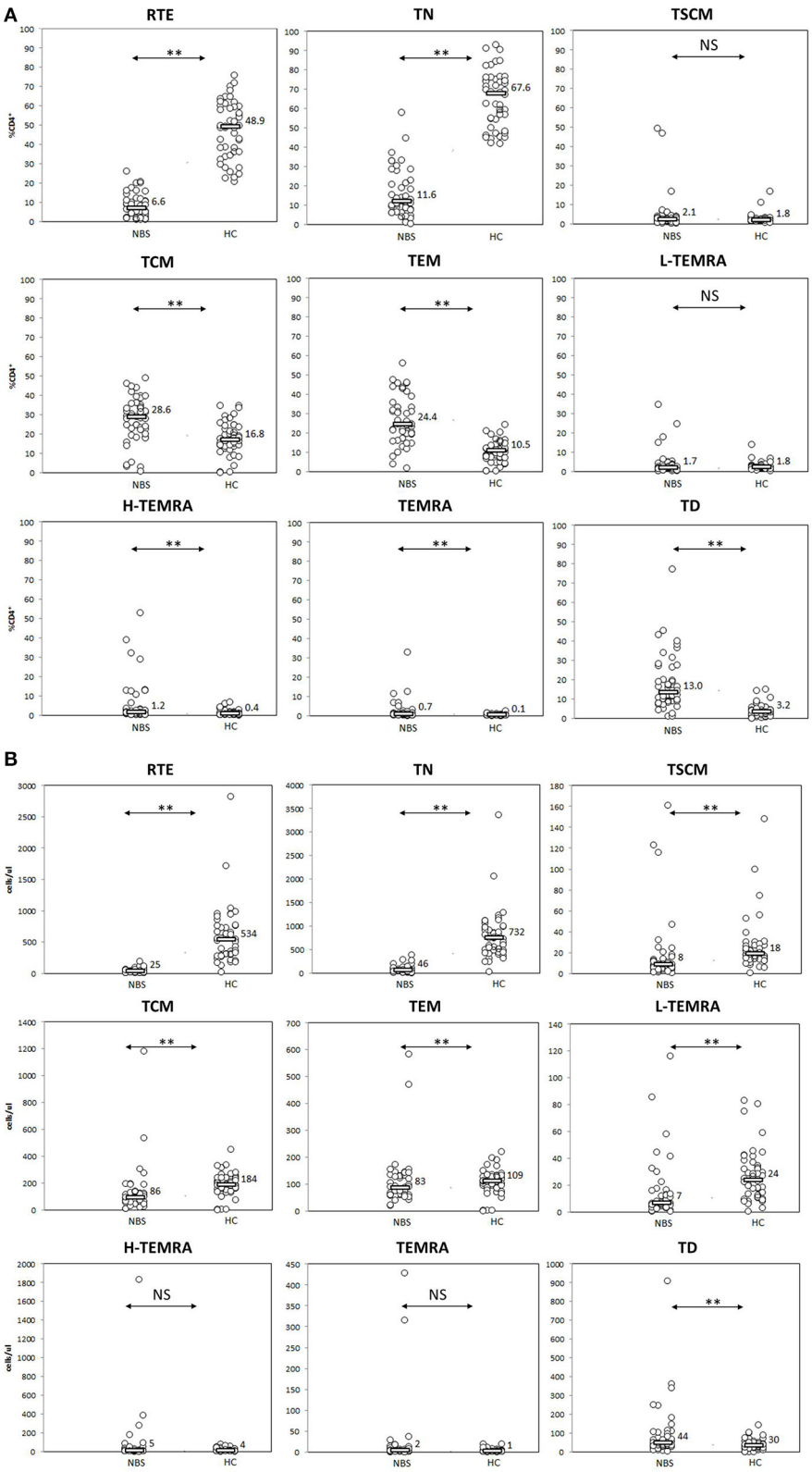

FIGURE 3 | Peripheral T helper cell maturation was significantly disturbed. Individual results in respective study cohorts are presented as circles. Median values are presented as bars with numerical values. Statistical significance: NS, not significant, ** <0.01. **(A)** Median relative counts of T helper cell subsets in NBS patients in relation to normal control. Patients with NBS demonstrated significantly lower proportions of RTE and naïve helper cells, and significantly higher proportions

(Continued)

FIGURE 3 | of TCM, TEM, H-TEMRA, TEMRA, and TD lymphocytes. There was no statistical difference between TSCM and L-TEMRA. **(B)** Median absolute counts of T helper cell subsets in relation to normal control. Patients with NBS demonstrated significantly different absolute counts of all analyzed T helper subsets, except for H-TEMRA and TEMRA.

with the DNA double-strand break repair defect caused by mutations in *NBN* gene.

The distribution of relative and absolute counts of basic lymphocyte subsets, i.e., T, B, NK, $CD4^+$, and $CD8^+$ T lymphocytes in the cohort under study did not differ from previous reports (7, 14, 15, 19, 50, 53). It was found that the naïve T lymphocytes subpopulation in NBS patients was significantly smaller in comparison to healthy controls, both within $CD4^+$ and $CD8^+$ cells, as expected in view of the previously reported reduced expression of CD45RA (19, 53). Our data demonstrated also, that thymic production of T helper lymphocytes measured by proportions and absolute counts of $CD31^+CD45RA^+$ cells was ineffective (50, 53) and resulting in significantly lower number of naïve T $CD4^+$ cells in NBS patients than in control subjects (**Figure 3**). The population of T $CD8^+$ lymphocytes described by $CD31^+CD45RA^+$ immunophenotype was significantly more numerous than of naïve CD8+ cells (**Figure 4**). Even though $CD31^+CD45RA^+$ within T $CD8^+$ are not limited to recent thymic emigrants (40), significantly lower proportions of $CD31^+CD45RA^+$ and TN indicate that thymic production of naïve T $CD8^+$ lymphocytes is significantly affected by the mutated variant of nibrin.

Unexpectedly, TSCM were found to compose similar proportions of $CD4^+$ and $CD8^+$, with significantly smaller absolute number of cells in NBS patients than in controls. Unaware of their existence, authors of previous reports included TSCM among naïve $CD45RA^+$ cells, as sharing common phenotypic characteristics ($CD27^+CD45RA^+CD45RO^-CD197^+$) (19, 53). Their functional properties are however completely different, as TSCM are antigen-experienced, and they exhibit effector activity in contrast to quiescent naïve T lymphocytes (36, 54, 55). Therefore, it seems that despite low thymic production, T lymphocytes in patients with NBS have enough potential to differentiate from naïve into more mature TSCM, but it is not sufficient to overcome low thymic production. Yet, this increased proliferative potential in comparison to physiology, might in turn potentially lead to the increased susceptibility to malignancies of lymphoid origin observed in NBS patients (9, 10).

Homeostatic proliferation ensures the longevity of TCM in absence of cellular differentiation or activation. After proliferation, TCM can efficiently differentiate into effector cells (56). These processes seem not to be affected negatively by mutations in *NBN*, as TCM compose significantly greater proportion of both $CD4^+$ and T $CD8^+$ lymphocytes. TEM, which are potent effectors in healthy subjects, are also generated in greater proportions in NBS patients in comparison to healthy controls, but only within the $CD4^+$ T lymphocyte population (**Figure 3**).

Similarly as in case of naïve cells, neither TCM, TEM, nor L-TEMRA within T $CD4^+$ helper cells reached absolute counts observed in healthy controls. H-TEMRA and TEMRA composed similar populations, while TD $CD4^+$ were even more numerous in NBS patients than in controls. Among $CD8^+$ T lymphocytes, TEM, and L-TEMRA were significantly less numerous, while TCM, H-TEMRA, TEMRA, and TD reached similar absolute counts as in healthy controls (**Figure 4**). The explanation of important differences in maturation and proliferative potential between CD4+ and CD8+ lymphocytes reflected by the number of cells within individual populations requires more detailed molecular and functional analyses.

Preliminary data regarding age-related distribution of individual cell populations indicate that thymic output is deeply defective in almost all age groups. The defect seems to be more pronounced in younger children. In several patients, the absolute counts of all antigen primed CD4+ lymphocyte populations reached normal or almost normal counts. Adults appear to be an exception from this general observation, as recent thymic emigrants and naïve cells may reach low normal limits in some patients (**Supplementary Figure 1A**). This however does not mean that more cells are produced, as low limits of normal ranges in adults are lower than in young patients. Yet, more cells appear to be generated in several adults for antigen-primed populations. CD8+ T lymphocytes appear to behave differently in many terms (**Supplementary Figure 1B**). Deep defect in thymic production was observed in children and most adolescents, but not in adults. Children below 2 years of age seem to generate normal cell counts since reaching H-TEMRA maturation stage, while several patients from other age groups produce almost normal or normal counts of earlier antigen-primed cell populations. Moreover, several adolescents and adults seem to generate even higher than normal cell counts from the analyzed cell populations. These observations and their clinical context need to be verified in relation to larger group of healthy controls, when age related normal values will be established (study in progress).

Results of our study demonstrate that evaluation of the T lymphocyte maturation process in NBS patients by differential expression of only CD45RA/RO isoforms is misleading. Despite unsatisfactory thymic production, the generation of effector cells seemed quite effective and probably explaining relatively low incidence of clinical manifestations associated with cellular immune deficiency (17). Despite differences in biological properties, both TCM and TEM were correctly included in previous reports within the increased $CD45RO^+$ population (19). The same applied to the terminally differentiated populations, which were generated in significantly greater proportions in NBS patients in comparison to controls. TEMRA, which develop in parallel with TD (57), represent a revertant population that re-express CD45RA, but also express CD45RO isoform. They must have been incorrectly included in both naïve and memory

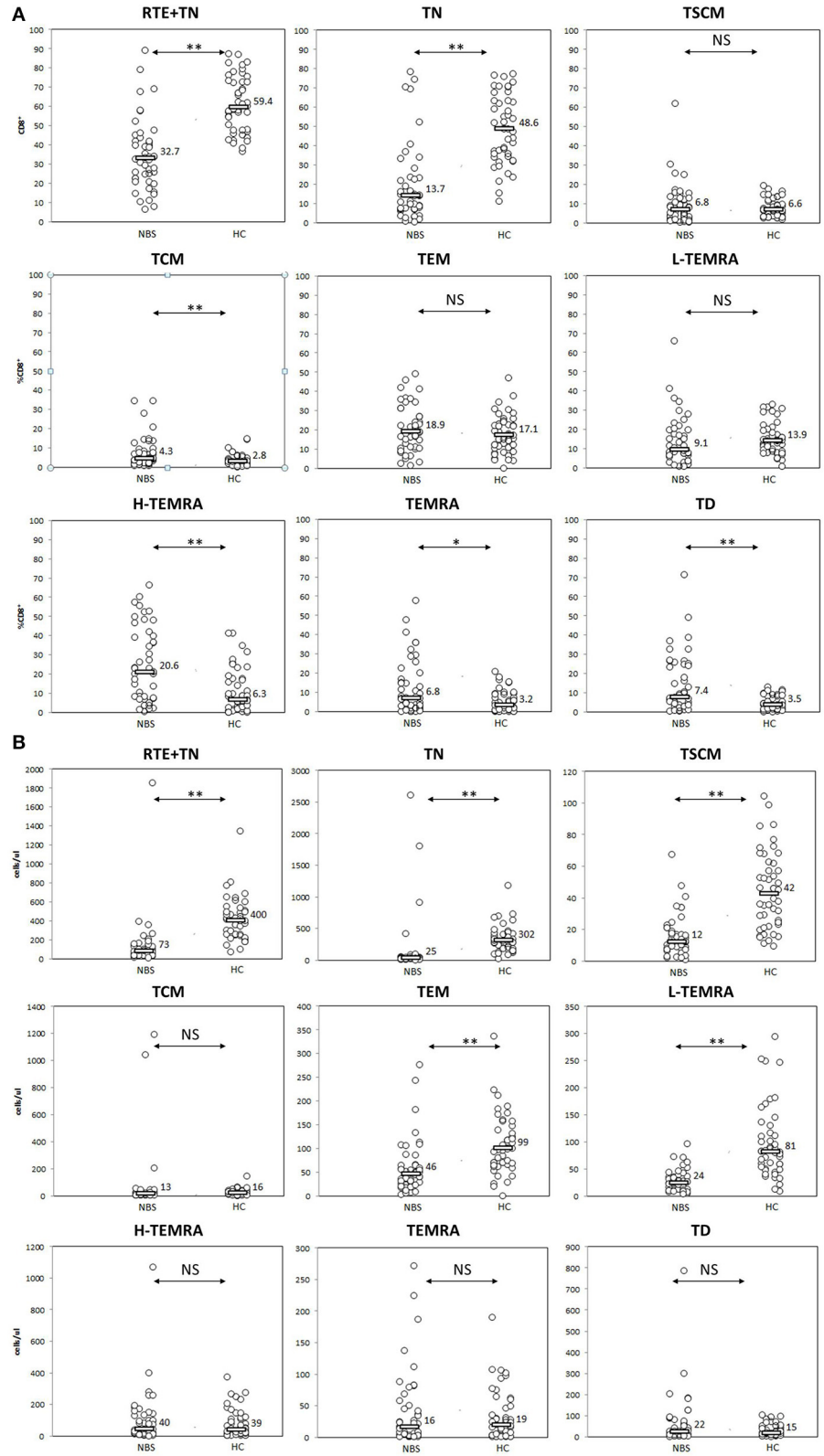

FIGURE 4 | Peripheral T CD8$^+$ lymphocyte maturation was significantly disturbed. Individual results in respective study cohorts are presented as circles. Median values are presented as bars with numerical values. Statistical significance: NS, not significant, * <0.05, ** <0.01. **(A)** Relative counts of T CD8$^+$ cell subsets in NBS patients in relation to normal control. Patients with NBS demonstrated significantly lower proportions of T CD8 cells with phenotype corresponding to RTE

(Continued)

FIGURE 4 | ($CD31^+CD45RA^+$) and naïve cells, and significantly higher proportions of TCM, H-TEMRA, TEMRA, and TD lymphocytes. There was no statistical difference in relative distribution of TSCM, TEM, and L-TEMRA. **(B)** Absolute counts of individual studied $CD8^+$ T lymphocyte populations. Significantly smaller populations of $CD31^+CD45RA^+$, TN, TSCM, TEM, and L-TEMRA cells were observed in NBS patients in comparison to controls. TEM, H-TEMRA, TEMRA, and TD composed similar populations in NBS and healthy cohorts.

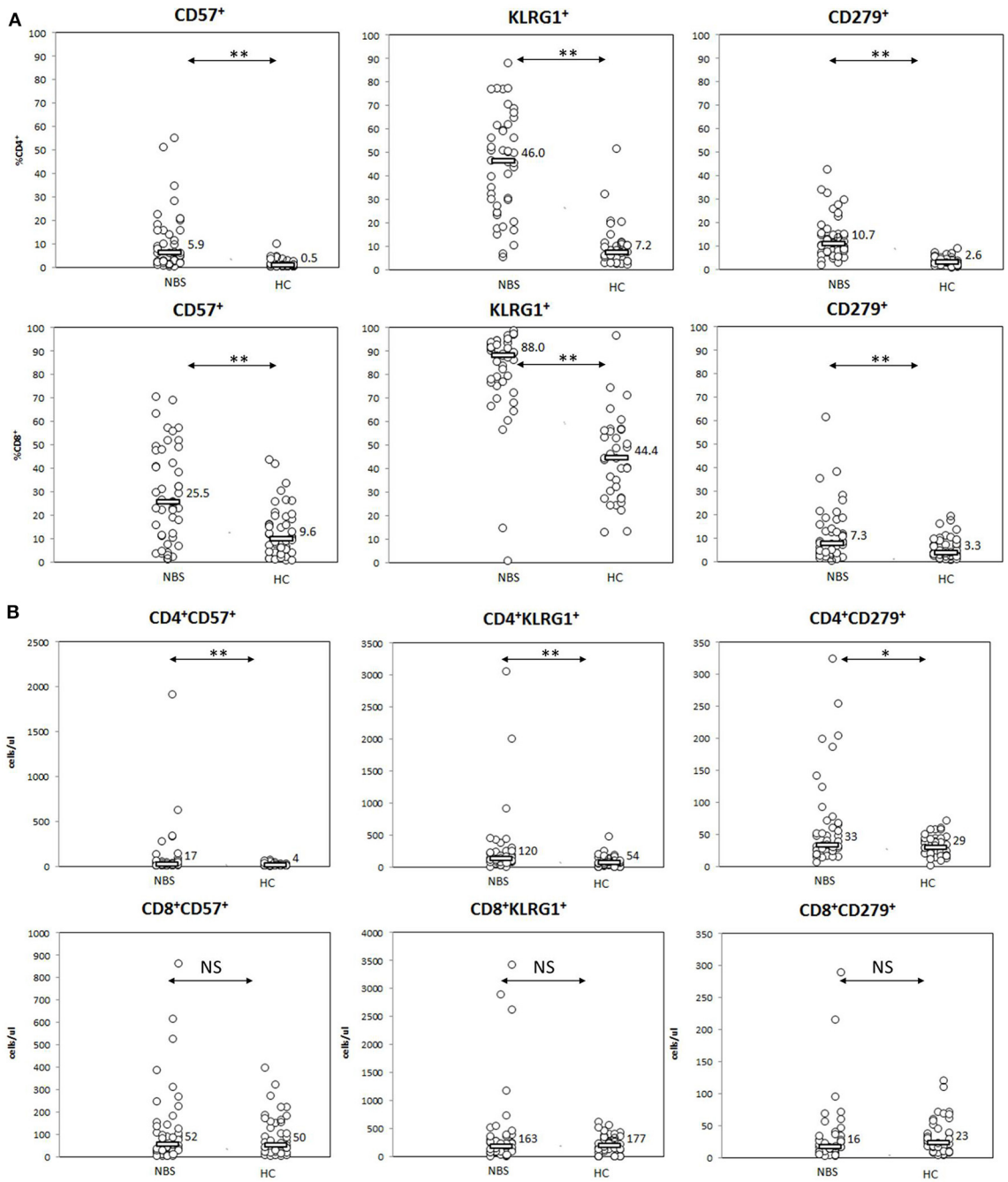

FIGURE 5 | Expression of senescence (CD57, KLRG1) and senescence (PD1=CD279) cell markers. Statistical significance: NS, not significant, * <0.05, ** <0.01. **(A)** Patients with NBS demonstrated significantly elevated proportions of T lymphocytes (from both $CD4^+$ and $CD8^+$ subsets) with features of senescence (expression of CD57 and KLRG1), as well as exhaustion (CD279) than healthy controls. **(B)** T helper cells composed significantly more numerous populations of cells expressing (CD57, KLRG1, and CD279) in NBS patients than healthy controls. No statistical difference in absolute counts within the studied populations was found among T $CD8^+$ lymphocytes.

populations (19, 53). Therefore, previous reports overestimated both the population of naïve cells defined as $CD45RA^+$, and memory population of $CD45RO^+$ cells (19, 53).

Mutations in the *NBN* gene are known to be associated with the telomere-initiated senescence (58, 59). We analyzed the features of pre-term senescence of T lymphocytes in NBS patients by evaluating the expression of CD57 and KLRG1. Although $CD57^+$ T lymphocytes are known to demonstrate cytotoxic abilities (49, 60, 61), the expression of CD57 is also associated with proliferative instability, correlating directly with the number of cell divisions and inversely with telomere length (43, 62). We found significantly increased proportions of $CD57^+$ T lymphocytes both among $CD4^+$ and $CD8^+$ populations (**Figure 5**). This, however, could not be correlated (or solely correlated) to increased proportions of cytotoxic cells raised during viral infections, as no difference in CD57 expression between patients demonstrating chronic EBV viremia or EBV-free were observed (study in progress).

Surface expression of an inhibitory killer-cell lectin-like receptor G1 (KLRG1) identifies T lymphocytes that have undergone a large number of cell divisions (63). In healthy subjects, predominant expression of KLRG1 is observed on TEM and TEMRA cells (64). Both populations demonstrate potent effector functions, but are unable to proliferate (65). We found significantly increased proportions of $KLRG1^+$ cells, corresponding to significantly increased proportions of TEM and TEMRA lymphocytes in patients from the study group (**Figure 3**). Similar suggestion regarding preterm T lymphocyte senescence in NBS was previously made by Meijerset al., based on results of studies performed in a significantly smaller group of patients (50). Therefore, considering the reported association of CD57 and KLRG1 with immune senescence (49, 66) and results of our flow cytometric experiments we conclude that patients from the study group demonstrate features of preterm senescence.

Functional impairment of T lymphocytes termed "exhaustion" is associated with an increased expression of PD1 (24, 66). We have observed significantly higher proportions of $PD1^+$ T lymphocytes in NBS patients, both within T $CD4^+$ and $CD8^+$ subpopulations, which is in line with increased proportions of lymphocytes at the terminal differentiation stage. To our knowledge this feature has not been studied yet. Considering the features of an excessive proliferative history reflected by increased proportions of terminally differentiated and $KLRG1^+$ T lymphocytes and increased proportions of $PD1^+$ cells found in the study group in comparison to healthy controls, we conclude that T lymphocytes from NBS patients demonstrate features of exhaustion.

The difference between absolute counts of $CD4^+$ and $CD8^+$ lymphocytes demonstrating CD57, KLRG1, and PD1 expression appears to correspond with the observed differences in distribution and cell counts of the studied cell subsets (24, 67, 68). Significance of this discrepancy requires further analysis in context of clinical data.

We are aware of the limitations of the study. All experiments have been performed by flow cytometry and did not include either functional or molecular studies. Moreover, all references to functional properties of individual cell populations are based on published data. Such approach resulted mainly from limitations of the available research material, as large proportion of patients included minors, among them several below 2 years of age. Although functional properties of cells expressing CD57 or KLRG1 have not been evaluated in this study, premature senescence and shortened telomeres have been demonstrated *in vitro* in cultured *NBN*-mutated cells (69–71). Therefore, we feel the conclusion regarding premature senescence in NBS is justified.

In summary, we found significant aberration in peripheral T lymphocyte maturation process in NBS patients in comparison to physiological process. Despite low thymic production, the identified aberrancies and functional properties of individual T lymphocyte subpopulations lead to generation of significantly larger populations of effector T cells in NBS patients than in healthy people. Although this hypothesis needs further investigation, such properties might be related to an increased susceptibility to malignancy and milder clinical course than expected in view of T cell lymphopenia in patients with NBS.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Bioethical Committee at the Children's Memorial Health Institute, Warsaw, Poland. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BP designed and supervised the flow cytometry experiments, analyzed and interpreted the data, and wrote the draft. BW-K designed the study, and collected and reviewed medical data. EH-P and AW collected and reviewed medical data. KT and UG performed the flow cytometry experiments. HG supervised the study. All authors critically revised and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01319/full#supplementary-material

Supplementary Figure 1 | Preliminary data regarding differences in age-related distribution of individual cell populations. Comparison of individual results of absolute counts of analyzed T lymphocyte populations between NBS patients (black circles) and healthy controls (white circles) in age-related groups **(A)**. Thymic output is deeply defective in almost all age groups, although it is more pronounced in younger children. In several patients, the absolute counts of all antigen primed $CD4^+$ lymphocyte populations may reach normal or almost normal counts. Adults appear to be an exception from this general observation. Such phenomenon is observed already for recent thymic emigrants and naïve cells, which in some patients may reach lower normal limits **(B)**. $CD8^+$ T lymphocytes appear to behave differently. Deep defect in thymic production is observed in children and most adolescents, but not in adults. Children below 2 years of age seem to generate normal counts since reaching H-TEMRA maturation stage, while many patients from other age groups produce almost normal or normal counts of antigen-primed cell populations. Several adolescents and adults seem to generate in several cases even higher than normal cell counts from the analyzed cell populations.

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PIK3AP1 and *SPON2* Genes Are Differentially Methylated in Patients With Periodic Fever, Aphthous Stomatitis, Pharyngitis, and Adenitis (PFAPA) Syndrome

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Periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) syndrome is the most common autoinflammatory disease in children and is often grouped together with hereditary periodic fever syndromes, although its cause and hereditary nature remain unexplained. We investigated whether differential DNA methylation was present in DNA from peripheral blood mononuclear cells (PBMC) in patients with PFAPA vs. healthy controls. A whole-epigenome analysis (MeDIP and MBD) was performed using pooled DNA libraries enriched for methylated genomic regions and identified candidate genes, two of which were further evaluated with methylation-specific restriction enzymes coupled with qPCR (MSRE-qPCR). The analysis showed that the *PIK3AP1* and *SPON2* gene regions are differentially methylated in patients with PFAPA. MSRE-qPCR proved to be a quick, reliable, and cost-effective method of confirming results from MeDIP and MBD. Our findings indicate that a B-cell adapter protein (*PIK3AP1*), as the PI3K binding inhibitor of inflammation, and spondin-2 (*SPON2*), as a pattern recognition molecule and integrin ligand, could play a role in the etiology of PFAPA. Their role and the impact of changed DNA methylation in PFAPA etiology and autoinflammation need further investigation.

Keywords: PFAPA, differential methylation, PIK3AP1, SPON2, MSRE-qPCR, MeDIP, MBD

INTRODUCTION

Periodic fever syndrome with adenitis, pharyngitis, and aphthous stomatitis (PFAPA) belongs to the group of autoinflammatory diseases (AID). This group of disorders is marked by increased inflammation associated with the innate immune system, and most of the disorders are inherited in a Mendelian pattern (1, 2). Of the various autoinflammatory diseases, many now have a confirmed known genetic cause. However, PFAPA syndrome still has an unknown genetic background and pathogenesis (3).

PFAPA was first described by Marshall et al. (4). Its most common feature is periodic fever, while its other features are more variable: pharyngitis, aphthous stomatitis, and cervical

adenopathies (5, 6). Episodes have an early onset, usually in patients around 2–3 years of age, mainly before the age of five. Episodes can reoccur for several years, followed by disease remission, and leave no long-term health consequences (5–7). The syndrome is considered sporadic and has been described as a non-inherited syndrome; however, familial cases of PFAPA have been reported, suggesting a potential genetic origin (3, 8–11). PFAPA could be caused by cytokine dysregulation linked to genetic variants of autoinflammatory disease-associated genes (12). Studies also point to altered complement activation and IL-1 production in PFAPA patients (13), as well as to IL-1β dysregulation (14). Since PFAPA shares clinical similarities with monogenic fever syndromes, several studies have investigated the possible involvement of the genes responsible for Familial Mediterranean Fever (*MEFV* gene), TNF-Receptor Associated Periodic Syndrome (*TNFRSF1A* gene), Mevalonate kinase deficiency (*MVK* gene), and Cryopyrin-Associated Periodic Syndrome (*NLRP3* gene) in PFAPA cohorts (15). A variant in *CARD8* has been associated with more severe cases of PFAPA. CARD8 acts as a negative regulator of the inflammasome; its decreased ability to bind NLRP3 could partly contribute to exacerbated inflammasome responses, although probably not on its own (16). Genes known to be involved in inflammation or in autoinflammatory disorders seem to contribute to a predisposition to PFAPA syndrome, suggesting complex genetic inheritance and interaction with non-genetic factors (12, 14, 17–20).

Several inflammasome-related genes have been found to have increased expression when demethylated during the differentiation of monocytes to macrophages, specifically *AIM2*, *NLRC5*, *PYCARD*, *CASP1*, and *PSTPIP2*, and their targets *IL1A*, *IL1B*, and *IL1RN*. Furthermore, untreated patients with Cryopyrin-Associated Periodic Syndrome (CAPS) have exacerbated DNA methylation-dependent regulation of the inflammasome product genes *IL1B*, *ILR1N*, *NLRC5*, and *PYCARD*, while patients with CAPS undergoing anti-IL-1β treatment have displayed demethylation levels in stimulated monocytes similar to those seen in healthy subjects (21). Different methylation levels of the *MEFV* gene have been observed in Familial Mediterranean Fever patients compared to healthy controls (22). Additionally, DNA methylation plays an important role during hematopoietic differentiation to a myeloid vs. a lymphoid lineage (23).

Since studies suggest that DNA methylation plays a role in other autoinflammatory diseases, we hypothesized that specific methylation patterns may be aberrant in at least a small portion (population) of peripheral blood mononuclear cells (PBMC) in PFAPA patients. To identify the potentially relatively small change in the methylation patterns due to the specifics of a PBMC-derived DNA sample and to confirm whether the PFAPA cohort differs in DNA methylation patterns from healthy controls, a whole-epigenome analysis was performed using pooled DNA libraries enriched for methylated genomic regions using Methylated DNA Immunoprecipitation (MeDIP) and Methyl-CpG-binding domain (MBD). We identified several candidate genes with differential methylation, two of which (*PIK3AP1* and *SPON2*) were chosen based on their involvement in the inflammation pathways and were further evaluated with MSRE-qPCR. To our knowledge, no research had previously been performed regarding differentially methylated DNA in PFAPA patients.

MATERIALS AND METHODS

Participants

Clinical data and samples of 75 patients (44 boys and 31 girls) with PFAPA syndrome at the University Children's Hospital Ljubljana were collected from 2008 to 2016. The median age of the patients when blood was taken for the DNA analysis was 4.1 [Interquartile range, IQR 3–5.8] years. Patients were not in an active state of the disease when samples for DNA isolation were taken. Prior to donating blood for DNA analysis, 15 (20%) had received methylprednisolone in the past but not in the month prior to blood donation (except for one individual, who had received treatment 2 weeks prior). Samples from 65 apparently healthy children (35 boys and 30 girls) of Slovenian ethnicity whose median age was 5.3 [IQR 5.2–5.4] were included in the study as healthy controls. The parents of each child included in the study were informed about the aim of the study and signed a written informed consent form for inclusion in the study. The study was approved by the Ethics Committee of the Republic of Slovenia and was conducted according to the principles of the Helsinki Declaration.

Samples

Five ml of peripheral blood was taken for DNA isolation. Blood was taken from patients during routine venipuncture at follow-up visits. Blood of the healthy controls was taken during routine health examinations of the children. DNA isolation was performed using the FlexiGene isolation kit (Qiagen, Germany) according to the recommended protocol. The DNA was stored at 4°C.

MeDIP and MBD Data Analysis

For each sample pool (PFAPA vs. healthy), three separate NGS libraries were generated for each enrichment method (MBD or MeDIP). All 36 libraries were combined and simultaneously sequenced on the MiSeq Illumina sequencer [MiSeq Reagent Kit v3 (150-cycle)] by a 2 × 75 paired-end run. Sequencing was repeated until each separate library had acquired at least 10 million pair-end reads. The acquired dataset was filtered for low-quality reads and duplicates aligned by the BWA-MEM aligner (24) and followed by MACS2 (25) analysis of narrow and broad peak regions. The callable regions identified by the MACS2 algorithm were used to count reads in each peak region for each library by the featureCounts algorithm (26). Using DeSeq2 (27), the algorithm peaks were compared to identify differentially methylated regions via differences in normalized read counts. The Benjamini-Hochberg procedure, which controls false discovery rate (FDR), was used to identify true differentially methylated regions (DMRs). DMRs were annotated for the overlapping or proximal gene regions. DMRs associated with the immune system were further verified using MSRE-qPCR.

TABLE 1 | Primer sequences and PCR product length, genomic position and location within the gene.

Gene	Accession number	Primer	Sequence	Product size [bp]	GRCh37 position	Location
HBB	NM_000518	HBB F	GGATGAAGTTGGTGGTGAGG	231	11:5247959–5248189	Exon 1–2
		HBB R	CAGCATCAGGAGTGGACAGA			
PIK3AP1	NM_152309	PIK_1 F	AAAAGAGTTAAATAGGCCGGGCG	120	10:98425881–98426000	Intron 2
		PIK_1 R	GTTTCACCATGTTAGCCAGGATG			
		PIK_2 F	GATCACAAGGTCAGGAGATCGAGA	238	10:98425953–98426190	Intron 2
		PIK_2 R	TTTGTTTGTTTGTTTGAGATGGAGTC			
SPON2	NM_012445	SPON_1 F	TAATTACTGCTGCTCCTCAAGACG	174	4:1163329–1163502	Intron 5
		SPON_1 R	GGACTTCAGACTTTCCCGAGGA			
		SPON_2 F	CTCCTCGGGAAAGTCTGAAGTC	248	4:1163480–1163727	Intron 5
		SPON_2 R	CATTCTCCTAGCTCTTCCAGGC			

TABLE 2 | qPCR reaction efficiencies.

qPCR reaction	E [$10^{\frac{-1}{slope}}$]	Efficiency	R^2	Slope
HBB	1.945	0.945	0.998	−3.46
PIK_1	2.231	1.231	0.984	−2.87
PIK_2	2.117	1.117	0.961	−3.07
SPON_1	1.906	0.906	0.996	−3.57
SPON_2	1.771	0.771	0.992	−4.03

TABLE 3 | Recognition sites for methylation-sensitive enzymes (both New England Biolabs) used for MSRE-qPCR.

Enzyme	Recognition site
MspJI (R0661)	^{m}CNNR
McrBC (M0272)	Pu^{m}CG

^{m}C represents 5-methylcytosine or 5-hydroxymethylcytosine, N represents any of the nucleotides, and R and Pu represent purines A or G.

TABLE 4 | Amplicon CG content, number of CpGs, and candidate restriction sites within amplicons.

	Length [bp]	CG content [%]	Number of CpGs	Candidate restriction sites
HBB	231	51.1	0	0
PIK_1	120	55.0	5	5
PIK_2	238	53.4	10	9
SPON_1	174	62.1	6	6
SPON_2	248	63.3	8	6

Primer Selection and Evaluation, MSRE-qPCR Verification of DMRs

PCR primers were designed using the Primer 3 online tool (http://primer3.ut.ee/) and SNP Check (https://genetools.org/SNPCheck/snpcheck.htm) according to the established laboratory protocol, covering the whole sequence identified by MBD or MeDIP. Since the identified sequences were longer than is optimal for qPCR amplicons, primers were chosen in pairs to cover the first and second halves of the sequence (**Table 1**). The primers were first evaluated with PCR and 2% agarose gel electrophoresis (SYBR staining) and with a 50 bp DNA ladder (N0556S, New England Biolabs), figure of Electrophoresis gel is available in **Supplementary Table 2**. PCR was performed with Go Taq G2 Green Master Mix according to the protocol.

After successful evaluation for specificity and annealing temperature, evaluation of the qPCR assay was performed. We used Luna Universal qPCR Master Mix (New England BioLabs) with a fast cycling profile. Standard curves were prepared for each primer pair to determine the efficiency (E) of the designed primers. Efficiency calculations were calculated online with the NEBioCalculator for qPCR Quantification (https://nebiocalculator.neb.com/#!/qPCRGen) (**Table 2**).

Enzyme Restriction

The regions selected for MSRE-qPCR had multiple CpGs between forward and reverse primer annealing sites. The selected enzymes (MspJI and McrBC; **Tables 3**, **4**) cover almost all CpGs; however, for successful digestion and subsequent evaluation, at least one of the targeted CpGs must be methylated. Furthermore, the MspJI enzyme can also recognize methylated C in front of any base (**Table 3**). However, since, in humans, mostly CpGs are methylated, only CpGs were counted for the number of candidate restriction sites (**Table 4**). Beta globin amplicon, which does not contain any CpGs, was used as a reference. Equal amounts of DNA were used in control and restriction reaction. First, 200 ng of DNA was digested in accordance with the manufacturer's protocol by 2 units of MspJI and 2 units of McrBC (both New England BioLabs) in a 25-µl reaction with CutSmart buffer for 1 h at 37°C, and this was followed by heat inactivation for 20 min at 65°C. Samples from the restriction reaction were then purified using Sample Purification Beads from a TruSight® One Sequencing Panel Kit according to the manufacturer's protocol.

Quantitative PCR

The purified digested and undigested DNA samples were used for relative qPCR quantification. qPCR assays were run in triplicates in 96-well plates using Luna Universal qPCR Master Mix (SYBR). In addition, melt curves were performed. Five pairs of primers [two for PIK3AP1, two for SPON2, and one for normalization (HBB, beta globin)] were analyzed on digested and undigested

DNA samples for relative quantification using the $\Delta\Delta C_T$ method (28). This method analyzes relative changes in methylation of the target gene to the reference gene, and it assumes uniform PCR amplification efficiency across all reactions. Due to varying efficiencies in qPCR assays, we used efficiency corrected calculation (Equations 1–3) (28) for each obtained CT to produce more accurate estimates in relative quantification.

$$Ratio = \frac{E_{\text{target}}{}^{\Delta CT(\text{target})}}{E_{\text{reference}}{}^{\Delta CT(\text{reference})}} \quad (1)$$

$$\Delta CT\left(target\right) = CT\left(undigested,\ target\ gene\right) - CT(digested,\ target\ gene) \quad (2)$$

$$\Delta\text{CT}\left(\text{reference}\right) = \text{CT}\left(\text{undigested,\ reference gene}\right) - \text{CT(digested,\ reference gene)} \quad (3)$$

Calculations for the $\Delta\Delta C_T$ method were done in Excel. Statistical data analysis was performed in the GraphPad Prism 8 program. A Mann-Whitney test was performed; the data represent relative expression ratios obtained with Equation (1). $P < 0.05$ was considered statistically significant, and $p < 0.01$ was considered statistically highly significant.

RESULTS

Clinical Characteristics of PFAPA Patients

All included participants fulfilled the clinical criteria for PFAPA syndrome (5). Included in our study were 44 (58.7%) boys and 31 (41.3%) girls. All patients were asymptomatic during the afebrile period. Pharyngitis (94.7%) and adenitis (89.3%) were the most present symptoms during febrile episodes, followed by abdominal pain (61.3%) and aphthous stomatitis (58.7%) (**Table 5**). Included in our cohort were three pairs of siblings (twin brothers, twin sisters, and two brothers). Positive family history, meaning that at least one first-degree relative had recurrent fevers or a tonsillectomy, was found in 73.4% of patients. Positive family history, either in the first or second degree, was present in 87.5% of patients.

TABLE 5 | Demographic and clinical characteristics of PFAPA patients with family history and symptoms.

Demographic and clinical characteristics of PFAPA patients	
Total number of patients	75
Male	44 (58.7%)
Female	31 (41.3%)
Age at disease onset (mean ± SD)	2.1 ± 1.4 years
Age at giving a sample for DNA (mean ± SD)	4.5 ± 2.0 years
Family history	
Positive family history (first degree)	47/64 (73.4%)
Tonsillectomy in first-degree relative	32/65 (49.2%)
Tonsillectomy in first-degree relatives, more than one family affected	8/64 (12.5%)
Tonsillectomy in second-degree relative	19/64(29.7%)
Tonsillectomy in second-degree relative only, excluding those with tonsillectomy in first-degree relative	8/64 (12.5%)
Unknown	11/75 (14.7%)
Symptoms	
Pharyngitis	71 (94.7%)
Adenitis	67 (89.3%)
Abdominal pain	46 (61.3%)
Aphthous stomatitis	44 (58.7%)
Vomiting	28 (37.3%)
Joint pain	27 (36.0%)
Diarrhea	16 (21.3%)
Skin rash	9 (12.0%)

Identification of DMRs With MDB and MeDIP

13.8 M (12.9–14.8 M) reads per library were collected on average, with 20% (17.2–22.8%) of the duplicates per library excluded from further analysis. The mean insert size per library was 318 bp (305–331 bp), and 91% (88.6–93.6%) of reads had proper pairs identified. MBD enrichment generated libraries with significantly higher %GC (63.3% [62.8–63.8%]) compared to MeDIP enrichment (49% [48.7–49.3%]). After cleanup of the datasets—by eliminating duplication, multiple alignment, and misalignment reads—the MACS2 algorithm identified 352,451 potential peaks in the MeDIP dataset and 100,902 peaks in the MBD dataset. To reduce the number of false-positive results in DMR analysis using DeSeq2, the FDR cutoff value was modified in such a way that the potential DMR set contained up to 1 (one) false-positive result. Consequently, the FDR cutoff value was set to $q < 0.06$ for the MeDIP dataset and $q < 0.15$ for the MBD dataset. After count tables were generated by the featureCount algorithm, the DeSeq2 identified 17 DMRs in the MeDIP dataset and seven DMRs in the MBD dataset. There was no overlap between the identified DMRs from the MeDIP and MBD datasets. All identified DMRs were annotated for proximal genes or other location-specific elements (intergenic space, regulatory elements, ncRNAs, etc.). The identified genomic elements/regions, listed in **Supplementary Table 1**, were analyzed for their potential role in (auto)immune response and consequently selected for further evaluation. Out of 24 identified DMRs, one DMR per enrichment method (MeDIP or MBD) with the most significant signal located near or within genes that could be reliably associated with the autoinflammation through published literature and public database search was further evaluated by MSRE-qPCR.

Identification of DMRs With MSRE-qPCR

Of the differentially methylated sequences previously identified by using MDB and MeDIP, two regions of interest (ROIs) were chosen to perform our analysis based on the location and function of the gene. Of the top significant candidate genes, one was chosen from MeDIP (*PIK3AP1*, region 10:98425909–98426208, GRCh37) and one from the MBD method (*SPON2*, region 4:1163349–1163641, GRCh37). Relative quantification of digested and undigested DNA was performed with MSRE-qPCR in order to compare patients with healthy controls. The second intron region of *PIK3AP1* (10:98425909–98426208) was found to

TABLE 6 | Descriptive statistics of each ROI with exact two-tailed *P*-values of the unpaired Mann-Whitney tests.

	PIK_1 controls	PIK_1 patients	PIK_2 controls	PIK_2 patients	SPON_1 controls	SPON_1 patients	SPON_2 controls	SPON_2 patients
N	64	74	64	75	59	65	61	69
Mean	0.1576	0.1892	0.03239	0.06402	0.2561	0.1912	0.2382	0.2911
Std. deviation	0.08185	0.1486	0.01146	0.09392	0.1584	0.1139	0.1505	0.1656
Std. error of mean	0.01023	0.01728	0.001433	0.01085	0.02063	0.01412	0.01927	0.01994
Lower 95% CI of mean	0.1371	0.1548	0.02953	0.04241	0.2148	0.163	0.1997	0.2514
Upper 95% CI of mean	0.178	0.2236	0.03526	0.08563	0.2974	0.2195	0.2768	0.3309
P-value	0.508		<0.0001		0.001		0.019	

be more methylated in PFAPA patients by the MeDIP method and was verified by MSRE-qPCR, with a slight difference in the size of the differentially methylated region. MSRE-qPCR showed that only the second half (10:98425953–98426190) of the second intron region was over-methylated ($P < 0.0001$), while the first half (10:98425881–98426000) was not significantly different ($P = 0.5079$) compared to the healthy controls (**Table 6**).

Methylation results in the fifth intron region of the *SPON2* gene are conflicting. MBD defined the region of the fifth intron of *SPON2* (4:1163349–1163641) as more methylated in PFAPA patients, while MSRE-qPCR later showed that this is not the case for the whole region. The first half (4:1163329–1163502) of the fifth intron was, in fact, less ($P = 0.001$) methylated and the second half (4:1163480–1163727) was more ($P = 0.0191$) methylated in PFAPA patients compared to the healthy controls.

Results from both MeDIP and MBD were confirmed with MSRE-qPCR, with some differences. Firstly, MSRE-qPCR identifies smaller regions where differential methylation occurs, because qPCR reaction efficiency is limited with amplicon length. Secondly, because MSRE-qPCR has an amplicon size limit, it was able to reveal that the whole region of the fifth intron of the *SPON2* gene does not have higher methylation in PFAPA patients. Moreover, MSRE-qPCR requires only one methylated CpG at the ROI per single DNA molecule to detect a difference, as one cut site is enough to prevent PCR amplification of a particular DNA molecule.

An unpaired Mann-Whitney test with a 95% confidence level was performed. Patients vs. controls were compared for each ROI. Exact two-tailed *P*-values are listed in **Table 6**. The data are visualized with boxplots with added scatter plots and the *P*-value of the Mann-Whitney test in **Figure 1**. Each dot represents the relative expression ratio of each control or patient obtained with Equation (1). The number of controls and patients used for MSRE-qPCR analysis was lower than the initial 65 controls and 76 patients due to the limited amount of DNA for specific samples and difficulties with qPCR.

DISCUSSION

PFAPA syndrome is the most common pediatric fever syndrome, and its etiology is unknown. So far, no clear genetic causes have been found. In this study, we analyzed differential methylation patterns in a cohort of 75 PFAPA patients. All patients included in the study fulfilled the clinical criteria for PFAPA syndrome (5). The majority of patients analyzed were boys (58.7%). During the fever episode, pharyngitis and adenitis were the most common symptoms (94.7 and 89.3%, respectively). All three major symptoms (adenitis, pharyngitis, and aphthous stomatitis) were present in 52% of the patients. The included patients had previously been analyzed in an independent study, and genetic analysis was performed for four genes: *AIM2, NLRP3, MEFV,* and *MVK*. No clinically significant variants were found (18).

Relative quantification with the MSRE-qPCR method proved to be a quick and efficient way to estimate differentially methylated DNA regions, which were identified beforehand by MeDIP and MBD. Our results showed that there are two regions, one inside the second intron of the PIK3AP1 gene and a second one inside the fifth intron of SPON2, which have a statistically significant difference in methylation in PFAPA patients when compared to healthy controls.

Both MeDIP and MBD have the advantage of identifying regions with differential methylation over the whole genome, while MSRE-qPCR is more suitable to analyze the few previously identified candidate regions. MeDIP favors regions with low CpG density and MBD favors regions with higher CpG density (29), which was also observed in our case. All three methods are a suitable way to identify differentially methylated genomic regions, though they cannot determine how many and which CpGs are methylated specifically.

The *PIK3AP1* gene was identified as more methylated in PFAPA patients by MeDIP, which the MSRE-qPCR method confirmed, but only for a part of the said region. The MBD results identified a region of *SPON2* as more methylated in PFAPA patients, while MSRE-qPCR later revealed that this is not the case for the whole identified region. The first half had lower methylation levels in PFAPA patients, while the second half had higher methylation levels. A possible reason for such divergence with the results of the first half of *SPON2* could be the specifics of the process of broad peak identification by the MACS2 algorithm, where a wider area is incorporated into the final signal peak identification, partially influenced by the final NGS library insert size as well. Nevertheless, there is a measurable difference in the methylation pattern in the fifth intron region of the *SPON2* gene.

The B-cell adapter protein (BCAP, *PIK3AP1* gene) is a phosphoinositide 3-kinase (PI3K) binding protein (30) and

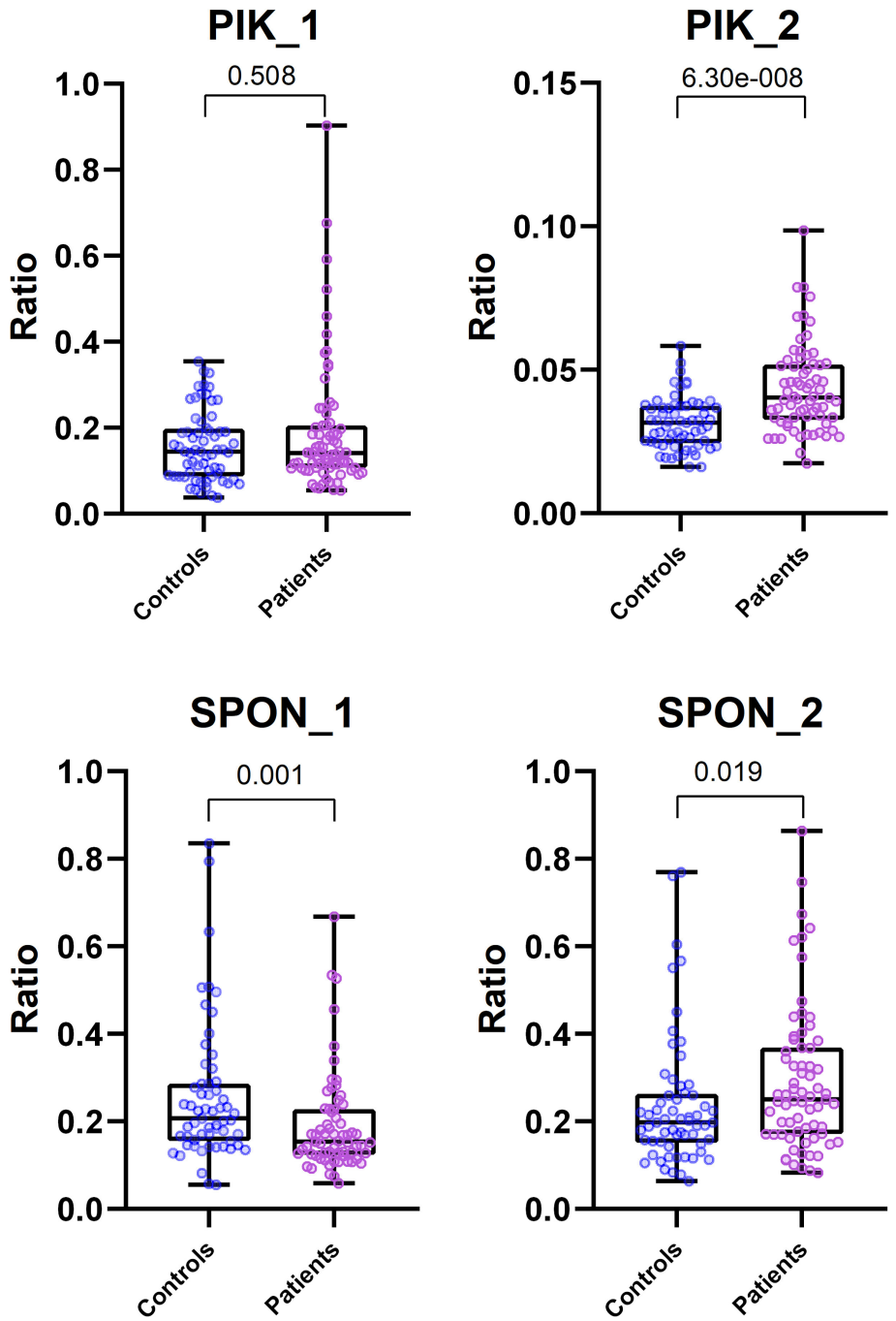

FIGURE 1 | Comparison of MSRE-qPCR ratios between Control and Patient group for each ROI. Boxplots include scattered dots that represent relative expression ratio of Control (blue) or Patient (pink) sample. Also included at the top are exact two-tailed *P*-values of unpaired Mann-Whitney tests. All scatter plots are plotted with all data, except the plot for PIK_2 ROI, which was plotted with data without outliers (ROUT method, Q = 0.1%) for visualization purposes. Mann-Whitney tests for all ROIs were performed with all data (outliers included).

an important inhibitor of proliferation and myeloid cell differentiation that works in a cell-intrinsic manner (31). The PI3K signaling cascade influences cell proliferation and survival, metabolic reprogramming, and cellular migration. As a PI3K adaptor, BCAP is a key regulator of PI3K signaling and T-cell development into effector and memory cells (32). It is expressed in lymphoid as well as in myeloid cell populations (31–34). BCAP regulates inflammatory response. BCAP deficiency results in exaggerated innate immune response, leading to higher $CD4^+$ activation (35) and to more proliferative cells (31). As a

macrophage signaling adaptor protein, it can dampen NLRP3 and NLRC4 inflammasome activation through interaction with the caspase-1 inhibitor (36). Altogether, BCAP could play a part in influencing systemic inflammation, which PFAPA syndrome is known for.

Spondin-2 (also called mindin, *SPON2* gene) is an extracellular matrix protein that functions as a pattern recognition molecule (PRM) for initiating innate immune responses, as well as an integrin ligand for inflammatory cell recruitment and T-cell priming (37–39). It has been shown that, *in vivo*, it is crucial for the efficient clearance of bacterial (37) and viral (40) infections. Spondin-2/mindin-induced signaling could be as important as other, better-defined signaling pathways, such as TLR signaling. He et al. have proposed that mindin-mediated carbohydrate recognition of microbial pathogens represents a secondary stimulation essential for activation of innate immune cells (37). Additionally, it has been suggested that it has a role in the immune response against tumor cell growth and migration (41). Little else is known of spondin-2 regarding immune regulation; however, as an extracellular PRM, it could have a role in inflammation, since extracellular PRMs are able to complement activation, opsonization, agglutination, neutralization, and regulation of inflammation (42).

Epigenetic modifications, including DNA methylation, can affect gene expression and, consequently, can be used as a disease biomarker (43). Methylation of CpG dinucleotides is one of the principal epigenetic mechanisms (44). Regions rich in CpGs are called CpG islands (CGI). Unmethylated promotor CGIs are generally associated with transcriptionally active genes, whereas hypermethylated promotor CGIs result in gene transcription repression. Furthermore, methylation levels inside the gene correlate with gene expression, especially the first intron methylation (45). The majority of research on disease-related DNA methylation has been done in the field of cancer (43); however, there is increasing evidence that epigenetic dysregulation plays a role in autoinflammatory diseases as well (46).

The role of DNA methylation of intragenic regions is less clear, though evidence exists that methylated intragenic regions influence gene transcription (47). Partial methylation of the coding region can inhibit gene expression (48, 49), and even a few methylated cytosines can inhibit a flanking promoter, but a threshold of modified sites is required (50). Elevated DNA methylation in intragenic regions usually correlates with silencing of the associated gene (51). Blattler et al. showed that changes in DNA methylation within bodies of genes played a much larger role than changes in promotor regions (52). The methylation level of the first intron is inversely associated with gene expression, and this association is conserved regardless of the species or tissue. Methylation tends to increase with distance from the first exon–first intron boundary, and its effect on gene transcription decreases with downstream distance from the first exon (45, 53). The study of DNA methylation during monocyte differentiation revealed that a high proportion of the changes occurred in non-promoter regulatory regions, mainly enhancers in gene bodies and intergenic regions (54). Methylation patterns in the *MEFV* exon in pediatric patients with Familial Mediterranean Fever correlated with expression of the same gene; the observed slight increase in DNA methylation of the second exon in patients correlated with decreased expression (22).

The observed differential methylation of the second intron in *PIK3AP1* (10:98425953–98426190) could be of great significance regarding changed expression. BCAP functions as a checkpoint to restrict TLR signaling and production of inflammatory cytokines (55). We hypothesize that the higher methylation found in the second intron of *PIK3AP1* in PFAPA patients could lead to lower expression of the BCAP protein and cause a disrupted inhibition of inflammation, leading to exaggerated inflammation or response to environmental stimuli (for example an infection). The cause of this particular differential methylation is unknown, and DNA methylation can be influenced by a range of external factors, such as diet, drugs, and infections (56, 57). There is evidence of infection-induced hypermethylation of *PIK3AP1* promoter and downregulation of its expression (58) and evidence of hypomethylation and increased expression of *PIK3AP1* triggered by low levels of folic acid (59). Infections are proposed contributors to the pathogenesis of PFAPA (15, 60).

The second half of the spondin-2 region (4:1163480–1163727, GRCh37) that was investigated and showed higher methylation in PFAPA patients is also part of a CTCF binding region (4: 1163601–1163800, GRCh37). CTCF is a transcription factor, chromatin organizer, and insulator protein that was initially discovered as a transcriptional repressor; however, its exact mechanisms remain unknown (61). We hypothesize that the observed increased methylation could potentially inhibit CTCF binding. As an insulator protein, CTCF can influence gene transcription by restricting the binding of transcription enhancers. Its binding sites are located far from the transcriptional start sites, and their distribution is strongly correlated with genes (62). However, without gene expression analysis, we cannot confirm whether the changed methylation in *PIK3AP1* and *SPON2* influences their expression and in what way.

DNA was isolated from whole blood, meaning we analyzed methylation in DNA from the whole blood cell population with unknown ratios. Samples were collected during an afebrile period, patients were asymptomatic, and we assumed that blood cell populations were normal and comparable based on the facts that changes in white blood cell counts are associated with febrile episodes (13, 63) and that the concentrations of white blood cells in afebrile PFAPA patients are comparable to those in healthy children (63). Our results indicate that there is a portion of blood cells with differentially methylated DNA in these two genes and that the number of cells or the specific clonal subpopulation of cells was high enough to generate a measurable signal. Currently, there is no clear evidence which cell subpopulation is involved. High monocytes and high neutrophils seem to be the most consistently reported changes in blood cell populations during PFAPA febrile attacks (13, 63, 64) and could be the possible source of the differential DNA methylation we observed.

A limitation of our study, in addition to the mixed cell populations as our source of DNA, is the fact that 15 patients (20%) had previously received methylprednisolone, which could

affect their DNA methylation patterns. We do not know if it affected the regions in question or in what way and for how long. Steroids do have an effect on the disease, but since their effect is not permanent, they only stop the episode and not the disease itself (65–67). In order to overcome this limitation, we re-analyzed data excluding patients that received steroid treatment and obtained similar results. Significance did not change, except for the second half of the *SPON2* region, which crossed the significance line but still showed a tendency of hypermethylation. Loss of significance could also be partly attributed to reduced sample size. Additional studies investigating the effect of methylprednisolone should be conducted. Another limitation would be that the test and control groups were not of the same age (4.1 and 5.3 years, respectively). The median age of our control group was intentionally above five, which is when the fevers generally begin to appear (5–7). Altogether, the results from our methods do not inform us of the identity and number of CpGs involved. We also cannot tell in which cells this difference in methylation occurs, since the source of our analyzed DNA was whole blood. For a more detailed look into the possible role of DNA methylation in the pathogenesis of PFAPA, DNA isolated from different cell lineages should be analyzed.

Both the B-cell adapter protein through the PI3K activation pathway and spondin-2 as an extracellular pattern recognition molecule are involved in the primary stages of immune responses. BCAP acts as an inhibitor of receptor signaling, mainly TLR and spondin-2, possibly as a recruiter or activator of T cells, or by working as an opsonin that activates complement. The role of changed DNA methylation in autoinflammation certainly needs further investigation, as does the role of BCAP and spondin-2 in the etiology of PFAPA. Differential methylation of genes *PIK3AP1* and *SPON2* is not reported in association with other similar autoinflammatory diseases; however, *PIK3AP1* was associated with autoimmunity (68), and its increased expression was shown to promote TLR7-driven lupus-like disease (69). Differential expression and methylation of both genes have been linked with cancer. Changed methylation of *PIK3AP1* is associated with neuroblastoma (70), and its upregulation is associated with Waldenström macroglobulinemia (71). Hypomethylation of the promoter of *SPON2* and its increased expression is associated with prostate cancer (72, 73); its upregulation is also associated with colorectal cancer (74).

As far as the clinical impact of these data is concerned, this kind of change in methylation would not be detected by Sanger sequencing, because the proportion of differentially methylated DNA molecules is too small. Therefore, a more advanced quantification method is needed, such as MSRE-qPCR. Nevertheless, the cutoff values for the positive MSRE-qPCR results should be carefully examined, set, and validated.

CONCLUSIONS

Whole-genome methylation screening analysis methods, such as MeDIP and MBD are a useful tool for identifying differentially methylated genomic regions. MSRE-qPCR proved to be a reliable, quick, and cost-effective method of confirming results and showed potential applicability in translation of the research into clinical practice. The changed methylation patterns in PIK3AP1 and SPON2 that we observed in PFAPA patients point to novel and still unknown roles of BCAP and spondin-2 in the etiology of PFAPA. Furthermore, likely transient changes of DNA methylation patterns are potentially a novel direction in research into the molecular mechanisms leading to PFAPA development.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Republic of Slovenia. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

EL, JK, TT, and TR contributed the conception and design of the study and performed the actual experiments and analysis. EL wrote the first draft of the manuscript. JK wrote the sections of the manuscript. NT and DP selected the included patients and collected data and samples. MD and TA contributed equally and were responsible for the final approval of the submitted version. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01322/full#supplementary-material

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Primary Immunodeficiencies in Russia: Data From the National Registry

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Introduction: Primary immunodeficiencies (PID) are a group of rare genetic disorders with a multitude of clinical symptoms. Characterization of epidemiological and clinical data via national registries has proven to be a valuable tool of studying these diseases.

Materials and Methods: The Russian PID registry was set up in 2017, by the National Association of Experts in PID (NAEPID). It is a secure, internet-based database that includes detailed clinical, laboratory, and therapeutic data on PID patients of all ages.

Results: The registry contained information on 2,728 patients (60% males, 40% females), from all Federal Districts of the Russian Federation. 1,851/2,728 (68%) were alive, 1,426/1,851 (77%) were children and 425/1,851 (23%) were adults. PID was diagnosed before the age of 18 in 2,192 patients (88%). Antibody defects (699; 26%) and syndromic PID (591; 22%) were the most common groups of PID. The minimum overall PID prevalence in the Russian population was 1.3:100,000 people; the estimated PID birth rate is 5.7 per 100,000 live births. The number of newly diagnosed patients per year increased dramatically, reaching the maximum of 331 patients in 2018. The overall mortality rate was 9.8%. Genetic testing has been performed in 1,740 patients and genetic defects were identified in 1,344 of them (77.2%). The median diagnostic delay was 2 years; this varied from 4 months to 11 years, depending on the PID category. The shortest time to diagnosis was noted in the combined PIDs—in WAS, DGS, and CGD. The longest delay was observed in AT, NBS, and in the most prevalent adult PID: HAE and CVID. Of the patients, 1,622 had symptomatic treatment information: 843 (52%) received IG treatment, mainly IVIG (96%), and 414 (25%) patients were treated with biological drugs. HSCT has been performed in 342/2,728 (16%) patients, of whom 67% are currently alive, 17% deceased, and 16% lost to follow-up. Three patients underwent gene therapy for WAS; all are currently alive.

Conclusions: Here, we describe our first analysis of the epidemiological features of PID in Russia, allowing us to highlight the main challenges around PID diagnosis and treatment.

Keywords: primary immunodeficiency, epidemiology, genetics, PID registry, HSCT, IVIG

INTRODUCTION

Primary immunodeficiencies (PID)—also referred to as "inborn errors of immunity"—are rare disorders characterized by susceptibility to infection and a preponderance of autoimmunity, allergy, autoinflammation, and malignancies. According to the latest update of the International Union of Immunological Societies Experts Committee (IUIS) (1) classification, germline

mutations in 430 genes cause 404 distinct phenotypes of immunological diseases, divided into 10 groups according to the type of immunological defect. Wide introduction of the molecular genetic techniques, including next-generation sequencing (NGS) (2), has led to the description of novel PID genes. This allows for a more precise assessment of clinical prognosis and for the choice of targeted therapy—or even gene therapy—as well as for family counseling (3).

Generally, PID are described as rare diseases. Yet their reported prevalence varies greatly in different countries, depending on many factors: from data collection methodology to objective epidemiological features. In European countries, the estimated prevalence of PID ranges from 2.7/100,000 in Germany, to 4.16-5.9/100,000 in Switzerland and the United Kingdom (UK), to 8/100,000 in France (3–7). These numbers are in the range of the "orphan diseases" category. Yet recent findings, in patients with mendelian susceptibility to mycobacterial diseases (MSMD) (8), suggest that the actual prevalence is much higher.

National PID registries (2, 3, 5), along with registries combining data for geographical regions (9, 10), have proven to be an important tool for assessing the clinical and epidemiological features of PID—as well as an instrument for facilitating PID collaboration and research, both within and between countries.

Several PID cohort study reports from Russia (11, 12) have been published recently, yet little has been known about the overall epidemiological features of PID in the heterogeneous Russian population. The aim of this study is to describe PID epidemiology in Russia, using a national registry.

MATERIALS AND METHODS

Registry Structure

The Russian PID registry was established in 2017, as an initiative of the National Association of Experts in PID (NAEPID)—a non-profit organization facilitating collaboration amongst leading specialists in the field of primary immunodeficiencies in Russia. The registry is a secure on-line database, developed, and designed with the aim of collecting epidemiological, clinical, and genetic data of PID in Russia. It includes demographic data, clinical and laboratory details, molecular diagnosis, and treatment aspects of PID patients of all ages. Regular information updates allow for the collection of prospective data. The data is entered via an online registry form only; no paper-based documentation is needed. A group of trained managers at federal centers and doctors at regional hospitals enter the data in the database.

This article analyzes the data input into the registry from its inception until February 1, 2020.

At the time of the data analysis, PID variants were grouped according to the IUIS 2015–2017 classification (1) and did not include the newly added category of bone marrow failure (13). The database structure includes the following obligatory fields: demographic data, family history, diagnosis, genetic testing results, and ages of disease onset and diagnosis. The extended universal fields—including detailed clinical description and treatment data—are not mandatory at the time of the first registry of a patient, but are eventually requested. New entries are reviewed automatically, and no duplicate entries can be created. Human-factor errors are prevented by built-in quality assurance measures.

Patients can only be registered if the documenting center is part of the registry's collaborative team. Written informed consent is given by all registered patients or their legal guardians. Regularly updated reports on PID epidemiological data are published on the NAEPID Registry website http://naepid-reg.ru.

Registry Platform

The software platform used in the study was developed by Rosmed.info, using the PHP programming language. For database management, the Maria DB relational system (offshoot of the MySQL system) was utilized. Server Version: the 10.1.40-Maria DB Server and replication mechanism were used for back-up and improved performance; the server's contour and physical protection were compliant with Russian law regarding personal information protection.

Centers

Russia is divided into 85 regions, which are grouped geographically into eight federal districts. Data on the PID patients residing in 83 of the federal regions has been accumulated in the registry, with the input of regional and tertiary centers. No patients residing in the other two regions (Chukotka and Tuva) were registered in the database. At the time of analysis, 69 regional medical centers and 5 university clinics—located in all 8 federal districts—have contributed to the collaborative work. Three tertiary immunology centers located in Moscow serve as the main reference centers. The diagnosis of the majority of the patients (2,488/2,728, 91%) has been confirmed in at least one of the tertiary centers.

Patients

PID diagnosis was made according to the ESID diagnostic criteria (13). Patients with secondary immune defects were excluded. Although the registry collects data on all PID, 233 patients with selective IgA deficiency, and 106 patients with PFAPA (periodic fever, aphthous stomatitis, pharyngitis, adenitis) were not included in the current analysis.

The entire cohort of patients (2,728) was included in the epidemiological analysis—while, for the treatment description, we used only the updated information available for the 1,851 alive patients.

Genetic testing has been performed using the main molecular techniques, including Sanger sequencing, targeted next-generation sequencing (NGS), whole-exome and whole-genome sequencing, fluorescent *in situ* hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), and chromosomal microarray analysis (CMA), according to standard protocols.

Data Verification

All data entered into the registry undergoes automatic verification for typing errors and is regularly checked by the database monitor for consistency and completeness.

Terminology and Definitions

The actual age distribution was calculated only for the patients with updated information; the age of each patient was determined

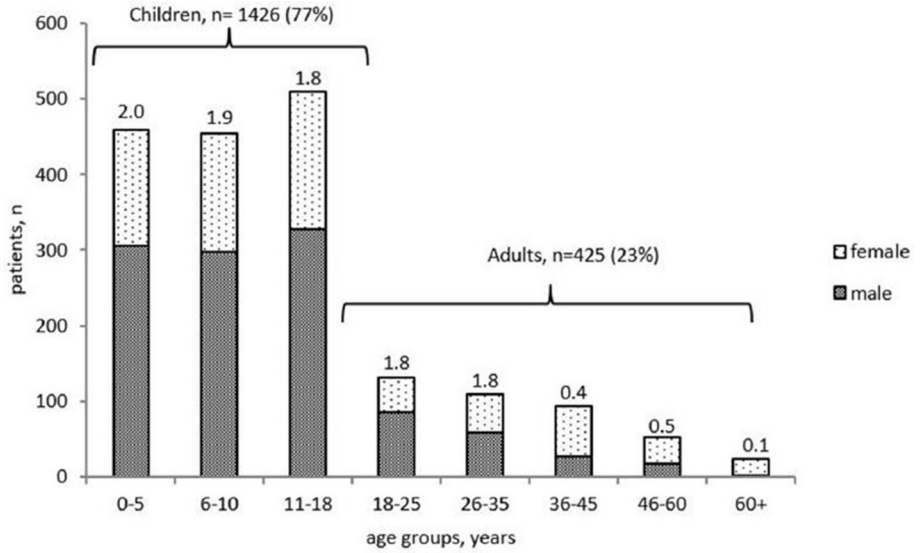

FIGURE 1 | Age distribution and gender ratio of PID patients registered in the Russian PID registry (living 492 patients only, $n = 1851$).

as the difference between their date of birth and the date of the last update.

Patients without any contact within the last 2 years were marked as "lost to follow-up."

The diagnostic delay was estimated for all registered patients, in the nine most common PID categories, as the difference between the date of disease onset and the date of clinical diagnosis of PID.

Prevalence was estimated as the number of all registered PID cases, divided by the population of Russia or of each federal district; information was obtained from open resources[1].

Incidence was estimated as the number of new PID cases diagnosed during each year, divided by the number of live births during that year in Russia; information was obtained from open resources.

Prevalence and incidence were expressed as the number of cases per 100,000 people.

Mortality rate, expressed in percentage, was estimated as the number of deceased patients divided by the number of all updated PID cases; lost-to-follow-up patients were excluded.

The category of "fully recovered" was not available at the time of analysis.

Patients from birth to 17 years, 11 months, and 29 days were counted as children. The rest were considered adults.

Statistical Analysis

Demographic and epidemiological characteristics were described as average for the categorical variables, and median and range for the quantitative variables. To compare the prevalence of the diseases, the chi-squared test was used and a p-value of <0.05 was considered statistically significant. The average immunoglobulin (IG) dose was expressed as mean ± standard deviation. Statistical analysis was performed using XLSTAT Software (Addinsoft).

[1]Available online at: https://showdata.gks.ru/finder/descriptors/274848 (accessed March 1, 2020).

RESULTS

Demographics and PID Distribution

Information on 2,728 PID patients was available for analysis. Of these patients, 1,851 (68%) were marked as alive and 200 (7%) as dead. The remaining 677 (25%) were not updated during the last year or were lost to follow-up. The male-to-female ratio was 1.5:1, with 1,657 male patients (60%) and 1,071 female (40%).

Of the 1,851 living patients, 1,426 (77%) were children, and 425 (23%) were adults. The majority of the children (913 of 1,426, 64%) were under 10 years old. The male-to-female ratio varied from 2:1 in children, to 1:1 in the group of adults under the age of 30 and 0.4:1 in the older patients (**Figure 1**).

PID was diagnosed before the age of 18 years (in childhood) in 2,192 patients (88%), predominantly in the first 5 years of life (1,356, 54%; **Figure 2**). The distribution of patients among the main PID groups varied greatly between children and adults. All forms of PID were observed in children and in young adults (under the age of 25 years). Yet the majority of older patients belonged to just two categories—common variable immunodeficiency (CVID) and hereditary angioedema (HAE).

Overall, primary antibody deficiencies (PAD; 699; 26%) and syndromic PID (591; 22%) were the most common disorders in Russia. These were followed by five PID groups, in similar proportions: complement deficiencies (342; 12%), phagocytic defects (262; 10%), combined T and B cell defects (368; 13%), autoinflammatory disorders (221; 8%), and immune dysregulation (196; 7%; **Figure 3**). Somatic phenocopies (6; <1%) and defects of innate immunity (43; 1.5%) were very rare.

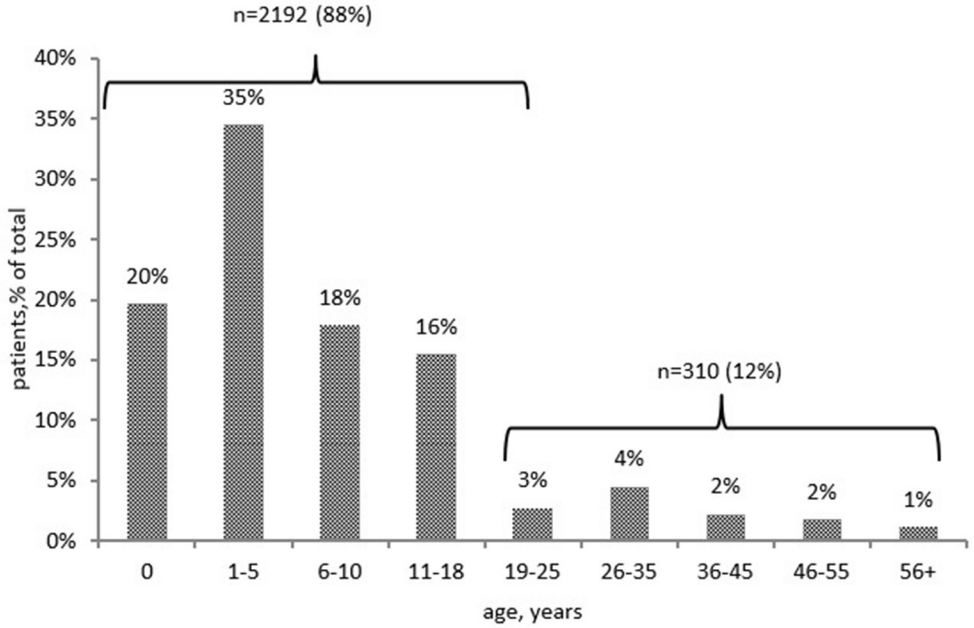

FIGURE 2 | Patients' distribution by the age of PID diagnosis (n = 2,502).

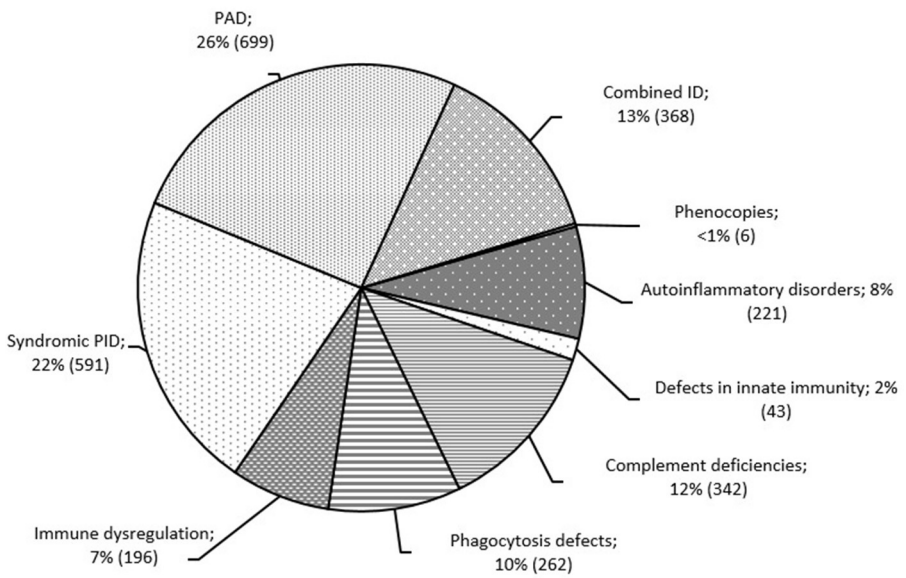

FIGURE 3 | Distribution of patients among the PID groups (n = 2,728). PID groups are shown according to IUIS classification, 2017 (1). Total number of patients and percentage of all registered patients are shown for each group.

The most frequent PID categories in Russia, which cumulatively accounted for 53% of all registered patients, were: HAE type 1 and 2 (n = 341), CVID (n = 317), Wiskott–Aldrich syndrome (WAS; n = 154), X-linked agammaglobulinemia (XLA; n = 155), Chronic granulomatous disease (CGD; n = 135; of them 92 patients with X-linked CGD (X-CGD), Severe combined immunodeficiency (SCID; n = 137; of them 47 patients with X-linked SCID (X-SCID), DiGeorge syndrome (DGS; n = 130), Ataxia-telangiectasia (AT; n = 127) and Nijmegen breakage syndrome (NBS; n = 88; **Table 1**).

To assess mortality, we analyzed the cohort of 2,051 patients whose status was known (including 1,851 alive and 200 deceased patients). The overall mortality rate was estimated at 9.7%. The precise date of death was known for 136 of the 200 deceased

TABLE 1 | Distribution of patients by PID groups [according to IUIS classification, 2017 (1)].

PID Category	Patients, *n*	Gender		Living status			Family cases	HSCT
		Female	Male	Alive	Deceased	Lost to follow-up		
Autoinflammatory disorders	221	101	120	186	1	34	23	6
Defects in intrinsic and innate immunity	43	19	24	32	1	10	8	7
Complement deficiencies	342	215	127	210	3	138	99	–
Congenital defects of phagocyte number or function	262	71	191	186	14	62	41	66
Diseases of immune dysregulation	196	65	131	134	18	44	24	41
Combined immunodeficiencies with associated or syndromic features	591	214	377	405	59	127	57	106
Predominantly antibody deficiencies	699	238	461	453	21	225	36	5
Immunodeficiencies affecting cellular and humoral immunity	368	145	223	248	83	37	22	111
Phenocopies	6	3	3	6	–	–	–	–
Total number of patients	2,728	1,071	1,657	1,851	200	677	310	342

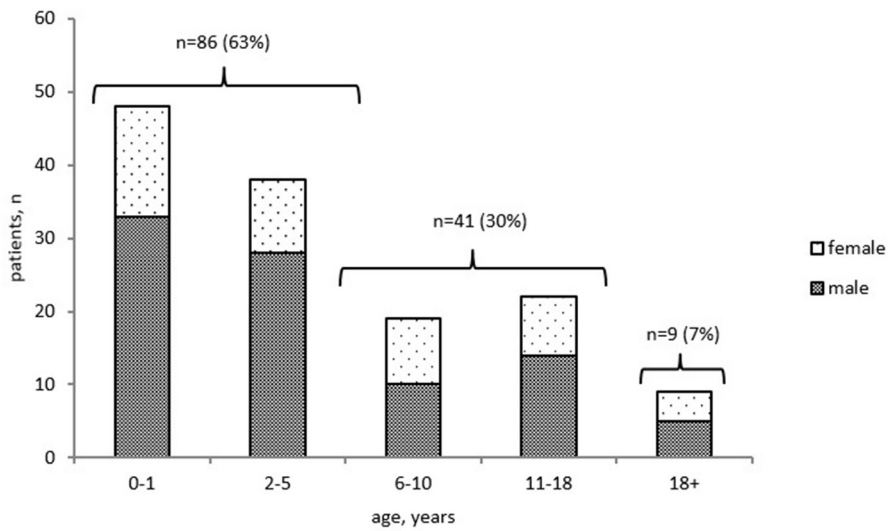

FIGURE 4 | Distribution of deceased PID patients by age at death and gender ($n = 136$). Only patients with known date of death were included.

patients: 127 (93%) children and 9 (7%) adults (**Figure 4**). The mortality rate ranged from 2 to 42% in different age groups; the highest rate was found in children in their first 2 years of life (**Figure S1**). The majority of infant deaths occurred in SCID patients (39 of 48, 81%; **Figure S1**). In the next age group (2–5 years), mortality was highest in the following four PID groups, in almost equal proportions: T and B cell defects (12/38, 32%) and syndromic PID (11/38, 29%), followed by phagocytic defects (7/38, 18%), and immune dysregulation (7/38, 18%). In total, 63% (86/136) of all PID-related deaths occurred in patients within the first 5 years of life. In older children (12–14)[1], (15–17), mortality was associated predominantly with syndromic PID (55%), immune dysregulation (9%), and PAD (13%)—whereas, in adults, it was associated only with PAD (78%) and HAE (22%; **Figure S1**).

[2]Available online at: https://cci-reporting.uniklinik-freiburg.de/#/ (accessed March 1, 2020).

Diagnostic Delay

Substantial PID diagnostic delay has been noted in Russia—with a median of 2 years for the whole group, but over a broad age range (0–68 years). No difference in diagnostic delay was observed, between patients diagnosed during the last 5 years ($M = 2$; 0–63, 997 patients) and before 2015 ($M = 2$ years; 0–68, 1,400 patients). Among the most common PID, the shortest diagnostic delay was observed in SCID ($M = 4$ months, 0–68), followed by the WAS ($M = 8$ months, 0–144), DGS ($M = 10$ months, 0–144), and CGD ($M = 1$, 0–17 years; **Figure 5A**). In X-linked agammaglobulinemia (XLA) patients, time to diagnosis varied greatly—from 0 to 141 months, with a median of 28 months. The DNA repair disorders NBS and AT were diagnosed with a median of 2.5 years (0–23) and 3.0 years (0–14), respectively (**Figure 5B**). The longest diagnostic delay was observed in CVID ($M = 6$ years, 0–52) and HAE ($M = 11$ years, 0–68; **Figure 5C**).

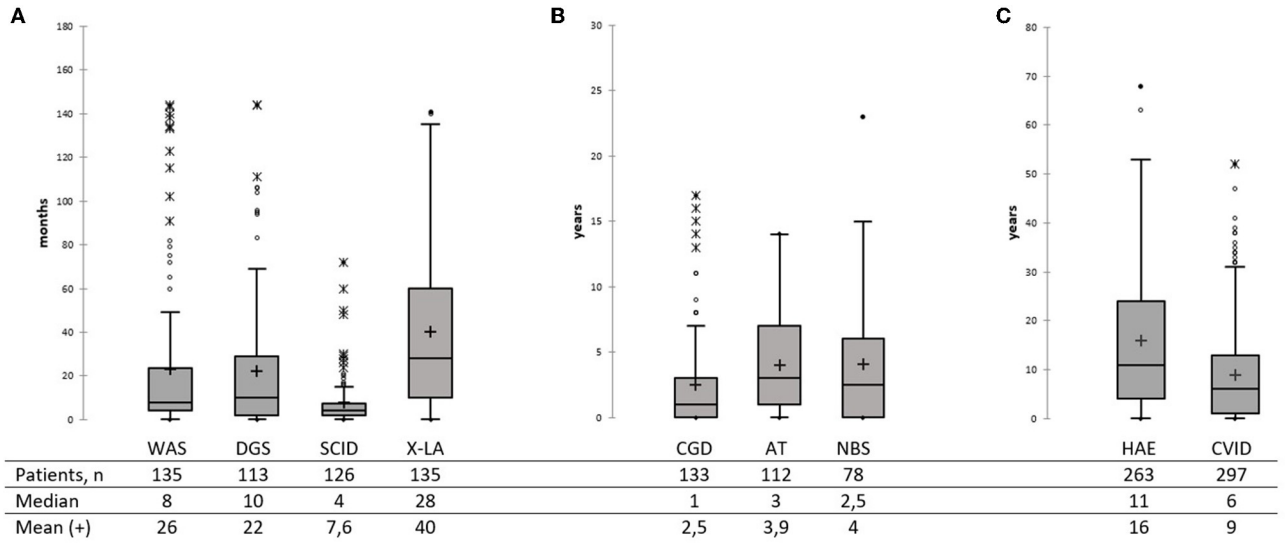

FIGURE 5 | Diagnostic delay in the main PID categories **(A–C)**. Total numbers of patients, median, and mean are shown below the graph. Median is marked as a black line; mean is marked as a cross. **(A)** Diagnostic delay in WAS, SDG, SCID, and XLA patients. **(B)** Diagnostic delay in CGD, AT, and NBS patients. **(C)** Diagnostic delay in HAE and CVID patients.

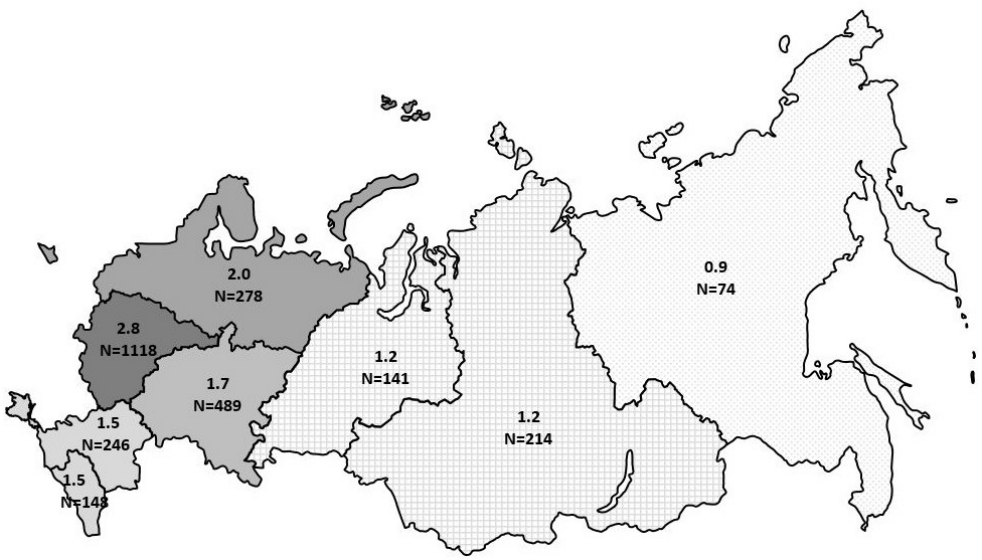

FIGURE 6 | Prevalence of PID in Russia by federal district. The numbers represent prevalence per 100,000 people and total number of registered PID patients in each district. The registered number includes living and deceased patients.

Just a few PID patients were diagnosed before the clinical onset of the disease, due to their family history; genetic testing was carried out for each of them. These included seven children with mutations in *SERPING1*, two with *BTK*, one with *WAS,* and one with *JAK3* defects. Genetic diagnosis led to an early start on IVIG therapy in the XLA patients, and to successful HSCT in the WAS and SCID patients.

Family History

The registry contained 310/2,728 (11%) familial PID cases, originating from 150 families (**Table 1**), with the most frequent familial PIDs being HAE, WAS, and XLA. Consanguinity, as reported by the parents, was documented in 45 families. A family history of at least one death suspected to be due to PID was documented for 275 patients. These included infection-related deaths, in 185 cases, and malignancy-related deaths in 49 cases.

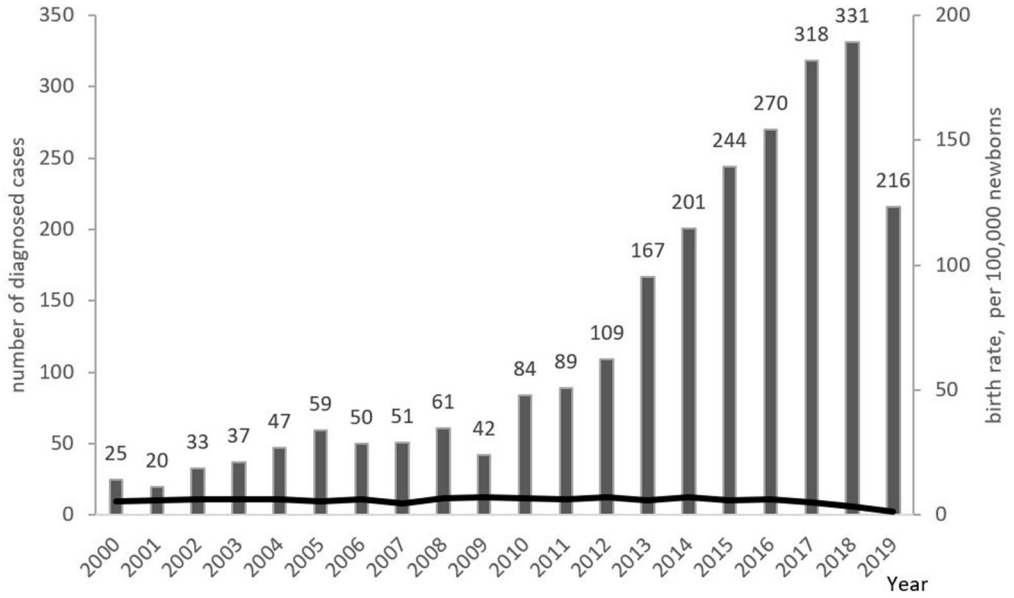

FIGURE 7 | Annual PID incidence and numbers of newly diagnosed PID cases. Incidence is presented as the number of PID born each year per 100,000 newborns (shown as black line); newly diagnosed PID cases (columns) are presented as the number of patients registered in each year. Note that the lower number of patients in 2019 represents a lag in patients' registration into the database.

Epidemiology

The minimum overall PID prevalence in the Russian population was estimated at 1.3:100,000 people, with drastic variations among the federal districts (from 0.9 to 2.8 per 100,000; **Figure 6**). The average annual PID incidence was estimated to be 5.7 ± 0.6 in 100,000 live births. This ranged from 4.4 to 7.1:100,000, over the period from 2000 to 2019. During this period, the average number of newly diagnosed PID cases per year increased from 201 to 331 (**Figure 7**).

Prevalence was estimated only for those PIDs frequently found in the adult group and with a low number of deaths registered in the database—CVID and HAE, with 0.22 and 0.23 per 100,000 people, respectively. This represents population frequency rates of 1 case per 430,000–450,000 people.

Genetic Defects

Genetic testing has been performed for 1,740 patients, with genetic defects confirmed in 1,344 (77%). PID diagnosis has been genetically confirmed in 86% of the children, yet in only 12% of the adults.

Disease-causing genetic defects were detected by the following genetic methods: by direct Sanger sequencing in 903 patients (67%) and by next-generation sequencing (NGS) methods in 323 (24%) patients [including targeted panels, in 278; whole exome sequencing (WES), in 30; Clinical exome, in 13; and whole genome sequencing (WGS), in 2]. In the remaining 118 (9%) patients, cytogenetic methods and MLPA were used. Deletion of 22q.11 was confirmed via the FISH method in 80 patients, and by CMA in 26. In 6 cases, various chromosomal abnormalities resulting in syndromic forms of PID were confirmed by CMA.

Mutations were found in 98 PID genes and in three genes that are not currently included in the PID classification (*NTRK1*, *SCN9A*, *XRCC4*) (**Table 2**).

As expected, the highest number of genetic defects were found in genes underlying the most frequent "classical" PID: mutations in *SERPING1* were found in 178 of 341 HAE cases (52.2%), WAS in 154 (100%) of WAS patients, *BTK* in 114 of 155 X-LA (73.5%), *CYBB* in 98 (73%) of CGD 135 cases, *NBN* in 75/88 (85%) of NBS patients and *ATM* in 55/127 (43%) of AT patients. 106/130 DGS patients had del22q.11 confirmed. At least 20 patients (for each disease) had mutations in the following genes: *MEFV*, *MVK*, *NLRP3*, *ELANE*, *SBDS*, *FAS*, *STAT3 LOF*, *IL2RG*, and *CD40LG*. Rare defects, with 4–20 patients for each gene, affected predominantly recently described genes: *PSTPIP1*, *TNFRSF1A*, *CXCR4*, *STAT1*, *CYBA*, *STXBP2*, *FOXP3*, *CTLA4*, *AIRE*, *XIAP*, *SH2D1A*, *SMARCAL1*, *RMRP*, *SPINK5*, *KMT2D*, *NFKB1*, *PIK3CD*, *PIK3R1*, *TNFRSF13B*, *RAG1*, *RAG2*, *ADA*, *ARTEMIS*, *JAK3*, *LIG4*, and *KRAS*. The remaining 57 genes had mutations recorded for single (1–3) patients (**Table 2**).

The proportion of patients with genetically confirmed diagnoses was highest among those with syndromic PIDs, reaching 77% (457/591) (**Table 1**). Within the phagocytic defect and innate immunity defect groups, 71% (185/262) and 63% (27/43) of the patients, respectively, had a genetic diagnosis. PID genetic confirmation showed about half of all patients in the groups to have immune dysregulation (56%; 109/196), autoinflammatory disorders (49%; 109/221), and complement deficiencies (52%; 179/342)—the last of these due mainly to HAE. The proportion of patients with genetic diagnoses showing T- and B-cell defects was 33% (123/368). The lowest number of patients with verified mutations, at 21% (144/699),

TABLE 2 | Distribution of patients by individual disorders/genetic defects.

PID Category	Patients, *n* With genetic diagnosis/total (% of total)
Autoinflammatory disorders	**109/221 (49%)**
Defects affecting the inflammasome	
MEFV	31
MVK	26
NLRP3	22
PSTPIP1	8
TNFRSF1A	8
polygenic: PSMB8, PSMA5, PSMC5	1
NLRP1	1
POMP	1
PLCG2	1
Non-inflammasome-related conditions	
IL36RN	1
Type 1 interferonopathies	
ADA2	3
IFIH1, GOF	3
TMEM173	2
ADAR1	1
Defects in intrinsic and innate immunity	**27/43 (63%)**
Epidermodysplasia verruciformis (HPV)	
CXCR4	9
Mendelian susceptibility to mycobacterial disease (MSMD)	
STAT1, AD LOF	1
STAT1, AR LOF	1
IL12RB1	3
IFNGR2	1
IFNGR1	1
Predisposition to mucocutaneous candidiasis	
STAT1, GOF, del3p25.3*	1
STAT1, GOF	6
Predisposition to severe viral infection	
STAT2	1
Other inborn errors of immunity related to non-hematopoietic tissues	
NBAS	2
TCIRG1	1
Complement deficiencies	**179/342 (52%)**
CFHR3	1
HAE	178/341
SERPING1	178
Congenital defects of phagocyte number or function	**185/262 (71%)**
Congenital neuntropenias	79/107 (74%)
ELANE	32
GFI1	1
G6PC3	1
SBDS	38
USB1	1
SMARCD2	1
CSF3R	1
Tafazzin (TAZ)	1
WAS, GOF	2
SLC37A4	1
Defects of respiratory burst	101/135 (75%)
CYBB	91
CYBB, 4XXY*	1
CYBA	6
NCF1	2
NCF2	1
Other non-lymphoid defects	
GATA2	3
Defects of motility	
ITGB2	1
RAC2	1
Diseases of immune dysregulation	**109/196 (56%)**
Familial hemophagocytic lymphohistiocytosis (FHL syndromes)	
PRF1	1
UNC13D	8
STXBP2	4
FHL syndromes with hypopigmentation	
LYST	2
RAB27A	1
Regulatory T cell defects	
FOXP3	8
CTLA4	11
CTLA4, del2q.33.2*	1
LRBA	2
STAT3, GOF	4
Autoimmunity with or without lymphoproliferation	
AIRE	11
Autoimmune lymphoproliferative syndrome	
CASP10	2
FAS	25
Immune dysregulation with colitis	
IL10RA	1
Susceptibility to EBV and lymphoproliferative conditions	
XIAP/ BIRC4	16
SH2D1A	10
RLTPR	2
Combined immunodeficiencies with associated or syndromic features	**457/591 (77%)**
Immunodeficiency with congenital thrombocytopenia	
WAS	154
Anhidrotic ectodermodysplasia with immunodeficiency	
IKBKG	3

(Continued)

TABLE 2 | Continued

PID Category	Patients, *n* With genetic diagnosis/total (% of total)
IKBA	1
DNA repair defects	
NBN	75
ATM	53
polygenic: ATM, NFKB1	1
ATM, dup4p16.3*	1
BLM (RECQL3)	2
DNMT3B (ICF1)	2
MRE11	1
ZBTB24 (ICF2)	2
Thymic defects with additional congenital anomalies	
22q11.2DS	106
CHD7	1
SEMA3E	1
Immuno-osseous dysplasias	
SMARCAL1	5
RMRP	4
Hyper IgE syndromes (HIES)	
STAT3, LOF	21
SPINK5	4
Other defects	
CCBE1	1
KMT2D	15
Chromosomal microdeletions	
10p.13-10p.14DS	1
11q23del	1
psu dic (21;Y)(q22;q11.1); 21q11.1, 21q21.1-q22.12, 21q22.3 (including IL10RB, IFNAR2)	1
46XX-21	1
11q13.5-q23.1	1
Predominantly antibody deficiencies	**144/699 (21%)**
X-LA	114/155 (74%)
BTK	113
BTK, del11p*	1
NFKB1	6
NFKB1, del 4q22.3-q25*	1
NFKB2	1
PIK3CD, GOF	8
PIK3R1, GOF	5
AICDA	1
TCF3	2
TNFRSF13B (TACI)	5
TRNT1	1
Immunodeficiencies affecting cellular and humoral immunity	**127/368 (35%)**
SCID	92/137 (67%)
T-B- SCID	
RAG1	16
RAG2	4
ADA	6
ARTEMIS	8
T-B+ SCID	
IL2RG	44
IL7RA	2
JAK3	6
LIG4	4
NHEJ1	1
CORO1A	1
Combined immunodeficiency (CID), generally less profound than SCID	31
PNP	1
CARD11	1
CD40LG	24
DOCK2	1
DOCK8	3
RFXANK	1
Not classified	
NTRK1	1
SCN9A	2
XRCC4	1
Phenocopies	**6**
KRAS	5
NRAS	1

(Continued)

TABLE 2 | Continued

**Patients with complex phenotype; GOF, gain-of-function variant; LOF, loss-of-function variant.*

was observed in the PAD group (**Table 1**); BTK abnormalities prevailed among them (114/155; 73.5%).

Somatic mutations in KRAS and NRAS were confirmed in six patients.

The segregation of genetic defects by mode of inheritance was nearly equal: 469 patients (38.4%) with an X-linked (XL) diseases had mutations in 10 genes, 383 (31.4%) patients with autosomal dominant (AD) diseases had mutations in 29 genes, and 369 (30.2%) patients with autosomal recessive (AR) diseases had mutations in 58 genes.

In the group of AR PID patients, 218 (59%) had compound heterozygous mutations and 151 (41%) had homozygous mutations; the majority (74; 49%), as expected, were NBS patients with the "Slavic" mutation in the *NBN* gene (**Figure 8**). Homozygous mutations were also found in the genes with the known "hot-spots": *MEFV* (11; 7%) and *AIRE* (5; 3%). Another "Slavic" mutation—*RAG1* c.256_257delAA p.K86fs, in a compound heterozygous or homozygous state—was reported in 7/16 patients with *RAG1* defects, putting this allele frequency at 25%.

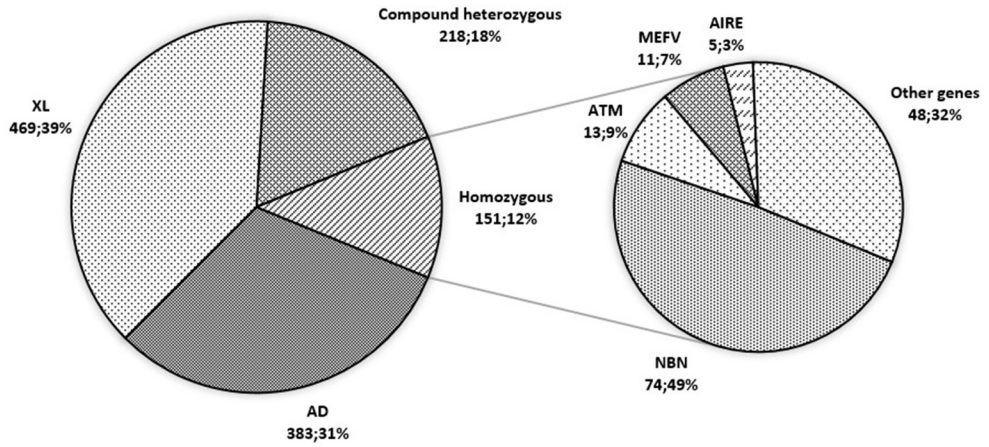

FIGURE 8 | Inheritance of PID in Russia and most frequent genes with homozygous mutations.

Testing for prenatal PID diagnosis (PND) was performed in 40 pregnancies among 37 families with previously known PID-causing genetic defects. Embryonic/fetal material was obtained by chorionic villi sampling at 10–12 weeks of gestation in 37 cases; by amniocentesis in the second trimester, in two cases; and by cordocentesis, in one. No serious complications were noted, during or after the procedures. 30/40 embryos were mutation-free. In six cases, a PID diagnosis was given; all families chose to terminate the pregnancies. Four embryos were heterozygous carriers of recessive PID mutations—all these pregnancies were carried to term. Two more sibling heterozygous carriers were born after preimplantation diagnosis.

Symptomatic Treatment

Treatment of PID symptoms, as documented in the registry, has been divided into three categories: immunoglobulin (IG) substitution, biologicals, and "other." There was updated information for 1,622 patients, regarding prescribed or on-going therapy. Half of the patients (843/1,622, 52%) received IG substitution. Of these, only 32 patients (4%) have ever had an experience with subcutaneous IG (SCIG); all others received intravenous IG (IVIG), with an average dose of 0.46 ± 0.09 g/kg per month. Regular IG substitution therapy was recorded in 279/369 patients (76%) with syndromic PID, in 296/433 (68%) PAD patients and in 173/270 patients (64%) with combined PID. At least single (but not regular) IG use was recorded for 15/29 patients (52%) with defects of innate immunity, 61/124 patients (49%) with immune dysregulation, 49/172 patients (28%) with phagocytosis defects, and 25/171 patients (15%) with autoinflammatory disorders.

414/1,622 (25%) patients were treated with various biological drugs. Updated information was available for 91 HAE patients, of whom 70/91 (77%) received either a C1 inhibitor or a selective antagonist of bradykinin receptors during attacks, including 51 patients who had experience with both drugs. In other PIDs, the rate of biological treatment was highest in the group of patients with autoinflammatory disorders: 86/186 (46%). This was followed by the group of immune dysregulation, with 48/134 (36%); and of combined PID, with and without syndromic features: 63/405 (16%) and 27/242 (18%), respectively. Patients with disorders of innate immunity and PAD were treated with biologicals only, in 3/32 (9%) and in 43/453 (6%) cases, respectively.

Curative Therapies

Three patients in the cohort underwent gene therapy for WAS; all are currently alive.

Information was available for 342/2,728 (16%) patients who underwent HSCT. Of these, 60 were deceased, 228 alive and 54 had not been updated during the prior 2 years (**Table 1**). All transplanted patients were diagnosed with PID as children. Yet, in 5/342, HSCT was performed after 18 years of age. HSCT has been performed in 106/591 (18%) patients with PIDs with syndromic features (18% of all syndromic PIDs), including 92/106 (88%) with WAS and 25/88(28%) with NBS; in 111 patients with combined T- and B-cell defects (30% of all CID), including 79/137 SCID (58%); in 66/262(25%) patients with phagocytic defects, including 47/135 CGD (35%) and 14/107 SCN (13%); in 41/196 (21%) patients with immune dysregulation; in 5/699 (0.7%) patients with PAD [four with activated PI3K syndrome (APDS) and 1 with XLA]; in 6/221(3%) patients with autoinflammatory disorders; and in 7/43 (16%) patients with defects of innate immunity.

DISCUSSION

The current study represents the first attempt to systematically assess clinical and epidemiological data on patients with PID in Russia, using the online registry.

At the time of analysis, 2,728 PID patients were registered, representing all districts of the country—thus making this study a valid assessment of the PID cohort in Russia. Since reporting

patients in the registry was not mandatory for the treating physicians, we expect underreporting of about 15–20% and are therefore able to discuss only the minimum epidemiological characteristics of PID in Russia. Though PID prevalence in the central part of Russia (2.8 per 100,000 people) is comparable to that of most European countries (2–8 per 100,000 people) (4–6, 15–17), the overall prevalence of 1.3 per 100,000 is quite low. This reflects significant under-diagnosis, especially in regions with low population density and economic status.

The male-to-female ratio in our various age groups does not differ greatly from previous observations, with males predominating amongst children and females in adulthood (4, 16, 17) (**Figure 1**).

Our study demonstrates a high mortality rate in the Russian PID cohort—as high as 9.8%—as compared with the most recently published German and Swiss registry. Yet it is comparable to the 8.6% (641/7,430) in the previously published ESID registry (18) and the 8% (2,232/27,550) provided by the online ESID reporting website[2]. Significantly, half of reported PID deaths occur within the first 5 years of life. This stresses the importance of early PID diagnosis and quick referral to transplanting centers, as SCID and other CIDs account for the majority of early PID deaths. In light of these statistics, unrecognized infant PID mortality may significantly contribute to the low prevalence of PID in Russia, as patients die before they are diagnosed with PID. Thus, future introduction of neonatal PID screening utilizing TREC/KREC detection may substantially improve PID verification (19, 20).

Children represent the majority (77%) of PID patients in the registry. Comparing this data to other registries—where patients over 18 years old represent up to 55% of all PID (4),[2] (18)—we can conclude that adults with PID are the most under-diagnosed category in Russia. This statement is confirmed by the low proportion of PAD defects in the Russian registry (21 vs. 56% in the ESID registry) (9),[2] (18, 21). This, in turn, reflects low numbers of patients with CVID, the main PID affecting adults worldwide. The estimated prevalence of CVID in Russia is 0.2 per 100,000 people—whereas, in other registries, CVID prevalence reaches 1.3 per 100,000 people (22).

In the recent years, Russia developed a relatively good network of pediatric immunologists, yet adult immunologists are scarce. NAEPID and the registry team have an educational and organizational plan aimed at improving adult PID diagnosis and care. The registry will be a good tool to assess success of the project in the next 5 years.

Combined immunodeficiencies with syndromic features constitute the most prominent PID group in the registry (22%), presumably due to the well-defined phenotype and the high awareness of these disorders among various medical specialists. Patients with WAS and DGS have the shortest diagnostic delay and the highest proportion of genetic confirmation. Overall, the majority of genetic defects were confirmed in the clinically or analytically well-defined and well-described PID (HAE, WAS, XLA, CGD, and NBS). Most studies also have the highest genetic confirmation rate in the group of combined PID (4, 6), though an Iranian study describes a predominance of genetic defects in the dysregulation group (17).

The patients' distribution amongst PID groups differs from that of most published registries in other aspects, as well. Though PAD are underrepresented, we have relatively large groups of autoinflammatory disorders (AID) and complement defects (predominantly HAE). This is because the Russian PID database collects data on all IUIS classified PIDs, in contrast with some other countries—where AID cases are followed and reported predominantly by rheumatologists, and HAE cases predominantly by allergists (23, 24). In our registry, HAE patients contribute 12% of all PID cases and have a high rate of genetic confirmation, though diagnostic delay in these cases is still quite high.

Overall, diagnostic delay amongst the predominant forms of PID varied from 4 months in SCID—which is similar to data reported by others (22, 25)—to 141 months in XLA patients. Obviously, such long diagnostic delays lead to a number of unrecognized PAD deaths and contribute to the low proportion of humoral deficiencies in the registry.

Diagnostic delay amongst NBS patients was shorter (median 2.5 years) than that reported previously in a smaller cohort of Russian NBS patients (median 5.0 years) (11). Yet the range of diagnostic delay is rather substantial: some patients were diagnosed as teenagers only after the onset of a malignancy, in spite of continuous follow-up by neurologists.

Sadly, with the increase of PID diagnosis in the last 5 years, there has been no improvement in diagnostic delay. This, yet again, raises the question of neonatal screening. Wider availability of next-generation sequencing methods, which were routinely introduced in Russia only in 2017, may also change this dynamic.

Unsurprisingly, 67% of the genetic defects in our cohort were detected via Sanger sequencing, in the most frequent and well-defined PID (2). A significant proportion of the mutations in the recently described genes were confirmed only with the advent of NGS techniques (26–29). NGS has allowed us to detect mutations in as many as 80 PID genes, sometimes with only one or a few patients per gene. The application of NGS to PID diagnosis has revolutionized the field by identifying novel disease-causing genes and allowing for the quick and relatively inexpensive detection of defects therein (27, 29). Adult PIDs show a substantially lower rate of genetic confirmation than that seen in children. This is partially because genetic defects are often not found in CVID, even using NGS methods (30, 31). Yet it also represents the fact that adults are less likely to pay for genetic testing since, in Russia, it is not covered by the state or by medical insurance.

As described by others (4, 6) the majority of the genetic defects were found in males, due to the fact that a lot of the "old" PID have X-linked inheritance.

In highly consanguineous populations, AR PIDs represent 70–90% of cases (32, 33). Interestingly—though the Russian population is very heterogeneous, with low numbers of consanguineous marriages (45 families, 1.9%)—AR genetic defects comprised 30% of all defects described in the cohort, with 40% of these being homozygous for the respective mutations. This is due to the "founder effect," known for affecting several PID genes in the Slavic population. The majority of NBS patients—74

(98.7%)—were homozygous for the "Slavic" mutation (11). A high frequency of the RAG1 c.256_257delAA p.K86fs mutation is also typical for Slavic populations, as previously noted (34).

Other homozygous mutations were reported in patients with defects in the *MEFV* and *AIRE* genes, and known for the hot-spot mutations. (35–39).

Our cohort included a group of patients with large aberrations, involving at least one PID gene. Therefore, we conclude that patients with complex phenotypes require implementation of not just Sanger sequencing and/or NGS methods, which can only indirectly point to a large aberration, but also cytogenetic methods, including CMA. Moreover, even well-described PIDs like HAE often require a combination of genetic methods, including MLPA, to detect large deletions frequent in this disease (2).

Our first analysis of the Russian PID population demonstrates substantial genetic diversity and high rate of genetic diagnosis confirmation-−49% of all registered patients. This is comparable to 36–43% of genetic PID confirmation in patients from French and German registries (4, 6).

The importance of genetic defect verification cannot be underestimated, as it influences overall treatment approach (HSCT vs. conservative treatment) and targeted therapy validation. It is also crucial for prenatal/preimplantation testing—which, if implemented, allows families to have healthy children. This is especially important for families with currently incurable PIDs, like AT and some others.

As previously published (40), the main treatment strategy for most PID patients (52% in the current study) is regular IG replacement. Additionally—in contrast to European data (4, 16)—the vast majority of patients in Russia are treated with IVIG, with only 4% of the patients having experience with subcutaneous IG replacement. Hence, IG substitution in Russia requires systemic modifications, i.e., wider availability of SCIG and home IVIG infusions that are not available at this time.

To our knowledge, the Russian PID registry is the first to analyze the use of monoclonal antibodies and other biologics in the treatment of PID symptoms. The number of patients treated with this kind of therapy in this cohort is rather high, reaching 25%.

Finally, 12% of patients underwent curative treatment, predominantly HSCT—a number comparable to the German registry (4). The proportions of transplanted patients with phagocytic disorders and with immune dysregulation were also similar in both registries. Yet, in comparison with the German registry—where one third of all HSCT was performed in CID patients—the predominant HSCT group in Russia consisted of patients with syndromic PIDs (18%) This reflects a significant prevalence of NBS patients, for whom HSCT has shown to be a successful and safe treatment strategy (11).

In conclusion, the current study has summarized the epidemiological features of PID patients in Russia and highlighted the main challenges for the diagnosis and treatment of patients with PID. As with all other rare disease registries, the Russian PID registry is a powerful tool—not just for data collection but also to help improve PID patient care, especially in the setting of a large country with highly diverse regional features.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the ethics committee of the Dmitry Rogachev National 474 Center of Pediatric Hematology, Oncology, and Immunology (approval No 2∋/2-20). All patients or their 475 legal guardians gave written informed consent for participation in the registry.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01491/full#supplementary-material

Figure S1 | Mortality rate by PID group and age of the deceased patients. Mortality rate in each age group is shown on the top of the stacked bars as a ratio of deceased patients to all patients of the relevant age. Textures represent different PID groups. Numbers next to the plots show the number of patients and the ratio of the deceased patients by PID group to the total number of the deceased patients of the age group.

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Interstitial Lung Disease in Children With Selected Primary Immunodeficiency Disorders—A Multicenter Observational Study

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Primary immunodeficiencies (PIDs) are rare disorders of the immune system encompassing inborn errors of immunity. Primary antibody deficiencies constitute the largest group of PID with common variable immunodeficiency (CVID) being the most common symptomatic form. Combined immunodeficiencies (CID) accompanied by antibody deficiency can mimic CVID and these patients need the verification of the final diagnosis. Respiratory involvement, especially interstitial lung disease (ILD), poses a relevant cause of morbidity and mortality among patients with PID and in some cases is the first manifestation of immunodeficiency. In this study we present a retrospective analysis of a group of children with primary immunodeficiency and ILD - the clinical, radiological, histological characteristics, treatment strategies and outcomes. Eleven children with PID-related ILD were described. The majority of them presented CVID, in three patients CID was recognized. All patients underwent detailed pulmonary diagnostics. In eight of them histological analysis of lung biopsy was performed. We noted that in two out of 11 patients acute onset of ILD with respiratory failure was the first manifestation of the disease and preceded PID diagnosis. The most common histopathological diagnosis was GLILD. Among the analyzed patients three did not require any immunosuppressive therapy. All eight treated children received corticosteroids as initial treatment, but in some of them second-line therapy was introduced. The relevant side effects in some patients were observed. The study demonstrated that the response to corticosteroids is usually prompt.

However, the resolution of pulmonary changes may be incomplete and second-line treatment may be necessary.

Keywords: computed tomography, children, CVID, interstitial lung disease, primary immunodeficiency, GLILD

INTRODUCTION

Primary immunodeficiencies (PIDs) are inherited disorders of the immune system encompassing inborn errors of immunity. The clinical spectrum of PIDs is broad, ranging from relatively mild disorders selectively affecting immune defense mechanisms to serious, life-threatening diseases characterized by profound lack of immune functions. According to a report by the International Union of Immunological Societies (IUIS), over 400 distinct disorders are recognized (1). Estimates by Bousfiha et al. suggest that ∼6 million people may be affected worldwide, though only ∼60,000 cases of PIDs are currently definitively diagnosed (2). Ten phenotypic categories of PIDs are recognized according to the underlying immune defects (3). Primary antibody deficiencies (PADs) are the largest group of PIDs, accounting for ∼55% of PIDs in Europe (www.esid.com) and up to 78% in the USA (4). PADs predominantly result from a primary defect in B cells, but can also be caused by defects in T cells or other immune cell populations that contribute to B-cell or plasma cell development and function. Antibody deficiencies are characterized by a malfunctioning antibody response, which is reflected in low or undetectable levels of immunoglobulin(s).

Common variable immunodeficiency (CVID), with an incidence of 1:25,000–1:50,000, is one of the most prevalent symptomatic PADs (5–7). Some well-defined disorders (e.g., combined immunodeficiencies—CID) accompanied by antibody deficiency can mimic CVID, and only new diagnostic tools, including next-generation sequencing (NGS), enable the verification of the final diagnosis (8, 9).

Patients with antibody deficiency present with a broad spectrum of manifestations. In addition to recurrent infections, non-infectious complications such as interstitial lung disease (ILD), gastrointestinal inflammatory disease, hematologic or organ-specific autoimmunity, lymphoproliferation, and lymphoma may occur (5–8). Respiratory diseases are a cause of morbidity and mortality among PID patients, and in some cases lung disease may play an important role in diagnosis as the first manifestation of PID (10–14). Lung involvement in patients with PID can be infection-related, immune-mediated, or associated with the occurrence of neoplastic diseases. In cases of infection-related lung disease, effective protection can be provided by immunoglobulin replacement therapy (IgRT); however, it does not prevent non-infectious pulmonary involvement (6, 12). ILDs represent one of the most significant immune-mediated complications of PID (14). The most common form of PID-related ILD is granulomatous-lymphocytic ILD (GLILD), an umbrella term that encompasses a spectrum of lung pathologies: various forms of pulmonary lymphoid hyperplasia [lymphocytic interstitial pneumonia (LIP), follicular bronchiolitis (FB), nodular lymphoid hyperplasia, and granulomatous disease], as well as organizing pneumonia (10, 14, 15). The risk of death in CVID patients with non-infectious complications like ILD has been shown to be several times higher than in patients with infectious complications only (16). CVID-related GLILD significantly shortens life expectancy (17).

Previous reports concerning PID-related ILD are mainly limited to adults; data regarding disease specificity and exceptionality of pediatric patients available in the literature are insufficient. Herein we present a retrospective analysis of the clinical, radiological, and histological characteristics, as well as treatment strategies and outcomes, in a unique group of children with PID-related ILD, predominantly CVID patients.

MATERIALS AND METHODS

Retrospective analysis of clinical data from children with PID-related ILD consulted or treated consecutively in the Department of Immunology at the Children's Memorial Health Institute (CMHI) of Warsaw and the Department of Pediatric Pneumonology and Allergy at the Medical University of Warsaw between 2012 and 2019 is presented.

The major inclusion criterion was the diagnosis of PID, mostly CVID. Diagnosis of CVID was established according to ESID diagnostic criteria (18). Since CVID encompasses a group of heterogenous antibody deficiencies with many monogenic forms, detailed immunophenotyping and genetic studies using next-generation sequencing (NGS), Sanger sequencing, or fluorescence *in situ* hybridization (FISH) were performed in six individuals to better characterize the study group. In five patients genetic tests were not performed because of: technical problems with genetic testing and consent not given by parents. Following laboratory and/or molecular studies, combined immunodeficiency (CID) was diagnosed in three individuals: one with Nijmegen breakage syndrome (NBS), one with DiGeorge Syndrome (DGS), and one with LRBA.

Diagnosis of PID-related ILD was based on (1) clinical signs and symptoms [tachypnea [defined as respiratory rate >90th percentile (19)], retractions, crackles]; (2) typical computed tomography (CT) findings [multiple nodules of different densities with perilymphatic or interlobular distribution predominantly in the middle and lower zones of the lungs and hilar or mediastinal lymphadenopathy (transverse dimension of the lymph nodes >1 cm)]; and (3) histopathology of lung biopsy with findings consistent with GLILD, cryptogenic organizing pneumonia (COP), LIP, FB, or lymphoid hyperplasia.

Exclusion criteria were other ILDs, coexistence of other chronic lung diseases (e.g., cystic fibrosis), and acute or chronic respiratory infections.

Data Collection

Prespecified data on all children with PID and related ILD were collected. These included (1) demographic data; (2)

clinical signs and symptoms such as dyspnea, tachypnea, cough, cyanosis, crackles, wheezing, respiratory failure, finger clubbing, splenomegaly, hepatomegaly, lymphadenopathy, hemolytic anemia, thrombocytopenia, and history of chronic lung diseases; (3) the results of the following laboratory tests: total blood count, detailed immunological screening (including immunoglobulin level and immunophenotyping of lymphocytes, with B- and T-cell functional tests and genetic testing in some cases); (4) the results of pulmonary function tests, including spirometry, body plethysmography, and diffusing capacity for carbon monoxide (DL_{CO}); (5) the results of lung high-resolution computed tomography (HRCT) and chest magnetic resonance imaging (MRI); (6) histopathology of lung biopsy; and (7) treatment regimens [corticosteroids, other immunosuppressive drugs (e.g., azathioprine, mycophenolate mofetil, rapamycin, cyclosporin), IgRT, and hematopoietic stem cell transplantation (HSCT)], duration, and outcomes.

The immune status of patients was assessed based on serum immunoglobulin concentration, antibody response to vaccines and/or isohemagglutinins titer, immunophenotyping of lymphocytes with switch memory B cells numbers, and lymphoproliferation tests as described previously [(20–22); data not shown]. The sources of data were an electronic database of patients with PID and medical records of patients with PID-related ILD.

Imaging Studies

In eight children, volumetric CT examinations were performed with either a 320-row multidetector computed tomography scanner (Aquilion ONE, Toshiba, Japan; three children) or a 128-row multidetector computed tomography scanner (SOMATOM Definition AS, Siemens, Germany; five children) and images were reconstructed using a high-resolution reconstruction algorithm. In the remaining two patients, CT scans performed in local hospitals were used for analysis. Contrast medium was administered in eight patients. Pulmonary involvement in the child with NBS with a known radiosensitivity was monitored with chest MRI.

Pulmonary Function Tests

Spirometry tests were obtained in nine cooperating individuals and included forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV1). All pulmonary function tests were performed in the sitting position using a spirometer (Lungtest 1000 MES, Poland, or JAEGER MasterScreen, Germany) according to the ERS (European Respiratory Society) guidelines for lung function testing (23). All spirometric results were presented as percentiles (pc). FVC, FEV1, and FEV1/FVC $\geq$5th pc were considered normal. Obstruction was diagnosed if the FEV1/FVC ratio was below the 5th pc. DL_{CO} was measured using the single-breath method using a Vmax22 spirometer (SensorMedics, USA), or MasterScreen (CareFusion, USA) and results between the 5th and 95th pc were considered to be within the normal range. The whole-body plethysmography was performed using a Lungtest 1000 (MES, Poland), or MasterScreen (CareFusion, USA). Total lung capacity (TLC) and residual volume (RV) were calculated for each patient. TLC lower than the 5th pc was considered to indicate restriction. Air trapping was defined as a RV/TLC ratio above the 95th pc.

Histological Assessment

Lung biopsies were performed in eight of the 11 patients. The obtained material was then fixed in 10% buffered formalin solution and paraffin embedded (FFPE). Microscopic slides with 4-μm FFPE sections were stained with hematoxylin and eosin (H + E) and in some cases immunohistochemical markers were used (CD3, CD20, CD5, CKAE1/AE3, and Ki-67; Ventana Roche, USA). Immunohistochemical tests were performed using an automated Ventana Benchmark GX Slide Staining System according to the manufacturer's protocol. In two cases, mediastinal lymph nodes biopsies were analyzed.

Statistical Analysis

The results of the study were summarized using standard descriptive statistics. Data are presented as median and range.

The studies involving human participants were reviewed and approved by The Bioethics Committee of the Children's Memorial Health Institute in Warsaw (approval no.9/KBE/2020). All parents or legal guardians, as well as patients older than 16 years of age, gave their written consent to participate in the study.

RESULTS

Demographic and Clinical Data

Eleven children with interstitial lung disease were selected from the database of 796 children with PID followed-up between 2012 and 2019. Detailed immunological and genetic diagnostics enabled the diagnosis of CVID in nine patients however, in one of these patients, finally an NGS panel revealed an *LRBA* mutation. The other two patients with a provisional diagnosis of PAD were eventually diagnosed with CID with associated features (NBS in the patient with hydrocephaly and DGS in the patient without an expressive dysmorphia). In three patients with CVID no mutations were confirmed.

All of the children diagnosed with PID-related ILD were boys.

The median age at the diagnosis of antibody deficiency was 9.5 years (range 4–17 years). Six patients had never developed respiratory symptoms (except respiratory tract infections). Chest imaging studies were performed for other reasons (e.g., generalized lymphadenopathy) and revealed pulmonary involvement. Five patients were symptomatic in terms of respiratory involvement. In two children, the symptoms of ILD preceded the recognition of immunodeficiency, so the median age at the onset of ILD symptoms was 6.5 years (range 2–16). The severity of ILD symptoms was diverse, with one child presenting with a mild cough (patient No. 4; **Table 1**) and three others with life-threatening respiratory failure (patients No. 3, 7, and 8).

Detailed demographic data and clinical symptoms are presented in **Table 1**.

Computed Tomography

Lung CT was performed in 10 patients. One child with NBS had been diagnosed and followed with chest MRI to avoid radiation exposure.

TABLE 1 | Baseline clinical characteristics at diagnosis.

Patient	1	2	3	4	5	6	7	8	9	10	11
Age, sex	13, M	19, M	13, M	19, M	5, M	16, M	10, M	12, M	11, M	18, M	17, M
Age at symptom onset	4 yr	11 yr	11 yr	3 yr	3 yr	16 yr	2 yr	8 yr	5 yr	10 yr	9 yr
Presenting symptoms	Splenomegaly, thrombocyte-penia,	Pain in the lower limbs, lymphadenopathy, splenomegaly, anemia, leucopenia	Dyspnea, crackles, acute respiratory failure	Generalized lymphadenopathy	Skin nodules, generalized lymphadenopathy, hepatospleno-megaly, pancytopenia	Hepatospleno-megaly, anemia, lymphadenopathy	Dyspnea, crackles, acute respiratory failure	Severe viral enterocolitis, parakeratosis, facial dysmorphia, hydrocephalus, strabismus, hypospadias, cryptorchidism	Hepatospleno-megaly, pancytopenia	Lymphadeno-pathy, hepatospleno-megaly	Recurrent pneumonia, lymphadeno-pathy
Recurrent respiratory infections	+	–	–	–	–	–	–	–	+	+	+
Age at ILD diagnosis	8 yr	17 yr	11 yr	13 yr	4 yr	16 yr	2.5 yr	10 yr	9 yr	10 yr	14 yr
Age at PID diagnosis	5 yr	17 yr	12 yr	12 yr	4 yr	16 yr	7 yr	4 yr	5 yr	15 yr	14 yr
Immunodeficiency	CVID	CVID	CVID	CVID	LRBA	CVID	CVID	NBS	CVID	DGS	CVID
Genetic testing	–	–	–	+ No changes	+ LRBA	–	+ No changes	+ NBS	+ No changes	+ DGS	–
ILD manifestation:											
Chronic cough	+	–	–	+	–	–	–	+	–	–	–
Dyspnea	–	–	+	–	–	–	+	+	–	–	–
Crackles	+	–	+	+	–	–	+	+	–	–	–
Clubbed fingers	–	–	–	–	–	–	+	–	–	–	–
Acute respiratory insufficiency	–	–	+	–	–	–	+	+	–	–	–
Extra-pulmonary manifestation:											
Hepatomegaly	+	+	+	–	+	+	+	–	+	+	–
Splenomegaly	–	+	+	–	+	+	+	–	+	+	–
Lymphadenopathy	+	+	–	+	+	+	–	–	+	+	+
Cytopenia:											
Anemia	–	+	–	–	+	+	+	–	–	–	–
Leukopenia	–	+	–	–	+	+	–	–	+	–	–
Thrombocytopenia	+	–	–	–	+	+	+	–	+	–	–
Lung HRCT findings*:											
Nodules	+	+	+	+	+	+	+	+	+	+	+
Perilymphatic distribution	+	+	+	+	+	+	–	–	–	+	+
Middle and lower zone predominance	+	+	+	+	–	+	–	+	–	+	–
Intrathoracic lymhadenopathy	+	–	–	+	+	–	+	+	–	+	+

(Continued)

TABLE 1 | Continued

Patient	1	2	3	4	5	6	7	8	9	10	11
Ground-glass opacities	–	–	+	+	–	+	+	–	–	–	–
Lung consolidations	+	–	+	+	+	–	+	–	–	–	+
Lungbiopsyresult	GLILD/LIP	GLILD/LIP	GLILD	GLILD/LIP	GLILD/LIP	GLILD	GLILD/LIP	Granulomas	–	–	–
Lymph node biopsy result						Granulomas				Granulomas	

**Patient 8 had only chest X-ray and chest MRI done. Due to increased risk of cancer after radiation exposure, he was disqualified from chest CT.*

PID, primary immunodeficiency; CVID, common variable immunodeficiency; LRBA, mutation in the lipopolysaccharide-responsive and beige-like anchor protein (LRBA) gene; NBS, Nijmegen breakage syndrome; ILD, interstitial lung disease; HRCT, high resolution chest tomography; GLILD, granulomatous-lymphocytic interstitial lung disease; FB, follicular bronchiolitis; LIP, lymphocytic interstitial pneumonia; COP, cryptogenic organizing pneumonia.

Multiple, mostly poorly defined pulmonary nodules were revealed in all children assessed. The diameter of the nodules ranged from 2 to 30 mm and their density varied from solid to part-solid to ground glass opacity (**Figures 1A**, **2**, **3**). Solid nodules were surrounded by a ground-glass halo in four patients. In most of the children, nodules had a perilymphatic distribution with a predilection for the middle and lower lung zones. The lesions formed rosettes and a "tree-in-bud" pattern in one patient (**Figure 2**). Interlobular septal thickenings or irregular linear opacities were found in one case each. The changes described above were accompanied by hilar and/or mediastinal lymphadenopathy in seven patients (**Figure 4**).

During the exacerbations of ILD, progression of lung involvement in imaging studies was observed, with an increase in the number, size, and density of lung nodules as well as extensive areas of ground-glass opacities and parenchymal lung consolidations (**Figures 1A,C**).

Pulmonary Function Tests

Pulmonary function tests (PFTs) were performed in nine children. Two children were unable to perform PFT, one due to the young age and the other due to intellectual disability. All of the PFTs were performed in the stable phase of the ILD. The results of PFT are shown in **Table 2**. We found one patient (No. 3) with a restrictive ventilatory pattern and five with air trapping (No. 2, 4, 9, 10, and 11). DL_{CO} was abnormal in two patients. A mild reduction of DL_{CO} was found in patient No. 2 (74% of predicted value) and a moderate reduction was observed in patient No. 3 (50% of predicted value).

The results of the PFTs are presented in **Table 2**.

Lung Biopsy Results

The most common histopathological diagnosis was GLILD (seven cases), with a predominance of the LIP pattern (five cases; **Figure 5**). Microscopic features of LIP were dominated by abundant diffuse interstitial infiltrates of lymphoid cells composed of CD3+ and CD20+ lymphocytes. In some patients, the inflammatory infiltration formed clusters with single, small lymph nodules. In addition to the intensive interstitial infiltration, a marked peribronchial and perivascular chronic inflammation was present (**Figure 6**).

Foci of COP and FB were found in addition to LIP features in one of the patients (**Figure 7**).

Interstitial lymphocytic infiltrates were accompanied by the presence of multiple non-necrotizing granulomas in two children.

In the child diagnosed with NBS, the only histopathological abnormality in the lung biopsy was non-necrotizing granulomas.

In two patients (No. 6 and 10), a mediastinal lymph node biopsy was performed and revealed non-caseating granulomas.

Treatment Strategies

In eight children with PID-related ILD, the immunosuppressive treatment was introduced (No. 1–8). In three patients (No. 3, 7, and 8) severe pulmonary symptoms were the only reason for initiating the treatment. Patients 1, 4, 5, and

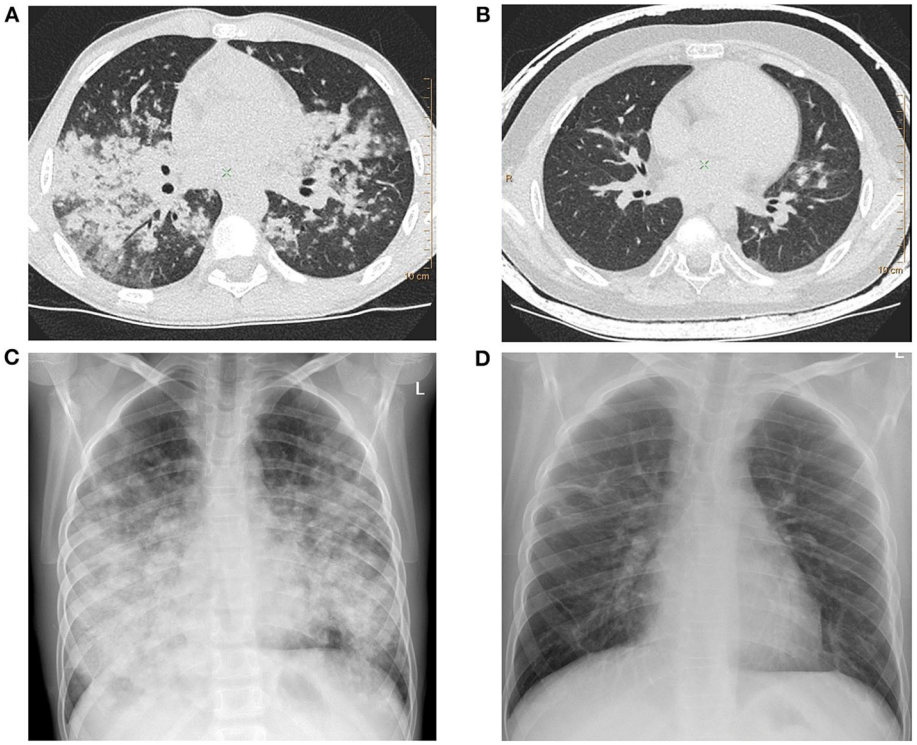

FIGURE 1 | (A) Lung high-resolution computed tomography (HRCT): multiple, poorly defined nodules merging in larger areas of ground-glass opacities and consolidations. **(B)** Lung HRCT: complete regression of pulmonary changes; residual irregular linear opacities are visible. **(C)** Chest X-ray: diffuse poorly defined nodules creating larger areas of lung consolidations with adjacent areas of ground-glass opacities. Middle and lower zone predominance is clearly marked. **(D)** Chest X-ray: complete resolution of pulmonary opacities with residual irregular linear thickenings.

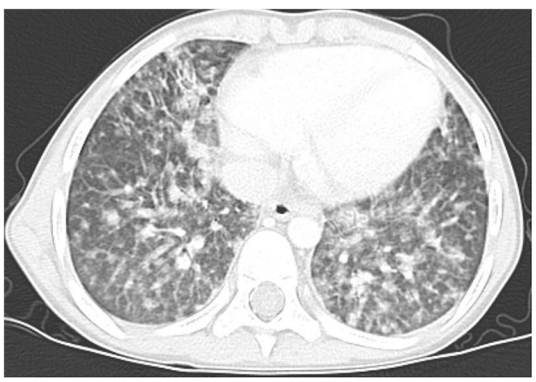

FIGURE 2 | Lung high-resolution computed tomography: multiple ground-glass and solid nodules scattered across the lungs, with perylimphatic distribution. Larger clusters of nodules and tree-in-bud pattern are visible peripherally.

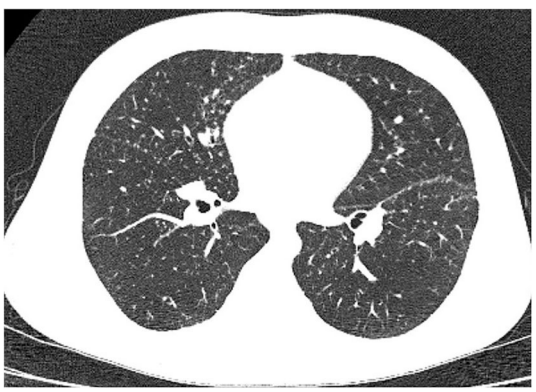

FIGURE 3 | Lung high-resolution computed tomography: multiple small pulmonary nodules with perilymphatic distribution (clearly seen along pleural fissures).

6 (one symptomatic, three asymptomatic) were treated due to autoimmune cytopenia and/or lymphoproliferation with coexisting extensive lung involvement. Patient No. 2, who didn't present with respiratory symptoms, was qualified for treatment because of decreased DL_{CO} results, progression of lung infiltrates in chest HRCT and features of fibrosis in the lung biopsy.

Three patients, due to a lack of clinical symptoms and stable, low-intensity changes in the lung HRCT, did not qualify for any immunosuppressive therapy (No. 9, 10, and 11).

Systemic glucocorticoids (GCs) were introduced in all eight children at the onset of treatment. Two children were initially

treated with corticosteroid pulses. GCs were the only drugs used in four patients (No. 2, 4, 6, and 7). In one of the patients (No. 4), initially a recurrence of symptoms was observed during GC dose reduction. It is worth noting that despite the treatment discontinuation after the patient's decision at the age of 18, he remains clinically and radiologically stable. In patient No. 7, a recurrence of ILD occurred after an asymptomatic period of several months. Patient No. 6 (clinically asymptomatic) started treatment with GCs due to autoimmune hemolytic anemia. Hematological improvement after 3 months of GC therapy was achieved, without resolution of radiological findings.

GC treatment resulted in rapid improvement of general condition and resolution of respiratory failure in patient No. 3. However, exercise intolerance, significant radiological abnormalities, and abnormal PFT results persisted despite the therapy.

Two boys were additionally treated with azathioprine (No. 1 and 3), achieving a meaningful clinical improvement, but due to serious side effects of the therapy (**Table 3**), the treatment was discontinued. In patient No. 1, mycophenolate mofetil was introduced, but the treatment was ineffective (thrombocytopenia did not resolve) so the boy was eventually qualified for HSCT. The post-transplant period was complicated by infections caused by HHV-6, *Pneumocystis jiroveci* and *Streptococcus viridans* shortly after transplantation. After HSCT a resolution of respiratory symptoms and regression of pulmonary abnormalities in imaging studies were achieved. Some months after the transplantation, due to the refractory thrombocytopenia, splenectomy was performed with a satisfactory effect. Despite the discontinuation of azathioprine, patient No. 3 maintained the significant clinical and radiological improvement obtained on the therapy (**Figures 1B,D**). In patient No. 8, the resolution of ILD symptoms was achieved after adding cyclosporin to GCs.

All of the patients remain alive. A clinical and radiological improvement or stability of the lung disease is observed in all the patients.

Significant side effects were observed during the immunosuppressive treatment. Six children treated with GCs presented with symptoms of Cushing syndrome. In patient No. 3, compression fractures of the lumbar vertebrae occurred due to post-steroid osteoporosis.

The azathioprine treatment was complicated by pancreatitis in patient No. 1 and by liver damage with cholestasis in patient No. 3. In both patients, signs of drug-induced organ damage resolved after discontinuation of the treatment. Transient cytopenia and elevated transaminase levels were observed during the treatment with rapamycin (second-line therapy, patient No. 5). In this case, almost total resolution of radiological changes was observed.

All of the children were receiving IgRT during the study.

The treatment strategies are presented in **Table 3**.

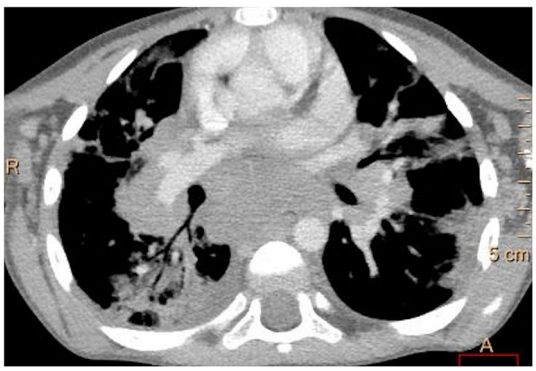

FIGURE 4 | Contrast-enhanced lung computed tomography (soft tissue window): marked enlargement of mediastinal and hilar lymph nodes with areas of subpleural lung consolidations.

DISCUSSION

Clinical presentations of PIDs are diverse, and PIDs are often a diagnostic challenge. This is due in part to pulmonary changes in patients with PIDs, as these changes may have different etiologies and varied clinical as well as radiological presentations, including ILD. Although our study showed a low prevalence of PID-related ILD in children, it highlights various clinical courses of ILDs, including life-threatening complications. Importantly, the study demonstrated that ILD may precede PID diagnosis and that the response to corticosteroids is

TABLE 2 | Results of pulmonary function tests.

Pulmonary function tests	Patient	1	2	3	4*	5	6	7*	8	9	10	11
Spirometry	**FEV1**, percentile	94	15.64	0.03	11.39	ND	75	22	ND	29.96	25.43	97.03
	FVC; percentile	10	18.50	0.02	7.08	ND	50	13	ND	48.97	13.91	80.49
	FEV1/FVC; percentile	38	36.29	84.84	46.88	ND	70	80	ND	20.11	60.37	94.58
Plethysmography	**RV**; percentile	ND	100	0.07	99.98	ND	97	ND	ND	100	100	100
	TLC; percentile	ND	99.96	0.0	90.35	ND	>99	ND	ND	99.82	98.94	99.97
	RV/TLC; percentile	ND	99.91	0.78	99.62	ND	73	ND	ND	99.94	99.82	99.17
Diffusing capacity for carbon monoxide	**DLCO**; percentile	ND	0	0	55.08	ND	41	ND	ND	97.39	90.13	ND

*****Lung function tests were performed in a stable phase of the disease, during treatment.*

ND, No data.

DLCO, diffusing capacity for carbon monoxide; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity.

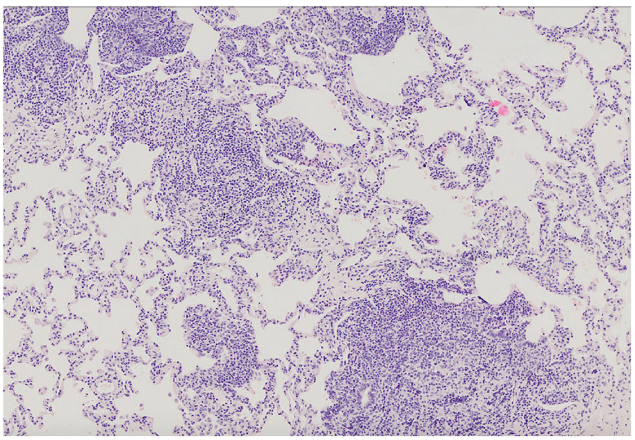

FIGURE 5 | Lung parenchyma with diffuse interstitial and peribronchiolar lymphocytic infiltration. Microphotograph: hematoxylin and eosin stain.

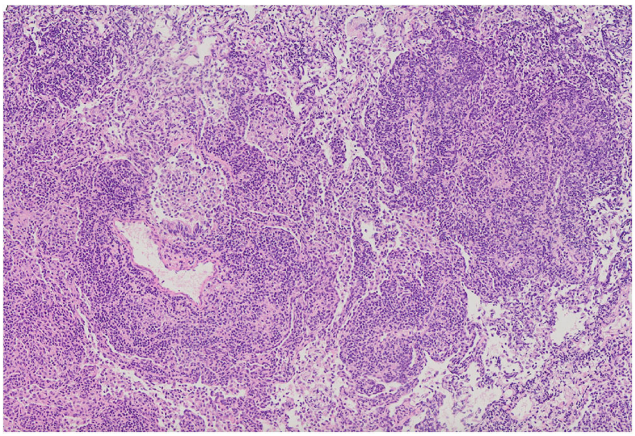

FIGURE 6 | Diffuse intensive lymphocytic infiltration, both interstitial and around blood vessels. Alveolar spaces are filled with numerous macrophages. Microphotograph: hematoxylin and eosin stain.

usually prompt. However, the resolution of pulmonary changes may be incomplete and a second-line treatment may be necessary in some patients. It should be highlighted that in 3 patients, the initial symptoms suggested CVID. However, further observation and the occurrence of other symptoms, including those suggestive of ILD, led to an in-depth diagnostics and the final diagnoses were established based on genetic testing. The achieved diagnoses resulted in changing both, the diagnostic (e.g., NBS) and therapeutic (e.g., DGS and LRBA) management. We would like to emphasize that this is the largest case series on PID-related ILDs ever reported in a pediatric population. Thus, we believe our findings may add to the existing literature on the topic.

GLILD was originally described in patients with CVID (17). Currently, there is a growing number of studies reporting this disease in other PIDs, including DGS and LRBA deficiency (24–26). Moreover, interstitial lymphocytic lung disease was recognized in 4 NBS patients (11%) described by Deripapa et al. (27). However, in the largest group of NBS patients, published by Wolska-Kuśnierz et al., infections clearly dominated among pulmonary complications (28).

The clinical picture of PID-related ILD in our study was very diverse. It should be stressed that in two of the 11 children (No. 3 and 7), ILD was the first presentation, documented even before PID diagnosis. Similar sequences of symptoms and diagnosis were recently reported in adults (29). Importantly, in both children, the presenting symptoms were severe and progressed to respiratory failure. The first child had four episodes of ILD exacerbations with respiratory failure and biopsy-proven LIP diagnosis 3 years before CVID diagnosis. In the second child, pulmonary signs and symptoms, which were initially interpreted as pneumonia, deteriorated rapidly to respiratory failure (**Figures 1A,C**). The clinical picture, imaging studies, and lung biopsy were consistent with GLILD, but the definitive diagnosis of CVID was established only 7 months later. Respiratory failure also occurred in the other child, but in contrast to the patients mentioned above, ILD symptoms appeared 3 years after the diagnosis of NBS.

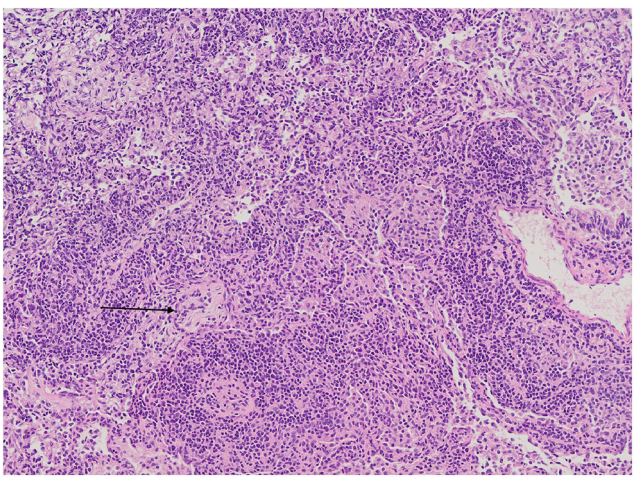

FIGURE 7 | Diffuse interstitial inflammation with small focus of organizing pneumonia (black arrow). The surrounding alveoli are filled with numerous macrophages. Microphotograph: hematoxylin and eosin stain.

In contrast to the three patients with severe lung disease, the majority of children with PID-related ILD were asymptomatic or presented mild respiratory symptoms, but had extensive pulmonary involvement in imaging studies. Thus, we strongly agree with the British Lung Foundation/United Kingdom Primary Immunodeficiency Network Consensus (BLF/UKPINC) that patients with CVID should be screened for pulmonary complications despite the absence of symptoms. HRCT, lung function tests, and other diagnostic procedures are recommended as a part of the diagnostic workup (30). Regular clinical monitoring for lung involvement should also apply to patients with other PIDs (31).

TABLE 3 | Treatment strategies.

Patient (lung biopsy)	Treatment line	Treatment effect	Treatment complications	Comments
1(GLILD/LIP)	First Line: DEX 1.0 mg/kg 10 days, PRED, initial dose: 1.0 mg/kg, maintenance dose: 0.2 mg/kg	Clinical and radiological improvement, but persistence of residual lesions in lung HRCT	Cushing syndrome	Introduction of second-line treatment
	Second Line (1): AZA 2mg/kg	Clinical stabilization	Pancreatitis	Due to pancreatitis, AZA was changed to MMF
	Second Line (2): MMF 500 mg/day	Clinical and radiological progression	Progression of lung infiltrates, persistent lymphadenopathy, splenomegaly, leukopenia, and hypogammaglobulinemia on a low dose of steroids and MMF	Patient qualified for HSCT, infectious complications after HSCT (HHV-6, Pneumocystis jiroveci, Str. viridans), splenectomy due to refractory thrombocytopenia and finally clinical and radiological improvement
2 (GLILD/LIP)	PRED initial dose: 0.75 mg/kg, maintenance dose: 0.33 mg/kg for 6 months	Radiological improvement	–	Patient had no clinical respiratory symptoms
3 (GLILD)	First Line: MPRED i.v. pulses 3 days (30 mg/kg), PRED initial dose: 2.0 mg/kg, maintenance dose: 0.4 mg/kg	Resolution of respiratory failure, incomplete clinical (exercise intolerance) and radiological (partial resolution of nodular lesions) improvement	Cushing syndrome, osteoporosis with multiple compression fractures of lumbar vertebrae	Clinical and radiological deterioration observed while reducing the steroid dose; therefore, AZA was introduced
	Second Line: AZA 2.0–3.5 mg/kg	Significant clinical and radiological improvement	Hepatitis with liver injury and cholestasis	AZA dose was modified depending on the 6-thioguanine levels; due to adverse effects, treatment was discontinued
4 (GLILD/LIP)	PRED initial dose: 2.0 mg/kg, maintenance dose: 0.6 mg/kg	Resolution of clinical symptoms, partial radiological improvement	Cushing syndrome	Recurrence of radiological changes while trying to discontinue the drug
5 (GLILD/LIP)	First Line: PRED initial dose: 2.0 mg/kg, maintenance treatment: MPRED 0.1 mg/kg in tapered doses	Almost total resolution of radiological changes	Severe steroid-dependent thrombocytopenia	
	Second Line (1): MMF 400 mg/day	Almost total resolution of radiological changes		MMF was introduced almost simultaneously with PRED
	Second Line (2): RAP 1.6–1.4 mg/kg		Transient leucopenia, hypertransaminasemia	Due to thrombocytopenia, MMF was replaced with RAP
6 (GLILD)	PRED initial dose: 0.7 mg/kg, maintenance dose: 0.5 mg/kg	Without radiological improvement	Cushing syndrome, depression	The patient reports periodically a subjective feeling of dyspnea
7 (GLILD/LIP)	PRED initial dose: 2.0 mg/kg, maintenance dose 0.2 mg/kg	Significant clinical and radiological improvement	Cushing syndrome	
8 (G)	First Line: PRED Initial dose: 1.0 mg/kg, maintenance dose: 0.2 mg/kg	Clinical and radiological improvement	Cushing syndrome	
	Second Line: CsA 2.5–3.0 mg/kg	Clinical stabilization		

AZA, azathioprine; CsA, cyclosporine A; DEX, dexamethasone; G, granulomas; GLILD, granulomatous-lymphocytic interstitial lung disease; HSCT, hematopoietic stem cell transplantation; HRCT, high-resolution chest tomography; LIP, lymphocytic interstitial pneumonia; MMF, mycophenolate mofetil; MPRED, Methylprednisolone; PRED, prednisone; RAP, rapamycin.

In addition to respiratory symptoms, patients with PIDs present with a wide spectrum of symptoms involving other organs. In a significant number of patients, autoimmune cytopenias, especially autoimmune thrombocytopenia, are observed as the first manifestation of the disease (32). This was the case of two our patients with autoimmune cytopenia, in whom autoimmune cytopenia was the first presenting symptom. Quinti et al. described autoimmune cytopenia as the only complication in 2.3% of CVID patients and autoimmune phenomena as the most common complication [17%; (33)]. Lymphadenopathy, hepatomegaly, and splenomegaly were the most common presentations in our study group. The other non-infectious manifestations included chronic diarrhea, allergic diseases, lymphoid hyperplasia, and failure to thrive.

GLILD can be also found as a serious complication of PIDs in adults. As in children, the clinical course of PID-related ILD in adults can be asymptomatic. Symptomatic patients usually present with dyspnea, tachypnea, cough, exercise intolerance of various severity, and crepitations. Similar to children, hypoxemic respiratory failure may occur in the most severe cases (17, 29, 34).

According to the BLF/UKPINC, the most typical radiologic findings in GLILD are solid or semisolid nodules, ground-glass

opacities, and enlarged thoracic lymph nodes (30). These data are consistent with our observations. Nodules of different sizes and densities were found in all children, with perilymphatic distribution (along pleura, fissures, interlobular septa, and bronchovascular bundles) predominating in eight out of 11 patients. A similar distribution of nodules was previously observed in patients with CVID-related ILD (35–38). However, other radiographic patterns were also reported, including random distribution (35–37, 39, 40) and mixed distribution with randomly scattered small nodules (<5 mm) and peribronchial localization of larger nodules (39).

Ground-glass opacities were identified in four patients with PID-related ILD. In GLILD, patchy areas of ground-glass opacities are usually located peripherally and in peribronchial and subpleural regions. These changes may reflect organizing pneumonia areas (10, 35, 39, 41, 42). "Halo sign" was found in two patients in our study group. Bouvry et al. reported that this sign was a common feature of GLILD and was associated with the presence of granulomas, which may be surrounded by foci of organizing pneumonia (37).

In two of the children in this study, CT revealed a tree-in-bud pattern. This pattern may reflect a spectrum of endo- and peribronchiolar disorders, including mucoid impaction. Tree-in-bud sign was reported by Bang et al. in CVID patients with pulmonary infections (43). This sign may also reflect non-infectious peribronchial or bronchiolar inflammation and was found in patients with follicular bronchiolitis (44) or, less commonly, in organizing pneumonia (44, 45).

In our patients, lung HRCT did not reveal reticular abnormalities, which appear to be common features of GLILD in adults (17, 39–41, 46). As reticular opacities seem to be features of more advanced stages of ILD (47), we propose that the presence of a reticular pattern in adults is associated with more prolonged lung involvement resulting in irreversible fibrotic changes in the lung architecture.

The data on pulmonary function in patients with PID-related ILDs mainly come from adult studies. Restrictive or mixed obstructive and restrictive patterns, as well as decreased DL_{CO}, were the most frequently observed abnormalities. However, it must be underlined that the results of PFT can be completely normal in the majority of patients (14, 35, 43). Similar observations were made in the very few studies on children. A restrictive pattern was reported by Tillman et al. in a 13-year-old girl with GLILD and by van de Ven et al. in eight children with CVID and CVID-like disease (48, 49). In our study, restrictive pulmonary dysfunction was demonstrated in one child (patient No. 3). Interestingly, an increased RV/TLC ratio indicating air trapping in the absence of FEV1/FVC reduction was the most common finding in our study. According to some authors, an increased RV/TLC ratio may be a more sensitive indicator of airflow obstruction than the FEV1/FVC ratio (50, 51). This parameter is thought to reflect distal airway narrowing or closure resulting from inflammation (50, 51). As nodular peribronchiolar lymphocytic inflammation is common in CVID patients with GLILD (52), we can hypothesize that our findings reflect small airway obstruction caused by an increased number of lymphocytes in this region. Decreased DL_{CO}, which is an early indicator of ILD (35, 36), was noted in two of our patients (No. 2 and 3).

In the light of data demonstrating that GLILD is associated with significantly increased mortality in patients with CVID, identifying an effective treatment is a critical issue (16). Although no standard treatment has been established, the consensus statement of the BLF/UKPINC (30) recommends optimizing IgRT as an initial step in the treatment of CVID. However, despite adequate IgRT, complications like CVID-related ILD may occur in some patients, making additional therapy necessary.

According to the aforementioned consensus statement, systemic corticosteroids should be applied as the first-line therapy in symptomatic patients. In our group, GCs were administered to all eight patients who required an intervention in addition to IgRT. In four patients decision about treatment was made due to the coexistence of autoimmune cytopenia or lymphoproliferation and extensive lung involvement in imaging studies. In patient No. 2 the treatment with GCs was started owing to significant progression in imaging studies, decreasing DL_{CO}, as well histopathological features of irreversible lung scarring. The use of GCs as monotherapy led to rapid improvement in almost all the treated individuals. Their effectiveness was particularly evident in patients with the most severe cases of ILD (No. 3, 7, and 8), whose respiratory failure resolved in response to the treatment. However, in the four patients who did not fully respond to the first-line therapy, developed significant side effects, or presented with disease exacerbation during steroid tapering, second-line treatment was applied. The second-line treatment drugs that were effective in our group were mycophenolate mofetil, azathioprine, cyclosporin A and rapamycin. These agents improved the clinical course of GLILD in three of our patients. However, it should be stressed that immunosuppressive treatment was associated with several significant side effects. Among the most relevant were: leucopenia after MMF treatment; pancreatitis, liver injury with hypertransaminasemia and cholestasis after azathioprine therapy; leucopenia and hypertransaminasemia after rapamycin treatment and advanced osteoporosis with compression fractures of lumbar vertebrae after GCs therapy (**Table 3**). In our study, we used either GCs alone (four patients) or a combination of immune-modulating agents (four patients). Since the second-line drugs rapamycin and azathioprine are directed toward T cells, they are effective in GLILD patients, whose lung biopsies are known to contain infiltrates with T cells. We found that this type of therapy brought about subjective and objective resolution of ILD in PAD patients. As reported by other authors, similar effects may be obtained with the CTLA-4 fusion protein abatacept. The effectiveness of this treatment has been documented, particularly in patients with LRBA deficiency (53–55). Our patient with LRBA deficiency was treated with GCs as a first-line therapy, and mycophenolate mofetil and rapamycin as a second-line therapy, but did not receive abatacept treatment.

As lung infiltrates also consist of CD20+ B cells, clinical improvement in GLILD may also be achieved after administration of the anti-CD20 antibody rituximab as monotherapy (56) or in combination with azathioprine, as reported by some groups (48, 57).

HSCT is not considered to be a standard therapeutic option in patients with GLILD (5). However, HSCT was reported to be applied to some patients with malignancies, refractory autoimmune cytopenias, ILD/GLILD, and/or autoimmune enteropathy, among others in patients with LRBA deficiency (58). As reported by Tesch et al., HSCT in these patients was associated with an increased risk of death in the course of early post-transplant complications, but HSCT survivors achieved remission of disease symptoms (including ILD), in contrast to patients who had not undergone transplantation (55). The overall survival rate of patients undergoing HSCT was 70.8%, and the vast majority (70.6%) didn't require further immunosuppressive treatment (55).

Since only one of our patients was treated with HSCT due to refractory autoimmune cytopenia, we did not feel entitled to enclosing a more extensive discussion or drawing any binding conclusions.

Strength and Limitations of the Study

To our knowledge, this is the largest study on children with PID-related ILD (mostly CVID patients) published so far, presenting detailed description of clinical, radiological, and histological signs of ILD/GLILD. Additionally, the analysis of the first- and second-line treatments is presented.

However, this study also has some limitations. First of all, it is a retrospective analysis. As a consequence of the study design, there are further drawbacks, including genetic diagnosis in only a few patients and non-uniform assessment and treatment.

It is important to point out that two of the three children with the most advanced lung disease were not able to perform PFT due to young age or intellectual disability. Finally, the type of ILD was not confirmed by surgical lung biopsy in three of the children.

We believe that our study clearly points out the need for developing a uniform and commonly accepted diagnostic and therapeutic algorithm for patients with PID and coexisting ILD. Special attention should be paid to early radiological detection of lung involvement with the use of HRCT. This is supported by our finding that some asymptomatic patients with PID-related ILD may present with advanced structural lung alterations. It also seems necessary to propose a more uniform therapeutic approach and to gather more long-term observations on the relationship between treatment regimen and disease outcomes.

CONCLUSIONS

Our analysis showed that the clinical presentation of PID-related ILD can be diverse, ranging from asymptomatic presentation to life-threatening disease. It should be emphasized that ILD symptoms may precede the diagnosis of PID and that aggressive treatment, especially in patients with a rapid clinical course, provides fast but sometimes incomplete improvement. Although corticosteroids seem to be effective in the treatment of PID-related ILD, even in severe cases, the optimal treatment of PID-related ILD patients is hindered by the lack of clinically proven treatment regimens for these diseases. Hence, there is a need for a well-designed, multicenter study that includes an adequate number of patients, including pediatric population, to determine the most effective treatment strategies and the optimal duration of the therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Bioethics Committee of the Children's Memorial Health Institute in Warsaw. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written, informed consent was obtained from all individuals AND/OR their parents for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MPa: conceived the idea for the study. MPa, TB, KG, and KK: study design, coordination and supervision of data collection, data analysis, and manuscript preparation. RL: preparation and assessment of histopathological preparations and manuscript edition. JK: radiological assessment and manuscript preparation. MPr: preparation and assessment histopathological preparations and critical review. BP and KT: contributed to immunologic data collection and critical review. SK, ND-L, KB-S, HD, AP-N, BM, EB, and IW-B: contributed to clinical data collection and critical review. All authors: contributed to the article and approved the submitted version and agree to be accountable for all aspects of the work.

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Newborn Screening for SCID and Other Severe Primary Immunodeficiency in the Polish-German Transborder Area: Experience From the First 14 Months of Collaboration

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In 2017, in the Polish-German transborder area of West Pomerania, Mecklenburg-Western Pomerania, and Brandenburg, in collaboration with two centers in Warsaw, a partnership in the field of newborn screening (NBS) for severe primary immunodeficiency diseases (PID), mainly severe combined immunodeficiency (SCID), was initiated. SCID, but also some other severe PID, is a group of disorders characterized by the absence of T and/or B and NK cells. Affected infants are susceptible to life-threatening infections, but early detection gives a chance for effective treatment. The prevalence of SCID in the Polish and German populations is unknown but can be comparable to other countries (1:50,000–100,000). SCID NBS tests are based on real-time polymerase chain reaction (qPCR) and the measurement of a number of T cell receptor excision circles (TREC), kappa-deleting recombination excision circles (KREC), and beta-actin (ACTB) as a quality marker of DNA. This method can also be effective in NBS for other severe PID with T- and/or B-cell lymphopenia, including combined immunodeficiency (CID) or agammaglobulinemia. During the 14 months of collaboration, 44,287 newborns were screened according to the ImmunoIVD protocol. Within 65 positive samples, seven were classified to immediate recall and 58 requested a second sample. Examination of the 58 second samples resulted in recalling one

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newborn. Confirmatory tests included immunophenotyping of lymphocyte subsets with extension to TCR repertoire, lymphoproliferation tests, radiosensitivity tests, maternal engraftment assays, and molecular tests. Final diagnosis included: one case of T-B^{low}NK+ SCID, one case of atypical T^{low} B^{low}NK+ CID, one case of autosomal recessive agammaglobulinemia, and one case of Nijmegen breakage syndrome. Among four other positive results, three infants presented with T- and/or B-cell lymphopenia due to either the mother's immunosuppression, prematurity, or unknown reasons, which resolved or almost normalized in the first months of life. One newborn was classified as truly false positive. The overall positive predictive value (PPV) for the diagnosis of severe PID was 50.0%. This is the first population screening study that allowed identification of newborns with T and/or B immunodeficiency in Central and Eastern Europe.

Keywords: newborn screening, SCID, TREC, KREC, RareScreen, PID, NGS

INTRODUCTION

Newborn screening (NBS) tests enable identification of infants with life-threating disorders, which require early intervention shortly after birth.

NBS was initially implemented in the early 1960's in the United States for the detection and treatment of phenylketonuria (1). Within the next years, this test was introduced in many other countries worldwide. Many rare genetic diseases, including inborn errors of metabolism and endocrine disorders, were successfully implemented to newborn screening programs later on. Different analytical techniques were used. These included bacterial inhibition tests, radioimmunoassays, immunoassays with colorimetric, fluorometric, or luminometric measurements and, starting from the late 1990's, tandem mass spectrometry followed soon after by DNA-based technologies (2, 3). In 2005, McGhee et al. and Chan and Puck described the first-time application of the DNA–based assay (T-cell receptor excision circles—TREC) colorimetric NBS for severe combined immunodeficiency (SCID) and other forms of T-cell lymphopenia (4, 5). Some years later, KREC (kappa–deleting recombination circles) was proposed for a combined TREC—KREC screening approach for severe forms of T- and/or B-cells deficiencies, such as SCID, late-onset adenosine deaminase deficient SCID (ADA-SCID), combined immunodeficiency (CID), or different forms of agammaglobulinemia (XLA) (6, 7). Additionally, in ADA-SCID the purine metabolites and adenosine deaminase activity can be determined using tandem mass spectrometry (8, 9).

Primary immunodeficiency diseases (PID) are a heterogenous group of inborn errors of immunity affecting ∼1 in 10,000 to 1 in 50,000 births (10). Currently, over 400 different genetic mutations and diseases are recognized, yet the collective prevalence is likely to be higher (11–13). Patients with PID are classified into one out of 10 groups, such as predominant antibody deficiency, cellular deficiency, combined immunodeficiency, and others (13). The leading symptom of the majority of them is a predisposition to life-threating infections.

SCID is the most severe form of PID and represents a group of rare inborn defects of the immunity with either known (about 20) or unknown gene defects. The most common feature is the lack or very diminished number of T-cells, accompanied by absent or non-functional B lymphocytes and NK cells (13, 14). Affected individuals appear to be healthy at birth. They start to present infections and failure to thrive at the age of ∼3–6 months. Severe and recurrent infections of bacterial, viral, fungal, and opportunistic origin, as well as secondary infections induced by life vaccines, are life-threatening with fatal outcomes within the first 1–2 years of life if untreated (15). Since the late 1960's, many advances have been made in the field of treatment, including hematopoietic stem cell transplantation (HSCT), enzyme therapy, or gene therapy for different genetic forms of SCID (16, 17).

As a condition which is potentially curable if recognized and treated early, SCID and other severe PID meet Wilson's and Jungner's criteria for NBS (18, 19). TREC assay for NBS dedicated to the early diagnosis of SCID was implemented for the first time in the United States in 2008 (20). Several studies performed since that time revealed a SCID incidence of 1: 58,000 live births (21). Now it is screened throughout the United States (as one out of 35 primary screened disorders included in the recommended uniform screening panel), as well as in some countries in Europe and other continents, either reimbursed by governments or by scientific/pilot studies (2, 22).

Outside the United States, routine TREC screening is currently performed in Israel, New Zealand, Norway, Taiwan, several provinces in Canada, Switzerland, Iceland, Sweden, Italy (Tuscany), Spain (Catalonia), and in some regions in Australia (15). The overall implementation status EU-wide is very diverse. Considerations are still going on in various European countries. Pilot projects are currently underway or have been announced in France (23), Spain (24, 25), Norway, and the Netherlands (26).

In Germany, prior to the nationwide implementation of SCID NBS, only a few screening laboratories had experience with this procedure, such as laboratories in Munich (head: B. Olgemoeller), Heidelberg (head: G.F. Hoffmann), Hannover (head: N. Janzen), Leipzig (head: J. Thiery), and Greifswald (head: M. Nauck) (27). The whole process to include SCID in newborn screening began in 2012 with a local pilot trial in Leipzig. From 2014 to 2016, another pilot project was carried

out in Heidelberg. Finally, in August 2019 the SCID NBS was implemented nationwide (TREC only) (28). We present the results of the first 14 months of the trans-border cooperation in the field of NBS for SCID and other severe PID in the area of West Pomerania, Poland, and Mecklenburg-Western Pomerania and Brandenburg in Germany.

MATERIALS AND METHODS

The Polish-German transborder cooperation in the field of NBS for SCID and other severe PID was possible thanks to the Cooperation Program Interreg V A Mecklenburg-Vorpommern/Brandenburg/Poland and the project entitled "Innovative Polish-German cross-border program for early diagnosis and treatment of rare diseases in newborns–RareScreen" (INT10). The project was intended to expand the NBS panel for inborn errors of metabolism and other rare diseases including SCID. It was initiated on 01.05.2017 and will last till 30.10.2020.

The "RareScreen" project includes newborns born in the Euro-Pomerania region which covers West-Pomerania in Poland and Mecklenburg-Western Pomerania and part of Brandenburg in Germany. The population of the above-mentioned geographical regions in 2016 was as follows: 724,161 inhabitants in Mecklenburg-Vorpommern, 300,243 in Brandenburg (1,024,404 in total), and 1,708,174 in West Pomerania, Poland (29).

Three screening laboratories are involved in the project: Newborn Screening Laboratory Independent Public Clinical Hospital nr 1 PUM, Szczecin, Poland; Newborn Screening Laboratory, University Medicine Greifswald, Germany; and Newborn Screening Laboratory Charité University Medicine Berlin, Germany. The four other project partners are: University Medicine Greifswald, Germany; Pomeranian Medical University Szczecin, Poland; Institute of Mother and Child, Warsaw, Poland; and the Children's Memorial Health Institute, Warsaw, Poland (**Figure 1**).

The results presented here are based on samples which were collected between October 24, 2018 and December 31, 2019. The sample collection is currently still ongoing and will last until October 2020.

For all newborns, the TREC and KREC NBS assay was performed in the NBS Laboratory in the Independent Public Clinical Hospital nr 1 PUM Szczecin (West Pomerania, Poland).

The organization of efficient transborder transport of dry blood spots samples (DBS) was fundamental to the cooperation between the NBS laboratories. The DBS from Berlin to Greifswald were sent via regular post. Subsequently, DBS from both German centers were delivered via courier from Greifswald to Szczecin NBS Laboratory. The transport between Greifswald and Szczecin took place four times a week and the samples were transported and stored at 4°C until use. The samples reached Szczecin usually within 1–7 days after their arrival in the local laboratories. The results were electronically transferred from Szczecin to Greifswald and Berlin within two working days. Waiting time for repetition from the first screening card of German newborns took from 2 to 10 days. Repetitions of the test from the first screening card of Polish newborns were performed in the next protocol which took place on the next working day. If any further action was needed, e.g., the necessity of taking a second screening card or admission of a newborn to the hospital for confirmatory tests, the procedure took place according to the protocols of each center.

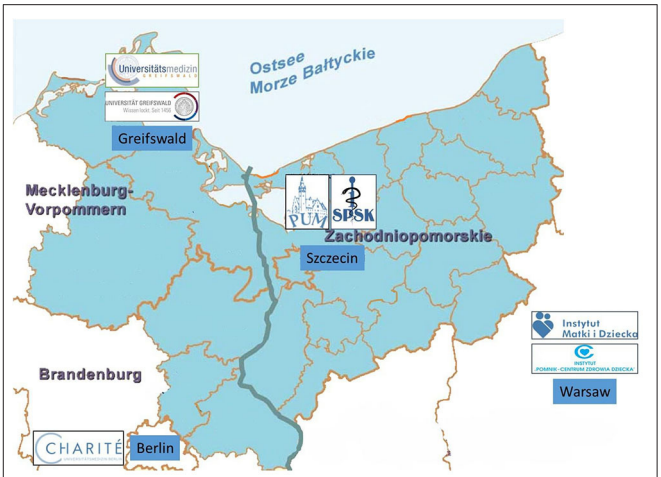

FIGURE 1 | The region covered by the Rare-Screen Project according to the Cooperation Program Interreg V A Mecklenburg-Vorpommern/ Brandenburg/ Poland with the participating German and Polish partner sites from Greifswald, Berlin (D), Szczecin, and Warsaw (PL).

Information brochures about the "RareScreen" program for parents and health care providers were distributed. Brochures included basic information about SCID, the goals and necessity of implementing this study, the advantages of participating in the project, and data protection. Additionally, informative meetings were organized for midwives, pediatricians, and other parties involved in the study. The aim of medical staff training and development of the brochures was to educate parents so that they could sign their informed consent to participate in the study.

No additional blood collection was needed from the newborns participating in the project apart from the blood usually taken for regular NBS. Heel prick blood samples were collected on filter paper (Whatman 903 for newborns from Poland and PerkinElmer 226 Ahlstrom for newborns from Germany) between the third and fifth day post-partum as part of the national NBS programs in Poland and Germany.

IT software (NeoBase) was adapted to transfer necessary data between centers involved in the project.

Demographic data, such as sex, birth date, data of sample collection, birth weight (BW), and gestational age (GA) of the newborns, was collected in the database (NeoBase). According to WHO definition, newborns were allocated to groups according to GA as follow: ≥38 weeks—born at term; ≥32–37 weeks—moderate preterm, ≥28–32 weeks—very preterm; <28 weeks—extremely preterm (30). Additionally, in the NeoBase other essential information about the newborns regarding their clinical condition, medicines taken (antibiotics, steroids), and blood

transfusions as well as the mothers' history and treatment during pregnancy, was registered.

The NBS tests were performed using a commercial kit—SPOT-it ™ TK (ImmunoIVD, Sweden). The screening laboratory in Szczecin was adapted and equipped with instruments for the PCR test method. Staff members were trained by the manufacturer of the TREC and KREC assay.

The 3.2 mm DBS were punched directly into 96-well-filter plate (ImmunoIVD) using a Wallac DBS puncher (Perkin Elmer). After DBS extraction and DNA elution, the qPCR reaction was performed (QuandStudio5, ThermoScience). The number of copies/μL for TREC, KREC, and beta-actin (ACTB) were calculated using the standard curves method. The amount of ACTB copy numbers indicates the efficacy of DNA extraction from DBS samples. Plates include three quality control punches with defined T- and B-cell ranges: T-cell depleted, B-cell depleted, and T-and B-cell depleted, as well as one blank control (DBS not soaked in blood) (QCs were provided also by ImmunoIVD).

Definition and Interpretation of the Results

The TREC and KREC assay is intended to screen newborns with the most severe forms of T- and B-cell lymphopenia (14, 31). According to the manufacturer's instructions, the cut off values are 6 copies/μL for TREC and 4 copies/μL for KREC.

In the case of abnormal results (TREC <6 copies/μL and/or KREC <4 copies/μL) or inconclusive results (ACTB <1,000 copies/μL), a sample was repeated (re-tested) in duplicate from the first DBS. In the case of a positive NBS result, the following procedure depended on the values obtained from the first screening card (3 punches). When the value of TREC was in range of 1–4 and/or KREC 1–6 copies/μL, parents or medical staff (if the child was still at the hospital) were informed by letter or by phone call about the necessity of taking the second blood sample. Numbers of TREC and/or KREC <1 copies/μL in the re-tested first DBS (urgent-positive) resulted in immediate recall of the newborn and admission to the Department of Pediatrics, Endocrinology, Diabetology, Metabolic Diseases, and Cardiology in Szczecin and/or the Department of Immunology, The Children's Memorial Health Institute, Warsaw, Poland or to the Department of Pediatric Pneumology and Immunology, Charité Universitaetsmedizin Berlin, Germany for a confirmatory diagnosis. The aim of our program was to detect severe forms of PID, SCID in particular (defined as T-cells <300 cells/μL), as well as to identify other severe forms of T- and/or B-cell lymphopenia, including CID, agammaglobulinemia, and secondary immunodeficiencies (32). The confirmatory diagnostics procedures included, beside routine pediatric examination and basic laboratory tests, detailed immunocytometry assay (T cells [CD3/CD4/CD8, T cell naivety [CD45RA and CD45RO], B cells [CD19], NK cells [CD3/CD16/CD56], recent thymic emigrants (RTE) [CD4/CD31/CD45RA], α/ß and γ/δ T cells [CD3/α/ß TCR/γ/δ TCR], lymphocyte proliferation tests (to PHA, anti-CD3, and Pansorbin), humoral immunity adjustment (immunoglobulin levels), cytogenetic tests (karyotype), molecular tests (New Generation Sequencing or single gene sequencing by Sanger), and, if available and needed, radiosensitivity tests, analysis of the TCR Vbeta repertoire, ADA and PNP enzyme activity levels, and anthropometry.

False-positive results were defined when values for TRECs or KRECs in NBS were over the established cut-offs in absence of SCID or other PID in the confirmatory diagnosis (**Figure 2**).

Statistical analyses were performed using Python with the SciPy package. Given the beta distribution of the data, the Mann–Whitney test and the Kruskal–Wallis test were used to compare continuous variables between the groups. Differences were considered statistically significant when the p-value was <0.05.

RESULTS

A total of 44,287 newborn samples were prospectively collected from two centers in Germany (Greifswald−11,114; Berlin−15,428) and one in Poland (Szczecin−17,745). In this group of newborns, 22,804 (51.5%) were males and 21 434 (48.4%) were females. Information regarding the sex was incomplete in 49 (0.1%) cases and for 138 newborns (0.3%) GA was not provided. 36 634 (82.7%) newborns were born at term and 7,515 were preterm (17.0%). In a group of preterm newborns, there were 6,646 (15.0%) moderate preterm born at ≤32–37 weeks of gestation, 621 (1.4%) very preterm born between ≤28–31 weeks, and 248 (0.56%) extremely preterm children born <28 weeks. The median value for GA was 39 weeks (min. 22, max. 42 weeks). The median value for birth weight for the whole group was 3,395 g (min. 450 g, max. 5,504 g) (**Table 1**).

Out of 44,287 samples, 321 (0.72%) were re-tested from the first DBS. Among the 321 re-tested samples, 168 had a low number of ACTB (<1,000 copies/μL) and, due to a lack of a sufficient amount of DNA in the sample, concomitant reduction of TRECs and KRECs were referred to as "inconclusive" and re-tested. The remaining 153 DBS with ACTB >1,000 copies/μL and TREC or KREC copy numbers below the respective cutoff values were considered "abnormal" and also re-tested. After re-testing, 256 newborns presented with normal results (TREC >6 copies/μL, KREC >4 copies/μL, ACTB >1,000 copies/μL); 65 neonates had positive results and required reevaluation. Out of this group, 11 (11/65; 16.92%) newborns had TREC value <6 copies/μL and 34 (34/66; 52.13%) retested children had KREC values <4 copies/μL. In two (2/65; 3.081%) newborns both TREC and KREC values were below the cut-off. In 18 (18/65; 27.70%) newborns, the poor-quality samples with undetected TREC and/or KREC copies and values of ACTB ≤1,000 copies/μL were detected, indicating insufficient efficacy of DNA extraction from DBS. In the case of eight newborns TREC and/or KREC copy values were below 1 copy/μL from the first blood samples (3 DBS punches). They were considered as urgent-positive and immediate admission to the hospital for clinical examination and flow cytometry was recommended, without waiting for the results from the second DBS. For the remaining 58 newborns, a second blood sample was requested. In the case of extremely and very preterm newborn (born <32 HBD) the second screening cards were taken when the child reached 32–34 weeks of gestational age. After re-testing the

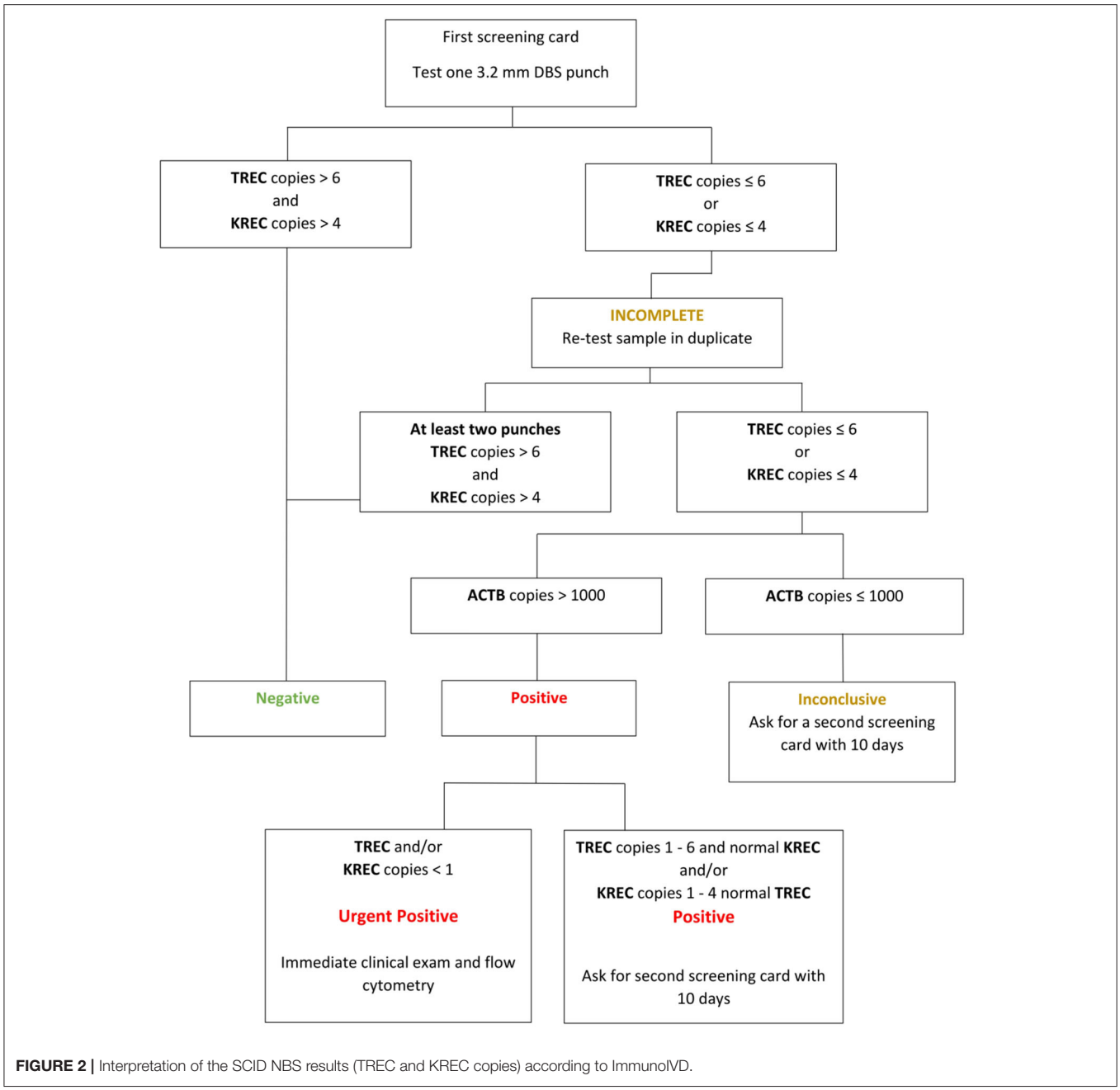

FIGURE 2 | Interpretation of the SCID NBS results (TREC and KREC copies) according to ImmunoIVD.

second DBS, one newborn had a positive result and was called to further immunological evaluation; 56 newborns had normal results (**Table 2**).

In the whole group of tested children, extremely premature newborns, born <28 weeks of gestation, had the lowest TREC values (median value−42 copies/μL) and the lowest spread of the results among the groups (**Figure 3A**). TREC values in 5% of the first DBS from this group were below the cut-off value of 6 copies/μL, however, they also tended to rise along with GA. In our study, the Kruskal Wallis test showed significant differences ($p < 0.05$) in the values of TREC median between the studied groups of newborns divided according to the GA.

Regarding the KREC values, we did not notice higher rates of KREC results below cut off in children born <28 weeks of GA. However, in extremely premature newborns the spread of KRECs values was greater than in the other groups. Children born between 32 and 37 and >38 weeks of gestation had similar distributions of KREC values (respectively median value: 41 and 42 copies/μL). The Kruskal-Wallis test shows a significant difference ($p < 0.05$) between the values of medians in the groups of newborns divided according to the GA (**Figures 3A,B**).

TABLE 1 | Demographic data and median values of TREC and KREC copies/μL in the study population.

	Sample size	TRECs, copies/μL, median (SD; range)	KRECs, copies/μL, median (SD; range)
All newborns	44,287	86 (54.8; 0–830)	42 (33.0; 0–734)
Sex			
Male*	22,804 (51.49%)	81 (52.0; 0–830)	40 (32.2; 0–734)
Female*	21,434 (48.40%)	92 (56.1; 0–758)	44 (33.7; 0–600)
Unknown sex	49 (0.11%)	94	48
Term ≥ 38 weeks; *n* (%)	36,634 (82.71%)	88 (54.8; 0–830)	42 (31.8; 0–400)
Moderate preterm, ≥32–37 weeks; *n* (%)	6,646 (15.01%)	80 (51.8; 0–758)	41 (34.2; 0–600)
Very preterm ≥28–31 weeks; *n* (%)	621 (1.40%)	68 (67.4; 0–498)	45 (57.30; 0–734)
Extremely preterm <28 weeks; *n* (%)	248 (0.56%)	42 (44.3; 0–341)	52 (61.4; 0–456)
Unknown term of birth	138 (0.32%)	94	45

**The Mann-Whitney test did not show a significant difference between males vs. females and TREC and KREC values.*

TABLE 2 | Number of newborns, retests from the first DBS, and recalls in the study population.

	Total	West pomerania szczecin	Mecklenburg-western pomerania greifswald	Brandenburg berlin
Newborns	44,287	17,745	11,114	15,428
Re-tested sample from the1st DBS	321*	116	105	100
Re-call for confirmatory diagnosis after re-testing 1st DBS	7	3	2	2
Second sample	58	23	15	20
Re-call for confirmatory diagnosis after re-testing 2nd DBS	1	1	0	0

**321 samples were re-tested from the first DBS (at least 2 punches): 168 "inconclusive"; results were related to low number of ACTB (<1,000 copies/μL) and subsequently reduced TREC and/or KREC values; - 153 "abnormal" results with ACTB >1,000 copies/μL had TREC and/or KREC copy numbers below the respective cutoff values.*

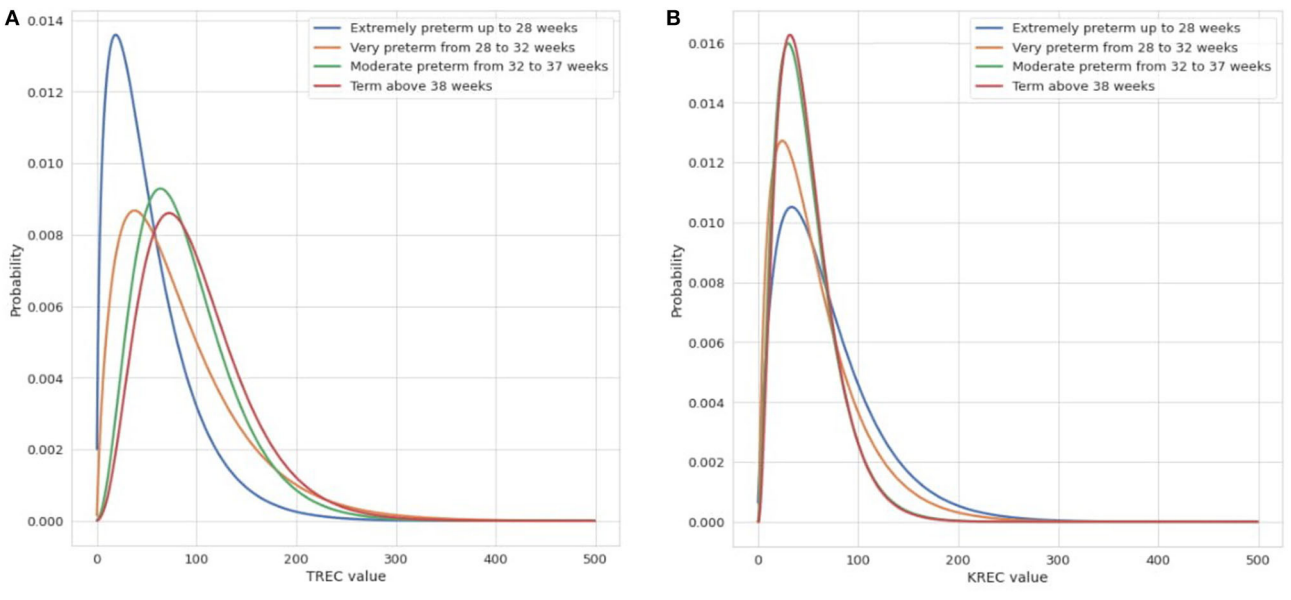

FIGURE 3 | The distribution of TREC copies/μL **(A)** and KREC copies/μL **(B)** in groups divided by GA. The fitted beta distributions of TREC (copies/μL) and KREC (copies/μL) values in four study groups: extremely preterm (blue line), very preterm (orange line), moderate preterm (green line), term newborn (red line).

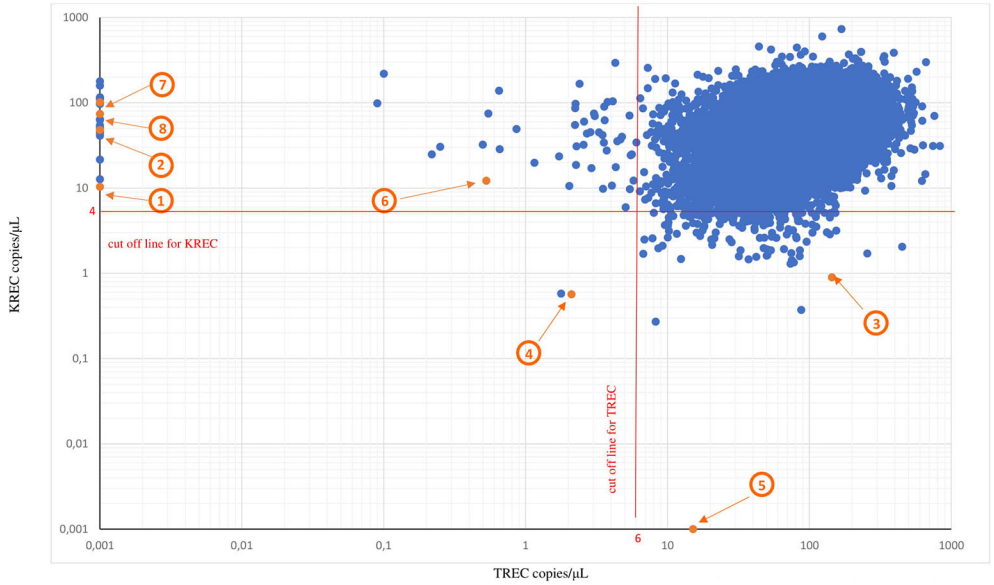

FIGURE 4 | TREC and KREC copy numbers/μL from the first DBS and final diagnosis in eight newborns with severe PID. TREC and KREC copy numbers from the first DBSs.* Red lines represent cutoff values for TREC copies/μL (cut off value 6 copies/μL) and KREC (cut off value 4 copies/μL), respectively. Orange dots represent eight positive results from the first DBSs with T and/or B cell lymphopenia confirmed on flow cytometry. 1—T-B^{low}NK+ SCID; 2—T^{low}B^{low}NK+ CID; 3—AR agammaglobulinemia; 4—Nijmegen breakage syndrome; 5—Transient B-cell lymphopenia due to mother's immunosuppression; 6—T- and B-cell lymphopenia due to prematurity; 7—Transient T- cell lymphopenia of unknown reason; 8—False positive of unknown reason.
*The samples which had a low number of ACTB (<1,000 copies/μL) and concomitant reduction of TREC and KREC copy numbers were referred to as "inconclusive" because of a lack of DNA and samples were excluded from the graph.

Final Diagnosis

During the first 14 months of the "RareScreen" project, 44,287 newborns were screened for SCID. After testing the first screening cards, among 65 positive results with low TREC and/or KREC values, seven neonates treated as urgent-positive were recalled to the hospital for confirmatory tests. Examination of second screening samples from 58 newborns resulted in recalling to the hospital of one newborn. Overall, eight newborns were referred for further immunological evaluation. Confirmatory procedures (mostly lymphocyte subsets analysis) revealed one case of T-B^{low}NK+ SCID with severe cartilage-hair hypoplasia (homozygous mutation in *RMRP* G.70A>G), one case of atypical T^{low}B^{low}NK+ CID without dysmorphic features and of unknown genetic defect, one case of autosomal recessive agammaglobulinemia, and one case of Nijmegen breakage syndrome. Among four other cases of T- and/or B-cell lymphopenia, one was related to the mother's immunosuppression during pregnancy and one to prematurity. The next one, a child with slight dysmorphic features, presented low TREC only, followed by persistent/repeatable T-cell lymphopenia in flow-cytometry with normal TCR repertoire and mitogen stimulation. However, due to persistent T-cell lymphopenia, a molecular test (NGS) was performed, revealing no underlying genetic defects. At the age of 7 months, T-cell lymphopenia almost resolved, however the child remains under observation. The last newborn was truly false positive with normal results of flow-cytometry. In children with SCID and CID (pt.1 and pt.2), higher proportions of TCR γ/δ expression and loss of naivety were observed. In patient 1, diminsihed lymphoproliferation to phytohemagglutinin was observed. In patient 4 with Nijmegen breakage syndrome, only standard karyotype was performed, which resulted in changes in chromosome 7 and 14 (characteristic for this disease).

Both children with SCID and CID underwent a successful HSCT at the age of 2 and 3.5 months of age, respectively. In the child with agammaglobulinemia, immunoglobulin replacement therapy (IgRT) was introduced. The child with Nijmegen breakage syndrome required two instances of IgRT and remains under regular immunological monitoring. The other children with transient T- and/or B-cell lymphopenia are doing well and the immunophenotyping results are either normal or at least definitely improved.

The overall positive predictive value (PPV) was 50.0% (**Table 3**, **Figure 4**).

DISCUSSION

Six decades have passed since Robert Guthrie developed the population newborn screening test for PKU (1). During this time, the NBS evolved from detection of phenylalanine levels on filter paper to application of DNA-based technics for early identification of a number of rare genetic disorders.

Many authors agree that SCID, and other severe forms of PID, are an important health problem with a known natural history and available treatment, which meets the Wilson and Junger criteria (18, 19).

TABLE 3 | Final diagnosis in eight newborns recalled for further immunological evaluation due to positive results in population newborn screening for severe PID.

	TREC copies from 1st screening card cut-off ≤ 6 copies/μL	KREC copies from 1st screening card cut-off ≤ 4 copies/μL	ACTB copies from 1st screening card cut-off > 1,000 copies/μL	Final diagnosis	Treatment	Flow cytometry			Outcome
						T cell	B cell	RTE cell	
						N^0 cells/μL / %	N^0 cells/μL / %	N^0 cells/μL / %	
1	0–0	10.4–11.4	2,918–3,589	T-B^{low}NK+ SCID Cartilage-hair hypoplasia Homozygous mutation in *RMRP* g.70A>G	HSCT at the age of 2 months	70 (ref. 2,300–7,000) 5 (ref. 60–85%)	130 (ref. 600–1,900) 9 (ref. 4–26%)	8.54 (ref. 49.78–79.60%)	Alive
2	0.0–0.0	48–88	3,020–8 895	$T^{low}B^{low}$NK+ CID Unknown genetic cause	HSCT at the age of 3.5 months	480 (ref. 2,300–7,000) 38 (ref. 60–85%)	240 (ref. 600–1,900) 18 (ref. 4–26%)	15.94 (ref. 49.78–79.60%)	Alive
3	144.5–163.4	0.9–0.6	6,186–10 409	AR agammaglobulinemia homozygous IGLL1-mutation *(IGLL1 c425C>T, p.Pro142Leu)* The parents are each heterozygous for this mutation	IgRT	4,170 (ref. 2,300–7,000) 93 (ref. 60–85%)	30 (ref. 600–1,900) 1 (ref. 4–26%)	80 (ref. 49.78–79.60%)	Alive
4.	2.1–3.1	0.6–0.7	4,104–4,356	Nijmegen breakage syndrome, homozygous deletion c.657_661del5 in *NBN* gene	IgRT	1 171 (ref. 1,700–3,600) 56.06 (ref. 58–67%) 1,581* (ref. 2,000–4,700) 49.9* (ref. 54.6–80.5%)	45 (ref 500–1,500) 2.17 (ref. 19–31%) 113* (ref. 700–2,400) 3.57* (ref. 10.0–30.7%)	45.57 (ref. 50–74%) 22.7* (ref. 50–74%)	Alive
5.	12.1–22.1	0–0.2	4,909–3,208	Transient B-cell lymphopenia due to mother's immunosuppression during pregnancy (Mycophenolate Mofetil—in the first gestation weeks, prednisone, azathioprine, and tacrolimus)—flow cytometry results normalized with age	No	2,420 (ref. 1 700–3,600) 95.61 (ref. 58–67%) 5,901* (ref. 2,000–4,700) 62.31* (ref. 57.1–72.4%)	10 (ref 500–1,500) 0.38 (ref. 19–31%) 2,870* (ref. 700–2,400) 30.3* (ref. 18.4–37.5%)	31.1 (ref. 50–74%) 56.6* (ref. 52–57%)	Alive

(Continued)

TABLE 3 | Continued

	TREC copies from 1st screening card cut-off ≤ 6 copies/μL	KREC copies from 1st screening card cut-off ≤ 4 copies/μL	ACTB copies from 1st screening card cut-off > 1,000 copies/μL	Final diagnosis	Treatment	Flow cytometry			Outcome
						T cell	B cell	RTE cell	
						N^0 cells/μL / %	N^0 cells/μL / %	N^0 cells/μL / %	
6.	0.5–0.1	12.18–7.0	755–1,117	T- and B-cell lymphopenia due to prematurity (GA—33 weeks). On molecular testing (WES)—no data for PID, in the *HBD* gene heterozygous pathogenic variant c.82G>T p. (Ala28Ser) was identified -the result is consistent with the genetic diagnosis of AD delta-thalassemia. Follow-up flow cytometry results improved with age	No	522 (ref. 1,700–3,600) 47.64 (ref. 58–67%)	222 (ref 500–1,500) 20.27 (ref. 19–31%)	45.57 (ref. 49.78–79.60%)	Alive
						1 133* (ref. 2,000–4,700) 30.4* (ref. 57.1–72.4%)	1 625* (ref. 700–2,400) 43.59* (ref. 18.4–37.5%)	27.1 (ref. 52–57%)	
						1 225** (ref. 1,400–2,000) 39,77** (ref. 66–76%)	813** (ref 300–500) 26,41** (ref. 12–22%)	29,57** (ref. 55–77%)	
7	0.0–1.0	101.3–80.1	1,334–2,528	T- cell lymphopenia of unknown reason—follow-up flow cytometry results improved with age Unknown genetic cause	No	1,250 (ref. 1,700–3,600) 56.14 (ref. 58–67%)	635 (ref. 500–1,500) 2.,53 (ref. 19–31%)	–	Alive
						637* (ref. 2,000–4,700) 37.7* (ref. 57.1–72.4%)	904* (ref. 700–2,400) 53.47* (ref. 18.4–37.5%)	56.1* (ref. 52–77%)	
						2,745** (ref. 2,800–5,700) 50.6** (ref. 58.5–77.1%)	2,240** (ref. 700–2,800) 41.3** (ref. 15.7–34.1%)	62.6** (ref. 55–77%)	
8	0.0–0.0	74.3–54.8	1 639–705	False positive	No	2,880 (ref. 2,300–7,000) 61 (ref. 60–85%)	940 (ref. 600–1,900) 20 (ref. 4–26%)	68.87 (ref. 60.9–80.6%)	Alive

Range of results from the first screening card includes 3 measurements (first test and repetition in duplicate).

Differences in age-related values of flow-cytometry parameters arise from differences in laboratory specific referential ranges of flow-cytometry.

SCID, severe combined immunodeficiency; CID, combined immunodeficiency; TREC, T-cell receptor excision circles; KREC, kappa-deleting element recombination circle; ACTB, beta-actin; HSCT, hematopoietic stem cell transplantation; AR, autosomal recessive trait of inheritance; IgRT, immunoglobulin replacement therapy; GA, gestational age; PID, primary immunodeficiency diseases; RTE, recent thymic emigrants; AD, autosomal dominant trait of inheritance; WES, whole exome sequencing; GA, gestation age.

**repeated flow cytometry in 2nd-3rd month of life.*

***repeated flow cytometry in 7nd-8th month of life.*

Since 2008, TREC analysis has been used as a screening method for severe forms of primary T-cell lymphopenia (2, 5, 20, 33). Further research enabled the extension of the qPCR method to simultaneous measurement of TREC and KREC values. The method, which includes KREC analysis to assess potential B-cell lymphopenia, allows for additional identification of patients with XLA, Nijmegen breakage syndrome, and purine nucleoside phosphorylase (PNP) deficiency (9, 34). However, TREC screening will not identify infants with SCID in which a molecular defect lies downstream of T-cell receptor rearrangement, including variants in *ZAP70*, MHC class II (major histocompatibility complex class II), and ADA (delayed-onset disease) (35–39).

Infants with SCID and other forms of PID are susceptible to life-threatening infections of different origins, as well as secondary infections induced by life vaccines (40). Early diagnosis was made possible by testing T- and/or B-cell lymphopenia with NBS procedures, thereby significantly improving the life of affected children. Detection of PID in the first weeks of life enables an effective treatment with HSCT, enzyme replacement therapy, gene therapy, and/or immunoglobulin replacement therapy (16, 17, 19).

Nowadays, more and more countries have already introduced or are considering the population NBS for PID using the evaluation of TREC alone or in combination with KREC analysis (19, 41).

In our study we used the combined TREC and KREC assay. It is similar to the methods used in other centers, including those in Sweden, Iran, and Spain (33, 34, 42, 43). The study by Borte et al. gave the first specific TREC/KREC cut-off values for SCID NBS after retesting DBS samples of known SCID patients. Their findings were supported by further studies which then allowed conclusions about specificity and sensitivity (34). Barbaro et al., performed a population wide NBS using a combined TREC/KREC test with a cohort study size of 58.834 Swedish newborns from Stockholm county (33). Their findings were supported by studies performed in Sweden, Spain, and Germany with a specificity of >99% and sensitivity of >95% for the detection of severe T- and /or B-cell lymphopenia in dry blood material (24, 34). The study from the Stockholm/Uppsala and Leipzig regions showed a PPV of 46% for severe T- and/or B-cell deficiency using the threshold values <6 copies/μL for TREC and <4 copies/μL for KREC. After evaluating the control-screening card, a PPV of 100% was found (34). In comparison, the following PPVs for severe T-cell deficiency was published based on data from the USA for TREC screening alone: State of Wisconsin 47%, State of California 31%, State of New York 18%, and State of Massachusetts 37%. Currently, the negative predictive value is 99% (44).

During the first 14 months of the RareScreen project, 44,287 newborns from Poland and Germany were screened. Overall, nine positive cases (including seven urgent-positive) were referred for immunological assessment starting with flow cytometry and followed by further investigations. Approximately, 1:5,536 of tested newborns underwent further confirmatory procedures in our cohort, while in the United States the number ranged from 1:735 to 1:7,500 depending on the state, and in Sweden it was 1:20,000 (21, 33).

Out of eight newborns, one case of SCID and one case of CID were diagnosed. The recognition of two patients with SCID/CID in the studied population of 44,287 newborns demonstrates the efficacy of NBS with TREC evaluation in early detection of affected individuals, but at the same time it requires careful consideration as the number of screened newborns is still very limited. The next child, presenting with normal TREC but low KREC levels, was diagnosed with autosomal recessive agammaglobulinemia. In another newborn, with low KREC and diminished TREC as well as microcephaly and dysmorphic features, Nijmegen breakage syndrome was recognized with a variant in the *NBN* gene typical for the Slavic population. These two cases prove that the combined TREC/KREC testing should be considered as a powerful tool for initial screening of not only SCID suspected patients but also individuals with other severe PID. The introduction of combined TREC and KREC tests can significantly advance early diagnosis of inborn errors of immunity, such as agammaglobulinemia, Nijmegen breakage syndrome, ataxia-teleangiectasia or DiGeorge syndrome, as well as late-onset ADA SCID (33, 45–48). It allows for the early introduction of the proper treatment and prophylaxis (ex. HSCT, IgRT) before infectious complications appear or to avoid X-ray exposition in the case of Nijmegen breakage syndrome, ataxia-teleangiectasia, or other radiosensitive syndromes.

One positive result with a low KREC value was related to the mother's immunosuppression during pregnancy. The mother was treated with Mycophenolate Mofetil (in the first weeks of gestation), prednisone, azathioprine, and tacrolimus after a liver transplant due to autoimmune hepatitis. It is known that azathioprine may cross the placenta and B lymphocytes are more sensitive to drug-induced apoptosis than T lymphocytes (49). Two similar cases were described by Filipe et al. in Spain (24). Although the patient's initial flow cytometry on the eleventh day of life revealed significant lymphopenia, including reduced numbers of CD19+B cells and milder diminished number of CD4+/CD3+ T cells and NK cells, repeated evaluation at the first and second month of age showed a normalisation of lymphocytes. This case shows that communication between neonatal/paediatric departments and screening laboratories is crucial in successful patient management. The knowledge about what medical treatment mother and/or the newborn underwent, may help to determine adequate follow-up steps.

In one premature neonate, born at the thirty-third week of gestation, initial low TREC resulted in recall for further evaluation. Although the first lymphocyte immunophenotyping revealed reduced numbers of CD19+B cells, CD3+, and CD4+naïve T cells as well as reduced T cell subpopulations, normalisation of CD19+B and significant improvement of CD3 subpopulation were noted in the repeated tests at the second and seventh month of life.It is worth underlining that TREC levels obtained in our study decreased with lower GA. It was especially apparent in children born <28 weeks of gestation. Such observations have been reported in several other publications (32, 50). Regarding the KREC values, we did not notice a higher rate of KREC results below cut off in children born <28 weeks

of GA. However, a significant difference between KREC values in the groups of newborns divided according to the GA was shown. In groups of extremely premature and very preterm newborns, the spread of KREC values was greater than in children born ≤32 weeks. In a study by Kanegae et al., KREC values did not vary according to GA and in the studies by Barbaro et al. and de Felipe et al. KREC levels did show a downward trend with decreasing GA (25, 50). Further evaluation is needed and may provide data to define the reason for these differences and their clinical implications.

In the whole study group, there was one truly false positive case (low TREC with normal T-cell in flow-cytometry) of unknown reason. Pilot screening performed by Holodnij et al. proved that blood collection is crucial for proper determination of TREC and KREC values. Newborn heel blood should be applied directly to the screening card. Blood applied from capillaries or heparinized blood collection tubes should be avoided as heparin is known to inhibit DNA polymerases resulting in low values of TREC and KREC and/or ACTB copies number (51). Furthermore, insufficiently soaked screening cards also tend to lead to low TREC/KREC results. The company providing SCID kits recommend using the center part of DBS, which could not be ensured for all used samples, especially in the beginning of our study.

In our study many assays, depending on necessity and availability, were used to confirm the diagnosis of severe PID following positive screening tests. The confirmation algorithms vary in different countries or even by state. In some centers they are routinely limited to complete blood count (CBC) and lymphocyte profile only with extension to TCR repertoire, mitogen stimulation, or, if needed, to maternally engrafted cells assessment (32, 52, 53). Rechavi et al. suggested an initial confirmatory panel with complete blood count, full lymphocyte profile, and TREC quantification in the peripheral blood (32). In our region CBC, lymphocyte subset with TCR, and mitogen tests are done routinely, followed by supplementary assays according to clinical and laboratory deviation.

In summary, we can conclude that newborn screening programs, including TREC and KREC, followed by detailed immunological assessment, are of great value to avoid complications in children with undiagnosed PID.

STRENGTHS AND LIMITATIONS OF THE STUDY

The strength of our study is the introduction of a combined TREC and KREC analysis, which allows the detection of not only SCID but also other severe PID with accompanying T- or B-cell lymphopenia. The recall frequency of 1: 5,536 is acceptable, however, we can expect that with growing experience (at the departments of neonatology and in NBS laboratories) and populations screened the frequency of recalls will be further reduced. Positive predictive values for SCID and other severe PID was 50.0% which was comparable to other studies (45).

As from August 2019, the national NBS program based on TREC has only been implemented in Germany and the comparison of pro and cons between both programs (with or without KREC) will be possible. Based on the literature data and our still very limited experiences, we believe that by determining KREC, patients with congenital severe B-cell lymphopenia can also be identified. With the help of KREC copies evaluation, compared to an isolated TREC screening, SCID patients who suffer from a prevalent B-cell deficiency at birth, such as the "delayed-onset" ADA or purine nucleoside phosphorylase (PNP) deficiency, can also be detected (9, 34, 39). In addition, using KREC screening newborns with agammaglobulinemia (e.g., Bruton disease) can be identified at birth and consequently treated before serious infections occur. This leads to the prevention of lethal and serious infections with irreversible organ damage (mostly bronchiectasis), which has a lasting impact on the development of the affected children (54). A combined TREC and KREC screening is also advisable in terms of the fact that it would not involve any technically complex changeover, however the established screening algorithm would need to be changed.

However, the small population of selected regions is a limitation of the study, not allowing conclusions on the exact prevalence and incidence of SCID and other severe PID in other countries. At the same time, the study shows that cross-border cooperation between several screening centers can very efficiently combine their complementary expertise on such rare diseases. This can compensate for the fact that corresponding capacities cannot be maintained at all locations.

This is the first population screening study that allowed identification of newborns with T and/or B immunodeficiency in Central and Eastern Europe. It shows that newborn screening for severe PID, including different types of SCID, is effective and should be implemented into population obligatory panels for NBS and, to this effect, international cooperation proved to be efficient.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request from the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethic Committee at the Pomeranian Medical University in Szczecin, Poland (EA2/119/18), Ethic Committee of the University Medicine Greifswald, Germany (BB 081/18), Ethic Committee of the University Medicine Charité, Berlin, Germany (EA2/119/18). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MG and MP designed the concept of the manuscript. TW, MG, MN, MO, MP, SS, OB, and TW designed the concept of the RareScreen project. MG, TW, and SS were coordinators for the project funding. TW, KD, OB, JK, EB, and NS did the laboratory work. KD and MO did the statistical analysis. MG, MP, KD,

TW, IO, OB, and MFP analysed and interpreted the data. KD drew figures and summarized the tables. MG, MP, KD, IO, HR, EK-Z, MFP, BB, EAB, HvB, CM, and MW collected the relevant information and references. MG, MP, KD, TW, IO, and MO wrote the manuscript with contribution from all co-authors. All authors read, critically reviewed, and approved the final version of the manuscript.

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