ADVANCES IN PRIMARY IMMUNODEFICIENCIES IN INDIA

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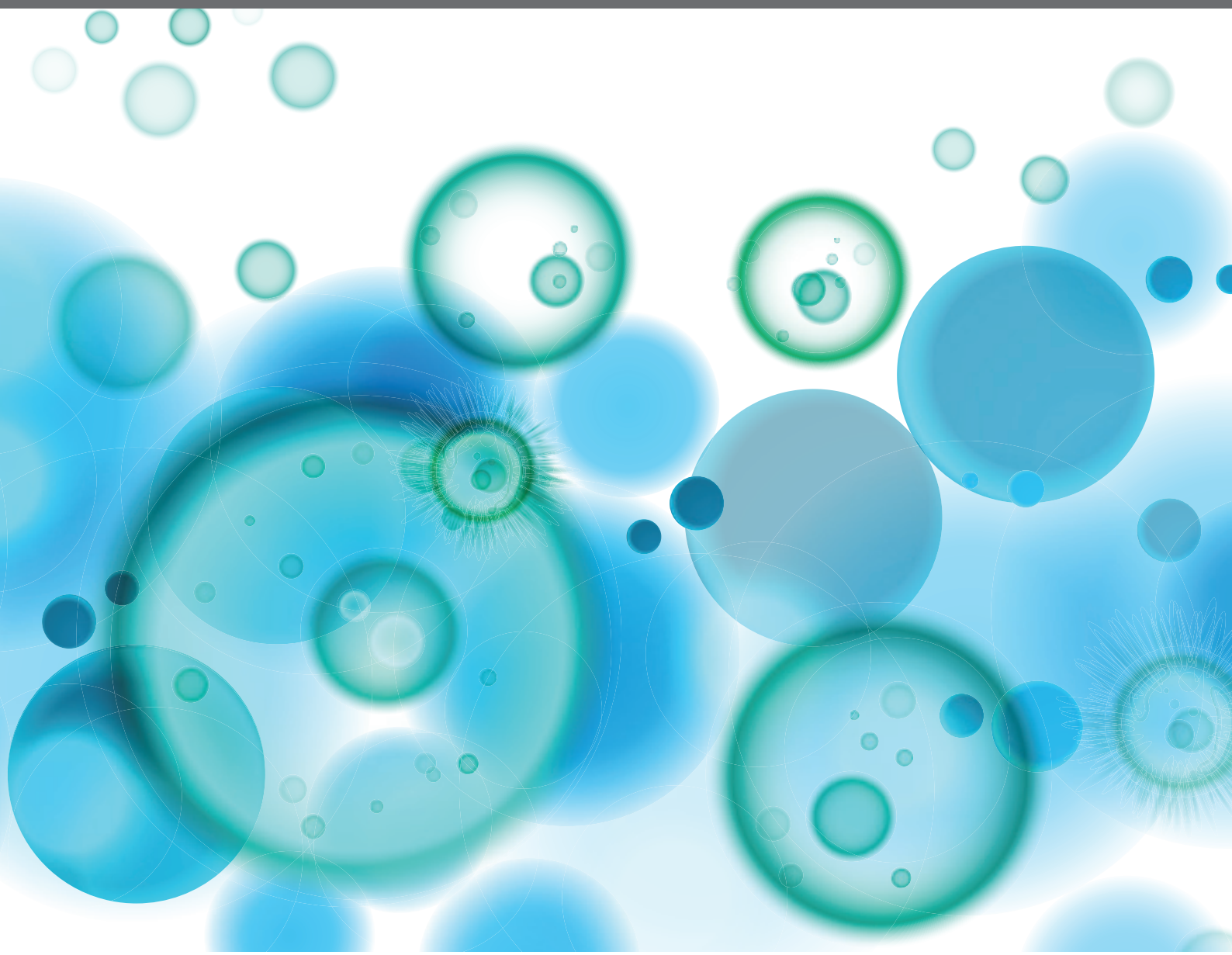

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ADVANCES IN PRIMARY IMMUNODEFICIENCIES IN INDIA

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EDITORIAL
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Editorial: Advances in Primary Immunodeficiencies in India

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Keywords: prenatal diagnosis, SCID, XLA, WAS, LAD, MSMD, HSCT, FLH

Editorial on the Research Topic

Advances in Primary Immunodeficiencies in India

Primary immunodeficiency diseases (PIDs) or inborn errors of immunity (IEI) are an ever-expanding universe of genetic defects of the immune system. In this editorial, we will be using PIDs and IEI interchangeably. In the last almost 70 years, since X-linked agammaglobulinemia (Bruton's agammaglobulinemia) was discovered by Col. Ogden Bruton, more than 460 IEI have been described and more than 400 mutated genes have been assigned to these disorders. This has been primarily due to advances in cellular and molecular technologies, including NGS, that are used to perform WES and GWS. We began with four categories of PIDs (Combined immune deficiency, Antibody deficiency, T cell deficiency, and Complement deficiency), and now IEI are classified into 10 different categories.

In India, the first description of PID was reported in two siblings with ataxia telangiectasia in 1975 exclusively on clinical presentation (1). Since then and until 2010, the majority of PIDs were described on clinical criteria and very little laboratory confirmation. In 2010, Sudhir Gupta established the Foundation for Primary Immunodeficiency Diseases (FPID; www.fpid.org) to improve awareness, provide education and training, and support treatment and research for PIDs in India. Currently, there are eight regional FPID diagnostic and Treatment Centers at eight different premier medical institutions spread all over India. Two of these centers are now designated as Center for Excellence in PIDs that are fully equipped with the capability of molecular diagnoses. In addition, there are several commercial laboratories, where gene mutation analyses are available. Increased awareness and availability of molecular diagnoses have resulted in increased referrals of patients suspected of IEI and therefore increased and early diagnosis. In the last 10 years, the landscape of IEI in India has changed dramatically. As recently as 10 years back, there were only two centers that were performing HSCT for PIDs. Currently, there are more than 15 center swhere HSCT for PIDs are actively performed. More than 250 scientific papers on IEI have been published from FPID centers. This special Research Article on "Advances in Primary Immunodeficiency Diseases in India" is a compilation of 11 articles presenting data on 1,539 patients with IEI and highlighting the progress made in the last decade in the diagnosis and treatment of PIDs. The 10 most common IEIs have been presented. Furthermore, the authors of these articles also emphasized financial, cultural, and institutional challenges that limit the diagnosis and therapy of patients with IEI.

India, unlike the US and some other Western countries, does not have a national newborn screening program. However, they are using prenatal diagnosis (PND) for IEI. Yadav et al. from the Madkaikar group discussed the PND services available and the economic, ethical, and cultural

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challenges associated with genetic counseling. Mutation detection in the index case and analysis of chorionic villous sampling or amniocentesis are the preferred procedures for PND and phenotypic analysis of cordocentesis sample is reserved for families with well-characterized index case seeking PND in the latter part of the second trimester of pregnancy. Of 112 families investigated for PID, PID was confirmed in 32 families. These included diagnoses of SCID, LAD, FHL, and CGD. Therefore, in families having a child affected with PID, genetic counseling and PND are the cornerstones of primary preventive management. Prenatal diagnosis provides a choice for the family to carry on or terminate the pregnancy. Needless to say, there are ethical issues involved.

There has been an exponential rise in the diagnosis of SCID. Vignesh et al. presented data on 254 patients with SCID; the molecular diagnosis was made in 192 patients, and, in the order of frequency, the mutations in genes included IL-2RG, RAG1, ADA, RAG2, JAK3, DCLRE1C, *IL7RA*, *PNP*, *RFXAP*, *CIITA*, *RFXANK*, *NHEJ1*, *CD3E*, *CD3D*, *RFX5*, *ZAP70*, *STK4*, *CORO1A*, *STIM1*, *PRKDC*, *AK2*, *DOCK2*, and *SP100*.

Rawat et al. presented a clinical and genetic profile of 137 patients with an established diagnosis of X-linked agammaglobulinemia. Missense variants in the *BTK* gene were the most common, followed by frameshift and nonsense variants. Most pathogenic gene variants were clustered in the distal part of the gene encompassing exons 14–19 encoding for the tyrosine kinase domain. There were delays in diagnosis because of the lack of availability of facilities for molecular diagnoses at several centers.

Missense variants are most commonly observed in patients with Wiskott Aldrich syndrome. Suri et al. presented clinical and molecular data from 81 patients with Wiskott-Aldrich syndrome (WAS). They reported 24 novel variants, most of these being frameshift and nonsense mutations resulting in premature termination of protein synthesis.

Saikia et al. reported seven novel STAT3 variants, including a rare linker domain nonsense variant and a CC domain variant in patients with Hyper IgE syndrome. Not surprisingly, because of endemic mycobacterial diseases in India, *Mycobacterial* diseases in HIES were more frequent compared to the Western world.

Clinical and molecular data on a large cohort of patients with chronic granulomatous disease (CGD) are presented (Rawat et al.). The proportion of patients with AR-CGD is higher as compared to Western cohorts; however, regional differences within India were observed. *CYBA* variants were documented only in Southern and Western parts of India; a common dinucleotide deletion in *NCF2* (c.835_836delAC) was noted only in the North Indian population.

Kambli et al. presented clinical and molecular data on a large cohort of 132 patients with leukocyte adhesion deficiency (LAD). Around 96% of patients were affected with LAD-1 and none with LAD-2. A total of 30 novel mutations were detected in the *ITGβ2* gene, and 4 novel mutations were detected in the *FERMT3* gene.

Clinical data on a large group of molecular-defined familial hemophagocytic lymphocytosis (FHL), and IEI characterized by immune dysregulation are reviewed (Shabrish et al.). The majority of patients were FHL-2 and FHL-3. Molecular characterization of respective genes revealed 76 different disease-causing mutations, including 51% novel mutations in the *PRF1, UNC13D, STX11*, and *STXBP2* genes.

Almost all types of autoinflammatory diseases are represented in the Indian population. Suri et al. presented clinical and molecular data on a large cohort of autoinflammatory disorders that included type 1 interferonopathies, diseases affecting inflammasomes, with non-inflammasome related conditions periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA). Type1 interferonopathies identified in the cohort included patients with deficiency of adenosine deaminase 2 (*DADA2)*, STING-associated vasculopathy with onset in infancy (SAVI), and spondyloenchondro-dysplasia with immune dysregulation (SPENCD). Diseases affecting inflammasomes included mevalonate kinase deficiency, cryopyrin-associated periodic syndromes (CAPS), NLRP12, familial mediterranean fever (FMF), autoinflammation, $PLCG_2$-associated antibody deficiency, and immune dysregulation (APLAID), TNF receptor-associated periodic syndrome (TRAPS), A20 haploinsufficiency, and deficiency of interleukin 1 receptor antagonist (DIRA).

Because of the practice of universal BCG vaccination, disseminated BCGosis is a common manifestation in certain PIDs. Taur et al. described BCGosis in more than 80% of patients with MSMD involving abnormalities of the IL-12/IL-23/ISG15/IFN-γ axis. Authors went on to suggest that all patients with BCGosis and suspected of MSMD, irrespective of age, should be investigated for abnormality of the IL-12/IL-23/ISG15/IFN-γ circuit.

During the last decade, the number of centers performing HSCT for PIDs has increased, and, therefore, the number of PID patients undergoing HSCT has exponentially increased. Raj et al. presented data on HSCT in 228 children with PIDs. The most common PIDs undergoing HSCT were SCID (25%) and HLH (25%). Others included WAS and CGD. In the last 5 years, the survival of children with PID undergoing HSCT in India has improved. HSCT protocols have been modified to reduce the cost without compromising survival that included doing away with T cell depletion.

India with a population of 1.3 billion is expected to have more than 1 million patients with IEI. In this compilation, data for just more than 1360 patients have been presented. Therefore, the prevalence of IEI in India is not known. However, with recent approval and funding of a national PID registry by the Indian Counsel for Medical Research, it should be possible. Although unprecedented advancements have been made in the diagnosis and treatment of patients with IEI in a short span of 10 years, a number of economical, ethical, and cultural challenges remain. India will need to continue to increase awareness, support education, training, and technology that would result in early and improved diagnosis and successful treatment. However, this would require a concerted effort between the Government of India, the State government, institutions, and NGOs. Recently, the Government of India in their National Rare Disease Policy has included PIDs. FPID is committed to continuing to provide

necessary academic, technical, political, and financial support for those with IEI.

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SG wrote major component of the Editorial. SS provided critical historical data of publications since 1975. All authors contributed to the article and approved the submitted version.

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Prenatal Diagnosis for Primary Immunodeficiency Disorders—An Overview of the Indian Scenario

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Prenatal Diagnosis (PND) forms an important part of primary preventive management for families having a child affected with primary immunodeficiency. Although individually sparse, collectively this group of genetic disorders represents a significant burden of disease. This paper discusses the prenatal services available for affected families at various centers across the country and the challenges and ethical considerations associated with genetic counseling. Mutation detection in the index case and analysis of chorionic villous sampling or amniocentesis remain the preferred procedures for PND and phenotypic analysis of cordocentesis sample is reserved for families with well-characterized index case seeking PND in the latter part of the second trimester of pregnancy. A total of 112 families were provided PND services in the last decade and the presence of an affected fetus was confirmed in 32 families. Post-test genetic counseling enabled the affected families to make an informed decision about the current pregnancy.

Keywords: prenatal diagnosis, chorionic villus sampling, maternal contamination, cordocentesis, flow cytometry, variants of unknown significance

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INTRODUCTION

Primary immunodeficiency disorders (PIDs) are a heterogeneous group of single-gene disorders of the immune system with more than 450 distinct PIDs described in literature. The overall prevalence rate may be as high as 1:1,000 to 1:5,000 in the general population (1). Despite an improvement in the understanding of the molecular basis of PIDs, its optimum management remains a challenge. Hematopoietic stem cell transplantation (HSCT) from a HLA matched sibling is an option available only to about 30% of cases (2). Other forms of supportive therapy like regular intravenous immunoglobulin transfusions and prophylactic antibiotics especially for patients with X-linked Agammaglobulinemia (XLA) and Chronic Granulomatous Disease (CGD) may result in long-term survival but are associated with a poor quality of life and a prohibitive cost. This poses a significant financial and emotional burden on the family and society as a whole. Therefore, in families having a child affected with PID, genetic counseling and PND are the cornerstones of primary preventive management. This paper highlights the PND services for PID available in India, the different techniques utilized, and their comparison, the challenges, and ethical considerations.

MATERIALS AND METHODS

Participant Details

We analyzed the PND services offered at eight centers across India to families with a known case of PID in the last decade. Clinical details of the index case, the underlying molecular defect in the index case, the technique used for PND, the result, and outcomes were studied.

Sample Collection Techniques and Sample Processing

Chorionic Villus Sampling

In CVS, fetal chorionic tissue was obtained by aspiration either by transabdominal or trans cervical route at 11–13 weeks' gestation. After aspiration, fetal villi were dissected from the decidual tissue, and then genetic testing was performed on the DNA extracted from the same.

Amniocentesis

In amniocentesis, exfoliated fetal cells in amniotic fluid were studied for molecular analysis. This was performed at 16–18 weeks' gestation. For samples collected by CVS or Amniocentesis, molecular analysis was performed by Sanger sequencing (for well-characterized mutation in the index case and parents) or Next generation sequencing. Maternal cell contamination (MCC) was ruled out by VNTR analysis.

Sampling of Fetal Blood or Cordocentesis

Cordocentesis was performed at 18–20 weeks of gestation in families in which the index case was immunophenotypically well-characterized and molecular analysis was not available. Also, situations where CVS or amniocentesis failed to provide a definite diagnosis, PND by cordocentesis was offered to affected families. The phenotypic evaluation comprised of lymphocyte subset analysis for Severe combined immunodeficiency (SCID), HLA-DR expression on lymphocytes for MHC class II deficiency, BTK expression on monocytes for XLA, CD18/CD11a integrin expression on leukocytes for Leukocyte adhesion defect (LAD1), and oxidative burst activity of fetal neutrophils by dihydrorhodamine assay for CGD. The interpretation of results was based on comparison with previously established reference ranges (3).

MCC was ruled out by Kleihauer–Betke test and confirmed by VNTR analysis. Before the aforementioned tests, another simple and rapid way for ruling out MCC in fetal blood was performed by checking high mean corpuscular volume (MCV) value (>110 fL) with a narrow, single red cell distribution curve before sample processing.

Ethical Approval

The study is approved by the Institutional Ethics Committee of ICMR-National Institute of Immunohaematology.

RESULT

Prenatal testing was performed in total of 112 affected families (n = 121 pregnancies). Of these, phenotypic prenatal diagnoses were performed in 44 and molecular analysis was performed in 77 cases. The year-wise number of prenatal tests conducted is depicted in **Figure 1**. Consanguinity was reported in 44% of the couples seeking PND.

The prenatal diagnostic techniques used for PND are summarized in **Figure 2**.

Prenatal diagnosis was most commonly sought for SCID, LAD, FHL and CGD. The diagnosis in the index case (proband) and the results of the investigations in the index cases and prenatal cases are summarized in **Figure 3** (**Supplementary Table 1**). Seven of the 112 families seeking PND had variants of unknown significance (VUS) identified in index case and parents.

Diagnosis following cordocentesis was offered to 43/44 families. In one family with LAD1(P42) in the index case, the diagnosis could not be offered due to significant MCC. Cord blood samples after delivery confirmed the accuracy of diagnosis in all but one case of LAD1(P4) in the child, and she expired at two months of age. Procedure-related complications were observed in two families—one pregnancy was aborted due to

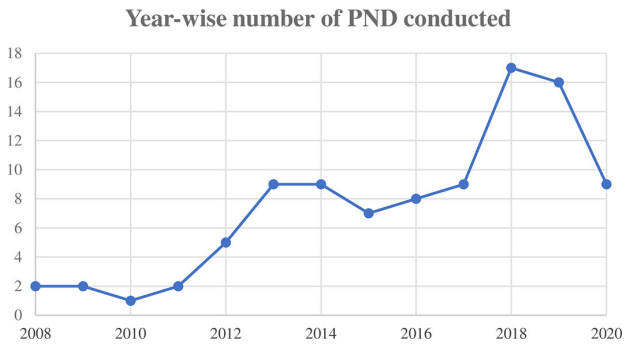


FIGURE 1 | Year-wise number of prenatal diagnosis (PND) conducted.

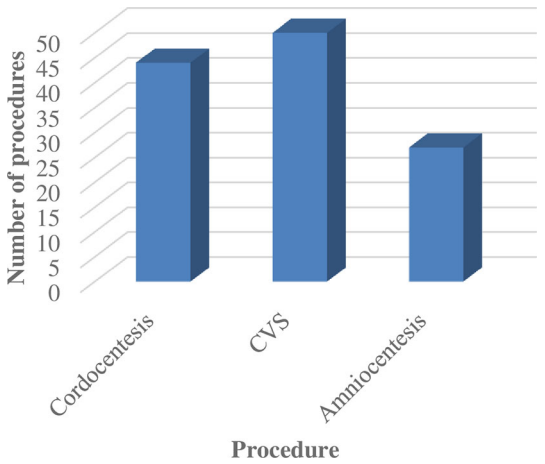


FIGURE 2 | Comparing the application of different prenatal diagnostic techniques.

infection and the second one due to rough travel soon after the cordocentesis procedure.

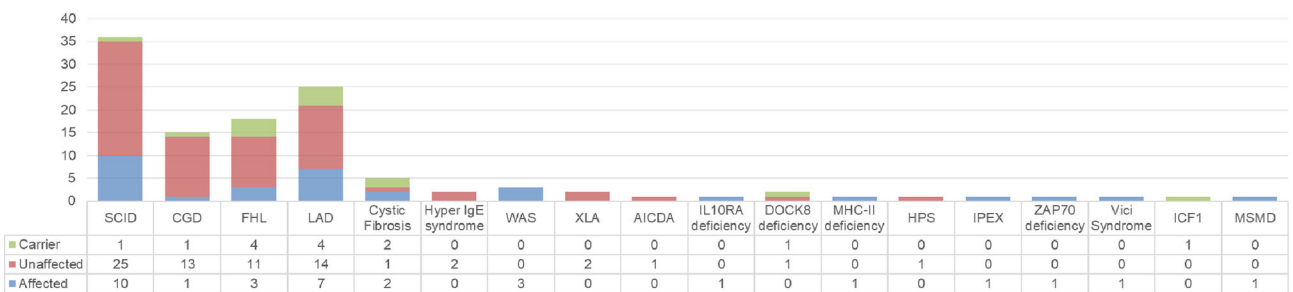
PND confirmed the presence of an affected fetus in 32 families, and 31/32 of these pregnancies were terminated; one family with a diagnosis of SCID decided to continue with the pregnancy, and it was confirmed after birth. Carrier status was detected in eight fetuses [one CGD family, one DOCK8 family, three LAD1, and one LAD3 family, one SCID family, and one Immunodeficiency, Centromeric instability, and Facial anomaly syndrome (ICF) family]; pregnancy was continued. In all cases with unaffected or carrier fetuses, the diagnosis was confirmed on cord blood samples after delivery and further follow-up of the children. Two such families are as yet on antenatal follow-up.

DISCUSSION

India has a significant collective burden of genetic disorders which can be attributed to a high rate of consanguinity, which is generally observed in approximately 14% marriages according to the National Family Health Survey (NFHS-4) report 2015–16 (4–6). In our cohort, consanguinity was reported by 44% of the total couples seeking PND. 33% of fetuses among consanguineous marriages were found to be affected compared to 22% affected fetuses in non-consanguineous marriages.

These services are commonly offered for various genetic disorders including hemoglobinopathies and Down's syndrome in India. The rising trend of PND sought for PID over the last decade shows an increasing awareness about the availability of these services in affected families and about PIDs itself. PND services including molecular diagnoses are being offered by some genetic centers both in the public and private sectors.

CVS was the technique most commonly deployed for sampling in our cohort followed by cordocentesis and amniocentesis. Mutation detection in the index case and analysis of CVS or amniocentesis remain the preferred sampling procedures for PND due to high sensitivity and specificity and allow a sufficient window for safe termination of pregnancy wherever required depending on the test results (7). As far as the ethics, the psychological distress and the health risk to the mother are concerned; the earlier the screening is performed, the better. In addition to the timing factor, the risk to the mother or fetus of the invasive techniques and the accessibility or cost of the techniques are important factors for consideration (8). The most dreaded complication after these invasive sampling procedures is fetal loss. The incidence of fetal loss subsequent to the procedure is highest with cordocentesis (1.4%), and comparable between CVS (0.2%) and amniocentesis (0.3%). Lower fetal losses are documented for centers having experience in performing these procedures. A higher number of attempts, presence of fetal structural abnormalities, underlying placental disorders, uterine malformations, and fibroids are associated with increased chances of fetal loss following the procedure (9, 10). The high risk of fetal loss with cordocentesis as reported in literature was consistent with our observation of fetal loss after two cordocentesis procedures. However, since most couples in India report for prenatal testing in the latter part of the second trimester (11), a phenotypic diagnosis does have a role, and cordocentesis is reserved only for such families. Due to the higher risk of fetal loss associated with cordocentesis, there is a need to stress upon reporting early in the next pregnancy for PND when genetic counseling is offered after diagnosis in the index case.



	SCID	CGD	FHL	LAD	Cystic Fibrosis	Hyper IgE syndrome	WAS	XLA	AICDA	IL10RA deficiency	DOCK8 deficiency	MHC-II deficiency	HPS	IPEX	ZAP70 deficiency	Vici Syndrome	ICF1	MSMD
Carrier	1	1	4	4	2	0	0	0	0	0	1	0	0	0	0	0	1	0
Unaffected	25	13	11	14	1	2	0	2	1	0	1	0	1	0	0	0	0	0
Affected	10	1	3	7	2	0	3	0	0	1	0	1	0	1	1	1	0	1

FIGURE 3 | Summary of diagnosis in index case (n) and the result of PND (affected, unaffected, carrier).

Cordocentesis followed by phenotypic analysis using flow cytometry is a sensitive, reproducible, and rapid technique for PND especially for patients seeking prenatal testing beyond 18 weeks of gestation as the results can be made available within 24 h of the procedure. We have established at our center, reference ranges on cordocentesis samples at 18 weeks of gestation as described in a previous paper (3, 12). The technique has an added advantage in detecting an affected fetus in the situation of partial maternal contamination, thereby precluding the need for a repeat procedure. However, phenotypic analysis has a few limitations (13, 14) as observed in one LAD1 family where the child was found to be affected at birth. As such, confirming the surface expression of a molecule may be misleading as the protein expression though normal might have a defective function and specifically for LAD1, checking for expression of CD11a in addition to CD18 becomes important as it is consistently abnormal in all patients with LAD1, whereas CD18 expression may be variable or functionally abnormal despite normal expression.

The presence of MCC in fetal samples can lead to prenatal misdiagnosis and must be ruled out in all PND. American College of Medical Genetics and Clinical Molecular Genetics Society have laid standards and guidelines for cytogenetic and molecular genetic testing that recommend MCC testing in prenatal diagnosis. For cordocentesis, looking at Kleihauer–Betke test and MCV value are quick methods to rule out MCC although confirmation by variable number tandem repeat (VNTR) remains the gold standard technique (15).

Next generation sequencing is being increasingly offered for the detection of mutation during PND (16, 17) especially in cases where a mutation is not identified in the index case. Since mutations in the Indian population are not well characterized, many pathogenic variants also get labeled as VUS which presents a challenge for genetic counseling. In our cohort, seven such families who had sought PND had VUS identified in the index case and parents. Once a VUS is identified, it needs to be correlated with the phenotype in the index case. All efforts should be made that the decision is made with a full understanding of the findings and its clinical relevance (18).

Utilization of non-invasive prenatal diagnosis through circulating cell-free fetal DNA (cffDNA) analysis in maternal circulation (19, 20) is being explored as it nullifies the risk of miscarriages, reduces the cost of the procedure, and may be utilized early in gestation. cffDNA is highly fragmented in nature (fragments of length <300 bp) and smaller than maternally derived sequences and the levels increase with increasing gestation (21). The quantitation of cffDNA is subject to variation in the gestational age, delay in processing time, extraction method used, and sensitivity of the detection technique. Currently, NIPT is mainly used to detect aneuploidy, fetal Rh D typing, X-linked genetic diseases, and some single gene inheritance diseases including Thalassemia (22) and has limited application in PID.

The provision of PND services benefits the high-risk family in making a decision about the current pregnancy. In the situation where the family makes an informed decision to continue with the pregnancy even though it would lead to the birth of an affected child, knowing the genetic diagnosis can lead to early intervention after birth. Affected infants diagnosed with SCID as neonates may have a better survival due to timely HSCT than those in whom disease detection is delayed.

On one hand, information obtained through genetic testing can be empowering in reproductive decision making, while on the other hand, the influence of this knowledge may result in psychological harm, stigmatization, and discrimination (23). To enhance decision making by a patient, ethical considerations include pretest counseling which should provide accurate information about the testing procedure and the risks involved, including the possibility of ambiguous results. Both the medical and social consequences of the proposed tests and their results should be discussed (24). Genetic counseling also has an important bearing in revealing carrier status to a mother in case of diagnosis of an X-linked condition in the index case as it can seriously affect her status within the family. This raises important concerns regarding the rights of individual privacy and familial privacy, and what information others outside the family can have access to especially biological relatives who also might be affected (25). Ethical concerns regarding genetic counseling also become especially important in the event of medically actionable incidental findings in parents and VUS in the fetus (26). Another ethical concern in that a substantial proportion of people in certain communities do not consider abortion as a morally acceptable option even in face of an affected pregnancy.

This study highlights the increasing awareness about PIDs and the knowledge about the availability of PND services for preventing the birth of an affected child. However, the uptake of these services is not yet sufficient to address the needs of all affected families. Although these conditions are amenable to diagnosis in the first trimester by analysis of CVS specimens, at present most are diagnosed by analyzing fetal blood taken at 18–20 weeks' gestation, and hence the need to counsel the affected families for seeking timely PND services.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ICMR-National Institute of Immunohaematology. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RMY compiled the data, wrote and edited the manuscript. Standardization of phenotypic analysis was done by MG, SSha, and AD. Molecular testing was done by GH, JA, MK, PKam, SSes,

SJ, BS, JR, MS, PG, SMah, PKad, SMit, AM, KT. RR, RU, UB, PTau, HL, PTam, VT, SB, PJ, GG, BG, and MD provided the clinical details, provided genetic counseling, and followed up the patients. MM supervised the study, reviewed and approved the final version of the manuscript. 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.2020.612316/full#supplementary-material

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Conflict of Interest: SMal, PG, SMah, and PKad were employed by the company MedGenome Labs Private Limited. SJ, BS, and JR were employed by the company Mediscan Systems.

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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Clinical, Immunological, and Molecular Features of Severe Combined Immune Deficiency: A Multi-Institutional Experience From India

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Background: Severe Combined Immune Deficiency (SCID) is an inherited defect in lymphocyte development and function that results in life-threatening opportunistic infections in early infancy. Data on SCID from developing countries are scarce.

Objective: To describe clinical and laboratory features of SCID diagnosed at immunology centers across India.

Methods: A detailed case proforma in an Excel format was prepared by one of the authors (PV) and was sent to centers in India that care for patients with primary

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immunodeficiency diseases. We collated clinical, laboratory, and molecular details of patients with clinical profile suggestive of SCID and their outcomes. Twelve (12) centers provided necessary details which were then compiled and analyzed. Diagnosis of SCID/combined immune deficiency (CID) was based on 2018 European Society for Immunodeficiencies working definition for SCID.

Results: We obtained data on 277 children; 254 were categorized as SCID and 23 as CID. Male-female ratio was 196:81. Median (inter-quartile range) age of onset of clinical symptoms and diagnosis was 2.5 months (1, 5) and 5 months (3.5, 8), respectively. Molecular diagnosis was obtained in 162 patients - *IL2RG* (36), *RAG1* (26), *ADA* (19), *RAG2* (17), *JAK3* (15), *DCLRE1C* (13), *IL7RA* (9), *PNP* (3), *RFXAP* (3), *CIITA* (2), *RFXANK* (2), *NHEJ1* (2), *CD3E* (2), *CD3D* (2), *RFX5* (2), *ZAP70* (2), *STK4* (1), *CORO1A* (1), *STIM1* (1), *PRKDC* (1), *AK2* (1), *DOCK2* (1), and *SP100* (1). Only 23 children (8.3%) received hematopoietic stem cell transplantation (HSCT). Of these, 11 are doing well post-HSCT. Mortality was recorded in 210 children (75.8%).

Conclusion: We document an exponential rise in number of cases diagnosed to have SCID over the last 10 years, probably as a result of increasing awareness and improvement in diagnostic facilities at various centers in India. We suspect that these numbers are just the tip of the iceberg. Majority of patients with SCID in India are probably not being recognized and diagnosed at present. Newborn screening for SCID is the need of the hour. Easy access to pediatric HSCT services would ensure that these patients are offered HSCT at an early age.

Keywords: severe combined immune deficiency, India, hematopoietic stem cell transplantation, newborn screening, BCG

INTRODUCTION

Severe Combined Immune Deficiency (SCID) is an inborn error of immunity characterized by defect in T lymphocyte development and function. Children with SCID often develop life-threatening opportunistic fungal, bacterial, or viral infections in early infancy. SCID is considered a medical emergency and affected children often succumb to severe infections if diagnosis and definitive treatment are delayed. The estimated incidence of SCID is 1 in 50,000 to 100,000 live births (1). Recent data also suggest an incidence of SCID as high as 1 in 3,000 live births in countries with high consanguinity rates (2). However, due to lack of awareness and diagnostic facilities in developing countries, diagnosis is often missed. Hematopoietic stem cell transplantation (HSCT) is the definitive management for SCID. Early diagnosis and management are essential for successful outcomes. Several countries such as United States of America, Israel, Germany, Switzerland, Sweden, Norway, Iceland, New Zealand, and Taiwan have initiated newborn screening for SCID based on quantification of T-cell receptor excision circles (TRECs) to facilitate early diagnosis (3).

Opportunistic infections in SCID are recurrent, typically start in early infancy, and result in failure to thrive. Common infection patterns seen in SCID include oral thrush, disseminated BCGosis, disseminated cytomegalovirus, and life-threatening bacterial and fungal infections. Non-infective manifestations of SCID include Omenn syndrome (OS), graft versus host reaction, autoimmunity, and hemophagocytic lymphohistiocytosis (4). CD3+ T lymphocyte numbers are usually decreased in SCID (T-). However, in cases of maternal T-cell engraftment or OS, CD3+ T cell numbers can be normal or increased. The expanded T cells are autoreactive in OS, whereas, they are alloreactive in cases with transplacental-acquired maternal T-cell engraftment. T lymphocyte function and naïve T cell numbers are reduced in such cases. T- SCID can be classified based on presence or absence of B lymphocytes and natural killer cells as T-B-NK+, T-B-NK-, T-B+NK-, and T-B+NK+. Combined immunodeficiencies (CID) are also characterized by presence of opportunistic infections and immune dysregulation; however, the age of onset is little older and have a milder immunodeficiency compared to SCID (5).

Until date, 58 different monogenic defects have been identified to result in immunodeficiencies affecting both cellular and humoral immunity and 18 amongst these are known to result in SCID (5). Molecular defects in SCID can be broadly classified as abnormalities in VDJ recombination (*RAG1, RAG2*, *DCLRE1C, NHEJ1, LIG4, PRKDC*), abnormalities of cytokine signaling (*IL2RG, JAK3, IL7RA*), toxic metabolite accumulation (*ADA, PNP*), defective survival of hematopoietic precursors (*AK2, RAC2*), abnormalities of T-cell receptor and signaling (*PTPRC, CD3D, CD3E, CD3Z, LAT*), and abnormalities of actin cytoskeleton (*CORO1A*). While X-linked SCID due to

defect in *IL2RG* is considered to be the commonest form of SCID in the US, Canada, and Europe, autosomal recessive form of SCID due to defects in *RAG1/2* are the commonest forms of SCID in countries where consanguinity rates are high (6–8). However, after initiation of newborn screening program, defects in *RAG1/RAG2* are now increasingly being identified even in countries like US and Canada where consanguinity rates are low (9).

Reports of clinical data and outcomes of SCID from developing nations are scarce. Being a tropical nation with universal coverage of BCG vaccination in newborns, microbiological pattern of infections in SCID in India is expected to be different from other cohorts. Molecular spectrum is also expected to be different considering high rates of consanguinity and endogamous marriages in India (6–8). A recent cohort of 57 patients from Mumbai, India showed a high incidence of autosomal recessive forms of SCID with *RAG1/2* defects being the commonest (7). We aim to describe the clinical, immunological, and molecular features of children with SCID in this large multicentric cohort from India.

METHODS

A detailed case proforma in an Excel format was prepared by one of the authors (PV) and was sent to centers that are recognized as Foundation for Primary Immunodeficiency Diseases (FPID) centers for care of primary immunodeficiencies in India. The format was also sent to tertiary-care centers that manage patients with primary immunodeficiency diseases (PIDs). Information on clinical, laboratory, and molecular details of patients with SCID and their outcomes was sought and collated. Twelve (12) centers provided details of 319 patients that were then compiled and analyzed. Fifteen (15) patients from 2 other centers with either flow-cytometry or mutation-proven SCID are not included in final analysis as data were incomplete. Twenty-three (23) children did not fulfil the criteria for clinical definition for SCID and were not included for analysis. Duplicate entries (n=4) were also noted and excluded.

Data of 277 children who had a clinical profile suggestive of SCID were taken for final analysis (**Supplementary Table 1**). Children were categorized as SCID/OS/CID/atypical SCID as per the European Society for Immunodeficiencies (ESID) working definition (10). Three (3) patients were classified as possible SCID as they did not fulfil the complete ESID definition, however, the treating team had a high index of suspicion based on clinical and immunological features (**Table 1**).

Clinical profile of all patients was obtained along with family history and other demographic details. Clinical features included number of infections, type of infections, site of infections, organism involved, age of presentation, age of onset, presence of skin rash, BCG ulceration, history of administration of vaccines and complications, if any. Basic hematological, biochemistry, and immunological investigations including immunoglobulin profile and lymphocyte subsets were also recorded.

Analysis of lymphocyte subsets by flow cytometry had been carried out in most patients. Methodology for laboratory assay of lymphocyte subsets, naïve, memory T cells, HLA-DR expression, CD132 expression, CD127 expression, and lymphocyte proliferation assays at Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh and National Institute of Immunohematology (NIIH), Mumbai have been previously described (11, 12). Other centers performed conventional lymphocyte subsets (CD3, CD19, CD4, CD8, CD56) by flow cytometry in private laboratories.

Adenosine deaminase (ADA) levels and percentage of deoxyadenosine nucleotides (%dAXP) from dried blood filter paper spot were assayed at Duke University, North Carolina for patients with ADA deficiency SCID who were diagnosed at PGIMER, Chandigarh.

Molecular Assays

Before the facility for in-house next-generation sequencing was made available in 2018, centre at PGIMER, Chandigarh had established academic collaborations with centers at Hong Kong (The University of Hong Kong), Japan (Kazusa DNA Research Institute, Kisarazu, Chiba; National Defense Medical College, Saitama), and USA (Duke University, North Carolina) for molecular work-up of patients. The centre at Hong Kong provided final molecular diagnosis for 12 patients (Pt. 8-10, Pt. 14-19, Pt. 21, Pt. 50-51) (**Table 1**). Molecular diagnosis for 4 patients was established at Kazusa DNA Research Institute, Japan (Pt. 3–6). Thirty-four (34) patients (Pt. 59–90, pt. 119, pt. 127) with SCID were worked-up for molecular diagnosis using NGS at National Defense Medical College, Saitama and Tokyo Medical and Dental University, Tokyo, Japan (Kato T et al. manuscript in submission). Final molecular diagnosis of a patient with *ADA* defect (pt. 22) was also established at Duke University, North Carolina.

Sanger sequencing for *IL2RG* and *RAG1/2* genes were initiated at PGIMER, Chandigarh (North India) in 2016. Sanger sequencing for patients with SCID at NIIH, Mumbai (West India) was previously described by Aluri et al. (7). Methodology for NGS at Christian Medical College, Vellore (South India) was described previously (13).

Next-Generation Sequencing (NGS) at PGIMER, Chandigarh

Next-generation sequencing (Ion Torrent, Thermo Fisher Scientific India Pvt. Ltd.) for clinical care was started in July 2018 at the Advanced Pediatrics Centre, PGIMER, Chandigarh. A targeted PID gene panel comprising 44 genes was used that covered 6 genes for SCID—*ADA*, *RAG1*, *RAG2*, *IL2RG*, *IL7RA*, and *LIG4*. Preparation of DNA target amplification reaction using 2-primer pools, amplification of target, combination of target amplification reactions, ligating adaptors to the amplicons and their purification was carried out as per the manufacturer's protocol using Ion AmpliSeq™ Library kit plus (Catalog numbers 4488990, A35121 A31133, A31136, A29751, 4479790). Amplified library was quantified using Qubit™ 2.0 fluorometer instrument. Dilution that results in a concentration of ~100pm was then determined. Template preparation on Ion

TABLE 1 | Clinical and immunological features of children with clinical features suggestive of SCID in our cohort.

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 1	8 months/ male	Recurrent episodes of diarrhea, failure to thrive, pneumonia, meningitis	Stool: *Clostridium difficile* toxin assay positive	2.260	IgG <1.64 g/L IgA <0.36 g/L IgM- 0.25 g/L	CD3- 0.3% (No: 6-7) CD19- 66% (No: 1492) CD56- 30% (No: 675)	Not done	SCID
Pt. 2	5 months/ male	Recurrent episodes of pneumonia, diarrhea, failure to thrive, elder male sibling expired at 6 months due to severe infections	Blood culture: *Alcaligens faecalis*	0.410	IgG <2.26 g/L IgA <0.1 g/L IgM<0.2 g/L	CD3- 0.15% (No: 0-1) CD19- 0% (No: 0) CD56- 84% (No: 345)	Not done	SCID
Pt. 3	6.5 months/ male	Recurrent episodes of pneumonia, meningitis, hepatosplenomegaly, pancytopenia, transaminitis (HLH), 3 elder male siblings died at early infancy due to recurrent infections	Blood culture: *Pseudomonas aeruginosa* Disseminated BCGosis and angioinvasive aspergillosis in lungs in autopsy	0.940	IgG<2.99 g/L IgA- 0.49 g/L IgM- 0.88 g/L	CD3- 0% CD19- 86% (808) CD56- 0.3%	*IL2RG*	SCID
Pt. 4	5 months/ male	2 episodes of pneumonia, recurrent diarrhea, umbilical sepsis, failure to thrive, 3 elder male siblings died at early infancy due to recurrent infections	N.A.	2.050	IgG- 2.64 g/L IgA <0.46 g/L IgM- 0.18 g/L	CD3- 0% CD19- 96.1% (1968) CD56- 0%	*IL2RG*	SCID
Pt. 5	3 months/ male	Erythroderma, generalized adenopathy, diarrhea, lymphocytosis, eosinophilia (Omenn syndrome), failure to thrive, elder male sibling died due to eczema and pneumonia at 3rd month	N.A.	18.540	N.A.	CD3- 70.94% (13,124) CD19- 0.1% CD56- 7% (1,295)	*RAG2*	Omenn syndrome
Pt. 6	6 months/ male	Persistent pneumonia, oral thrush, 7 maternal uncles died at early infancy due to recurrent infections	N.A.	2.322	IgG- 0.65 g/L IgA- 0.22 g/L IgM- 0.24 g/L	CD3- 0% CD19- 96.75% (2,245) CD56- 3.2% (74)	*IL2RG*	SCID
Pt. 7	3 months/ male	Recurrent episodes of pneumonia, diarrhea, meningitis, generalized erythroderma (incomplete Omenn), elder male sibling died at early infancy due to rash and pneumonia	N.A.	1.566	IgG- 2.14 g/L IgM- 0.24 g/L	CD3- 74.79% (1167) CD19- 0.27% (42) CD56- 23% (360) CD3+45RA+ 45RO-: 18.65% compared to 82% in control	Not done	Omenn syndrome
Pt. 8	10 months/ male	Recurrent episodes of diarrhea, pneumonia, otitis media, failure to thrive, BCG site ulceration, hepatosplenomegaly, generalized adenopathy, erythroderma, eosinophilia (Omenn syndrome), 5 maternal uncles died at early infancy due to recurrent infections	Disseminated BCGosis, disseminated *Mycobacterium avium*, disseminated CMV, and Aspergillus pneumonia in autopsy	3.600	IgG- 1.04 g/L IgA- 0.07 g/L IgM- 0.31 g/L IgE- 700 U/L (Normal: 0.-6 U/L)	CD3- 95.79% (3,448) CD19- 0.2% (7) CD56- 1% (36)	*IL2RG*	Omenn syndrome
Pt. 9	2 months/ female	Recurrent episodes of oral thrush, failure to thrive, 1 elder male sibling expired due to sepsis in early infancy	N.A.	0.648	IgG- 2.72 g/L IgA- 0.09 g/L IgM- 0.41 g/L	CD3- 1.1% (7) CD19- 0.2% (1) CD56- 93.6% (607)	*DCLRE1C*	SCID
Pt. 10	3 months/ male	Recurrent episodes of pneumonia, diarrhea, rickets, nephrocalcinosis, distal renal tubular acidosis, oral thrush, failure to thrive	N.A.	0.896	IgG- 0.88 g/L IgA <0.06 g/L IgM- 0.19 g/L	CD3- 1.1% (10) CD19- 92.2% (8,26)	*IL7RA*	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
						CD56- 6.4% (57)		
Pt. 11	6 months/ male	Pustulosis, hepatosplenomegaly, BCG site ulceration, transfusion-associated GVHD, elder male sibling died at 5 months due to pneumonia	Disseminated BCGosis, Blood culture: *Enterobacter* sp.	1.462	N.A.	CD3- 1.25% (183) CD19- 95% (1389) CD56- 0.45% (6-7)	No gene variants found in *IL2RG, JAK3, RAG1, RAG2*	SCID
Pt. 12	4 months/ male	Recurrent episodes of pneumonia, diarrhea, failure to thrive, meningitis, oral thrush, hepatosplenomegaly, rash, eosinophilia (Omenn phenotype), one elder female sibling expired in early infancy	*Pneumocystis jirovecii* pneumonia, disseminated CMV in autopsy	4.176	IgG- 2.06 g/L IgA- 0.08 g/L IgM- 0.41 g/L	CD3- 71.6% (2,993) CD19- 1.0% (42) CD56- 12% (504) CD3+45RO-45RA+: 24% as compared to 82% in control	Not done	Omenn syndrome
Pt. 13	6 months/ male	Persistent pneumonia, diarrhea, oral thrush, erythematous rash, hepatosplenomegaly (incomplete Omenn), nephrotic range proteinuria, two elder siblings (one male and other female) expired in early infancy	Blood culture: *Acinetobacter* sp.; Pneumonia and meningitis due to *Aspergillus* sp. and ventriculitis due to CMV in autopsy	1.404	IgG- 2.46 g/L IgA- 0.37 g/L IgM- 1.38 g/L	CD3- 93.7% (1,312) CD19- 0.2% (3) CD56- 5.6% (78)	Not done	Omenn syndrome
Pt. 14	7 months/ male	Recurrent pneumonia, failure to thrive, oral thrush	Endotracheal aspirate: *Klebsiella* sp.; RSV pneumonia and disseminated CMV in autopsy	0.156	IgG- 7.86 g/L IgA- 0.61 g/L IgM <0.11 g/L	CD3- 45.7% (72) CD19- 1.6% (2-3) CD56- 21.7% (34)	*PNP*	SCID
Pt. 15	6 months/ male	Recurrent episodes of pneumonia, failure to thrive, 5 elder siblings died at early infancy	N.A.	1.391	IgG <0.93 g/L IgA <0.16 g/L IgM <0.11 g/L	CD3- 0% CD19- 0% CD56- 92.2% (1,291)	*RAG2*	SCID
Pt. 16	6 months/ female	Recurrent pneumonia, diarrhea, failure to thrive, hepatosplenomegaly	Blood culture: *Candida* sp.	0.785	IgG- 6.33 g/L IgA- 0.07 g/L IgM <0.11 g/L	CD3- 0.2% (1-2) CD19- 38.9% (312) CD56- 52.2% (407)	*IL7RA*	SCID
Pt. 17	4 months/ male	Recurrent pneumonia, pus discharging sinuses in neck, generalized rash (incomplete Omenn), 3 elder siblings (one female and 2 male) died in early infancy	CMV PCR+, Blood culture: *Enterococcus cloacae*	1.800	IgG <0.95 g/L IgA <0.17 g/L IgM- 0.12 g/L	CD3- 83.3% (1,499) CD19- 0.2% (3-4) CD56- 14.3% (257) CD3+45RO-45RA+: 27.5% compared to 82% in control	*RAG2*	Omenn syndrome
Pt. 18	9 months/ male	2 episodes of pneumonia, failure to thrive, meningoencephalitis and hydrocephalus, MRI Brain: multiple tuberculomas noted over parietal and occipital area, 2 elder male siblings expired in early infancy	N.A.	0.612	IgG <0.95 g/L IgA <0.17 g/L IgM <0.15 g/L	CD3- 4.2% CD19- 0.2% CD56- 85%	*RAG1*	SCID
Pt. 19	2 months/ male	Recurrent episodes of pneumonia, failure to thrive	N.A.	0.655	IgG- 2.02 g/L IgA <0.16 g/L IgM <0.11 g/L	CD3- 0% CD19- 0.13% (1) CD56- 72% (468)	*RAG1*	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 20	5 months/ male	Generalized rash, alopecia, loose stools (incomplete Omenn), failure to thrive, meningitis	N.A.	1.372	IgA <0.17 g/L IgM <0.12 g/L	CD3- 69.6% (954) CD19- 0.15% (2) CD56- 10.7% (147)	*DCLRE1C*	Omenn syndrome
Pt. 21	5 months/ male	Younger sibling of Pt. 15, recurrent episodes of pneumonia, diarrhea, failure to thrive	CMV DNA PCR positive	0.480	IgG <0.94 g/L IgA- 0.18 g/L IgM <0.12 g/L	CD3- 21.7% (109) CD19- 1% (5) CD56- 86% (430)	*RAG2*	SCID
Pt. 22	1.5 months/ male	Persistent pneumonia, diarrhea, elder female sibling expired at early infancy	Blood culture: *Candida* sp.	0.328	IgG <2.02 g/L IgA <0.17 g/L	CD3- 75% (248) CD19- 8.3% (27) CD56- 7.1% (23)	*ADA*	SCID
Pt. 23	5 months/ male	Oral thrush, pneumonia, meningitis, one elder female sibling expired due to anemia and pneumonia in early infancy	Disseminated CMV and early invasive pulmonary aspergillosis in autopsy	0.788	IgG- 2.49 g/L	CD3- 0.79% (6) CD19- 1.02% (8) CD56- 92.7% (744)	*RAG1*	SCID
Pt. 24	2 years/ male	Recurrent pneumonia, diarrhea, otitis media, failure to thrive, esophageal candidiasis	N.A.	8.567	IgG- 5.19 g/L IgA <0.17 g/L IgM- 0.85 g/L	CD3- 25.76% (2,236) CD3+CD4+- 33.5% (737) CD3+CD8+- 50.2% (1,104) CD19- 51.95% (4,451) CD56- 11.6% (994) CD3+45RA+ 45RO-: 31.7% compared to 74% in control	No gene variants found	CID
Pt. 25	4 months/ male	Younger sibling of pt. 8, recurrent episodes of pneumonia and diarrhea, failure to thrive	N.A.	5.280	IgG <2.05 g/L IgM <0.25 g/L	CD3- 0.23% (12) CD19- 94.6% (4,995) CD56- 0.47% (25)	*IL2RG*	SCID
Pt. 26	10 months/ male	Recurrent episodes of pneumonia, failure to thrive	N.A.	0.378	N.A.	CD3- 2.7% CD19- 2.15% CD56- 85.2%	*DCLRE1C*	SCID
Pt. 27	2.5 months/ female	Recurrent pneumonia, otitis media, oral thrush, diarrhea, erythroderma, hepatosplenomegaly, eosinophilia (incomplete Omenn syndrome), elder female sibling expired in early infancy	N.A.	1.650	IgG- 1.23 g/L IgA <0.17 g/L IgM <0.25 g/L	CD3- 7.67% (127) CD19- 0.69% (11) CD56- 82.7% (1,365) CD3+45RA+ 45RO-: 6.42% compared to 72% of control	*RAG2*	SCID/ Omenn syndrome
Pt. 28	5 months/ male	Recurrent episodes of pneumonia, failure to thrive	BAL: *Pseudomonas* sp.; Blood culture: *Candida* sp.	0.360	IgG <2.05 g/L IgA- 0.07 g/L IgM <0.05 g/L	CD3- 2.3% (8) CD19- 3.8% (14) CD56- 92.2% (332)	*RAG1*	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 29	8 months/ female	Persistent pneumonia, recurrent episodes of diarrhea, failure to thrive, chorioretinitis, hepatosplenomegaly	Disseminated CMV; Blood culture: *Acinetobacter baumanii*	1.316	IgG- 4.17 g/L IgA- 0.22 g/L	CD3- 11.3% (149) CD19- 69.8% (921) CD56- 1.75% (23)	*JAK3*	SCID
Pt. 30	1.5 months/ male	Recurrent episodes of pneumonia, diarrhea, failure to thrive, elder male sibling died in early infancy	Blood culture: *Acinetobacter baumanii*	0.204	IgG- 1.96 g/L IgM <0.25 g/L	CD3- 54% (108) CD19- 24% (48) CD56- 20% (40)	*ADA*	SCID
Pt. 31	4 years/ male	Recurrent episodes of pneumonia since early infancy, failure to thrive	N.A.	0.116	IgG- 4.73 g/L IgA- 1.05 g/L IgM- 1.12 g/L	CD3- 64.8% (78) CD19- 4% (5) CD56- 7% (8-9)	*ADA*	Atypical SCID
Pt. 32	9 months/ male	Recurrent episodes of pneumonia, diarrhea, failure to thrive	N.A.	0.154	IgG- 2.27 g/L IgA- 0.27 g/L IgM <0.25 g/L	CD3- 44.4% (67) CD19- 38.5% (58) CD56- 5.7% (9)	Not done	SCID
Pt. 33	2 months/ female	Recurrent episodes of pneumonia, diarrhea, failure to thrive	N.A.	0.977	IgG- 2.45 g/L IgA- 0.23 g/L IgM- 0.29 g/L	CD3- 32% (314) CD19- 57% (559) CD56- 1.2% (12)	Not done	SCID
Pt. 34	5 months/ male	Recurrent diarrhea, failure to thrive, BCG site ulceration, pneumonia, erythroderma, eosinophilia, alopecia (Omenn syndrome)	Blood culture: Enterococcus sp.; *Pneumocystis jirovecii* pneumonia and disseminated BCGosis in autopsy	2.498	IgG <2.05 g/L IgM- 0.34 g/L IgE- 369 kU/L (up to 7.3)	CD3- 78.01% (1,950) CD19- 4.44% (110) CD56- 13.12% (325) CD3+45RA+ RO-: 2.26% compared to 83.7% in control CD3+HLA-DR+: 86.25% compared to 8.6% in control	No gene variants found	Omenn syndrome
Pt. 35	6 months/ male	Recurrent pneumonia, failure to thrive, elder male sibling expired in early infancy due to pneumonia	N.A.	0.868	N.A.	CD3- 3% (26) CD19- 94% (818) CD56- 0.4% (3)	*IL2RG*	SCID
Pt. 36	1.5 months/ female	Anasarca, nephrotic range proteinuria, pneumonia, failure to thrive, erythematous rash (incomplete Omenn), elder male sibling expired in early infancy	N.A.	0.722	IgG- 8.29 g/L IgA- 0.75 g/L	CD3- 89% CD3+CD4+- 8% CD3+CD8+- 85.1% CD19- 0.3% CD56- 0.8% CD3+45RA+ 45RO-: 30% compared to 90% in control CD3+45RA- 45RO+: 79.14% compared to 19.24% in control CD3+HLA-DR+: 90.13% compared to 12.7% in control	*ADA*	Omenn syndrome

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 37	5 months/ female	Recurrent pneumonia, diarrhea, failure to thrive, BCG site ulceration	N.A.	0.861	IgG <2.0 g/L IgA <0.17 g/L	CD3- 34% (292) CD3+CD4+- 29.7% (89) CD3+CD8+- 55.3% (165) CD19- 45% (387) CD56- 12.1% (103)	Not done	SCID
Pt. 38	5 months/ male	Recurrent pneumonia, diarrhea, failure to thrive	N.A.	0.140	N.A.	CD3- 9.6% (13) CD19- 8.7% (12) CD56- 80% (112)	*RAG1*	SCID
Pt. 39	2 months/ male	Recurrent episodes of diarrhea, failure to thrive, sacral abscess, 2 elder siblings died in early infancy due to repeated infections	Blood culture: Staphylococcus aureus; Disseminated CMV in autopsy	0.06	N.A.	CD3- 50% (30) CD19- 7.7% (4-5) CD56- 34.6% (21)	*ADA*	SCID
Pt. 40	2.5 months/ male	Recurrent pneumonia, otitis media, failure to thrive, 6 maternal uncles and 2 elder male siblings died at early infancy due to repeated infections	N.A.	1.406	N.A.	CD3- 0.07% (01) CD19- 91.5% (1,598) CD56- 1.8% (33)	Not done	SCID
Pt. 41	4 years/ female	Eczematoid eruptions and chronic otitis media since early infancy, autoimmune hemolytic anemia, generalized adenopathy	N.A.	1.922	IgG- 21.56 g/L IgA- 4.77 g/L IgM- 0.57 g/L IgE- 933 U/L (Normal: up to 60)	CD3- 24.79% (912) CD3+CD4+- 21.2% (193) CD3+CD8+- 55% (500) CD19- 42.3% (812) CD56- 2.7% (58) CD3+45RA+ RO-: 45% compared to 76% in control CD3+CD4+ 45RA+RO-: 14.9% compared to 67% in control CD3+CD8+ 45RA+45RO-: 35.8% compared to 72% in control	*STK4*	CID
Pt. 42	4 months/ male	Recurrent pneumonia, diarrhea, failure to thrive, oral thrush, 1 maternal uncle died at 2 years due to repeated infections	Blood culture: *Moraxella* sp.	1.302	N.A.	CD3- 1.3% (17) CD19- 85.16% (1,109) CD56- 2.9% (37)	Not done	SCID
Pt. 43	2.5 months/ male	Chronic diarrhea, failure to thrive, esophageal candidiasis, maternal cousin (male) expired at early infancy due to pneumonia	N.A.	0.415	N.A.	CD3- 3.8% (15) CD19- 84% (336) CD56- 3% (12)	*IL2RG*	SCID
Pt. 44	3 months/ male	Recurrent pneumonia, diarrhea, failure to thrive, erythroderma, eosinophilia, hepatosplenomegaly (maternal T cell	Blood culture: *Weisella confusa*	7.457	N.A.	CD3- 15.9% (1,192) CD19- 76.4%	*IL2RG*	Atypical SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
		engraftment), 1 maternal uncle died at early infancy due to pneumonia				(5,692) CD56- 1.9% (142) CD3+45RA+ RO-: 5.43% compared to 59% in control CD3+45RA- 45RO+: 96.9% compared to 60% in control CD3+HLA-DR+: 83.5% compared to 15.7% in control		
Pt. 45	4 months/ male	Recurrent pneumonia, diarrhea, failure to thrive, oral thrush, BCG site ulceration	N.A.	2.831	IgG <0.87 g/L IgA <0.16 g/L	CD3- 0.2% (5-6) CD19- 97.7% (2,765) CD56- 0.48% (13-14)	No gene variants found	SCID
Pt. 46	5 months/ male	Recurrent fever, BCG site ulceration, hepatosplenomegaly, oral thrush	Disseminated BCGosis	2.086	IgG <1.46 g/L	CD3- 0.6% (13) CD19- 97.8% (2,044) CD56- 0.2% (4)	Not done	SCID
Pt. 47	15 days/ male	Younger sibling of pt. 31, pneumonia, recurrent diarrhea, failure to thrive	Blood culture: *Candida* sp.	0.094	N.A.	CD3- 42% (38) CD19- 40% (36) CD56- 16% (14)	*ADA*	SCID
Pt. 48	4 months/ male	Younger sibling of pt. 27, recurrent pneumonia, diarrhea, failure to thrive, erythroderma, hepatosplenomegaly, eosinophilia (Omenn syndrome)	N.A.	1.896	N.A.	CD3- 74% (1,406) CD19- 0.4% (8) CD56- 22% (418) CD3+45RA+ 45RO-: 16% compared to 71% in control	*RAG2*	Omenn syndrome
Pt. 49	3 years/ male	Recurrent sinopulmonary infections, diarrhea, failure to thrive, 1 episode of liver abscess, intra-cranial B cell lymphoma, defective T lymphocyte proliferation on stimulation with PHA.	N.A.	3.265	IgG- 4.02 g/L	CD3- 45.14% (1,467) CD3+CD4+- 6.9% (103) CD3+CD8+- 70.3% (1,033) CD19- 6.83% (222) CD56- 25.01% (816) CD3+45RA+ 45RO-: 71.06% compared to 64% in control CD3+CD4+ 45RA+45RO-: 3.6% compared to 72% in control CD3+CD8+ 45RA+45RO-: 75.3% compared to 68% in control	*CORO1A*	Atypical SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 50	6 months/ male	Recurrent episodes of pneumonia, failure to thrive	N.A.	0.411	IgG <0.95 g/L IgA <0.17 g/L IgM <0.25 g/L	CD3- 20% (80) CD19- 73% (292) CD56- 1.4% (5-6)	*JAK3*	SCID
Pt. 51	10 months/ male	Pneumonia, diarrhea, failure to thrive, meningoencephalitis	Endotracheal aspirate: *Klebsiella pneumoniae*	0.810	IgG <0.95 g/L IgA <0.17 g/L IgM <0.25 g/L	CD3- 4% (32) CD19- 95% (760) CD56- 1% (8)	*IL2RG*	SCID
Pt. 52	3 months/ male	Recurrent pneumonia, diarrhea, failure to thrive	N.A.	0.199	N.A.	CD3- 0.82% (2) CD19- 1.17% (2-3) CD56- 88.9% (178)	*DCLRE1C*	SCID
Pt. 53	5 months/ male	Pneumonia, failure to thrive, complicated otitis media with facial nerve palsy, transfusion-associated GVHD	N.A.	0.292	N.A.	CD3- 0.2% (0-1) CD19- 29% (87) CD56- 60% (180)	Not done	SCID
Pt. 54	3.5 years/ male	Severe eczema since early infancy, pustules, otitis media, pneumonia, chest wall abscess, eosinophilia (incomplete Omenn)	Pus culture- *Staphylococcus aureus*	1.244	IgG- 1.64 g/L IgA- 1.56 g/L IgE- 4269 kU/L (upto 32) IgG1- 1.01 g/L IgG2- 0.95 g/L IgG3- 0.23 g/L IgG4- 0.71 g/L	CD3- 60% (744) CD3+CD4+- 17.3% (128) CD3+CD8+- 71.5% (529) CD19- 2.3% (28) CD56- 15% (186) CD3+45RA-45RO-: 36.6% compared to 65% in control CD3+45RA-45RO+: 67% compared to 31% in control CD3+HLA-DR+: 64.2% compared to 19.3% in control	No gene variants found	Omenn syndrome
Pt. 55	5 months/ male	Recurrent pneumonia, diarrhea, failure to thrive, hyperferritinemia, hypofibrinogenemia, pancytopenia (HLH)	ET aspirate: *Klebsiella pneumoniae*, *Acinetobacter baumanii;* PCR positivity for H1N1	1.547	IgG- 2.32 g/L IgA <0.2 g/L IgM- 0.22 g/L	CD3- 1.74% (27) CD19- 91.6% (1,426) CD56- 5% (78)	IL2RG	SCID
Pt. 56	6 months/ female	Pneumonia, failure to thrive, diarrhea, BCG site ulceration	N.A.	1.098	IgG- 0.54 g/L IgA <0.2 g/L IgM <0.17 g/L	CD3- 0% (0) CD19- 2% (22) CD56- 79% (869)	*DCLRE1C*	SCID
Pt. 57	7 months/ male	Pneumonia, diarrhea, failure to thrive, hepatosplenomegaly, BCG site ulceration	N.A.	0.855	N.A.	CD3- 5.1% (43) CD19- 77.5% (667) CD56- 17% (146)	Not done	SCID
Pt. 58	11 months/ male	Recurrent pneumonia, failure to thrive, hepatosplenomegaly, generalized adenopathy, BCG site ulceration, erythematous rash (incomplete Omenn), meningitis with hydrocephalus	Disseminated BCGosis, CMV DNA PCR positive, Endotracheal aspirate: *Klebsiella pneumoniae*	1.832	IgG- 7.74 g/L IgA- 0.36 g/L IgM- 2.42 g/L	CD3- 68% (1,244) CD3+CD4+- 7.8% (97) CD3+CD8+- 45.1% (558) CD19- 5.6%	*RAG1*	Omenn syndrome

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
						(102) CD56- 23.5% (430) CD3+CD4+ 45RA-45RO+: 90.4% compared to 30.2% in control CD3+HLA-DR+: 67.9% compared to 5.8% in control		
Pt. 59	5 months/ male	BCG site ulceration, persistent diarrhea, generalized papular rash	*M. bovis*	0.931	IgA<0.10 g/L	CD3- <1% CD19- 97% (902) CD56- <1%	*IL2RG*	SCID
Pt. 60	6 months/ male	BCG site ulceration, oral thrush, septicemia	*Candida* sp.	2.129	N.A	CD3- 29% (617) CD19- 62% (1320) CD56- 8% (170)	No gene variants identified	SCID
Pt.61	5 months/ female	BCG site ulceration, pneumonia, erythroderma, alopecia, CMV DNA PCR-positive	CMV, *M. bovis*	1.144	IgG-9.03 g/L IgA-0.17 g/L IgM-0.41 g/L	CD3-70.70% (806) CD19-0.14% (2) CD56-17.70% (202) CD3+45RA+ -12.57% compared to 86% in control	*RAG2*	Omenn syndrome
Pt. 62	4 months/ male	Severe pneumonia, CT chest: diffuse bilateral ground glass opacities with multifocal consolidation	Nil	0.507	IgG- <2.02 g/L IgA- <0.17 g/L IgM- <25 g/L	CD3- 57.23% (290) CD19-0.05% (1) CD56-35.08% (179)	*RAG1*	SCID
Pt. 63	5 months/ male	Severe pneumonia, CT chest: bilateral small random nodules	Nil	1.236	IgG- <2.03 g/L IgA- <0.17 g/L IgM- <0.25 g/L	CD3- 0.28% (4) CD19-96.20% (1193) CD56-0.51% (6)	*IL2RG*	SCID
Pt. 64	5 months/ female	Persistent pneumonia- pneumothorax, oral thrush	*Candida* sp.	0.180	IgG- <2.03 g/L IgA- <0.17 g/L IgM- 0.33 g/L	CD3-0.16% (1) CD19-0.16% (1) CD56-74.40% (134)	*DCLRE1C*	SCID
Pt. 65	1.5 months/ female	Left ear complicated otitis media, pneumonia, diarrhea	*S. aureus*	2.443	IgG-8.04 g/L IgA-0.75 g/L IgM-1.38 g/L	CD3-22.87% (559) CD19-73.60% (1776) CD56-1.43% (34)	No gene variants identified	SCID
Pt. 66	7 months/ male	BCG adenitis, encephalitis	*M. bovis*	0.506	IgG- <2.05 g/L IgA- <0.17 g/L IgM- <0.26 g/L	CD3-18.19% (93) CD19-0.08% (1) CD56-77.24% (394)	*DCLRE1C*	SCID
Pt. 67	8 months/ female	Recurrent diarhea, failure to thrive, pneumonia, axillary adenopathy	Nil	6.864	IgG-<2.03 g/L IgM->4 g/L	CD3-69.75% (4785) CD3+CD4+ - 32% of CD3+ lymphocytes (1530) CD3+CD8+ -	No gene variants identified	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
						62% of CD3+ lymphocytes (2967) CD3+45RA+ - 24.3% compared to 85% in healthy control CD19-8.95% (617) CD56-2.57% (178)		
Pt. 68	10 months/ male	recurrent gastroenteritis, pneumonia, DCT+ autoimmune hemolytic anemia	CMV	2.200	IgG-3.33 g/L IgA- <0.17 g/L IgM- 1.19 g/L	CD3-86.75% (1910) CD19-0.64% (13) CD56-6.50% (143) CD3+CD45RA+ -38.2% compared to 79% in control	*NHEJ1*	Atypical SCID
Pt. 69	5 months/ male	persistent pneumonia, absent BCG scar	Nil	0.287	IgG-3.47 g/L IgA- 0.21 g/L IgM-0.76 g/L	CD3-51.62% (150) CD19-31.30% (91) CD56-9.46% (28)	No gene variants identified	SCID
Pt. 70	4 months/ male	Recurrent pneumonia, diarrhoea, generalized erythematous macular rash, CMV retinitis, seizures, GVHD skin lesions	CMV	4.921	IgG- <1.99 g/L IgA- <0.36 g/L IgM- <0.25 g/L	CD3-0.54% (25) CD19-0.54% (25) CD56-91.74% (4512)	*DCLRE1C*	SCID
Pt. 71	6 months/ male	Recurrent pneumonia, otitis media, ulceration at BCG site, hepatosplenomegaly	*Enterococcus* sp.	0.816	IgA-0. 56 g/L	CD3-1.39% (12) CD19-90.95% (746) CD56-5.4% (44)	*IL2RG*	SCID
Pt. 72	11 months/ male	Skin pustule and abscess, generalized erythematous macular rash, oral thrush	Nil	1.118	IgG-<0.90 g/L IgA- <0.21 g/L	CD3-24.80% (278) CD19-6.20% (69) CD56-67.6% (757)	*NHEJ1*	SCID
Pt. 73	3.5 months/ male	Recurrent pneumonia, diarrhoea, generalized erythematous macular rash	Nil	2.420	IgG- 1.62 g/L IgA- 0.09 g/L IgM- 0.48 g/L	CD3-5% (121) CD19- 47% (1137) CD56- 42% (1016)	No gene variants identified	SCID
Pt. 74	3 months/ male	Recurrent pneumonia, diarrhoea	Nil	1.643	IgG- 3.09 g/L IgA- <0.07 g/L	CD3-87.90% (1442) CD19-1.7% (28) CD56-2.2% (33) CD3+45RA+ -1.6% compared to 78% in control	*ADA*	Atypical SCID
Pt. 75	3 months/ male	Recurrent pneumonia, diarrhoea, failure to thrive	Nil	2.862	IgG- 2.14 g/L IgA- <0.20 g/L	CD3-35.90% (1026) CD19-3.11% (89)	No gene variants identified	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
						CD56- 38% (1087)		
Pt. 76	12 months/ female	Recurrent pneumonia, diarrhoea, oral thrush	Nil	4.300	IgG- 3.28 g/L IgA- 1.46 g/L IgM- 2.99 g/L	CD3-3.29% (142) CD19-79.37% (3414) CD56-9.63% (413)	No gene variants identified	SCID
Pt. 77	1.5 months/ female	Recurrent pneumonia, otitis media, generalized erythematous macular rash	*Pichia fermentans; E. coli*	4.720	IgG- 4.27 g/L IgA- <0.16 g/L IgM- 0.35 g/L	CD3-49.03% (2303) CD19-1.27% (61) CD56-37.44% (1765) CD3+45RA+ - 1.46% compared to 73% in control	*RAG1*	Omenn syndrome
Pt. 78	6 months/ male	Recurrent pneumonia, diarrhoea, generalized erythematous macular rash	Nil	1.808	IgG- <2.02 g/L IgA- 0.20 g/L IgM-1.71 g/L	CD3-95.65% (1732) CD19-1.78% (32) CD56-0.53% (9) CD3+45RA+ - 11% compared to 86% in control	*IL2RG*	Atypical SCID
Pt. 79	6.5 months/ male	Recurrent pneumonia, diarrhoea	Nil	0.600	IgG- 3.65 g/L IgA- 0.38g/L IgM- 0.41 g/L	CD3-29.54% (177) CD19-41.13% (247) CD56-18.87% (114)	No gene variants identified	SCID
Pt. 80	15 months/ male	Recurrent pneumonia, diarrhoea, oral thrush	*Klebsiella pneumoniae*, CMV	7.191	IgG-2.02 g/L IgA-0.18 g/L IgM-0.46 g/L	CD3-12.36% (892) CD19-0.74% (53) CD56-51.5% (3703) CD3+45RA+ - 14.29% (decreased)	No gene variants identified	SCID
Pt. 81	42 months/ female	Recurrent pneumonia, oral thrush	Nil	1.615	IgG-21.77 g/L IgA-1.23 g/L IgM-1.81 g/L	CD3-37.40% (606) CD4- 5.7% CD8- 14.7% CD19-22.6% (366) CD56- 46% (743)	No gene variants identified	SCID
Pt. 82	3 months/ male	Recurrent pneumonia, failure to thrive, oral thrush, one elder female sibling expired due to pneumonia in early infancy	Nil	2.492	IgG-2.72 g/L IgA-0.09 g/L IgM-0.73 g/L	CD3-30% (747) CD19-9.10% (227) CD56- 41% (1021)	No gene variants identified	SCID
Pt. 83	3 months/ female	Recurrent pneumonia, diarrhoea, otitis media, oral thrush	Nil	2.608	IgG-<2.02 g/L IgA-<0.17 g/L IgM-0.90 g/L	CD3-32.68% (853) CD19-29.79% (783) CD56-33.41% (872)	No gene variants identified	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
						CD3+ 45RA+ -2.23% (decreased)		
Pt. 84	4 months/ male	Recurrent pneumonia, diarrhoea, otitis media, ulceration at BCG site	Nil	0.663	IgG-<2.02 g/L IgA-<0.17 g/L IgM-<0.25 g/L	CD3-87.66% (579) CD19-0.05% (1) CD56-10.22% (66)	*RAG1*	SCID
Pt. 85	4 months/ male	Recurrent pneumonia, severe erythroderma, developmental delay	Nil	1.441	IgG-<2.02 g/L IgA-<0.17 g/L	CD3-0.16% (3) CD19-94.81% (1365) CD56-0.67% (10)	*IL2RG*	SCID
Pt. 86	15 months/ male	Recurrent pneumonia, generalized eczematoid macular rash, developmental delay, myopathy	Nil	14.84	IgG-13.16 g/L IgA-1.70 g/L IgM<0.26 g/L IgE- 8423 U/L	CD3-92.20% (13,683) CD19-2.85% (416) CD56-3.21% (475) CD4+45RA+ - 12.17% compared to 56% in control CD8+45RA+ - 18.6% compared to 72% in control	*STIM1*	CID
Pt. 87	6 months/ male	Recurrent pneumonia, extensive eczematoid rash	CMV	6.556	IgG-13.75 g/L IgA-0.42 g/L IgM-1.88 g/L IgE- 622 U/L	CD3-53.90% (3536) CD4- 4.9% (320) CD8- 30.2% (1968) CD19-25.9% (1706) CD56-5.6% (368)	No gene variants identified	Omenn syndrome
Pt. 88	2.5 months/ female	Recurrent pneumonia, diarrhoea, BCG site abscess	Nil	0.055	IgG-2.33 g/L IgA<0.17 g/L IgM<0.25 g/L	CD3-95.58% (48) CD19-0.07% (1) CD56-0.79% (1)	*ADA*	SCID
Pt. 89	4 months/ female	Recurrent diarrhoea, otitis media, generalized erythematous macular rash, ulceration at BCG site	*Enterococcus faecalis*	2.352	IgG<2.02 g/L IgM<0.21 g/L	CD3-68.95% (1621) CD19-0.05% (1) CD56-25.37% (597) CD3+ 45RA+ -6.79% compared to 70% in control HLA DR in CD3+ - 74.2% compared to 15% in control	*RAG1*	Omenn syndrome
Pt. 90	4.5 months/ male	Recurrent pneumonia,	Nil	0.724	IgG-<0.95 g/L IgA<0.17 g/L	CD3-2.41% (17) CD19-0.45% (4) CD56-90.91% (655)	*DCLRE1C*	SCID
Pt. 91	4 months/ male	Recurrent pneumonia, chorioretinitis, failure to thrive, 3 maternal uncles died at early infancy due to severe infections	Blood CMV PCR positive	1.760	IgG < 2.7 g/L IgA <0.4 g/L IgM- 1.07 g/L	CD3- 1% (18) CD19- 61%	*IL2RG*	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 92	4 months/ male	Oral thrush, pneumonia, failure to thrive	N.A.	0.650	N.A.	(1,098) CD56- 1% (18) CD3- 0.4% (2-3) CD19- 97% (631) CD56- 0.4% (2-3)	*IL2RG*	SCID
Pt. 93	6 months/ female	Recurrent pneumonia, CMV chorioretinitis	Blood CMV PCR positive	2.200	IgG <2.7 g/L IgA <0.4 g/L IgM <0.25 g/L	CD3- 7.6% (167) CD19- 1% (22) CD56- 40% (880)	*RAG2*	SCID
Pt. 94	4.5 months/ male	Persistent pneumonia, failure to thrive, elder sibling died at 6 months due to severe pneumonia	N.A.	1.750	IgG <1.37 g/L IgA <0.26 g/L IgM <0.16 g/L	CD3- 4% (70) CD19- 47% (823) CD56- 25% (438)	*IL7RA*	SCID
Pt. 95	6 months/ male	Recurrent pneumonia, failure to thrive	N.A.	2.000	IgG <1.37 g/L IgA <0.26 g/L IgM- 0.53 g/L	CD3- 20% (400) CD19- 80% (1,600) CD56- 0.1% (2)	*IL2RG*	SCID
Pt. 96	3 months/ female	Oral thrush, septicemia	N.A.	0.720	IgG- 0.8 g/L IgA <0.26 g/L IgM <0.18 g/L	CD3- 5% (36) CD19- 7% (50) CD56- 53% (382)	*RAG2*	SCID
Pt. 97	5 months/ male	Recurrent episodes of pneumonia, diarrhea, failure to thrive, oral thrush, BCG site ulceration, elder female sibling expired at 6 months due to recurrent infections	N.A.	0.850	IgG <2.7 g/L IgA <0.4 g/L IgM <0.25 g/L	CD3- 5% (43) CD19- 2.3% (20) CD56- 46% (391)	Not done	SCID
Pt. 98	3 months/ female	Recurrent pneumonia, failure to thrive	N.A.	0.700	IgG <2.7 g/L IgA <0.4 g/L IgM <0.25 g/L	CD3- 6.5% (46) CD19- 3.6% (25) CD56- 34% (238)	Not done	SCID
Pt. 99	3 months/ female	Recurrent pneumonia, failure to thrive	N.A.	0.680	IgG- 0.65 g/L IgA <0.5g/L IgM <0.25 g/L	CD3- 2% (14) CD19- 4% (27) CD56- 53% (360)	*DCLRE1C*	SCID
Pt. 100	3.5 months/ male	Persistent pneumonia, failure to thrive	N.A.	4.911	IgG- 0.82 g/L IgA <0.26 g/L IgM <0.18 g/L	CD3- 9.4% (461) CD19- 0.4% (20) CD56- 90% (4,410)	Not done	SCID
Pt. 101	6 months/ female	Persistent pneumonia, failure to thrive	N.A.	5.756	IgG- 6.7 g/L IgA <0.26 g/L IgM- 0.78 g/L	CD3- 2% (116) CD19- 41% (2,362) CD56- 47% (2,707)	Not done	SCID
Pt. 102	7.5 months/ male	Recurrent pneumonia, diarrhea, failure to thrive	N.A.	0.750	IgG- 0.76 g/L IgA- 0.08 g/L IgM- 0.08 g/L	CD3- 26% (195) CD19- 67% (503) CD56- 6% (45)	*JAK3*	SCID
Pt. 103	1 month/ male	Erythroderma, loss of eyelashes, eosinophilia (incomplete Omenn), failure to thrive, two siblings (one male and one female) expired in early infancy due to erythroderma, generalized lymphadenopathy, and severe infections	N.A.	3.358	IgG- 5.86 g/L IgA <0.26 g/L IgM- 0.36 g/L IgE >2,500 U/L	CD3- 9% (302) CD19- 48% (1,613) CD56- 11% (370)	No variants identified	Omenn syndrome

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 104	5 months/ female	Recurrent pneumonia, otitis media, failure to thrive, oral thrush, pancytopenia, hepatosplenomegaly, seizures, encephalopathy (HLH)	N.A.	1.890	IgG <2.7 g/L IgA <0.4 g/L IgM <0.25 g/L	CD3- 32% (605) CD3+CD4+- 87% (528) CD3+CD8+- 13% (78) CD19- 65% (1,229) CD56- 3% (57)	*SP110*	SCID
Pt. 105	11 months/ male	Chronic diarrhea, failure to thrive	Stool culture: *Acinetobacter* sp.	0.691	IgG- 3.0 g/L IgA- 0.52 g/L IgM- 0.39 g/L	CD3- 13.6% (94) CD19- 56% (386) CD56- 28% (193)	*RAG1*	SCID
Pt. 106	4.5 months/ male	Oral thrush, recurrent pneumonia, diarrhea, failure to thrive, BCG site ulceration, 3 maternal uncles died at early infancy due to repeated infections	N.A.	0.900	IgG- 1.1 g/L IgA- 0.05g/L IgM- 0.07g/L	CD3- 5% (45) CD19- 91% (819) CD56- 2% (18)	*IL2RG*	SCID
Pt. 107	5 months/ male	Recurrent pneumonia, oral thrush, failure to thrive, BCG site ulceration, encephalopathy	N.A.	1.120	IgG <1.4g/L IgA <0.17g/L IgM <0.19g/L	CD3- 6% (66) CD19- 92% (1,012) CD56- 1% (11)	*JAK3*	SCID
Pt. 108	8 months/ male	Recurrent pneumonia, diarrhea, failure to thrive	N.A.	0.380	IgG <1.4g/L IgA <0.17g/L IgM <0.19g/L	CD3- 0% CD19- 94% (357) CD56- 4% (15)	*IL7R*	SCID
Pt. 109	4.5 months/ male	Persistent pneumonia, failure to thrive	N.A.	2.092	IgG <1.4g/L IgA <0.17g/L IgM- 0.24g/L	CD3- 0.8% (17) CD19- 98% (2,048) CD56- 0%	Not done	SCID
Pt. 110	4.5 months/ male	Persistent pneumonia, recurrent diarrhea, skin abscess, failure to thrive, situs inversus, one elder sibling died at early infancy due to pneumonia	N.A.	0.780	IgG- 1.92 g/L IgA- 0.04 g/L IgM- 0.02 g/L	CD3- 0.04% CD19- 0.29% CD56- 96%	Not done	SCID
Pt. 111	5.5 months/ female	Recurrent pneumonia, failure to thrive, 3 elder male siblings died within first year of life due to severe infections	N.A.	1.425	IgG- 0.42 g/L IgA <0.03 g/L IgM- 0.34 g/L	CD3- 1.2% (17) CD19- 71% (1,012) CD56- 25% (356)	*CD3D*	SCID
Pt. 112	6 months/ female	Recurrent pneumonia, failure to thrive	N.A.	0.336	IgG <1.36 g/L IgA <0.25 g/L IgM <0.18 g/L	CD3- 0.2% (0-1) CD19- 0% CD56- 99% (335)	Not done	SCID
Pt. 113	3.5 months/ female	Recurrent pneumonia, failure to thrive, one elder female sibling died at 4 months due to a probable infection	N.A.	2.210	IgG <1.36 g/L IgA <0.25 g/L IgM <0.18 g/L	CD3- 0.8% (18) CD19- 97.4% (2,153) CD56- 1% (22)	No variants identified	SCID
Pt. 114	8 months/ male	Recurrent pneumonia, BCG site ulceration, failure to thrive, one elder male sibling died at early infancy due to pneumonia	Disseminated BCGosis	1.650	IgG- 0.15 g/L IgA <0.24 g/L IgM- 0.2 g/L	CD3- 0% CD19- 61% (1,007) CD56- 38% (627)	*IL7R*	SCID
Pt. 115	7 months/ female	Persistent pneumonia, failure to thrive, two elder siblings (one male, one female) died in early infancy due to severe infections, one had disseminated BCGosis	N.A.	0.870	IgG <2.0 g/L IgA <0.3 g/L IgM <0.2 g/L	CD3- 15% (131) CD19- 0% CD56- 40% (350)	*DCLRE1C*	SCID
Pt. 116	7 months/ female	Persistent pneumonia, failure to thrive, autoimmune hemolytic anemia	Disseminated CMV, pulmonary aspergillosis	1.700	IgG- 3.2 g/L IgA- 0.38 g/L IgM- 0.4 g/L	CD3- 4% (68) CD19- 0% CD56- 72% (1,224)	*RAG2*	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 117	8 months/ male	Recurrent pneumonia, persistent diarrhoea, BCG site ulceration	Disseminated BCGosis	2.400	IgG- 2.9 g/L IgA- 0.32 g/L IgM- 0.24 g/L	CD3- 0% CD19- 70% (1,680) CD56- 24% (576)	*CD3E*	SCID
Pt. 118	5 months/ male	Recurrent pneumonia, failure to thrive	*Pneumocystis jirovecii* from endotracheal aspirate	3.200	IgG- 3.64 g/L IgA- 0.42 g/L IgM- 0.38 g/L	CD3- 2% (64) CD19- 64% (2,048) CD56- 1% (32)	*IL2RG*	SCID
Pt. 119	3.5 months/ male	Recurrent pneumonia, septicemia	None	0.064	IgG- 1.49 g/L IgA- <0.26 g/L IgM- <0.16 g/L	CD3- 63% (27) CD19- 2.4% (1) CD56- 2.4%	*ADA*	SCID
Pt. 120	1 month 8 days/ female	Recurrent pneumonia, cupping of ribs with blunting of lower end of scapula in radiology	None	0.160	IgG- 3.54 g/L IgA- <0.05 g/L IgM- <0.03 g/L	CD3- 32% (51) CD19- 8.9% (14) CD56- 58% (93)	*ADA* (probable); Gene sequencing not done	SCID
Pt. 121	10 months/ male	Recurrent pneumonia, persistent diarrhoea, oral candidiasis	Adenovirus	0.582	IgG- 3.54 g/L IgA- <0.05 g/L IgM- <0.03 g/L	CD3-11% (47.8) CD19-68.7% (298.7) CD56-18% (79.2)	*JAK3*	SCID
Pt. 122	7 months/ female	Recurrent pneumonia, persistent diarrhoea, septicemia	Rhinovirus, Blood- *Candida* sp.	0.952	IgG- 16.55 g/L IgA- 0.29 g/L IgM- 1.12 g/L	CD3-4.7% (12) CD19-0% CD56-91% (231)	Not done	SCID
Pt. 123	6 months/ male	Recurrent pneumonia, persistent diarrhoea	Nil	0.780	IgG- 3.06 g/L IgA- 0.26 g/L IgM- 0.30 g/L	CD3-85% (665) CD19-3% (26) CD56-11% (87)	*ADA*	SCID
Pt. 124	6 months/ male	Recurrent pneumonia, persistent diarrhoea, cellulitis, hepatosplenomegaly, panniculitis	*M. bovis*	0.370	IgG- 0.19 g/L IgA- <0.01 g/L IgM- 0.16 g/L	CD3-4.94% (22) CD19-84% (404) CD56-0.09% (3)	Not done	SCID
Pt. 125	36 months/ male	Recurrent pneumonia, persistent diarrhoea	Nil	0.480	IgG- 11.80 g/L	CD3-33.3% (156.5) CD19-33.4% (156.3) CD56- 28.3% (112)	Not done	SCID
Pt. 126	7 months/ female	Recurrent pneumonia, persistent diarrhoea, septicemia	Blood- *Acinetobacter baumanni, Candida* sp.	1.090	IgG- 9.80 g/L IgA- 0.17 g/L IgM- 0.43 g/L	CD3-0.35% (4) CD19-82.7% (1048) CD56- 4.56% (58)	Not done	SCID
Pt. 127	11 months/ male	Recurrent pneumonia, persistent diarrhoea, otitis media	Ear pus- *P. aeruginosa*	0.824	IgG- 12.40 g/L	CD3-3.0% (26) CD19-84% (682) CD56- 3.56% (48)	Not done	SCID
Pt. 128	4 months/ female	Recurrent pneumonia, persistent diarrhoea, otitis media, cellulitis	BAL- Adenovirus	0.160	IgG- 7.30 g/L IgA- 0.34 g/L IgM- 0.92 g/L	CD3-0% CD19-30% (75) CD56- 42% (103)	Not done	SCID
Pt. 129	8 months/ male	Recurrent pneumonia, persistent diarrhoea, otitis media, septicemia	Blood- *S. aureus, P. aeruginosa*	0.340	IgG- 3.30 g/L IgA- 0.24 g/L IgM- 0.17 g/L	CD3-2.0% (6) CD19-93% (310) CD56- 4% (18)	*JAK3*	SCID
Pt. 130	22 months/ male	Recurrent pneumonia, persistent diarrhoea, septicemia	Blood- *Streptococcus pneumoniae*	0.357	IgG- 18.80 g/L IgA- 1.62 g/L IgM- 0.85 g/L	CD3-5% (18) CD19-27% (97) CD56- 72% (222)	Not done	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 131	60 months/ female	Recurrent pneumonia, persistent diarrhoea, septicemia, microcephaly	Nil	0.760	IgG- 13.80g/L IgA- 0.34 g/L IgM- 1.20 g/L	CD3-4.0% (24) CD19-92.0% (696) CD56- 3% (18)	Not done	SCID
Pt. 132	4 months/ male	Recurrent pneumonia, persistent diarrhoea	Nil	1.378	IgG- 9.60 g/L IgA- 0.22 g/L IgM- 0.55 g/L	CD3-4.0% (44) CD19-91% (986) CD56- 5.3% (58)	Not done	SCID
Pt. 133	30 months/ male	Recurrent pneumonia, persistent diarrhoea, otitis media, septicemia	CMV	0.357	IgG- 10.70 g/L IgA- 0.30 g/L IgM- 0.79 g/L	CD3-5.0% (17.5) CD19-12% (93) CD56- 34.6% (124)	Not done	SCID
Pt. 134	8 months/ male	Recurrent pneumonia, otitis media, septicemia	Nil	3.485	IgG- 6.80 g/L IgA- 0.31 g/L IgM- 0.43 g/L	CD3-1.0% (6) CD19-82% (2830) CD56- 18% (654)	Not done	SCID
Pt. 135	5 months/ male	Recurrent pneumonia, persistent diarrhoea, septicemia	*Candida* sp.	3.240	IgG- 2.80 g/L IgA- 0.18 g/L IgM- 0.26 g/L	CD3-12% (388) CD19-0% CD56- 86% (2786)	Not done	SCID
Pt. 136	6 month/ male	Two elder male sibling death at early infancy	Nil	0.300	N.A.	CD3-0.7% (1) CD19-97.6% (290) CD56-0.4% (1)	*IL2RG*	SCID
Pt. 137	6 months/ male	Recurrent pneumonia, septicemia, eczematoid rash	*Candida* sp.	2.436	IgG- 2.70 g/L IgA- 0.35 g/L IgM- 0.36 g/L IgE- 24,200 U/L	CD3-66% (1610) CD19-26% (634) CD56-8% (195) CD3+45RO+ - 97.5% (elevated)	*CD3D*	Omenn syndrome
Pt. 138	2 months/ female	Recurrent pneumonia, cellulitis, OS, abscess	*Candida* sp.	32.600	IgG- <0.33 g/L IgA- <0.06 g/L IgM- <0.04 g/L	CD3-87% (28,362) CD19-0% CD56-7.6% (2478)	Not done	Omenn syndrome
Pt. 139	1 months/ female	Cellulitis, rash	N.A	1.230	IgG- 9.40 g/L IgA- <0.25 g/L IgM- N.A	CD3-1% (12) CD19-N.A. CD56-N.A.	Not done	SCID
Pt. 140	36 months/ male	Recurrent pneumonia, persistent diarrhoea	Clostridium difficle, CMV	3.024	IgG- 12.90 g/L IgA- 1.53 g/L IgM- 0.56 g/L	CD3-56% (1680) CD19-1.4% (42) CD56-26% (780)	*RAG1*	Atypical SCID
Pt. 141	6 months/ female	Recurrent pneumonia, septicemia	*Candida* sp., *Staphylococcus* sp.	2.405	IgG- <0.75 g/L IgA- 0.24 g/L IgM- N.A	CD3-0.6% (14) CD19-62.4% (1504) CD56-22.9% (552)	Not done	SCID
Pt. 142	8 months/ male	Recurrent pneumonia, persistent diarrhoea, septicemia	candida	2.075	IgG- 0.09 g/L IgA- <0.26 g/L IgM- <0.16 g/L	CD3-1% (21) CD19-93% (1934) CD56-0.2% (4)	Not done	SCID
Pt. 143	8 months/ male	Recurrent pneumonia	Nil	2.650	IgG- 7.62 g/L IgA- 0.25 g/L IgM- 0.64 g/L	CD3-18.36% (488) CD19-5% (133) CD56- N.A.	Not done	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 144	7 months/ female	Recurrent pneumonia, persistent diarrhoea, BCG site ulceration	Nil	1.090	IgG- 0.10 g/L IgA- 0.02 g/L IgM- N.A	CD3- 1.1% (12) CD19- 0% CD56- 22% (231)	Not done	SCID
Pt. 145	5 months/ male	Recurrent pneumonia,	Nil	0.060	IgG- 1.08 g/L IgA- 0.10 g/L IgM- 0.14 g/L	CD3-0.01% (1) CD19-NA (151) CD56- 62.33% (206)	Not done	SCID
Pt. 146	7 months/ male	Recurrent pneumonia,	Nil	4.200	N.A	CD3-18.31% (838) CD19-51.69% (2682) CD56- 15.9% (826)	Not done	SCID
Pt. 147	9 months/ female	Recurrent pneumonia, Septicemia, disseminated BCGosis	*E. coli*, *M. bovis*	0.994	IgG- 0.06 g/L IgA- 0.26 g/L IgM- 0.30 g/L	CD3-1.53% (10) CD19-84.69% (692) CD56- 2.76% (23)	Not done	SCID
Pt. 148	16 months/ male	Recurrent pneumonia, Septicemia	*Candida* sp.	1.316	IgG- 12.20 g/L IgA- 1.08 g/L IgM- 6.54 g/L	CD3-0.54% (2) CD19-0.64% (3) CD56- 10.93% (46)	Not done	SCID
Pt. 149	5 months/ male	Recurrent pneumonia, persistent diarrhea, BCG site ulceration	Nil	1.5	IgG- 0.02 g/L IgA- 0.37 g/L IgM- 0.21 g/L	CD3- 0.1% (2) CD19- 0% CD56- 95% (1425)	Not done	SCID
Pt. 150	7 months/ male	Recurrent pneumonia,	BAL – *M.* tuberculosis, *Pseudomonas* sp.	3.000	IgG- <2.0 g/L IgA- 0.10 g/L IgM- 0.90 g/L	CD3-0.20% (6) CD19-70% (2100) CD56- 36% (1080)	*CD3E*	SCID
Pt. 151	6 months/ male	Recurrent pneumonia, oral thrush	Enterococcal sepsis	0.6	IgG- 1.24 g/L IgA- <0.01 g/L IgM- <0.01 g/L	CD3- 47.2% (283) CD19- 0.1% (1) CD56- 46% (276)	*RAG1*	SCID
Pt. 152	5 months/ male	Recurrent pneumonia, persistent diarrhoea	Nil	1.099	IgG- <0.75 g/L IgA- <0.10 g/L IgM- 0.35 g/L	CD3-0% CD19-93% (1015) CD56- 2% (23)	*IL2RG*	SCID
Pt. 153	2 months/ male	Recurrent pneumonia, septicemia	*Candida* sp. (blood)	0.080	IgG- 1.90 g/L IgA- <0.05 g/L IgM- <0.05 g/L	CD3-10.4% (2.4) CD19-5.6 (1.29) CD56- 64% (14.84)	*ADA*	SCID
Pt. 154	3 months/ male	Acute fever, cough	Nil	0.323	IgG- <1.46 g/L IgA- <0.24 g/L IgM- 0.97 g/L	CD3-26% (84) CD19-65% (202) CD56-40% (129)	*ADA*	SCID
Pt. 155	7 months/ male	Persistent diarrhoea	Nil	2.538	IgG-1.59 g/L IgA- <0.24 g/L IgM- <0.17 g/L	CD3-0% (0) CD19-86% (2183) CD56%-11% (279)	*IL7R*	SCID
Pt. 156	21 months/ male	Meningoencephalitis, right chorioretinitis, left vitreal hemorrhage	CMV	0.508	N.A.	CD3-5% (25) CD19-12% (61) CD56-62% (315)	*PNP*	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt. 157	5 months/ male	Pneumonia	*Citrobacter* sp.	1.520	IgG- <1.34 g/L IgA- <0.28 g/L IgM- 0.25 g/L	CD3-0% (0) CD19-0% (0) CD56-96% (146)	*RAG1*	SCID
Pt. 158	24 months/ male	Recurrent pneumonia, diarrhoea, meningoencephalitis	*E. coli*	16.624	N.A.	CD3-85% (14130) CD4- 7% (1164) CD8- 70% (11637) CD19-10% (1662) CD56- 4% (665) HLA-DR expression on B cells- 0%	*RFXANK*	CID
Pt. 159	5 months/ male	Pneumonia, diarrhoea, rash	*S. epidermidis*	0.320	N.A.	CD3-1% (1) CD19-32% (102) CD56-14% (45)	*ADA*	SCID
Pt. 160	5 months, female	Pneumonia	*P. jirovecii*, H1N1	1.967	N.A.	CD3-2% (39) CD19-0% (0) CD56-95% (1869)	Not done	SCID
Pt. 161	3 months, female	Pneumonia, diarrhoea	Nil	0.203	N.A.	CD3-0% (0) CD19-0% (0) CD56- 82% (166)	Not done	SCID
Pt. 162	12 months/ male	Recurrent diarrhoea, left empyema	Nil	1.958	IgG- 15.7 g/L IgA- 3.94 g/L IgM- 2.13 g/L	CD3-43% (842) CD4- 2% (39) CD8- 30% (387) CD19-15% (294) CD56-38% (744)	Not done	SCID
Pt. 163	7 months, male	Pneumonia, global developmental delay	*M. tuberculosis*	0.979	NA	CD3-7% (69) CD19-86% (842) CD56-3% (29)	Not done	SCID
Pt. 164	2 months, male	Scaly erythrodermic rash (OS)	Nil	3.854	IgG-1.89 g/L IgA-0.28 g/L IgM-2.08 g/L	CD3-55% (2120) CD4- 33% (1272) CD8- 11% (424) CD4+ 45RA+ - 3% (decreased) CD19-18% (694) CD56-25% (964)	Not done	Omenn syndrome
Pt. 165	12 months, male	Abscesses in lung, liver, oral thrush	Nil	0.548	IgG-5.63 g/L IgA- <0.70 g/L IgM- <1.07 g/L	CD3-42% (230) CD19-18% (694) CD56-20% (110)	Not done	SCID
Pt. 166	2 months, male	Recurrent pneumonia, rash	*Acinetobacter* sp.	1.620	N.A.	CD3-68% (1102) CD4- 12% (194) CD8- 36% (583) CD4+ 45RA+ - 0% CD19-21%	Not done	Omenn syndrome

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
						(340) CD56-6% (97)		
Pt. 167	10 months, male	Chronic fever, pneumonia, hepatomegaly, pancytopenia	Nil	0.954	IgG- <1.34 g/L IgA- <0.28 g/L IgM- <0.17 g/L	CD3-24% (229) CD19-63% (601) CD56-7% (67)	Not done	SCID
Pt.168	12 months, male	N.A.	N.A.	3.080	IgG- <0.29 g/L IgA- 0.64 g/L IgM- 0.34 g/L	CD3- 4.5% (138) CD19- 71.3% (2197) CD56- 1.9% (60)	*IL2RG*	SCID
Pt.169	60 months, male	NA	NA	0.870	IgG- <0.10 g/L IgA- <0.001 g/L IgM- <0.01 g/L	CD3- 0.5% (6) CD19- 89.7% (1076) CD56- 7.8% (94)	*IL2RG*	SCID
Pt.170	7 months, male	NA	NA	0.330	IgG- 0.06 g/L IgA- 0.001 g/L IgM- 0.002 g/L	CD3- 7% (23) CD19- 0.3% (1) CD56- 80.9% (267)	*RAG1*	SCID
Pt.171	48 months, male	NA	NA	1.160	IgG-0.59 g/L IgA-0.005 g/L IgM-0.007 g/L	CD3- 31.6% (367) CD19- 35.3% (410) CD56- 31.4% (364)	*RAG1*	SCID
Pt.172	12 months, female	NA	NA	7.220	IgG-2.36 g/L IgA-0.002 g/L IgM-0.01 g/L	CD3- 0.3% (22) CD19- 0.7% (54) CD56- 56% (4044)	*RAG2*	SCID
Pt.173	48 months, male	Otitis media, recurrent pneumonia since early infancy	NA	1.800	IgG-1.16 g/L IgA-0.008 g/L IgM-0.006 g/L	CD3- 9.4% (169) CD19- 58.3% (1049) CD56- 22.5% (406)	*DOCK2*	CID
Pt.174	4 months, female	Chronic diarrhoea, pneumonia, failure to thrive, absent thymus	*E. coli*, Cryptosporidium	1.63	IgG- 5.35 g/L IgA- 0.31 g/L IgM- 1.82 g/L	CD3- 89.7% (1462) CD19- 1.2% (19) CD56- 4% (65)	Not done	Possible SCID***
Pt.175	30 months, male	Recurrent pneumonia, diarrhoea, failure to thrive	Nil	0.35	IgG-7.02 g/L IgA-1.31 g/L IgM- 0.82 g/L	CD3- 56.3% (197) CD19- 0.8% (3) CD56- 39.4% (138)	Not done	SCID
Pt.176	9 months, male	Chronic diarrhoea, pneumonia, failure to thrive	*P. aeruginosa*, *Candida* sp.	0.60	IgG- 0.99 g/L IgA-0.7 g/L IgM-0.4 g/L	CD3- 35.8% (215) CD19- 6.7% (40) CD56- 47.8% (287)	Not done	SCID
Pt.177	14 months, female	Recurrent pneumonia, diarrhoea, failure to thrive	Nil	2.63	IgG-1.46 g/L IgA- <0.25 g/L IgM- <0.18 g/L	CD3- 74% (1947) CD4- 14% (368) CD8- 34% (895) CD19- 2% (53) CD56- 23% (605)	Not done	CID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt.178	3 months, male	Recurrent pneumonia, fungal skin infection, 2 early sibling death	*P. aeruginosa, Streptococcus* sp.	2.12	IgG-2.52 g/L IgA- <0.25 g/L IgM- 1.12 g/L	CD3- 57.3% (1215) CD4- 0.5% (11) CD8- 56.3% (1194) CD19- 38.2% (810) CD56- 1% (21)	Not done	SCID
Pt.179	7 months, male	Pneumonia, scaly erythrodermic rash	Nil	0.42	IgG- 0.18 g/L IgA-0.52 g/L IgM-0.42 g/L	CD3- 83.3% (350) CD19- 1% (4) CD56- 1% (4)	Not done	Omenn syndrome
Pt.180	14 months, male	Recurrent pneumonia, eczematoid rash, failure to thrive	Cryptosporidium	2.19	IgG- 2.16 g/L IgA-1.21 g/L IgM-1.22 g/L	CD3- 41% (897) CD4- 4% (88) CD8- 17% (372) CD19- 1% (22) CD56- 17% (372)	Not done	CID
Pt.181	6 months, male	Chronic diarrhoea, failure to thrive, septicemia	*E. coli, Candida* sp.	1.37	IgG-9.52 g/L IgA-1.79 g/L IgM-0.26 g/L	CD3- 87.1% (1194) CD4- 34% (466) CD8- 53.1% (727) CD19- 0.2% (3) CD56- 12% (165)	Not done	SCID
Pt.182	3 months, male	Meningitis, pneumonia, oral thrush, early sibling death	*P. aeruginosa*	3.55	N.A.	CD3- 35% (1244) CD4- 10% (355) CD8- 15% (533) CD19- 0.1% (4) CD56- 56% (1990)	Not done	SCID
Pt.183	16 months, male	Pneumonia, eczematoid rash, Varicella infection, early sibling death due to pneumonia	*Acinetobacter* sp., *Pseudomonas* sp.	2.55	N.A.	CD3- 46% (1174) CD4- 14% (357) CD8- 33% (842) CD19- 4% (102) CD56- 50% (1276)	Not done	CID
Pt.184	3 months, female	Pneumonia, abdominal distension, diarrhoea, failure to thrive	Nil	1.80	IgG-1.63 g/ IgA- <0.06 g/L IgM- <0.16 g/L	CD3- 35% (630) CD19- 0.8% (14) CD56- 63% (1134)	Not done	SCID
Pt.185	3 months, male	Pneumonia, failure to thrive	*M. tuberculosis*	0.50	IgG-9.03 g/L IgA- 0.39 g/L IgM-2.23 g/L	CD3- 45% (315) CD19- 50% (350) CD56- 1.4% (10)	Not done	SCID
Pt.186	9 months, male	Persistent diarrhoea, pneumonia, left forearm abscess	Nil	2.43	IgG-4.0 g/L IgA-0.74 g/L IgM- 1.1 g/L	CD3- 43.9% (1068) CD3+CD4+- 26% (631) CD3+CD8+- 14% (340) CD19- 54.9% (1335) CD56- 1% (24)	Not done	Possible SCID***

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt.187	4 months, male	Developmental delay, pneumonia, diarrhoea, failure to thrive, 1 early sibling death	*E. coli*	1.50	IgG-2.95 g/L IgA-0.07 g/L IgM- 1.04 g/L	CD3- 44.9% (674) CD19- 44.9% (674) CD56- 10% (150)	Not done	Possible SCID***
Pt.188	3 months, female	Otitis media, oral thrush, failure to thrive	Nil	1.86	IgG-2.95 g/L IgA-0.07 g/L IgM- 1.04 g/L	CD3- 9% (167) CD19- 0.5% (9) CD56- 87.8% (1633)	Not done	SCID
Pt.189	96 months, female	Recurrent pneumonia, ear discharge, failure to thrive	Nil	1.21	IgG-2.14 g/L IgA- 7.05 g/L IgM-1.54 g/L	CD3- 39% (473) CD19- 16% (194) CD56- 41.2% (498)	Not done	CID
Pt.190	24 months, female	Ear discharge, diarrhoea, scaly rash (Omenn phenotype)	Nil	8.75	N.A.	CD3- 80% (7003) CD4- 5% (438) CD8- 30% (2626) CD19- 2% (175) CD56- 14% (1226)	Not done	CID
Pt.191	1 month, female	Septicemia, 3 early siblings died at early infancy	Nil	2.89	N.A.	CD3- 63.9% (1847) CD4- 55.9% (1616) CD8- 8% (231) CD19- 18% (520) CD56- 15% (433)	Not done	SCID
Pt.192	6 months, male	Multiple hypodense lesions in liver and spleen, necrotic retroperitoneal lymph nodes	Nil	0.01	N.A.	CD- 0 CD19- 0 CD56- 0	Not done	SCID
Pt.193	7 months, male	Recurrent pneumonia, diarrhoea, early sibling death due to disseminated BCGosis	Acid-fast bacilli, *Candida* sp. (BAL)	2.15	N.A.	CD3- 0% CD19- 98.9% (2128) CD56- 0.3% (6)	Not done	SCID
Pt.194	2.5 months, male	Diarrhoea, ear discharge, pneumonia, dermatitis, knee joint swelling, axilla abscess, 1 elder sibling expired due to SCID	Blood, pus: *S. aureus* (Methicillin sensitive)	2.85	N.A.	CD3- 57% (1624) CD4- 17% (484) CD8- 31% (883) CD19- 0.3% (9) CD56- 40% (1140)	Not done	SCID
Pt.195	NA, male	Pneumonia, otitis media, septicemia	*Pseudomonas* sp.	1.51	IgG-4.0 g/L IgA-0.52 g/L IgM-0.32 g/L	CD3- 0.3% (5) CD19- 0 CD56- 4% (62)	Not done	SCID
Pt.196	3 months, male	Pneumonia, oral thrush	Nil	0.01	N.A.	N.A.	*ADA*	SCID
Pt.197	3 months, male	Recurrent pneumonia, 1 early sibling death	Nil	2.86	N.A.	N.A.	*IL2RG*	SCID
Pt.198	2 months, female	Pneumonia, colitis	Nil	4.40	IgG-0.89 g/L IgA- <0.24 g/L IgM- <0.17 g/L	CD3- 0.5% (22) CD19- 87.3% (3839) CD56- 2% (88)	*JAK3*	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt.199	1.5 months, female	Pneumonia, oral thrush, 2 elder female siblings died at early infancy	Nil	1.50	N.A.	CD3- 0 CD19- 63.1% (947) CD56- 34.7% (521)	Not done	SCID
Pt.200	8 months, male	Recurrent pneumonia, BCGosis	Nil	N.A.	N.A.	CD3- 0% CD19- 64% (443) CD56- 31% (214)	*IL7RA*	SCID
Pt.201	8 months, female	Recurrent pneumonia, oral thrush, BCGosis	Nil	0.55	IgG- <0.06 g/L IgA- <0.24 g/L IgM- <0.17 g/L	CD3- 0 CD19- 16.7% (92) CD56- 65.3% (359)	Not done	SCID
Pt.202	7 months, female	Recurrent pneumonia, diarrhoea	CMV viremia, *Candida* sp.	2.48	IgG-0.97 g/L IgA-1.82 g/L	CD3- 70.8% (1755) CD4- 2.6% (65) CD8- 59% (1463) CD19- 30.2% (748) CD56- 22.3% (553)	Not done	SCID
Pt.203	24 months, male	Recurrent pneumonia, otitis media	*S. aureus* (Methicillin resistant)	1.60	IgG-1.61 g/L IgA-0.29 g/L IgM-0.29 g/L	CD3- 22% (352) CD19- 58% (928) CD56- 13% (208)	Not done	SCID
Pt.204	24 months, female	Chronic diarrhoea, pneumonia	Corona virus 229E, Alpha hemolytic streptococci (blood), esophageal candidiasis	1.16	IgG-1.14 g/L IgA-0.15 g/L IgM-0.27 g/L	CD3- 23.4% (272) CD19- 9.1% (105) CD56- 42.3% (491)	*RAG1*	SCID
Pt.205	192 months, male	Recurrent pneumonia, varicella infection, madarosis, Hodgkin lymphoma	Epstein Barr viremia	N.A.	N.A.	N.A.	*RAG1*	Atypical SCID
Pt.206	5 months, male	Recurrent pneumonia, diarrhoea, elder male sibling died in early infancy, 4 maternal uncles expired < 6 months age	Adenovirus	N.A.	N.A.	NA; CD132 expression very low in monocytes (0.2%) compared to normal expression in controls	Not done	SCID
Pt.207	5 months, male	Pneumonia, diarrhoea, ear discharge, oral thrush, rash, early sibling death	VAPP in stool, Enterovirus, Klebsiella (BAL), CSF- Enterovirus, Mycoplasma	0.39	IgG- <1.46 g/L IgA- <0.28 g/L IgM- 0.17 g/L	CD3- 28% (109) CD19- 1% (4) CD56- 68% (265)	*RAG2*	SCID
Pt.208	20 days, male	Pneumonia, diarrhoea, rash, renal abscess	Corona OC43, Rhinovirus	0.25	N.A.	CD3- 19% (47) CD19- 0 CD56- 24.4% (61)	*ADA*	SCID
Pt.209	5 months, female	Chest wall abscess, recurrent pneumonia, oral thrush, diarrhoea	*P. jirovecii*, Rotavirus (stool), Mycoplasma (nasopharyngeal aspirate)	0.97	IgG- <1.46 g/L IgA- <0.17 g/L IgM- <0.28 g/L	CD3- 1.3% (13) CD19- 0 CD56- 60% (581)	*RAG2*	SCID

(Continued)

TABLE 1 | Continued

S No	Age/ Sex	Clinical features	Organisms isolated	Absolute lymphocyte count	Immunoglobulin profile	Lymphocyte subsets	Molecular defect	ESID Working Definition
Pt.210	6 months, male	Recurrent pneumonia, diarrhoea, scalp abscess, 1 male sibling death	CMV, Rhinovirus, Enterovirus	N.A.	IgG-0.26 g/L IgA-0.02 g/L IgM- 1.70 g/L	N.A.	*CIITA*	CID
Pt.211	84 months, female	Recurrent diarrhoea, oral ulcer, pneumonia, colitis	Nil	0.84	IgG-4.97 g/L IgA- <0.67 g/L IgM-1.7 g/L	CD3- 77% (649) CD19- 15.5% (130) CD56- 3% (28) HLA-DR expression in B cells- 0%	*RFX5*	CID
Pt.212	18 months, male	Recurrent pneumonia, diarrhoea, failure to thrive	VDPV, *M. tuberculosis*, Cryptosporidium, *Enterobacter* sp. (blood)	3.75	IgG- <1.41 g/L IgA- <0.24 g/L IgM-0.20 g/L	CD3- 53.04% (1989) CD4- 22% (826) CD19- 4% (150) CD56- 42% (1576)	Not done	CID
Pt.213	132 months, female	Recurrent pneumonia, diarrhoea, oral thrush, otitis media, meningitis	*Hemophilus influenzae* (CSF)	2.94	IgG-0.22 g/L IgA- <0.24 g/L IgM-0.44 g/L	CD3- 34.7% (1022) CD4- 16.7% (490) CD8- 13.7% (405) CD19- 34% (1001) CD56- 2.2% (64)	Not done	CID
Pt.214	4 months, male	Failure to thrive, recurrent pneumonia, diarrhoea	Nil	1.22	NA	NA	*JAK3*	SCID
Pt.215	7 months, male	Otitis media, septicemia	Staphylococcus aureus	6.23	IgG- <0.3 g/L IgA- <0.05 g/L IgM- 0.11 g/L	NA	*IL2RG*	SCID
Pt.216	8 months, male	Pneumonia, diarrhoea, rash	Nil	5.02	IgG- <0.11 g/L IgA- <0.05 g/L IgM- <0.11 g/L	NA	*IL2RG*	SCID
Pt.217	1 month, male	Failure to thrive, persistent diarrhea, perianal rash	Nil	0.97	IgG- 0.42 g/L IgA- 0.06 g/L IgM- 0.59 g/L	CD3- 4% (39) CD19- 39% (378) CD56- 54% (524)	Not done	SCID
Pt.218	2 months, female	Recurrent episodes of pneumonia and diarrhoea, failure to thrive, doing well after HSCT	Nil	NA	NA	CD3- 3476 (Very low CD4 counts with CD4/CD8 reversal) CD19- 1765 CD56- 156	Probable MHC Class 2 defect	CID
Pt.219	1 month, female	Recurrent episodes of pneumonia and diarrhoea	Nil	NA	NA	NA	*IL7R*	SCID
Pt.220	1 month, male	Recurrent episodes of diarrhoea and failure to thrive	Nil	NA	NA	NA	*IL2RG*	SCID

ESID, European Society for Immunodeficiencies; CMV, Cytomegalovirus; BCG, Bacillus Calmette-Guerin; BAL, Bronchoalveolar lavage; CSF, Cerebrospinal fluid; OS, Omenn syndrome; PJP, Pneumocystis jirovecii pneumonia; EBV, Epstein-Barr virus; VDPV, Vaccine-derived polio virus; VZV, Varicella zoster virus; AIHA, Autoimmune hemolytic anemia; VAPP, Vaccine-associated paralytic polio; CID, Combined Immune Deficiency.

Clinical details of patients 221-277 are previously reported (7).

****Possible SCID is categorized if patients did not fulfil the complete ESID definition, however, the treating team had a high index of suspicion based on clinical and immunological features.*

One Touch™ Instrument, recovery, washing and enrichment of template-positive ISPs was done as per the manufacturer's protocol using Ion 520™ and Ion 530™ Kit-OT2 (catalog number A27751). Ion S5™ sequencer instrument was then initialized. Annealing of primers to enriched ISPs and chip loading was carried out using Ion 520 and 530 Loading Reagents OT2 Kit. Sequencing run was initiated and Torrent Browser was used to review results. Raw data were analyzed on Ion Reporter software and on integrative genome viewer.

NGS using a targeted gene panel was also performed for some patients (n = 6) in private laboratories (Medgenome Labs Pvt. Ltd., India).

NGS at Other Centers

Other centers in India obtained molecular testing results from private laboratories (Medgenome Labs Pvt. Ltd., India; Strand Genomics Pvt. Ltd., India; Neuberg Anand Diagnostics Pvt. Ltd., India). Illumina platform was used for sequencing in private laboratories with coverage of >80X. Sanger sequencing was used to confirm variants obtained by NGS.

Multiplex Ligation Probe Amplification (MLPA) Assay for DCLERC1 Exon 1-3 Deletion at PGIMER, Chandigarh

SALSA MLPA probe-mix P368 DCLRE1C kit was used in this protocol. MLPA was performed according to the instructions provided by the manufacturer (MRC Holland). 50–100ng/μL of DNA was denatured in thermocycler and hybridized with 1.5 μL of probe-mix along with 1.5μL of MLPA buffer. Content was mixed and incubated for 1 min at 95°C followed by incubation at 60°C for 18 h. After hybridization, probes were ligated using a ligase mix at 54°C for 15 min. Ligase was inactivated at 98°C for 5 min. PCR was performed using PCR primers, polymerase, buffers and required amount of water. Following conditions were used for amplifications—95°C for 20 s, 65°C for 80 s, for 35 cycles, followed by a final extension for 20 min at 72°C. ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was used for capillary electrophoresis. Later, 0.7μL of PCR reaction, 8.9μL of HI-DI formamide, and 0.4μL of DNA standard LIZ 600 provided by GeneScan were mixed and then denatured for 2 min at 95°C. The sample was then loaded and MLPA data were analyzed using a Coffalyser software.

RESULTS

Current study included data of patients diagnosed and managed at centers in Northern, Southern, and Western parts of India. Amongst the 277 patients, 254 were categorized as SCID (208 – SCID; 17 – atypical SCID; 26 – OS; 3 – possible SCID) and 23 as CID (**Table 1**). A steady increase in number of diagnosed cases was noted over last 10 years. The unit at PGIMER, Chandigarh (North India) diagnosed its first case of SCID in year 2001. Only 14 cases of SCID were identified until 2011 and an exponential rise in number of cases was noted after 2011 (**Figure 1**). Rise in number of cases over years paralleled the expansion of available manpower resources and laboratory facilities for pediatric immunology at Advanced Pediatrics Centre, PGIMER (North India). Ninety (90) children (Pt. 1-90) with SCID have been diagnosed at PGIMER, Chandigarh until date. Fifty-eight (58) and 27 cases of SCID were enrolled from Bai Jerbai Wadia Children's Hospital, Mumbai (West India) and Aster CMI, Bengaluru (South India), respectively.

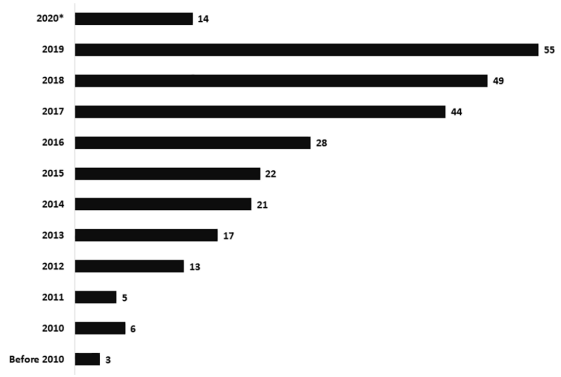

FIGURE 1 | Bar graph depicting the rise in number of cases diagnosed over last 10 years.

Male-female ratio was 196:81 (**Table 1**). Median [inter-quartile range (IQR)] age of onset of clinical symptoms and diagnosis was 2.5 months (1, 5) and 5 months (3.5, 8), respectively. Consanguinity was noted in 78 families (28.2%), and was noticeably more in Southern region (32.3%) of our country compared to Northern (22.4%). Family history of early childhood deaths was noted in 120 children (43.3%). Median (IQR) age at diagnosis in children who had a positive family history was 4.5 months (3, 6) compared to 6 months (4, 9) in children who did not have a family history, $p<0.05$ (Mann-Whitney U test).

Opportunistic infections were the presenting manifestation in most patients. These included pneumonia (82%), diarrhoea (43.7%), oral thrush (18.4%), BCG site ulceration (17%), otitis media (12.6%), and meningitis (4%) (**Figures 2**, **3**). Blood-culture proven septicemia was seen in 63 children (23%)—*Candida* sp. (16), *Staphylococcus* sp. (10), *Escherichia coli* (5), *Acinetobacter* sp. (5), *Pseudomonas aeruginosa* (8), *Klebsiella pneumoniae* (5), *Enterococcus* sp. (3), *Enterobacter* sp. (2), *Streptococcus* sp. (1), *Pichia fermentans* (1), *Burkholderia cepacia* (1), *Chryseobacterium* sp. (1), *Bacillus subtilis* (1), *Citrobacter* sp. (1), *Moraxella* sp. (1), *Alcaligens faecalis* (1), and *Weisella confusa* (1). Bacteria isolated from respiratory tract included *Mycobacterium bovis* (15), *Klebsiella pneumoniae* (5), *P. aeruginosa* (4), *M. tuberculosis* (3), atypical mycobacterium (1), *E. coli* (1), *Staphylococcus aureus* (1), and *Acinetobacter* sp. (1). Microbiology proven disseminated BCG infection was noted in 27 patients (9.7%). Apart from oral thrush and candidemia, other fungal infections noted were pneumonia due to *Pneumocystis jirovecii* (8), invasive aspergillosis (5), esophageal candidiasis (5), and pulmonary cryptcoccosis (1).

Disseminated cytomegalovirus (CMV) infection was documented in 23 (8.3%) children and 6 amongst these had evidence of CMV retinitis. Intestinal lymphangiectasia due to CMV was noted on autopsy of a child with X-linked SCID (pt.8). Prolonged excretion of vaccine-derived poliovirus was documented in a child with leaky SCID at Mumbai (14, 15). Vaccine-associated paralytic poliovirus strain was also isolated in a child with *RAG1* defect at Mumbai. He had presented with persistent diarrhea, developmental delay, and hypotonia.

Clinical features of OS were seen in 33 children (11.9%)—classical OS in 11 and incomplete OS in 22 (**Figure 3**). Molecular defects associated with OS include *RAG1* (7), *RAG2* (5), *ADA* (2), *NHEJ1* (1), *IL2RG* (1), *JAK3* (1), *STIM1* (1), *CD3D* (1), *DCLRE1C* (1), and *RFXANK* (1). Two children with *IL2RG* defect had features of engraftment of transplacental-acquired maternal T cells that mimicked clinical features of OS (**Figure 4**). Warm autoimmune hemolytic anemia (AIHA) requiring immunosuppressive medications was observed in 5 children. While anemia responded to intravenous (IV) methylprednisolone pulses in 2 patients (*RAG1* and *NHEJ1* defect each), pt.42 with *STK4* defect received IV rituximab (375 mg/m^2 2 doses) for control of AIHA and she did not have further relapse of AIHA for next 1.5 years. Transfusion-associated graft-vs-host reaction was documented in 4 patients (2 X-linked SCID; 2 AR-SCID); all had development of rash and transaminitis following transfusion of non-irradiated blood products. Four (4) children had features of hemophagocytic lymphohistiocytosis (HLH). Possible triggers for HLH included disseminated BCG (2) and H1N1 (1) infections. The child with *SP110* defect did not have any identifiable trigger for HLH (pt.104). Hodgkin lymphoma and intra-cranial B cell lymphoma were noted in children with *RAG1* and *CORO1A* defects, respectively.

Four of 18 children with *ADA* defect were noted to have radiographic abnormalities—scapular spurring and flattening of lower end of scapula (**Figure 3**). Glomerular involvement was seen in 4 children—3 children with OS and 1 with atypical/leaky SCID. Nephrotic range proteinuria was noted in 3 patients and one child (pt.13) had features of mesangial sclerosis on autopsy. Another child (pt. 12) with OS had features of focal segmental glomerulosclerosis on autopsy. One child (pt.10) with *IL7RA* defect had features of distal renal tubular acidosis and nephrocalcinosis. This patient had deletion of exons 2–5 of *CAPSL* along with exon 4–8 deletion of *IL7RA* in chromosome 5p13.2. A child with *PNP* defect (pt.14) had evidence of horse-shoe kidney at autopsy (16).

Median (IQR) absolute lymphocyte count (ALC) observed was 1.33 × 10^9/L (0.6, 2.5). Normal ALC (≥ 3 × 10^9/L) was observed in 51 children (18.4%)—of these 26 had OS, 2 had transplacental-acquired maternal T-cell engraftment, and 23 had leaky SCID/combined immunodeficiency. Eosinophilia was observed in 37 children, and 26 amongst these had features of OS. One child (Pt. 105) with *RAG1* defect had unexplained monocytosis (2.7-3.0 × 10^9/L) that resolved after HSCT. Results of immunoglobulin profile was available for 198 children. Fifty-five (55) children had normal or elevated levels of IgM levels—30 in SCID (14.2%), 7 in atypical SCID (41.2%), 8 in OS (30.8%),

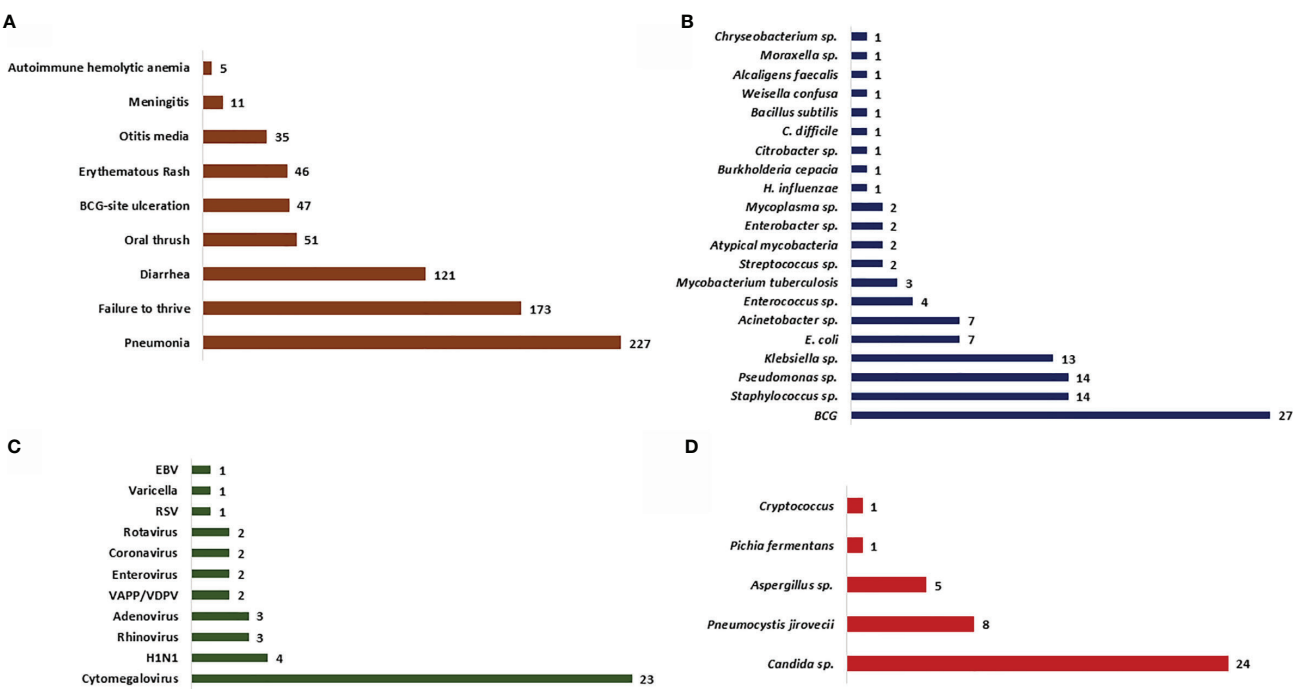

FIGURE 2 | Bar graph depicting the clinical manifestations and microbiological profile. **(A)** Clinical manifestations noted at first clinical presentation; **(B–D)** Microbiological profile of the organisms isolated—bacteria **(B)**, fungi **(C)**, and viruses **(D)**.

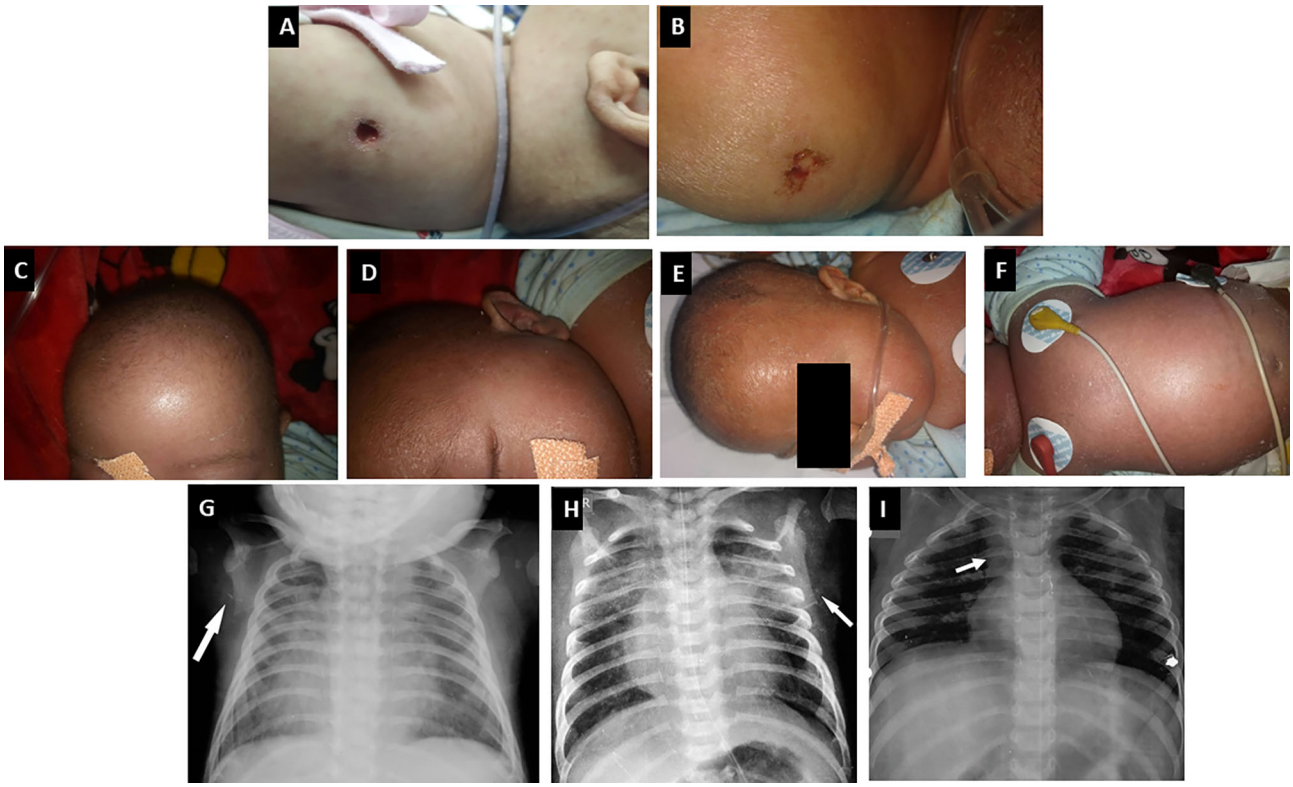

FIGURE 3 | Clinical manifestations of children with SCID. **(A**, **B)** BCG site ulceration and pus discharge (Pt. 46 and 34); **(C–F)** Features of Omenn syndrome such as generalized erythema, scaling, loss of hair, and eyebrows (Pt. 34); **(G**, **H)** Chest radiograph of a child with ADA SCID showing radiological abnormalities—scapular spur and flattening of lower border of scapula (Pt. 39); **(I)** Chest radiograph of a child with *CORO1A* defect showing normal thymus shadow (Pt. 49).

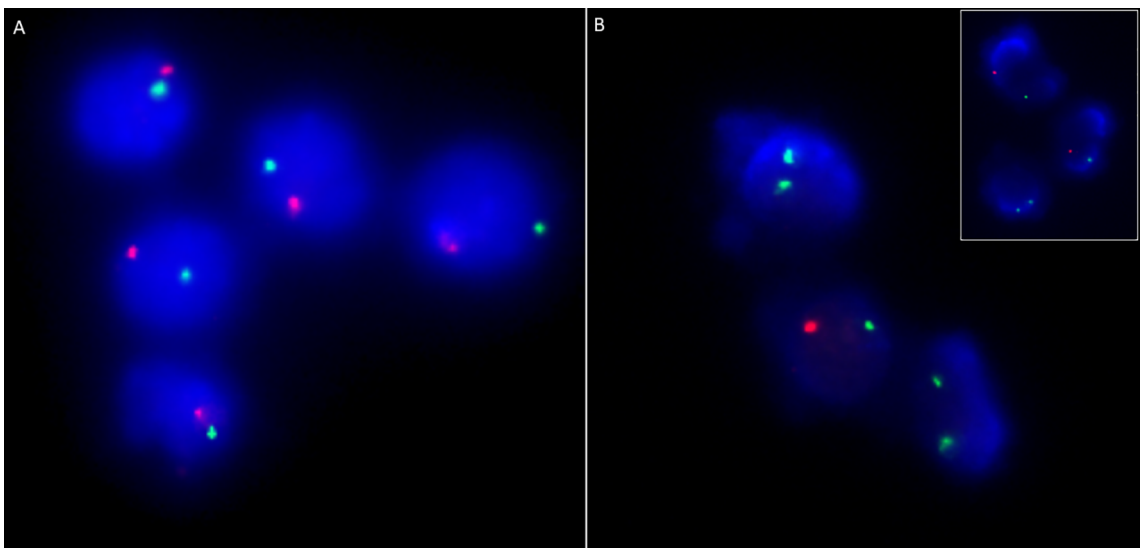

FIGURE 4 | Chimerism analysis using dual colour FISH probes targeting centromeres of X (DXZ1; green) and Y (DYZ1, orange) chromosomes in a male child suspected with transplacental-acquired maternal T cell engraftment (Pt. 44). **(A)** Immunomagnetically sorted CD19 positive cells (B cells) showing XY pattern in all cells while; **(B)** Immunomagnetically sorted CD3 positive cells showing XX pattern in two out of three cells suggesting maternal T cell engraftment. Inset shows XX pattern in a lymphocyte and XY pattern in neutrophils.

and 10 in CID (43.5%). We observed elevated levels of IgE in 12 children—8 had OS, 1 had eczema and *STK4* defect, and 3 had unexplained eosinophilia.

Immunophenotyping by flow cytometry showed the following distribution: T-B-NK- (32), T-B+NK- (67), T-B+NK+ (33), T-B-NK+ (84). T+ SCID is observed in 20 children with OS—T+B-NK+ (17), T+B-NK- (2), T+B+NK+ (1) and 2 children with transplacental-acquired maternal T-cell engraftment—T+B+NK- (1), T+B-NK- (1). Genetic defects observed under each category are summarized (**Supplementary Table 2**). We observed decreased naïve (CD3+CD45RA+) and elevated memory (CD3+CD45RO+) CD3 lymphocytes in 24 children with OS. We noted elevated HLA-DR expression in CD3+ lymphocytes in 15 children with OS. CD132 expression by flow cytometry showed reduced expression in lymphocytes or monocytes in 8 children with suspected X-linked SCID (**Table 2**) (17–19). Levels of ADA and %dAXP were measured in 7 children with *ADA* SCID and 2 heterozygous carriers of *ADA* mutation (**Table 3**).

Molecular diagnosis was obtained in 162 patients—*IL2RG* (36), *RAG1* (26), *ADA* (19), *RAG2* (17), *JAK3* (15), *DCLRE1C* (13), *IL7RA* (9), *PNP* (3), *RFXAP* (3), *CIITA* (2), *RFXANK* (2), *NHEJ1* (2), *CD3E* (2), *CD3D* (2), *RFX5* (2), *ZAP70* (2), *STK4* (1), *CORO1A* (1), *STIM1* (1), *PRKDC* (1), *AK2* (1), *DOCK2* (1), and *SP100* (1). Of the 176 molecular variants, 51 were identified to be novel in this study (**Table 4**, **Supplementary Table 3**) (7, 13, 17–49). A novel variant in *RAG1* (c.1758_1760delinsGAATC) was identified in 2 unrelated North Indian families. Deletion of exons 1-3 (8947bp) in *DCLRE1C* was observed in 11 children (9 from North and 2 from South India). MLPA confirmed EX1_EX3del in *DCLRE1C* in 7 children from North India (**Figure 5**). Targeted clinical exome sequencing by NGS did not identify pathogenic variants in 25 patients. Whole exome sequencing was performed in 5 children, and pathogenic variants were detected in 2 amongst these (pt. 50 and 51).

Majority of patients (n=198) in this cohort succumbed to overwhelming infections as HSCT could not be carried out in them (**Figure 6**). Twenty-three patients (8.3%) underwent hematopoietic stem cell transplantation (HSCT) and 11 are doing well post-HSCT. The centre at South India (Apollo Children's Hospitals, Chennai) has performed HSCT for 32 children with SCID until now and 17 are alive and doing well on follow-up. However, only 4 children are included in this analysis, as flow cytometry and mutation details were not available for other children. Another centre in South India (Aster CMI Hospitals, Bengaluru) has carried out HSCT for 9 children with SCID in the last 3 years (**Table 5**).

DISCUSSION

We describe the largest multi-centric cohort of patients with SCID from India. We included patients from 12 different tertiary care centers located in Northern, Southern, and Western parts of India. Patients from Eastern parts of India are usually referred to the centers located in other areas of India due to lack of availability of facilities for immunological investigations in that region. We witnessed an exponential rise in the number of cases with SCID after 2013 at multiple centers across India. We attribute this steady increase in cases to 2 factors—establishment of Indian Council of Medical Research Centers for Advanced Research in PIDs at PGIMER, Chandigarh (North India) and NIIH, Mumbai (West India) and expansion of laboratory facilities for pediatric immunology at other centers. The Pediatric Immunology and Bone Marrow Transplant Unit at Aster CMI Hospital, Bengaluru (South India) was established in 2017. Twenty-seven cases of SCID (Pt. 60–84) were diagnosed between 2017 and 2020, reflecting rise in awareness amongst referring pediatricians and better availability of diagnostic facilities at Bengaluru (South India).

Based on data from Sample Registration System of India, we estimated around 221 million live births from January 2011 to June 2020 (50). An estimated 257 patients with SCID have been diagnosed in this time period, which suggests a rough incidence of SCID at 0.12 per 100,000 live births. Though we have included data from most of the centers that care for patients with SCID in India, the estimated incidence from this study may not reflect true incidence of the country because of retrospective nature of the study and some patients diagnosed at other centers may have been missed. Nation-wide registry for SCID is needed for an accurate estimation of incidence. Nevertheless, if we extrapolate our current data on to the U.S. incidence figures of SCID (i.e. 1:58,000 live births), estimated number of children with SCID in India would be around 3,822 during the period 2011–June 2020 (1). Moreover, incidence of SCID in India is expected to be even higher than the U.S. considering high rates of consanguinity within the country. This suggests that though we have been increasingly diagnosing these children over the last few years, the diagnosis is still missed in almost 93% of these children. This is clearly unacceptable and mandates urgent intervention of health care professionals.

We observed a higher incidence of autosomal recessive forms of SCID (78.4%) compared to X-linked SCID. This is similar to reports from several other countries where consanguinity rates are high (**Table 6**) (7, 8, 51–59). Though consanguinity rate of 28.2% observed in our study is lower than that of Saudi Arabia and Iran, practice of endogamous and intra-community marriages is, perhaps, responsible for high proportion of autosomal recessive forms of SCID in India (2, 6). Median age at diagnosis of SCID in our study is 5 months. This is similar to reports from other countries such as China, Turkey, and U.S.A (**Table 6**). Children who had a family history of SCID had an earlier age of diagnosis (median:4.5 months) compared to children who did not have a suggestive family history (median:6 months). Our observation is similar to the report by Luk et al. that suggested the importance of family history for an early diagnosis of SCID (17).

Opportunistic infections in SCID are life-threatening and must be identified and treated adequately before HSCT. We documented a higher incidence of microbiologically-proven infections in our cohort compared to a previous report published from India (7). Amongst the bacterial infections,

TABLE 2 | CD132 expression by flow cytometry in children with X-linked SCID.

Patient	Molecular defect in *IL2RG*	Protein change	Type of mutation	Novel or previously reported	Clinical and Immunological phenotype	CD132 expression in case			CD132 expression in control		
						Lymphocyte	Monocyte	Neutrophils	Lymphocyte	Monocyte	Neutrophils
Pt. 25	c.202G>T (hemizygous); Mother - heterozygous carrier	p.E68X	Nonsense	Previously reported (17)	X-linked family history (5 maternal uncles died at early infancy), 1 elder male sibling (pt. 8) died at early infancy. T-B+NK- SCID	41.5%	94.1%	62.9%	50.9%	78.2%	39%
Pt. 35	c.170T>A (hemizygous); Mother -heterozygous carrier	p.L57H	Missense	Novel	Male child, T-B+NK- SCID, low CD132 expression	–	12.2%	–	–	87%	–
Pt. 40	–	–	–	–	X-linked family history (6 maternal uncles died at early infancy), 2 elder male siblings died at early infancy. T-B+NK- SCID; low CD132 expression	30.4%	60.3%	22.1%	84.1%	87.6%	30.5%
Pt. 43	c.455T>C (hemizygous); Mother -heterozygous carrier	p.V152A	Missense	Previously reported (18)	X-linked family history. Cousin brother of pt. 78. T-B+NK- SCID with low CD132 expression	–	15.8%	–	–	88.2%	–
Pt. 44	c.752C>G (hemizygous); Mother -heterozygous carrier	p.S251X	Nonsense	Novel	1 elder male sibling died at early infancy due to opportunistic infections. T+B+NK- SCID with low naïve CD3 cells and low CD132 expression	24.3%	25.8%	26.8%	48.3%	81.7%	77.5%
Pt. 46	c.596_598delinsTGGATTAT (hemizygous); Mother -heterozygous carrier	p.E199VfsX76	Frameshift	Novel	Male infant with T-B+NK- SCID with low CD132 expression	25.2%	98.2%	17.5%	83.5%	99.5%	66.3%
Pt. 59	c.8_9insA (hemizygous); Mother -heterozygous carrier	p.P4AfsX31	Frameshift	Novel (Kato et al., Manuscript in submission)	Male infant with T-B+NK- SCID with low CD132 expression; low naïve CD3 cells	51.1%	67.2%	66%	95.2%	98.9%	99.8%
Pt. 63	c.854G>A (hemizygous); Mother -heterozygous carrier	p.R285Q	Missense	Previously reported (19) (Kato et al., Manuscript in submission)	Male infant with T-B+NK- SCID with low CD132 expression	48.8%	50.4%	21.7%	84.1%	90.7%	85.1%
Pt. 78	c.455T>C (hemizygous); Mother -heterozygous carrier	p.V152A	Missense	Previously reported (18) (Kato et al., Manuscript in submission)	X-linked family history. Cousin brother of pt. 43. T-B+NK- SCID	67.8%	59.7%	24.8%	88.4%	89%	29.3%
Pt. 85	c.116-1G>T (hemizygous); Mother -heterozygous carrier	–	Splice-site	Novel (Kato et al., Manuscript in submission)	X-linked family history – 2 maternal uncles died at early infancy with severe infections. T-B+NK- SCID with low CD132 expression	53.7%	55.7%	55.1%	96.1%	92%	90.8%

TABLE 3 | Erythrocyte ADA levels and % dAXP measured in dried blood spots.

Patient	Molecular defect in *ADA*	ADA levels (nmol/h/mg)	% dAXP	PNP levels (nmol/h/mg)
Normal levels		26.4 ± 10.0	<1.0	1354 ± 561
Pt. 22	c.301C>T c.461 G>T	0.1	63.9	1025
Mother of pt. 22	c.461 G>T (heterozygous carrier)	10.8	0	808
Father of pt. 22	c.301C>T (heterozygous carrier)	9.6	0	834
Pt. 30	c.646G>A	0	51.4	1264
Pt. 31	c.478+6T>A	0.3	6.8	1151
Pt. 36	c.407G>A	0	21.1	1532
Pt. 39	c.845G>T	0	54.2	1316
Pt. 74	c.466C>T	0	NA	NA
Pt. 88	c.845G>T	0	NA	929

ADA, adenosine deaminase; AXP (dAXP), total adenosine (deoxyadenosine) nucleotides; PNP, purine nucleoside phosphorylase; NA, Not available.
% dAXP= (dAXP/AXP+dAXP) x100.

BCG was the commonest organism isolated. BCG-site ulceration has been noted in 47 children, however, disseminated BCGosis could be proven in 27 children only. BCG adenitis was noted in one child at D+90 post-HSCT as a part of immune reconstitution inflammatory syndrome. Lack of microbiological confirmation of BCG infection in many patients could have accounted for low rates of disseminated BCGosis in our cohort (**Table 6**) (51, 52). Infants with SCID who had received BCG vaccination and had not developed disseminated infection, are generally started on prophylactic medications—isoniazid and rifampicin at age-appropriate doses, that is generally continued until successful engraftment following HSCT.

Septicemia due to unusual organisms such as *W. confusa* and *A. faecalis* was also noted in our cohort. These are environmental bacteria and usually do not cause invasive infections in immunocompetent hosts. We also noted a high rate of disseminated CMV infection (8.3%) in our cohort. However, several amongst these were identified only on autopsy (60). This underscores the importance of vigilant screening and preventive measures for CMV infection in children with SCID. Cytomegalovirus infection, in our cohort, was possibly transfusion-acquired as most of the children received blood transfusions that are not always leuko-depleted and screened for active CMV infection. Though many patients had clinical features suggestive of *P. jirovecii* pneumonia (tachypnea, hypoxemia, interstitial pneumonia) and were treated for the same, microbiological or histopathological confirmation was possible in only 8 of them.

Thirty-three children had features of OS in our cohort. One child with OS (pt.54) was being treated as severe eczema for 3 years with multiple topical and systemic immunosuppressive agents, and diagnosis of SCID was made only after he developed severe infections. This highlights the importance of early identification of clinical phenotype of OS based on clinical features (generalized erythematous rash with scaling and partial loss of scalp hairs and eyebrows) and referral for appropriate immunological workup. Twenty-eight (28) children with OS had normal or high ALC and 2 children with transplacental-acquired maternal T-cell engraftment had elevated ALC. Laboratory assay of naïve T cells, memory T cells, and HLA-DR expression in T lymphocytes necessary for the diagnosis of OS are currently being performed only in two centers (PGIMER, Chandigarh and NIIH, Mumbai).

Twenty-three children in our cohort who did not have OS had normal ALC (>3 × 10^9/L). However, lymphocyte subsets and naïve T cell estimation revealed diagnosis of SCID in them, thereby highlighting the importance of clinical suspicion and immunological investigations in infants with severe and life-threatening infections even if ALC is normal. Expansion of B cells or NK cells, engraftment of transplacental-acquired maternal T cells, or partial genetic defects allowing selective clone of T cell expansion could be the possible reasons for normal ALC in SCID. Aluri et al. have previously highlighted the importance of assessment of naïve T helper and cytotoxic T cells in children with severe infections and normal ALC to characterise MHC class II and *ZAP70* defects, respectively (7). A child with *IL7RA* defect in our cohort had a T-B+NK- phenotype, similar to the report by Aluri et al. (7). Also, two children with *IL2RG* defect had a T+B-NK- phenotype (1-OS, 1- transplacental-acquired maternal T cell engraftment). A possible explanation for low B cells is the depletion of B cells due to high inflammatory milieu secondary to OS and severe infections (61).

CD132 expression by flow cytometry is currently carried out at only two centers—PGIMER, Chandigarh (North India) and NIIH, Mumbai (West India). At PGIMER, Chandigarh, we found low CD132 expression in lymphocytes by flow cytometry as an inexpensive and rapid method of confirmation of diagnosis of X-linked SCID in 7 children. Two (2) children with X-linked SCID and previously reported variants in *IL2RG* (pt. 25 and pt. 78) had a normal expression of CD132 in lymphocytes (**Table 2**). We could not assay phosphorylated STAT5 in activated T-cells by flow cytometry to determine the functionality of IL2Rγ in many patients due to absent or very low amounts of T cells, however, naïve T cells by flow cytometry and TREC levels by RT-PCR have been assayed in some of them (**Table 2**). Only a handful centers in India (e.g. PGIMER, Chandigarh, North India, and NIIH, Mumbai, West India) have the wherewithal to perform functional studies. Both the centers have performed flow cytometry tests for samples received from other centers, however, timely transportation of viable blood samples from far off places, especially during hot

TABLE 4 | Molecular defects in genes associated with SCID/CID in our cohort.

Pt No	Gene	Type of mutation	Exon	cDNA position	Protein change	Novel or previously reported	References
1. SCID							
Pt. 3	*IL2RG*	Hemizygous- missense	Exon 4	c.515T>G	p.L172R	Novel	Current study
Pt. 4	*IL2RG*	Hemizygous- nonsense	Exon 5	c.737G>A	p.W246X	Previously reported	(20)
Pt. 6	*IL2RG*	Hemizygous- missense	Exon 2	c.185G>A	p.C62Y	Novel	Current study
Pt. 8 and Pt. 25	*IL2RG*	Hemizygous- nonsense	Exon 2	c.202G>T	p.E68X	Previously reported	(17)
Pt. 35	*IL2RG*	Hemizygous- missense	Exon 2	c.170T>A	p.L57H	Novel	Current study
Pt. 43 and Pt. 78	*IL2RG*	Hemizygous- missense	Exon 4	c.455T>C	p.V152A	Previously reported	(18); (Kato T et al. Manuscript in submission)
Pt. 44	*IL2RG*	Hemizygous- nonsense	Exon 5	c.752C>G	p.S251X	Novel	Current Study
Pt. 46	*IL2RG*	Hemizygous- frameshift	Exon 5	c.596_598delinsTGGATTAT	p.E199VfsX76	Novel	Current study
Pt. 51	*IL2RG*	Hemizygous- nonsense	Exon 7	c.865C>T	p.R289X	Previously reported	(21)
Pt. 55	*IL2RG*	Hemizygous- nonsense	Exon 8	c.964C>T	p.Q322X	Previously reported	(22)
Pt. 59	*IL2RG*	Hemizygous- frameshift	Exon 1	c.8_9insA	p.P4AfsX31	Novel	(Kato T et al. Manuscript in submission)
Pt. 63	*IL2RG*	Hemizygous- missense	Exon 2	c.854G>A	p.R285Q	Previously reported	(19); (Kato T et al. Manuscript in submission)
Pt. 71	*IL2RG*	Hemizygous- splice site	Exon 4	c.594+5G>T		Previously reported	(22); (Kato T et al. Manuscript in submission)
Pt. 85	*IL2RG*	Hemizygous- splice site	Exon 2	c.116-1G>T		Novel	(Kato T et al. Manuscript in submission)
Pt. 91	*IL2RG*	Hemizygous- missense	Exon 2	c.854G>A	p.R285Q	Previously reported	(19)
Pt. 92	*IL2RG*	Hemizygous- nonsense	Exon 4	c.505C>T	p.Q169X	Novel	Current study
Pt. 95	*IL2RG*	Hemizygous- missense	Exon 5	c.677G>A	p.R226H	Previously reported	(23)
Pt. 106	*IL2RG*	Hemizygous- nonsense	Exon 1	c.67delC	p.L23X	Novel	Current study
Pt. 118	*IL2RG*	Hemizygous- splice-site	Intron 2	c.269+1G>T		Novel	Current study
Pt. 152	*IL2RG*	Hemizygous- missense	Exon 4	c.520T>A	p.W174R	Novel	Current study
Pt. 168	*IL2RG*	Hemizygous- missense	Exon 5	c.664C>T	p.R222C	Previously reported	(19)
Pt. 169	*IL2RG*	Hemizygous- missense	Exon 3	c.314A>G	p.Y105C	Previously reported	(24)
Pt. 197	*IL2RG*	Hemizygous- frameshift	Exon 3	c.359dupA	p.E121GfsX47	Novel	Current study
Pt. 216	*IL2RG*	Hemizygous- missense	Exon 5	c.670C>T	p.R224W	Previously reported	(22)
Pt. 220	*IL2RG*	Hemizygous- missense	Exon 5	c.664C>T	p.R222C	Previously reported	(19)
Pt. 18	*RAG1*	Homozygous- frameshift	Exon 2	c.1758_1760delinsGAATC	p.D587NfsX5	Novel	Current study
Pt. 19	*RAG1*	Homozygous- frameshift	Exon 2	c.908delC	p.P303LfsX42	Novel	Current study
Pt. 23	*RAG1*	Homozygous- frameshift	Exon 2	c.1758_1760delinsGAATC	p.D587NfsX5	Novel	Current study
Pt. 28	*RAG1*	Homozygous- missense	Exon 2	c.2147G>A	p.R716Q	Previously reported	(25)
Pt. 38	*RAG1*	Homozygous- frameshift	Exon 2	c.1178delG	p.G393AfsX10	Previously reported	(17)
Pt. 58	*RAG1*	Compound heterozygous- frameshift, missense	Exon 2 Exon 2	c.2849delT c.1421G>A	p.I950MfsX28 p.R474H	Previously reported Previously reported	(7)
Pt. 62	*RAG1*	Homozygous- missense	Exon 2	c.2210G>A	p.R737H	Previously reported	(26); (Kato T et al. Manuscript in submission)
Pt. 77	*RAG1*	Homozygous- missense	Exon 2	c.2923C>T	p.R975W	Previously reported	(27); (Kato T et al. Manuscript in submission)
Pt. 84	*RAG1*	Homozygous- missense	Exon 2	c.2923C>T	p.R975W	Previously reported	(27); (Kato T et al. Manuscript in submission)
Pt. 89	*RAG1*	Homozygous- missense	Exon 2	c.1211G>A	p.R404Q	Previously reported	(28); (Kato T et al. Manuscript in submission)
Pt. 105	*RAG1*	Homozygous- nonsense	Exon 2	c.310C>T	p.Q104X	Novel	Current study
Pt. 140	*RAG1*	Homozygous- missense	Exon 2	c.2333G>A	p.R778Q	Previously reported	(27)
Pt. 151	*RAG1*	Homozygous- missense	Exon 2	c.1331C>T	p.A444V	Previously reported	(29)
Pt. 157	*RAG1*	Homozygous- missense	Exon 2	c.1871G>A	p.R624H	Previously reported	(30)
Pt. 170	*RAG1*	Homozygous- missense	Exon 2	c.2326C>T	p.R776W	Previously reported	(31)
Pt. 171	*RAG1*	Homozygous- nonsense	Exon 2	c.424C>T	p.R142X	Previously reported	(32)
Pt. 204	*RAG1*	Compound heterozygous- missense, missense	Exon 2	c.1421G>A; c.1442G>A	p.R474H; p.C481Y	Previously reported; Novel	(29)
Pt. 205	*RAG1*	Compound heterozygous- missense, missense	Exon 2	c.323G>A; c.1228C>T	p.R108Q; p.R410W	Previously reported; Previously reported	(33, 34)
Pt. 5	*RAG2*	Homozygous- missense	Exon 2	c.1247G>T	p.W416L	Previously reported	(35)
Pt. 15 and Pt. 21	*RAG2*	Homozygous- nonsense	Exon 2	c.921G>A	p.W307X	Previously reported	(29)

(Continued)

TABLE 4 | Continued

Pt No	Gene	Type of mutation	Exon	cDNA position	Protein change	Novel or previously reported	References
Pt. 17	*RAG2*	Homozygous- missense	Exon 2	c.1247G>T	p.W416L	Previously reported	(35)
Pt. 27 and Pt. 48	*RAG2*	Homozygous- missense	Exon 2	c.1247G>T	p.W416L	Previously reported	(35)
Pt. 61	*RAG2*	Homozygous- missense	Exon 2	c.1247G>T	p.W416L	Previously reported	(35) (Kato T et al. Manuscript in submission)
Pt. 93	*RAG2*	Homozygous- missense	Exon 2	c.95G>A	p.G32E	Novel	Current study
Pt. 96	*RAG2*	Homozygous- missense	Exon 2	c.608G>A	p.G203E	Novel	Current study
Pt. 116	*RAG2*	Homozygous- missense	Exon 2	c.644C>T	p.T215I	Previously reported	(36)
Pt. 172	*RAG2*	Homozygous- frameshift	Exon 2	c.1056delA	p.D353MfsX91	Previously reported	(13)
Pt. 207	*RAG2*	Homozygous- missense	Exon 2	c.329T>C	p.M110T	Novel	Current study
Pt. 209	*RAG2*	Compound heterozygous- missense, frameshift	Exon 2	c.303T>G; c.171delG	p.N101K; p.K58SfsX73	Novel; Previously reported	Current study; (7)
Pt. 9	*DCLRE1C*	Homozygous- large deletion	Exon 1-3	EX1_EX3del		Previously reported	(37)
Pt. 20	*DCLRE1C*	Homozygous- large deletion	Exon 1-3	EX1_EX3del		Previously reported	(37)
Pt. 26	*DCLRE1C*	Homozygous- large deletion	Exon 1-3	EX1_EX3del		Previously reported	(37)
Pt. 52	*DCLRE1C*	Homozygous- large deletion	Exon 1-3	EX1_EX3del		Previously reported	(37)
Pt. 56	*DCLRE1C*	Homozygous- large deletion	Exon 1-3	EX1_EX3del		Previously reported	(37)
Pt. 64	*DCLRE1C*	Homozygous- large deletion	Exon 1-3	EX1_EX3del		Previously reported	(37); (Kato T et al. Manuscript in submission)
Pt. 66	*DCLRE1C*	Homozygous- large deletion	Exon 1-3	EX1_EX3del		Previously reported	(37); (Kato T et al. Manuscript in submission)
Pt.70	*DCLRE1C*	Homozygous- large deletion	Exon 1-3	EX1_EX3del		Previously reported	(37); (Kato T et al. Manuscript in submission)
Pt. 90	*DCLRE1C*	Homozygous- large deletion	Exon 1-3	EX1_EX3del		Previously reported	(37); (Kato T et al. Manuscript in submission)
Pt. 99	*DCLRE1C*	Homozygous- frameshift	Exon 10	c.874dupA	p.M292NfsX33	Novel	Current study
Pt. 115	*DCLRE1C*	Homozygous- large deletion	Exon 1-3	EX1_EX3del		Previously reported	(37)
Pt. 22	*ADA*	Compound heterozygous- missense, missense	Exon 4 Exon 5	c.301C>T c.461 G>T	p.R101W p.C154F	Previously reported; Novel	(38); Current study
Pt. 30 and Pt. 47	*ADA*	Homozygous- missense	Exon 7	c.646G>A	p.G216R	Previously reported	(39)
Pt. 31	*ADA*	Homozygous- splice-site	Intron 6	c.478+6T>A		Novel	Current study
Pt. 36	*ADA*	Homozygous- missense	Exon 5	c.407G>A	p.G136D	Novel	Current study
Pt. 39	*ADA*	Homozygous- missense	Exon 9	c.845G>T	p.R282L	Previously reported	(40)
Pt. 74	*ADA*	Homozygous- missense	Exon 5	c.466C>T	p.R156C	Previously reported	(41); (Kato T et al. Manuscript in submission)
Pt. 88	*ADA*	Homozygous- missense	Exon 9	c.845G>T	p.R282L	Previously reported	(40); (Kato T et al. Manuscript in submission)
Pt. 119	*ADA*	Homozygous- deletion	Exon 2	EX2_del		Novel	(Kato T et al. Manuscript in submission)
Pt. 123	*ADA*	Homozygous- missense	Exon 11	c.1028T>C	p.L343P	Previously reported	(42)
Pt. 153	*ADA*	Homozygous- splice-site	Intron 10	c.975+2T>G		Novel	Current study
Pt. 154	*ADA*	Compound heterozygous- nonsense, missense	Exon 6 Exon 8	c.523C>T; c.716G>A	p.Q175X; p.G239D	Previously reported; Previously reported	(7, 43)
Pt. 196	*ADA*	Compound heterozygous- nonsense, missense	Exon 6 Exon 8	c.523C>T; c.716G>A	p.Q175X; p.G239D	Previously reported; Previously reported	(7, 43)
Pt. 208	*ADA*	Homozygous- nonsense	Exon 6	c.523C>T	p.Q175X	Previously reported	(7)
Pt. 29	*JAK3*	Compound heterozygous- missense, missense	Exon 8 Exon 6	c.1048C>T; c.704T>C	p.R350W; p.M235T	Novel Novel	Current study Current study
Pt. 50	*JAK3*	Compound heterozygous- frameshift, missense	Exon 2 Exon 10	c.115delC c.T1289C	p.Q39SfsX108 p.I430T	Novel Novel	Current study Current study
Pt. 102	*JAK3*	Homozygous- nonsense	Exon 19	c.2605C>T	p.Q869X	Novel	Current study
Pt. 107	*JAK3*	Homozygous- frameshift	Exon 22	c.3049_3050delCT	p.L1017VfsX24	Novel	Current study
Pt. 121	*JAK3*	Homozygous- missense	Exon 11	c.1442A>G	p.E481G	Previously reported	(44)
Pt. 198	*JAK3*	Homozygous- missense	Exon 13	c.1765G>T	p.G589C	Novel	Current study
Pt. 10	*IL7RA*	Homozygous- large deletion	Exon 4-8	EX4_EX8del		Novel	Current study
Pt. 16	*IL7RA*	Homozygous- nonsense	Exon 5	c.616C>T	p.R206X	Previously reported	(45)
Pt. 94	*IL7RA*	Homozygous- frameshift	Exon 5	c.623delT	p.I208TfsX244	Novel	Current study
Pt. 108	*IL7RA*	Homozygous- splice-site	Intron 5	c.707-1G>T		Novel	Current study

(Continued)

TABLE 4 | Continued

Pt No	Gene	Type of mutation	Exon	cDNA position	Protein change	Novel or previously reported	References
Pt. 114	*IL7RA*	Homozygous- missense	Exon 4	c.509G>C	p.R170P	Novel	Current study
Pt. 155	*IL7RA*	Homozygous- large deletion	Exon 4-8	EX4_EX8del		Novel	Current study
Pt. 200	*IL7RA*	Homozygous- missense	Exon 3	c.324T>G	p.C108W	Novel	Current study
Pt. 219	*IL7RA*	Homozygous- nonsense	Exon 5	c.616C>T	p.R206X	Previously reported	(45)
Pt. 14	*PNP*	Homozygous- nonsense	Exon 3	c.244C>T	p.Q82X	Previously reported	(46)
Pt. 156	*PNP*	Homozygous- splice-site	Intron 3	c.286-18G>A		Previously reported	(47)
Pt. 111	*CD3D*	Homozygous- nonsense	Exon 2	c.158C>A	p.S53X	Novel	Current study
Pt. 137	*CD3D*	Homozygous- splice-site	Intron 2	(IVS2-2A>G)		Previously reported	(48)
Pt. 117	*CD3E*	Homozygous- nonsense	Exon 6	c.288T>A	p.Y96X	Novel	Current study
Pt. 150	*CD3E*	Homozygous- splice-site	Intron 6	c.352+1G>A		Novel	Current study
Pt. 68	*NHEJ1*	Homozygous- frameshift	Exon 5	c.544_545delGA	p.E182TfsX3	Novel	(Kato T et al. Manuscript in submission)
Pt. 72	*NHEJ1*	Homozygous- frameshift	Exon 3	c.221_222delGT	p.C74SfsX4	Novel	(Kato T et al. Manuscript in submission)
Pt. 49	*CORO1A*	Homozygous- splice-site	Intron 7	c.862-2A>G		Novel	Current study
2. CID							
Pt. 210	*CIITA*	Homozygous- nonsense	Exon 16	c.3122C>A	p.S1041X	Novel	Current study
Pt. 158	*RFXANK*	Homozygous- frameshift	Exon 6	c.430dupC	p.L144PfsX37	Novel	Current study
Pt. 211	*RFX5*	Homozygous- missense	Exon 7	c.446G>A	p.R149Q	Previously reported	(49)
Pt. 173	*DOCK2*	Homozygous- nonsense	Exon 34	c.3430C>T	p.R1144X	Previously reported	(13)
Pt. 41	*STK4*	Homozygous- nonsense	Exon 10	c.1165C>T	p.Q389X	Novel	Current study
Pt. 104	*SP110*	Homozygous- nonsense	Exon 8	c.855G>A	p.W285X	Novel	Current study
Pt. 86	*STIM1*	Homozygous- missense	Exon 10	c.1285C>T	p.R429C	Novel	(Kato T et al. Manuscript in submission)

Molecular analysis results of patients 221–277 are previously reported (7).

summers remains a significant problem (11, 12). Lack of state-of-the-art facilities to do functional assays in all patients with SCID is one of the limitations of our study. Establishment of more clinical immunology laboratories, training of necessary manpower, and improvement in existing laboratory services are needed to overcome these barriers (11, 12).

Genetic confirmation of diagnosis of SCID is necessary for identification of pattern of inheritance and genetic counselling of affected families. Eighty-two (82) patients did not undergo a molecular analysis for confirmation of diagnosis due to lack of easy access to molecular diagnostics and financial difficulties. With the establishment of commercial NGS laboratories and reduction in costs involved for genetic sequencing over last few years, NGS-based diagnostics have become feasible in India (7, 13). In-house NGS facility for molecular diagnosis of PID is currently available only at PGIMER, Chandigarh (North India) and Christian Medical College, Vellore (South India). Most of the patients with SCID present in a critically-ill state and convincing families for genetic studies is often challenging due to significant financial and social constraints. It must be noted that expenses for molecular diagnosis are borne by the families in India most of the times as it is not covered by state or insurance schemes. Despite these challenges, we have been able to perform genetic studies in 195 patients. Academic collaborations with institutes at Hong Kong, Japan, and USA helped the centre at PGIMER, Chandigarh (North India) to get free molecular diagnosis for the families who cannot afford for costly molecular tests. We prefer to store blood samples in terminally-ill patients and later call the family for counselling to undergo genetic tests, as confirmation of molecular diagnosis has helped the families to undergo antenatal testing in subsequent pregnancies.

Defects in *RAG1/2* were found to be commonest in our cohort followed by *IL2RG*, *DCLRE1C*, and *ADA*. This is similar to the previous reports from Turkey, Iran, and Serbia (**Table 6**) (8, 52, 54). MHC Class II defect and defects in *STIM1*, *DOCK2*, *SP110*, *ZAP70*, and *STK4* genes are categorized as combined immunodeficiencies as per the 2019 International Union of Immunological Societies Expert Committee classification of human inborn errors of immunity (IEI) (5). However, we have included children with these defects in our cohort because they had severe infections from early infancy mimicking the clinical presentation of SCID (7).

Clinical phenotype of patients with *RAG1/2* defects in our cohort was very heterogenous. This included classical SCID, OS, atypical/leaky SCID phenotype, autoimmunity in form of AIHA, and development of hematological malignancy such as Hodgkin lymphoma. Wide spectrum of clinical manifestations could be due to difference in VDJ recombination activity or influence of other genetic or environmental factors (34, 62). Other reported clinical phenotypes in *RAG1/2* such as cutaneous granulomas, CVID-like phenotype or elevated γδ T cells were not seen in our cohort.

Low or undetectable ADA levels and elevated %dAXP levels were seen in 7 and 5 children with *ADA* defect, respectively. We noted that %dAXP levels in 2 children (pt. 31 and 36) were lower compared to other 3 children. While pt.31 had a milder clinical phenotype, pt.36 had features of OS. This suggests that low levels

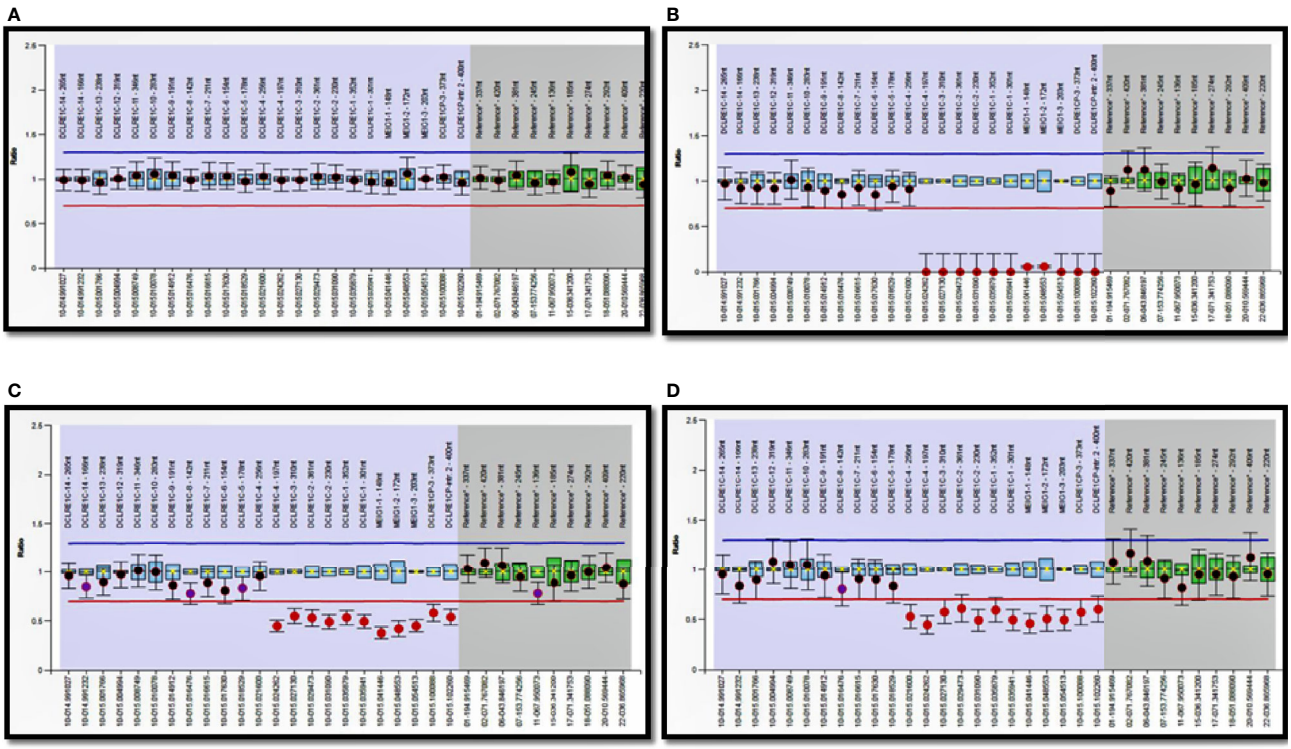

FIGURE 5 | MLPA data was analyzed using Coffalyser software. **(A)** Healthy control sample having a Dosage Quotient (DQ) between 0.80 and 1.20; **(B)** A patient with T-B-NK+ SCID (pt. 66) showing a homozygous deletion and DQ=0; **(C, D)** Parents of index child showing a heterozygous deletion with DQ Score in range of 0.40 to 0.65.

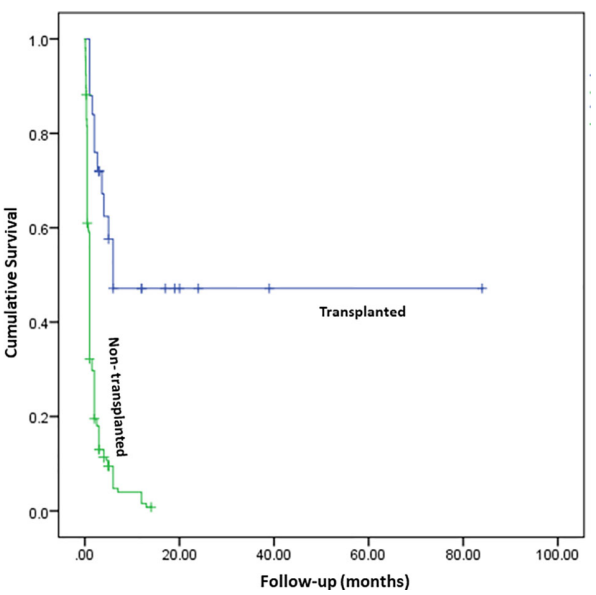

FIGURE 6 | Survival curve comparing the outcome of children who underwent HSCT (n=23) and children who were not transplanted (n=213), $p < 0.001$ (Log Rank Mantel-Cox). Total person follow-up months—629.13 months.

of accumulation of dAXP with residual ADA activity in lymphocytes may play a role in development of restricted T-cell clones that could be responsible for partial immunity and development of OS (63).

One child with *ADA* defect (pt. 36) had evidence of nephrotic syndrome along with OS. Renal abnormalities described with *ADA* defect (such as diffuse mesangial sclerosis) could result in nephrotic syndrome (64). However, renal involvement in OS manifesting as nephrotic syndrome has also been previously reported (65). We could not perform renal biopsy in this child due to severe ascites. Two other children with OS in our cohort also had renal involvement at autopsy—focal segmental glomerulosclerosis and mesangial sclerosis. Both of them also had severe infections—disseminated CMV in one and invasive aspergillosis in other. Whether the renal abnormalities are the result of genetic defect, inflammatory phenotype of OS, or severe infections is not clear and further research is needed in this regard.

Identification of radiosensitive forms of SCID is essential in B-NK+ SCID before HSCT as these children are prone to toxicity by chemotherapeutic drugs and radiation (66). Amongst the radiosensitive forms of SCID, molecular defects are predominantly noted in *DCLRE1C* in our cohort. Moreover, only mutation in *DCLRE1C* observed in North Indian children (n=9) was EX1_EX3 del. Initial MLPA screening for *DCLRE1C*

TABLE 5 | Details of hematopoietic stem cell transplantation of 13 children with SCID.

Patient	Type of SCID	Molecular defect	Centre	Age at transplantation	Donor characteristics	Outcome
Pt. 19	T-B-NK+	*RAG1*	PGIMER, Chandigarh	3.5 months	Father who is a complete HLA match with child	Developed BCG IRIS post-HSCT (D+90)- successfully treated with isoniazid, rifampicin, and ethambutol. He successfully engrafted and is currently doing well at 3rd year follow-up.
Pt. 31	T-B-NK-	*ADA*	Diagnosed at PGIMER, Chandigarh; transplanted at Apollo Hospitals, Chennai	4 years	Fully matched unrelated donor	Engrafted and doing well at 1st year follow-up.
Pt. 80	T-B-NK+	-	PGIMER, Chandigarh	18 months	Haploidentical donor	Developed graft failure. Underwent second transplant at 3 years (details not available).
Pt. 94	T-B+NK+	*IL7RA*	Aster CMI Hospitals, Bengaluru	5 months	Haploidentical (Mother)	Delayed graft failure (6 months post-HSCT). Underwent second HSCT with father being donor. Successfully engrafted and doing well at 20 months follow-up.
Pt. 103	T-B+NK+	N.A.	Aster CMI Hospitals, Bengaluru	1.5 months	Haploidentical (Mother)	Failed engraftment. Underwent second HSCT with father being donor- successfully engrafted, however, died after 1.5 months due to fulminant sepsis.
Pt. 105	T-B-NK+	*RAG1*	Aster CMI Hospitals, Bengaluru	11 months	Haploidentical (Mother)	Successfully engrafted. Doing well 1.5 years post-HSCT.
Pt. 106	T-B+NK-	*IL2RG*	Aster CMI Hospitals, Bengaluru	5 months	Haploidentical (Mother)	Successfully engrafted. Doing well 1.4 years post-HSCT.
Pt. 108	T-B+NK-	*IL7RA*	Aster CMI Hospitals, Bengaluru	8.5 months	Fully matched sibling	Died D+20 of HSCT- MDR Klebsiella sepsis.
Pt. 110	T-B-NK+	N.A.	Aster CMI Hospitals, Bengaluru	5 months	Haploidentical (Mother)	Successfully engrafted. Doing well 10 months post-HSCT.
Pt. 111	T-B+NK+	*CD3D*	Aster CMI Hospitals, Bengaluru	6 months	Haploidentical (Mother)	Expired D+9 due to pulmonary haemorrhage.
Pt. 114	T-B+NK+	*IL7R*	Aster CMI Hospitals, Bengaluru	9 months	Haploidentical (Father)	Developed severe gut GVHD and died D+60.
Pt. 115	T-B-NK+	*DCLRE1C*	Aster CMI Hospitals, Bengaluru	7.5 months	Haploidentical (Father)	Successfully engrafted. Doing well 2 months post-HSCT.
Pt. 152	T-B+NK-	*IL2RG*	Aditya Birla Memorial Hospital, Pune	5 months	Matched family donor	Doing well at 7th year follow-up.

exon 1-3 deletion before NGS in children with B-NK+ SCID was found to be more cost-effective than subjecting these children to NGS without a MLPA screen. The former approach is preferred at Chandigarh (North India) because of two reasons—NGS can miss large deletions and patients identified to have EX1_EX3 del in *DCLRE1C* by MLPA do not need to undergo NGS that is four to five times more expensive than MLPA in India. We also describe molecular defects in *STK4*, *CORO1A*, *CD3D*, *CD3E*, and *SP110* for the first time in India. Clinical phenotype of eczema, AIHA, and CD4 lymphopenia noted in *STK4* defect (pt. 41) has been previously described (67). Moshous et al. have described EBV-induced B cell lymphoma and naïve T-cell lymphopenia in patients with a hypomorphic missense variant in *CORO1A* (c.717G>A) (68). Our patient (pt. 49) with a novel splice-site defect in *CORO1A* (c.862-2A>G) had CD3 and CD4 lymphopenia, and developed an intracranial B cell lymphoma at 3.5 years of age.

A significant proportion of children (n=254) could not be subjected to HSCT due to medical and social reasons and succumbed to the illness. Presence of fulminant infections at time of diagnosis and lack of financial support dissuaded many families to undergo a costly procedure like HSCT. At present, facilities for pediatric HSCT for IEI are available at very few centers in India. Two centers in India have carried out most of the transplants for SCID – Apollo Children's Hospitals, Chennai (South India) and Aster CMI Hospitals, Bengaluru (South India). Establishment of such dedicated pediatric HSCT units and development of manpower for HSCT services across the country are the need of the hour to ensure easy access to these services for affected patients. Provision of financial support from the government to affected families to undergo HSCT will also be required for successful outcomes. Studies from Western countries have shown that children with SCID transplanted below the age of 3.5 months of age had a significantly better outcome compared to children who underwent transplantation later (58). Though the age at diagnosis in our cohort is similar to countries where newborn screening has not been initiated, delayed referrals, presence of life-threatening infections at presentation, and lack of easy access to pediatric HSCT accounted for the unacceptable mortality rates in our cohort (**Figure 6**) (**Table 6**) (51, 52). We also realise that diagnosis of SCID is still being missed in most babies in India. Institution of universal newborn screening for SCID would provide more accurate estimates of incidence of SCID in our country and would also facilitate early diagnosis and treatment. However, financial implications and cost-effectiveness of implementing such a programme in a country as large, and as diverse, as India need to be worked out by health planners (69).

To conclude, we describe the largest multicentric cohort of SCID from India and document several novel mutations. Number of children with molecular diagnosis and those who

TABLE 6 | Comparison of our cohort with published multicentric studies on SCID from other countries in last 10 years.

Study (Year)	Place	No. of patients	Age of onset and diagnosis	Clinical manifestations	Molecular defects	Outcome
Yao et al. (51)	Shanghai, China	44; Male: female – 40:4	Mean age of onset – 3.56 ± 3.91 months Mean age at diagnosis – 7.1 ± 7.96 months	BCG-related complications noted in 14 children (31.8%). Three (3) had disseminated BCG infection. Two (2) had CMV infection	Defect in *IL2RG* noted in 25 children (56.8%).	Mortality seen in 37 children (84%). Six (6) children underwent HSCT and 1 of them had survived.
Pasic et al. (52)	Serbia and Montenegro	21	Median age of onset – 2 months	BCG-related complications in 41%. Pneumonia noted in 15 children (PJP- 5, CMV- 3, BCG-2, respiratory virus- 5). OS noted in 6 children.	17 had proven molecular defect (81%). *RAG1/2* commonest (12) followed by *IL2RG* (3), *JAK3* (2), *DCLRE1C* (1)	Mortality seen in 16 children (76.2%). Eight (8) children underwent HSCT and 5 of them survived.
Lee et al. (53)	South East Asia	42; Male: female – 30:12	Median age of onset and diagnosis – 2 and 4 months, respectively.	BCG-related complications in 10 children (23.8%) – 6 had localized reaction; 3 had regional adenitis; 3 had disseminated BCGosis. Oral thrush (12), CMV (2), and PJP (2) are other documented infections. OS noted in 4 children.	26 had proven molecular defect (61.9%). *IL2RG* commonest (19) followed by *IL7RA* (2), *JAK3* (2), *RAG1/2* (2), *DCLRE1C* (1)	12 children underwent HSCT and 8 of them survived.
Abolhassani et al. (54)	Iran	169; Male: female – 96:73	Mean age of onset and diagnosis – 4.2 and 8.6 months, respectively.	BCG-related complications noted in 23 (13.6%). Other infections noted are PJP (13), CMV (15), EBV (8), VDPV (6), Cryptococcus (6), and VZV (6). OS noted in 11 children.	37 had proven molecular defect (21.9%). *RAG1/2* commonest (19) followed by *IL2RG* (3), *JAK3* (3), *DCLRE1C* (3), *ADA* (2), *IL7RA* (2), *CD3E* (1), *CD3D* (1), *PRKDC* (1), *NHEJ1* (1), *PTPRC* (1)	NA
Rozmus et al. (55)	Canada	40	Mean age at diagnosis – 4.2 months.	Oral thrush (8), CMV (6), PJP (6), RSV (1), and adenovirus (1) are the infections noted.	20 had proven molecular defect (50%). *ADA* commonest (10) followed by *IL2RG* (4), *RAG1* (2), *ZAP70* (2), and MHC Class II defects (2).	Mortality observed in 12 children (30%). Fifteen (15) underwent HSCT and 10 of them survived.
Ikinciogullari et al. (8)	Turkey	234 (transplanted patients); Male: female – 145:89	Median age at diagnosis – 5 months.	Infections noted are oral thrush (51.5%), CMV (13.5%), bacterial infections (7.4%), BCG-related complications (2.2%), and respiratory viruses (4.4%)	42.3% had proven molecular defects – *RAG1/2* (15.4%), *JAK3* (6.8%), *IL2RG* (6%), *DCLRE1C* (5.6%)	Survival at 20 years is 65.7%
Mazzucchelli et al. (56)	Brazil	70; Male: female – 49:21	Mean age of onset and diagnosis – 3.3 and 6.7 months, respectively.	BCG-related complications seen in 39 children (55.7%) – disseminated form in 29 and localized in 10. Features of OS noted in 8 children.	NA	Mortality seen in 35 patients (50%). Thirty (30) underwent HSCT and 18 of them survived.
de Pagter et al. (57)	Netherlands	43	Median delay in diagnosis in typical and atypical SCID – 2 and 27 months, respectively.	Infections noted are bacterial sepsis (11), PJP (11), CMV (8), and BCGitis (6). AIHA seen in 5 children with atypical SCID.	*IL2RG* (21%), *RAG1* (21%), *RAG2* (5%), *ADA* (12%), *DCLRE1C* (7%), *PNP* (7%), and *IL7RA* (5%)	Mortality observed in 18 children (41.9%). Thirty-two (32) underwent HSCT and 24 of them survived. Two (2) underwent gene therapy and 1 survived.
Haddad et al. (58)	USA and Canada	662 (transplanted patients); Male: female – 471:191	Median age at diagnosis – 141.5 days (4.7 months)	NA	*IL2RG* (187), *RAG1/2* (52), *ADA* (45), *IL7RA* (40), *DCLRE1C* (28), *JAK3* (24), CD3 receptor defects (7), *PNP* (1), *AK2* (1), *CD45* (1)	Survival is better in children transplanted less than 3.5 months. Survival at 10 years is 71% and is higher with matched sibling

(Continued)

TABLE 6 | Continued

Study (Year)	Place	No. of patients	Age of onset and diagnosis	Clinical manifestations	Molecular defects	Outcome
						donors compared to other donor types.
Micho et al. (59)	Greece	30; Male: female – 19:11	Median age at diagnosis – 6.2 months	NA	*DCLRE1C* (3), *IL2RG* (2), *JAK3* (2), *RAG1* (2), *ADA* (2), *PNP* (1)	Mortality is observed in 15 children (50%). Twenty-two (22) underwent HSCT and 14 of them are doing well.
Aluri et al. (7)	India	57; Male: female – 40:17	Median age of onset and diagnosis – 2 and 5.1 months, respectively	Infections observed include oral thrush (21%), BCG-related complications (12%), and PJP (1). OS noted in 4 children	49 children had proven molecular defects (86%). *RAG1/2* commonest (12), followed by *JAK3* (9), *IL2RG* (9), MHC Class II defects (6), *ADA* (5), *DCLRE1C* (2), *ZAP70* (2), *IL7RA* (1), *PRKDC* (1), *PNP* (1), and *AK2* (1)	Mortality observed in 47 children (82.5%). Four (4) underwent HSCT and none survived.
Present study (2020)	India	277 (23 CID, 254 SCID); Male: female – 196:81	Median age of onset and diagnosis – 2.5 and 5 months, respectively	BCG-related complications in 47 patients (17%) – localized form (20) and disseminated BCGosis (27). Other common infections include bacteria (72), CMV (23), Candida sp. (23), PJP (8), Aspergillus sp. (5), VAPP/VDPV (2). OS noted in 33 children. AIHA and lymphoreticular malignancy observed in 5 and 2 children, respectively.	162 patients had proven molecular defects (58.5%) - *RAG1/2* (43), *IL2RG* (36), *ADA* (19), *JAK3* (15), *DCLRE1C* (13), *IL7RA* (9), *PNP* (3), *CIITA* (2), *RFXAP* (3), *RFXANK* (2), *NHEJ1* (2), *CD3E* (2), *CD3D* (2), *RFX5* (2), *ZAP70* (2), *STK4* (1), *CORO1A* (1), *STIM1* (1), *PRKDC* (1), *AK2* (1), *DOCK2* (1), and *SP100* (1)	Mortality noted in 210 children (75.8%). Twenty-three (23) underwent HSCT and 11 of them are doing well.

BCG, Bacillus Calmette-Guerin; CMV, Cytomegalovirus; HSCT, Hematopoietic stem cell transplantation; OS, Omenn syndrome; PJP, Pneumocystis jirovecii pneumonia; EBV, Epstein-Barr virus; VDPV, Vaccine-derived polio virus; VZV, Varicella zoster virus; AIHA, Autoimmune hemolytic anemia; VAPP, Vaccine-associated paralytic polio; CID, Combined Immune Deficiency; SCID, Severe Combined Immune Deficiency.

have undergone HSCT has increased significantly in last decade. However, we are only too aware of our limitations. Improvement in awareness amongst physicians and pediatricians, expansion of diagnostic laboratories, institution of newborn screening, development of pediatric HSCT services, and financial support to the families to undergo HSCT are essentially needed for a better diagnosis and outcome of affected patients in the country.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

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 minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PV, AS, AGum, JN, AJ, DS, AGup, AlK, MD, PT, VG, AP, SagB, SR, RC, MeS, DM, SarB, ArR, AA, FN, BJ, AM, HL, RU, RR, SanB, and SuS—Clinical management of patients; provided necessary clinical details for compilation. AmR, RK, MaS, AnK, BS, RM, KaS, AD, NJ, PK, MM, AV, KoS, SrS, YO, TK, KI, KC, DL, OO, SN, MH, and Y-LL—Laboratory work-up of patients; provided necessary laboratory results for compilation. KG—Provided necessary histopathology details. PV, RK, AS, AGum, MaS, AnK, and JN—Compiled the data and framed the initial draft and editing of manuscript. PV, RK—Literature search. PV, AmR, and SuS—Editing of manuscript at all stages of preparation and final approval. 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.2020.619146/full#supplementary-material

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Clinical and Genetic Profile of X-Linked Agammaglobulinemia: A Multicenter Experience From India

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Background: There is paucity of literature on XLA from developing countries. Herein we report the clinical and molecular profile and outcome in a multicenter cohort of patients with XLA from India.

Methods: Data on XLA from all regional centers supported by the Foundation for Primary Immunodeficiency Diseases (FPID), USA and other institutions providing care to patients with PIDs were collated. Diagnosis of XLA was based on European Society for Immunodeficiencies (ESID) criteria.

Results: We received clinical details of 195 patients with a provisional diagnosis of XLA from 12 centers. At final analysis, 145 patients were included (137 'definite XLA' and eight 'probable/possible XLA'). Median age at onset of symptoms was 12.0 (6.0, 36.0) months and median age at diagnosis was 60.0 (31.5, 108) months. Pneumonia was the commonest clinical manifestation (82.6%) followed by otitis media (50%) and diarrhea (42%). Arthritis was

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seen in 26% patients while 23% patients developed meningitis. Bronchiectasis was seen in 10% and encephalitis (likely viral) in 4.8% patients. *Pseudomonas aeruginosa* was the commonest bacterial pathogen identified followed by *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. Molecular analysis revealed 86 variants in 105 unrelated cases. Missense variants in *BTK* gene were the most common (36%) followed by frameshift (22%) and nonsense variants (21%). Most pathogenic gene variants (53%) were clustered in the distal part of gene encompassing exons 14–19 encoding for the tyrosine kinase domain. Follow-up details were available for 108 patients. Of these, 12% had died till the time of this analysis. The 5-year and 10-year survival was 89.9% and 86.9% respectively. Median duration of follow-up was 61 months and total duration of follow-up was 6083.2 patient-months. All patients received intravenous immunoglobulin (IVIg) replacement therapy. However, in many patients IVIg could not be given at recommended doses or intervals due to difficulties in accessing this therapy because of financial reasons and lack of universal health insurance in India. Hematopoietic stem cell transplant was carried out in four (2.8%) patients.

Conclusion: There was a significant delay in the diagnosis and facilities for molecular diagnosis were not available at many centers. Optimal immunoglobulin replacement is still a challenge

Keywords: arthritis, *BTK* gene, intravenous immunoglobulin, neutropenia, X-linked agammaglobulinemia

INTRODUCTION

X-linked agammaglobulinemia (XLA) is one of the most frequent inborn errors of immunity (IEI). Patients typically present with recurrent sinopulmonary and gastrointestinal infections (1). Meningitis, sepsis, arthritis, skin and soft tissue infections and enteroviral encephalitis are the other common clinical manifestations seen in these patients. Autoimmune and inflammatory complications have also been reported rarely (2). XLA results from loss of function variants in Bruton Tyrosine Kinase (*BTK)* gene (3, 4) which is located on long arm of X chromosome (Xq21.3 to Xq22) (5). Pathogenic variants in *BTK* gene lead to maturation arrest of developing B-lymphocytes in the bone marrow at pre-B cell stage (3, 4). Diagnosis is based on presence of pan-hypogammaglobulinemia and absence of mature B-lymphocytes in peripheral blood. Confirmation of diagnosis requires evidence of reduced expression of Btk protein on flow cytometry (or on Western blot) and *BTK* gene sequencing.

There is paucity of literature on XLA from developing countries. This is largely because of lack of awareness and dearth of appropriate diagnostic facilities (6–8). We reported a single center experience on 36 patients with XLA from Chandigarh, India in 2016 (9). Apart from this, literature on XLA from India is limited to single cases report and a small case series (10–19). Clinical profile of patients with XLA may vary from one country to another (20). This could be because of regional differences in infection profile or differences in genetic profile among populations. For instance, vaccine associated paralytic poliomyelitis (VAPP) has been reported from countries where live oral poliovirus is still being used (21). Survival rates of patients with XLA beyond 20 years vary from 70% in developed regions of the world (e.g. North America, Europe and Australia) to 40% in developing countries in Asia and Africa (20, 22).

Herein we report the clinical and molecular profile and outcome in a multicenter cohort of patients with XLA from India. This is the first attempt at nationwide data collection on XLA.

METHODS

Data on XLA from all regional centers supported by the Foundation for Primary Immunodeficiency Diseases (FPID), USA and other institutions providing care to patients with PIDs were collated. These centers included Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, North India; Bai Jerbai Wadia Hospital for Children, Mumbai, West India; National Institute of Immunohematology (NIIH), Mumbai, West India; Government Medical College, Kozhikode, Kerala, South India; Aster CMI Hospital, Bengaluru, South India; Sanjay Gandhi Postgraduate Institute of Medical Sciences (SPGIMS), Lucknow, North India; Apollo Hospitals, Chennai, South India; Kasturba Medical College (KMC), Mangalore, South India; Sir Ganga Ram Hospital (SGRH), New Delhi, North India; Kanchi Kamakoti Childs Trust Hospital, Chennai, South India; Nizam's Institute of Medical Sciences, (NIMS) Hyderabad, South India, and Niruj Rheumatology Clinic, Ahmedabad, Gujarat, West India (**Figure 1**). All centers were contacted *via* email and requested to provide details of their patients with XLA on a pre-designed Microsoft Excel sheet. Details included demographic information, clinical manifestations, immunological investigations, genetic diagnosis, treatment and

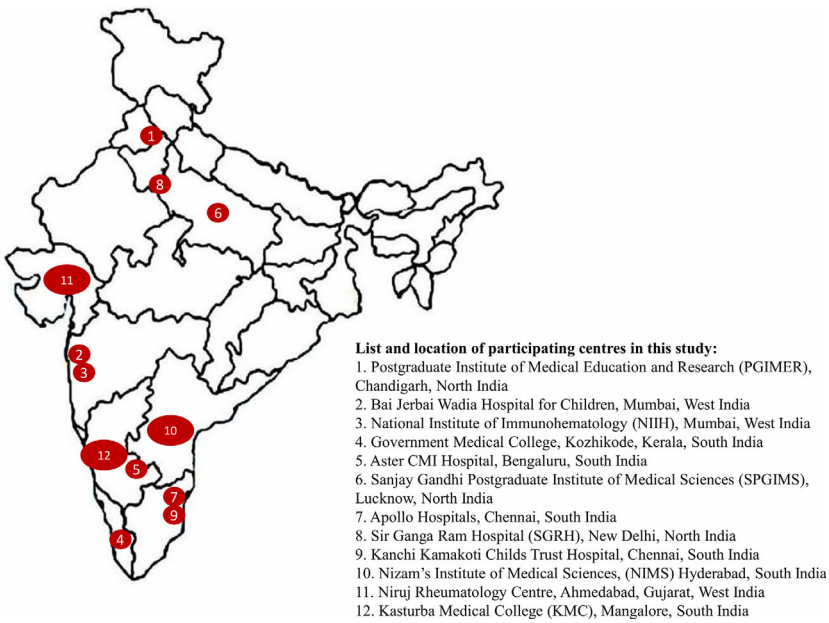

FIGURE 1 | Shows list and locations of centers that participated in this multicenter study.

follow-up. The central data collection center was PGIMER, Chandigarh (Review board no.: 194-20)

Assay of serum immunoglobulins (IgG, IgA, and IgM) are now being carried out by nephelometry or turbidimetry at most centers in India. In the past, radial immunodiffusion (RID) was the preferred method for assay. Lower limit for detection of serum immunoglobulins varied depending on type of technique and type of kit used. For instance, in Chandigarh the lower limits for detection of IgG, IgA, and IgM are 2.02 gm/l, 0.17 gm/l, and 0.25 gm/l. Serum immunoglobulins were reported to be 'below lower limit of detection' in several patients and absolute value was not given. Serum IgE was assessed using enzyme linked immunosorbent assay (ELISA). Lymphocyte subset analysis and Btk protein estimation on peripheral blood monocytes was performed using flow cytometry (9).

BTK gene analysis was carried out using Sanger sequencing, targeted next generation sequencing or whole exome sequencing depending on availability and preference at individual centers.

Diagnosis of XLA was based on European Society for Immunodeficiencies (ESID) criteria (23). The ESID criteria defines '*Definitive XLA*' as a male patient with less than 2% CD19+ B cells and at least one of the following: 1) Mutation in *BTK* gene; 2) Absent Btk mRNA on northern blot analysis of neutrophils or monocytes; 3) Absent Btk protein in monocytes or platelets; 4) Maternal cousins, uncles or nephews with less than 2% CD19+ B cells. '*Probable XLA*' is defined as a male patient with less than 2% CD19+ B cells in whom all of the following are positive: 1) Onset of recurrent bacterial infections in the first 5 years of life; 2) Serum IgG, IgM and IgA more than 2SD below normal for age; 3) Absent isohemagglutinins and /or poor response to vaccines; 4) Other causes of hypogammaglobulinemia have been excluded. '*Possible XLA*' is defined as a male patient with less than 2% CD19+ B cells in whom other causes of hypogammaglobulinemia have been excluded and at least one of the following is positive: 1) Onset of recurrent bacterial infections in the first 5 years of life; 2) Serum IgG, IgM and IgA more than 2 SD below normal for age; 3) Absent isohemagglutinins.

For purposes of the present study, male patients with hypogammaglobulinemia and decreased or absent B cells and X-linked family history (i.e. involvement of male siblings, maternal cousins, maternal uncles or nephews) were labeled as *'probable'* or *'possible'* XLA. Patients with hypogammaglobulinemia and low B cell numbers who had no suggestive X-linked family history and in whom Btk protein expression was either not performed or was normal and *BTK* gene sequencing could not be carried out, were excluded from final analysis.

Statistical Analysis

Data were obtained on a predesigned worksheet (Excel, Microsoft Office) which were analyzed using the Statistical Package for the Social Sciences software (SPSS, version 23, IBM Corporation). As most variables had a non-parametric distribution, continuous variables were summarized as median (25^{th}, 75^{th} percentile) [n], where 'n' represents the total number of patients for whom the said data were available. Nominal variables were expressed as percentages [x] [n=y], where 'x' represents the absolute number and 'n' represents the total number of patients for whom the said data were available.

RESULTS

In this multicenter cohort from 12 centers in India, we initially received clinical details of 195 patients who had

hypogammaglobulinemia and a provisional diagnosis of XLA was considered by the treating physician. On initial analysis, five patients were excluded as their B cell numbers were found to be normal; 13 patients had to be excluded as complete clinical details were not available. Of the remaining 177 patients, 32 patients could not be taken up for final analysis as *BTK* gene mutation or Btk protein expression had not been carried out in these patients. They also did not have family history suggestive of X-linked inheritance. Therefore, in the final analysis, 145 patients were included (137 *'definite XLA'* and eight *'probable/possible XLA'*) (**Figure 2**).

Clinical Profile

Median age at onset of symptoms was 12.0 (6.0, 36.0) months [n=130]. Median age at diagnosis was 60.0 (31.5, 108) [n=144] months. Median delay in diagnosis was 42.0 (15.8, 72.0) [n=130] months. 59.2% [77] [n=130] patients had onset of symptoms on or before the age of 1 year; however, only 6.2% [8] [n=130] were diagnosed before their first birthday. Details about family history were available in 87 patients. Of these 52 (60%) had a family history suggestive of an X-linked inheritance. There was no significant difference in the duration of diagnostic delay or the outcomes when patients with family history were compared with those who lacked a family history. In children diagnosed in the 1st year of life, family history was negative in 50% [4] [n=8], positive in 25% [2] [n=8], and unavailable in the rest [2].

Pneumonia was the commonest clinical manifestation seen in 82.6% [119] [n=144] patients. Complications of pneumonia in form of empyema was seen in 13.4% [16] [n=119] patients. Other common manifestations were recurrent otitis media (50.7% [73] [n=144]), recurrent diarrhea (42.4% [61] [n=144]), and skin and soft tissue infections (35.4% [51] [n=144]) including suppurative lymphadenitis. Arthritis was seen in 26% (36 [n=144]) patients while 23% (34 [n=144]) patients developed meningitis. Bronchiectasis was seen in 10% (14 [n=144]) patients. Encephalitis (likely viral) was observed in 4.8% (7 [n=144]) patients. Clinical profile of our cohort is summarized in **Figure 3A**.

Neutropenia (defined as absolute neutrophil count <1.5x10^9/L) was observed in 4.1% (6 [n=145]) patients. Clinical profile of patients with neutropenia was not found to be different from other patients. However, *Pseudomonas aeruginosa* was cultured from three of these six patients (50%) - from blood culture in two and from pus culture in one.

Miscellaneous clinical manifestations are summarized in **Table 1**.

Microbiological Profile

Body fluid cultures were obtained from sites of overt infection. These included pus (from ear discharge, empyema/effusions, skin/soft tissue infections) in 29% (21/73), blood cultures in 18% (13/73) and bronchoalveolar lavage/endotracheal aspirate cultures in 8% (6/73) (**Figure 3C**). *Giardia lamblia*, *Entamoeba histolytica* and *Cyclospora spp.* were identified in stool microscopy examination in 9.5% (7/73).

An organism was identified in 37.9% [55] [n=145] patients (66 episodes of bacterial infections and seven episodes of parasitic infections).

Pseudomonas aeruginosa was the commonest bacterial pathogen identified (29% [19] [n=66]), followed by *Streptococcus pneumoniae* (18% [12] [n=66]), *Staphylococcus aureus* (15% [10] [n=66]) and *Klebsiella pneumoniae* (14% [9] [n=66]) (**Figure 3B**). About one-third of *S. aureus* infections were caused by MRSA (30% [3] [n=10]). Among parasitic infections, *Giardia lamblia* was identified in five patients, and

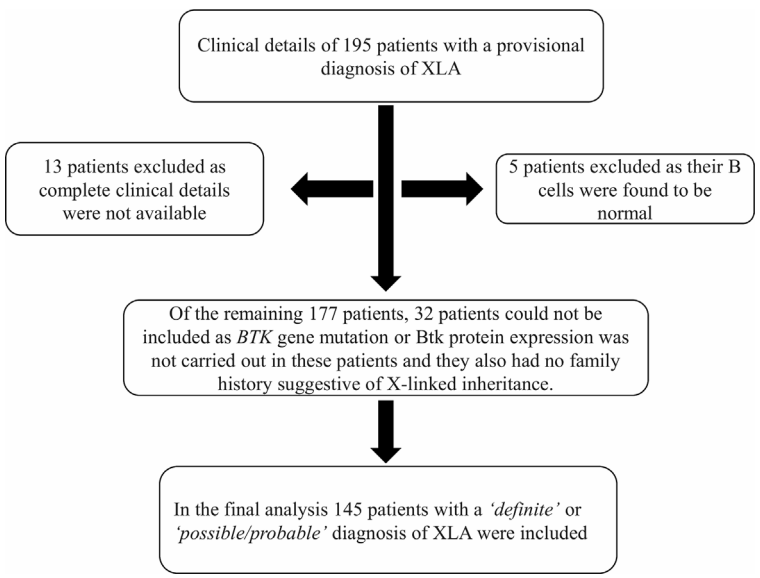

FIGURE 2 | Schematic diagram showing inclusion and exclusions of cases in the study. Abbreviations: XLA, X-linked agammaglobulinemia; BTK, Bruton tyrosine kinase.

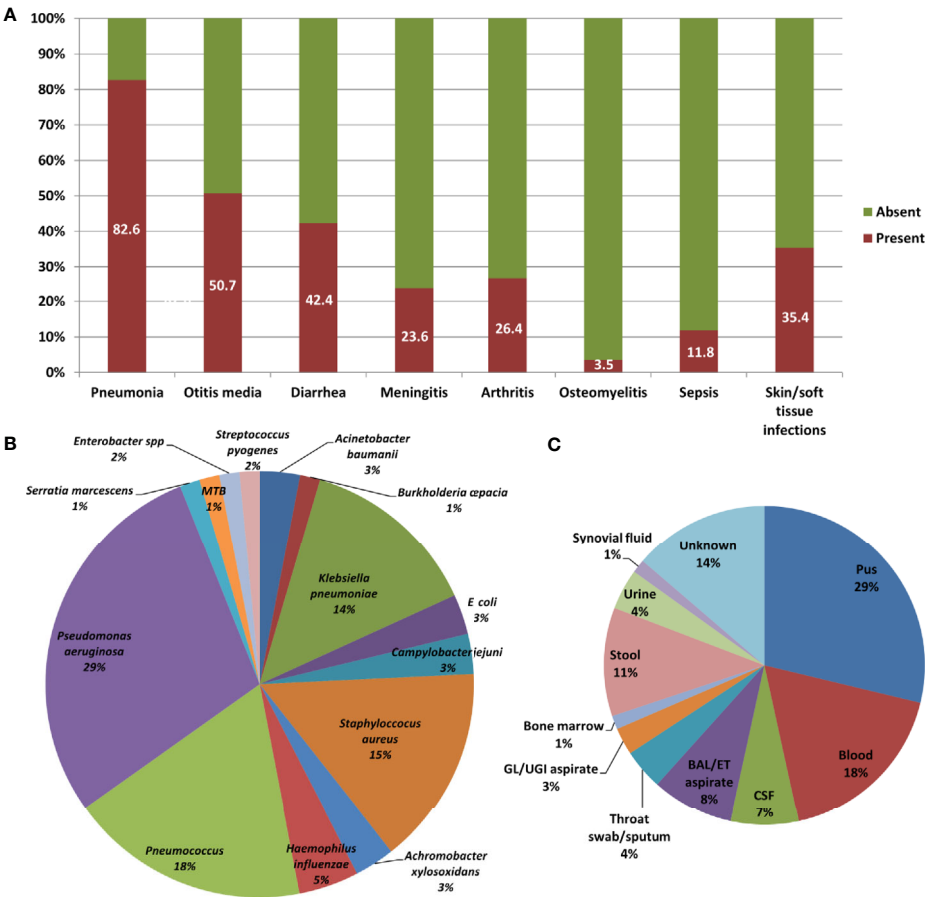

FIGURE 3 | Clinical profile of patients with X-linked agammaglobulinemia (XLA). **(A)** Bar chart showing infections that were seen in patients with XLA. **(B)** Pie chart showing various pathogenic organisms that were isolated from patients with XLA. **(C)** Pie chart showing various techniques used for isolating the pathogenic organisms.

TABLE 1 | Miscellaneous clinical manifestations in patients with X-linked agammaglobulinemia (XLA).

S. no.	Clinical manifestation	Number of patients
1.	Kawasaki disease	2
2.	Brain abscess	1
3.	Spondylodiscitis	1
4.	Psoas abscess	1
5.	Bacillus Calmette–Guérin (BCG) site ulceration and BCG adenitis[a]	1
6.	Pulmonary tuberculosis[b]	4
7.	Anterior horn myelitis	1
8.	Haemolytic Uremic syndrome (HUS)	1
9.	Immune thrombocytopenia	3
10.	Pyo-pericardium	1
11.	Vaccine associated paralytic poliomyelitis (VAPP)	1
12.	Pyoderma gangrenosum-like ulcer possibly caused by *Helicobacter sp.*[c] (**Figure 4**)	1
13.	Growth hormone deficiency[d]	1

[a]*BCG is administered universally at birth in India. BCG adenitis is a common complication and may not be related to underlying XLA.*

[b]*There is likely no causal relationship between XLA and pulmonary tuberculosis as the latter is endemic in the region.*

[c]*Helicobacter sp. could not be grown from skin. However, patient responded to 6 months of therapy with a combination of azithromycin and doxycycline. Skin biopsy showed dense neutrophilic rich dermal infiltrates and no evidence of an infective etiology (Gram stain, Grocott stain, PAS stain, ZN stain and modified ZN stain negative). Immunofluorescence showed negative IgG, IgM, IgA and C3.*

[d]*Received growth hormone therapy.*

Entamoeba histolytica and *Cyclospora spp.* were identified in one patient each.

Immunoglobulin Profile

Levels of IgG, IgA, and IgM were reported to be below the lower limit of detection in 45.5% [66] [n=145], 68.8% [88] [n=128], and 51.5% [67] [n=130] respectively. Hence, an absolute level of immunoglobulins for these patients was not available. In the remaining patients, median IgG, IgA, and IgM levels were 1.20 (0.52, 2.90), 0.18 (0.06, 0.25), and 0.20 (0.10, 0.45) g/L respectively. Normal IgA and IgM were seen in 0.8% [1] and 8.3% [12] respectively; elevated IgA and IgM were seen in 2.3% [3] and 1.5% [2] respectively. This group with normal or elevated IgM or IgA levels comprised of 17 patients (one patient had elevation of both IgA and IgM). There was no significant difference in the mutational profile, B-cell counts, proportion of patients with undetectable IgG levels, or outcome when the group with normal or elevated IgM or IgA levels was compared to the group with low IgA and IgM. However, in patients with detectable IgG levels, the group with normal or elevated IgA or IgM had IgG levels of 4.00 (1.86, 4.94) g/L as compared to IgG levels of 1.00 (0.47, 2.75) g/L in the group with low IgA and IgM ($p = 0.008$, Mann-Whitney test). Serum IgE levels were measured in 53 patients and reported to be below the lower limit of detection in 53% [28]. Median IgE level in remaining patients was 5.30 (4.15, 26.25) kIU/L. Serum IgE levels were reported to be elevated in 3.7% [2].

Lymphocyte Immunophenotyping

In our multcentric cohort, the marker panel utilized for performing basic lymphocyte immunophenotyping consisted of CD45 (Leukocyte common antigen), CD3 (T-cells), CD19 (B-cells), and CD56 (NK-cells). Btk protein expression was analyzed on $CD14^+$ monocytes and was run as a separate experiment.

Median percentages of $CD3^+$ T, $CD19^+$ B, and $CD16^+56^+$ NK cells (of the total lymphocytes) were 92.58 (89.00, 95.75) [n=93], 0.07 (0, 0.8) [n=140], and 4.60 (3.00, 7.07) [n=67] respectively. Median absolute counts ($\times 10^9$/L) for $CD3^+$ T, $CD19^+$ B, and $CD16^+56^+$ NK cells were 4.42 (2.35, 5.90) [n=66], 0.001 (0, 0.005) [n=70], and 0.21 (0.11, 0.36) [n=49] respectively. Data on Btk protein expression were available in 42 patients with median percentage positivity (percentage of monocytes expressing Btk by flowcytometry) of 17.7 (1.6, 59.0). However, the data on MFI was available in only one-third of these 42 patients.

Median percentage of $CD4^+$ and $CD8^+$ T cells (of the total lymphocytes) was 45.47 (37.00, 57.58) [n=27] and 41.02 (30.00, 46.46) [n=27] respectively. Median CD4:CD8 ratio was 1.22 (0.82, 1.90) [n=27]. Median absolute $CD4^+$ and $CD8^+$ T cell counts ($\times 10^9$/L) were 2.02 (1.42, 3.22) [n=27] and 1.81 (1.09, 3.33) [n=27] respectively. Absolute $CD4^+$ T cell counts $<1.000\times10^9$/L were seen in two patients aged between 1–5 years; however, none had counts $<0.500\times10^9$/L.

Molecular Analysis

Molecular analysis for *BTK* gene was available for 111 patients. Of these, four were siblings or maternal cousins whereas two comprised a patient with his maternal uncle. Molecular analysis revealed 86 pathogenic variants in 105 unrelated cases. Of these, 90 patients (86%) had variants in coding part of the gene whereas 15 (14%) had intronic splice-site variants. All 86 coding and non-coding *BTK* gene variants are depicted in **Figure 5**. Missense variants were the most common [38/105 (36%)] followed by frameshift [23/105 (22%)] and nonsense variants [22/105 (21%)]. Frameshift variants due to small deletions were seen in 17.14 % (18 cases), whereas nucleotide insertions occurred in 3.80% (4 cases). Duplication induced frameshift variant was detected in 1 case (**Figure 6A**). Two synonymous exonic variants, i.e., c.240G>T; p.80G(=) and c.1104 A>G; p.368G(=) were also detected. However, these variants had a pathogenic effect by virtue of their being proximal to donor splice site and distal to the acceptor splice site respectively thereby adversely affecting splicing. Although variants were found to be distributed throughout the coding and non-coding regions of the *BTK* gene, most variants [53% (56/105)] were located in the distal exons (exon 14–19) encoding for the tyrosine kinase domain. (**Figure 6B**). Of all exons of *BTK* gene, most patients had variants in exon 15 [15/105 (14.28%)] (**Figure 6C**). In addition, 15 intronic splice- site variants were also observed - five of these variants were located in distal portion of *BTK* gene. Of the 86 variants, nine were novel (**Table 2**) and the remaining 77 had been reported previously.

Founder variants could not be suspected based on the data. Two variants (c.1581_1584delTTTG and c.1922G>A) were found four cases each. Five variants (c.1559G>A; c.1594C>T; c.1696C>T; c.215_215delA and IVS18-8G>A) were detected in three cases each. In the group with normal or elevated IgA or IgM, mutational details were available in 59% [10] [n=17] and are as follows: c.392-1G>C; c.1516T>C, p.C506R; c.1567-2A>C; c.1581_1584delTTTG, p.C527Wfs*2; c.1594C>T, p.Q532*; c.215_215delA, p.N72Ifs*49; c.752G>A, p.W251*, c.83G>A, p.R28H; Del-exon 9-12; IVS1+1G>A.

Genotype-Phenotype Analysis

Patients with missense mutations (36% [40] [n=111]) had 11 episodes of infection in which an organism could be identified, whereas, patients with other non-missense mutations (non-sense, frameshift, splice-site, deletions) had 43 such episodes. This translated to a 2.8-fold (95% CI 1.2–6.8, p=0.02) higher risk of identification of an organism in patients with non-missense mutations as compared to patients with missense mutations. Additionally, *Pseudomonas aeruginosa* infection was seen almost exclusively in patients with non-missense mutations. However, there was no significant difference in the syndromic diagnosis (pneumonia, diarrhea, otitis media, etc.) of infections. There was no difference in the age at onset of symptoms, age at diagnosis, the duration of delay in the diagnosis, B-cell proportions, or immunoglobulin levels between the two groups.

When mutations were stratified as per their severity (based upon previous reports or *in-silico* predictions), patients with less severe mutations (7.2% [8] [n=111] had 2 episodes of infection in which an organism could be identified, whereas patients with severe mutations (92.8% [103] [n=111]) had 52 such episodes. However, there was no difference in the age at onset of symptoms, age at diagnosis, duration of delay in the diagnosis,

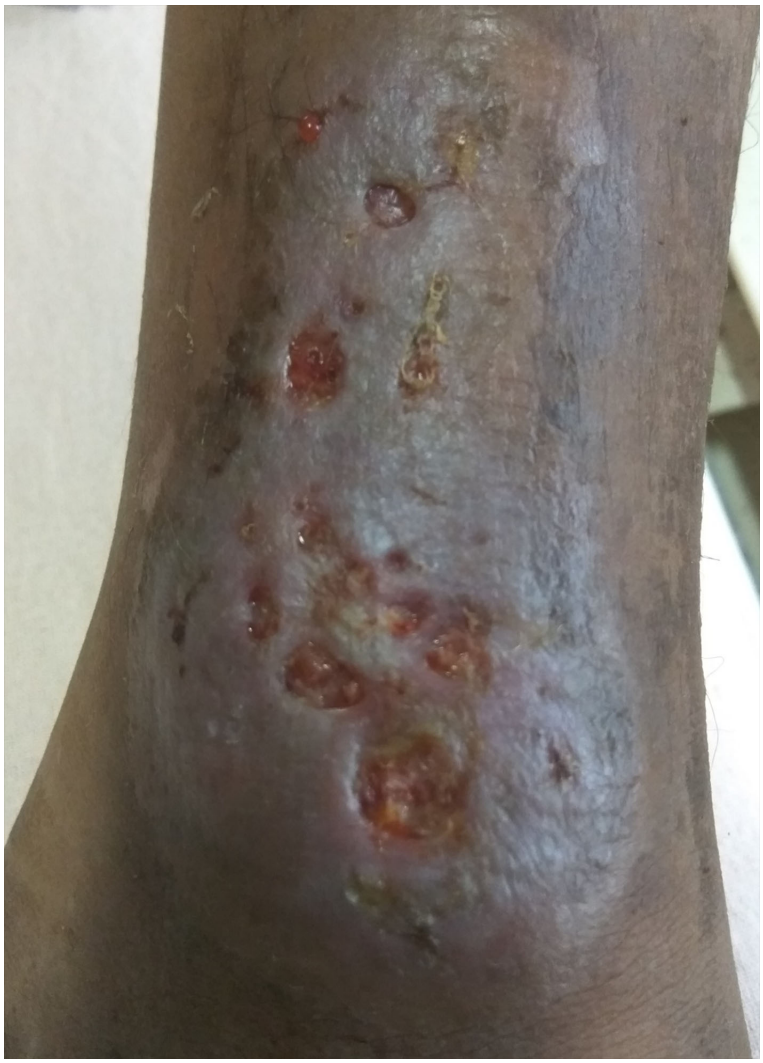

FIGURE 4 | Clinical photograph of ulcerative skin lesion of a patient with X-linked agammaglobulinemia (XLA) suspected to be related to *Helicobacter sp.* infection.

B-cell proportions, or immunoglobulin levels, or overall survival between the two groups. Extensive genotype-phenotype correlation could not be carried out in our cohort due to lack of data regarding Btk protein expression (data regarding Btk protein expression was available in only 28.9% of the cohort).

Treatment and Outcome

In India, all patients with XLA are presently being treated with intravenous immunoglobulin (IVIg, at a dose of ~400 mg/kg/month) as subcutaneous immunoglobulin is not available. Many patients had difficulties in accessing this therapy because of financial reasons and lack of universal health insurance in India. Therefore, IVIg could not be given to several patients at recommended doses and intervals. Breakthrough infections were managed using antimicrobials based on culture reports and/or clinical judgment of treating physicians. Cotrimoxazole prophylaxis (5 mg/kg/day of trimethoprim component) is also prescribed to several patients. Many patients with XLA in India who are on replacement IVIg, are being supported by FPID, various state governments and philanthropic organizations. In addition, a few patients were procuring IVIg on their own. Four patients in the cohort also underwent hematopoietic stem transplantation (HSCT) at Apollo Hospitals, Chennai. None of these four patients were on regular IgRT prior to HSCT. The decision to transplant was based on the caregivers' preferences (life-long IgRT was considered to be more cumbersome and costly as compared to one time potentially curative treatment, although, with significant risk of mortality). In our setup, the cost of life-long IgRT is approximately 7–10 times the cost of HSCT. The details of the transplant procedure have been presented in **Supplementary Table 1**.

Follow-up details were available for 108 patients. Of these, 12% [13] had died till the time of this analysis (four patients died in the 1st decade, seven patients during 2nd decade and two

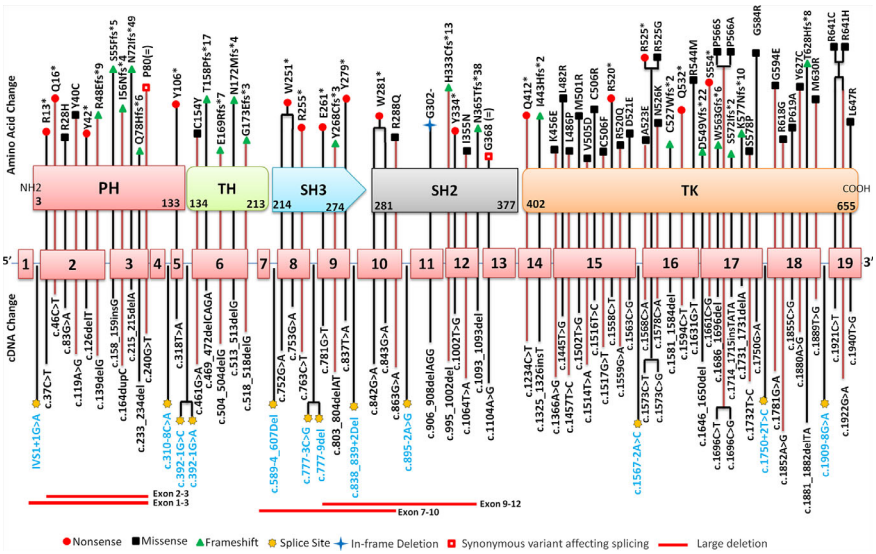

FIGURE 5 | Distribution of pathogenic variants on various exons, exon-intron junctions and corresponding domains of *BTK* gene. cDNA is mentioned in the lower panel while amino acid change is mentioned in the upper panel. Corresponding amino acid changes in the Bruton tyrosine kinase (BTK) protein domains has also been highlighted using various symbols. PH, Pleckstrin homology domain; TH, Tec homology; SH, Src homology; TK, Tyrosine Kinase.

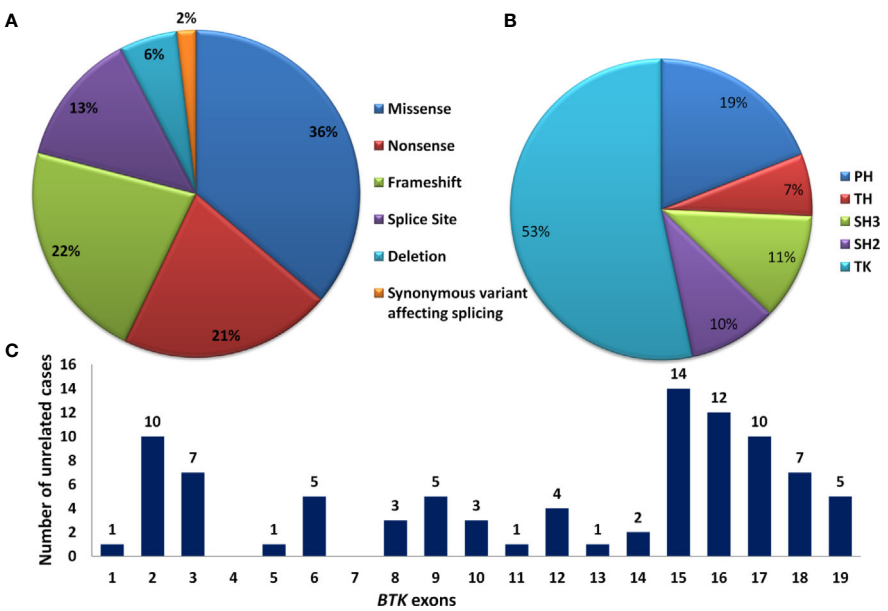

FIGURE 6 | **(A)** Pie chart showing type of mutations in the *BTK* gene. **(B)** Pie chart showing affected domains on the *BTK* gene. **(C)** Bar chart showing number of variants located over individual exons of *BTK* gene. BTK, Bruton tyrosine kinase; TK, tyrosine kinase domain; PH, Pleckstrin homology; TH, Tec homology; SH, Src homology.

patients during 3rd decade). We calculated 5-year and 10-year survival in patients for whom the follow-up data were available. The 5-year survival was 89.9% and 10-year survival was 86.9%. **Figure 7** depicts the survival curve of 108 patients in the present cohort. Thirty-four patients are surviving beyond the age of 10 and nine patients are surviving beyond the age of 20. The 20-year survival was 47.9%, however, only two patients in the entire cohort have been followed-up for 20 years or more. Median duration of follow-up was 61.0 (24.0, 120.6) [n=78] months and total duration of follow-up was 6083.2 patient-months (506.9 patient-years) [n=78]. All four patients who underwent HSCT had attained B cell reconstitution and were doing well at 7, 15, 18,

TABLE 2 | Novel variants seen in patients with X-linked agammaglobulinemia (XLA).

S. no.	Variant	Variant Change
1.	c.158_159insG	S55Ffs*5
2.	c.1325_1326insT	F442fs*444
3.	c.164dupC	I56Nfs*4
4.	IVS6-1C>T	c.392-1G>A
5.	c.518_518delG	G173Efs*3
6.	c.513_513delG	N172Mfs*4
7.	c.233_234delAG	Q78Hfs*6
8.	c.1646_1650delATGAA	D549Vfs*22
9.	c.1104 A>G	G368(=)

and 48 months of follow-up. None of these four patients were receiving IVIg replacement at time of this analysis.

DISCUSSION

We report a multicenter cohort of 145 patients with XLA from India. As there is no national registry for IEIs in India, collection of data on various PIDs from different centers may give information on nationwide burden of these diseases.

Median age at onset of symptoms and diagnosis was 1 and 5 years respectively with a median delay in diagnosis 3.5 years. In our previous study we had reported a mean delay of 4.2 years Delays in diagnosis are primarily a reflection of lack of awareness about PIDs among medical professionals as well as the laity. Diagnostic delays of upto several years have been noted in series from Latin America, Africa and the Middle East (24–27) (**Table 3**). However, a greater delay in the diagnosis was noted in our scenario (20) emphasizing the need for concerted efforts to shorten this delay.

In the present study, pneumonia and other respiratory tract infections and otitis media were the most common clinical manifestations. Similar results have also been reported in several other cohorts including one previously reported series from Chandigarh, North India (9, 22, 24, 26, 28, 30, 31) (**Table 3**).

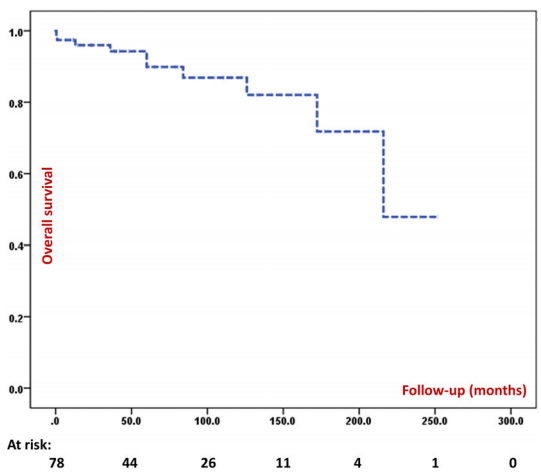

FIGURE 7 | Kaplan Meir survival curve of the entire cohort.

Arthritis was seen in 26% patients in the present cohort. Arthritis is an important clinical manifestation in patients with XLA. It may be infectious or non-infectious and in the latter case may mimic juvenile idiopathic arthritis (JIA) (15, 36). Six patients in present cohort had polyarthritis—one among these has been reported previously (15). In a previously reported single center series on XLA from Chandigarh, arthritis was observed in 42% patients. Joint involvement in patients with XLA in present series was observed to be higher than that reported from United States, Argentina, Italy and Iran (24, 26, 28, 30) but similar to that reported from Hong Kong (29%) (31, 37), Mexico (38%) (35), Africa (37%), Turkey (22%) (27) and China (22%) (29) (**Table 3**). It has been observed that patients with XLA who fail to maintain adequate trough levels despite IVIg replacement therapy are at higher risk of developing arthritis. A study published from Chandigarh, India in 2017 measured the serial trough levels in patients with XLA. In this study, it was observed that the mean trough IgG level (435 mg/dl) is much less as compared to the trough levels of IgG that are seen in western countries (38). This could have been a reason for higher proportion of children with arthritis seen in our cohort. Another likely reason for higher proportion of children with arthritis is delay in diagnosis of XLA in India.

Patients with XLA are predisposed to develop pyogenic infections caused by *Streptococcus pneumoniae, H. influenzae, S. aureus* and *P. aeruginosa.* In the present cohort, *Pseudomonas aeruginosa* was the most common organism isolated followed by *Streptococcus pneumoniae*. While *Pseudomonas aeruginosa* is not a signature organism for patients with XLA, it has been found that patients with XLA who have episodes of neutropenia are at increased risk of pseudomonas sepsis (20). In a recently reported series from Taiwan, Pseudomonas sepsis was the most common clinical presentation (34). Winkelstein et al. reported data on XLA from United States registry in 2006. In this study, commonest causes of sepsis were *Pseudomonas sp.* (29%) and *Streptococcus pneumoniae* (24%) (28). However, the most common cause of bacterial infection was *Streptococcus pneumoniae* followed by *Haemophilus influenzae*. Similar results were also reported by Basile et al from Argentina (24). In a previously reported series from India (9), only one of 36 patients had *Pseudomonas aeruginosa* as the etiological agent for sepsis. Similarly, in a study from Mexico by García et al. (35), *Pseudomonas sp.* was identified in 1/12 patients as a cause of ecthyma gangrenosum. In our multicenter cohort, we observed an unusually high percentage of patients with XLA in whom *P. aeruginosa* was identified as a cause of any infection. Even though neutropenia has been identified as a risk factor for pseudomonas sepsis in patients with XLA, only 4% patients in the present series had neutropenia. Nonetheless, pediatricians, internists, and hematologists evaluating children for neutropenia should consider XLA among the differential diagnosis.

Although, viral infections are not usually seen in patients with XLA, viral encephalitis (often caused by polio and non-polio enteroviruses) is an uncommon but potentially life-threatening

TABLE 3 | Comparison of clinical profile of patients with X-linked agammaglobulinemia (XLA) in the present series from previously reported studies.

S. no.	Author (Country), Year, (Reference)	No. of patients	Age at presentation (years)	Age at diagnosis (years)	Family history	Pneumonia	Diarrhea	Skin and soft tissue infections	Arthritis	Neutropenia	Meningitis	Encephalitis	Most common organism	Mortality
1	Rawat et al. (India), Present study	145	1 (median)	5 (median)	60%	82.6%	42.4%	35.4%	26%	4.1%	23%	4.1%	*Pseudomonas aeruginosa* (overall) Giardia (diarrhea)	13%
2	Winkelstein et al. (USA), 2006 (28)	201	<1 (50% patients)	<2 (50% patients)	41%	62%	23%	18%	7%	11%	12%		Pneumococcus (pneumonia) Giardia (diarrhea) Pseudomonas (sepsis)	8.5%
3	Chen et al. (China), 2016 (29)	174	1 (median)	7.09 (mean)	34.71%	77%	18.97%	8.05%	18.75%	5.17%	19.65%	NR	NR	
4	Lougaris et al. (Italy), 2020 (22)	168	NR	7 (mean) before 2000; 23 months (mean) after 2000	39.3%	39.9%	19%	20.8%	9.5%	NR	4.8%	0.6%	*Hemophilus influenzae* followed by *Streptococcus pneumoniae*	7.7%
5	Plebani et al. (Italy), 2002 (30)	73	1 (median)	3 (median)	39.7%	53.4%; 50% (after IVIg))	13% (4% after IVIg)	27%	10%	1%	4%	1% (after IVIg therapy)	NR	1.4%
6	Lee et al. (Hong Kong), 2010 (31)	62	1 (median)	7 (median)	35%	72.6%	29%	25.8%	29%	NR	12.9%		NR	NR
7	Basile et al. (Argentina), 2009 (24)	52	<1 (>50% patients)	3.5 (median)	55%	61%	27%	31%	8% (osteo-articular)	22%	16%		Pneumococcus (pneumonia) Giardia (diarrhea) Pseudomonas (sepsis)	6.1%
8	Aadam et al. (2016), North Africa (25)	50	9 months (median)	3 (median)	32%	55%	42%	15%	37.5%	NR	27.5%		*Pseudomonas aeruginosa* (overall)	NR
9	Aghamohammadi et al. (Iran), 2006 (26)	37	10 months (median)	4 (median)	52%	86%	78%	NR	24%	2.5%	24%	11%	NR	14%
10	Singh et al. (India), 2016 (9)	36	1 (median)	5 (median)	57%	86%	44%	25%	42%	11%	25%		NR	19%
11	Moin et al. (2004), Iran (32)	33	8 months (median)	4 (median)	NR	81.8%	75.8%	6.1%	21%	3%	24.2%	12.1%	NR	15%
12	Esenboga et al. (Turkey), 2019 (33)	32	9.5 months (median)	3.5 (median)	47%	53.1%	53.1%	3.1%	6.2%	9.3%	8.3%	12.4%	NR	NR
13	Yeh et al. (2020), Taiwan (34)	29	1.2 (median)	5 (median)	NR	68.4%	13.8%	17.2%	3.4%	3.4%	3.4%	NR	*Pseudomonas aeruginosa* (overall)	6.8%
14	García et al. (2016), Mexico (35)	26	NR	NR	NR	69.2%	19.2%	23%	38.4%	NR	38.5%	NR	*Pseudomonas sp.* (overall)	NR
15	Dogruel et al. (2019), Turkey (27)	22	5 months (median)	15.5 months (median)	68.1%	86.3%	36.4%	NR	18%	NR	NR	4.5%	NR	0%

IVIg, intravenous immunoglobulin; NR, not reported.

complication. In an international survey of patients with XLA reported in 2019, this complication was seen in 4.6% of patients. Meningoencephalitis has been observed in up to 1/3rd of all patients with XLA in various series. However, etiological agent is not identified in most cases (9, 20, 24, 26, 28, 30, 31). In the present cohort, 4.8% patients developed encephalitis. The etiology for encephalitis could not be determined in most and diagnosis was largely based on clinical and radiological findings (**Figure 8**). However, in one patient who had fatal encephalitis, the brain specimen on autopsy showed features suggestive of enteroviral encephalitis (39). In another patient, *S. pneumoniae* was the cause of meningoencephalitis like presentation. VAPP was seen in one patient from Mumbai, West India. VAPP is still a significant problem for patients with primary B cell and combined immunodeficiency in countries such as India, where live oral polio vaccine is being used (21).

Other viral infections have rarely been reported in patients with XLA (40–46). Extensive molluscum contagiosum infection was seen in one patient in the present series (12).

Mycobacterial infections are not usually seen in patients with XLA. It has been observed in patients with XLA from Argentina and India (20). In the present cohort, four patients developed pulmonary tuberculosis and one had BCG adenitis (**Table 1**).

Autoimmune and inflammatory complications have also been reported in patients with XLA (2). Common ones include inflammatory arthritis, inflammatory bowel disease, thrombocytopenia and Kawasaki disease (11). In the present series, inflammatory arthritis was seen in six patients, immune thrombocytopenia in four, haemolytic uremic syndrome in one and Kawasaki disease in two (11).

In our cohort, missense variants in *BTK* gene involving tyrosine kinase domain were the most common mutations. Similar results have also been reported from China, Africa, Hong Kong, Argentina and Italy (22, 24, 25, 29, 31) (**Table 4**). Most variants were located in exon 15 as has also been reported from China and Italy (22, 29). However, most variants were found to be located on exon 2 in a study from Hong Kong (29). Nine of 86 variants in our study were novel.

Because of non-availability of subcutaneous immunoglobulin therapy in India, IVIg is the cornerstone of management. However, access to IVIg therapy is not easy for most patients due to high cost of treatment and absence of universal health insurance. In last

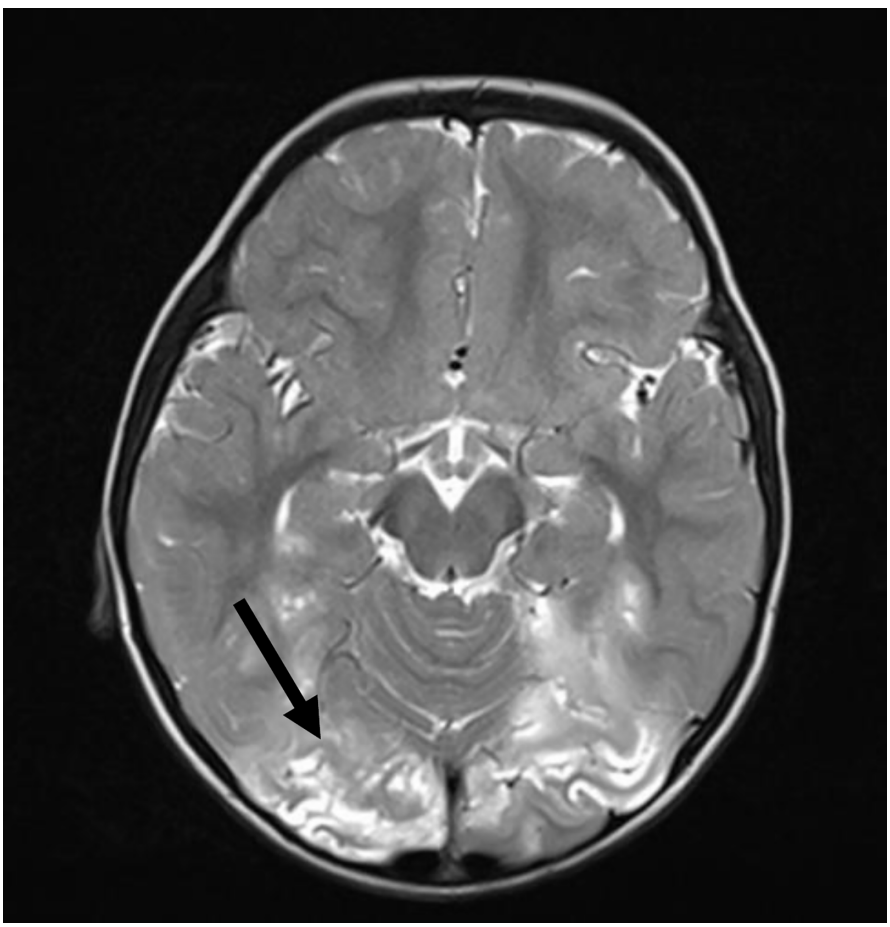

FIGURE 8 | Magnetic resonance imaging shows T2-weighted cortical and subcortical hyperintensities in bilateral occipital region.

TABLE 4 | Comparison of genetic profile of patients with X-linked agammaglobulinemia (XLA) in the present series from previously reported studies.

S.no	Author (Country), Year, (Reference)	No. of variants ((*) = genotype-phenotype correlation available)	Types of mutations	Most commonly affected domain of *BTK* gene	Most commonly affected exon of *BTK* gene	Novel variants
1	Rawat et al. (India), present study	86 variants from 105 patients (*)	Missense mutations (37%) Frameshift mutations (21%) Splice-site mutations (14%) Nonsense mutations (21%) Deletions (5%) Synonymous variants affecting splicing (2%)	TK (55%) PH (19%) TH (6%) SH3 (11%) SH2 (9%)	15	9
2	Chen et al. (China), 2016 (29)	127 variants from 124 families were found to have *BTK* gene mutation (*)	Missense mutations (n=48) Frameshift mutations (n=29) Splice-site mutations (n=23) Nonsense mutations (n=21) Deletions (n=5)	TK domain (n=64) PH domain (n=28) SH2 domain (n=16) SH3 domain (n=15) TH domain (n=3)	15	45
3	Lougaris et al. (Italy), 2020 (22)	104 different variants from 125 families (*)	Missense mutations (49%) Indels (18%) Nonsense mutations (17%) Splice-site mutations (12%) Large deletions (4%)	NR	15	18
4	Broides et al. 2006, USA (47)	94 different variants (*)	Missense mutations (36.4%) Frameshift mutations (10%) Premature stop codons (15.5%) Splice-site mutations (25.4%) Gross deletions (5.4%) Duplication/inversions (2.7%) Complex mutations (3.6%) Retroposon insertion (0.9%)	NR	NR	NR
5	Lee et al. (Hong Kong), 2010 (31)	56 different variants in 57 families (*)	Missense mutations (n=20) Insertion mutations (n=1) Splice-site mutations (n=12) Nonsense mutations (n=6) Deletions (n=13) Promoter (n=1) Complex mutations (n=3)	TK domain (n=23) PH domain (n=16) SH2 domain (n=12) SH3 domain (n=4) TH domain (n=1)	2	15
6	Aadam et al. (2016), North Africa (25)	33 different variants from 35 families (*)	Missense mutations (n=12) Frameshift mutation (n=4) Splice-site mutations (n=6) Nonsense mutations (n=6) Deletions (n=2), in frame deletion (n=1), complex mutation (n=2; one indel and one double mutation)	TK domain (n=17) PH domain (n=6) SH2 domain (n=4) SH3 domain (n=3) TH domain (n=2) 1 frameshift mutation was predicted to affect both SH2 and TK.	NR	17
7	Basile et al. (Argentina), 2009 (24)	29 different variants from 35 families (*), [a]	Missense mutations were most common followed by non-sense mutations	NR	NR	9
8	Aghamohammadi et al. (Iran), 2006 (26)	18 different variants in 21 families (*), [a]	Missense mutations (n=7) Splice-site mutations (n=5) Nonsense mutations (n=3) Deletions (n=3)	TK domain (n=10) PH domain (n=4) SH2 domain (n=1) SH3 domain (n=1) TH domain (n=1) SH1 domain (n=1)	NR	13
9	García et al. (2016), Mexico (35)	12 different variants (*),[b]	Missense mutations (n=4) Splice-site mutations (n=7) Deletion mutation (n=1)	SH2 (n=6) SH1 (n=3) PH (n=1) SH3 (n=1) One mutation located in both SH1 and SH2	NR	4
10	Dogruel et al. (2019), Turkey (27)	12 variants from 12 families (*), [a]	Missense mutations (n=3) Splice-site mutations (n=3) Nonsense mutations (n=4) Deletions (n=2)	PH domain (n=2) SH1 domain (n=6) SH2 domain (n=2) SH3 domain (n=2)	11	2

BTK, Bruton tyrosine kinase; TK, tyrosine kinase domain; PH, Pleckstrin homology; TH, Tec homology; SH, Src homology; NR, not reported.
[a]*Genotype-phenotype analysis based on individual mutations rather than the severe-mild classification commonly used for such analysis.*
[b]*Genotype-phenotype analysis based on the domain of Btk affected.*

5 years, several state governments in India (e.g. Governments of Punjab, Haryana, Madhya Pradesh, Delhi, Himachal Pradesh, Karnataka, Kerala, Tamil Nadu and West Bengal) have started supporting IVIg therapy to patients with PIDs. FPID and other philanthropic organizations have been supporting the treatment of several patients with hypogammaglobulinemia across the country for last 10 years. Despite the generous support provided by aforementioned government and non-government organizations, several patients still find it difficult to get their monthly doses of IVIg. As a result of these constraints, most patients do not get the recommended doses of IVIg and fail to maintain trough levels of IgG above the recommended levels (38).

Survival rates of XLA vary from country to country. These are largely determined by availability of diagnostic facilities and ease of access to treatment. While more than 70% survival beyond the age of 20 has been reported from United States, Europe and Australia, corresponding figures are less than 40% from Asia and Africa (20). Mortality rate in the present cohort was 12%. Prognosis of XLA in the developed countries is much better (e.g. 8.5% mortality United States in 2005 (28); 6.1% in Argentina in 2009 (24) and 1.4% in Italy in 2002) (30) (**Table 3**).

There are several difficulties in diagnosis and management of XLA in India. Because of lack of awareness, several patients are diagnosed late and have already developed chronic lung disease by the time they start receiving their immunoglobulin replacement therapy. Diagnostic facilities such as nephelometry, flow cytometry and genetic sequencing are available only in tertiary level institutes or medical schools. Access to IVIg is not easy for several of these families and most patients do not get recommended doses. As a result of this, patients present with frequent breakthrough infections that adds to their morbidity and mortality. Despite all difficulties, it is noteworthy that 9 of the patients in the present series have now reached their 3rd decade of life and are doing well on immunoglobulin replacement therapy.

HSCT was performed in four patients at a single center with expertise in transplant facility (48, 49). There have been rare reports of successful HSCT in patients with XLA (50, 51). All 4 children in the present series underwent successful HSCT and are off IVIg. HSCT has been performed sparingly in patients with XLA. The conditioning regimens and the outcomes have been variable (52). As noted in our scenario, HSCT utilizing treosulfan-based conditioning regimens have provided promising outcomes. Till date, reports describing successful gene therapy in patients with XLA are lacking. However, studies on gene therapy in murine models of XLA have shown encouraging results with full B-cell recovery (53).

Strengths of this study are that this is the first multicenter cohort on XLA from India and one of the largest cohorts in world. Limitations of this study are that complete data were not uniformly available from all centers. Details of molecular analysis were not available for approximately 20% patients. In India, as there is a disparity in the availability of diagnostic testing, recognition of important clinical findings (absence of tonsils and lymph nodes) is essential for diagnosing XLA. However, data on these findings was available from only a few centers due to the retrospective design of our study.

CONCLUSION

We report a multicenter cohort of patients with XLA from India. *Pseudomonas aeruginosa* was the most common organism isolated, even in the absence of neutropenia. VAPP is still a significant problem for patients with primary B cell and combined immunodeficiency in countries where live oral polio vaccine is being used. In addition, few patients in this XLA cohort also had mycobacterial infections. However, India being endemic for tuberculosis, it would be difficult to ascribe these mycobacterial infections to the underlying antibody defect. There is a significant delay in the diagnosis of XLA because of a lack of awareness among pediatricians and internists. Delay in the diagnosis does not only lead to a greater burden of infection, but it may also result in organ damage and a higher burden of autoimmune complications in patients with XLA. Facilities for molecular confirmation of diagnosis are not available at many centers in the country. Provision of replacement immunoglobulin therapy is a daunting task for families with affected children. However, with financial support provided by FPID; some state governments in India and several philanthropist organizations, several patients have been able to access immunoglobulin replacement therapy now.

DATA AVAILABILITY STATEMENT

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

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.

AUTHOR CONTRIBUTIONS

AR, AKJ: Data collection, writing of initial draft, editing of manuscript at all stages of its production, patient management, review of literature. DS, PV, AG, BS, RM, MS, MD, PT, AP, VG, SS-D, MG, AD, MM, AA, RR, RU, SB, AJ, HL, LR, DM, MK, AS: Data collection, management of patients, review of the final manuscript. AB, RT, KA, VJ, SM, JS: Data collection, writing of initial draft, review of literature. RSa, RSh, RG: Patient management, review of literature. KI, SN, OO, PL, KC, Y-LL: Genetic evaluation, review of the final manuscript. SS: Data collection, patient management, review of literature, editing and critical revision of manuscript at all stages of its production, final approval of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Wiskott Aldrich Syndrome: A Multi-Institutional Experience From India

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Background: Wiskott Aldrich syndrome (WAS) is characterized by bleeding manifestations, recurrent infections, eczema, autoimmunity, and malignancy. Over the last decade, improved awareness and better in-house diagnostic facilities at several centers in India has resulted in increased recognition of WAS. This study reports collated data across major primary immunodeficiency diseases (PID) centers in India that are involved in care of children with WAS and highlights the varied clinical presentations, genetic profile, and outcomes of patients in India.

Methods: Request to share data was sent to multiple centers in India that are involved in care and management of patients with PID. Six centers provided requisite data that were compiled and analyzed.

Results: In this multi-institutional cohort, clinical details of 108 patients who had a provisional diagnosis of WAS were received. Of these, 95 patients with 'definite WAS' were included Fourteen patients were classified as XLT and 81 patients as WAS. Median age at onset of symptoms of patients was 3 months (IQR 1.6, 6.0 months) and median age at diagnosis was 12 months (IQR 6,48 months). Clinical profile included bleeding episodes (92.6%), infections (84.2%), eczema (78.9%), various autoimmune manifestations (40%), and malignancy (2.1%). DNA analysis revealed 47 variants in 67 cases. Nonsense and missense variants were the most common (28.4% each), followed by small deletions (19.4%), and splice site defects (16.4%). We also report 24 novel variants, most of these being frameshift and nonsense mutations resulting in premature

termination of protein synthesis. Prophylactic intravenous immunoglobulin (IVIg) was initiated in 52 patients (54.7%). Hematopoietic stem cell transplantation (HSCT) was carried out in 25 patients (26.3%). Of those transplanted, disease-free survival was seen in 15 patients (60%). Transplant related mortality was 36%. Outcome details were available for 89 patients. Of these, 37% had died till the time of this analysis. Median duration of follow-up was 36 months (range 2 weeks- 12 years; IQR 16.2 months- 70 months).

Conclusions: We report the first nationwide cohort of patients with WAS from India. Bleeding episodes and infections are common manifestations. Mortality continues to be high as curative therapy is not accessible to most of our patients.

Keywords: thrombocytopenia, X-linked thrombocytopenia, microplatelets, hematopoetic stem cell transplant, WASP, autoimmunity, bleeding, malignancy

INTRODUCTION

Wiskott–Aldrich syndrome (WAS; OMIM#301000) is an X-linked immune deficiency disorder with an estimated incidence of 3.7- 4.1 per 1 million live births, and is characterized by micro-thrombocytopenia, eczema, combined immunodeficiency, and increased risk for autoimmunity, and malignancy (1–4).

This syndrome is caused by mutations in *WAS* gene that contains 12 exons and is located on short arm of X chromosome (Xp11.23) (5). *WAS* gene encodes Wiskott Aldrich syndrome protein (WASp), which is a 502-amino acid protein, and a key molecule for actin cytoskeleton polymerization (6–9). WASp is expressed by all hematopoietic cells (10) and has essential cellular functions like formation of immunological synapses (11–15), release of secretory granules (16, 17), phagocytosis (18, 19), cellular migration (20, 21), and motility (22).

Review of literature revealed occasional case reports with limited information on genetic abnormalities in WAS from India (23–32). We published a small series of eight patients in 2012 highlighting that under-reporting was mainly due to lack of awareness amongst medical fraternity and nonavailability of diagnostic and therapeutic facilities (23). In 2011, a dedicated society for PID (Indian Society for Primary Immune Deficiency, ISPID) was founded. ISPID has been working toward increasing awareness regarding PIDs and establishment of diagnostic support and research centers in the country. ISPID with the support of Foundation of Primary Immunodeficiency Diseases (FPID), USA organized national, international level conferences for sensitization, and further research in field of PIDs. The Indian Council of Medical Research (ICMR) helped set up the Centre for Advanced Research (CAR) facility in PIDs at PGIMER, Chandigarh in 2015 and subsequently at the National Institute of Immunohaematology (NIIH), Mumbai in 2017. There seems to be a paradigm shift in number of patients diagnosed with PID in India after these CAR facilities were started (33). With improved awareness and advent and availability of better genetic diagnostic tests, patients with WAS and other PIDs are now being diagnosed at several centers.

This study reports data across major centers in India that are involved in care of children with PID and highlights the clinical manifestations and genetic profiles. It also emphasizes the difficulties likely to be encountered in management of these patients in context of a developing country.

PATIENTS AND METHODS

All members of ISPID were also contacted *via* email to share data of patients with WAS on a predesigned excel sheet by the lead author (DS). Different centers supported by the FPID, USA, and other institutions involved in care of patients with PID across India were also contacted. Data including demographics, prominent clinical manifestations, laboratory investigations, genetic results, treatment regimens and long-term outcomes were collated on an excel sheet. (appendix1) Participating centers included Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, North India (60 patients); Apollo Hospitals, Chennai, South India (19 patients); Bai Jerbai Wadia Hospital for Children, Mumbai, West India (16 patients); Aster CMI Hospital, Bengaluru, South India (5 patients); Kasturba Medical College, Manipal Academy of Higher Education, South India (4 patients); and King George's Medical University, Lucknow, North India (1 patient). Ethical approval was obtained from the Institute's Ethics Committee (INT/IEC/1216).

European Society of Immunodeficiencies (ESID) definitions were used to categorize patients for this analysis (34). The term 'definite WAS' was used when there was congenital thrombocytopenia and small platelets and at least one of the following: 1. Mutation in WAS gene; 2. Absent WASp mRNA on northern blot analysis of lymphocytes; 3. Absent WASp protein in lymphocytes; 4. Maternal cousins, uncles or nephews with small platelets and thrombocytopenia). 'Probable WAS' was diagnosed, if patients had congenital thrombocytopenia, small platelets and at least one of the following: 1. Eczema; 2. Abnormal antibody response to polysaccharide antigens; 3. Recurrent bacterial or viral infections; 4. Autoimmune diseases; 5. Lymphoma, leukemia, or brain tumor (34).

The WAS severity scoring system was used to differentiate between patients with mild and severe clinical phenotypes (35). Patients with 'mild' WAS phenotype or XLT had

thrombocytopenia with absent or isolated and intermittent eczematous lesions and absence of recurrent or chronic infections (WAS score 1 or 2). Patients with classic WAS had extensive and difficult-to-treat eczema or had recurrent infections requiring frequent antimicrobial agents (score 3 or 4). Patients who developed autoimmunity or malignancy received a score of 5 (35). The collated data include demographic profile, clinical presentation, investigation details including platelet counts, mean platelet volume (MPV), and serum immunoglobulins (IgG, IgA, IgM, IgE). MPV value between 7.5 -11 fL was considered normal, and microplatelets were defined if MPV was < 7.5 fL.

DNA analysis of WAS was carried out to confirm the diagnosis in patients with clinical suspicion of WAS and in those who had low MPV or reduced WASp expression, or suggestive X-linked family history.

PGIMER, Chandigarh was designated as a Centre for Advanced Research for Primary Immunodeficiency Disorders by ICMR in 2015 and was soon followed by NIIH, Mumbai. Facilities for flow cytometry and genetic studies are available at both these centers. Most patients diagnosed at Bai Jerbai Wadia Hospital for Children (BJWHC), Mumbai, underwent investigations in collaboration with NIIH, Mumbai. At most other centers, flow cytometry and NGS based molecular test are outsourced to commercial laboratories. Hematopoietic Stem Cell Transplant (HSCT) was carried out at PGIMER, BJWHC, Apollo Hospitals and Aster CMI Hospital, Bengaluru.

Laboratory Investigations at PGIMER, Chandigarh

Platelet count and size (MPV) was estimated by automated analyzers (COULTER® HmX AL Analyzer, Beckman Coulter, United States or COULTER® LH780 Hematology Analyzer, Beckham Coulter, United States) standardized for MPV estimation. A dedicated peripheral smear examination to look for platelet morphology was also carried out by an experienced hematologist as automated analyzers can dismiss small thrombocytes as debris leading to erroneously low platelet count and high MPV. Serum IgG, IgM and IgA were estimated by nephelometer (MININeph, semiautomated nephelometer, The Binding Site, United Kingdom) while serum IgE was estimated by enzyme immunoassay.

WASp Quantification by Flow Cytometry

Intracellular staining of WASp using phycoerythrin (PE)-labeled anti human WASp antibody (sc-13139PE (clone: B-9), Santa Cruz Biotechnology, United States), was carried out on non-erythroid blood cells in peripheral blood. Cells were gated using side scatter vs CD45 labeled with fluorescein isothiocyanate (FITC) (A07782; Beckman Coulter Life Sciences, United States). Cells were acquired on an in-house flow cytometer (Navios™ EX System, Beckman Coulter, United States). Median fluorescence intensity (MFI) and Stain Index (SI) in stimulated and unstimulated samples were calculated.

DNA Amplification and Sequencing

Prior to 2015, DNA analysis for WAS was carried out in collaboration with overseas centers, which included: Service d'Hématologie, d'Immunologie et de Cytogénétique, Hôpital de Bicêtre, Le Kremlin-Bicêtre, France; National Defense Medical College, Saitama, Japan; and Department of Paediatrics & Adolescent Medicine, The University of Hong Kong, Hong Kong.

After 2015, DNA analysis was performed in-house in Pediatric Immunology laboratory, Advanced Pediatrics Centre by Sanger sequencing using specific primers. After obtaining informed consent from parents or caregivers, genomic DNA was isolated from peripheral blood samples using Qiagen kits (QIAMP DNA Blood Mini Kit, 51106, Qiagen Ltd., United States). All 12 exons of *WAS* gene were amplified by polymerase chain reaction (PCR) using 9 sets of specific primers. Primer sequences and PCR conditions are attached as Appendix1.

Direct sequencing was performed at Central Sophisticated Instrument Centre on a genetic analyzer (Applied Biosystems® 3500 Series Genetic Analyser, Thermo Fisher Scientific, United States). Sequences were aligned with known *WAS* gene sequence (NCBI reference sequence: NG_007877.1). Sequence variations were described according to reference sequences (Ensembl ID: ENST00000376701.5), and cDNA nucleotides were counted from the first ATG translation initiation codon. *WAS* gene variants were confirmed with Ensembl, Genome Aggregation Database (gnomAD) and Human Gene Mutation Database (HGMD).

Since 2018, we have started performing targeted next generation sequencing (NGS) for PID patients using a gene panel comprising of 44 genes including the WAS gene on IonS5 platform, (Ion torrent S5, Thermo Fisher Scientific, United States). However, variants in regulatory regions and the polyadenylation sites of the *WAS* gene were not covered by the panel that we have been using.

Laboratory Investigations at Other Centers

At other centers, WASp expression could only be carried out in some patients due to non-availability of this specialized flow cytometric test. Genetic tests were performed based on clinical suspicion. NGS using a targeted gene panel was also performed by most centers through commercial laboratories (MedGenome Laboratories Pvt Ltd). Illumina platform was used for sequencing in the commercial laboratories with a coverage of >80X. Sanger sequencing was used to confirm the variants obtained by NGS.

Management and Treatment

Appropriate antimicrobial therapy was initiated to treat breakthrough infections based on clinical manifestations, microbiological susceptibility, and clinical judgment of the treating physician. Patients with classic WAS were initiated on cotrimoxazole prophylaxis and monthly intravenous immunoglobulin (IVIg) therapy, while patients with XLT phenotype were managed symptomatically. Most patients with XLT were not initiated on any prophylactic therapy. Indications

for replacement IVIg were: WAS score 3-5, frequent infections despite antimicrobial prophylaxis, patients awaiting HSCT. Corticosteroids were used for treatment of skin vasculitis and autoimmune hemolytic anemia (AIHA), and malignancies were treated using standard chemotherapeutic protocols. HSCT was carried out in patients for whom a suitable donor could be arranged, at a center of their choice.

Statistical Analysis

Patient demographics, disease and treatment related variables were summarized using descriptive statistics such as medians and ranges for continuous variables and counts and percentages for categorical variables. Mann Whitney U test was used to compare continuous variables between groups. Categorical variables were compared by using the x^2 test or Fisher's exact test wherever needed. The Kaplan-Meier method was used to estimate probability of overall survival (OS). Death from any cause was considered as an event, and surviving patients were censored at last follow-up. Log-rank test was used to compare overall survival probability between different groups of patients. All p-values were 2-sided, and a p value of <0.05 was considered significant.

RESULTS

Data of 108 patients with WAS, collated from 6 centers, were included and analyzed. Ninety-eight patients had 'definite WAS', and 10 patients had 'probable WAS'. Patients with 'probable WAS' and 3 patients with 'definite WAS' with insufficient clinical and demographic details were excluded from analysis. The study thus included 95 patients with WAS for further analysis (**Figure 1**). Complete variant details were available for 67 of 86 patients who had been confirmed by genetic tests. The cohort included 14 patients of XLT (WAS score 1- 7 children; score 2- 7 children), while remaining 81 children had classic WAS (WAS score 3- 29 patients; score 4- 13 patients; and score 5- 39 patients).

Median age at onset of symptoms was 3 months (range birth -14 years; interquartile range, (IQR) 1. - 6 months) while median age at diagnosis was 12 months (range 2 month – 32 years; IQR 6- 48 months) (**Table 1**). Median age of alive children at the time of analysis was 86.5 months (IQR: 52- 168 months, range: 10-418 months). Median delay in diagnosis was 8 months (range 1 month – 16.5 years; IQR 3- 30.2 months) and median follow-up was 36 months (range 2 weeks- 12 years; IQR 16.2 months- 70 months). Family history of WAS or X-linked inheritance was

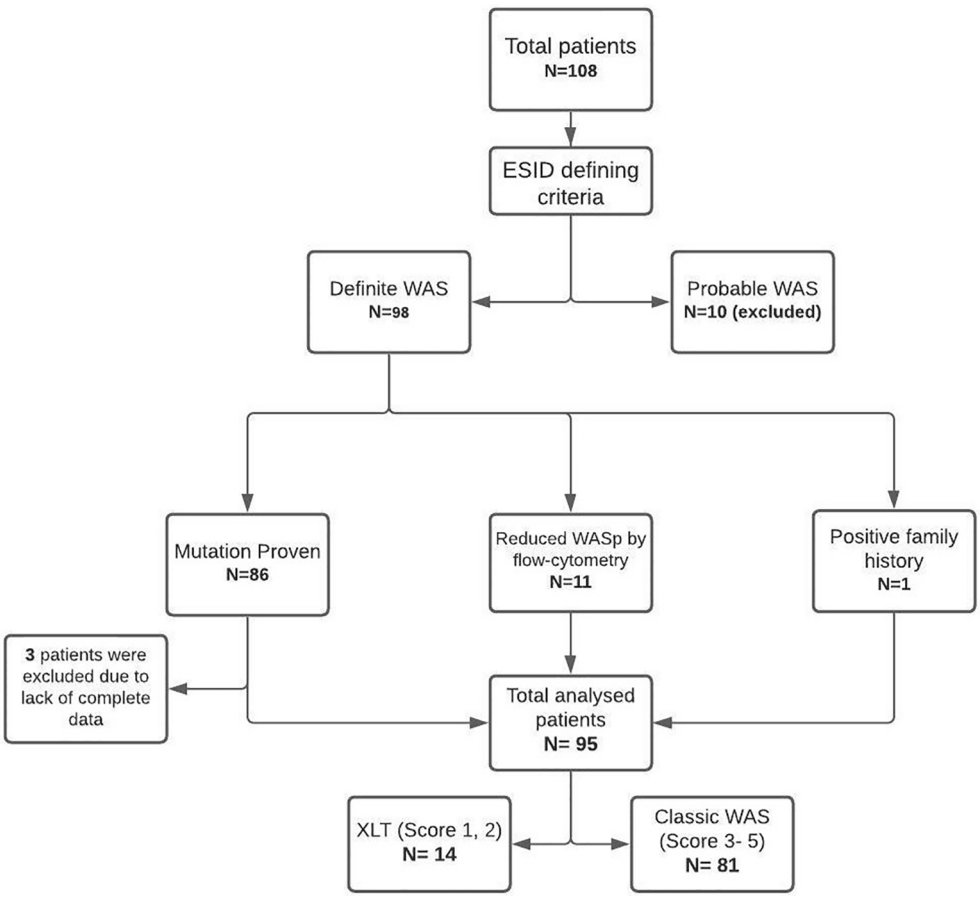

FIGURE 1 | STROBE diagram of collated data, stratification and inclusion of study subjects.

TABLE 1 | Baseline characteristics of the included patients.

Parameter	Entire cohort (n=95)	XLT (n=14)	WAS (n=81)	p value*
Median age at onset of symptoms (IQR, months)[a]	3 months (1.6- 6)	6 months (2.5- 48)	3 months (1.25- 6)	0.044
Median age at diagnosis (IQR, months)[a]	12 months (6- 48)	51 months (10 – 102)	12 months (5.5- 36)	0.019
Median delay in diagnosis (IQR, months)[a]	8 months (3- 30.2)	31 months (6.5- 69)	7 months (3- 24)	0.021
Median follow-up (IQR, months)[a]	36 months (16.2- 70)	40 months (26.5- 75)	36 months (12.2- 70)	0.496
Median value of MPV (IQR)[a]	5.6 fL (5- 7)	5.4 fL (4- 6.9)	5.6 fL (5- 7)	0.702
Median platelet count (IQR)[a]	$20x10^9$/L (12- $33x10^9$/L)	$22x10^9$/L (5- 43 $x10^9$/L)	$20x10^9$/L (12- $31x10^9$/L)	0.943
Median WASp SI (IQR)[a]	0.33 (0.24- 0.60)	0.36 (0.13- 0.49)	0.32 (0.25- 0.61)	0.259
Number of patients with mortality[b]	33	1	32	0.028

IQR, Interquartile range.
[a]Mann-Whitney U test.
[b]Fisher's exact test or chi-squared test.
**p value comparison between XLT and WAS, < 0.05 is significant.*

present in 29 patients (30.5%). Details of median age at onset of symptoms and age at diagnosis in patients with XLT and WAS have been summarized in **Table 1**. Patients with classic WAS earlier onset of symptoms, were younger at time of diagnosis and had less delays in establishment of diagnosis in comparison to patients with XLT (p values were 0.044, 0.019, and 0. 021 respectively) (**Table 1**).

Clinical Manifestations at First Presentation

Most children with WAS (71, 74.7%) had bleeding manifestations at time of presentation, commonest being gastrointestinal bleeding in form of blood-stained stools (47, 49.4%), followed by eczema and infections (**Table 2**). Clinical triad of bleeding, eczema, and infections was seen in 15 patients (15.7%) at time of presentation, though complete triad evolved over time in 70 patients (73.6%). Three patients had presented with autoimmune manifestations (AIHA 2 patients; skin vasculitis 1 patient) and were later diagnosed to have WAS (**Table 2**).

TABLE 2 | Clinical manifestations at the time of presentation in the cohort.

Presenting Manifestations	No. of patients (n=95)	%
Bleeding	71	74.7
Blood in stools	47	49.4
Skin bleeds	19	20
Skin and mucosal bleed	5	5.3
Epistaxis	5	5.3
Hematuria	4	4.2
Hematemesis	2	2.1
Bleeding from >1 site	11	11.5
Eczema	44	46.3
Infections	41	43.1
Pneumonia	19	20
Otitis media	5	5.3
CMV infection	2	2.1
Chronic diarrhea	3	3.1
Infection at >1 site	6	6.3
Autoimmune Manifestations	3	3.1
AIHA	2	2.1
Skin vasculitis	1	1.0

CMV, Cytomegalovirus; AIHA, autoimmune hemolytic anemia.

Data of provisional diagnosis proffered to patients before referral was available in 42 patients. Eight of these 42 patients (19%) were initially diagnosed and managed as ITP. Diagnosis of WAS (WAS: 5 patients; XLT: 3 patients) was later suspected on based family history (2 patients), non-responsiveness to treatment (6 patients), development of infections (2 patients) or eczema (1patient).

Clinical Manifestations on Follow-Up

Spectrum of clinical features of our cohort over the follow- up period was summarized in the **Table 3**.

Bleeding Manifestations

Bleeding manifestations were encountered in 88 children (92.6%) with blood-stained stools (67; 70.5%) and skin bleeds (54; 56.8%) being the common symptoms (**Table 3**). Other manifestations included epistaxis (11 patients), hematuria (6 patients), hematemesis (2 patients), hemoptysis (1patient) and bleeding from auditory canal (1 patient). Intracranial bleeding was observed in 5 patients (5.3%). Bleeding from multiple sites (more than 1) was found in 45 patients (47.3%) during follow-up.

Infections

Eighty patients (84.2%) had evidence of infections with pneumonia being the most common (52, 54.7%), followed by otitis media in 34 patients (35.7%). Septicemia, meningitis, septic arthritis, and skin and soft tissue abscesses were other infections encountered in these patients. Detailed microbiological profile of these infections was not available, as many patients had been treated at peripheral healthcare facilities and records were not available.

Cytomegalovirus (CMV) infection was documented in 7 (7.3%) patients. Four amongst these had been treated as congenital CMV infection. Immunological investigations ($CD3^+$, $CD\ 19^+$, and $CD56^+$ cell populations and serum immunoglobulins) were non-contributory. Diagnosis of WAS was suspected much later when they continued to have persistent thrombocytopenia despite treatment, suggestive family history and/or eczema. One patient developed skin vasculitis in at 11 months of age and further work-up led to diagnosis of WAS.

TABLE 3 | Clinical manifestations encountered in patients over 217 patient years of follow-up (n=95).

Clinical Manifestations	No of patients (n=95)	%
Bleeding	88	92.6
Blood in stools	67	70.5
Skin bleed	54	56.8
Bleeding >1 site	45	47.3
Epistaxis	11	11.5
Hematuria	6	6.3
Hematemesis	2	2.1
Hemoptysis	1	1
Ear canal bleed	1	1
Intracranial bleed	5	5.2
Infections	80	84.2
Bacterial		
Pneumonia	52	54.7
Otitis Media	34	35.7
Septicemia	4	4.2
Meningitis	4	4.2
Others*	9	9.4
Viral		
Cytomegalovirus infection	7	7.3
Molluscum contagiosum	5	5.2
Epstein Barr virus infection	2	2.1
Varicella	4	4.2
Eczema	75	78.9
Atopy[#]	4	4.2
Autoimmune Manifestations	38	40
Autoimmune hemolytic anemia	9	9.5
Positive direct Coombs test	9	9.5
Antinuclear antibodies	9	9.5
Skin vasculitis	9	9.5
Takayasu arteritis	1	1.1
Guillain Barre syndrome	1	1.1
Autoimmune lymphoproliferative Syndrome-Like	1	1.1
Primary sclerosing cholangitis	1	1.1
>1 autoimmune manifestation	14	14.7
Others[##]	5	5.2
Neoplasms	2	2.1

**Chronic infective diarrhea (3); Suppurative lymphadenitis (3); Gluteal abscess (1); Septic arthritis (1).*
[#]Asthma (2); Lactose intolerance (1); Cow milk protein allergy (1).
[##]Hypothyroidism (2); Perinicious anemia (1); Alopecia (1); Nephritis (1).

Molluscum contagiosum (MC) *virus* infection was seen in 5 patients - infection was difficult to eradicate in one of these. Varicella infection was seen in 4, of which 2 had severe hemorrhagic manifestations. *Epstein Barr* virus infection was recorded in 2 patients.

Eczema and Atopy

Eczema was observed in 75 patients (78.9%). Other allergic manifestations included bronchial asthma (2 patients), lactose intolerance (1 patient) and cow milk protein allergy (1 patient).

Autoimmunity

Autoimmune manifestations were observed in 38 patients (40%). AIHA was the commonest manifestation with Direct Coombs test (DCT) being positive in 9 children (9.5%). Two children, aged 6 months and 3.5 years, presented with AIHA and it was only due to high index of suspicion based on other clinical findings, that the diagnosis of WAS could be established. Skin vasculitis was recorded in 9 children (9.5%). Diagnosis of WAS was made because of persistent micro-thrombocytopenia. Incidental antinuclear antibodies (ANA) were detected in 9 patients (9.5%). A 17 years old patient developed Takayasu arteritis after 12 years of follow-up.

Malignancy

Malignancy developed in 2 patients and both succumbed to it. A child diagnosed to have WAS at 8 months developed high grade intracranial non-Hodgkin lymphoma as early as 3.5 years (27) while the second child succumbed to non-Hodgkin lymphoma at 18 years of age. He had persistent monoclonal gammopathy without overt malignancy for 8 years prior to clinical evidence of the disease (28).

Laboratory Investigations

Thrombocytopenia

Thrombocytopenia is a hallmark of WAS. Median platelet count in our cohort was 20 $x10^9$/L (range 1 $x10^9$/L to 142 $x10^9$/L; IQR: 12 $x10^9$/L – 32.2 $x10^9$/L) was recorded in our cohort. Life threatening intracranial bleeding and gastrointestinal was recorded in 5 and 2 patients, respectively.

Mean Platelet Volume

MPV values were available for 65 patients and median MPV was 5.6 fL (range 3.1- 10.8 fL; IQR 5 - 7 Fl),. An MPV value of >7.5 fL was seen in 10/65 patients (15.3%) which could be attributed to differences in laboratory methods and techniques.

Serum Immunoglobulins

Details of estimated serum immunoglobulins (Ig) levels were available in 45 patients. Variable immunoglobulin profile was observed in these patients. Serum IgG was normal in 28 (62,2%), increased in 15 (33.4%) patients and was reduced in 2 (4.4%) patients. Low serum IgM was noted in 13 (28.8%) patients. Serum Ig A and Ig E were elevated 23 (51.1%) and 39 (87%) patients, respectively.

Lymphocyte Subset Analysis

Flow cytometric data of lymphocyte subset (CD3+ T cells, CD19+ B cells, CD56+ NK cells and CD3+CD56+ NKT cells) was available for 28 patients (**Supplementary Table 1**). Absolute lymphocyte counts (ALC) were low in 5/23 (%) patients, who had concomitant CMV infection or autoimmune hemolytic anemia or hemophagocytic lymphohistiocytosis (HLH), Median CD3+ T lymphocyte counts were 2.017 $x10^9$/L (67.3%) (IQR 1.48- 3.59 $x10^9$/L, range: 0.43 - 10.6 $x10^9$/L), while median CD19+ B lymphocyte counts were 0.38$x10^9$/L (10.44%) (IQR 0.154 - 0.716 $x10^9$/L, range: 0.04 - 2.17 $x10^9$/L). Median CD56+ NK lymphocyte counts were 0.436 $x10^9$/L (12.24%) (IQR 0.176- 1 $x10^9$/L, range: 0.1- 1.46 $x10^9$/L. B lymphocytes were reduced in 11/20 (55%) patients while 8 patients had elevated levels of NK cells (36). Detailed immunophenotyping of subsets and T cell proliferation assay were not routinely carried out at most centers due to nonavailability.

WASp Expression by Flow Cytometry

Results of WASp expression on lymphocytes were available in 34 of 95 patients (6 patients with XLT and 28 patients with WAS). Reduced WASp expression (SI 0.29, 0.00 - 0.64) was seen in 30/34 patients (88.2%). Of these, 24 patients had WAS and 6 patients had XLT phenotype. Normal WASp (SI 0.75, 0.74 - 0.83) expression was observed in remaining 4 children. Of these 4 patients with normal WASp, 2 had intronic splice site mutation, and the remaining 2 patients had frameshift mutation in exon 10 (C-terminal end) of *WAS* gene.

Genetic Analysis

Eighty-six patients underwent DNA analysis; however, complete variant details were available for 67 patients at time of analysis of data. We identified 47 *WAS* gene variants in these 67 patients, 24 of which were novel and are being reported here for the first time (**Supplementary Table 2**). Mutations were found in all exons, except exons 8 and 11. Commonest mutations were single nucleotide substitutions (39/67) [nonsense (19 patients, 28.4%); missense (19 patients, 28.4%); and stop-loss (1 patient, 1.5%)]; followed by frameshift deletions (n=13, 19.4%); splice-site defects (n=11, 16.4%); small insertions (n=3, 4.5%); and large deletions (n=1, 1.5%). (**Supplementary Table 3** and **Figure 2**). Fifteen of 19 missense mutations were found in EVH1 (Drosophila enabled/vasodilator-stimulated phosphoprotein homology 1) domain (exons 1- 4). Hotspot mutations identified in our cohort included (p.R86H (n=5), p.R13X (n=4), p.R34X (n=4). It is interesting to note that, of the 6 frameshift deletions in exon 10, 5 resulted in termination of protein at 444 position (Patient no 03, 32,84,96,98,25,26 in **Supplementary Table 2**).

Novel Variants

Twenty-four novel variants (marked with bold in **Supplementary Table 2** and **Figure 3**) were found in 27 patients. These included deletions (10); splice-site (5); insertions (3); nonsense (3); and missense (3). Four novel variants were found in proline rich region (exon 10) while 3 were in EVH1 domain (exon 4). Of the remaining 17 variants, 12 were exonic while 5 were intronic. Combined Annotation Dependent Depletion (CADD) Scores for single nucleotide variants (3 missense and 4 splice site) were calculated and was found to be more than 24 suggesting deleterious effect (**Supplementary Table 2**). Sixteen novel variants (frameshift deletions, frameshift insertions and nonsense) resulted in premature termination (PT) protein synthesis and were thus classified as pathogenic.

Missense and splice-site variants (7/9) occurred in the coiled-coil region of WASp protein. Missense variants (p.G40R, p.T48N and p.V75L) and splice-site variant (c.415+5G>C) affected WH1 domain responsible for binding of WASp interacting protein (WIP) and stabilization of WASp. Variant (p.G40R) resulted in substitution of non-polar amino acid by a polar amino acid at 40^{th} position placed adjacent to α-helix and resulted in reduction of Wasp protein. Stability of β-strands was affected by variants (p.T48N and p.V75L) as substituted amino acids were neither β-branched nor aromatic. Variant (p.T48N) also resulted in low WASp expression. Intervening sequence (IVS) variant (c.832+3_+6delGAGT) caused splicing defect in Cdc42-binding site and Rac- interactive binding (CRIB) domain and reduced expression of WASp in expression.

Deletion (c.1564_1567delAGTG) affected Verprolin homology sequence (V) that binds monomeric actin and central (C) and acidic (A) sequence (VCA) domain caused

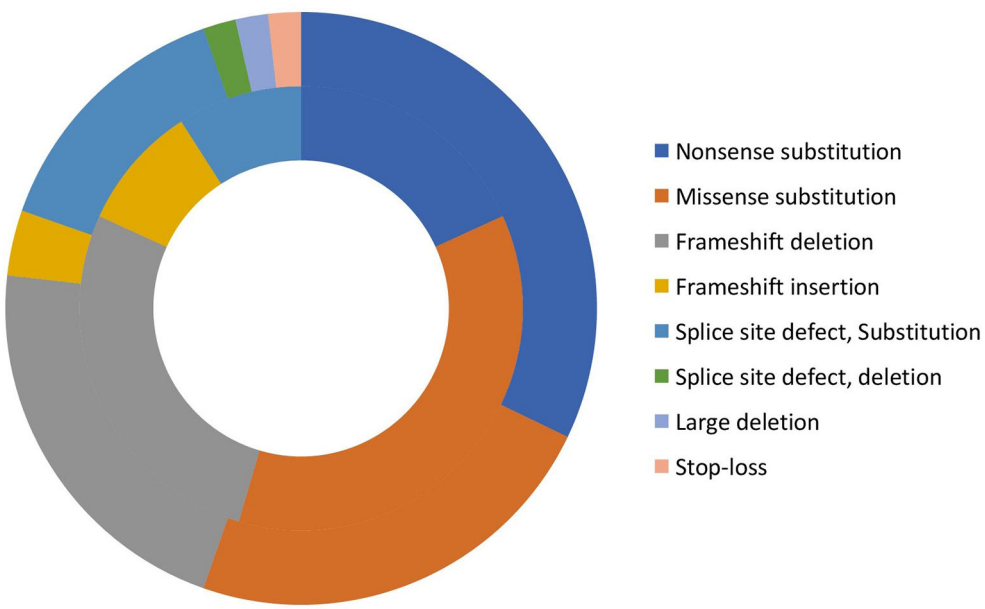

FIGURE 2 | Distribution of variants in XLT, and classic WAS patients (Inner circle: XLT, and outer circle: WAS).

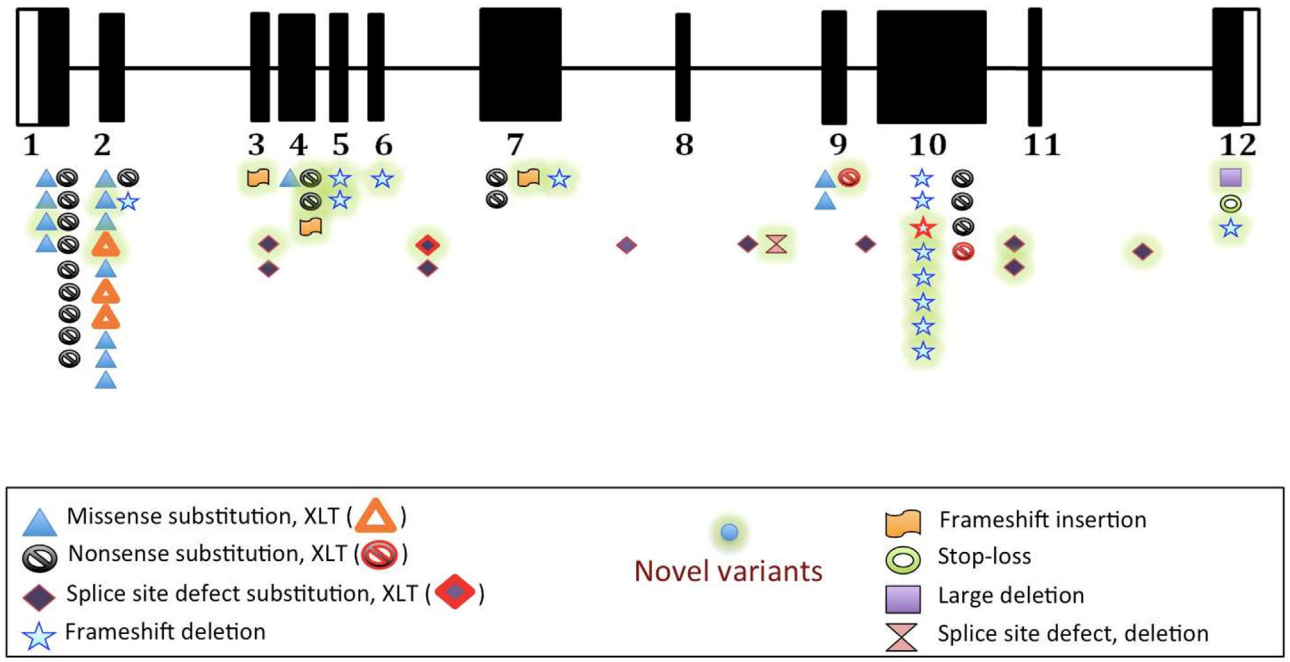

FIGURE 3 | Distribution of variants on various exons, and intervening sequences of *WAS* gene in study population (n=67).

extension of WASp beyond termination codon, binding of Actin related protein 2/3 (Arp2/3) complex resulting in instability of WASp. Variant (c.1507+2T>A) caused splicing defect and affected central (C) sequence essential for nucleation and interaction with Arp2/3 complex disturbing the stability of WASp and also affected WASp protein expression in the patient.

Genotype-Phenotype Correlations

Of 67 patients in whom genetic analysis details were available, 56 had WAS and 11 had XLT (**Supplementary Table 3**). Nonsense variants (32.1%) were the most common mutations found in patients with WAS while missense mutations were most common mutations found in patients with XLT (4/11, 36.4%). Premature termination (non-sense, frameshift, and splice site mutation) was more frequent in patients with WAS phenotype than those with XLT [39/56 (69.6%) versus 7/11 (63.6%)]. Though, missense mutation were more common in XLT, 15 patients (26.7%) with WAS were found to have missense mutations.

Treatment

Treatment details were available for 66 of 95 patients in our cohort. Of these 57 patients (86.3%) were commenced on cotrimoxazole prophylaxis. IVIg was administered in prophylactic dose (400 mg/kg/month) in 52 patients (78.8%). Twenty-eight patients (28/66, 42.4%) had received corticosteroids. Major indications of corticosteroid treatment included autoimmune manifestations like AIHA (8 patients), skin vasculitis (8 patients), and colitis (2 patients). Eight patients had received corticosteroids in variable doses and duration for refractory thrombocytopenia as they were initially diagnosed and followed up as ITP. Rituximab was used in the patient with Takayasu arteritis (and WAS) in addition to IVIg and methyl prednisolone. Splenectomy was performed only in one patient with refractory thrombocytopenia.

HSCT was performed in 25 patients (26.3%) (**Supplementary Table 4**). Haploidentical transplants were carried out in 10 patients while matched unrelated transplant were performed in 4 patients. Five patients each underwent matched related, and cord blood donor transplantation (**Supplementary Table 4**). Conditioning regimens included fludarabine (n= 19), busulfan (n=14), and anti-thymocyte globulin (n = 10). Acute graft versus host disease (GVHD) occurred in 10 of 24 transplant recipients and 9 of these were managed successfully with systemic corticosteroids. One child succumbed to acute GVHD. Complete donor chimerism (whole blood donor cell chimerism of >95%) was attained in 15/25 recipients (60%), while mixed chimerism occurred in 3 patients. Successful lymphoid and myeloid engraftment was achieved in 14 patients, and they were maintaining a disease-free survival course (DFS) till date. One child was awaiting a second HSCT. Nine patients succumbed to transplant related complications- 4 had sepsis; 2 had GVHD; 1 each had CMV disease, pulmonary hemorrhage, acute respiratory distress syndrome, and immune cytopenia.

Outcomes

Outcome details were available for 89 of 95 patients (93.7%), while follow-up duration was available for 56 patients with a

median follow-up was 36 months (range 2 weeks- 12 years; IQR 16.2 months- 70 months). Of 89 patients in whom follow up data were available, 33 (37%) had died (classic WAS 32; XLT 1) at the time of analysis. Causes of death included intracranial hemorrhage (5 patients), transplant related complications (9 patients, **Supplementary Table 4**), malignancy (2 patients), severe gastrointestinal bleed (1 patient), HLH(1 patient), infections (7 patients including 2 patients with pneumonia). Cause of death could not be ascertained in remaining 8 patients.

Survival Analysis

Overall survival of our cohort is shown in **Figure 4A**. Cumulative survival on Kaplan- Meier analysis was not statistically significant between the following parameters: XLT versus classic WAS (P 0.154) (**Figure 4B**); presence or absence of autoimmune manifestations (p 0.410) (**Figure 4C**); HSCT versus conservative management (p 0.543) (**Figure 4D**); and missense mutations versus other types of mutations (p 0.083) (**Figure 4E**).

DISCUSSION

WAS is one of the oldest PIDs described, yet there are hardly any data on this condition from India. We report the first-ever multicentric cohort of children with WAS from India.

Clinical presentation of patients with WAS/XLT is extremely variable. Patients often present to different pediatric sub-specialties, and are often misdiagnosed as chronic ITP, colitis, or atopic dermatitis (37). It is therefore important that General pediatricians, as well as those working in pediatric sub-specialties should be aware of this disorder for early diagnosis. The classical triad of eczema, thrombocytopenia, and immune deficiency may not be evident at first presentation (38) but usually evolves over time. The triad was noted only in (15.7% patients in our cohort also at the time of first presentation.

The median age of diagnosis was as low as 1.75 months in an initial cohort from USA (38) while it ranged from 11months (1-42 months) to 24 months (1-132 months) in studies from China and Turkey respectively (37, 39). Mean age at diagnosis was 31.9 months (1-108 months) at our center in the year 2008 (23). However, median age at diagnosis in this multicentric cohort was found to be 12 months (5.5-36 months). This suggests improved recognition and early diagnosis of patients with WAS which can be ascribed to increased sensitization and improving diagnostic facilities in our country.

Thrombocytopenia is a universal feature of WAS and XLT, usually manifesting as petechiae, spontaneous or prolonged bleeding in first year of life. Most common presenting manifestations observed in our study was bleeding from gastrointestinal tract and skin bleeds as reported previously in other studies also (37, 38, 40). As us uniform graded data about bleeding was not available from all centers, A comparative profile of clinical manifestations with various published cohorts have been summarized in **Supplementary Table 5**.

Patients with WAS are more susceptible to infections due to impairment in both cellular and humoral immunity. Sino-pulmonary infections were the commonest infection in our cohort. Viral infections from Varicella-zoster virus (VZV), Herpes simplex virus (HSV), EBV, CMV, and Human papillomavirus (HPV) can be extremely severe CMV infection in WAS needs particular attention and often poses a diagnostic dilemma (38, 41–43). Thrombocytopenia may be erroneously ascribed to CMV infection and underlying diagnosis of WAS is often missed or delayed (41, 42). Life threatening hemorrhagic varicella was observed in 2 patients. Complicated varicella infection is often seen in children in India, as vaccination is optional and not part of universal immunization program. It is essential to highlight that all patients with WAS had received BCG vaccine at birth (as part of the national immunization schedule); however, BCG adenitis or disseminated BCG disease was not seen in any patient. BCG infection in patients with WAS per se is extremely rare (44).

Autoimmune manifestations are seen in up to 70% of WAS patients (37–40, 45–48) and can even occur post HSCT (49). A multitude of autoimmune complications affecting all organs (**Supplementary Table 5**) are described though autoimmune cytopenia (including hemolytic anemia, neutropenia, and thrombocytopenia) are the most reported complications. Autoimmune manifestations were seen in 40% of children, with AIHA being the commonest. Unusual autoimmune complication seen in our cohort were Takayasu arteritis (50) and Guillain Barre syndrome, which have been rarely described before (50).

Children with WAS have increased risk to develop malignancies. Overall incidence of tumors is reported in up to 13% (38, 46) while in our cohort it was 2.1%. Most reported malignancies are EBV related B cell lymphomas or leukemia (2, 38). As most children with WAS are transplanted early in developed countries malignancies are seen much less commonly. However, they are still encountered in patients in developing countries and are important contributors to mortality. Both the children with malignancy in our cohort succumbed to their illness.

Differentiation between XLT and WAS is based on the clinical scoring system (35). Clinical observations indicate that age of patient at diagnosis may influence the score assignment. Bleeding may be the only clinical manifestation in early infancy, suggesting a milder phenotype. These patients may evolve to develop serious infections, autoimmune manifestations, and malignancy later in childhood. Patients with milder phenotypes of WAS may go on to develop serious infections, autoimmune manifestations, and malignancy later in childhood. Also, clinical observations indicate that age of patient at diagnosis may influence the score assignment. Thus, it has been proposed to assign a score of 5 to patients with age below 2 years who present with life-threatening severe refractory thrombocytopenia (51). Five patients who were labeled as XLT evolved into the WAS phenotype on follow-up. Moreover, WAS expression or genotype is not considered in the scoring system.

Intracellular staining of WASp by flow cytometry is a quick test to establish diagnosis if protein expression is completely absent or markedly reduced. However, interpretation of result is difficult in patients with residual expression. Genetic analysis is needed in clinically suspected patients with residual WASp

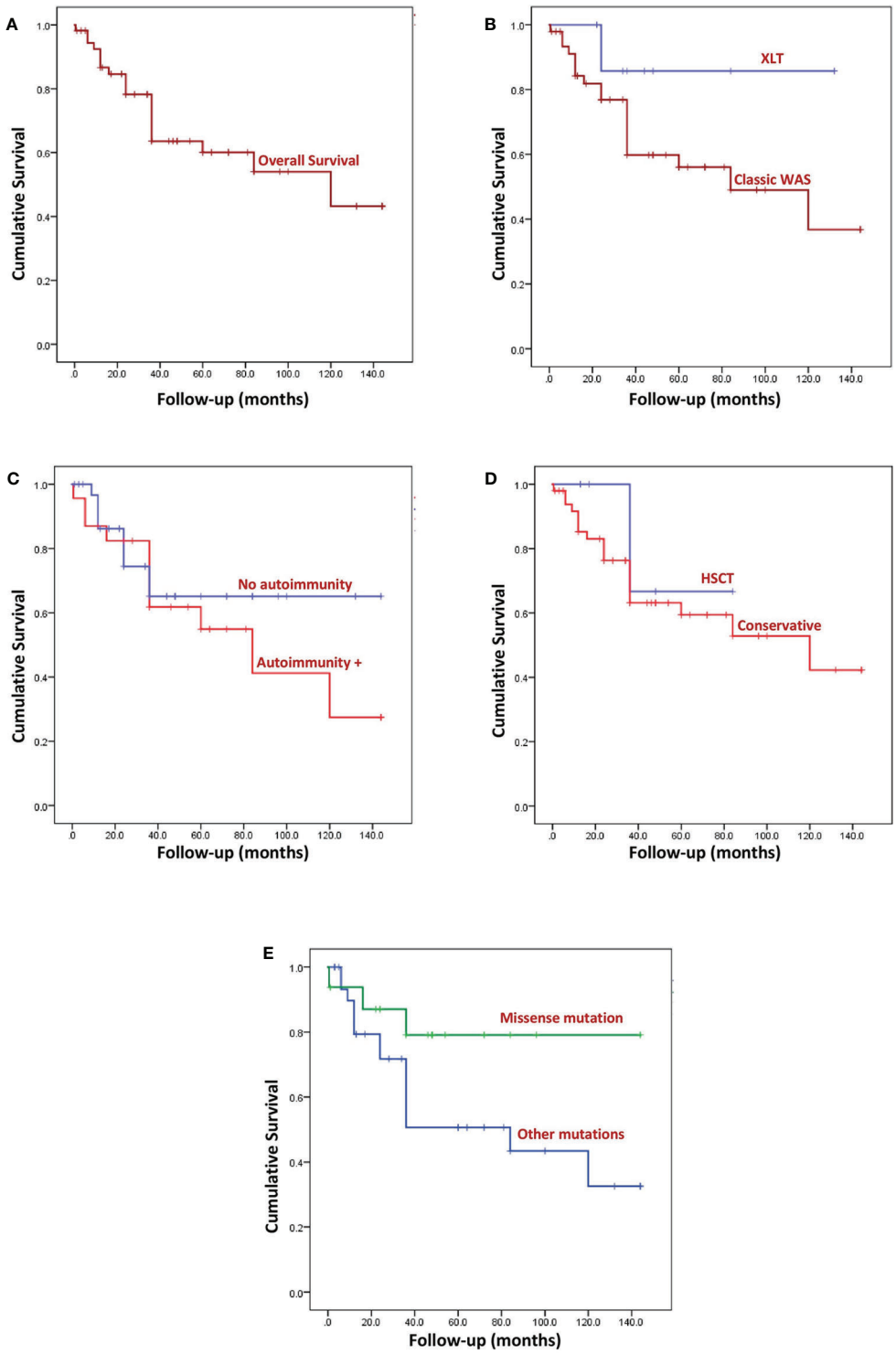

FIGURE 4 | Kaplan Meier survival curves. **(A)** Overall survival (OS) of the entire cohort. Median follow-up was 36 months (range: 2 weeks- 12 years). **(B)** Comparison of OS between XLT and WAS, (p 0.154). **(C)** Comparison of OS of patients with autoimmune manifestation and without autoimmunity, (p 0.410). **(D)** Comparison of OS of patients who underwent HSCT and patients who were not transplanted, (p 0.543). **(E)** Comparison of OS of patients who had missense variants and those with variants other than missense mutation, (p 0.083).

expression for confirmation of diagnosis. Despite expanding availability of flow cytometry-based test, this specialized test is not easily available at all centers in India. WASp expression may be preserved in patients with milder phenotypes with missense variants (46) and mutations involving C terminal end of *WAS* gene. WASp antibody usually recognizes and binds to epitope in the region between amino acid 146-265 thus resulting in normal WASp expression in some patients (52). Normal WASp levels could also be seen in patients with revertant mutations. Two patients with intronic mutations and two patients with frameshift mutation in C terminal (exon 10) had normal WASp expression in our cohort.

Identification of mutation in the *WAS* gene is essential for confirmation of diagnosis of WAS. Sanger sequencing technique is an easy, accessible and effective method for determining variants in the 12 exons and intron/exon boundaries of the WAS gene. Coverage of proximal and distal promoter regions of WAS gene is important as variants are often encountered in these regions and can be missed by NGS panels. We found 10 unique intronic mutations in 11 families in our cohort.

We found 47 unique mutations of *WAS* gene in 67 patients in our cohort. Of these, 24 proved to be novel. Fifty-six patients had exonic variants, with 31 patients (55.3%) having mutations localized to exons 1-4. Several cohorts have reported a frequency of 50%- 58.8% of these mutations in first 4 exons (40, 42–44, 51, 53, 54). This information is helpful for initial screening of patients by targeted sequencing. Studies suggested nonsense variants to be the commonest in their cohort (40, 42–44, 48, 51, 53–55), while others have found missense mutations to be more common (47, 56, 57). In our study both missense, and nonsense variants were equal.

Published literature suggests that deleterious mutations (i.e., non-sense, frameshift, and splice site mutations) are more common in classic WAS as compared to XLT phenotype (45). In our cohort, these mutations were 5.5 times more frequent in patients with classic WAS than XLT. However, this difference was not statistically significant (p 0.262). On the other hand, missense mutations which are reported more with XLT (45) were also found in 26.78% patients with WAS.

Management of patients with WAS must be individualized to specific clinical manifestations and degree of severity. Allogeneic HSCT has proven to be potentially curative and more recently, gene therapy is an important alternative in patients in whom HSCT cannot be performed (58). Conventionally, children with XLT, who have milder disease, do not require standard prophylactic interventions. However, with declining immune functions, development of clinically evident infections or defective antibody responses they too may require prophylactic measures such as cotrimoxazole prophylaxis or IVIg replacement. Risk of life -threatening complications and recognized life-long medical problems that affect the prognosis and quality of life of these patients, many centers now also support early HSCT for patients with XLT (59). However, risk-benefit of HSCT for patients with WAS mutations needs to be individualized in resource limited settings with high transplant related complications and mortality.

Decision to initiate chemoprophylaxis with cotrimoxazole (and whether it should include anti-viral and/or antifungal medications) depends on frequency, severity, and type of infections suffered by the individual patients. Most of our patients were initiated on cotrimoxazole prophylaxis (57 patients, 86.3%).

Prophylactic immune globulin treatment IVIg needs to be given even if serum IgG levels are within normal limits, as these patients have functional defects. Most of our patients at PGIMER, were initiated on replacement therapy depending upon frequency and severity of infections. Subcutaneous immunoglobulin is not available in India at present. Providing regular IVIg is a daunting task in financially deprived situations. Of late, support from state Governments and well as non-governmental philanthropic organizations has been very helpful is recognized in providing this therapy to children with PID.

WAS was among the first few diseases to be treated by HSCT (1968) (60). HSCT results in complete reversal of the disease and reported outcomes from developed countries are extremely encouraging. The ESID Registry of 170 patients with WAS have reported an overall survival of 87% with HLA-matched sibling donors (MSD), 71% with MUD, and 52% with mismatched donors (61). There are occasional reports of successful HSCT in patients with WAS from various centers in India (25, 26, 29). Only 25 patients (26.3%) could undergo HSCT in our cohort, and transplant related mortality was 36%. HSCT still remains out of reach for most patients in developing countries. There are many barriers to successful HSCT in resource limited settings. Limited centers offer this specialized therapy and there is a need for more robust donor registries. Delays in diagnosis often results in transplant being performed much later in life-this itself affects the outcomes adversely.

Mortality (33 patients, 37%) continues to be high in our cohort. Like threatening bleeding episodes, infections, and development of complications like malignancy and autoimmunity remain important causes of death.

Several limitations of the study are recognized. Complete uniform data was not available from all centers especially with respect to bleeding, infections, and treatment. Laboratory data including immunophenotyping of lymphocyte subsets, WASp expression and genetic analysis could not be performed in all patients. Moreover, the median follow-up was only 3 years only, thereby limiting the data about long term complications and, and outcomes.

The authors are aware of other centers in the country that could not participate in the study and have not been thus reported.

CONCLUSIONS

To conclude, we document the first nationwide study on clinical and genetic features of 95 patients with WAS from India. We report wide spectrum of clinical manifestations and 24 novel variants in our cohort. Mortality continues to be high as definitive therapy is not accessible to all patients. There is an urgent need to increase awareness of WAS amongst internists

and pediatricians and improve pediatric HSCT services in our country.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institute Ethics Committee, PGIMER, Chandigarh. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

DS, RRi, AJ, PV, AG, MS, AKh, RRa, RU, MD, PT, VG, AP, MM, HL, SB, HK, SV, and SS: Clinical management of patients and provided necessary clinical details for compilation. AR, MM, JA, RR, PB, AKa, JS, PT, KI, KC, PL, OO, SN, and YL: Laboratory work-up of patients and provided necessary laboratory results for compilation. DS, RRi, MS, AKa, and JS: compiled the data and framed the initial draft and editing of manuscript. DS, RRi, MS, and AKa: literature search. DS and SS: editing of manuscript at all stages of preparation and final approval. 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.2021.627651/full#supplementary-material

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Clinical Profile of Hyper-IgE Syndrome in India

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Introduction: Hyper-IgE Syndrome (HIES) is a rare inborn error of immunity (IEI) characterized by a constellation of symptoms related to susceptibility to *Staphylococcal* skin and pulmonary infections, eczema, raised serum IgE (>2,000 IU/ml), craniofacial anomalies, and recurrent bone fractures. Data on HIES from the Indian subcontinent is scarce and restricted to small case series and case reports. This is the first compilation of national data on HIES.

Materials and Methods: A total 103 cases clinically diagnosed and treated as HIES were analyzed from nine centers. Cases with clinical and/or molecular diagnosis of DOCK8 deficiency were not included. Patients were divided into two groups: group I for whom a heterozygous rare variant of STAT3 was identified, and group II, with clinical features similar to those of AD STAT3 deficiency, but without any genetic diagnosis.

Results: Genetic diagnosis was available in 27 patients (26.2%) and all harbored rare variants in the STAT3 gene. Majority of these STAT3 HIES patients presented with recurrent skin abscesses (77.7%) or pneumonia (62.9%) or both (59.2%). Other features included eczema (37%), candidiasis (55.5%), facial dysmorphism (55.5%), recurrent fractures (11.1%), and retained primary teeth (7.4%). *Mycobacterial* infections were seen in a significant 18.5%. Mortality was seen in three subjects (11.1%). A similar trend in the clinical presentation was observed when all the 103 patients were analyzed together. Twenty percent of patients without a rare variant in the STAT3 gene had an NIH score of ≥40, whereas, 51.9% of STAT3 HIES subjects had scores below the cut off of ≥40. TH17 cell numbers were low in 10/11 (90.9%) STAT3 HIES tested. Rare variants observed were 8 in exon 21; 8 in exon 13; 3 in exon 10; 2 in exon 15, and one each in exon 6, 16, 17, 19, 22, and splice site downstream of exon 12. Seven variants were novel and included F174S, N567D, L404Sfs*8, G419 =, M329K, T714I, R518X, and a splice site variant downstream of exon 12.

Conclusions: The report includes seven novel STAT3 variants, including a rare linker domain nonsense variant and a CC domain variant. *Mycobacterial* diseases were more frequent, compared to western literature.

Keywords: hyper-IgE syndrome, India, STAT3 LOF, multi-centric study, rare variants

INTRODUCTION

Hyper-IgE Syndrome (HIES) represents a heterogenous group of disorders majorly resulting from impaired STAT3 signaling. Characteristic features include *staphylococcal* "cold" skin abscesses, *staphylococcal* pneumonia with *pneumatocele* formation, early onset eczema, muco-cutaneous candidiasis, retained primary dentition, recurrent fractures, osteoporosis, and a raised serum IgE (>2,000 IU/L). Dominant negative heterozygous STAT3 loss-of-function (LOF) mutations accounts for majority of the autosomal dominant (AD) and sporadic forms of HIES. (1, 2). Lack of TH17 cells, resulting from a defective STAT3 signaling probably accounts for only a minor fraction of the HIES disease spectrum because, inborn errors of immunity involving the IL-17 axis results in isolated chronic mucocutaneous candidiasis without any other features of HIES (3). Another feature that has emerged over the recent years is the presence of cranio-facial and dental anomalies in defects involving IL-6ST (gp130) (4–6) apart from STAT3 (1, 2). Occurrence of similar cranio-facial anomalies in IL-11R deficiency (7), that functions upstream of STAT3 through the common gp130 receptor chain (7, 8) but lack of the same in IL-6R deficiency (9) points toward a defect in IL-11/STAT3 mediated signaling as the cause for the craniofacial anomalies. While ZNF341 is required for transcription of STAT3 (10, 11), ERBB21P functions through formation of Stat3/erbin/Smad2/3 complex (12). Other molecules like PGM3 (13), and CARD11 (14) are not etiologies of bonafide HIES as they lack many features of typical HIES (15).

Diagnosis of HIES, like many other IEIs is a combination of clinical and laboratory parameters, and there is no single specific test which clinches the diagnosis. Whereas absolute eosinophil count (AEC) and serum IgE levels can be part of routine laboratory testing, TH17 cell numbers, pSTAT3 assay, and memory B cell numbers are tests that can be done only in specialized laboratories. Molecular diagnosis hence becomes imperative for clinching the diagnosis. With the advent of commercially available NGS platforms providing molecular diagnosis at a reasonable cost, it has become possible for clinicians to make a diagnosis in a suspected case even without the specialized laboratory tests. These functional tests are however still relevant even if a reverse diagnostic approach starting with NGS is considered.

The first case of HIES from India was reported in 1994 by Pherwani et al. (16). Salaria et al. (17, 18) reported three cases in two separate reports in 1997 and 2001. Pherwani and Madnani (19) reported six patients with prominent cutaneous and respiratory features, but only one had familial involvement. Patel et al. (20) reported 10 cases in 2018 but all these reports were without a molecular diagnosis. The first series of six patients of STAT3 LOF HIES with a documented genetic defect was reported by Saikia et al. (21) in 2014, that included a novel variant. This was followed by another case report with a novel variant in 2017 (22). Publications in the form of original research papers followed from the center at Chandigarh subsequently (23, 24). More research is currently being undertaken at this center with funding from Indian Council of Medical Research, New Delhi, and Jeffrey Modell Foundation, USA, but there has been a stark silence from any other center in the country except for a case report by Govindaraj et al. (25) in 2018. In a report by Gupta et al. (26) in 2012, HIES accounted for 4.9 and 16.3% of all IEIs diagnosed at two major centers in India. This manuscript is the first effort to compile data on Hyper-IgE Syndrome at a national level. Cases reported in references 19, 20, and 23 ($n = 6$) are included in this report.

MATERIALS AND METHODS

Patients and Clinical Presentation

The cohort consisted of a total of 103 subjects. Data was compiled from from all regional centers supported by the Foundation for Primary Immunodeficiency Diseases (FPID), USA, and other centers (federal government run as well as from the private sector) providing clinical care to patients with IEIs using a common proforma that was circulated by email. These nine centers included Postgraduate Institute of Medical Education and Research, Chandigarh (number of cases contributed, $n = 34$); BJ Wadia Hospital for Children, Mumbai ($n = 26$); Indian Council of Medical Research-National Institute of Immunohaematology (ICMR-NIIH), Mumbai ($n = 15$); Kasturba Medical College, Mangalore ($n = 13$); Department of pediatric hematology, oncology, blood and marrow transplantation, Apollo hospitals, Chennai ($n = 5$); Aster CMI Hospital, Bangalore ($n = 5$); Niruj Rheumatology Clinic, Ahmedabad ($n = 2$); Sir Ganga Ram Hospital, New Delhi ($n = 2$); and Government Medical College, Kozhikode, Calicut, Kerala ($n = 1$). Cases with a clinical diagnosis of HIES with or without a molecular diagnosis and under treatment/follow-up in these centers were recruited. The following information was obtained from each participating center: age at presentation of index patient, gender, highest serum IgE levels, highest absolute eosinophil count (AEC), NIH score, family history, skin infection (with pathogen Isolates), pulmonary infections (pneumonia) with or without pneumatocele, associated TB if present, fungal infections, facies, connective tissue, and skeletal abnormalities (retention of primary teeth, minor trauma fractures, osteopenia, scoliosis, hyperextensible joints), vascular abnormalities (aneurysms, dilation of arteries), associated autoimmunity/malignancy,

pSTAT3 (%), Th17 cells (%), Memory B cells (%), treatment and follow up, gene variant: gene, exon, nucleotide change, amino acid change, ACMG Classification, and whether a known or a novel rare variant. Cases with a suspected and/or molecular diagnosis of DOCK8 deficiency were not included in the study.

Immunologic Investigations

TH17 cell enumeration, Memory B cell numbers, and pSTAT3 assay is available only at PGI Chandigarh, and were hence done in the cases that were assessed here. Five cases were however evaluated on transported samples from various centers.

TH17 Cell Enumeration

PBMCs were isolated with Ficoll-Hypaque density centrifugation (Sigma Aldrich, St Louis, Mo). T_H17 cells were identified by means of intracellular staining of $CD4^+$ T cells for the production of IL-17. Briefly, 1×10^6 cells from patients and an age matched healthy control subject were stimulated for 6 h with 10 ng/ml phorbol 12-myristate 13-acetate and 1 ug/ml ionomycin (Sigma-Aldrich, St Louis, Mo) in the presence of GolgiPlug (BD Biosciences, San Jose, CA). After cell-surface staining with PerCP-conjugated anti-CD4 (BD Biosciences, San Jose, CA), cells were fixed, permeabilized (Cytofix/Cytoperm, BD Biosciences, San Jose, CA), and stained with Alexa Fluor 647-conjugated anti-IL-17A (BD Biosciences, San Jose, CA). Immunoglobulin isotype control was used as a background control. $CD4^+$ T cells were also evaluated for IFN-γ production (FITC-conjugated anti-IFN-γ; BD Biosciences, San Jose, CA). $CD4^+IL17^+IFN\text{-}\gamma^-$ cells were taken as TH17 cells (**Supplementary Figure 1A**).

Phospho-STAT3 Assay

One-hundred microliters of fresh whole blood were incubated with IL-6 for 15 min. Cells were simultaneously fixed and RBCs lysed using BD fix and lysing solution (BD Biosciences, USA). Cells were then permeabilized for 20 min using Perm III solution (BD Biosciences, USA) and subsequently incubated with Alexa Fluor 647 phospho-STAT3 antibody against phospho-Y705 (BD Biosciences, USA) for 30 min at room temperature. After washing twice with stain buffer, cells were suspended in 1% paraformaldehyde for acquisition (**Supplementary Figure 1B**).

Memory B Cells

Memory B cells were assessed as CD19+ CD27+ cells using CD19-FITC and CD-27 APC antibodies (BD Biosciences) using standard surface staining protocols. All flow cytometry assays were performed on a BD LSR Fortessa instrument (BD Biosciences) and analyzed with Cell Quest Pro software (BD Biosciences).

Molecular Analysis

Molecular analysis was performed either by NGS or Sanger sequencing as per availability at the referral centers. The NGS panels included a limited 44 gene panel and a 320 gene panel. The former included STAT3 and DOCK8 as the HIES associated genes while the latter included STAT3, DOCK8, TYK2, and CARD11. Sanger sequencing for the STAT3 gene was performed using a set of previously published primers. The sequencing data were analyzed using Codon Code Aligner software. Polymorphism Phenotyping program (*PolyPhen*, http://genetics.bwh.harvard.edu/pph) and Combined Annotation Dependent Deletion (*CADD*, http://cadd.gs.washington.edu) programs were used to predict the effect of the identified STAT3 rare variants.

Statistical Analysis

Descriptive methods of statistical analysis were used using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, version 15.0 for Windows).

RESULTS

Of the 103 individuals, 76 were males and 27 were females, with age at diagnosis ranging from 6 months to 35 years (mean age 7.4 years, median 5 years). For analysis, patients were divided into two groups: patients of group I, for whom a heterozygous rare variant of STAT3 has been identified ($n = 27$), and patients of group II, with clinical features similar to those of patients with AD STAT3 deficiency, but without any genetic diagnosis ($n = 76$).

Group I, HIES With Rare Variants in STAT3 Gene

Genetic diagnosis was available in 27 patients (26.2%): 19 males and 8 females from 25 kindreds with a mean age at diagnosis of 9.8 years (range 8 months to 35 years). A positive family history in the form of sib death or sibling with similar symptoms was elicited in six kindreds of which two kindreds had a clear cut autosomal dominant pattern of inheritance with an affected parent. Serum IgE levels (available in 26 subjects) of >2,000 IU/mL was observed in 19 (73%), whereas it was in the range of 1,000–2,000 IU/mL in three subjects (11.5%). Blood eosinophilia of ≥700/Cumm was seen in 63.6%. An NIH score of ≥40 was present in 13 (48.1%), and more importantly, the rest (51.9%) had a score between 20 and 39, which was below the cut-off of ≥40. Majority of the patients presented with recurrent skin abscesses (21/27, 77.7%). Pneumonia was seen in 17 (62.9%) of which *pneumatoceles* were seen in 7 (25.9%), (pyo)-pneumothorax in 4 (14.8%), and empyema in 3 (11.1%). Pneumonia with recurrent skin abscesses was seen in 16 subjects (59.2%). Other atypical sites included psoas abscess ($n = 2$), sternoclavicular abscess ($n = 1$), and orbit ($n = 1$). Eczema was seen in 10 patients (37%). Connective tissue and skeletal abnormalities were observed as follows: facial dysmorphism 15 (55.5%), hyperextensible joints 6 (22.2%), recurrent fractures 3 (11.1%), retained primary teeth 2 (7.4%), and scoliosis 2 (7.4%).

The commonest pathogen encountered was *Staphylococcus aureus* (20/27, 74%). Associated candidiasis was seen in 15 (55.5%) of which, majority were oral ($n = 12$), followed by nail ($n = 3$), lungs ($n = 3$), and skin ($n = 1$). A combination of oral candidiasis and onychomycosis was seen in two subjects. One patient had a mediastinal mass due to *Aspergillus Niger*. Other notable pathogens isolated included *Mycobacterium Abscessus complex* ($n = 1$), injection site BCG infection ($n = 2$), and *Mycobacterium tuberculosis* (MTB; $n = 2$): a case each of Pott's spine and a subcutaneous abscess in the arm, the latter showing presence of acid-fast bacilli on pus aspirate. Probable autoimmunity was encountered in 1 patient who developed anal

and oral ulcerations with evidence of immune complex vasculitis on biopsy. One case of early CMV pneumonia at 2 months of age was encountered.

Absolute lymphocyte count values were available in 13 subjects and were all within normal range. $CD4^+IL17^+$ TH17 cell numbers were performed in 11 subjects and was found to be low (<0.5%) in 10 (90.9%). pSTAT3 was performed in eight subjects and was low in 3 (37.5%). Memory B cells were done in five subjects and were low in all.

All 27 patients harbored rare variants in the STAT3 gene: eight in exon 21; eight in exon 13; three in exon 10; two in exon 15, and one each in exon 6, 16, 17, 19, 22, and splice site downstream of exon 12 (**Figure 1**, **Supplementary Table 1**). Of these, three were picked up by whole exome sequencing, 16 by the 320 gene NGS panel, one by the limited 44 gene NGS panel, and seven by Sanger sequencing for STAT3 gene (**Supplementary Table 2**). Majority were missense variants ($n = 23$), and one each of frameshift, nonsense, synonymous, and splice site rare variants. A total of 18 rare variants were identified from 25 kindreds, of which two kindreds were AD with an affected parent harboring the same STAT3 variant. Ten variants were in the DNA binding domain (DBD), four in the SH2 domain, two in the linker domain (LD), and one each in the trans-activation (TA) and coiled-coil (CC) domains (**Figure 1**). Seven rare variants were novel and they included F174S, N567D, L404Sfs*8, G419 =, M329K, T714I, R518X, and a splice site variant downstream of exon 12. The latter alteration was predicted to result in a broken WT Donor Site alteration, most probably affecting splicing and categorized as disease causing [(HSF Donor site (matrix GT) chr17:42329749 AGGGTAAGT>AGGGTAAAT 93.76>84.19 (−10.21%); MaxEnt Donor site chr17:42329749 AGGGTAAGT>AGGGTAAAT 10.45>5.83 (−44.21%)].

There were two kindreds with familial AD LOF-STAT3-HIES. One (*kindred 2*, **Supplementary Table 1**) was an 8 months old female child presenting with recurrent oral thrush with history of CMV pneumonia at 2 months of age and had a raised serum IgE of 1,200 IU/mL. Genetic analysis revealed a known pathogenic heterozygous variant p.V637M in exon 21 of the STAT3 gene. The same variant was found in the mother. The mother was however relatively asymptomatic and gave history only of occasional pyoderma in childhood. The second kindred (*kindred 16*, **Supplementary Table 1**) were a family where a 2 months old child presented to the hospital with severe pneumonia, pneumothorax, pyoderma, had typical facies and serum IgE of 2,449 IU/mL. The child however died before he could be investigated further. The father of the child, 35 years of age, had coarse facies and gave history of recurrent pneumonias since childhood. Genetic analysis of the father revealed a known pathogenic heterozygous variant p.K340Q in exon 10 of STAT3. The variant was found in another son, 5 years of age, who had recurrent upper respiratory infections and itchy skin lesions. This family has been previously reported (21). They were however lost to follow-up.

p.F174S (*kindred 1*, **Supplementary Table 1**) was a CC domain heterozygous rare variant in a 15 years old male with history of atopic eczema in childhood, allergic rhinitis and cellulitis in the cheek at 13 years of age. He had oral candidiasis and hyperextensible joints but his serum IgE was not raised. He developed mediastinal and abdominal lymphadenopathy with splenomegaly and abdominal lymph node biopsy showed *Mycobacterium abscessus complex*. A possibility of Mendelian Susceptibility to Mycobacterial Disease (MSMD) was considered, but no variants were found in the MSMD related genes (**Supplementary Data: Case Report 1**).

R518X (*kindred 20*, **Supplementary Table 1**) was a *de novo* heterozygous rare variant detected in a 2 years old boy presenting with pneumonia and extremely high absolute eosinophil count (21,432/Cumm). The variant was predicted to result in a truncated STAT3 protein lacking both the SH2 (required for dimerization) and the transactivation domain (containing the Y705 phosphorylation). Both the parents, and the 3 siblings were STAT3 wild type (**Supplementary Data: Case Report 2**).

Follow-Up and Treatment

Follow up data was available in 23 subjects and 4 were lost to follow up. Majority of the patients were on antimicrobial prophylaxis ($n = 15$) and doing well on follow-up. IVIG was instituted in 3 subjects. The patient with p.F174S variant had an associated Klinefelter's Syndrome (46XXY). The patient with p.T714I variant developed a refractory *E coli* psoas abscess that required multiple surgical drainage. Patient with pR518X variant developed pulmonary symptoms for which a bronchoalveolar lavage was done that showed plenty of eosinophils and diagnosis of eosinophilic pneumonitis was considered and the patient put on oral steroids to which he responded. Mortality was observed in three male subjects (11.1%) aged 2, 4, and 15 years, all due to pneumonia and related complications. One patient with pD661V variant had a peripheral T cell Lymphoma and underwent a matched unrelated HSCT at Apollo Hospital, Chennai and is currently in remission, 3 months post HSCT.

Group II, HIES Without Documented Genetic Diagnosis

There were 76 patients where a clinical suspicion of HIES was entertained but a rare variant could not be demonstrated on genetic analysis or where genetic studies was not done. Genetic analysis was attempted in 30 subjects: 25 by sanger sequencing for STAT3 gene, four using the limited 44 gene NGS panel, and one by whole exome sequencing. The limited 44 gene NGS panel however did not contain the more recently described genes associated with HIES viz. IL-6R, IL-6ST, ZNF341, PGM3, CARD11, and ERBB21P.

There were 57 males and 19 females with age at diagnosis ranging from 6 months to 27 years (mean 6.5 years). A positive family history was elicited in 16 (21%). Serum IgE levels of >2,000 IU/mL was seen in 61/75 (81.3%), whereas it was between 1,000 and 2,000 IU/mL in 5/75 (6.6%). Blood eosinophilia of ≥700/Cumm was found in 39/68 (57.3%). Eczema was seen in 44 (57.8%). An NIH score of ≥40 was encountered in 20 (26.3%) and in majority (53; 69.7%), the score ranged from 20 to 39. Connective tissue and skeletal anomalies recorded included facial dysmorphism in 50 (65.7%), hyper-extensible joints 15

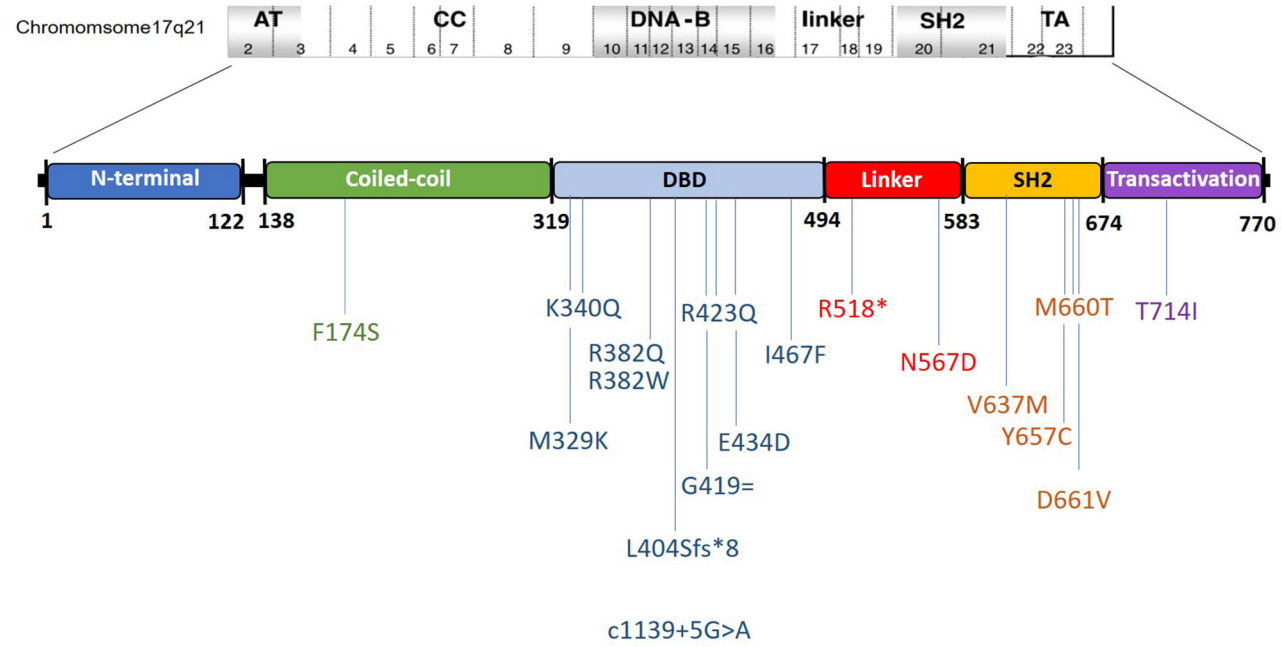

FIGURE 1 | Schematic representation of the observed mutations and their location in the STAT3 gene in the present cohort. Ordinal position of nucleotide in the exon–intron boundaries according to https://www.ncbi.nlm.nih.gov/nuccore/NG_007370.1.

(19.7%), high arched palate in 4 (5.2%), retained primary teeth in 2 (2.6%), recurrent fractures in 2 (2.6%), and scoliosis in 2 (2.6%).

Majority presented with history of recurrent skin abscesses (46; 60.5%) and/or pneumonia (45; 59.2%), both being present in 43 (56.5%). Pulmonary complications were seen as follows: pneumatocele in 6 (7.8%), bronchiectasis in 3 (3.9%), and pneumothorax in 2 (2.6%). One patient had associated psoriasis. Other manifestations included recurrent diarrhea, pyomyositis, renal abscess, and liver abscess (one case each).

Pathogen isolates included *S. aureus* (48; 63.1%) and one case each of *Streptococcus pyogenes, Pseudomonas* spp., *Acenatobacter*, and *E. coli.* Candidal infections were seen in 18 subjects (23.6%) and included 15 oral, three nail, and one case each of gastro-intestinal, skin and lung candidiasis. *Aspergillus* sinusitis was seen in one case. *Mycobacterial* infections were seen in 5: pulmonary MTB infection in 4 and local site BCG infection in 1. TH17 cells were examined in 19 cases and were found to be low in 11 (57.8%). pSTAT3 assay was done in 21 subjects and was low in 9 (42.8%).

Follow-Up and Treatment

Follow-up data were available in 52 subjects, and 24 were lost to follow-up. Of those with follow-up, 49 were on antimicrobial prophylaxis and doing well. Three patients, 2 females and a male aged 6 months, 1 year and 8 years died due to pneumonia and related complications.

Clinical features of the STAT3 HIES group, the group without a genetic diagnosis and the entire cohort combined is summarized in **Table 1**.

DISCUSSION

Though HIES has been recognized and reported from India as early as 1994, when genetic cause of the disease was still unknown, 103 cases compiled from the entire country with a population of 1,366 million (2019 census) clearly indicates it is an under recognized and under reported entity. With organizations like FPID and Indian Society for Primary Immune Deficiency (ISPID) involved in awareness campaigns amongst the medical fraternity for nearly the entire last decade, cases are now being recognized more often. With availability of commercial as well as federal government run NGS facilities, clinicians are now at a position to get a molecular diagnosis even without resorting to functional assays. The ICMR advanced center for diagnosis of PID at PGIMER, Chandigarh has taken forefront in diagnosis and research in HIES and has been providing services for assays like TH17 and pSTAT3. However, conducting these assays on transported samples from distant centers under hot and humid conditions prevailing through major part of the year has been largely frustrating.

This cohort of patients did not include patients with DOCK8 mutations in keeping with the fact that DOCK8 deficiency is considered a combined immunodeficiency and hence classified therein (27). Since STAT3 deficiency accounts for more than 90% of all autosomal dominant and sporadic forms of HIES, it's over representation with only STAT3 defect in the current cohort is understandable. However, non-representation of other genetic variants associated with HIES could be because of the lack of genetic testing in majority, and even in those who were tested, the gene panels employed did not contain the relevant genes and hence were likely to be missed.

TABLE 1 | Summary of clinical features.

Clinical feature	HIES with mutation in STAT3		HIES without mutation demonstrated		HIES, all	
Mean age at diagnosis	9.8 years		6.5 years		7.4 years	
	Number	**%**	**Number**	**%**	**Number**	**%**
NIH score ≥ 40	13/27	48.1	20/76	26.3	33/103	32.0
Serum IgE ≥ 2,000 IU/mL	19/26	73	61/75	81.3	80/101	79.2
Eosinophilia ≥ 700/Cumm	14/22	63.6	39/68	57.3	53/90	58.8
Recurrent skin abscesses	21/27	77.7	46/76	60.5	67/103	65.0
Recurrent pneumonia	17/27	62.9	45/76	57.8	62/103	60.1
Pneumatoceles	7/27	25.9	6/76	7.8	13/103	12.6
Eczema	10/27	37%	44/76	57.8	54/103	52.4
Facial dysmorphism	15/27	55.5	50/76	65.7	65/103	63.1
Hyper-extensible joints	6/27	22.2	15/76	19.7	21/103	20.3
Retained primary teeth	2/27	7.4	2/76	2.6	4/103	3.8
Recurrent fractures	3/27	11.1	2/76	2.6	5/103	4.8
Staphylococcal infection	20/27	74	48/76	63.1	68/103	66.0
Candidiasis	15/27	55.5	18/76	23.6	33/103	32.0
Mycobacterial infections	5/27	18.5	5/76	6.5	10/103	9.7
Autoimmunity	1/27	3.7	1/76	1.3	2/103	1.9
Malignancy	1/27	3.7	–	–	1/103	0.9
Mortality	3/23	13	3/52	5.7	6/75	8.0

We compared the clinical profile of our cohort to two published large cohorts: the USIDNET (28) and the French cohort (29). While the former was a clinical cohort of 85 patients without reference to their gene variants, and hence a heterogenous group with or without genetic diagnosis, the French cohort was a cohort of 67 patients from 47 kindreds with exclusively the STAT3 defect, autosomal dominant as well as sporadic. For the sake of uniformity, we compared our cases with a rare variant in the STAT3 gene with the French cohort and then our entire cohort with the USIDNET cohort. Two Chinese cohorts (30, 31) were included as Asian cohorts for comparison.

Majority of our patients with STAT3 rare variants presented with recurrent skin abscesses (77.7%) and pneumonia (62.9%) which were seen in 73 and 38% of the French cohort. Early onset eczema was 37% in our STAT3 HIES cohort but was observed in larger numbers the French cohort (48%). *S. aureus* was the commonest pathogen isolated (74%) which was seen in 94% of the French cohort. Candida was seen in 55.5% of our patients, which was 85% of the French cohort. Though majority of the patients without a genetic diagnosis had an NIH score between 20 and 39, 20% had a score ≥40. This was seen in 56% of the cases of suspected HIES without a STAT3 rare variant in the study by Woellner et al. (32). On the other hand, majority (51.9%) of our patients with a documented STAT3 rare variant had scores below cut off of ≥40.

Tuberculosis is an endemic disease in India and accounts for more than 27% of tuberculosis worldwide [*Global Tuberculosis report 2019. World Health Organization (WHO), P1,2*]. Hence, unlike western literature, presence of TB in the pathogen spectrum of HIES in India is not unexpected. Similar to other Asian cohorts, in our cohort, TB and related mycobacterial infections was seen in 18.5% of the STAT3 cohort. This included MTB as well as atypical mycobacteria (*Mycobacterium abscessus complex* and *BCG*). In two different Chinese cohorts (30, 31), 37.5 and 38.8% incidence of BCG complications have been reported that included local BCG site abscess/ulceration as well as disseminated *BCGosis*. BCG related complications were observed in 2 of our STAT3 HIES patients. *Mycobacterial* infections in HIES have been observed in other studies as well (33, 34). Malignancy in the form of a Non-Hodgkins' Lymphoma (NHL) was observed in one of our STAT3 HIES patients (0.9%). Malignancy has been observed in 7% of the patients in the French cohort all of which were NHL.

Comparison of our entire cohort of 103 patients with the USIDNET cohort showed similar trends: Staphylococcal skin abscesses 65 vs. 74.4%; pneumonia 60.1 vs. 72%; eczema 52.4 vs. 57.3%. *S. aureus* was the commonest organism isolated (66 vs.

72.3%), followed by candida (32 vs. 25.9%). Molluscum was seen in five of our 103 patients which was seen in four patients in the USIDNET cohort. Mycobacterial infections accounted for 9.7% when the entire cohort was considered.

Majority of the reported STAT3 rare variants are described in the DBD and SH2 domains which are known mutation hotspots in the STAT3 gene (1, 2, 29). DBD and SH2 domain rare variants together comprised 77.8% of the variants in our present cohort. Rare variants were however observed through all the five domains in the present cohort, including the LD and CC domain. R382Q/W was the commonest variant seen in our cohort, comprising 7/27 patients (25.9%) followed by V637M, seen in 4/27 patients (14.8%) and F174S, seen in 2/27 patients (7.4%). R382Q/W accounted for 34% of the rare variants in the French kindreds and 22.8 and 45% of the Chinese cohorts. V637M similarly accounted for 17.4% in the Chinese and 10.6% in the French cohort.

STAT3 Linker domain mutations are rare (35, 36) and constitute <2% in the larger series (1, 2, 29, 37) and few publications as case reports are found in the literature (35). Majority of reported variants in STAT3 are missense variety, and non-sense variants in the STAT3 gene are not frequent (1, 2, 29, 37). This made the R518X variant in our cohort an extremely rare variant; a null variant in the linker domain of STAT3. As the predicted truncated STAT3 protein lacking both the SH2 and TA domains was not expected to exert a dominant negative effect, haploinsufficiency (HI) as a possible disease mechanism was contemplated. This would however need functional assessment of the rare variant which is being currently carried out. HI as a disease mechanism has been proposed previously by Natarajan et al. (38) in a c.1140-3C>G; p.S381* null variant. Mutations in the CC domain of STAT3 hasn't yet been reported in literature and the p.F174S in our cohort hence is an extremely rare variant.

Cranio-facial, dental, and skeletal features were seen in a minority of our patients: facial dysmorphism in 55.5% of the STAT3 and 63.1% of the entire cohort, retained primary teeth in 7.4 and 3.8%, and recurrent fractures 11.1 and 4.8%. While delayed shedding and retained primary dentition indicates decreased osteoclast (OC) function, recurrent fractures and osteoporosis denotes increased OC activity, which are contradictory. Studies conducted at the Chandigarh center to look at the pathogenesis of cranio-facial and dental manifestations in HIES by looking at genes involved in bone homeostasis revealed *osteopontin* (OPN) as a candidate gene that was altered significantly in patients with HIES and STAT3 deficient cell lines (24). The OPN gene was also shown to have hitherto undescribed STAT3 response elements in its promoter region by *in silico* studies (24). Interestingly, differential expression of OPN was observed in patients with HIES even before the STAT3 era (39). Though patients with STAT3 rare variants do not show obvious alterations in OC morphology, differential expression of genes like NFaTc1, STAT3, and OPN has been observed in OCs from HIES subjects and mutant cell lines (unpublished observations).

pSTAT3 assay by flowcytometry is considered to be an important functional analysis in STAT3 LOF HIES and assesses the canonical STAT3 pathway mediated through pY705 phosphorylation in the TA domain. The assay however can be normal in a significant majority and hence a normal pSTAT3 expression doesn't rule out the disease. pSTAT3 was done in 8 of the 27 STAT3 HIES patients in our cohort and was normal in a majority (62.5%). The non-canonical pathway of STAT3, that do not require pY705 phosphorylation (unphosphorylated STAT3, uSTAT3) has been shown to act through NFkB mediated RANTES, IL-8 and other IFN-γ response elements (40, 41). Investigation showed a downregulation of RANTES, IL-8, and IFNβ genes in patients with HIES (unpublished observations) which could further contribute to the immune deficiency in these patients.

CONCLUSIONS

We report here a multi centric cohort of 103 HIES patients from India, of which 27 were STAT3 HIES. Though molecular diagnosis was available only in 27 patients, the 18 STAT3 variants detected included seven novel rare variants, including a rare LD nonsense variant and a CC domain variant. *Mycobacterial* diseases were more frequent, similar to other Asian cohorts, and hence need to be considered in the pathogen spectrum of HIES in India in addition to the usual *Staphylococcus* and *candida* infections. Notably, more than half of our STAT3 HIES subjects had low NIH scores, that was below the cut off of ≥40. With increasing awareness and better availability of molecular diagnostic facilities, more and more cases of HIES are likely to be reported and diagnosed.

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 Institute Ethics Committee, PGIMER, Chandigarh. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BS collected the data, did the analysis, and wrote the paper. SmS, AK, and AD did the flowcytometry experiments and analyzed the genetic analysis data. SuS, DS, AR, MD, PT, AP, VG, MM, AD, RM, HL, RR, RU, VV, SB, GC, HK, AS, MK, and GG provided patient data and conducted clinical exploration and treatment of the subjects. RM, AR, DS, VP, and SuS did a critical review of the manuscript. 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.2021.626593/full#supplementary-material

Supplementary Figure 1 | (A) Representative flow cytometry contour plots for TH17 ($CD4^+IL17^+IFN\text{-}\gamma^-$) cell estimation. Upper panel is a healthy control's plot (TH17 cells = 2.4%) and lower panel from a suspected patient of HIES (TH17 cells = 0.4%). ST, Stimulated with PMA-Ionomycin; US, Unstimulated. **(B)** Representative flow cytometry histogram plots for pSTAT3 assay. Upper panel is from a healthy control sample (MFI 479) and lower panel from a HIES subject (MFI 228). ST, Stimulated with IL-6; US, Unstimulated.

Supplementary Table 1 | List of STAT3 mutations with their CADD and SIFT scores.

Supplementary Table 2 | List of genes in the targeted 44 gene NGS panel and the 320 gene NGS panel.

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Clinical, Immunological, and Molecular Profile of Chronic Granulomatous Disease: A Multi-Centric Study of 236 Patients From India

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Background: Chronic granulomatous disease (CGD) is an inherited defect in phagocytic respiratory burst that results in severe and life-threatening infections in affected children. Single center studies from India have shown that proportion of autosomal recessive (AR) CGD is more than that reported from the West. Further, affected patients have high mortality rates due to late referrals and difficulties in accessing appropriate treatment. However, there is lack of multicentric collaborative data on CGD from India.

Objective: To describe infection patterns, immunological, and molecular features of CGD from multiple centers in India.

Methods: A detailed proforma that included clinical and laboratory details was prepared and sent to multiple centers in India that are involved in the care and management of patients with inborn errors of immunity. Twelve centers have provided data which were later pooled together and analyzed.

Results: Of the 236 patients analyzed in our study, X-linked and AR-CGD was seen in 77 and 97, respectively. Male female ratio was 172:64. Median age at onset of symptoms and diagnosis was 8 and 24 months, respectively. Common infections documented include pneumonia (71.6%), lymphadenitis (31.6%), skin and subcutaneous abscess (23.7%), blood-stream infection (13.6%), osteomyelitis (8.6%), liver abscess (7.2%), lung abscess (2.9%), meningoencephalitis (2.5%), splenic abscess (1.7%), and brain abscess (0.9%). Forty-four patients (18.6%) had evidence of mycobacterial infection. Results of molecular assay were available for 141 patients (59.7%)—*CYBB* (44.7%) gene defect was most common, followed by *NCF1* (31.9%), *NCF2* (14.9%), and *CYBA* (8.5%). While *CYBA* variants were documented only in Southern and Western parts of India, a common dinucleotide deletion in *NCF2* (c.835_836delAC) was noted only in North Indian population. Of the 174 patients with available outcome data, 67 (38.5%) had expired. Hematopoietic stem cell transplantation was carried out in 23 patients, and 12 are doing well on follow-up.

Conclusions: In India, proportion of patients with AR-CGD is higher as compared to Western cohorts, though regional differences in types of AR-CGD exist. Clinical profile and mortality rates are similar in both X-linked and AR-CGD. However, this may be a reflection of the fact that milder forms of AR-CGD are probably being missed.

Keywords: Chronic Granulomatous Disease, India, *Mycobacterium tuberculosis*, Bacillus Calmette Guerin

INTRODUCTION

Chronic granulomatous disease (CGD) is an inborn error of immunity (IEI) characterized by a defective respiratory burst in phagocytes, resulting in defective clearance of phagocytosed microorganisms (1). It is caused by mutations in genes encoding different protein subunits of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex (2). Clinical manifestations vary from an immunodeficient phenotype with repeated infections (3, 4) to those characterized by uncontrolled hyper-inflammation (5) and other autoimmune manifestations. Common patterns of infections in CGD include pneumonia, lymphadenitis, hepatosplenomegaly, and abscesses (6).

Laboratory diagnosis of CGD can be made by the nitroblue tetrazolium dye reduction test (NBT) (7, 8) or by flow-cytometry based dihydrorhodamine (DHR) assay (9, 10). The DHR assay is now considered to be the preferred screening test due to its higher reproducibility, sensitivity, rapidity, and ability to detect X linked carriers (11, 12). While several centers in India have published their individual experiences on CGD (13–16), countrywide data have never been collated before. Such collaborative efforts are the need of the hour and are especially important for uncommon conditions like CGD, for which there is paucity of data on disease burden in the country. The present work is the first multi-centric study in India to provide data on clinical, immunological, and molecular features of CGD.

METHODS

We contacted all centers that are recognized as Foundation for Primary Immunodeficiency Diseases (FPID) regional centers for diagnosis or treatment for primary immunodeficiencies in India, and also other Indian institutions involved in care of patients with IEI. All centers were requested to provide clinical and laboratory details of patients with CGD on a pre-designed Excel sheet. Data collection was completed by July 2020. Details included demographic information, family history including consanguinity, clinical manifestations, immunological investigations, genetic diagnosis, treatment, and follow-up.

Participating institutes included—Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, North India (80 patients); Bai Jerbai Wadia Hospital for Children, Mumbai, West India (57 patients); Aster CMI Hospital, Bengaluru, South India (22 patients); National Institute of Immunohematology (NIIH), Mumbai, West India (22 patients); Kanchi Kamakoti Childs Trust Hospital, Chennai, South India (21 patients); Apollo Hospitals, Chennai, South India (17 patients); Christian Medical College, Vellore, South India (five patients); Kasturba Medical College, Mangalore, South-West, India (five patients); Sir Ganga Ram Hospital (SGRH), New Delhi, North India (four patients) and one patient each from R G Kar Medical College, Kolkata, East India; Indraprastha Apollo Hospital, New

Delhi, North India; and Medens Hospital, Haryana, North India. Data were collated and subsequently translated on to a database and analyzed (**Figure 1**). Before the analysis, all patient identity details were anonymized. Three (3) female patients had a probable skewed X-linked inactivation. These patients have been described separately.

Nitroblue Tetrazolium and Dihydro-Rhodamine Tests

Diagnosis of CGD was based on an abnormal granulocyte oxidative burst evaluated by either NBT or flow-cytometry based DHR assay or both. Nitroblue tetrazolium test was available in almost all the centers. However, flow cytometry based DHR was available only in six centers. Analysis of NADPH oxidase components by flow cytometry was carried out only in two centers: National Institute of Immunohematology (NIIH), Mumbai, and Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh. Details of flow cytometry methods for DHR and NADPH oxidase components at NIIH, Mumbai (13, 14) and PGIMER, Chandigarh (15–17) have been described previously.

Molecular Assays

Diagnosis was confirmed by molecular analysis in 60% of the patients. At most centers, molecular analysis was carried out at private laboratories (Medgenome Laboratories Pvt. Ltd., India; Strand Genomics Pvt. Ltd., India; Neuberg Anand Diagnostics Pvt. Ltd., India). Illumina platform was used for Next-Generation sequencing (NGS) in these private laboratories with coverage of >80×. Sanger sequencing was used to confirm variants obtained by Next-Generation Sequencing (NGS).

Two centers had in-house facilities for molecular analysis—NIIH, Mumbai and PGIMER, Chandigarh. Molecular assays carried out at NIIH, Mumbai have been detailed in a previous publication (18). Molecular analysis for some patients at PGIMER, Chandigarh (before 2016) was performed at centers in Hong Kong (Department of Pediatrics and Adolescent Medicine, The University of Hong Kong) and Japan (Kazusa DNA Research Institute, Chiba; Tokyo Medical and Dental University, Tokyo; National Defense Medical College, Saitama).

Sanger Sequencing and Next-Generation Sequencing at PGIMER, Chandigarh

Genomic DNA was extracted from peripheral blood using DNA extraction kits (QIAamp DNA Blood mini kit, Germany). Depending upon flow-cytometry evaluation for NADPH oxidase component expression, polymerase chain reaction (PCR) was performed for respective genes *i.e. CYBB* (13 exons) for X-linked CGD and *NCF1* (11 Exons), *NCF2* (16 Exons), *CYBA* (six Exons) for AR-CGD. PCR products from genomic DNA were sequenced on an auto fluorescent sequencer (ABI 3500, Applied Biosystems™; Thermo Fisher Scientific, USA) using BigDye™ Terminator (V3.1 Applied Biosystems™). Sequencing primers were the same as those used for PCR. Upon sequencing the results were obtained in.*abi* format and were analyzed using Codon-code aligner for DNA sequence assembly (4.2.5/2013). Patient sequence was compared with reference human sequence obtained from Ensemble database (https://asia.ensembl.org/index.html), and novel variants were further assessed for their disease-causing effect on protein. Prediction tools such as SIFT, PolyPhen-2, Mutation Taster,

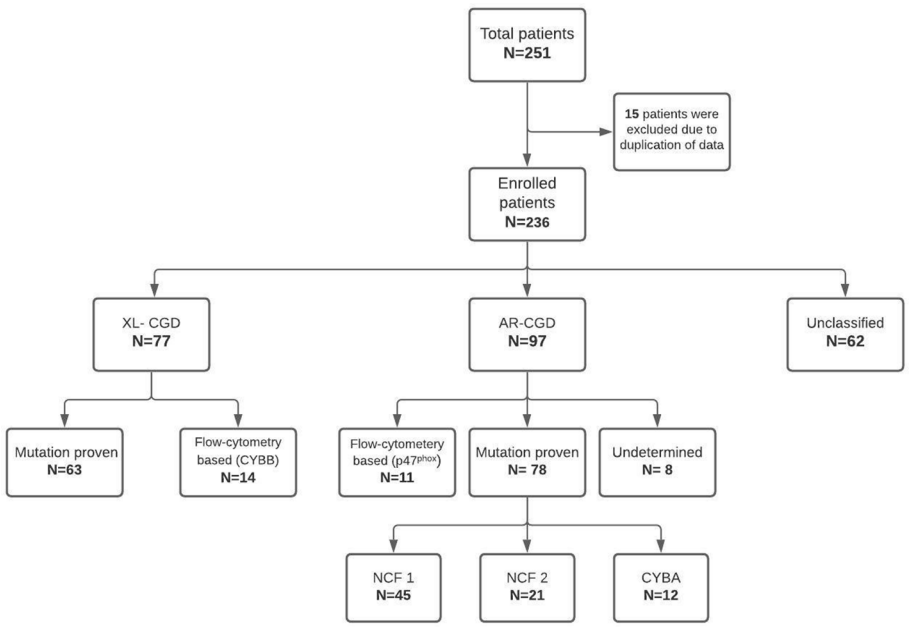

FIGURE 1 | Flowchart showing the classification of patients analyzed in the current study.

and CADD score were used to evaluate their pathogenicity. Human Splicing Finder tool was used for splice-site variants.

Since 2018, the center has started performing targeted NGS using a modest 44 gene panel for patients with primary immunodeficiency diseases. Genomic DNA was quantified using Qubit™ Fluorometer (ThermoFisher Scientific, USA) followed by target amplification using PID 2X 2-primer pool panel. Amplified products were partially digested followed by adapter ligation, barcoding, library purification, and amplification. Further, DNA fragments were immobilized on Ion sphere particles and clonally amplified using Ion One Touch™ 2 Instrument. This emulsion PCR results in beads containing amplified and cloned DNA fragment. Elimination of empty beads was carried out using a robotic enrichment system (Ion One Touch™ ES). Finally, the beads containing clonal population of DNA were loaded on to Ion530 Chip. Sequencing was done using Ion S5™ instrument and simultaneously processed on an Ion torrent server for further analysis. Variant calling and analysis were performed using Ion reporter software (ThermoFisher Scientific, USA). The identified variants were validated using Sanger sequencing.

Gene-Scan Analysis at PGIMER, Chandigarh

The most common defect in *NCF1* is a dinucleotide deletion, c.75_76delGT at the beginning of exon 2. Conventional Sanger Sequencing or NGS can miss this defect as *NCF1* has two flanking pseudogenes (*ΨNCF1*) with 99% sequence homology with functional gene. So, fluorochrome labeled primers were used to amplify *NCF1* gene and *ΨNCF1* using a method described earlier (Roos et al, 2001). The amplification yielded a mixture of labeled amplicons from the *NCF1* gene and *ΨNCF1* differing by 2 bp in length. This product mixture was analyzed on an Applied Biosystems 3500 Genetic Analyzer using GeneMapper software (Life Technologies, Carlsbad, CA, USA). The amplification product from the pseudogene, being shorter than that from the functional gene, had a shorter retention time and could thus be resolved as distinct peak from the one resulting from the amplicon from the functional gene. Ratio between the two peak heights denotes the relative number of genes and pseudogenes in a test sample.

Statistical Analysis

Descriptive tests such as medians and ranges were used for continuous variables. Counts and percentages were used for categorical variables. Mann–Whitney U test was used to compare continuous variables between groups. Categorical variables were compared by using the chi-square (x^2) test or Fisher's exact test wherever needed. Wilcoxon rank sum test was used to assess efficacy of intervention in a single group. Kaplan–Meier method was used to estimate the survival analysis. Death from any cause was considered as an event, and log-rank test was used to compare the groups. All p values were two-sided and considered significant when $p < 0.05$.

RESULTS

Patient Profile

Of the 236 patients, 172 (72.8%) were boys and 64 (27.1%) girls. Details about consanguinity were available for 91 patients, and 40 of them had a history of consanguineous marriage (43.9%). XL-CGD was seen in 77 patients, AR-CGD in 97 children, and the remaining 62 patients could not be categorized as X-linked or AR due to insufficient details of flow cytometry results or molecular analysis (**Figure 1**). AR-CGD was the commonest type in our cohort (XL: AR ratio, 0.8). Three female patients who had skewed X-linked inactivation have been described separately and were not included in the survival analysis.

Median age of onset of initial symptom was 8 months (interquartile range [IQR]: 3–24, range: 0.1 months–20 years), and median age of establishing the diagnosis was 24 months (IQR: 8–60 months, range: 2 weeks–35 years). Median delay in diagnosis was 8 months (IQR: 1.9 months–24 months, range: 0–33.2 years). Follow-up data were available for 98 patients, and median duration of follow-up was 1 year (IQR: 0.1–3 years, range: 1 month–24 years).

Comparison of Clinical and Laboratory Characteristics Between XL-CGD and AR-CGD

Median age at diagnosis was earlier in XL-CGD than AR-CGD (p = 0.002). However, there was no difference in median age of onset symptoms between these groups (p = 0.074) (**Table 1**). Similarly, median values of stimulation index (SI) derived from DHR assay were also comparable between both groups (p = 0.741) (**Table 1**).

Number of episodes of pneumonia was more in AR-CGD (n = 70) than XL-CGD (n = 45) (p = 0.003). Number of patients with >three episodes of pneumonia was also higher in AR-type as compared to XL-CGD (p 0.007). There was no statistical difference between AR-CGD and XL-CGD with regard to following parameters—episodes of superficial abscess (p = 0.462); deep abscess (p = 0.488); lymphadenitis (p = 0.272); and osteomyelitis (p = 0.510). Mortality rate was also comparable in both groups (p = 0.489) (**Table 1**).

On comparing the individual subtypes (*CYBB, NCF1, NCF2*, and *CYBA* defects), age of onset of symptoms and age at diagnosis were earlier in XL-CGD compared to other subtypes (**Table 2**). Mortality rates were low in *NCF1* defect (13.3%) compared to other forms (p 0.040) (**Table 2**).

Clinical Characteristics of $p47^{phox}$ Defect in Comparison to $p67^{phox}$- Defect

Median age at symptom onset was higher in $p47^{phox}$ defect (14 months) when compared to $p67^{phox}$ defect (5 months). The difference was, however, not statistically significant. Median age at diagnosis and median delay in diagnosis was comparable between two groups (**Supplementary Table 1**). There was also no statistical difference between $p47^{phox}$ and $p67^{phox}$ deficiency with regard to the following parameters: number of patients with >three infectious episodes (p 0.326); number of patients with more than three episodes of pneumonia (p = 0.545); and colitis (p 0.441) (**Supplementary Table 1**).

Infection Profile

We document 559 episodes of infections in 236 patients over 298.6 patient–years of follow-up (**Table 1**). Median number of episodes of infections was two (IQR 1–3 episodes).

TABLE 1 | Comparison of clinical, and laboratory characteristics between XL-CGD, and AR-CGD.

Parameter	XL (n = 77)	AR (n = 97)	p value
Median age at onset of infections (IQR)[a]	6 months (3–20)	12 months (3–35)	0.074
Median age at diagnosis[a]	12 months (7–24)	30 (9–86)	0.002
Median delay in diagnosis of CGD[a]	6 months (1.5–19)	9.2 (1.5–33)	0.353
Median follow-up[a]	1.16 year (1–2.76)	1 year (0.10–2.8)	0.735
Median stimulation index in DHR[a]	1.2 (1–2.76)	1.34 (0.98–2.2)	0.741
Number of patients with >3 infectious episodes[b]	19	15	0.100
Episodes of pneumonia[b]	45	70	0.003
Number of patients with >3 episodes of pneumonia[b]	9	3	0.007
Episodes of superficial abscesses[b]	20	22	0.462
Episodes of deep abscesses[b]	6	7	0.488
Episodes of lymphadenitis[b]	20	27	0.272
Episodes of osteomyelitis[b]	9	9	0.510
Episodes of liver abscesses[b]	6	10	0.500
Episodes of septicemia[b]	8	6	0.540
Number of patients with mycobacterial disease[b]	10	20	0.105
Number of patients with mortality[b]	18	23	1.000
Median Stimulation index <1.5 in DHR[b]	31	44	0.672

[a]*Mann–Whitney U test.*
[b]*Fisher's exact test or chi-squared test.*

TABLE 2 | Comparison of clinical, and laboratory characteristics between *CYBB, NCF1, NCF2*, and *CYBA* defects.

Parameter	CYBB (n = 63)	NCF1 (n = 45)	NCF2 (n = 21)	CYBA (n = 12)	P value
Median age at onset of infections (IQR)[a]	6 months (3–20)	14.5 months (8–36)	5 months (1–26)	6.5 months (2.2–31.5)	0.037
Median age at diagnosis[a]	12 months (7–30)	36 months (12–96)	21 months (4–90)	30 months (5.5–61.5)	0.027
Median delay in diagnosis of CGD[a]	7 months (1.5–19.5)	12 months (0.7v39)	5.5 months (1.5–26.2)	16 months (2–31.2)	0.781
Median follow-up[a]	1 year (0.1–3.1)	1.3 year (0.1–7.5)	0.5 year (0.01–2)	0.2 year (0.1–5.3)	0.321
Median stimulation index in DHR[a]	1.18 (1–2.09)	1.23 (0–2.01)	1.3 (1.0v2.16)	2.15 (0.29–5.08)	0.626
Number of patients with >3 infectious episodes[b]	19 (30.1%)	7 (15.5%)	6 (28.5%)	0	0.051
Episodes of pneumonia[b]	41 (65%)	32 (71.1%)	16 (76.1%)	8 (66.6%)	0.012
Number of patients with >3 episodes of pneumonia[b]	9 (14.2%)	3 (6.6%)	0	0	0.058
Episodes of superficial abscesses[b]	17 (26.9%)	8 (17.7%)	10 (47.6%)	0	0.343
Episodes of deep abscesses[b]	5 (7.9%)	1 (2.2%)	1 (4.7%)	2 (16.6%)	0.825
Episodes of lymphadenitis[b]	19 (30.1%)	13 (28.8%)	7 (33.3%)	2 (16.6%)	0.608
Episodes of liver abscesses[b]	6 (9.5%)	4 (8.8%)	4 (19%)	0	0.408
Episodes of septicemia[b]	8 (12.6%)	6 (13.3%)	3 (14.2%)	0	0.145
Number of patients with Mycobacterial disease[b]	8 (12.6%)	10 (22.2%)	4 (19%)	3 (25%)	0.144
Number of patients with mortality[b]	17 (26.9%)	6 (13.3%)	8 (38%)	6 (50%)	0.042
Number of patients with stimulation index <1.5 in DHR[b]	28 (44.4%)	18 (40%)	12 (57.1%)	3 (25%)	0.462

[a]*Mann–Whitney U test.*
[b]*Fisher's exact test or chi-squared test.*

Localization of Infection

Lung

Pneumonia was seen in 169 patients (71.6%), and the total number of episodes of pneumonia was 242. Due to indolent presentation of pneumonia in CGD, and India being an endemic country for tuberculosis, several patients with difficult to treat pneumonia were empirically started on antitubercular therapy (ATT) before the diagnosis of CGD could be ascertained. Fifty-three (54%) of 96 patients with persistent pneumonia had received empirical ATT in our cohort. Number of ATT courses ranged from one to three. Three patients with CGD had extensive bronchiectasis with pulmonary hypertension at time of diagnosis—all three had received multiple courses of empirical ATT for many months before the diagnosis of CGD was confirmed in them.

In our cohort, fungal pneumonia was commonest (26.8%), followed by bacterial (23.9%) and mycobacterial infections (11.5%) (**Table 3**). The commonest organisms were *Aspergillus* sp., *Mycobacterium* sp., *Staphylococcus aureus*, *Pseudomonas* sp., *Klebsiella* sp., and *B. cepacia* (**Table 2**). Confirmed fungal etiology either on lung histopathology or on microbiological cultures from affected tissues was documented in 18 of 62 episodes of suspected fungal infection. The remaining patients also probably had a fungal infection as they had positivity for biomarkers such as galactomannan and/or beta-D-glucan. Twenty patients (8.4%) required mechanical ventilation due to severe lung disease or associated septicemia. Other pulmonary complications include lung abscesses (n = 7; 2.9%), bronchiectasis (n = 5; 2.1%), and contiguous rib osteomyelitis (n = 5; 2.1%).

Mycobacterial infections were documented in 44 patients (18.6%)—*Mycobacterium tuberculosis* in 25, Bacillus Calmette Guerin (BCG) related complications in 17 (localized disease in 13, and disseminated disease in four), and infection due to non-tuberculous mycobacteria in two (**Table 4**). Two patients with

TABLE 3 | Profile of microorganisms in patients with pneumonia.

Microorganisms	Number of episodes (n = 242)		%
Bacterial organisms	90		37.1
• *Staphylococcus aureus*	14		5.7
• Mycobacterial disease	28		11.5
• *Mycobacterium tuberculosis*	24		9.9
• Disseminated BCG	4		1.6
• *Pseudomonas* sp.[#]	11		4.5
• *Klebsiella pneumonia*	8		3.3
• *Burkholderia cepacia*	8		3.3
• CONS	9		3.7
• *Acinetobacter* sp.	3		1.2
• *Nocardia* sp.	2		0.8
• Others*	5		2
Fungal organisms	63		26
	Probable	**Proven**	
• *Aspergillus* sp.	38	10	19.8
• *Aspergillus* sp. (subspecies: not known)		4	1.6
• *A. fumigatus*		4	1.2
• *A. flavus*		2	0.8
• *Candida* sp. (subspecies: not known)	9	1	4.1
• *Candida tropicalis*		1	0.4
• *Candida lusitanie*		1	0.4
• *Fusairum dimerium*	–	1	0.4
• *Mucor* sp.		2	0.8

**Streptococci sp. (n=2); Chryseobacterium gleum (n=1); Fransciella noatuensis (n=1); Proteus mirabilis (n=1).*
CONS, Coagulase Negative Staphylococcus sp.[#]Pseudomonas aeruginosa (n=6).

disseminated tuberculosis had a stormy course—one patient had anterior chest wall cold abscess, hepatosplenomegaly, and computed tomography (CT) guided lung biopsy showed granulation tissue with AFB positivity; one patient presented with pericardial and pleural effusions. Both of them also had lymphadenopathy and rib osteomyelitis at time of diagnosis. While one improved after initiation of conventional ATT, the other succumbed to the illness due to concomitant *Mucorales* infection in the lung.

Blood-Stream Infections

Though patients with CGD usually do not have a proclivity for dissemination of infection, 21 patents (9%) in our cohort had septicemia. Blood-stream infections (BSI) were found in 75 of 551 infectious episodes (**Table 4**). Bacterial infections were commonest (70/75 episodes, 93.3%). Some of the signature organisms that were isolated included *S. aureus* (21.3%); *Burkholderia cepacia* (13.3%); *Pseudomonas* sp. (9.3%); *Klebsiella* sp. (8%); followed by other organisms (**Table 5**).

TABLE 4 | Spectrum of mycobacterial disease in our cohort.

Mycobacterial disease	No of patients (Organism positive) (n = 44)	%
***Mycobacterium* sp.**	27 (2, non-tuberculous)	61.3
• Pneumonia	24	54.5
• Lymphadenitis	6	13.6
• Abdominal	2	4.5
• Osteomyelitis	2	4.5
• CNS	1	2.2
• Skin (Lupus vulgaris)	1	2.2
• Disseminated tuberculosis	9	20.4
BCG infection	17	38.6
• Localized BCG adenitis	13	29.5
• Disseminated BCG	4	9

Lymphadenitis

Lymphadenitis was seen in 74/236 patients (31.6%), and 94 episodes were documented in our cohort. Microorganisms were isolated from lymph node aspirate in 25/74 patients (33.7%). The commonest organisms isolated were *Staphylococcus* sp. (40%), *Mycobacterium* sp. infections (36%) followed by *Burkholderia* sp., *Klebsiella* sp., and *Pseudomonas* sp. (**Table 6**). Two patients underwent surgical resection for recurrent lymphadenitis that was recalcitrant to medical therapy.

Skin and Subcutaneous Abscesses

Skin and subcutaneous abscesses were found in 56 patients (23.7%), and a microorganism could be isolated from pus culture in 17/56 patients (30.3%) (**Table 7**). Majority (88.2%) were due to bacterial infections and among these, *S. aureus* was isolated in eight patients (53.3%). Perianal abscesses were found in six patients (2.5%) with CGD.

Liver Abscess

Seventeen patients presented with liver abscesses (7.2%), and one among these had a recurrent liver abscess (19). Four patients with liver abscess had concurrent other site abscesses (staphylococcal skin pustules in one patient, *Pseudomonas aeruginosa* cervical abscess in one patient, and multiple deep-seated abscesses noted in two other patients).

S. aureus was isolated from pus in 5/17 patients (29.4%). Three patients with liver abscess were administered corticosteroids besides intravenous antibiotics. Rupture of liver abscess warranting surgical intervention was documented in one child who had not received corticosteroids.

TABLE 5 | Isolation of microorganisms in blood stream infections.

Microorganisms	No of episodes isolated (n = 75)	%
Bacteria	70	93.3
• *Staphylococcus aureus*	16	21.3
• *Klebsiella pneumoniae*	6	8
• *Pseudomonas* sp.[#]	7	9.3
• *Burkholderia* sp.^	10	13.3
• CONS	7	9.3
• *Entercoccus* sp.	4	5.3
• *Salmonella* sp.[##]	6	8
• *Acinetobacter* sp.[^^]	5	6.6
• Others*	7	9.3
• Gram negative septicemia	2	2.6
Fungal	5	6.6
• *Aspergillus fumigatus*	2	2.6
• *Candida* sp.	2	2.6
• *Candida tropicalis*	1	1.3

**Streptococci sp. (n=2); E. coli (n=2); Bacillus subtilis (n=1); Neisseria meningitidis (n=1); Citrobacter freundi (n=1); Fransicella sp. (n=1).*
CONS, Coagulase Negative Staphylococcus sp.
[#]Pseudomonas aeruginosa (n=3); Pseudomonas stutzeri (n=2).
^Burkholderia cepacia (n=3); B. cenocepacia (n=1).
[##]Non-typhoidal Salmonella.
[^^]Acinetobacter baumanni (n=3).

TABLE 6 | Organism profile in lymphadenitis.

Microorganisms	Number of events (n = 25)	%
Bacteria	24	96
• *Staphylococcus aureus*	10	40
• Mycobacterial infection	9	36
• *M. tuberculosis*	2	8
• BCG adenitis	7	28
• *Bulkholderia cepacia*	3	12
• *Klebsiella pneumoniae*	1	4
• *Pseudomonas aeruginosa*	1	4
Fungal		
• *Candida parapsilosis*	1	4

TABLE 7 | Spectrum of microorganisms in patients with skin and subcutaneous abscesses.

Microorganisms	Number of patients (n = 56)	%
Bacteria	**15**	**26.7**
• *Staphylococcus aureus*	8	14.2
• *Klebsiella pneumoniae*	2	3.5
• *Pseudomonas* sp.*	3	5.3
• *Burkholderia cepacia*	2	3.5
• *Acinetobacter baumanii*	1	1.7
Fungal	**2**	**3.5**
• *Candida* sp.	1	1.7
• *Basidiobolus* sp.	1	1.7

**Pseudomonas aeruginosa (n=2).*

Other Deep-Seated Abscesses

Seven patients had lung abscess—*Nocardia* sp. and *Aspergillus* sp. was isolated in one patient each. Splenic abscess was found in four patients. Two patients developed brain abscesses, and *Aspergillus fumigatus* was isolated from the pus in both. One child also had retropharyngeal and parapharyngeal abscess.

Bone

Infective osteomyelitis was found in 20 patients (8.6%). Site of involvement was variable: ribs—five; lower extremities [tibia (n = 2), fibula (n= 1)]; vertebrae—one; radius—one; skull—one; and small bone osteomyelitis of the hands in two patients. *Aspergillus fumigatus* was isolated from four patients; *Enterobacter* sp., *Chromobacterium violaceum*, *A. terreus*, and *Serratia marcescens* were isolated in one patient each (16, 19, 20). Mycobacterial infection was responsible for two cases of osteomyelitis (one with *M. tuberculosis*, and another with atypical mycobacterial disease). Surgical resection of ribs was required in two patients, while other patients were managed conservatively.

Other Sites

Meningoencephalitis was seen in six patients. Two children had complicated meningitis with hydrocephalus. *Aspergillus nidulans* was isolated from CSF from one of them. Ten children had otitis media (4.2%). Urinary tract infection (UTI) was seen in 12 patients (5.1%).

Non-Infectious Manifestations

Twelve patients (5.1%) had colitis. Twenty-three episodes of colitis were noted in the cohort, and one patient had had six relapses. One child had been diagnosed to have inflammatory bowel disease elsewhere. He had received infliximab for recalcitrant colitis before the diagnosis of CGD and had developed severe pneumonia following the therapy. Glucocorticoids, mesalamine, and azathioprine were used in seven, five, and three patients respectively. A child with XL-CGD had complete remission of colitis following hematopoietic stem cell transplantation (HSCT).

Lung granuloma was documented in 16 patients (6.8%), and liver granuloma in two patients. Secondary hemophagocytic lymphohistiocytosis (HLH) was documented in six patients (2.5%) (21). Common infective triggers identified were blood stream septicaemia (n = 3) [*Francisella noatuensis* (one), *Burkholderia cenocepacia* (one), *Candida albicans* (one)], pneumonia (n = 4) [*Nocardia* sp. (n = 1), probable *Aspergillus* sp. (n = 1)], and disseminated BCGosis (n = 1) (21). Other non-infective manifestations found in our cohort included chilblains (n = 2), HLA-B27 related arthritis (n = 1), Kawasaki disease with coronary artery aneurysm (n = 1), unexplained chronic kidney disease (n = 1), and intestinal obstruction in one patient.

Molecular Diagnosis

Results of genetic analysis were available for 141 patients (59.7%). Pathogenic variants in the *CYBB* were the most common (63/141; 44.7%), followed by *NCF1* gene variants (45/141; 31.9%) whereas genetic variants in *NCF2* and *CYBA* comprised 14.9% (21/142) and 8.5% (12/141) of all the variants, respectively (**Supplementary Table 2**) (22–30). Fifteen of the 141 genetic variants were novel; 10 in the *CYBB* gene, three in the *NCF2* gene, and two in the *NCF1* gene. Nonsense variants comprised majority of *CYBB* gene variants (21/63; 33.3%), followed by missense variants (11/63; 17.4%), splice-site variants (10/63; 15.8%), deletions (9/63; 14.3%), insertions (5/63; 7.9%), duplications (4/63; 6.3%), and promoter-site variant (n = 1) (**Figure 2**). Five patients had a previously reported synonymous variant c.252G>A; p.A84(=) which is located proximal to the splice donor site of intron 3 and results in skipping of exon 3 of *CYBB*. Most patients with autosomal recessive CGD due to gene variants in the *NCF1* gene had the common dinucleotide deletion in exon 2 of the gene. However, variants were also detected in other exons of the gene including a large deletion involving exons 2–10 of the *NCF1* gene and nonsense variants in exon 7 in two other patients (**Figures 3**, **4**). *NCF2* gene variants were more common in patients from North India (15/22; 68.1%) compared to patients from West or South India (7/22; 31.9%). Majority of the patients with *NCF2* gene variants from North India (10/15; 66.7%) had a common dinucleotide deletion in exon 9 (c.835_836delAC; p.Thr279GlyTer16) (31) of the gene resulting in alteration of the reading frame and termination (**Figures 3**, **4**). *CYBA* gene variants in contrast were exclusively seen in patients from South and West India. Most of the *CYBA* gene variants were located in exon 4 of the gene (6/12; 50%) and one patient had a large deletion involving exons 2–4 of the *CYBA* gene (**Figure 3**).

Three X-linked carriers of *CYBB* defect had manifestations of CGD in form of severe infections probably as a result of skewed inactivation (32, 33). A carrier of X-linked CGD developed manifestations of severe lupus malar rash, arthritis, positivity for anti-nuclear and anti-double stranded DNA antibodies,

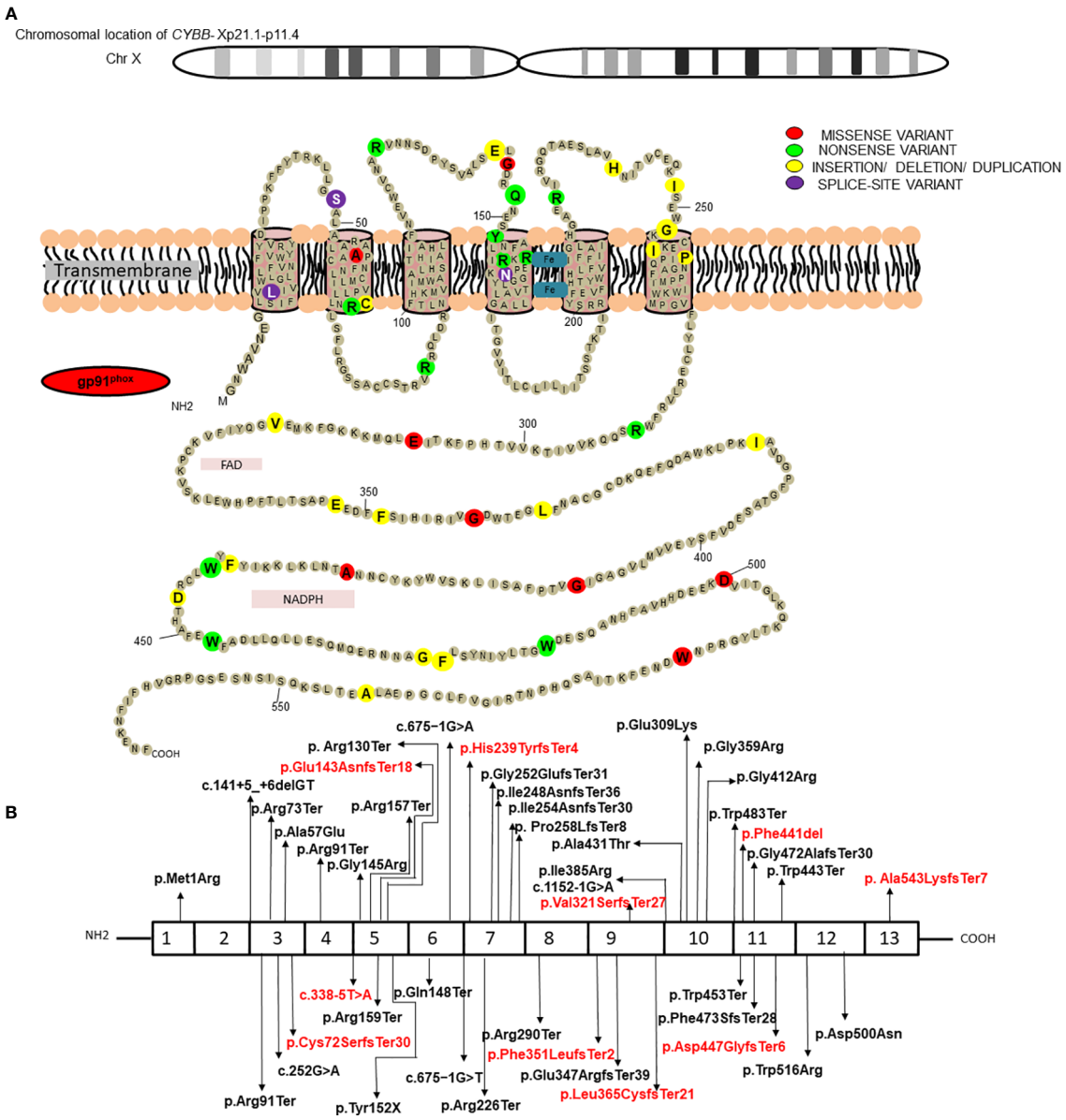

FIGURE 2 | **(A)** Position of protein change in gp91^{phox} for the molecular variants in *CYBB* identified in current study; **(B)** Molecular variants identified in different exons of *CYBB* and corresponding protein domains. *Novel variants have been highlighted in red.*

seizures, and demyelinating lesions in the brain and spinal cord (21). She ultimately succumbed to her illness.

Treatment

All patients were started on prophylactic antimicrobials, usually a combination of cotrimoxazole and itraconazole. Data on breakthrough infections after initiation of antimicrobial prophylaxis were available for 78 children. Statistical analysis showed a significant reduction in episodes of pneumonia; lymphadenitis ($p = 0.003$); skin, and subcutaneous abscesses ($p < 0.001$); deep-seated abscesses ($p = 0.007$); osteomyelitis ($p = 0.035$); liver abscess ($p = 0.02$); and septicemia ($p = 0.007$) in those compliant to antimicrobials (**Table 8**). Corticosteroids were used in four children with liver abscess, two patients with pneumonia, and in two patients with HLH.

Twenty-three patients (9.7%) in the present cohort underwent an HSCT [X-linked—12; AR—three (one each in *NCF1*, *NCF2*, and *CYBA*); unclassified—eight]. Eight patients underwent transplantation with matched related donors. Haploidentical transplantations were carried out in seven patients, while matched unrelated transplantations were performed in three patients, and umbilical cord blood transplantation in one patient. Acute graft *versus* host disease (GVHD) occurred in seven of 23 transplant recipients, and two patients succumbed

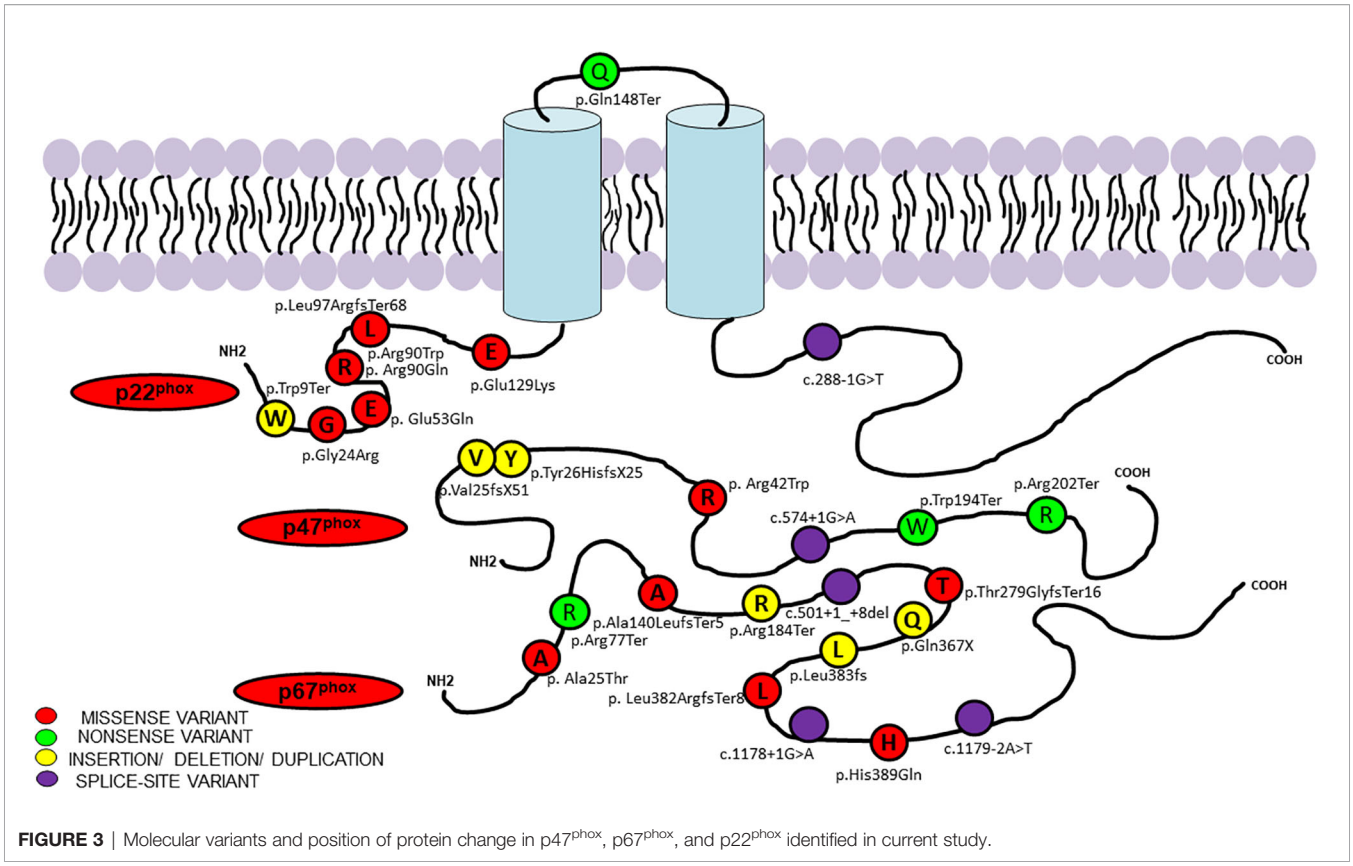

FIGURE 3 | Molecular variants and position of protein change in $p47^{phox}$, $p67^{phox}$, and $p22^{phox}$ identified in current study.

to acute GVHD. Complete donor chimerism was attained in 16/23 recipients (68.1%). Six patients succumbed to transplant related complications (two patients had GVHD; one patient had primary graft failure; and one patient had CNS complications).

Survival Analysis

Outcome details were available for 174 patients (73.7%), and follow-up duration was available for 98 patients (292.6 patient-years of follow-up). Sixty-seven of 174 patients (38.5%) had expired at the time of analysis (XL-CGD: 27; AR-CGD: 28; and undetermined: 12) (**Figure 5A**). The cumulative survival of XL-CGD and AR-CGD was comparable on Kaplan–Meier analysis (p: 0.152) (**Figures 5B, C**). Cumulative survival comparisons between individual subtypes (*CYBB, NCF1, NCF2*, and *CYBA*) showed better survival in NCF1 defect compared to other subtypes (p 0.012) (**Figures 5D, E**).

Median age at diagnosis in individuals who succumbed to illness was 14 months (IQR 7–40 months), and the median age at time of death was 19 months (IQR 7.8–53.6 months). All patients were under antibiotic prophylaxis. Details of terminal events were available only for 37 out of the 61 patients who did not undergo HSCT. Terminal events that were observed include pneumonia in 30 patients (81%); septicemia (10 patients, 27%); brain abscess (two patients, 5.4); lung abscess, liver abscess, and meningitis in one patient each (2.7%). Three patients (8.1%) also had concomitant HLH at terminal illness.

Details of microbiological profile were available in 22 of 37 patients (59.4%). *Aspergillus* sp. was isolated in eight patients (26.6%) including A. *fumigatus* in two patients and A. *flavus* in one patient; *M. tuberculosis*, Mucorales, *Nocardia* sp. in two patients each (6.6%); *Chryseobacterium gleum*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Candida lusitanea*, and cytomegalovirus were isolated in one patient each (3.3%). Out of 10 patients with septicemia, microorganisms could be isolated in eight patients [*S. aureus* (two), *Candida* sp. (one), *C. tropicalis* (one), *P. aeruginosa* (one), *P. stutzeri* (one), *Acinetobacter* sp. (one), *B. cepacia* (one), *B. cenocepacia* (one), and *K. pneumoniae* (one)]. *Aspergillus fumigatus* was isolated in a patient with brain abscesses, and *A. nidulans* was isolated in cerebrospinal fluid in a patient who succumbed to meningitis.

DISCUSSION

CGD was first described in four male children from Minnesota in the year 1954 (34) and was later referred as 'fatal granulomatosus of childhood' by Berendes et al. in 1957 (35). Subsequently, molecular mechanism of disease pathogenesis (36) and genetic defect (*CYBB*) was discovered in the years 1967 and 1986 (37, 38), respectively. CGD is now one of the commonly recognized IEI in children. Prognosis of affected children has improved significantly over the last few decades with the introduction of long-term antimicrobial prophylaxis and initiation of curative

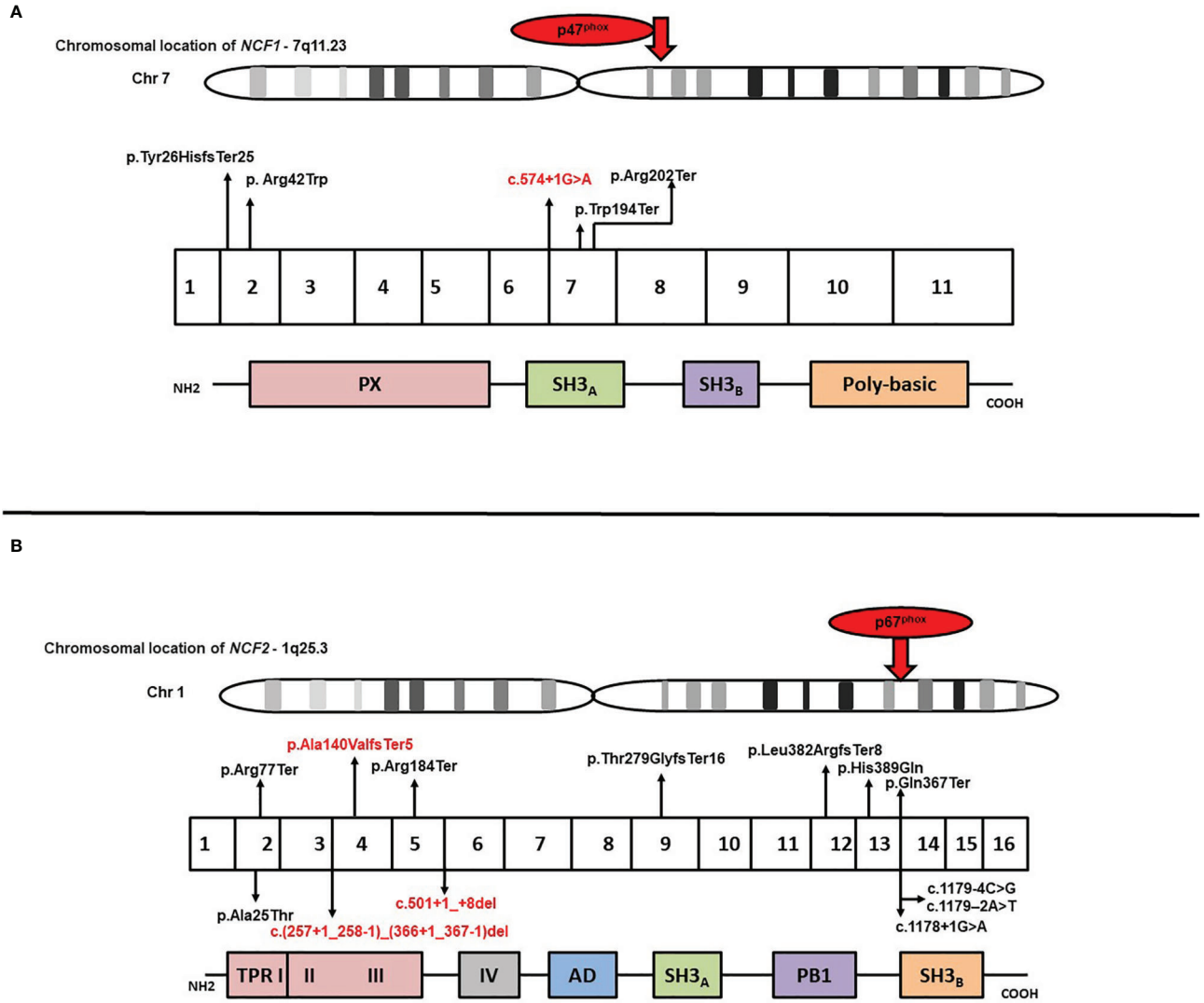

FIGURE 4 | **(A)** Molecular variants identified in different exons of *NCF1* and corresponding protein domains; **(B)** Molecular variants identified in different exons of *NCF2* and corresponding protein domains. *Novel variants have been highlighted in red.*

therapies such as HSCT. Incidence of CGD differs between populations, and it ranges from an estimated one per 200,000 lakh live births in the US and Europe (1, 6), to 1.5 per 100,000 live births among Arabs in Israel where consanguinity rates are high (39).

Initial reports of CGD from India date back to late 1990s (40). Subsequently, the two Indian Council of Medical Research Centres for Advanced Research in Primary Immunodeficiency Diseases (PGIMER, Chandigarh and NIIH, Mumbai) published large case series on CGD from their respective centres (13, 15, 16). Several case reports and case series from other centers have also emanated in the last few years. The present multi-centric study is an effort to collate the data of patients with CGD diagnosed at multiple centers across the country to understand epidemiology, profile of infections, and molecular spectrum of CGD in India. For this study, we obtained data on 236 patients with CGD diagnosed at 12 different centers in India.

We observed a higher proportion of AR forms of CGD as compared to X-linked forms. This is consistent with the previously published data on CGD from India (15). Higher proportion of AR-CGD may be a reflection of high rates of consanguinity and endogamous marriages in the country. Median age at diagnosis in our cohort was 24 months (IQR: 8–60 months), which is comparable with data from other large multi-centric studies from the USA, Europe, and China (1, 6, 41, 42) (**Supplementary Table 3**). Patients with X-linked CGD have an earlier age of presentation and are reported to have a more severe illness as compared to patients with AR-CGD. We also observed that age at onset of infections and diagnosis of AR-CGD was significantly higher than XL-CGD. However, unlike

TABLE 8 | Comparison of infectious episodes before and after prophylactic therapy in 78 patients.

Pattern of infections	Pre-diagnosis n = 78	Post-diagnosis n = 78	p value*
Pneumonia	115	25	0.001
Lymphadenitis	37	10	0.003
Skin and subcutaneous abscess	32	2	0.001
Osteomyelitis	9	2	0.035
Deep abscesses (excluding liver abscess)	15	0	0.007
Liver abscess	7	1	0.020
Cellulitis	2	0	0.157
Septicemia	9	1	0.007
Otitis Media	4	1	0.180
Diarrhea	4	0	0.157

**Fisher's exact test or chi-squared test.*

reports from several other countries (1, 43), there was no difference in survival and mortality rates between AR and XL types of CGD. We attribute this difference to three factors—delays in diagnosis of patients with AR-CGD, a probable missed diagnosis of milder forms of AR-CGD in many patients probably due to lack of awareness especially among the adult physicians, and lack of appropriate laboratory facilities for diagnosis of CGD at many hospitals in India. At present, NBT test can be performed at most centers involved in care of IEI and DHR is currently performed only at six centers. However, many tertiary care hospitals in the country still do not have the facility to perform either NBT or DHR. Median follow-up duration of our cohort is 1 year suggesting that majority of patients are only diagnosed only in the recent years. Increase in awareness of IEI and upscaling of laboratory facilities for carrying out basic immunological investigations are the needs of the hour.

Among the breakthrough infections, pneumonia was most common followed by abscesses (skin, subcutaneous, and deep abscesses, inclusive of liver abscesses), lymphadenitis, and BSI. India, being a country endemic for tuberculosis, it is not surprising that many children with persistent pneumonia (54%) in our cohort had received empirical ATT in our cohort. This has led to significant delays in diagnosis of CGD. Pediatricians and internists practising in developing countries

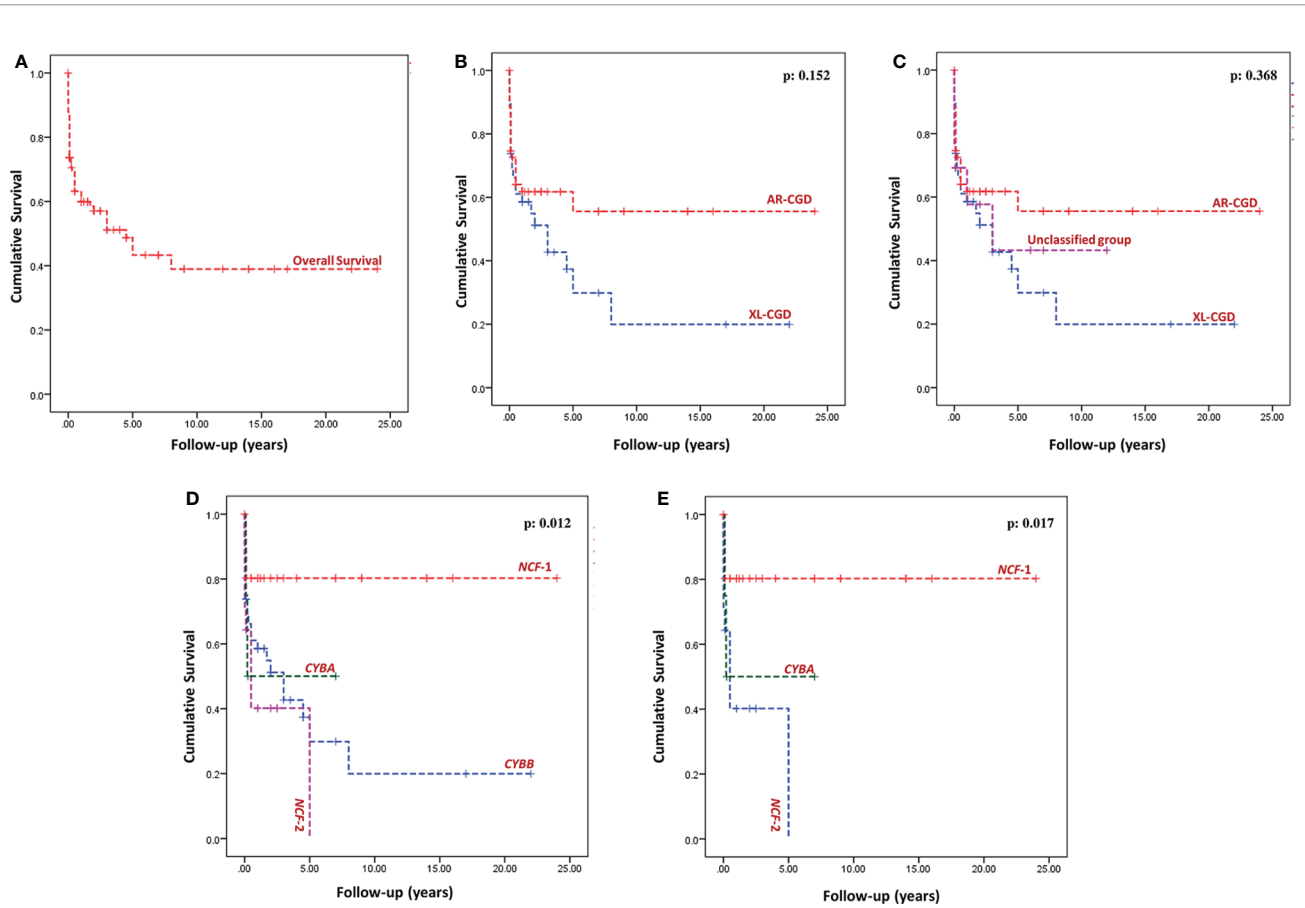

FIGURE 5 | Kaplan–Meier survival curves—**(A)** Overall survival of the entire cohort; **(B)** Comparison of overall survival between AR-CGD and XL-CGD, p = 0.152 (Log Rank Mantel–Cox); **(C)** Comparison of overall survival between Unclassified group, AR-CGD and XL-CGD, p = 0.368 (Log Rank Mantel-Cox); **(D)** Comparison of overall survival between individual subtypes (*CYBB, NCF1, NCF2*, and *CYBA*), p = 0.012 (Log Rank Mantel–Cox); **(E)** Comparison of overall survival between *NCF1, NCF2*, and *CYBA* defects p = 0.017 (Log Rank Mantel–Cox).

such as India need to keep in mind that persistent pneumonia may be a presentation of CGD. Empirical ATT may not be warranted in such cases.

Aspergillus sp. is the most common etiology for pneumonia in our cohort followed by gram-negative bacteria, *Mycobacterium* sp., and *Staphylococcus* sp. Microbiological profile of pneumonia is similar to cohorts from China (44), Iran (45), and Turkey (43). Pneumonia spreading to contiguous tissues such as ribs and vertebra has been documented only with pneumonia due to fungal or mycobacterial etiology (Aspergillus sp.—four; *Mucorales* sp. —one; *Mycobacterium* sp. —two). Pulmonary infection due to *Mucor* sp. was documented in two patients who had not been exposed to corticosteroids. Delays in diagnosis, malnutrition, and prolonged antibiotic therapy for pneumonia could be the reasons for the development of *Mucorales* infection in our cohort. *Nocardia* sp. has been isolated in two patients—one with a lung abscess and the other with persistent pneumonia. Unusual bacteria documented with pneumonia include *C. gleum*, *Citrobacter* sp., and *Francisella noatuensis*. A patient with persistent pneumonia had grown *C. lusitaniae* from the lung tissue which was resistant to amphotericin B.

A wide spectrum of microorganisms that have been isolated in CGD patients with pneumonia suggests the importance of microbiological isolation of organism and appropriate targeting of anti-microbials. We suggest that patients with severe pneumonia in CGD must be managed in tertiary-care centers with facilities for advanced microbiological testing such as MALDI-TOF, 16S rRNA PCR, fungal cultures, and PCR.

Apart from signature organisms such as *Aspergillus* sp. and *Staphylococcus* sp., we also documented a high incidence of mycobacterial infections (18.5%) in our cohort. Most common presentation of mycobacterial infection was pneumonia followed by disseminated forms and lymphadenitis (**Table 4**). Localized or disseminated infections due to BCG have been documented in 8% of patients in our cohort. Abnormal BCG response has been documented at higher rates in other cohorts such as Iran (55.9%) (45), Turkey (22.5%) (43), Latin America (29.6%) (46), and China (64%) (41) that also administer BCG to all children. We do not investigate all patients with BCG adenitis for CGD. It is possible that milder forms of CGD may present only as BCG adenitis and that such patients may have been missed in our cohort. Incidence of *Serratia* infection in our cohort is very low (2/236, 0.8%) when compared to studies from North America and Europe (47). Possible reasons for this observation include tropical climate patterns and low microbiological isolation rates. Lee et al. have previously reported that infection due to *Burkholderia pseudomallei* and *Chromobacterium violaceum* in CGD have been predominantly noted in tropical countries (48). Melioidosis is common in Southern India, and we have also documented melioidosis in a patient from South India who had X-linked CGD (49). Septicemia due to *C. violaceum* has also been documented in a child with *NCF1* defect who hailed from Central India. It is possible that many patients with melioidosis are not screened for CGD in the country and diagnosis of CGD may be missed in them.

Staphylococcus aureus was the commonest organism isolated in suppurative lymphadenitis and skin abscess. Other bacteria isolated in such infections included gram-negative organisms like *Burkholderia* sp., *Klebsiella* sp., and *Pseudomonas* sp. Fungi such as *Candida* sp. and *Basidiobolus* sp. were only occasionally isolated in such infections. The microbiological spectrum in these infections is similar to reports from other countries (1, 6, 43, 50). Among the BSI, the microbiological spectrum was almost similar—*Staphylococcus* sp. was the commonest, followed by *Burkholderia* sp. and other gram-negative bacteria. However, non-typhoidal *Salmonella* (8%) was isolated more frequently from blood as compared to other sites. This suggests the importance of frequent handwashing and avoidance of ingestion of raw food items in patients with CGD, as these factors increase the risk of acquiring non-typhoidal *Salmonella*. *S. aureus* is the only organism documented in patients with liver abscess. Oral prednisolone was successfully used in three children with liver abscess for enhanced resolution, and none of them required surgical intervention. Our experience is in line with recent evidence concerning use of corticosteroids in liver abscess in CGD (51).

Common non-infective manifestations documented in our cohort include inflammatory bowel disease-like colitis, visceral granuloma, and secondary HLH. We document a lower rate of colitis (5%) in our cohort compared to studies from North America, Europe and China. However, our rates are comparable to cohorts from Turkey, Iran, and Israel, where autosomal recessive forms of CGD are predominant compared to X-linked CGD (**Supplementary Table 3**) (43, 45). Two patients who had colitis since early infancy were managed as inflammatory bowel disease elsewhere, and were diagnosed to have CGD at 5 and 7 years, respectively. One of the children also developed fulminant pneumonia following infliximab therapy for IBD-like colitis, similar to the report by Uzel et al. (52). This suggests that work-up for CGD must be considered in children with early-onset colitis – pediatricians and gastroenterologists need to be aware of this presentation. Unlike reports from other studies (1, 53), children with AR-CGD had a higher incidence of colitis in our cohort compared to X-linked forms. The difference is probably due to predominance of AR forms of CGD in the present series. While four patients achieved remission with only oral prednisolone and mesalamine, three achieved partial control with azathioprine. Complete remission of colitis following HSCT was documented in a child with X-linked CGD. This shows that immunological reconstitution following HSCT is also beneficial for the inflammatory component of CGD.

Flow cytometry-based evaluation of NADPH oxidase components is a surrogate marker for identifying the genetic defect in CGD. However, this assay can be currently performed in only in two centers in India—PGIMER, Chandigarh and NIIH, Mumbai. We identified regional heterogeneity in molecular spectrum of CGD within India. Overall, *NCF1* is the commonest molecular defect identified in patients with AR-CGD, similar to the cohorts from other parts of world. However, *CYBA* is the second common type of AR-CGD in patients from Southern and Western India, whereas, *NCF2* defect

is commoner in patients from North India. Moreover, a common dinucleotide deletion in *NCF2* (c.835_836delAC) was observed in 10 patients from North India, suggesting a Founder effect in this population. Observed regional differences in molecular spectrum could be due to heterogeneity in genetic background of the population in India. Genescan is considered to be the preferred mode for identification of common dinucleotide deletion in Exon 2 of *NCF1* (c.75_76delGT). This modality is, however, currently available only at Chandigarh and Mumbai. The preferred molecular approach for work-up of patients with CGD at Mumbai has been detailed in a previous publication. At Chandigarh, we follow a similar approach, except that Sanger sequencing for *NCF2* c.835_836delAC variant is carried out first for patients who have decreased $p67^{phox}$ expression by flow cytometry, as this variant is commonly and exclusively observed in North Indian population. This is followed by NGS whenever required. We have not identified $p40^{phox}$ (*NCF4*) defect in our cohort. However, patients with *NCF4* defect can have near normal neutrophil stimulation in the conventional DHR assay done using phorbol myristate acetate (PMA) as the stimulant (54). It is possible that this defect has been missed in many patients in India, as all patients suspected to have CGD were screened with DHR assay using stimulation with PMA. Recently, *EROS* (*CYBC1*) defect resulting in CGD has also been described (55). We have not screened for this defect in our cohort.

Most of the patients in our cohort were kept on antimicrobial prophylaxis—cotrimoxazole and itraconazole. Interferon-gamma was not used in any of our patients as it is not available in India. Number and severity of infections, especially those due to *Staphylococcus* sp., significantly came down after initiation of antimicrobial prophylaxis (**Table 8**). However, many patients had documented breakthrough infections even while being continued on prophylactic medications. Overall mortality in our cohort was 38.5% which is similar to reports from other countries such as Mexico and China (41, 50, 56, 57). Our mortality rates are, however, much higher than those reported from the USA, Iran, and European countries. Delays in diagnosis, severe infection at the presentation, and lack of widespread availability of pediatric HSCT services in the country accounted for high mortality rates in our cohort. However, we also document significant decrease in number of infections following initiation of antibiotic prophylaxis (**Table 8**). This suggests that patients with CGD in developing countries, especially AR forms (*NCF1* defect), can have a reasonable quality of life provided they are diagnosed early and continued on long-term antimicrobial prophylaxis (**Figure 5**).

HSCT could be carried out in only 23 patients in our cohort. Most of the HSCTs have been carried out in Apollo Children's Hospitals, Chennai. Only five other centers (BJ Wadia Hospitals for Children, Mumbai; PGIMER, Chandigarh; SGRH, New Delhi; Aster CMI Hospitals, Bangalore; Apollo Indraprastha Hospitals, New Delhi) have carried out HSCT for CGD until date. Successful engraftment was documented in 15 patients who received either bone marrow or peripheral blood-derived stem cells. A child who received cord-blood transplantation failed to develop early myeloid engraftment and succumbed to severe infection. This is similar to the experience reported by Morio et al. (58). Four out of six children who received haploidentical transplantation have had a successful engraftment, indicating that haploidentical transplantation is a potential life-saving option in children when completely matched donors are not available. We reiterate that establishment of dedicated pediatric HSCT centres, and government support for patients undergoing HSCT are essential for state-of-the-art management of CGD in developing countries.

To conclude, we document the first multicentric study on clinical and molecular features of 236 patients with CGD from India. To the best of our knowledge, ours is the third largest cohort of patients with CGD documented till date after the multicentric reports from the USA (n = 368) and Europe (n = 429). We have documented a wide spectrum of bacterial and fungal infections and a high incidence of mycobacterial infections in our cohort. Though AR-CGD predominates in our cohort, significant regional differences in molecular spectrum have been observed. Reasons for a high mortality rate (30%) include lack of awareness of CGD among internists and pediatricians, lack of easy access to immunological tests, and paucity of pediatric HSCT services in our country. Establishment of a nation-wide registry for a rare disease like CGD will be needed in future to decipher precise estimate of disease burden in the country and for better identification of barriers in the existing diagnostic and management strategies.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

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 individuals for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PV, MSu, DS, AJi, AnG, SL, PP, AK, PT, AP, VG, MD, SB, CG, HK, DM, MSi, RR, RU, FN, BG, HL, MKa, AS, SSe, TS, AmG, and SSi—clinical management of patients and follow-up and contribution of clinical data. AR, MSh, JS, GK, SC, BS, RM, VA, MKu, GH, UB, PK, MM, AJa, FN, KV, MD, KC, KI, OO, SN, and YL—laboratory work-up of patients and contribution of laboratory data. PV, MSu, MSh, and SL—preparation of first draft and literature review. AR, PV, and SSi—critical review and

editing of manuscript. AR and PV—final approval of manuscript. 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.2021.625320/full#supplementary-material

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Clinical and Genetic Spectrum of a Large Cohort of Patients With Leukocyte Adhesion Deficiency Type 1 and 3: A Multicentric Study From India

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Leukocyte adhesion deficiency (LAD) syndrome is a group of inborn errors of immunity characterized by a defect in the cascade of the activation and adhesion leading to the failure of leukocyte to migrate to the site of tissue injury. Three different types of LAD have been described. The most common subtype is LAD type 1 (LAD1) caused due to defects

in the *ITGβ2* gene. LAD type 2 (LAD2) is caused by mutations in the *SLC35C1* gene leading to a generalized loss of expression of fucosylated glycans on the cell surface and LAD type 3 (LAD3) is caused by mutations in the *FERMT3* gene resulting in platelet function defects along with immunodeficiency. There is a paucity of data available from India on LAD syndromes. The present study is a retrospective analysis of patients with LAD collated from 28 different centers across India. For LAD1, the diagnosis was based on clinical features and flow cytometric expression of CD18 on peripheral blood leukocytes and molecular confirmation by Sanger sequencing. For patients with LAD3 diagnosis was largely based on clinical manifestations and identification of the pathogenic mutation in the *FERMT3* gene by next-generation Sequencing. Of the total 132 cases diagnosed with LAD, 127 were LAD1 and 5 were LAD3. The majority of our patients (83%) had CD18 expression less than 2% on neutrophils (LAD1°) and presented within the first three months of life with omphalitis, skin and soft tissue infections, delayed umbilical cord detachment, otitis media, and sepsis. The patients with CD18 expression of more than 30% (LAD1^{+}) presented later in life with skin ulcers being the commonest manifestation. Bleeding manifestations were common in patients with LAD3. Persistent neutrophilic leukocytosis was the characteristic finding in all patients. 35 novel mutations were detected in the *ITGβ2* gene, and 4 novel mutations were detected in the *FERMT3* gene. The study thus presents one of the largest cohorts of patients from India with LAD, focusing on clinical features, immunological characteristics, and molecular spectrum.

Keywords: Leukocyte Adhesion deficiency, CD18, CD11, *FERMT3*, *ITGβ2*

INTRODUCTION

Leukocyte adhesion deficiency (LAD) is a rare phagocytic disorder characterized by a defect in the trafficking of leukocytes from the blood vessels to the site of tissue injury (1–4). These patients usually present in infancy with delayed separation of the umbilical cord, omphalitis, and necrotic infections of the skin and mucosal surfaces (5). The absence of pus and persistent marked neutrophilic leukocytosis are the hallmarks of LAD. Three types of LAD have been described, with LAD type 1 (LAD1) being the most common form. LAD1 is caused due to defect in the *ITGβ2* gene encoding the common beta subunit of β2 integrins (CD18) (5–9). β2 integrins form a heterodimer by non-covalently binding to the different subunits including, αL (CD11a), αM (CD11b), αX (CD11c), and αD (CD11d) (1, 4, 9). In a setting of strong clinical suspicion, the immunological workup for diagnosis of LAD1 involves studying the flow cytometric expression of CD18 and CD11 on leukocytes followed by molecular confirmation. Depending on the CD18 expression on neutrophils LAD1 patients are classified into severe (CD18 expression <2%), moderate (2%–30%), and mild (>30%) (1, 2, 5, 10). LAD type 2 (LAD2) is caused due to mutations in the *SLC35C1* gene leading to defective expression of cell surface fucosylated glycan structures (11, 12). These patients suffer from recurrent bacterial infections, severe mental, and growth retardation characterized by distinct facial characteristics (12). Flow cytometry demonstrates the absence of SLeX (CD15a) expression on cell-surface glycoproteins, along with deficiency of H antigen on erythroid cells, resulting in the Bombay phenotype (13). LAD type 3 (LAD3) is caused by a mutation in the *FERMT3* gene that encodes protein kindlin-3 which plays a crucial in integrin activation (14–16). These patients also have severe recurrent bacterial infections, persistent leukocytosis, and delayed umbilical cord fall with a platelet aggregation defect that results in severe bleeding manifestation (17). Though individual case reports and small case series are available from India (18–23), there is a paucity of data on the clinical, immunological, and molecular spectrum in LAD. In this study, we report a retrospective cohort study of 132 patients LAD patients from 28 different centers of India.

MATERIALS AND METHODS

Patients with a clinical suspicion of LAD referred to the Indian Council of Medical Research-National Institute of Immunohaematology (ICMR-NIIH) and other tertiary care centers in India between 1990 and 2020 were retrospectively analyzed in this study. The clinical and laboratory information about the age of presentation, age at diagnosis, site of infections, organisms isolated, umbilical cord complications, family history, consanguinity, complete blood count (CBC), immunological investigations were collected from the data available. The study was approved by the institutional ethics committee of ICMR-NIIH.

As a part of the diagnostic workup of LAD the flow cytometric expression of CD18, CD11 markers on leukocytes was assessed. Based on the CD18 expression on neutrophils, LAD1 was sub-classified into three phenotypes viz. severe (LAD1°) with CD18

expression <2% moderate (LAD1$^-$) phenotype with CD18 expression 2%–30%, and mild (LAD1$^+$) phenotype with CD18 expression ≥30%. CD11a expression data were available for 89 patients. We analyzed our data by looking at different parameters like median fluorescence intensity (MFI) and stain index (SI) index on different populations of leukocytes via, neutrophils, lymphocytes, and monocytes.

Molecular confirmation was performed was done using Sanger sequencing for LAD1 and next-generation sequencing (NGS) for LAD3. The candidate variants identified by NGS were confirmed by Sanger sequencing in the index and family members.

Graph pad prism version 5.03 statistical software was used to perform statistical analysis. The descriptive variables were expressed as percentage counts and the median-interquartile range (IQR) were used. The groups in this study were compared using the one-way ANOVA. The test was performed at a 95% confidence interval (95% CI), and $p<0.05$ was considered statistically significant. Kaplan–Meier evaluation was used to predict the survival probabilities of the patients.

RESULTS

Patients Characteristics

In this study, we analyzed a total of 127 cases from 125 families with LAD1 and 5 cases from 4 families with LAD3. LAD2 were not reported in our cohort. The clinical and demographic features of the patients are as shown in **Table 1**. Consanguinity was seen in 51% of the patients. Male preponderance was seen in our cohort (62%). The genetic diagnosis was available in 80% of cases (n=105) cases. White blood count (WBC) and absolute neutrophil count (ANC) were noted in all the cases with a median of 53 x10^3/μl (14–167 x10^3/all) and 36 x10^3/μl (22–137x10^3/μl), respectively. It was observed that the ANC was higher in LAD1° 40 × 10^3/μl (11–136× 10^3/μl) cases as compared to LAD1$^-$ 25 × 10^3/μl (16–74× 10^3/μl), LAD1$^+$ 27 × 10^3/μl (10–91× 10^3/μl), and LAD3 21 × 10^3/μl (10–38× 10^3/μl).

Clinical and Genetic Characteristics of Leukocyte Adhesion Deficiency 1

On the basis of absent or abnormal CD18 expression on the surface of neutrophils, three distinct phenotypes were observed in our cohort. The commonest being LAD1° seen in 83% (n=106) individuals, followed by LAD1$^-$ phenotype seen in 9% (n=11) of the cases and LAD1$^+$ in 8% (n=10). The mean CD18 expression in LAD1$^-$ and LAD1$^+$ patients was 9.6 ± 5% (3%–18%) and 68 ± 24% (32%–99%), respectively. The median fluorescence intensity was noted in 76/127 LAD1 patients. **Figure S1**, presents the percentage, MFI, and SI on neutrophils, monocytes, and lymphocytes in LAD1°, LAD1$^-$, and LAD1$^+$ cases. Although the expression of CD18 was >30% in LAD1$^+$ cases, the CD11a was significantly reduced in 80% (8/10) of the cases. The median age of presentation was 0.3 months (0.03–12 months) for the LAD1° patients, 1 month (0.09–72 months) for LAD1$^-$ and 2.5 months (0.5–132 months) for patients with LAD1$^+$. The median age of diagnosis was 3 months (0.1–48 months) for LAD1°, 5 months (1–78 months) for LAD1$^-$ and 84 months (3–168 months) for LAD1$^+$.

Umbilical cord related complications like omphalitis (64%) and delayed separation (62%) were the most common manifestation seen in the LAD1° and LAD1$^-$ cases. Other frequent infections included lower respiratory tract infection (LRTI) in 41% (43/106), sepsis in 37%. Necrotic skin ulcer was the most common infection in LAD1$^+$ which may mimic pyoderma gangrenosum. The perianal region was the commonest site in LAD1$^-$ cases (27%). Infectious organisms were isolated from 69 cases comprising predominantly bacterial infections including *Pseudomonas aeruginosa* (n=28), *Staphylococcus aureus* (n=17), and *Klebsiella pneumonia* (n=11), and fungal infections were noted in 7 patient. Also, unusual organisms like *Proteus* sp., *Citrobacter* sp., *Stingomonas*

TABLE 1 | Clinical characteristics of patients with leukocyte adhesion deficiency (LAD).

	LAD1°		LAD1$^-$		LAD1$^+$		LAD3	
Total patient (n)	106		11		10		5	
Age of presentation in months Median (Range)	0.3 (0.03–12)		1 (0.09–72)		2.5 (0.5–132)		0.8 (0.03–1)	
Age of diagnosis in months Median (Range)	3 (0.1–48)		5 (1–78)		84 (3–168)		34 (3–168)	
Median WBC count (Range)	57 (16.5–167)		38.6 (19.2–140)		32.5 (14.7–102)		30 (18–99.6)	
Median ANC × 10^3/μl (Range)	40 (11–136)		25 (16–74)		27 (10–91)		21 (10–38)	
	n	%	n	%	n	%	n	%
Gender (Males)	63	59	7	63	8	80	4	80
Family History	38	36	1	9	3	30	2	40
Consanguinity	54	51	5	45	5	50	2	40
Umbilical cord complication	65	61	7	64	2	20	3	60
LRTI	43	41	5	45	3	30	–	–
Sepsis	39	37	3	27	2	20	–	–
Skin infections	55	52	4	36	7	70	–	–
Periodontal infections	11	10	–	–	–	–	1	20
Otitis media	22	21	2	18	1	10	1	20
Diarrhea	9	8.5	1	9	–	–	–	–
Failure to thrive	31	29	3	27	4	40	–	–
Meningitis	5	5	–		–		–	–
Bleeding Manifestation	–		–		–		5	100

paucimobilis, and Acinetobacter baumani were noted in a few patients.

Direct Sanger sequencing of the *ITGβ2* gene revealed 57 disease-causing variants in 105 patients (**Table S2**), including 30 patients we have previously reported (18). These mutations were clustered mostly in exons 6 (22%) and exon 7 (11%). The spectrum of mutation has been shown in **Figure 1**. The frequency of mutation c.533C>T (p.Pro178Leu) and c.817G>A (p.Gly273Arg) was high. The majority of the patients (n=95) had homozygous mutations, while compound heterozygous mutations were identified in only 10 patients. These compound heterozygous mutations were seen only LAD1° cases. Missense mutations (40%) were the most common mutations identified in our cohort followed by nonsense (21%), splice site (19%), and frameshift (19%). 54% of the mutations were located in exon 5–9, a highly conserved region of the extracellular domain of CD18 followed by cysteine-rich repeat region (CRR) domain (32%), Mid region (7%), plexins, semaphorins, and integrins domain (PSI) domain (5%) and transmembrane domain (TM) region (2%). Missense and nonsense mutations were frequently seen in LAD1° and LAD1$^-$ patients. On the other hand, splice site mutations affected almost 60% of LAD1$^+$ patients.

Clinical and Genetic Features of Leukocyte Adhesion Deficiency 3

Recurrent infections and severe bleeding manifestation were seen in all patients within 1 month of life. The median age of diagnosis was 34 months (3–168 months). Omphalitis was seen in 3/5 patients. CD18 expression on the surface of the leukocytes was normal in all patients. The molecular diagnosis using NGS technology identified four novel pathogenic variants in the *FERMT3* gene in five LAD3 patients which were conserved across the species. Out of these 3 were missense mutations and one was nonsense mutations.

Outcome

Follow up data were available for 124 patients with the median follow up duration of 7 months (0.033–216 months), 6 months (2–120 months), 182 months (5–276 months), and 60 months (11–192 months) for LAD1°, LAD1$^-$, LAD1$^+$, and LAD3, respectively. Out of these, 81% of patients expired due to severe infections in absence of hematopoietic stem cell transplant (HSCT). The majority of them died within the first year of life (n=70). The overall survival in our LAD1 cohort is only 14% while that of LAD3 is 83%. Mortality was higher in LAD1° patients with only 6% survival beyond 2 years as compared to 16% of the LAD1$^-$ and 60% of the LAD1$^+$ patients in our cohort (**Figure 2**). Twelve patients underwent HSCT. Of these, ten patients underwent stem cell transplant (SCT) from HLA-matched related donors, whereas two patients received graft from matched unrelated donor. Three patients expired due to severe graph versus host disease (GVHD) and secondary complications.

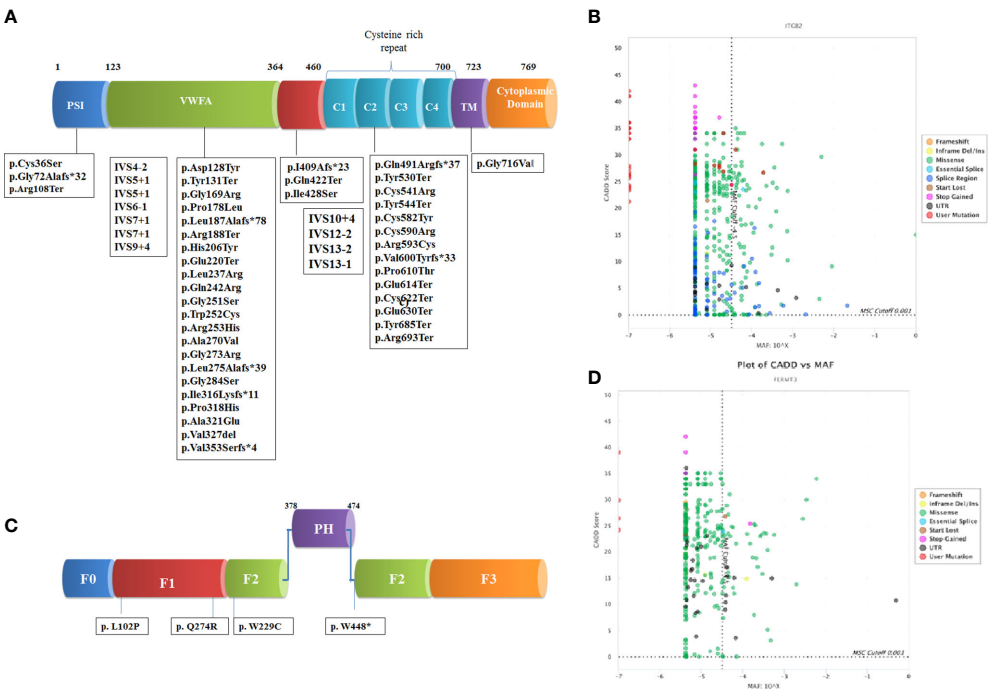

FIGURE 1 | Schematic representation of domain wise distribution of the mutations identified in our LADpatients **(A)** *β2* integrin **(B)** kindlin-3 protein. Combined annotation–dependent depletion (CADD) and Minor allele frequency (MAF) scores of the variants reported in gnomAD and novel variants identified in our cohort for the genes **(C)** *ITGβ2* and **(D)** *FERMT3* using PopViz software (24). The mutation significance cutoff (MSC) with 99% confidence interval is shown in dotted line.

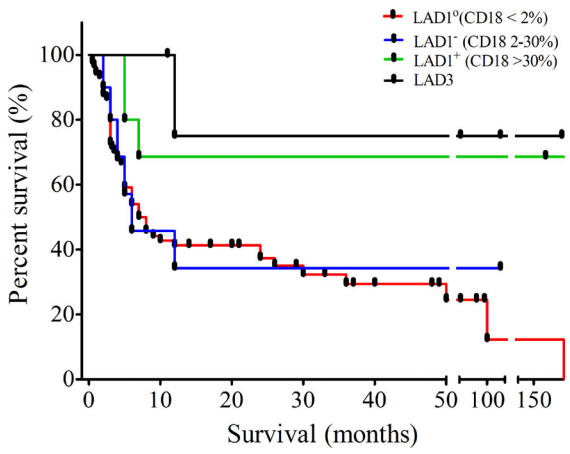

FIGURE 2 | Outcome of leukocyte adhesion deficiency (LAD) patients: Kaplan-Meier curve showing survival of patients diagnosed with LAD in our cohort.

DISCUSSION

Leukocyte adhesion deficiency is a rare phagocytic disorder associated with defective neutrophil recruitment, rolling, and adhesion (1, 25–27). To the best of our knowledge, this is the largest comprehensive study on clinical, immunological, and molecular findings of LAD from India. Most of our patients presented with recurrent bacterial infections with a history of umbilical cord related complications. The infective spectra was similar to other reported cohorts with LRTI and sepsis being the commonest (5, 8, 9, 28–37). Delayed umbilical cord fall was seen in only 66% of the cases. Periodontal infections including gingivitis and oral ulcers are reported in 24% of severe and 52% of moderate LAD1 patients (5). In our cohort, it was observed in 10% of LAD1° patients. This might be because of under-diagnosis of mild LAD1 phenotype. Late- onset autoimmune complications have been reported previously (38). However, it was not observed in our patients as most of them expired in infancy.

The degree of severity of infections in LAD1° and LAD1$^-$ patients was high with poor survival of 8% and 33% (beyond 24 months), respectively. On the other hand, patients with LAD1$^+$ had a more variable clinical phenotype with a significant difference between the age of onset and diagnosis. We observed that the mortality rate was high in LAD1° as well as LAD1$^-$ compared to LAD1$^+$.

The clinical suspicion was strengthened with the presence of marked neutrophilic leukocytosis observed in all patients with total WBC count >25 x 10^3/µl in 86% cases and ANC of >15 x 10^3/µl in 82% of the cases. The median WBC count was higher in LAD1° as compared to the other subtypes and LAD3 patients, however, there was no significant correlation ($r<0.1$) between the WBC count and CD18 expression on neutrophil for the entire LAD1 cohort and the three phenotype as seen in the earlier studies (**Figure S2**).

The diagnosis of LAD1 often relies on the percentage of positive neutrophils expressing CD18. For severe forms of LAD1 where the expression is <2% diagnosis is easy and reliable. The expression may vary from patient to patient despite the same underlying disease-causing mutation (5, 30). It was observed that positive predictive value (PPV) of the assay significantly increased from 98.51 to 100% when the MFI of patients and healthy controls are compared ($p<0.001$) (data not shown). It is known that α- subunit of LFA-1 cannot be efficiently expressed unless it first associates with the β subunit. Previous studies have also reported that the expression of CD11a is abnormal in all the patients of LAD1 irrespective of CD18 expression (7, 32, 37). In P20 and P28, the CD18 expression was 50 & 90% and CD11a expression was 70 & 50%, respectively; SI observed was comparatively below laboratory lower limits obtained from the SI of healthy controls (SI of CD18/CD11a: P20- 1.81/1.59 & P28-1.61/1.75). This concurs that the addition of the CD11a marker to the assay may increase the diagnostic accuracy (32).

We identified 57 mutations in 105 patients of which 35 were novel suggesting heterogeneity in the mutation spectrum for LAD1. 51% (29/57) of the mutations were identified in the VWFA domain followed by 16% (n=9) in cysteine-rich region and 3% (n=2) PSI domain, 4% (n=2) mid-region and 2% (n=1) in TM. The common mutation identified included c.533C>T (n=9), c.817G>A (n=9), c.751G>A (n=5), c.1224+4A>G (n=5), and c.2077C>T (n=5) in different domains. Out of the total mutations identified in the VWFA domain, 89% of them resulted in absent expression of CD18 on PMNs causing severe infection in LAD1° and LAD1$^-$ cases.

50% of LAD1$^+$ had a splice site mutation. These patients presented later in life with recurrent skin lesions like pyoderma gangrenosum. All of them had the same c.1224+4A>G mutation and 4 of these patients have been reported by us earlier (39). In contrast to other patients with pyoderma gangrenosum, there is a paucity of neutrophils in the dermis of the skin lesions in patients with LAD1$^+$ and they also show only partial and temporary response to steroids. Though the pathogenesis of these inflammatory lesions is not clear, partial expression of CD18 resulting in an aberrant oscillation of integrins on the neutrophil surface and Th17 mediated aberrant inflammatory response may be responsible for these inflammatory skin lesions (40, 41).

LAD3 was diagnosed in 5 patients classically presenting with recurrent infection of skin, ear, and mucosal surfaces, and bleeding from gums and skin. There was a significant variation in the age of diagnosis and presentation in these patients. Unfortunately, platelet aggregation studies were not available for these cases. Unlike LAD1, the surface expression of CD18/CD11 expression on leukocytes was normal in these patients. This disorder has mostly been reported in patients of Arab Maltese, Turkish, or African American origin (15, 16, 20, 42, 43).

The overall outcome in our cohort was poor with 81% mortality. The time taken for the patients from the diagnosis to treatment is critical and many patients are lost before they reach the stage of transplant. HSCT was possible in only 12 cases as most of the patients included in the study were from the last decade when limited transplantation facilities were available in India. However, with the increase in the number of HSCT centers, this scenario may change in the near future. Recent

advancements in gene therapy for LAD1 may change the course of management (30).

This study describes the clinical and molecular spectrum of a large cohort of patients of LAD from India. It highlights the importance of analyzing MFI and SI of CD11a along with CD18 for accurate diagnosis of LAD1. It reports a large number of previously unreported mutations in the *ITGβ2* and *FERMT3* gene. Knowledge of the nature and frequency of these mutations is not only important for providing accurate diagnosis and genetic counseling to the families but will also help in the future for planning gene editing and gene therapy strategies for these rare genetic disorders.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Indian Council of Medical Research National Institute of Immunohematology. 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 minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PK analyzed the data and wrote the manuscript. UB and RM helped in procuring the clinical details and follow-up of the patients. MD, AR, PT, VG, AP, AG, KA RKP, HC, PK, AA, SunK, SagB, SR, RCP, VinG, RR, RU, MSi, DM, HP, MKa, AnuS, AviS, SarB, GG, ShoK, RN, MamM, GS, AbhS, ICK, ParG, SwaK, SPM, VK, ShN, and SNem supervised the management and follow up of the patients. PK, MG, AD, GH, PS, MKe, SS, NJ, NN, AmrD, PalG, ShwS, ShoK, and AJ performed the laboratory investigations for the different cases. PK, PK, MG, MSh, and VJ were involved in the molecular analysis of the different patients. MM supervised the study and reviewed the manuscript. 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.2020.612703/full#supplementary-material

SUPPLEMENTARY FIGURE S1 | The percentage expression, median fluorescence intensity (MFI), stain index (SI- ratio of intensity of stain/unstain) of CD18 and CD11a on a) Neutrophils b) Lymphocytes, and c) monocytes.

SUPPLEMENTARY FIGURE S2 | Correlation between WBC count and CD18 expression on neutrophils a) all LAD patients b) LAD1° c) LAD1$^-$ d) LAD1$^+$ e) LAD3.

SUPPLEMENTARY TABLE S1 | Clinical summary of all the patients diagnosed with LAD.

SUPPLEMENTARY TABLE S2 | Mutation spectrum of *ITGβ2* gene and *FERMT3* genes.

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The Spectrum of Clinical, Immunological, and Molecular Findings in Familial Hemophagocytic Lymphohistiocytosis: Experience From India

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Hemophagocytic lymphohistiocytosis (HLH) is a syndrome of immune dysregulation characterized by hyperactivation of the immune system, excessive cytokine secretion and severe systemic inflammation. HLH is classified as familial (FHL) when associated with mutations in *PRF1*, *UNC13D*, *STX11,* and *STXBP2* genes. There is limited information available about the clinical and mutational spectrum of FHL patients in Indian population. This study is a retrospective analysis of 101 molecularly characterized FHL patients over the last 10 years from 20 different referral centers in India. FHL2 and FHL3 together accounted for 84% of cases of FHL in our cohort. Patients belonging to different FHL subtypes were indistinguishable based on clinical and biochemical parameters. However, flow cytometry-based assays viz. perforin expression and degranulation assay were found to be specific and sensitive in diagnosis and classification of FHL patients. Molecular characterization of respective genes revealed 76 different disease-causing mutations including 39 (51%) novel mutations in *PRF1*, *UNC13D*, *STX11,* and *STXBP2*

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genes. Overall, survival was poor (28%) irrespective of the age of onset or the type of mutation in our cohort. Altogether, this article sheds light on the current scenario of FHL in India. Our data reveal a wide genetic heterogeneity of FHL in the Indian population and confirms the poor prognosis of FHL. This study also emphasizes that though mutational analysis is important for diagnostic confirmation of FHL, flow cytometry based assays help significantly in rapid diagnosis and functional validation of novel variants identified.

Keywords: familial hemophagocytic lymphohistocytosis, perforin, degranulation, HLH-targeted therapy, flow cytomertry, NGS

INTRODUCTION

Familial hemophagocytic lymphohistiocytosis (FHL) is a disorder of immune dysregulation characterized by persistent high-grade fever, progressive cytopenias, hepatosplenomegaly and systemic inflammation. It is an autosomal recessive disorder and affects mostly infants and young children, but has also been reported in adolescents and adults (1, 2). So far, based on the gene mutations observed, FHL is categorized as FHL2 (*PRF1)*, FHL3 (*UNC13D)*, FHL4 (*STX11),* and FHL5 (*STXBP2)* encoding for Perforin, Munc13-4, Syntaxin11, and Syntaxin binding protein 2, respectively (3). These proteins play a fundamental role in lymphocyte cytotoxicity. FHL1 (9q21.3-22) was identified by homozygosity mapping of four inbred families of Pakistani origin (4); though, the disease-causing gene in this locus is yet been unknown.

As the clinical features are similar to those with various infections and inflammatory disorders, diagnosis can often be missed. Though, Histiocyte Society has established HLH diagnosis criteria, which includes clinical manifestations and laboratory findings; it does not help in classifying FHL patients and identifying the underline genetic defect.

Impaired function of NK cells and cytotoxic T lymphocytes (CTLs) due to inherited defect in the granule mediated cytotoxicity is the hallmark of FHL patients (5). Thus, for the diagnosis of these patients, evaluating the function of NK cell and CTLs is crucial. In recent years, assays based on flow cytometry have been developed for evaluating NK cell and CTL cell functions viz. measurement of intracellular perforin expression and degranulation assay determined by upregulation of CD107a expression (6, 7). Granule release assay (GRA) is a screening test for detection of FHL3, FHL4, and FHL5 patients. Measurement of intracellular perforin levels serves as a phenotypic assay in identifying FHL2 patients. These tests serve as rapid screening tests for identifying FHL patients. However, molecular characterization of the respective genes is essential for the final diagnostic confirmation. Identifying underlying genetic defect is also important for offering genetic counseling and prenatal diagnosis in affected families.

The incidence of the four FHL subtypes varies significantly in different ethnic groups; also certain mutations are unique or commonly seen in a particular population (1, 8–10). Understanding this pattern of mutation in a particular population not only helps in cost-effectively designing strategies for mutation screening but may also have epidemiological implications. However, very limited data is available on FHL from India. Thus, in this retrospective study, we report one of the largest series on general clinical features; immunological and molecular findings and outcome of FHL in 101 patients from 20 different referral centers of India over the last 10 years.

MATERIALS AND METHODS

Enrollment of Patients and Ethics Statement

This study was approved by Institutional Ethics committee (IEC) for Human subjects of ICMR-National Institute of Immunohematology (NIIH). Ethical clearance was obtained at each center and patients received research information and provided written informed consent to participate in this study. Patients fitting into HLH criteria of the Histiocyte Society (5) referred to ICMR-NIIH or collaborating FPID centers or other tertiary care centers in India from 2010 to 2020 were included in this study. Detailed clinical and family history was recorded for these patients. All the procedures involving human subjects were performed in accordance with the international ethical standards.

Diagnosis of HLH

As a part of diagnostic workup of HLH, perforin expression and degranulation assay on NK cells were performed as previously described (6, 7, 11). Lymphocyte subset analysis of patient samples was performed using Multitest 6-color TBNK reagent (Becton Dickinson).

Further, confirmation of molecular diagnosis was done either by direct Sanger sequencing (in perforin deficient patients) or by targeted Next-Generation sequencing (NGS) or clinical whole-exome sequencing (WES) (in patients with abnormal degranulation).

Bioinformatics and Statistical Analysis

The sequences obtained were compared with the reported gene structures (*PRF1*, *UNC13D*, *STX11*, *STXBP2*: NCBI) using the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST). Nature of any novel sequence variant was analyzed by using different prediction software i.e., PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (www.http://sift.jcvi.org/). For splice region variants, Human Splicing Finder tool is used (https://hsf.genomnis.com/).

Data was presented in terms of median and percentages. For a comparison of >2 groups, One-way analysis of variance (ANOVA) test was used. Mann-Whitney U-test was used for comparing groups with non-parametric data. The *p*-values ≤0.05 were considered statistically significant. GraphPad Prism (Chicago, IL, USA) version 5 was used for statistical calculations.

RESULTS

The Pattern of Familial HLH

In this study, we have included 101 molecularly characterized FHL patients from the year 2010–2020. Of these, 50 patients were identified as FHL2, 35 were FHL3, seven were FHL4 and nine were FHL5 based on the defective genes. Thus, in our Indian cohort, perforin deficiency was found to be the commonest (49%) followed by Munc13-4 (35%) deficiency.

Patient Characteristics

The baseline characteristics of these patients are detailed in **Table 1**, **Supplementary Table 1**, which also defines the patients according to their genotype. Of these patients, 67% of patients were male, with a male: female ratio of 2:1. The median age of diagnosis for the entire cohort was 12 months (range: 8 days−33 years) with around 55% of the patients presenting within 1 year of age. Consanguinity was observed in 43% (37/86) patients and 28% (22/80) patients had a family history of an affected sibling. No significant difference in age of presentation was observed amongst the FHL groups.

In our cohort, trigger for FHL development could be identified in 18 patients. Viral infection was found to be the most common trigger in nine patients while bacterial infections are associated with FHL in four patients. Parasitic and fungal infection was seen in one patient each. Three FHL patients had hematological malignancy. However, in rest of the patients, information of triggers for FHL development was not available. 74% of patients fulfilled at least 5/8 HLH diagnostic criteria; whereas 22% of patients fulfilled 4/8 criteria. A pre-symptomatic diagnosis in view of strong family history was achieved in 4% patients. The common clinical presentations seen in all groups of FHL were fever (94%) and hepatosplenomegaly (97%). Hyperferritinemia was observed in 93% cases while 88% patients presented with hemophagocytosis on bone marrow examination. 83.5 and 69% of the patients had hypertriglyceridemia and hypofibrinogenemia, respectively. Deranged liver enzymes were observed in 69% patients. CNS manifestations were seen in 32% patients. Seizure disorder was the commonest CNS manifestation while cerebral palsy and peripheral neuropathy was observed in one patient each. Erythematous skin rash was observed in 19% patients. No significant difference in clinical features was observed between the different subtypes of FHL patients (**Table 1**).

All FHL patients, except six patients (two FHL2, three FHL3, and one FHL5), had serum ferritin levels >500 ng/ml. Both the FHL2 patients had a family history and they were asymptomatic when referred to our laboratory. Three FHL3 patients (P58, P73, and P74) with <500 ng/ml ferritin had borderline ferritin levels (471, 474, and 488 ng/ml, respectively). Overall, 65% FHL patients had ferritin levels between 500 and 10,000 ng/ml while 17% of FHL2 and 11% of FHL3 patients had ferritin levels >20,000 ng/ml. Ferritin levels did not differ amongst the FHL subtypes. sCD25 levels were available in 27 patients and were elevated in 96% patients.

IMMUNOLOGICAL FINDINGS

Lymphocyte Subset Analysis

Lymphocyte subset analysis (enumerating T cells, Tc cells, Th cells, NK cells, and B cells) was performed in 37 patients (18 FHL2, 11 FHL3, 2 FHL4, and 6 FHL5). Though lymphopenia was observed in 49% of the patients, the frequency and absolute counts of lymphocyte subsets did not differ amongst the subsets of FHL (**Supplementary Figure 1**).

Perforin Expression

Perforin expression could be checked by flow cytometry in 40 out of 50 FHL2 and 30 out of 51 FHL3/4/5 patients. 38/40 FHL2 (95%) had significantly lower expression of perforin on NK cells (median 2%; reference range 72 ± 2%) than the healthy controls (median 92%) and FHL patients in other groups (median 88%) ($p < 0.001$) (**Figure 1A**). 92.5% of the FHL2 patients had ≤10% perforin expression on NK cells with 62.5% having ≤2% expression on NK cells. The cut-off of <10% perforin expression on NK cells to identify patients with biallelic mutations compared to patients with normal sequencing results yielded a sensitivity of 92.5% the specificity of 100%, the positive predictive value of 100%, and negative predictive value of 93.75% with an accuracy of 97% (AUC of 0.9852) (**Supplementary Figure 2A**).

Degranulation Assay

CD107a degranulation assay could be performed in 20 out of 50 FHL2, 18 out of 35 FHL3, five out of seven FHL4 and four out of nine FHL5 patients. Defective degranulation was defined as ≤10% CD107a expression on stimulated NK cells. All FHL4 and FHL5 (100%) and 12/18 FHL3 patients (79%) patients had defective degranulation assay (**Figure 1B**). Three FHL3 patients (P56, P59, and P61) had borderline CD107a expression on NK cells (22, 18, and 17%, respectively), while three FHL3 patients (P62, P63, and P69) had degranulation within a normal range (Median 37.5%; 22–54%; reference range 28 ± 8%). Additionally, three FHL2 patients (P27, P36, and P39) had defective degranulation on NK cells (**Figure 1B**). The cut-off of <10% CD107a expression on NK cells for diagnosis of FHL patients with degranulation defect yielded a sensitivity of 73.91%, the specificity of 88.24%, the positive predictive value of 89%, and negative predictive value of 81% with an accuracy of 85% (AUC, 0.8939) (**Supplementary Figure 2B**).

Mutation Spectrum

Out of 102 molecularly characterized FHL patients, details of the mutation spectrum were available in 88 FHL patients (44 FHL2, 29 FHL3, six FHL4, and nine FHL5). This manuscript involves retrospective data from multiple centers all over India of last 10years. Thus, retrieving data of exact annotation of mutations of very old patients (six FHL2, seven FHL3, and one FHL4)

TABLE 1 | Comparison of patient characteristics in different groups of FHL patients.

Genotype	FHL2	FHL3	FHL4	FHL5
Characteristics				
Number of patients	50	35	7	9
Male	31 (62%)	23 (64%)	6 (86%)	8 (89%)
Family history	12/37 (32%)	8/32 (27%)	1/6 (17%)	1/5 (20%)
Consanguinity	16/40 (40%)	15/32 (48%)	2/7 (28.5%)	4/7 (57%)
Age of diagnosis				
<3 months	12 (24%)	10 (28%)	0 (0%)	1 (11%)
3–6 months	13 (26%)	6 (19%)	1 (14%)	2 (22%)
6–12 months	4 (8%)	5 (14%)	1 (14%)	1 (11%)
1–4 years	7 (14%)	9 (25%)	2 (29%)	0 (0%)
>4 years	14 (28%)	5 (14%)	3 (43%)	5 (56%)
Median age of presentation (range) (in months)	6 (0.25–384)	6 (1–396)	18 (3–144)	48 (2–168)
Fever	46/50 (92%)	31/33 (94%)	7/7 (100%)	7/7 (100%)
Hepatosplenomegaly	45/46 (98%)	30/32 (94%)	7/7 (100%)	9/9 (100%)
Bicytopenia	29/40 (72.5%)	16/23 (69.5%)	6/6 (100%)	7/9 (78%)
Hyperferritinemia	39/41 (95%)	25/28 (89%)	7/7 (100%)	6/7 (86%)
Elevated sCD25 levels*	19/19 (100%)	5/6 (83%)	2/2 (100%)	ND
Hemophagocytosis	38/40 (95%)	18/25 (72%)	6/6 (100%)	5/5 (100%)
Hypertriglyceridemia	30/39 (77%)	23/27 (85%)	6/6 (100%)	7/7 (100%)
Hypofibrinogenemia	21/33 (64%)	18/22 (82%)	3/4 (75%)	2/5 (40%)
CNS symptoms	9/32 (28%)	9/20 (45%)	2/6 (33%)	1/3 (33%)

sCD25 levels compared to age-matched healthy controls.

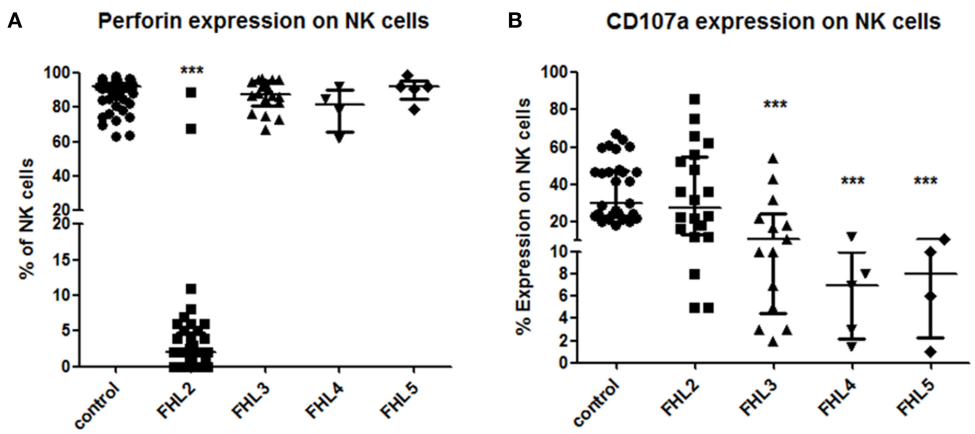

FIGURE 1 | Comparison of Perforin expression and CD107a expression on stimulated NK cells in different groups of FHL patients **(A)** Perforin expression **(B)** CD107a expression. One way ANOVA test was used to evaluate differences between activation markers in different groups. $^{***}P < 0.001$.

was difficult. However, on records, these patients have proven mutations with details of underlying defective gene mentioned. Also for these patients' transplant details and outcome could be traced and hence are included in this study.

Total of 76 different disease-causing mutations were identified in four HLH-related genes (**Table 2**). Of these 76 mutations identified; 42 (55%) were missense mutations, 7 (9%) were nonsense mutations, 18 (24%) were frameshift mutations and 9 (12%) were intronic and splice site mutations (**Table 2**). 63 (72%) patients were detected to have homozygous mutations, 12 (14%) had compound heterozygous variants and 12 (14%) had a single heterozygous mutation (**Figure 3A**). In total, 39 (51%) novel mutations were identified that included 15 missense mutations, four nonsense mutations, 16 frameshift mutations and four splice site and intronic mutation.

In the 44 FHL2 patients, we identified 33 different *PRF1* mutations with a total of 17 novel variants (**Table 2**). Homozygous mutations were found in 62% of FHL2 patients.

TABLE 2 | Different mutations identified in patients with FHL.

	FHL2 ($n = 44$)	FHL3 ($n = 28$)	FHL4 ($n = 6$)	FHL5 ($n = 9$)
No of mutations	33	30	5	8
Novel mutations	17 (51%)	18 (60%)	2 (40%)	2 (25%)
Type of mutation				
Missense	24 (73%)	11 (37%)	3 (60%)	5 (62%)
Nonsense	5 (15%)	1 (3%)	1 (20%)	0 (0%)
Frameshift	4 (12%)	12 (40%)	1 (20%)	0 (%)
Splice-site and intronic	0 (0%)	6 (20%)	0 (0%)	3 (38%)

The most frequent mutations were c.386G>C (p.W129S) which was present in five patients; c.1349C>T (p.T450M) which was present in five patients; c.528_529delinsAA (p. C176X) which was present in three patients; and c.673C>T (p.R225W) which was also seen in three patients. Overall, missense mutations were commonly found in FHL2 patients (48%) in our cohort.

In the 28 patients with FHL3, 30 different mutations with 18 novel variants were identified (**Table 2**). Homozygous mutations were seen 62% of FHL3 patients. Frameshift, intronic and splice site mutations were more frequently observed in our FHL3 patients (59%). The most frequent mutations were c.762delC (p.C255AfsX73) (frameshift deletion); and c.1822del (V608CfsX16) (frameshift deletion); and c.858+1G>A (intronic splice site mutation) which were present in two patients, respectively.

Also, in the six FHL4 and nine FHL5 patients, we identified five and eight different mutations, respectively (**Table 2**). c.173T>C (L58P) was found in two FHL4 patients. Homozygous intronic splice site variant 1247_1G>C in STXBP2 gene was observed in three FHL5 patients.

Genotype-Phenotype Correlation

Irrespective of FHL subtype, 53% patients harboring homozygous mutations presented below 1 year of age (median age of presentation was 10 months) while patients with compound heterozygous mutations had later onset of disease (median age of presentation was 3 years). Similarly, 12 patients with a monoallelic mutation in FHL genes had a median age of presentation of 10 months (**Figures 2**, **3A,B**).

Irrespective of the genetic background i.e., presence of homozygous/compound heterozygous/monoallelic mutations, FHL2 patients had perforin expression ≤10% except for patient P34 (with homozygous mutation) and patient P41 (with compound heterozygous mutation). P34 and P41 had 68 and 89% perforin expression on NK cells, respectively (**Figures 2A**, **3C**). However, in P41, mean fluorescence intensity (MFI) of perforin expression was very low (1.83) as compared to healthy control (19.83); indicating that not only expression but MFI of staining also has to be considered during interpretation of the results.

From 43 patients of FHL3/4/5 for whom mutation details were available; 32 patients had homozygous mutations in the corresponding genes (**Figures 2B**, **3D**). Seven patients of FHL3/4/5 had compound heterozygous mutations in the respective genes while the remaining four patients had a monoallelic mutation in the respective genes. Five out of these seven patients with compound heterozygous variants had CD107a degranulation on NK cells ≤10%, one patient P61 had borderline degranulation and one patient P69 had degranulation in the normal range. Additionally, two FHL3 patients (P62 and P63) with homozygous frameshift mutations had normal degranulation assay. Amongst the four patients with monoallelic mutations, CD107a degranulation could be performed in two patients and it was found to be abnormal.

Although majority of the patients had typical HLH manifestations, four FHL patients (three FHL2 i.e., P6, P7, P14, and one FHL5 i.e., P93) are identified with atypical clinical presentations like lymphoma, leukemia, or autoimmune diseases. All of these patients received treatment for the respective pathological conditions and then were diagnosed with HLH. As there was a delay in diagnosis of FHL due to the unusual clinical presentations, all these patients succumbed to the disease.

Treatment and Outcome

Of the 101 cases of FHL, 13 patients were lost to follow up and the outcome and remission status could be reported in 88 patients. Seven patients expired before starting the HLH treatment, while 51 patients expired even after receiving HLH-2004/94 protocol. Of the latter, 43 patients did not achieve remission and expired; another eight showed a relapse of the disease post-remission and could not be salvaged. Hematopoietic cell transplantation (HCT) being the definitive treatment, 18 patients underwent BMT. Thirteen are currently doing well while five patients expired due to post-transplant complications. Twelve patients are currently in remission and awaiting transplantation. The treatment and outcome of HLH patients in our study are summarized in **Table 3**.

DISCUSSION

FHL is a genetically heterogeneous disorder of immune dysregulation affecting the cytotoxic function of lymphocytes (3, 5). The pattern of FHL has been reported to vary significantly in different ethnic groups (8–10, 12), however, its prevalence in Indian population is not known. In this study, we have comprehensively evaluated clinical, immunological and molecular findings in 101 molecularly characterized FHL patients from 20 different centers of India. To the best of our

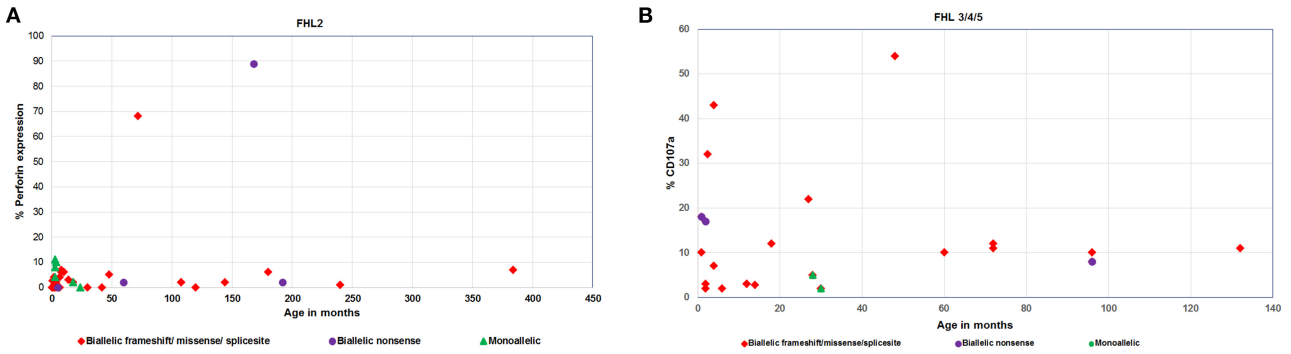

FIGURE 2 | (A) Perforin expression and **(B)** CD107a degranulation in FHL patients with different age categories and genetic background.

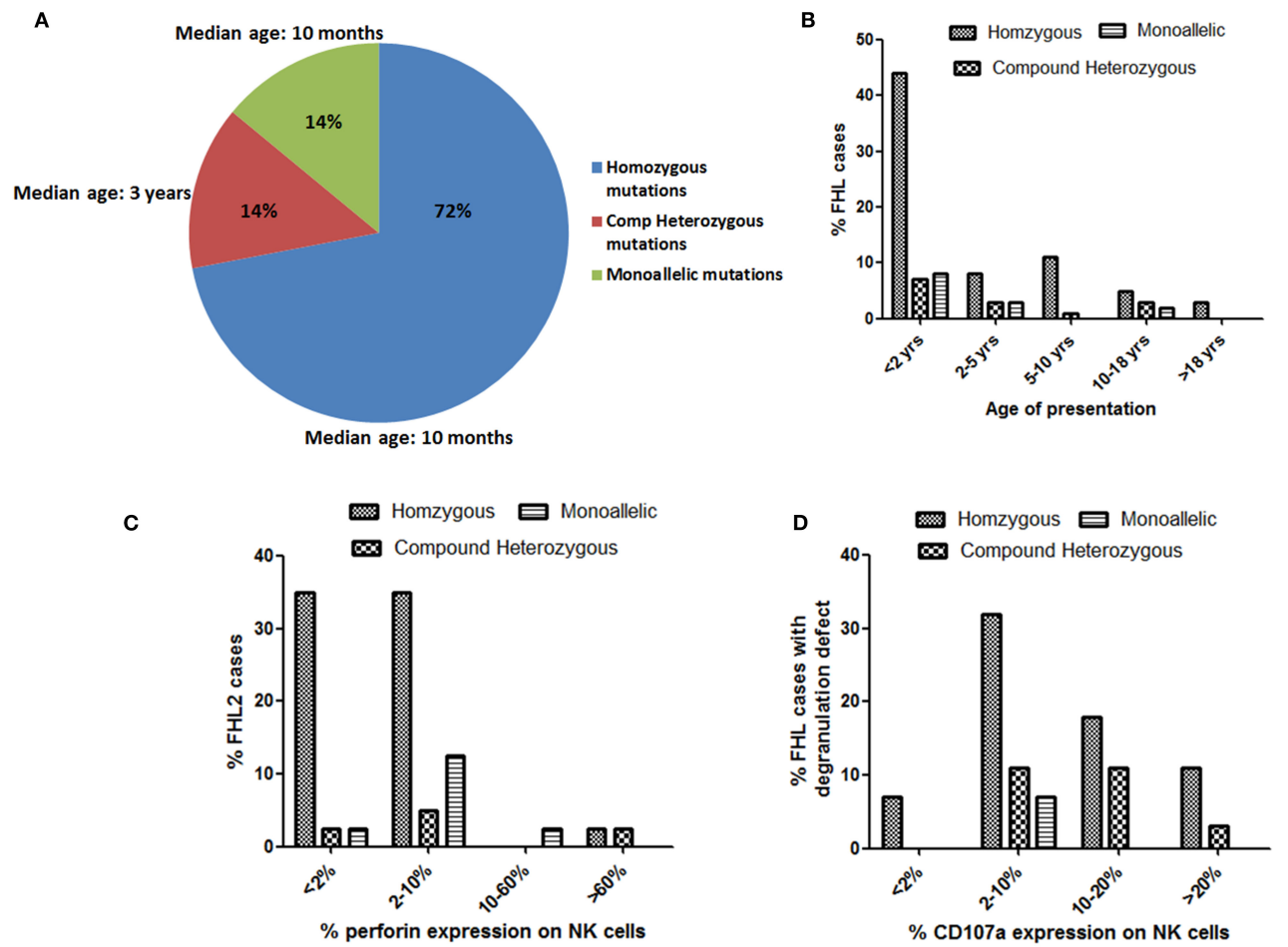

FIGURE 3 | Evaluation of age of presentation of disease **(A,B)**, Perforin expression **(C)** and CD107a expression **(D)** on stimulated NK cells in different groups of FHL patients with associated genetic profiles.

knowledge, this is one of the largest studies on the spectrum of FHL in Indian population. This study also evaluates the utility of flow cytometry based assays for efficient diagnosis of FHL patients.

In accordance with previous studies (1, 3, 9, 10, 13), 84% of our FHL patients were either FHL2 or FHL3 (50 and 35%, respectively); while the remaining 16% were FHL4 and FHL5 patients. Also as reported in various retrospective studies

TABLE 3 | Outcome of therapy seen in different FHL groups.

	FHL2 *n* = 50	FHL3 *n* = 35	FHL4 *n* = 7	FHL5 *n* = 9	Total *n* = 101-
Expired before receiving the protocol	6	0	0	1	7
Expired even after receiving the protocol	28	16	3	4	51
In remission while on protocol and awaiting transplantation	3	5	2	2	12
Well post-HCT	4	7	1	1	13
Expired post-HCT	3	2	0	0	5
Lost to follow-up	6	5	1	1	13

in other populations, in our cohort as well prolonged fever, hepatosplenomegaly, hemophagocytosis and hyperferritinemia were the most common clinical presentations (3, 9, 12, 14). CNS manifestations were more common in our FHL3 patients, which is consistent with that reported in literature (15, 16). Although FHL has widely been considered as a disease of infancy (3, 10, 14), 43% of our FHL patients had onset beyond 1year of age, with 24% of FHL2 patients presenting beyond 4 years of age and had severe disease. This suggests the importance of screening for underlying genetic defect in all HLH patients irrespective of their age.

As reported in previous literature (5, 12, 14), 22% of the patients in our cohort did not fulfill the Histiocyte society criteria (5) suggesting certain limitations of existing diagnostic criteria. Many patients may not meet these criteria early during the disease, and some patients may never meet criteria including those with atypical clinical presentations like isolated central nervous system disease (17–19). In some patients, information on levels of sCD25 or NK-cell cytotoxicity may not be available. So in such scenario, measurement of additional screening markers like flow cytometric detection of perforin, CD107a degranulation, T cell upregulation of HLA-DR, serum levels of CXCL9, IL18 are gaining attention for HLH diagnosis and differentiating HLH patients from patients with rheumatological diseases (20).

Nowadays, molecular analysis using targeted NGS or clinical WES is the preferred mode of FHL diagnosis (21). But such molecular diagnosis can be challenging in terms of cost, genetic heterogeneity, analytic difficulty, turnaround time and availability. Thus, in this scenario performing functional screening of cytotoxic cells by flow cytometry is complementary, as the results are available within 72 h. Currently, there are only two Centers of Excellence (COE) in India which provide Immunoassays like flow cytometry based detection of perforin expression and CD107a degranulation on NK cells. Immunoassays (at least one) could be performed in 70 patients in our cohort. In majority of the cases, results are available within 48 h except for the few traveled samples where additional time is required for transport. There are several peripheral centers which send the samples simultaneously for phenotypic and molecular analysis. In recent years, assays based on flow cytometry for evaluating NK cell and CTL cell functions are developed (6, 7, 22–24). In addition to cytotoxicity assays, quantification of perforin, SAP and XIAP proteins which are involved in granule-mediated exocytosis are useful in the diagnosis of primary HLH patients (22, 25–27). Along with NK cells, evaluation of CD107a degranulation on cytotoxic T lymphocytes (CTL) is recently reported in the diagnosis of FHL (28, 29). In our cohort, perforin expression and degranulation assay both had high specificity and sensitivity for distinguishing patients with HLH-associated mutations, as seen in previous studies (11, 23, 24).

Molecular characterization of patients in our cohort, revealed 39 novel variants (51%), highlighting the diverse molecular findings in Indian population compared to other populations reported in the literature (1, 12, 30, 31). The mutations identified in HLH-related genes viz. *PRF1*, *UNC13D*, *STX11,* and *STXBP2* genes were widely spread across the coding regions of the respective genes and no founder mutation could be identified in our population. As consanguinity is mainly seen (43%) in our Indian cohort, homozygous mutations in FHL genes are majorly found in the study.

The minimal or complete absence of perforin protein is commonly associated with the most detrimental *PRF1* gene mutations while, compound heterozygous *PRF1* gene missense mutations may encode partially active perforin and are predominantly identified in older patients with milder clinical manifestations (1, 32, 33). In our cohort, most patients (82.5%) harboring homozygous mutation had perforin expression <10% (**Figures 3B,C**). In two patients viz. P34 and P41, perforin expression was found to be 68 and 89%, respectively, and had later onset of disease. Patient P34 had c.136G>A (p.E46K) which is a reported missense mutation. This mutation leads to a defect in the MACPF domain of perforin protein which consists of ∼349 amino acids stretching in the middle of the protein and is thought to be involved in pore formation. In P41, though perforin expression was 89%, MFI of staining was low. This patient carried compound heterozygous mutation including missense mutation (c.1349 C>T; p.T450M) and nonsense mutation (c.1519G>T; p.E507X) affecting the C2 domain of perforin protein which is involved in Ca^{2+} and membrane binding. Both these patients had late onset of the disease (6 and 14 years, respectively) probably attributing to residual perforin expression. Despite the presence of perforin expression on NK cells and late-onset of disease, these patients presented with severe clinical manifestations. Mutations identified in these patients might be affecting the function or structure of perforin protein but not its expression, thus highlighting the fact that presence of a protein does not rule out its functional or structural defect. So, in case of high clinical

suspicion, molecular analysis along with functional assays should be considered.

All FHL2 patients had normal degranulation pattern except patient P27, P36, and P39. P27 was only 45 days old and abnormal degranulation pattern could be attributed to the immature immune system at this early age (34). Unfortunately, degranulation assay could not be repeated in any of these patients. Majority of the patients with a homozygous mutation in either *UNC13D* or *STX11* or *STXBP2* had either defective (≤10%) degranulation on stimulated NK cells (**Figure 3D**). Borderline NK degranulation (22, 18, 10, and 17%) was observed in patients 56, 59, 60, and 61, respectively. Three of these patients (56, 59, and 61) harbored novel frame-shift mutation in homozygous state in *UNC13D* gene leading to premature stop codon causing truncated protein formation while patient 60 harbored compound heterozygous missense mutation (one novel and one reported mutation) in *UNC13D* gene. Although these patients had degranulation ≥10%, it was lower than the healthy controls (22–54%). Thus, indicating that these mutations definitely had effect on the NK cell degranulation pattern. Amongst the FHL3 patients, three patients (62, 63, and 69) harboring novel frameshift mutations in *UNC13D* gene had NK cell degranulation within normal range. NK cell degranulation assay though has high sensitivity and specificity, it is a screening test and hence can have false negative results. Similar findings were observed in study by Bryceson et al. (11) wherein degranulation assay was evaluated for diagnosis of FHL and was found that 4% of the patients with genetically determined degranulation defects had NK cell degranulation >10% while 7% had above 20% (11). Thus, indicating that, in case of high suspension or family history, one has to go ahead with molecular analysis irrespective of the outcome of functional assays. Evaluation of parents' status in these patients and *in vitro* functional assays in future may help in assigning pathogenicity in such novel variants.

Interestingly, in our cohort 12 patients (eight FHL2, four FHL3) though harbored monoallelic mutations, had severe clinical manifestations. Phenotypic assays could be performed in nine patients and were abnormal in eight of them. Clinical follow up was available in seven patients. Six of them expired while one required HCT because of relapse and currently well. Of these patients, mutations in three patients (P76–P78) were identified by Whole exome sequencing thus ruling out the possibility of compound heterozygous mutations in these patients. Presence of monoallelic mutations has also been observed in previous studies and there is a growing evidence that monoallelic variants can also contribute to FHL (16). Though involved in the pathogenesis of the disease, monoallelic variants may not be sufficient to initiate the disease phenotype alone. Additional unidentified genetic defects or possibly even environmental factors may contribute to the development of HLH (2, 35). The digenic mode of inheritance (35) and deep intronic variants [c.118-308C>T, c.118-307G>A and 253-kb inversion] in UNC13D gene (36, 37) are described in FHL patients carrying monoallelic variants support this inference. The simple possible explanation is that the variants like promoter mutation, deep intronic variants and big deletions can be missed with the commonly used molecular techniques. Another possibility is that some patients (especially with degranulation defects) might possess pathogenic variants in additional genes that contribute to the development of HLH (35, 38). On the other hand, few monoallelic mutations may confer dominant-negative function to the encoded protein interfering with the cytotoxic function of lymphocytes although the exact mechanism and clinical relevance of these monoallelic mutations need to be explored (39).

Since cellular cytotoxicity plays a crucial role in immune surveillance and tolerance, it gives rise to an association of FHL with cancer, autoimmune susceptibility and other manifestations (16). In our cohort, we identified four FHL patients with atypical initial presentations, namely, two FHL2 patients with malignancy (P6 and P7), one FHL5 patient with ALPS (P93), and one FHL2 patient with isolated neurological relapse (P14). Particularly, isolated CNS manifestations in FHL can be challenging to diagnose (17, 40, 41). These findings highlight some of the atypical manifestations in FHL which may delay diagnosis in selected cases.

Management of HLH is mainly focused on treating the infectious trigger, suppression of hyperactive immune system and correction of an underlying genetic defect. Prompt initiation of immunosuppressive chemotherapy is essential for improved outcome. This can be achieved by increasing the awareness about the availability of immunoassays for diagnosis of HLH helping in early initiation of therapy and preparation for HCT. The underlying triggering infections must be extensively evaluated as the disease activity can be improved by managing the etiology. Some biomarkers like sCD25 levels and sCD163 levels are available at only in limited centers. Awareness to incorporate these biomarkers along with the fall in ferritin levels must be used actively in the management of these patients. In cases of refractory HLH, other novel therapies like Emapalumab or Alemtuzumab are recommended but they are currently not easily available in India (20, 42). In our study cohort, 63 patients expired either before initiating therapy or when on therapy or post-HCT. Thirteen patients are well post-HCT while twelve patients are in remission on HLH protocol and awaiting bone marrow transplantation. Two FHL2 patients having late onset of disease, relapsed when treatment was tapered and hence, emphasizing on the fact that even patients with adult-onset FHL need HCT for better outcome (13). Thus, though early initiation of treatment is necessary for the survival of HLH patients; HCT is the only curative therapy for patients with a known genetic defect and also for patients with recurrence/relapse of the disease despite adequate therapy (though the genetic cause is not known). HLA-matched sibling donor is the preferred donor for FHL patients due to the minimized risk of GVHD. However, various reports on variation in the age at onset within each family, case studies reporting the adult FHL patients and also symptomatic patients harboring monoallelic mutations add to the dilemma of choosing a donor for HCT (43, 44). The studies from our lab have shown that parents and siblings of few FHL2 patients were asymptomatic carriers for the respective mutations in a heterozygous state and had partial (<50%) perforin expression (unpublished data)

as has been reported previously (16). Thus, in such cases, the decision of whether to go ahead with HCT from these potential donors is a dilemma especially, in a country like India where transplantation procedures are often not feasible either due to unavailability of HLA-matched donor or the unaffordable cost of therapy.

Thus, to summarize, this is one of the largest studies of retrospective data on the clinical, immunological and molecular spectrum of FHL patients in Indian population. Availability of flow cytometry based assays and NGS have significantly improved the diagnosis and management of FHL patients. Both flow cytometry based assays and molecular studies are complementary to each other and aid in key management decisions. Forthcoming advances in FHL syndrome recognition to minimize delays in diagnosis and modified treatment and transplant approaches will continue to improve patient outcomes.

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 Institutional Ethics Committee (IEC) for human subjects of ICMR-National Institute of Immunohematology (NIIH). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SS and MKe analyzed the data and wrote the manuscript. RM and UB wrote the manuscript and obtained clinical details. MGu, ADa, JA, MKu, SSh, GH, PS, NJ, and NN performed the laboratory investigations. RV, SS-D, PKa, ADh, and PG involved the molecular investigations. PT involved in collection of samples and maintaining clinical details. VG, AP, RR, RU, RS, PKi, MS, DM, PV, AB, AR, AA, UP, MGi, AC, AS, DJ, NC, NS, FJ, SC, SK, BA, SSe, and ML supervised the clinical care and management of patients. MD and MM supervised the study and reviewed the manuscript. 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.2021.612583/full#supplementary-material

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Spectrum of Systemic Auto-Inflammatory Diseases in India: A Multi-Centric Experience

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Background: Systemic autoinflammatory diseases (SAID) are rare inherited disorders involving genes regulating innate immune signaling and are characterized by periodic or chronic multi-systemic inflammation.

Objective: To describe spectrum of clinical, immunological, molecular features, and outcomes of patients with SAID in India.

Methods: Request to share data was sent to multiple centers in India that are involved in care and management of patients with Inborn Errors of Immunity. Six centers provided requisite data that were compiled and analyzed.

Results: Data on 107 patients with SAID were collated—of these, 29 patients were excluded due to unavailability of complete information. Twelve patients (15%) had type 1 interferonopathies, 21 (26%) had diseases affecting inflammasomes, 30 patients (41%) had non-inflammasome related conditions and 1five patients (19%) had Periodic Fever, Aphthous Stomatitis, Pharyngitis, Adenitis (PFAPA). Type1 interferonopathies identified in the cohort included patients with Deficiency of Adenosine Deaminase 2 (*DADA2)* (six patients; five families); STING-associated vasculopathy infantile-onset (SAVI) (three patients, one family); Spondyloenchondro-dysplasia with Immune Dysregulation (SPENCD) (two patients). Diseases affecting inflammasomes include Mevalonate Kinase Deficiency (eight patients); Cryopyrin-Associated Periodic Syndromes (CAPS) (seven patients); NLR Family, Pyrin domain-containing 12 **(**NLRP12) (two patients); Familial Mediterranean fever (FMF) (two patients); Autoinflammation and $PLCG_2$-associated antibody deficiency and immune dysregulation (APLAID) (two patients). TNF receptor-associated periodic syndrome (TRAPS) (three patients); A20 haploinsufficiency (four patients); Deficiency of Interleukin 1 Receptor Antagonist (DIRA) (two patients) were categorized as non-inflammasome related conditions. There were significant delays in

diagnosis Corticosteroids and other immunosuppressive agents were used for treatment as anti-IL-1 drugs and other biological agents were and still are not available in India. Eight (16.3%) patients had so far succumbed to their illness.

Conclusions: This is the first nationwide cohort of patients with SAID from India. Clinical manifestations were diverse. Overlapping of clinical features with other relatively common rheumatological disorders often resulted in delays in diagnosis. More nationwide efforts are needed to enhance awareness of SAID among health care professionals and there is an urgent need to make targeted immunotherapies universally available.

Keywords: systemic autoinflamatory diseases, India, deficiency of adenosine deaminase 2, NOMID/CINCA, hyper IgD syndrome, A20 (TNFAIP3), inflammasome, Type I interferonopathies

INTRODUCTION

Systemic autoinflammatory diseases (SAID) are complex inherited disorders caused by defects in several genes regulating innate immune signaling and are characterized by periodic or chronic multisystem sterile inflammation (1–3).

The term "autoinflammatory disorders" was coined in 1999 by Daniel Kastner's group when they proposed a new group of immunological diseases (4). The paper described genetic background of familial Hibernian fever, and rechristened it as "TNF receptor-associated periodic syndrome (TRAPS)." It also linked it with previously described mutations in Pyrin (*MEFV)*gene that causes familial Mediterranean fever (FMF) (4–6). In 2010, Kastner et al. defined autoinflammatory diseases as "clinical disorders marked by abnormally increased inflammation, mediated predominantly by cells and molecules of the innate immune system with a significant host predisposition" (1, 7). Euro fever registry and Pediatric Rheumatology International Trials Organization (PRINTO) have also proposed classification criteria for different hereditary recurrent fever syndromes (8).

SAIDs can be monogenic and polygenic or multifactorial (9, 10). Monogenic SAID (e.g., TRAPS, FMF) follow Mendelian inheritance and result from pathogenic variants in a single gene. On the other hand, disorders such as systemic juvenile idiopathic arthritis, Periodic Fever, Aphthous Stomatitis, Pharyngitis, Adenitis (PFAPA) syndrome and Adult-Onset Still Disease have polygenic or multifactorial etiology. The 2019 International Union of Immunological Societies (IUIS) Expert Committee classified monogenic SAID into 3 major groups: Type 1 interferonopathies, defects affecting the inflammasome and non-inflammasome-related conditions (11).

Over the last 2 decades due to an increasing awareness and availability of high throughput genetic sequencing techniques, there has been an exponential increase in discovery of genes responsible for SAID (2, 12, 13). Further, molecular insights of these disorders have provided the basis for new therapeutic interventions leading to improved outcomes and long-term survivals. There is paucity of data on SAID from India with published literature comprising of only anecdotal case reports (14–21). In this manuscript we describe clinical features, molecular profile, treatment and outcome in patients with monogenic SAID from six centers in our country. This paper reports nationwide cohort on SAID.

PATIENTS AND METHODS

Centers supported by the Foundation for Primary Immunodeficiency Diseases (FPID), USA, and other institutions involved in care of patients with Inborn Errors of Immunity (IEI)across India were contacted to share details of patients with SAID on a template designed by lead author (DS). Data including demographics, prominent clinical manifestations, laboratory investigations, molecular results, treatment regimens, and long-term outcomes were collated on predesigned Microsoft Excel sheet. Findings of radiology and histopathology were also recorded. Participating centers included Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, North India (52 patients); Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow, North India (25 patients); Christian Medical College, Vellore (12 patients), Bai Jerbai Wadia Hospital for Children (BJWHC), Mumbai, West India (10 patients); and ASTER CMI Hospitals, Bengaluru, South India (seven patients), Lilavati Hospital and Research Center,Mumbai, West India (one patient).

DEFINITION OF SAID

Several definitions have been proposed for SAID (4, 8, 11, 22). For the purpose of this study we have used European Society for Immunodeficiencies (ESID) working group definition for the categorization of SAID. ESID has defined "unclassified autoinflammatory diseases" to be characterized by recurrent fever (temperature >38°C) having occurred on at least six occasions with exclusion of other known infective/inflammatory autoimmune disorders and documented evidence of increased inflammatory markers [erythrocyte sedimentation rate (ESR), C-reactive protein (CRP)], age of onset under 40 years and predominantly but not exclusively with systemic symptoms (23). In the present study all patients who fulfilled ESID working group definition and had molecular confirmation of monogenic SAID were included. Patients with polygenic SAID (e.g., systemic

juvenile idiopathic arthritis, chronic non-infectious osteitis) and infantile inflammatory bowel disease were excluded.

All patients were further classified into three subtypes according to 2019 IUIS classification for SAID (11). Coatamer complex one protein alpha subunit (COPA) syndrome was classified as Type 1 interferonopathy (24, 25). Patients without molecular confirmation of diagnosis and/or could not be classified in accordance with IUIS classification were also excluded. Some patients included in this series have been reported earlier and these have been duly cited (14, 17, 19, 26, 27).

MOLECULAR INVESTIGATIONS

Molecular analysis of patients for PGIMER, Chandigarh was performed at Pediatric Allergy Immunology Laboratory at PGIMER, Chandigarh or in collaboration with international centers, namely Center for Autoinflammatory Diseases and Immunodeficiency, Genoa, Italy (1three patients) and National Institutes of Health (NIH), USA (three patients). Measurement of plasma adenosine deaminase 2 (ADA2) activity in extracts of dried plasma spots was performed in the laboratory of Dr. Michael Hershfield at Duke University School of Medicine, Durham NC, USA (28).

LABORATORY INVESTIGATION AT PGIMER, CHANDIGARH

Molecular analysis of Nucleotide binding oligomerization domain 2 (*NOD* 2) gene in patients suspected to have Blau syndrome (11/14 patients) and Adenosine deaminase 2(*ADA2)* gene for Adenosine Deaminase 2 (ADA2) deficiency was performed in-house in Pediatric Immunology Laboratory, Advanced Pediatric Center by Sanger sequencing. Exon-4 of *NOD2* gene was amplified using specified oligonucleotide primers and results were analyzed using Codon Code Aligner software (Codon Code Corporation, Massachusetts, USA). Screening of hotspot region (exon 2) of *ADA*2 gene was also performed in patients clinically suspected to have Deficiency of Adenosine Deaminase 2 (DADA2).

Molecular analysis in most patients at other centers was carried out at commercial laboratories that use targeted gene panel by Next Generation Sequencing (NGS) techniques. Sanger sequencing was used to confirm the variants obtained by NGS.

RESULTS

Data on 107 patients with SAID were collated from various centers in India. Of these, 19 patients had to be excluded as molecular confirmation was not available. Ten patients with variants of unknown significance (VUS) in genes associated with SAIDs were also excluded if found inconsistent with clinical profiles or non-pathogenic based on predictive analysis tools. Remaining 78 patients (**Figure 1**) included Periodic Fever, Aphthous Stomatitis, Pharyngitis, Adenitis (PFAPA) (15 patients (19%) from PGIMER); type 1 interferonopathies (1two patients, 15%); diseases affecting inflammasomes (21 patients, 26%); and non-inflammasome related conditions (30 patients, 38%%). Clinical details of patients with PFAPA (1five patients) and Blau syndrome (14 patients) are not being presented in the current manuscript (Suri et al., manuscript in submission).

CLINICAL PROFILE OF PATIENTS WITH TYPE 1 INTERFERONOPATHIES

Type 1 interferonopathies identified in the cohort included patients with DADA2 (six patients; five families); STING-associated vasculopathy infantile-onset (SAVI) (three patients from one family); Spondylo enchondro dysplasia with Immune Dysregulation (SPENCD) (two patients) and Coatamer complex one protein alpha subunit (COPA syndrome) (one patient) (**Table 1**).

DADA2

Age of onset of symptoms in patients with DADA2 ranged from 5 months to 17 years while age at diagnosis ranged from 9 months to 48 years. All patients with DADA 2 were diagnosed and managed as polyartritis nodosa (PAN). Family history was contributory in three patients (patient no. 2, 3, and 5). Predominant clinical features included fever (4/5), recurrent stroke (3/5), vasculitic rash (3/5), and retinal changes (2/5). Patient one had presented with hypertensive stroke at 3.3 years of aging 1992 and had second episode at the age of 16 years in 2002. The diagnosis of DADA2 was established in 2018 after three decades of follow-up.

Patient no 3 was diagnosed to have central retinal artery occlusion. Inflammatory markers were persistently normal. His sister (patient two) was under treatment and follow up for PAN. She had presented with recurrent abdominal pain with perforation peritonitis and catheter angiography had revealed microaneurysms in mesenteric arteries and renal arteries (**Figure 2**). In view of family historyDAD2 was suspected and mutation in *ADA2* gene was detected. Establishment of diagnosis lead to stoppage of aspirin and commencement of anti-TNF agents.

SAVI

A 10-year-old girl (patient no. 7), previously reported (19) had presented with fever, polyarthritis, and interstitial lung disease (ILD). Initial diagnosis of juvenile idiopathic arthritis with ILD was considered. Younger sibling (patient no. eight) and father (patient no. nine) of index patient also had similar symptoms. Father gave history of gangrene of both lower limbs with amputation of right midfoot and left 2nd toe. Exome sequencing revealed pathogenic variant in Transmembrane protein 173 (*TMEM173)* gene confirming the diagnosis of SAVI.

SPENCD

A 13-year-old girl (patient no 10) had persistent pyrexia, decreased vision with bilateral optic atrophy, hypertensive stroke, seizures, and proteinuria. Investigations showed hypergammaglobulinemia and positive antinuclear antibodies (ANA) with elevated anti-double stranded DNA (dsDNA) but normal complements. Initial diagnosis of systemic lupus

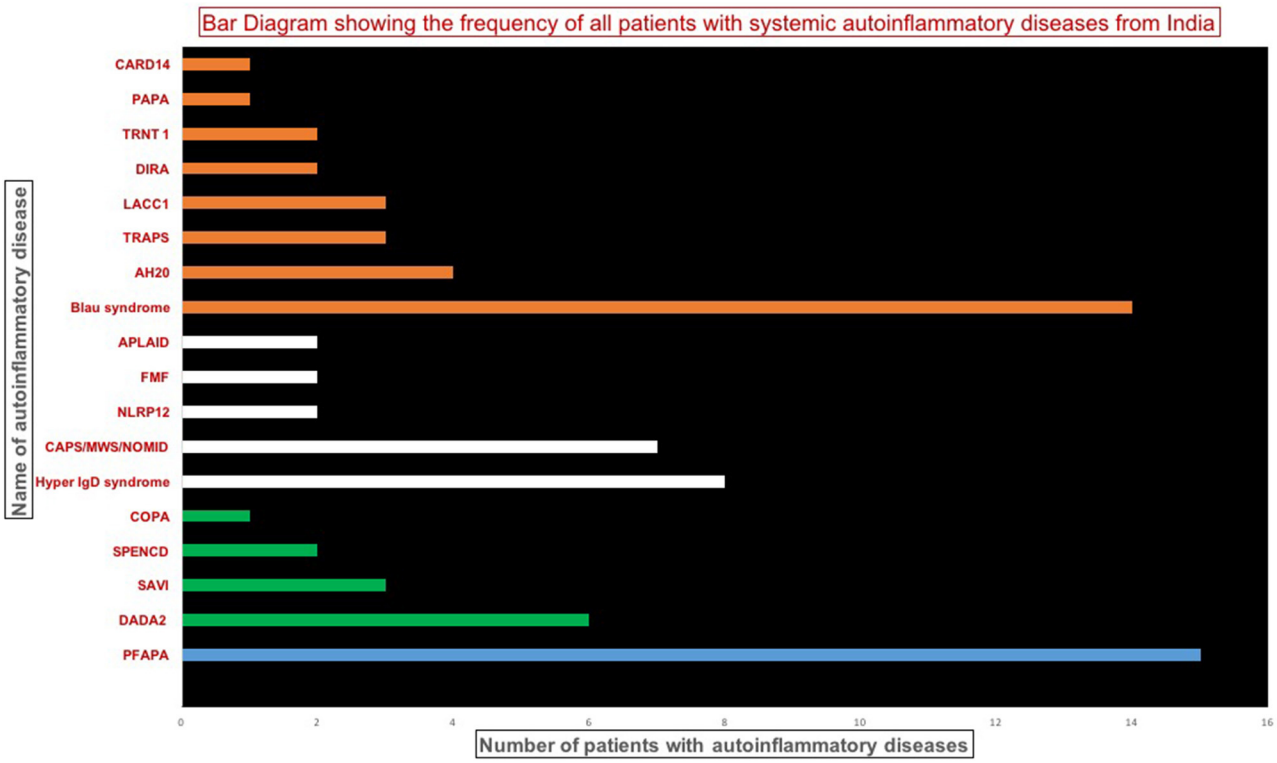

FIGURE 1 | Bar diagram showing the frequency of patients with various systemic autoinflammatory disease. DADA2, Deficiency of adenosine deaminase 2; SAVI, STING-associated vasculopathy infantile-onset; SPENCD, Spondyloenchondro-dysplasia with Immune Dysregulation; CAPS, Cryopyrin-Associated Periodic Syndromes; NLRP12, NLR Family, Pyrin domain-containing 12; FMF, Familial Mediterranean fever; APLAID, Autoinflammation and PLCG2-associated antibody deficiency and immune dysregulation; TRAPS, TNF receptor-associated periodic syndrome; HA20, A20 haploinsufficiency; LACC1, Laccase Domain Containing 1; DIRA, Deficiency of Interleukin 1 Receptor Antagonist; TRNT1, TRNA nucleotidyl transferase; PAPA, Pyogenic Arthritis; Pyoderma gangrenosum and Acne; COPA, Coatamer complex 1 protein alpha subunit (COPA) syndrome; CARD14, Caspase Recruitment Domain Family Member 14.

erythematosus was proffered. Renal biopsy revealed IgA nephropathy. Magnetic Resonance Imaging (MRI) brain showed basal ganglia calcifications. Exome sequencing revealed pathogenic variant in Acid phosphatase 5 (*ACP 5*)gene which was confirmed on Sanger sequencing.

A 4-year-old girl (patient no 11) had presented with bleeding manifestations (skin, mucosal and intracranial bleed) since infancy (**Figure 3**). She had steroid refractory anemia and thrombocytopenia with no autoantibodies and hypocellular bone marrow. She was later noted to have short stature and metaphyseal dysplasia along with bilateral basal ganglia calcification. Targeted gene panel revealed homozygous nucleotide deletion in exon 1 of *ACP5* gene.

COPA Syndrome

The index patient (patient no 12), previously reported (30) was diagnosed to have COPA syndrome when they had presented with rheumatoid factor positive deforming polyarthritis and interstitial lung disease. His father also had arthritis and had succumbed to progressive lung disease.

CLINICAL PROFILE OF PATIENTS WITH DEFECTS AFFECTING THE INFLAMMASOMES

Twenty-one patients were classified to have inflammasomopathies (n = 21, 26%). These included Mevalonate Kinase Deficiency (HyperIgD syndrome) (eight patients); Cryopyrin-Associated Periodic Syndromes (CAPS) (seven patients); NLR Family, Pyrin domain-containing 12 (NLRP12) (two patients); FMF (two patients); Autoinflammation and $PLCG_2$-associated antibody deficiency and immune dysregulation (APLAID) (two patients) (**Table 2**).

Hyper IgD Syndrome

Patients with Hyper Ig D syndrome (Patient no. 13–20) had onset of symptoms during infancy (15 days−1 year) with predominant clinical features being fever (7/8), rash (4/8), lymphadenopathy (4/8), hepatosplenomegaly (5/8), and anemia (4/8). Initial diagnosis of neonatal sepsis was considered in two patients (patient no. 13–14). Three patients were found to have the V377I Dutch founder variant and 2 had c.1129G>A variant which is fairly common in South India particularly Kerala (15).

TABLE 1 | Clinical manifestations, molecular profile, treatment, and outcome of patients with type I interferonopathies ($n = 12$).

Center	Patient (Age at diagnosis/ Sex)	Age of onset of symptoms	Clinical features	Laboratory features	Family history (Consanguinity/ siblings affected)	Initial Diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
Deficiency of Adenosine Deaminase 2 (DADA2) ($n = 6$)									
PGIMER	Pt. 1 (31y/M)	3.3y	• Fever • Rash • Hypertension • Recurrent strokes at 3 and 16 years of age	CRP: 32 mg/L ESR: 20 mm/h MRI brain: multiple infarcts right MCA territory and right posterior circulation CTA: microaneurysms in branches renal artery Muscle biopsy: healed arteritis	Third degree consanguinity	PAN	*ADA2* exon 2; c.140G>T; p.Gly47Val Homozygous; missense Previously reported: Yes	CS, AZR, enalapril, aspirin Change in treatment after diagnosis: Aspirin stopped, HCQs added and planned for ant-TNF	34 years and doing well
	Pt. 2 (13 y/F)	5 y	• Fever • Recurrent abdominal pain • Hypertension • Optic atrophy • Left hemiparesis and facial palsy intestinal perforation	CRP: 45 mg/L ESR: 40 mm/h DSA: multiple microaneurysms involving bilateral interlobar and segmental branches of renal artery, branches of gastroduodenal artery, distal branches of SMA and IMA GI Biopsy: Ulcer, ischemic, gangrene, perforation in ileum. Chronic inflammation in recto-sigmoid junction Plasma ADA2 activity: 1.1 mU/g protein mL Plasma ADA2 activity (Father): 42.5 mU/g protein mL Plasma ADA2 activity (Mother): 69.5 mU/g protein mL	Sister of Pt. 3	PAN	*ADA2* exon 2; c.139G>C; p.Gly47Arg Homozygous missense Previously reported: Yes	CS, CYC (10 pulses), AZR, aspirin Change in treatment after diagnosis: Aspirin stopped, HCQs added and planned for anti-TNF	8 year and doing well
	Pt.3 (18 y/M)	17 y	• Sudden Painless loss of vision • Raynaud phenomenon, CRAO	CRP: 10 mg/L ESR: 12 mm/h CTA: Normal study Plasma ADA2 activity: 0.3 mU/g protein mL	Brother of Pt. 2	PAN	*ADA2* exon 2; c.139G>C; p.Gly47Arg Same as the sibling (Pt. 2)	CS, CYC (6 pulses), AZR, aspirin, LMWH Change in treatment after diagnosis: Aspirin stopped, HCQs added and planned for anti-TNF	3 years and doing well
SGPGI	Pt. 4 (17 y/M) (29)	5 y	• Fever • Vasculitic ulcers • Seizures, • Recurrent stroke with neurological deficits • VIth Cranial Nerve palsy, median nerve neuropathy, • GI bleed	Skin Biopsy: Necrotizing cutaneous vasculitis	No	PAN	*ADA2* exon 2; c.139G>C; p.Gly47Arg; exon 2; c.278T>C; p.Ile93Thr Previously reported: Yes Homozygous missense variation	CS, AZR Change in treatment: anti-TNF commenced	1 year and doing well

(Continued)

TABLE 1 | Continued

Center	Patient (Age at diagnosis/ Sex)	Age of onset of symptoms	Clinical features	Laboratory features	Family history (Consanguinity/ siblings affected)	Initial Diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
	Pt. 5 (48 y/M) (29)	8 y	• Fever • Ulcers and rash • Recurrent stroke along with neurological deficits, mononeuritis multiplex, CRAO	CRP: 5.11 mg/L ESR: 30 mm/h C3/C4: 129/32.3		PAN	*ADA2* exon 2; c.139G>C; p.Gly47Arg Homozygous missense variation Previously reported: Yes	CS, MMF Change in treatment: Stopped aspirin	doing well
Aster CMI	Pt. 6 (0.9 y/F) (29)	5 months	• Fever • Anemia • generalized lymphadenopathy, splenomegaly	CRP: 102 mg/L ESR: 155 mm/h Bone marrow: Normocellular bone marrow with trilineage hematopoiesis IgG: 1,640 mg/dL IgA: 101 mg/dL IgM: 96 mg/dL IgE: 3.7 mg/dL	No	-	*ADA2* exon 2; c.139G>C; p.Gly47Arg Homozygous missense variation Previously reported: Yes	Injection etanercept	Doing well
STING-associated vasculopathy with onset in infancy (SAVI) (*n* = 3)									
PGIMER	Pt 7 (10 y/F) (19)	0.91 y	• Fever, • Failure to thrive, • Deforming inflammatory arthritis with contractures of small and large joints • ILD, corneal • Opacity in right eye	CRP: 97.23 mg/L ESR: 120 mm/h CT chest: ILD RA factor: positive ANA: 4+ RIM IgG: >2,535 (540–1,610) IgA: 436 (70–250) C3: 166 mg/dl (89–187) C4: 20 mg/dl (16–38) Anti ds-DNA: 10.8 IU/ml (<25-Negative) Serum IL-6: 3,700 pg/ml Serum IL-10: 13,900 pg/ml Interferon levels elevated	Brother and Father affected (Pt. 8 and Pt. 9)	JIA, COPA	*TMEM173* exon5; c.463G>A; p.Val155Met heterozygous missense variation Previously reported: Yes	CS, MTX, AZR, Naproxen, HCQ	Alive
	Pt 8 (3 y/M) (19)	2 y	• Fever • Polyarthritis (bilateral knee, small joints of the hands) • Rash ILD	CRP: 12.98 mg/L ESR: 108 mm/h CT chest: ILD RA factor: negative ANA: 2+ Speckled IL-6: 3,500 pg/ml IL-10: 14123 pg/ml Interferon levels elevated	B/o Pt. 7	JIA, COPA	*TMEM173* exon5; c.463G>A; p.Val155Met heterozygous missense variation Same as Pt. 7	AZR, MTX	Well

(Continued)

TABLE 1 | Continued

Center	Patient (Age at diagnosis/ Sex)	Age of onset of symptoms	Clinical features	Laboratory features	Family history (Consanguinity/ siblings affected)	Initial Diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
	Pt 9 (4y/-) (19)		• Deforming inflammatory polyarthritis involving small and large joints • ILD • Peripheral vascular disease of bilateral lower limbs with guillotine amputation of right midfoot and 2nd toe in the year 2008	ESR: 12 mm/h RA Factor: 6.49 mg/L ANA (IF): 3 + diffuse CT chest: Emphysematous changes and interstitial thickening in bilateral lungs consistent with ILD	F/o Pt. 7	RA	*TMEM173* exon5; c.463G>A; p.Val155Met heterozygous missense variation Same as Pt. 7	-	-
Spondyloenchondrodysplasia (SPENCD) (*n* = 2)									
SGPGI	Pt. 10 (15 y/F)	13 y	• Fever • Seizure • Stroke • Optic atrophy • Hypertensive • Short stature	MR brain: Basal ganglion calcification Renal biopsy: IgA nephropathy ANA–Positive Anti dsDNA: 67.5 IU C3/C4: 105 mg/dL/29.6 mg/dL IgG: 3,590 mg/dl IgA: 621 mg/dl IgM: 60.9 mg/dl	No	SLE	*ACP 5* exon 3; c.550C>T; p.Gln184* exon 4; c.740T>G; p.Leu247Arg	HCQs, antihypertensive drugs	NA
Lilavati Hospital	Pt. 11 (4y/F)	1y	• Fever • Bleeding (Skin, mucosal and intracranial) • Anemia • Facial dysmorphism (delay in motor and cognitive milestones, fronto-parietal bossing, hyperteleorism, low set ears	X-ray wrist: metaphyseal dysplasia CT brain: Symmetrical bilateral basal ganglion calcifications and gliotic area noted in left Parieto-Temporal area Bone marrow biopsy: hypercellular marrow with erythroid and megakaryocytic hyperplasia. Increased bone marrow fibrosis DCT ICT: strongly positive multiple antibodies Cold agglutinin: positive	No	Early onset Immune thrombocytopenia	*ACP 5* exon 1; c.136delc; p.R46Gfs*24 Homozygous nucleotide deletion Parents heterozygous for the same variant	Multiple packed cell transfusions and platelet transfusions IVIg, CS, dapsone, cyclosporine	Doing well

(Continued)

TABLE 1 | Continued

Center	Patient (Age at diagnosis/ Sex)	Age of onset of symptoms	Clinical features	Laboratory features	Family history (Consanguinity/ siblings affected)	Initial Diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
Coatomer protein complex subunit alpha (COPA) defect (*n* = 1)									
PGIMER	Pt. 12 (11 y/M) (30)	5 Y	Polyarthritis, ILD	CRP: 32 mg/L ESR: 23 mm/h HRCT: ILD IgG: 1,453 mg/dL IgA: 131 mg/dL IgM: 135 mg/dL RA, CCP: Positive ANA: 3+ speckled ANCA: negative	Father died due to progressive lung disease	Poly JIA	*COPA* exon 9 (intron 9-10 junction) c.841C>T, p.Arg281Trp Novel heterozygous splice-site mutation Sangers (PGI)	CS, MTX, HCQs	Alive

ACP5, Acid phosphatase 5; ANCA, Anti neutrophil cytoplasmic antibody; ADA2, Adenosine deaminase 2; ANA, Antinuclear antibodies; anti TNF, Tumor necrosis factor; Aster CMI, Aster CMI Hospital, Bengaluru, India; AZR, Azathioprine; BJWHC, Bai Jerbai Wadia Hospital for Children, Mumbai, India; CMC, Christian Medical College and Hospital, Vellore, India; CRAO, Central retinal artery occlusion; CRP, C-reactive protein; CS, Corticosteroids; CT, Computed tomography; CTA, Computed tomography angiography; CYC, intravenous pulse cyclophosphamide; DCT, Direct Coombs test; DSA, Digital subtraction angiography; ESR, Erythrocyte sedimentation rate; HCQS, Hydroxychloroquine; HRCT, High resolution computed tomography GI, Gastrointestinal; ILD, Interstitial lung disease; IMA, Inferior mesenteric artery; JIA, Juvenile idiopathic arthritis; Lilavati Hospital, Lilavati Hospital and Research Center, Mumbai, India; LMWH, Low molecular weight heparin; MCA, Middle cerebral artery; MRI, Magnetic resonance imaging; MTX, Methotrexate; MMF, Myocphenolate mofetil; PAN, Polyarteritis nodosa; PGIMER, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India; RA, Rheumatoid arthritis; SGPGI, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGI), Lucknow, India; SLE, Systemic lupus erythematosus; SMA, Superior mesenteric artery; TMEM173, Transmembrane protein 173; Y, years.

Cryopyrin-Associated Periodic Syndromes CAPS

In this cohort we report seven patients with CAPS caused by mutations in NLR family pyrin domain containing 3 (*NLRP3)* gene. All patients had been symptomatic since early infancy but there were significant delays in diagnosis. Age at diagnosis ranged from 15 months to 13 years. Most of these patients were initially diagnosed as JIA. Seven patients had classical phenotype of NOMID with infantile onset of fevers, urticarial rash, arthritis, and progressive deformities with bony overgrowths (**Figure 4**). Sensory neural hearing loss and headache was found in only1 patient. Of the seven patients, pathogenic variants in *NLRP3* gene were identified in four patients (patient no. 21–23, 27) while no mutation could be identified in patient no 24 on exome sequencing. Molecular studies of two patients (patient 25, 26) are awaited. Three patients (patient no 21,22, and 23) had developed amyloidosis when by the time diagnosis of CAPS was established and two. patients (patient no 21 and 23) succumbed to their illness. Drugs used for treatment included corticosteroids, thalidomide and colchicine as anti-interleukin 1 (anti-IL1) therapy was not easily accessible. 4/7 patients with CAPS died while 3 were alive at the time of this report. Patient no. 22 has been on thalidomide for 12 years which has resulted in normalization of inflammatory parameters but she continues to have significant growth retardation, deformities, and intermittent headaches.

NLRP12

Variants in NLRP12 gene were identified t in two patients (patient no 28, 29). Both children were symptomatic since early infancy. Patient no. 28 had presented with recurrent episodes of fever, and infections (skin and subcutaneous abscess, diarrhea, meningitis, pneumonia), arthritis, sensorineural hearing loss and hepatosplenomegaly while patient no. 29 in addition had urticarial rash, pustular skin lesions, and lymphadenopathy. Heterozygous mutation in exon 3 in NLR family pyrin domain containing12 (*NLRP12)* gene was identified. Corticosteroids were used for treatment in patient 28 and is currently well.

FMF

Clinical profile of patients (Patient no 30, 31) with variants in *MEFV* gene is summarized in **Table 2**. Patient no. 30 had presented with periodic fever, rash, and abdominal pain. Targeted panel revealed variants of unknown significance in *MEFV* gene and Phospholipase C Gamma 2 (*PLCG 2)* gene. The patient is doing well on colchicine.

Nine months old boy (patient 31) had presented with recurrent oral ulcers. In view of family history of oral ulceration exome sequencing was performed. Heerozygyous issensense mutation in MEFV gene was identified. Symptomatic improvement has been noted after initiation of colchicine.

APLAID

Clinical profiles of patients no. 32, 33 with *PLCG2* variants are summarized in **Table 2**. Patient no. 32 had erythematous macular

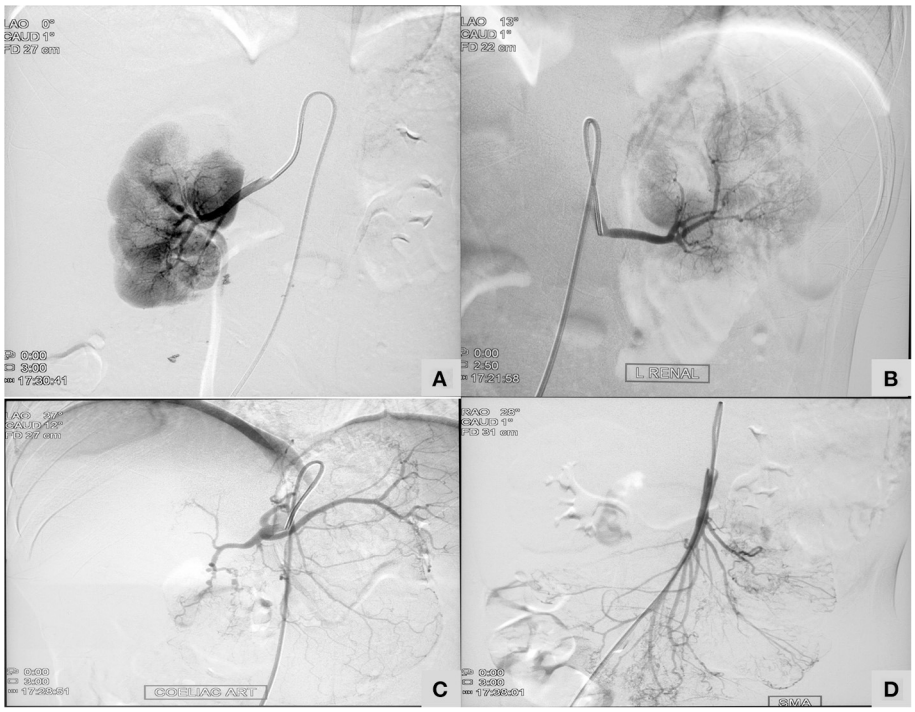

FIGURE 2 | A 13-year-old girl with Deficiency of Adenosine Deaminase 2 (**Table 1**; Patient two) showing micronaeurysm on intervation catheter angiography in bilateral renal arteries **(A,B)**, celiac artery **(C)**, and superior mesenteric artery **(D)**.

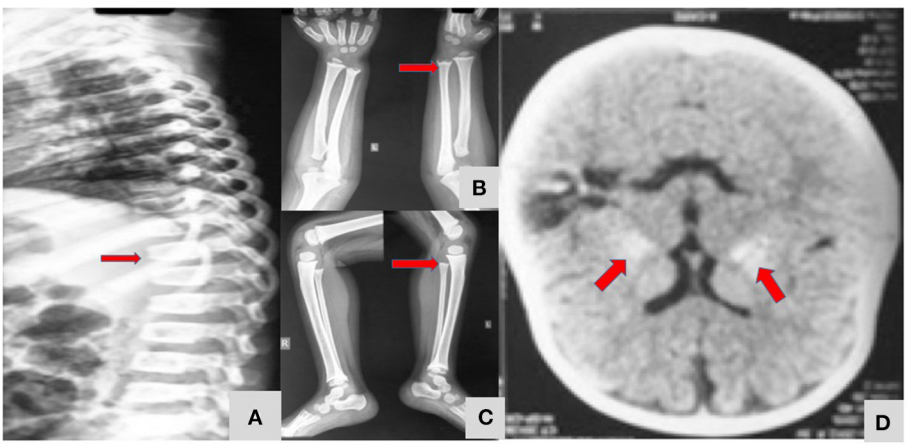

FIGURE 3 | A 4 year-old-girl with Spondyloenchondrodysplasia (**Table 1**; Patient 11) showing fish mouth vertebra **(A)**, metaphyseal dysplasia of long bones of upper and lower limbs **(B,C)**, and bilateral symmetrical basal ganglia calcification **(D)**.

rash (**Figure 5**), large joint arthritis, episodes of intussusception along with recurrent sinopulmonary infections. A *de-novo* heterozygous missense mutation in exon 22 of *PLCG2* gene that resulted in substitution of serine by asparagine at codon 798 (pAsn798Ser), was validated using Sanger sequencing. The Asn798Ser variant has a minor allele frequency of 0.08, 0.07, and 0.16% in the 1,000 genomes, ExAC and internal databases, respectively. The *in-silico* predictions of the variant were found damaging by PolyPhen-2 (HumDiv), damaging by Sorting Intolerant from Tolerant (SIFT), likelihood ratio test (LRT) and Mutation Taster 2.

Patients no. 33 had scaring photosensitive rash and a provisional diagnosis of Kindler syndrome was made (Mahajan et al. manuscript in submission). He was also detected to have same mutations in *PLCG2* gene as patient no. 31. Both these patients were unrelated and belonged to different ethnic backgrounds. They had multiple relapses and both succumbed to their illness.

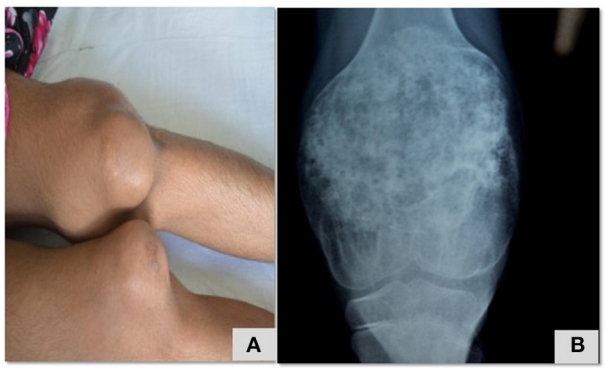

FIGURE 4 | Swelling of knee joint with enlarged, deformed femora and patellae due to overgrowth arthropathy **(A)**, heterogeneously calcified tumor-like protrusions originating from the growth plate **(B)** in a child with NOMID (**Table 2**; patient 22).

CLINICAL PROFILE OF PATIENTS WITH NON-INFLAMMASOME RELATED CONDITIONS

Thirty patients (38%) in our cohort were grouped under non-inflammasome related conditions that included TNF receptor-associated periodic syndrome (TRAPS) (three patients); Deficiency of the Interleukin 1 Receptor Antagonist(DIRA) (two patients); Pyogenic sterile Arthritis, Pyoderma Gangrenosum, Acne syndrome (PAPA) (one patient); A20 haploinsufficiency (four patient); CCA-adding transfer RNA nucleotidyl transferase (TRNT1) (two patients); Caspase recruitment domain-containing protein 14 (CARD 14) (one patients); and Laccase Domain Containing 1(LACC1) (three patients from one family) (**Table 3**). Patients with Blau syndrome (14 patients are not being presented in this paper (Suri et al., manuscript in submission). Patient with LACC1 has also been reported previously (27) (**Table 3**).

TRAPS

Patient no. 34 had recurrent episodes of fever lasting for 2–3 weeks every 3–4 months with rash since 18 months of age. These f episodes were associated with pain abdomen, myalgias, arthritis, periorbital edema (**Figure 6**), and subcutaneous swellings. She had received multiple courses of antimicrobials in view of marked polymorphonuclear leukocytosis. Her father was also symptomatic and used to have fever and intermittent subcutaneous swelling and rash. Father was diagnosed to have acute rheumatic fever in childhood. In view of periodic fever, with systemic manifestations and family history suggestive of autosomal dominant disorder, diagnosis of TRAPS was proffered and confirmed on exome sequencing. She was initially managed with corticosteroids followed by injection etanercept. She remains well at follow up.

A 45 years old female (patient no. 35) who had been under follow-up of Dermatology services was diagnosed to have pustular psoriasis since early adolescence. She would have intermittent flares with rash, fever and arthritis. I Her disease was refractory to methotrexate, cyclosporin and corticosteroids. In view of recurrent episodes of fevers, markedly elevated inflammatory parameters, sterile neutrophilic infiltrates on skin biopsy, a possibility of autoinflammatory disease was considered. Whole exome sequencing revealed variant in TNF Receptor Superfamily Member 1A(*TNFRSF*1A) mutation. She could not be initiated on biological agents due to financial constraints and succumbed to her illness 2 years after diagnosis was established.

A 10-year-old boy (patient no 36) had presented with high grade fever without focus. He reported having febrile episodes lasting for 3–4 weeks with variable afebrile periods since early childhood. These episodes were associated with rash over the trunk and limbs, myalgia and limp, abdominal pain, vomiting, and periorbital swelling. The inflammatory parameters were elevated and targeted panel for autoinflammatory diseases confirmed the diagnosis of TRAPS. The patient demonstrated partial response to etanercept which was changed to tocilizumab to which he responded well.

DIRA

Patient no 37 as has been previously reported (26), was the first Indian patient with large deletion in Interleukin 1 Receptor Antagonist (*IL1RN*) gene. She is doing well on Anakinra at 6 years of follow-up supported by National Institutes of Health, USA.

Patient no 38 had presented at day 7 of life with paucity of movement of both upper limbs. Inflammatory parameters were increased with sterile blood cultures. X-rays showed bilateral humerus, rib and clavicular involvement. He was treated with oral prednisolone 2 mg/Kg with slow tapper over 4 months. He responded dramatically and bone lesions healed. He developed pustules at follow up. Deletion in IL1RN gene as in patient no 37 was not detected on Western blot analysis. Results of whole exome sequencing are awaited. He is currently doing well and off corticosteroids. However, ESR remains elevated.

PAPA Syndrome

A 4-years old girl (patient no 39) had been unwell for 2.5 years when she presented with periodic fevers associated with painful oral ulcers, abdominal pain with hematochezia and colitis. Over the years, she developed multiple pyoderma gangrenosum lesion over extremities, angle of mouth and gluteal region that caused complete destruction of left cheek and lower lip. The lesion were difficult to heal and resulted in fistulae formation. She was initially suspected to have inflammatory bowel disease and oral prednisolone and azathioprine were initiated. Injection infliximab (3 doses) were also commenced. There was partial response in skin lesions and colitis initially. However, lesions reoccurred, and she succumbed to her illness.

A20 Haploinsufficiency

Patient 40 was 2 years old when she had presented with recurrent oral ulcers and genital ulcers (**Figure 7**). She had colitis, refectory ulcers requiring repeated hospitalization. Markers of inflammation were elevated, and Human Leucocyte Antigen 51 (HLA B 51) allele was detected. Considering a possibility of Bechet's disease, she was commenced on corticosteroid and azathioprine. At 5 years, she was readmitted with persistent

TABLE 2 | Clinical manifestations, molecular profile, treatment, and outcomes of patients with defect affecting the inflammasome ($n = 21$).

Center	Patient (Age of diagnosis (years)/sex)	Age of onset of symptoms (months)	Clinical features	Laboratory features	Family history (Consanguinity/ Family history)	Initial diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
Hyper IgD Syndrome/Mevalonate Kinase Deficiency (MVK) ($n = 8$)									
PGIMER	Pt. 13 (1.33 y/M)	2 months	• Fever • Jaundice • Cholestatic • hepatosplenomegaly anemia • Failure to thrive	CRP: 290 mg/L ESR: 110 mm/hr	Yes (Younger brother of patient 14)	Neonatal cholestasis with sepsis	*MVK* exon 9; c.803 T>C; p.Ile268Thr exon 10; c.976G>A; p.Gly326Arg Missense (phase unknown)	Thalidomide	Alive intermittent episodes of fever present
	Pt. 14 (4.5 y/M)	2 months	• Fever • Jaundice • Anemia,generalized lymphodenopathy, hepatosplenomegaly • Failure to thrive	CRP: 56 mg/L ESR: 38 mm/hr	B/o Pt 13	Sepsis	*MVK* exon 9; c.803 T>C; p.Ile268Thr exon 10; c.976G>A; p.Gly326Arg Missense Same as Pt. 13	Thalidomide	Alive and well
	Pt. 15 (3.5 y/F)	6 months	• Polyarthritis (wrist, elbows, knee), • abdominal pain, Diarrhea, colitis • Anemia, hepatosplenomegaly, generalized lymphadenopathy • Global developmental delay	CRP:160 mg/L ESR:109 mm/h Bone marrow biopsy: Dyserythropoiesis with lymphoid aggregates Gut biopsy: acute on chronic inflammation IgG: 2,079 mg/dL IgA: 303 mg/dL	3rd degree consanguinity, no similar illness in family	JIA/Blau/IBD arthritis	*MVK* exon 6; c.546G>T; p. Leu182Phe Homozygous, Missense	CS, MTX, AZA	Alive and well
SGPGI	Pt. 16 (15 y/M) Ref (14)AM	3 months	• Fever • Rash • Arthralgia • Pleuritis • Peritonitis • Hepatosplenomegaly, generalized lymphadenopathy	CRP: 80 mg/L ESR: 90 mm/hr IgG: 1,465 mg/dL IgA: 1,166 mg/dL IgM: 58.6 mg/dL IgD: 938 mg/dL	Sibling of Pt 17	AID ? HIGD syndrome	*MVK* Exon 11 c.1129G>A p.V3771	NSAIDs Change in treatment: DMARDs stopped	NA
	Pt. 17 (11y /M) (14)AM	2 months	• Fever • Rash • Arthralgia • Hepatosplenomegaly, generalized lymphadenopathy • Peritonitis, adhesions on laparotomy	IgG:1377mg/dL IgA:633mg/dL IgM:119.1mg/dL IgD: 1363mg/dL	Sibling of Pt 16	AID ? HIGD syndrome	*MVK* Exon 11 c.1129G>A p.V3771	NSAIDs Change in treatment: DMARDs stopped	NA

(Continued)

TABLE 2 | Continued

Center	Patient (Age of diagnosis (years)/sex)	Age of onset of symptoms (months)	Clinical features	Laboratory features	Family history (Consanguinity/ Family history)	Initial diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
BJWHC	Pt. 18 (3 y/M)	12 months	Fever Petechial rash Recurrent cervical adenitis Sinusitis Hepatosplenomegaly	-	-	-	*MVK* exon 2; c.10G>T; p.Glu4Ter (this is novel) exon 11; c.1129G>A; p.Val377Ile Het/AR (this is already known as common Dutch founder variant)	NA	Doing well
	Pt. 19 (0.91 y/M)	15 days	• Fever • Rash Failure to thrive • Dactylitis • Perianal abscess • Otomyocosis	IgG: 2,400 mg/dL IgA: 159 mg/dL IgM: 341 mg/dL CD3: 3,724 CD19: 1,375 CD56: 516 NBT: Normal (98%)	No	PID	*MVK* Exon11; c.1097A>G; Asp366Gly Novel and homozygous Not published	CS	NA
CMC Vellore	Pt. 20 (1y/F)	NA	Recurrent infections Fever Anemia Failure to thrive	IgG: 520mg/dL IgA: 43mg/dL IgM: 39mg/dL TG and Ferritin: increased Fibrinogen: normal Coombs: 1+ NBT: normal	NA	NA	*MVK* Exon7; c.644G>A; p.Arg215Gln Homozygous	NA	NA
Cryopyrin-Associated Periodic Syndromes (CAPS)/ Muckle –Wells Syndrome (MWS)/Neonatal-Onset Mutisystem Inflammatory Disease (NOMID) (*n* = 7)									
PGIMER	Pt. 21 (10 y /F) (31)AM	1 month	• Recurrent urticarial rash • Arthritis (ankle and wrist) • hypertension, • conjunctivitis, opticatrophy, • nephrotic range anasarca, proteinuria, • hypothyroidism • CSVT	CRP: 19.5 mg/L ESR: 51 mm/hr Renal biopsy: AA Amyloidosis IgG: 623 mg/dL; IgA: 253mg/dL IgM: 282 mg/dL	No	Atypical nephrotic syndrome	*NLRP3* (exon 3; c.1055C > T; p.Ala352Val) Substitution	CS, thalidomide, enalpril, amlodipine	Died due to amyloid associated renal failure
	Pt. 22 (13 y//F)	Infancy	• Fever • Rash • Arthritis with bony overgrowth • Headache, • Short Stature	CRP: 60 mg/L ESR: 98 mm/hr FNAC, abdominal fat pad, amyloidosis	-	Systemic JIA	*NLRP3* exon 3; c.913G>C; p.Asp305His	CS, thalidomide	Alive

(Continued)

TABLE 2 | Continued

Center	Patient (Age of diagnosis (years)/sex)	Age of onset of symptoms (months)	Clinical features	Laboratory features	Family history (Consanguinity/ Family history)	Initial diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
	Pt. 23 (11 y/M)	NA	• Fever • Arthritis • Amyloidosis, • renal failure	CRP: 58 mg/L ESR: 89 mm/hr FNAC, abdominal fat pad, renal biopsy, amyloidosis	-	Systemic JIA	*NLRP3* exon 3; c.1792C>T; p.Thr349Ile	CS	Died due to amyloid associated renal failure
	Pt. 24 (6.5 y/M) (32)AM	18 months	• Fever • Erythematous macular non itchy rash, later painful nodular • Seizures with meningitis, • SNHL	CRP: 65 mg/L ESR: 96 mm/hr Skin panniculitis, non specific perivascular dermatitis MR brain: Bilateral Bilateral cerebellar atrophy with mild hydrocephalus	No	Tubercular meningitis	*NLRP3* genetic screening negative for all exons	CS, thalidomide	Died
SGPGI	Pt. 25 (4 y/F)	Since birth	• Fever • Arthritis • Urticaria • Knee flexion contractures • Short stature • Hepatosplenomegaly	CRP:11.7 mg/L ESR: 30 mm/hr	No	Oligo JIA, NOMID	Mutation screening under process	Colchicine	Doing well
	Pt. 26 (5 y/M)	Since birth	• Fever • Arthritis • Urticaria, • Lymphadenopathy, hepatosplenomegaly	CRP:12 mg/L ESR: 90 mm/h IgG:1,590 mg/dL IgA: 275 mg/dL IgM: 109 mg/dL IgE: 409.8 mg/dL	No	NOMID	Mutation screening under process	Colchicine	Doing well
BJWHC	Pt.27 (1.33 y /M)	D1 of life	• Fever • Urticarial rash • Hepatosplenomegaly • Hypertelorism • Macrocephaly • Delay in cognitive milestones	CRP: 10 mg/L ESR: 140.5 mm/h MRI brain: Mild cerebral atrophy with dilated lateral ventricles and cisterns IgG: 1,472 mg/dL IgA: 124 mg/dL IgM: 181 mg/dL IgE: 785 mg/dL	No	AID	*NLRP3* exon 4; c.2263G>A/G>C; p.Gly755Arg	CS, NSAIDs	Died
NLR Family Pyrin Domain containing 12 (NLRP12) ($n = 2$)									
SGPGI (17)2)	Pt. 28 (4 y/F)	Since birth	• Fever • Diarrhea • Pneumonia • Arthritis	CRP: 54 mg/L ESR: 34 mm/hr	No	PID	*NLRP12* exon 9; c.2935A>G; p.Ser979Gly published	CS	NA

(Continued)

TABLE 2 | Continued

Center	Patient (Age of diagnosis (years)/sex)	Age of onset of symptoms (months)	Clinical features	Laboratory features	Family history (Consanguinity/ Family history)	Initial diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
			• Cervical lymphadenopathy and hepatosplenomegaly, • Skin pustules,subcutaneous abscess • Meningitis, SNHL	Gut biopsy: cryptitis with occasional crypt distortion NBT: Normal CD3, CD19, CD56: Normal					
	Pt. 29 (1 y/M)	1 month	• Fever • Urticarial rash, bullous eruptions over fingers, pustular skin lesion • Cervical and axillary lymphadenopathy	CRP: 72 mg/L ESR: 103 mm/hr USG: synovial thickening of joint and both radiocarpal joints. C3/C4: 1.73 mg/dL /0.25 mg/dL	-	AID	*NLRP12* exon3; c.779C>T; p.Thr260Met Heterozygous VUS Not published	CS	Well
Familial Mediterranean Fever (FMF) (*n* = 2)									
BJWHC	Pt. 30 (0.91y/F)	4 months	Fever Irritability Maculopapular rash Recurrent abdominal pain Hepatomegaly	CRP: 39 mg/L ESR: 53 mm/hr CD3: 4,084 CD19: 2,106 CD56: 128 NBT: 97%	No	AID	*PLCG2* exon 2;c.62C>T; p.Ala21Val het/AD/VUS het/AD/VUS *MEFV* exon 2; c.464G>C; p.Arg155Thr) Het/AD/VUS Not previously published. This is a non-confirmatory variant as per new Eurofever/ PRINTO classification criteria	Colchicine	Doing well
PGIMER	Pt. 31 (1.66 y/M)	9 months	Oral ulcers	CRP: 1.87 mg/L ESR: 37 mm/hr TH17/STAT3: reduced IgE: Normal NBT: Normal CD3, CD19, CD56,CD4, CD8: Normal	N/Yes (oral ulcers in father; Not Screened)	PID (TH17/PSTAT1 defects)	*MEFV* exon 10; c.2177T>C; p.Val726Ala heterozygous, missense. This is a non-confirmatory variant as per new Eurofever/ PRINTO classification criteria	Fluconazole Colchicine	Alive

(Continued)

TABLE 2 | Continued

Center	Patient (Age of diagnosis (years)/sex)	Age of onset of symptoms (months)	Clinical features	Laboratory features	Family history (Consanguinity/ Family history)	Initial diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
PLCG2 associated antibody deficiency and immune dysregulation (APLAID) (*n* = 2)									
PGIMER	Pt. 32 (9 y/F)	24 months	• Generalized Erythematous macular rash, bilateral knee and elbow arthritis, two episodes of intussception, otitis media • Pneumonia, • Recurrent vaginal bleeding • Short stature	HRCT: bilateral hyper inflated lung with fibrotic changes Skin Biopsy: non-specific perivasculitis with no immune deposits ANA: 2+ speckled and nucleolar C3/C4: 73 mg/dL/ <8mg/dL CH50: 166% (69–129) IgG: 1,683 mg/dL(540–1,610) IgA: >594mg/dL (50–240) IgM: 117 mg/dL(50–180) IgE: >10,000 IU/mL CD3: 51.8 (55–78) CD19: 40.89 (10–31) CD56: 3.11	Younger male sibling expired at 9 months, diarrhea, necrotic skin rash	SLE	*PLCG2* exon 22; c.2393 A>G; pAsn798Ser heterozygous, missense	CS, thalidomide, AZA	Died
	Pt. 33 (3 y/M)	4 months	• Fever • Rash (multiple supportive lesions, erythematous plaques pustular lesions, alopecia) along with scars • Photosensitivity • Flexural contractures at small joints of hand, claw hand • Bilateral corneal epithelial defect with corneal ulcers and corneal opacity • Phimosis	CRP: 70 mg/L ESR: 100 mm/hr Skin Biopsy: Epidermis shows Epidermis shows hyperkeratosis, focal neutrophilic crust over stratum corneum, basal cell vacuolation, perivascular infiltrates ANA: Negative IgA: >595 mg/dL IgM: 89 mg/dL IgE: 8,856 mg/dL CD3/CD19/CD56: normal NBT /TH17/STAT3: normal	No	Kindler syndrome, hyper IgE syndrome	*PLCG2* exon 22; c.2393 A>G; pAsn798Ser heterozygous, missense	CS, MTX, IVIg	Died

AID, Autoinflammatory disorder; ANA, Antinuclear antibodies; AZR, Azathioprine; BD, Behcet disease; BJWHC, Bai Jerbai Wadia Hospital for Children, Mumbai, India; CMC, Christian Medical College and Hospital, Vellore, India; CRP, C-reactive protein; CS, Corticosteroids; CSVT, Cerebral sinovenous thrombosis; CT, Computed tomography; CTA, Computed tomography angiography; CYC, intravenous pulse cyclophosphamide; ESR, Erythrocyte sedimentation rate; FNAC, Fine needle aspiration cytology; HCQS, Hydroxychloroquine; Ig, Immunoglobulin; JIA, Juvenile idiopathic arthritis; MRI, Magnetic resonance imaging; MTX, Methotrexate; MMF, Myocphenolate mofetil; MVL, Mevalonate kinase; NBT, Nitroblue tetrazolium; NLRP3, NLR family pyrin domain containing 3; NLRP12, NLR family pyrin domain containing 12; PID, Primary immunodeficiency disorder; PGIMER, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India; PLCG2, Phospholipase C gamma 2; Ref, Reference of previously reported paper; SGPGI, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGI), Lucknow, India; SNHL, Sensory neural hearing loss; TG, Triglycerides; Y, years.

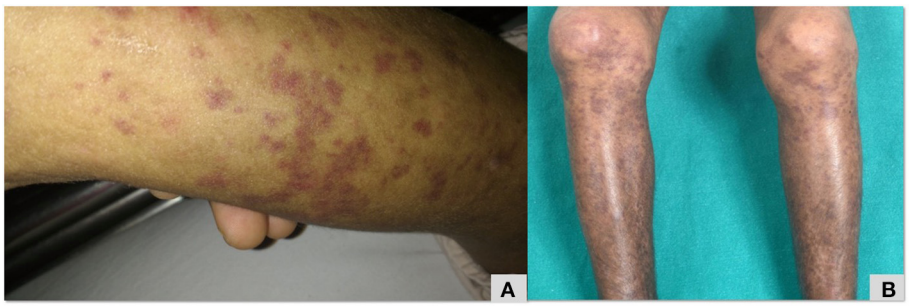

FIGURE 5 | Maculopapular erythematous rash over lower limb **(A,B)** that was initially diagnosed as Henoch Scholein purpura in a patient with PLCG2 (**Table 2**; patient no 32).

headache, blurring of vision and relapse of oro-genital ulcers. She had papilledema and magnetic resonance imaging (MRI) of brain revealed type 2 Arnold Chiari malformation. When younger brother also developed recurrent oral ulcers, genetic studies were performed in the index patent and targeted panel revealed a novel variant in Tumor necrosis factor alpha-induced protein 3 (*TNFAIP3)* gene (**Table 3**). She is doing well on follow up. Mother is carrier for the same variant while the younger sibling does not carry this variant. Patient 41 had also presented with early onset inflammatory bowel disease and oral ulcers and a novel variant in *TNFAIP3)* gene was found.

TRNT1 Deficiency

Patient no. 44, had presented with recurrent fever, and diarrhea. Two elder brothers earlier had died. He was evaluated for primary immune deficiency and was noted to have pan hypogammaglobulinemia. A provisional diagnosis of X-linked agammaglobulinemia was made and intravenous immunoglobulin replacement initiated. Exome sequencing revealed a compound heterozygous mutation in exon 2 of *TRNT1* gene. Similarly, patient no. 48 also had recurrent infections, bronchiectasis and hypogammaglobulinemia.

CARD 14: Patient 46had early onset difficult to treat psoriasis and was found to have mutations in CARD 14 gene.

DISCUSSION

SAID were first recognized in 1999 (4). Over the last two decades, knowledge and recognition of SAID has grown at an unprecedented speed and there have been a plethora of publications on this subject (3, 12, 13, 22). Significant improvement in understanding of genetic and pathogenic mechanisms of SAIDs has resulted in remarkable progress in their management. However, the darta from India is limited (14–18, 26, 27, 33–40). There is no national registry for SAID and there is lack of knowledge on nationwide burden of these diseases. This manuscript is the first attempt to collate data from various centers involved in care of patients with SAID and highlight diagnostic difficulties, treatment, and outcomes of such patients in India.

SAID display wide range of clinical manifestations and can affect almost every organ system. They are uncommon and difficult to diagnose clinically. Overlapping clinical features of these disorders with other relatively common rheumatological disorders often lead to delayin diagnosis. At Chandigarh, we diagnosed our first child with NOMID in 2005. This child was being managed as systemic onset JIA for over 10 years. Similarly, patients with Blau syndrome were initially treated as JIA with uveitis, and patients with DADA2 as PAN. Other initial diagnosis included systemic lupus erythematosus, inflammatory bowel disease and Bechet's disease. With improved awareness amongst internists and pediatrician along with availability of affordable diagnostic techniques, these syndromes are now being suspected and diagnosed early.

Diagnosis of most SAID is based on clinical suspicion, family history and demonstration of elevated inflammatory parameters (ESR, CRP, serum amyloid A protein). Distinct interferon signatures and cytokine patterns may be helpful biomarkers to stratify and monitor patients. However, interpretation and standardization of these tests is difficult. Despite expansion of various laboratory investigations, these investigations are not yet available for clinical use in India. Genetic analysis is needed in all patients for confirmation of diagnosis. In the past, most genetic studies were performed in collaboration with international centers. In the last 5 years, molecular diagnostic techniques were established at PGIMER Chandigarh and National Institute of Immunohematology Mumbai, which are Indian Council of Medical Research (ICMR) recognized Centers for Advanced Research (CAR) in Primary Immune Deficiency Diseases. However, diagnostic facilities are still limited. At PGIMER, among SAID, we can perform Sanger sequencing for *ADA2* and *NOD2* genes. In recent years, NGS based targeted autoinflammatory panels are available in commercial laboratories, albeit expensive. Interpretation of data and functional validation of variants of unknown significance (VUS) detected remains a challenge.

Management of SAID is aimed at suppression of systemic inflammation. Colchicine and glucocorticoids have been traditionally used to treat SAID. However, with improved knowledge and understanding of pathogenic mechanisms of autoinflammation and availability of specific targeted immunotherapies, treatment strategies have been completely revolutionized (41, 42). Anti-IL-1 drugs (anakinra, canakinumab, and rilonacept), have become standard of care for most

TABLE 3 | Clinical manifestations, molecular profile, treatment and outcomes of patients with non-inflammasome-related conditions ($n = 16$).

Center	Patient (age of diagnosis, years/sex)	Age of onset of symptoms	Clinical features	Laboratory features	Family history	Initial diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
Tumor necrosis factor receptor-associated periodic syndrome (TRAPS) ($n = 3$)									
PGIMER	Pt. 34 (2.75 y/F)	1 y 9 months	• Periodic fever • Subcutaneous swellings, rash, periorbital edema • Recurrent episodes of abdominal pain	CRP: 41 mg/L ESR: 120 mm/hr IgG: 1,301 mg/dL (270–1,580) IgA: 135 mg/dL (30–130) IgM: 250 mg/dL (50–220) NBT: Normal	Father affected; migratory lymphedema (same mutation)	Periodic fever	*TNFRSF1A* exon3; c.215G>A p.Cys72Tyr previously unreported	CS, NSAID Change in treatment: etanercept	Alive
	Pt. 35 (45 Y/F)	Since adoloscence	• Fever, • Arthralgia • Conjunctivitis, • Pustular psoriasis (recurrent sterile pustular lesions)	CRP: 87 mg/L ESR: 65 mm/hr Skin biopsy: neutrophilic infiltrate in upper spinous and subcorneal layers	No	Pustular psoriasis	*TNFRSF1A* exon9; c.902C>A p.Pro301His Missense Reported in gnomAD. Predicted to be pathogenic by polyphen and SIFT	CS, cyclosporine MTX	Died
Aster CMI	Pt. 36 (10 y/M)	3 months	• Recurrent fevers since early infancy (each episode for 3-4 weeks, afebrile intervals up to 10 days), • Rash over trunk and limbs, • Limb pains and limp, • Abdominal pain • Vomiting • Eye puffiness	CRP: 150 mg/L ESR: 120 mm/hr ANA: Negative	No	TRAPS	*TNFRSF1A* exon 9; c.146A>G; p.Tyr49Cys Previously reported	CS, antimicrobials Change in treatment: Etanercept - partial response Tocilizumab – responded	Alive and doing well
Deficiency of the interleukin-1 receptor antagonist (DIRA) ($n = 2$)									
PGIMER	Pt. 37 (5 months/F) (26)	21 days	• Reduced movement and pain of left hip, left shoulder, right wrist, bilateral elbows since early infancy (multifocal osteitis) • Pustules	CRP: 110.7 mg/L ESR: 113 mm/h Bone scan: increased uptake in multiple joints (bilateral hip, shoulders, and sternoclavicular joints, lower ribs near costochondral junction and left elbow) X-ray: osteolytic lesions at humerus, left proximal femur, ribs and clavicle Bone biopsy: Bone inflammation	No	DIRA	*IL1RN* deletion, at chr2_hg19_113,865,011 and chr2_hg19_113,887,227 homozygous 22,216bp deletion spans the first four exons of IL1RN, Parents carrier for same mutation (NM_173843) Homozygous deletion Exon 1-4 deletion	Change in treatment done: Anakinra	Well

(Continued)

TABLE 3 | Continued

Center	Patient (age of diagnosis, years/sex)	Age of onset of symptoms	Clinical features	Laboratory features	Family history	Initial diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
	Pt. 38 (2.58 y/M)	7 days	Paucity of bilateral upper limb movements since day 7 of life Pustular lesions	CRP: 1.8 mg/L ESR: 8 mm/hr X-ray: bilateral humerus, clavicle and rib metaphyseal widening,	No	DIRA	Mutation for ILRN deletion as in patient 36 screened but not found	CS	Alive, healed lesions
Pyogenic Arthritis, Pyoderma gangrenosum and Acne (PAPA) ($n = 1$)									
PGIMER	Pt. 39 (5 y/F)	2.5 y	• Fever • Pyoderma gangrensosum • Colitis • Multiple abscess • Pus drainage, fistula, oral ulcers, pustules • Abdominal pain • Recurrent diarrhea	CRP: 101 mg/L ESR: 35 mm/hr Platelets: 964 × 109/L Colonoscopy: ileocecal valve thickened and distorted. Ileum shows active ulceration, cobble stone appearance, pseudo-polyp. Alteration of vascular pattern in cecum and ascending colon. Few active ulcers in hepatic flexure, transverse colon, recto sigmoid junction with pseudo-polyps Impression: Crohn's disease or tuberculous colitis Gut biopsy: Crohn's disease ANA, ANCA: negative C3/C4: 182/23 IgG: 869 NBT, CD3: Normal	-	Crohn's disease	*PSTPIP1* exon3; c.203C>T; p.Thr68Met Missense Place: Gasilini, italy	ATT, CS, infliximab, AZA	Died
A20 haploinsufficiency (TNFAIP3) ($n = 4$)									
PGIMER	Pt. 40 (6 y/F)	6 M	• Recurrent fever • Oro-genital ulcers • Ocular inflammation, blurring of vision • Headache • Papilledema • Abdominal pain • Arthritis • Colitis	CRP: 73.9 mg/L ESR: 26 mm/hr MR brain: type 2 Arnold Chiari malformation, HLAB51: positive ANA, ANCA: negative Gut Biopsy: no vasculitis	Younger brother has recurrent oral ulcers since 8 months age; Mother heterozygous for same variant	Behcet disease	*TNFAIP3* exon 7;c.1504C>T; p.Arg502Trp Heterozygous missense	colchicine, AZA	Alive and well
CMC Vellore	Pt. 41 (7 y/M)	NA	• Autoinflammatory syndrome • Inflammatory ulcers duodenum to caecum, gastritis	IgG: NA IgA: 579 mg/dL IgM: NA IgE: NA CD3:487 CD19: 22 CD56: 410	NA	NA	*TNFAIP3* exon7; c.1316_1317del; p.Gly440ArgfsTer4 Heterozygous Novel Likely pathogenic	NA	NA

(Continued)

TABLE 3 | Continued

Center	Patient (age of diagnosis, years/sex)	Age of onset of symptoms	Clinical features	Laboratory features	Family history	Initial diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
CMC Vellore	Pt. 42 (7y/M)	NA	• AIHA, • Skin rashes • Immune deficiency	IgG: 2148mg/dL IgA: 145mg/dL IgM: 14mg/dL IgE: NA Direct coombs test 3+, Ferritin normal. No increase in Double negative TCRαβ+ T cells	NA	NA	*TNFAIP3* exon8; c.2036T>C; p.Ile679Thr Heterozygous VUS	NA	NA
CMC Vellore	Pt. 43 (3 y/M)	NA	Osteomyelitis/CGD	NA	NA	NA	• *TNFAIP3* • exon7; c.1807G>A; • p.Gly603Arg • Heterozygous • VUS	NA	NA
TRNT1 deficiency (Sideroblastic anemia, immune deficiency, periodic fever, delay) (SIFD) ($n = 2$)									
ASTER CMI	Pt. 44 (3y/M)	6 months	• Recurrent fever (each episode for 4-7 days and recur twice a month) • Diarrhea • Vomiting • Panhypogmmaglobulinmeia	IgG: 223 mg/dL IgA: 17mg/dL IgM: 24 mg/dL CD3: 77% (1927) CD19: 2.5% (62) CD56: 18% (460)	Two brothers had died within the first 2 years of life with recurrent fever	X-linked agammaglobulinemia	*TRNT1* exon 2; c.143_144insTT p.Thr49Ter and exon 7;c.1043A>T p.Asp348Val compound heterozygous mutation	Replacement IVIg	Doing well
CMC Vellore	Pt. 45 (5y/M)	NA	• Hypogammaglobulinemia • Bronchiectasis	IgG: 478 mg/dL IgA: 31 mg/dL IgM: 50mg/dL IgE: 22.8 mg/dL CD3: 2,897 CD19: 96 CD56: 747 Elevated ferritin	NA	NA	*TRNT1* exon5; c.569G>T; p.Arg190Ile Homozygous	NA	NA
CARD14 mediated psoriasis (CAMPS) ($n = 1$)									
CMC Vellore	Pt. 46 (8 y/M)	NA	Psoriasis	NA	NA	NA	*CARD14* exon7; c.458G>C; p.Cys153Ser homozygous	NA	NA

(Continued)

TABLE 3 | Continued

Center	Patient (age of diagnosis, years/sex)	Age of onset of symptoms	Clinical features	Laboratory features	Family history	Initial diagnosis	Molecular details	Treatment details	Follow-up duration and outcomes
Laccase Domain Containing 1 (LACC1) defect ($n = 3$)									
PGIMER	Pt. 47 (5.75y/F) (27)	9 M	• Polyarticular joint disease. • Joint symptoms with involvement of knee and ankle and rapidly progressed to involve small joints and cervical spine, multiple joint involvement, pain, deformities and contractures, bed bound, stunted, nail dystrophy, marked swelling, deformity of large and small joints	X-ray: osteopenia, erosion of vertebrae without any platyspondyly RA Factor: positive	Sibling of Pt. 49 and 50	Torg Winchester syndrome, Pseudorheumatoid chondrodysplasia and Familial inflammatory arthropathy	*LACC1* exon4; c. 832G>C, p.Ala278Pro Parents heterozygous for the same	Naproxen, CS, MTX	Doing satisfactory
	Pt. 48 (3y/F) (27)	9 M	• Polyarticular joint disease. • Joint symptoms with involvement of knee and ankle and rapidly progressed to involve small joints and cervical spine, multiple joint involvement, pain, deformities and contractures, bed bound, stunted, nail dystrophy, marked swelling, deformity of large and small joints	X-ray: osteopenia, erosion of vertebrae without any platyspondyly	Sibling of Pt. 48 and 50	Similar to Pt 48	Same as Pt 48	Naproxen, CS, MTX	Doing satisfactory
	Pt. 49 (0.91y/F) (27)	9 M	• Polyarticular joint disease. • Joint symptoms with involvement of knee and ankle and rapidly progressed to involve small joints and cervical spine, multiple joint involvement, pain, deformities and contractures, bed bound, stunted, nail dystrophy, marked swelling, deformity of large and small joints	X-ray: ostopenia, erosion of vertebrae without any platyspondyly	Sibling of Pt. 48 and 49	Similar to Pt 48	Same as Pt 48	Naproxen, CS, MTX	Doing satisfactory

ANA, Antinuclear antibodies; Aster CMI, Aster CMI Hospital, Bengaluru, India; AZR, Azathioprine; CARD14, Caspase recruitment domain family member 14; CMC, Christian Medical College and Hospital, Vellore, India; CRP, C-reactive protein; COPA, Coatamer complex 1 protein alpha subunit; CS, Corticosteroids; CT, Computed tomography; CTA, Computed tomography angiography; ESR, Erythrocyte sedimentation rate; HCQS, Hydroxychloroquine; IL1RN, Interleukin 1 Receptor Antagonist; IVIg, Intravenous immunoglobulin; JIA, Juvenile idiopathic arthritis; LACC1, Laccase domain containing 1; MRI, Magnetic resonance imaging; MTX, Methotrexate; PGIMER, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India; PSTPIP1, Proline-serine-threonine phosphatase interacting protein 1; Reference of previously reported paper; TNFAIP3, TNF alpha induced protein 3; TNFRSF1A, TNF receptor superfamily member 1 A; TRNT1, TRNA nucleotidyl tranferase 1; Y, year.

inflammasomopathies. These agents successfully control inflammation and improve growth and quality of life. Other biologic agents used include anti-TNF drugs (etanercept), anti-IL-6 drugs (tocilizumab), and Janus kinase inhibitors (tofacitinib, baricitinib, and ruxolitinib). Treatment of SAID is extremely challenging in resource constrained settings. Anti-IL-1 drugs are not readily available in India and other developing countries. These drugs have to be imported on a "named-patient-basis" and are exorbitantly expensive. Some biosimilar molecules like anti TNF (adalimumab, infliximab) and anti IL6 (tocilizumab) are available alternative therapies. Though these molecules are cost cheaper in India when compared to Western countries, yet they remain well beyond the scope of average Indian family. Thus, corticosteroids and other conventional immunosuppressive agents still form the main stay of therapy. Patients often require higher doses as the disease progresses and they often develop corticosteroid related side effects. Off late, hematopoietic stem cell transplant (HSCT) is also emerging as a curative option for some SAID (43, 44). However, none of our patients received HSCT.

AID related morbidity and mortality continues to be high. In our cohort (8/49) have died at the time of analysis due to non-availability of treatment and development of complications. Amyloidosis had already developed in four patients at the time of diagnosis and it remains an important cause of death.

There are several limitations of this study. It is a case-based record review report from major primary immunodeficiency diseases centers across the country. Data from various individual rheumatology units could not be collated. Moreover, shared data were not uniform from all centers. Patients with unclassified SAID and patients in whom molecular diagnosis could not be established were excluded from the study.

This is a first comprehensive multicentric report of patients with SAID from India. Varied clinical and molecular spectrum has been reported. Considerable delays in diagnosis were recognized. Application of NGS based targeted panels and whole exome sequencing has helped in identifying known as well as novel gene defects. Establishment of diagnosis in a patient enabled early diagnosis

FIGURE 6 | Unilateral periorbitaledema without conjunctivitis noted in child with TRAPS during flares (**Table 3**; Patient no 34).

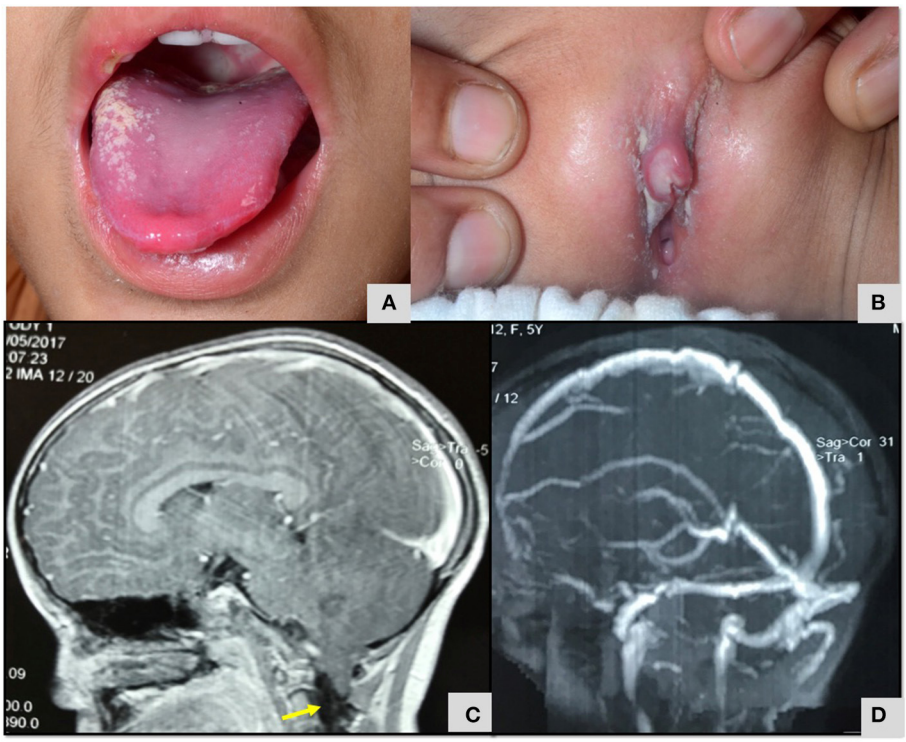

FIGURE 7 | (A,B) Oral cavity erythema and ulceration and genital ulcers in child with A20 haploinsufficiency (**Table 3**; Patient no 40). **(C)** MRI Brain T2 weighted sagittal section images demonstrating type 2 Arnold Chiari malformation (**Table 3**; Patient no 40). **(D)** MR venography of brain demonstrated normal flow and no evidence of cerebral sinus venous thrombosis (**Table 3**; Patient no 40).

of other family members and provided an opportunity for prenatal diagnosis. Although, ability to diagnose SAID has improved, non-availability of expensive immunotherapies remains a major drawback. In India corticosteroids and conventional immunosuppressive agents continue to remain corner stones for treatment. Lack of availability of targeted immunotherapies for treatment prevents the initiation of effective treatments that can change patients' lives. SAID continue to result in significant morbidity and mortality.

To conclude, more efforts are needed to enhance awareness of autoinflammatory diseases among health care professionals and there is an urgent need to make life saving drugs universally available.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institute Ethics Committee, PGIMER, Chandigarh (Ref No: INT/IEC/2021/SPL-264). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

DISCLOSURE

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AUTHOR CONTRIBUTIONS

DS, AR, AJ, PV, AG, and RP: data collection, writing of initial draft, editing of manuscript at all stages of its production, patient management, and review of literature. DS, AR, AJ, PV, AG, RP, VJ, KA, RK, GA, AA, SP, FN, BG, EE, MD, PT, VG, AP, SB, and SK: data collection, management of patients, and review of final manuscript. AR, VJ, KA, RK, SP, FN, BG, and ES: genetic evaluation and data collection. MG, IC, AAdJ, and RG-M: genetic evaluation, review of final manuscript, and critical revision. AR, SB, SK, MG, IC, AAdJ, RG-M, and SS: genetic evaluation and review of the final manuscript. MH: performed ADA2 levels in patients with DADA two patients. DS, AR, and SS: patient management, review of literature, editing and critical revision of manuscript at all stages of its production, and final approval of manuscript. All authors contributed to the article and approved the submitted version.

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Clinical and Molecular Findings in Mendelian Susceptibility to Mycobacterial Diseases: Experience From India

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Mendelian Susceptibility to Mycobacterial diseases (MSMD) are a group of innate immune defects with more than 17 genes and 32 clinical phenotypes identified. Defects in the IFN-γ mediated immunity lead to an increased susceptibility to intracellular pathogens like mycobacteria including attenuated *Mycobacterium bovis*-Bacillus Calmette-Guérin (BCG) vaccine strains and non-tuberculous environmental mycobacteria (NTM), *Salmonella*, fungi, parasites like *Leishmania* and some viruses, in otherwise healthy individuals. Mutations in the *IL12RB1* gene are the commonest genetic defects identified. This retrospective study reports the clinical, immunological, and molecular characteristics of a cohort of 55 MSMD patients from 10 centers across India. Mycobacterial infection was confirmed by GeneXpert, Histopathology, and acid fast bacilli staining. Immunological workup included lymphocyte subset analysis, Nitro blue tetrazolium (NBT) test, immunoglobulin levels, and flow-cytometric evaluation of the IFN-γ mediated immunity. Genetic analysis was done by next generation sequencing (NGS). Disseminated BCG-osis was the commonest presenting manifestation (82%) with a median age of presentation of 6 months due to the practice of BCG vaccination at birth. This was followed by infection with *Salmonella* and non-typhi *Salmonella* (13%), *Cytomegalovirus (CMV)* (11%), *Candida* (7%), NTM (4%), and *Histoplasma* (2%).

Thirty-six percent of patients in cohort were infected by more than one organism. This study is the largest cohort of MSMD patients reported from India to the best of our knowledge and we highlight the importance of work up for IL-12/IL-23/ISG15/IFN-γ circuit in all patients with BCG-osis and suspected MSMD irrespective of age.

Keywords: IL-12/IL-23/ISG15/IFN-γ axis, intracellular pathogens, BCG-osis, *Mycobacterium tuberculosis* complex, IL-12Rβ1 defect, anti-tubercular treatment

INTRODUCTION

Mendelian Susceptibility to Mycobacterial diseases (MSMD) now also known as Inborn Errors of IFN-γ immunity (IEI) are a group of innate or intrinsic immune defects localized to 17 genes and 32 clinical phenotypes identified (1–3). IL-12/23/ISG15-IFN-γ axis is the principal immunological pathway for intra-macrophagic pathogens (4, 5). Defects in this pathway lead to an increased susceptibility to intracellular pathogens like mycobacteria including attenuated *Mycobacterium bovis*-Bacillus Calmette-Guérin (BCG) vaccine strains and non-tuberculous environmental mycobacteria (EM), *Salmonella*, fungi, parasites like *Leishmania,* and some viruses, in otherwise healthy individuals (4, 6–9). Tuberculosis is the commonest public health problem in India with an estimated incidence of 2.4 million cases in 2019 (10). BCG vaccination is universally administered to all babies soon after birth (11), to protect against severe forms of tuberculosis. Adverse event following BCG immunization might be the presenting manifestation in MSMD.

In a setting of strong clinical suspicion, flow-cytometric evaluation of IL-12/23-IFN-γ pathway followed by molecular work-up for identifying the genetic etiology is warranted. Our first patient of IL-12Rβ1 defect was diagnosed with help from Dr. Dinakantha Kumararatne from Cambridge. Subsequently, our initial cases suspected with MSMD were evaluated by Dr. Jacinta Bustamante and Pr. Jean-Laurent Casanova at Paris; and a genetic cause could be identified in nine patients with three IL-12Rβ1 complete defects, two IFN-γR1 (partial dominant), two STAT1 (partial dominant, loss-of-function), one complete IFN-γR1 defect, and one complete IL-12p40 defect. In India, with increasing awareness about MSMD, pediatricians, infectious disease specialists, hemato-oncologists and those dealing with the menace of *Mycobacterium tuberculosis* (*M. tb*) started recognizing and appreciating genetic factors responsible for susceptibility to mycobacterial tuberculosis. With the ease of access to next generation sequencing (NGS) in recent years, the diagnosis of MSMD has increased.

There is a paucity of literature on MSMD from India with only a few published case reports (12–15). In this study, we report clinical, immunological, and molecular characteristics of a retrospective cohort of 55 MSMD patients from 10 centers across India.

MATERIALS AND METHODS

Ten participating of Primary immunodeficiencies (PID) centers from India contributed data for this retrospective analysis. The participating centers included Bai Jerbai Wadia Hospital for Children, Mumbai ($n = 14$), Christian Medical College; Vellore ($n = 11$), Indian Council of Medical Research—National Institute Immunohaematology; Mumbai ($n = 10$), ACPED (Advance Center for Pediatrics) PGI, Chandigarh ($n = 8$), ASTER CMI; Bangalore ($n = 3$), Narayana Hrudayalaya; Bangalore ($n = 3$), Kasturba Medical College and Hospital; Mangalore ($n = 2$), Apollo Hospital; Chennai ($n = 2$), Superspeciality Pediatric Hospital and Post Graduate Teaching Institute; Noida ($n = 1$), and Sir Ganga Ram Hospital, Delhi ($n = 1$). All centers were contacted *via* email and requested to provide details of their MSMD patients on a pre-designed Microsoft Excel datasheet. Details included demographic information, clinical manifestations, family history, microbiological and immunological investigations, genetic evaluation, management, and follow-up. Being a retrospective analysis of data, it was exempted from ethics approval.

Diagnosis of infection with *Mycobacterium tuberculosis* complex (MTBC) was confirmed by PCR in addition to histopathologic and microbiologic findings of acid-fast bacilli (AFB) detected from lymph nodes or broncho-alveolar lavage. Diagnostic approach for suspected MSMD included basic lymphocyte subset analysis, nitroblue tetrazolium (NBT) test, immunoglobulin levels, and flow-cytometric evaluation of the IFN-γR1 (CD119), IL-12Rβ1 (CD212), serum IFN-γ levels, STAT1, and STAT4 phosphorylation along with NGS for making a molecular diagnosis. NGS included PID targeted-gene panel and clinical Exome sequencing. Imaging studies including chest X-ray, ultrasound, and chest CT scan were used when internal organ involvement was suspected. The specialized assays were used for rapid identification of the candidate gene when molecular facilities were not freely available initially. Eventually with the easy availability of NGS facilities, the relevant assays were performed for functional validation of the identified mutation and has been carried out in majority of the cases. Some centers performed molecular workup directly after the basic immune work-up for the patients suspected with MSMD.

Statistical analysis of data was obtained on a predesigned worksheet (Excel, Microsoft Office) and analyzed using Graph pad Prism (Chicago, IL, USA) for Microsoft Windows. Kaplan Meier's analysis was used to estimate the risk of BCG related complications.

RESULTS

Patient Characteristics

In this study, we analyzed a total of 55 patients of MSMD of which 51 (93%) had a confirmed molecular diagnosis and four were evaluated by flow cytometry. The clinical and demographic

TABLE 1 | Demographic and clinical findings.

Patient ID	Age of presentation (in months)	Sex	Consanguinity	Family history	BCGosis	Salmonella	Other organisms	Type of MSMD Defect	Outcome
P1	0.5	F	+	+	+	−	*CMV*	*IFN-γR1*	D
P2	6	F	−	−	+	−	*Pseudomonas aeruginosa, CMV*	*IFN-γR1*	A
P3	3	M	+	−	+	−	−	*IL-12p40*	A
P4	6	F	+	−	+	−	−	*STAT1*	D
P5	3	F	+	+	+	−	−	*STAT1*	D
P6	11	M	+	−	+	−	−	*IFN-γR2*	A
P7	168	M	+	+	+	−	*Giardia, Histoplasma*	*IL-12Rβ1*	A
P8	120	M	−	+	+	+	−	*IL-12Rβ1*	A
P9	300	F	+	−	−	+	−	*IL-12Rβ1*	A
P10	18	M	+	−	+	−	*Candida*	*IL-12Rβ1*	D
P11	120	F	−	−	+	−	*Staphylococcus aureus, NTM, Molluscum* (*Coxsackie*)	*IFN-γR1*	A
P12	24	M	+	+	+	−	−	*IFN-γR1*	D
P13	180	M	+	−	−	−	*Mycobacterium leprae*	*IFNγR1*	D
P14	9	F	−	−	+	−	−	*IL-12Rβ1*	A
P15	27	F	−	−	+	−	−	*IL-12Rβ1*	Lost F/U
P16	48	M	−	−	+	−	*Candida*	*IL-12Rβ1*	Lost F/U
P17	12	F	+	+	+	−	−	*IL-12Rβ1*	A
P18	3	F	+	+	+	−	−	*IL-12Rβ1*	D
P19	132	M	+	−	+	−	−	*IL-12Rβ1*	A
P20	14	F	+	−	+	−	−	*IFN-γR2*	A
P21	5	F	+	+	+	−	−	*IFN-γR1*	Lost F/U
P22	3	M	+	−	+	−	−	*IFN-γR1*	Lost F/U
P23	4	F	−	−	+	−	−	*IFN-γR1*	A
P24	4	M	+	−	+	−	−	*IFN-γR2*	D
P25	2	F	+	−	+	−	−	*IL-12Rβ1*	D
P26	6	F	+	−	−	−	*Candida*	*RORC*	A
P27	72	F	−	−	+	−	*Varicella Zoster*	*IFNγR1*	A
P28	4	F	+	+	+	−	−	*IL-12Rβ1*	A
P29	3	M	+	+	+	−	−	*IFN-γR2*	D
P30	6	M	−	+	+	−	−	*STAT1*	A
P31	6	M		−	+	−	−	*IFNγR1*	Lost F/U
P32	7	F	+	−	+	+	−	*IL-12Rβ1*	A
P33	4	M	−	−	+	−	−	*IL-12Rβ1*	A
P34	2	F	−	−	+	−	−	*IL-12Rβ1*	Lost F/U
P35	13	M	+	−	+	−	*CMV, NTM*	*IFN-γR1*	A
P36	3	M	+	−	+	−	−	*IL-12Rβ1*	A
P37	3	M	+	−	+	−	*Streptococcus pneumoniae, Enterococcus, BOCA VIRUS*	*ISG15*	D
P38	3	M	−	−	+	−	−	*IFN-γR2*	D
P39	4	F	−	+	−	+	−	*IL-12Rβ1*	A
P40	1	F	−	−	+	−	*Staphylococcus aureus*	*IL-12Rβ1*	A

(Continued)

TABLE 1 | Continued

Patient ID	Age of presentation (in months)	Sex	Consanguinity	Family history	BCGosis	Salmonella	Other organisms	Type of MSMD Defect	Outcome
P41	1	M	–	–	+	–	–	*IL-12Rβ1*	D
P42	1	M	–	–	–	–	–	*STAT1*	A
P43	7	M	–	+	+	–	–	*IFN-γR2*	Lost F/U
P44	48	M	–	–	+	+	–	*IFN-γR1*	Lost F/U
P45	120	F	–	–	–	+	*Proteus*	*IL-12Rβ1*	Lost F/U
P46	120	M	–	+	+	+	*Clostridium difficile*	*IL-12Rβ1*	Lost F/U
P47	96	M	–	–	+	–	–	*IFNγR1*	Lost F/U
P48	36	F	+	–	+	–	*Streptococcus pyogenes*	*IFNγR1*	Lost F/U
P49	6	F	+	+	+	–	–	*IL-12Rβ1*	Lost F/U
P50	6	M	–	+	+	–	–	*IL-12Rβ1*	Lost F/U
P51	9	M	+	+	–	–	*CMV*	*IFN-γR2*	A
P52	6	M	+	+	–	–	*Neisseria, CMV*	*IFN-γR2*	D
P53	6	M	+	–	–	–	*Candida*	*IL-12Rβ1*	A
P54	3	M	–	–	+	–	*CMV*	*IFN-γR1*	A
P55	60	M	–	–	–	–	–	*IL-12Rβ1*	A

D, dead; A, alive; F/U, follow-up.

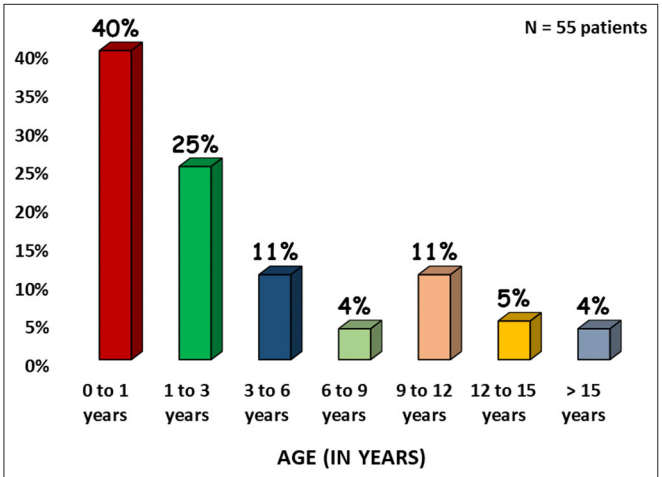

FIGURE 1 | Age-wise distribution at presentation.

details of the patients are presented in **Table 1**. There was a slight male preponderance with 56% males and 44% females. History of consanguinity was present in 58% families and 33% families had history of a previous affected sibling. The median age of presentation was 6 months (0.5–300 months). **Figure 1** shows the age-wise distribution at presentation.

The commonest clinical presentation was disseminated BCG-osis (82% patients) [ESID criteria for BCG-itis and BCG-osis was followed (16)], representative clinical images are shown in **Figure 2**. The average number of organs involved in patients with BCG-osis was three.

Microbiological Spectrum

All patients in the cohort had mycobacterial disease with MTBC (96%), and non-tuberculous mycobacteria (NTM) in (4%). Multisystem involvement with mycobacteria was the commonest followed by lymph node involvement, tubercular osteomyelitis, pulmonary, skin, and central nervous system (CNS) in the order of decreasing frequency (**Figure 3**).

Thirty-six percent of patients in cohort were infected by more than one organism. Other infections associated with our cohort were *Salmonella* and non-typhi *Salmonella* (13%), *Candida* (7%), *Histoplasma* (2%), *Nocardia* (2%), *Mycobacterium leprae* (2%) other bacterial infections like *Staphylococcus aureus* (4%), Streptococci (4%), *Pseudomonas aeruginosa* (2%), *Neisseria* (2%), *Proteus mirabilis* (2%), *Clostridium difficile* (2%), *Enterococci* (4%), and viruses *Cytomegalovirus* (CMV, 11%), Molluscum (2%), Varicella zoster (2%), *Bocavirus* (2%). The overall microbiological spectrum and distribution of infections with different families of micro-organisms are presented in **Figure 4**.

Immunological and Molecular Evaluation

Evaluation of the IL-12/23/IFN-γ pathway by flow-cytometry could be performed in 21 patients and the results were consistent with the underlying molecular defect. NGS analysis in 55 MSMD patients within 51 families led to the identification of 5 previously reported and 24 novel mutations in 7 different genes as presented in **Table 2**. The pathogenicity of novel mutations identified in our cohort was determined by *in silico* tools like Mutation Taster, SIFT and Polyphen-2, and genotype-phenotype correlation.

The spectrum of molecular defects identified in the patients is presented in **Figure 5**. The prevalence of different molecular defects in India is consistent with world literature as shown in **Figure 5** (21, 22).

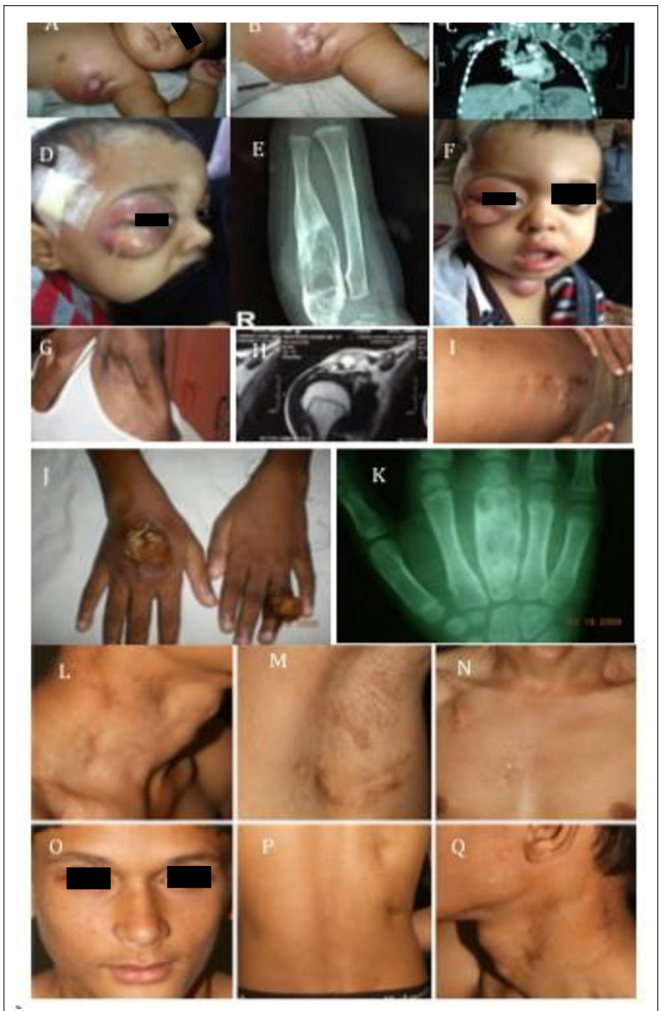

FIGURE 2 | Clinical images. **(A–C)** Show image of BCG-osis without failure to thrive: IL-12Rβ1 defect. **(D–F,J,K)** Represent multi-focal bone involvement seen in PD-IFNγR1 defect or AD-STAT1 defect. **(G–I)** Shows child with BCG-osis, shoulder, and disseminated *Histoplasmosis*: IL-12Rβ1 defect. **(L–Q)** Represent another case of BCG-osis with impetigo and lymphadenopathy whose culture grew non-typhi *Salmonella*: IL-12Rβ1 defect.

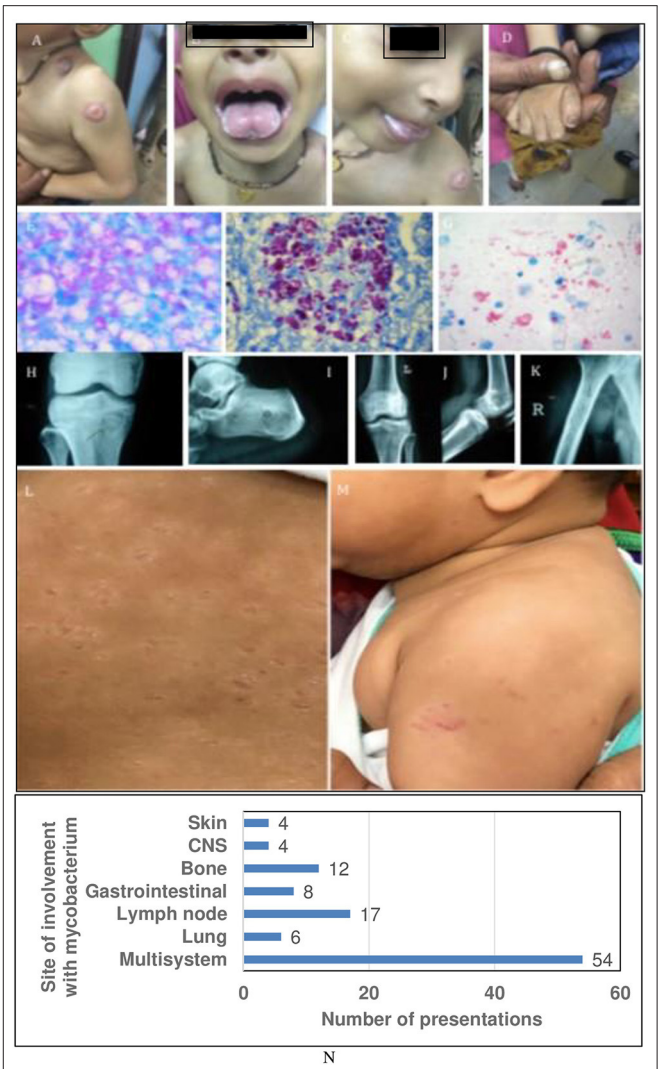

FIGURE 3 | Representative clinical images and sites of involvement of Mycobacteria. **(A–D)** Show BCG-osis with candidiasis: IL-12Rβ1 defect or ROR-γT defect. **(E–G)** Show exuberant growth of AFB in tissue Liver Biopsy **(F)** also shows *CMV* inclusions. **(H–K)** multifocal bone TB: IFN-γR1 or IFN-γR2 defect. **(L,M)** Show cutaneous BCG-osis. **(N)** Showing graph with sites of involvement with mycobacterium.

AR Complete IL-12Rβ1 Deficiency

The complete IL-12Rβ1 defect was identified in 25 patients presenting at a median age of presentation of 9 months (Range 1–300 months). 21/25 (84%) patients presented with BCG-osis; Six patients (P8, P9, P32, P39, P45, and P46) had recurrent *Salmonella* infection and three patients (P10, P16, and P53) had *Candidiasis* along with tuberculosis. Nineteen patients (76%) had only BCG-osis and did not develop any other infections during the period of follow up. Autoimmune manifestations in the form of inflammatory bowel disease was seen in three patients (P8, P45, and P46) and one patient (P46) had autoimmune hemolytic anemia, leukocytoclastic vasculitis and was ANA positive. The other infections identified included *Histoplasma* (P7), *Giardia* (P7), *Staphylococcus* (P40), *Proteus* (P45), *Enterococcus* (P45), and *Clostridium difficile* (P46). One patient (P7) with histoplasmosis and BCG-osis also had CD4 lymphopenia and portal vein thrombosis. Consanguinity was present in 12/25(48%) patients and there was history of a previous affected sibling in 7/25(28%) families. Flow cytometric evaluation of IL-12Rβ1 expression was found to be absent in 11 patients where it was evaluated.

IL-12Rβ1 was the predominant gene affected in our cohort with a homozygous mutation located in exon 9, c.962C>A being the commonest mutation identified (11/25) followed by another mutation Intron 15 c.1791+2T>G (6/25). Two of our patients had homozygous missense mutation in exon 16 of *IL12Rβ1,* c.1786A>G. This mutation was reported as benign in Clinvar, however flow cytometry confirmed deficient *IL-12Rβ1.*

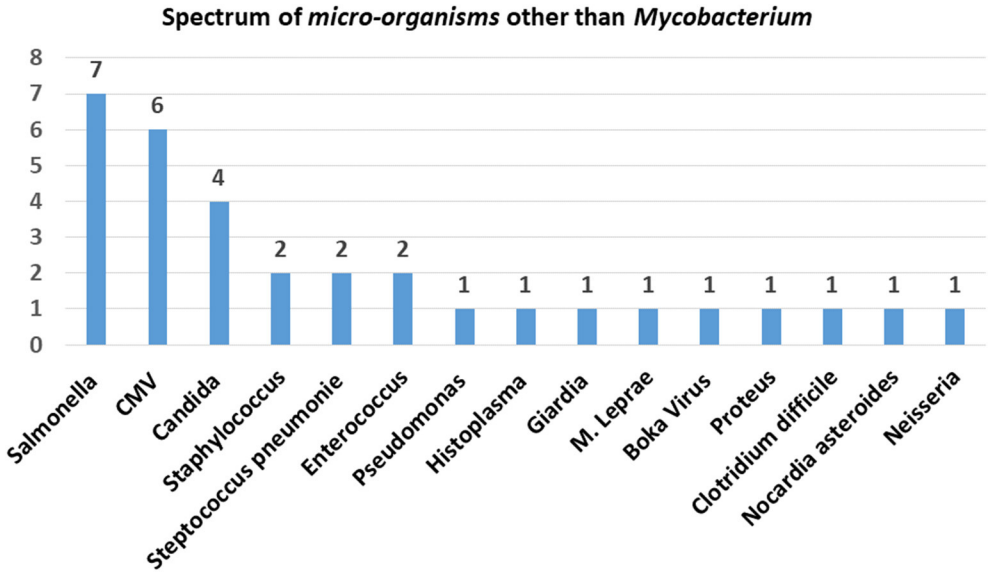

FIGURE 4 | Microbiological spectrum.

Carrier status in parents was confirmed in three families. All patients received anti-mycobacterial treatment with antibiotics. Two siblings also had an aspiration of pus for BCG-itis. Three patients of complete IL-12Rβ1 deficiency (P32, P53, and P55) underwent allogeneic hematopoietic stem cell transplantation (HSCT), which engrafted well and the patients are doing well. P32 and P53 had father as haploidentical donor while P55 had a fully matched sibling donor. Four (16%) patients (P10, P18, P25, and P41) expired.

Three of our patients developed inflammatory bowel disease (IBD) during follow up which is not reported in world literature. IL-12Rβ1 defect also results in TH17 pathway defect that can be associated with autoimmunity and auto inflammation which could be the cause of IBD in our patients. This needs to be investigated further.

AR Complete IL-12p40 Deficiency

IL-12p40 defect was identified in one patient (P3) who presented at three months of age with BCG-osis and also needed admission twice due to tubercular pleural effusion. The patient received ATT thrice and is doing well.

AR Complete IFN-γR1 Deficiency

Complete IFN-γR1 deficiency was identified in eight patients. The median age of presentation was 5.5 months (0.5–36 months). BCG-osis was the presenting manifestation in all patients. Additional infections with *Streptococcus* (P48), and CMV (P1, P35, and P54) were observed. Flow cytometric evaluation of IFN-γR1 was performed in four patients and found to be absent in three. One patient (P1) revealed a partial expression, however, baseline IFN-γ levels were very high and downstream STAT1 phosphorylation was found to be absent suggesting that although partial expression of IFN-γR1 was present, it was non-functional. All patients received ATT for mycobacterial infections. Two (25%) patients (P1 and P12) expired due to respiratory distress and hypersplenism with increased transfusion requirement. One patient (P54) with complete IFN-γR1 underwent haploidentical HSCT as the child had no matched family donor, nor any matched unrelated donor. He rejected his graft and had autologous reconstitution by day 28 post-HSCT. He continues to be on four ATT drugs. One patient (P35) is well on ATT and NTM prophylaxis. Four patients are (P21, P22, P31, and P48) are lost to follow up.

AD Partial IFN-γR1 Deficiency

Seven patients were diagnosed with a partial dominant (PD) IFN-γR1 deficiency with a median age of presentation of 72 months (4–180 months). BCG-osis was the presenting manifestation in 6/7(86%) patients. Severe forms of the disease in the form of multifocal osteomyelitis were identified in (71%) five patients (P2, P11, P13, P44, and P47) with PD IFN-γR1 defect which was similar to world literature (21). The organisms isolated included tubercular and non-tubercular mycobacteria. One patient (P11) also identified with PD IFN-γR1defect had recurrent lymphadenopathy and joint involvement and extensive cutaneous infection with *Mycobacterium avium* intracellulare (**Figure 6**). She also developed molluscum contagiosum of the vulvar region. Other infections associated with partial dominant defect were *Pseudomonas aeruginosa* (P2), *Staphylococcus aureus* (P11), *Mycobacterium leprae* (P13), *Salmonella* (P44), *CMV* (P2) leading to secondary hemophagocytic lymphohistiocytosis (HLH), and *Varicella* (P27). Flow cytometry was performed in all seven patients, and showed increased IFN-γR1 expression, and abnormal STAT1 phosphorylation suggestive of the PD IFN-γR1 deficiency. All four patients in whom mutation was available had c.818_821delTTAA in exon 6 of *IFNGR1*. All patients received anti-tubercular treatment for mycobacterial

infections. P27 needed three courses of ATT for recurrent lymphadenopathy. Two patients (P2 and P13) expired while two patients (P44 and P47) are lost to follow up. Rest three patients (P11, P23, and P27) are alive and doing well.

AR Complete IFN-γR2 Deficiency

IFN-γR2 deficiency was identified in eight patients (14%) which is higher than 3% reported in world literature (21). The median age of presentation is 6.5 months (3–14 months). All vaccinated patients presented with BCG-osis; two patients were not vaccinated due to previous affected sibling. One patient (P43) had a previously affected siblings, he had recurrent mycobacterial infection and needed three courses of ATT. Other infections identified included CMV infection in P51 and P52 and *Neisseria* in P52; both were siblings. Consanguinity was identified in 6/8 (75%) families. Three (37.5%) patients (P24, P29, and P38) expired before transplant. The siblings P51 and P52 underwent haplo-identical transplant; P51 is alive and well, while P52 expired 2 years later due to an unrelated cause.

STAT1 Deficiency

STAT1 deficiency was identified in four patients of whom two patients had an AR complete defect (P4 and P5) and two had a PD partial defect (P30 and P42). Both patients with a complete defect and one with a partial defect (P30) presented with BCG-osis and one patient with partial defect (P42) had history of recurrent tuberculosis. No other infections besides MTBC were identified in any patients. Both patients with complete defect were siblings and born of a consanguineous marriage. One patient with a partial defect (P30) had family history of father affected with cutaneous tuberculosis and paternal grandmother affected with Pott's spine, suggestive of autosomal dominant transmission. STAT1 phosphorylation by flow cytometry was found to be absent in both patients with complete STAT1 defect who were evaluated along with normal IFN-γ levels. Both patients with complete defect expired, one patient with PD defect (P42) is on antibiotic prophylaxis for recurrent respiratory tract infections and the other is on anti-mycobacterial treatment.

ISG15 Defect

One patient (P37) was identified with ISG15 defect, born of a third-degree consanguineous marriage had presented at 3 months of age with BCG-osis, he also had *Bocavirus* infection and recurrent Streptococcal pneumonia. A homozygous mutation was identified in exon 2 of the *ISG15* gene, c.454_455del, identified by NGS and confirmed by Sanger sequencing. The patient received ATT and antibiotics but succumbed to the disease.

RORγT Deficiency

One patient (P26) was identified with RORγT deficiency caused due to homozygous mutation in Exon 5 of the *RORC* gene, c.558T>G, identified by NGS and confirmed by Sanger sequencing by Dr. Anne Puel. The patient had presented with CNS tuberculomas, oral thrush, and onychomycosis. The patient received ATT and is currently well.

Treatment and Outcome

All patients were started on ATT comprising of a combination of four drugs -isoniazid, rifampicin, ethambutol, and pyrazinamide, which was altered based on sensitivity pattern. BCG strain was identified based on its inherent pyrazinamide resistance [Danish 1331 is pyrazinamide resistant (11)] hence in BCG-osis, pyrazinamide was not part of treatment protocol. Duration of treatment was 6 months to 2 years depending on the site of involvement. Eighty-five percent received one course of ATT, 7% received two courses, 7% received three courses and 2% received five courses of ATT. Second-line drugs were given for a longer duration for MDR tuberculosis. Other antibiotics and antifungals based on drug sensitivity reports were added depending on concurrent infections. 6/51 (12%) cases of molecularly confirmed MSMD underwent allogenic HSCT. Three patients of complete IL-12Rβ1 (P32, P53, and P55) engrafted well and the patients are doing well. P32 and P53 had father as haploidentical donor while P55 had a fully matched sibling donor. One patient with complete IFN-γR1 (P54) underwent haploidentical HSCT as the child had no matched family donor, nor any matched unrelated donor. He rejected his graft and had autologous reconstitution by day 28 post-HSCT. He continues to be on four drug ATT. The siblings P51and P52 with complete IFN-γR2 deficiency underwent haplo-identical transplant from father; P51 is alive and well, while P52 expired 2 years later due to an unrelated cause. Three patients (P51, P52, and P53) were transplanted at Narayana Hrudayalaya, two (P54 and P55) at Apollo Chennai and One (P32) at CMC Vellore. Among 41 patients who could be followed 66% (27/41) are alive and well. Fourteen patients (34%) expired of which four had IL-12Rβ1 defect, two had AR complete IFN-γR1 deficiency, one had PD IFN-γR1 defect, four had AR complete IFN-γR2 defect, two AR complete STAT1 deficiency, and one AR ISG15 defect.

DISCUSSION

Although Inborn Errors of Immunity (IEI) as a group have been traditionally described to cause susceptibility to a wide range of micro-organisms, MSMD causes predisposition to infection by selective intracellular organisms. BCG related complications may be the presenting manifestation in MSMD in 4–80% of the patients (15). The practice of vaccinating all babies soon after birth might be the reason for an early presentation seen in our cohort; in addition to other well-known social factors like high population density, high incidence of *M. tb* in the country, poor nutrition, and unhygienic conditions. Although the majority of our patients presented at <3 years of age, some patients presented later in life and their diagnosis was confirmed after several years. Like other inherited genetic IEI, MSMD too can present at any age and should be kept in the differential diagnosis of any child with the right clinical infection to suspect MSMD. This was observed in particular in two patients where the diagnosis was delayed for years before the infection gave a clue to MSMD. One child (P8) was diagnosed after a lymph node biopsy grew non-typhi *Salmonella* which was the clue to suspect MSMD and IL-12Rβ1 defect was confirmed. Another child (P7) with BCG-osis

TABLE 2 | Molecular findings.

Gene	Defect	Number of patients	Patient ID	Position	Nucleotide change	Amino acid change	Zygosity	Mutation type	Reported/novel
IL12Rβ1	Complete	1	P7	Exon 9	c.982_982delC	p.M328Cfs*41	Homozygous	Deletion	Novel
		2	P9, P25	Exon 16	c.1786A>G	p.K596E	Homozygous	Missense	rs567051378 (Reported Benign in Clinvar)
		11	P10, P15, P19, P28, P32, P34 P39, P40, P41, P46, P50	Exon 9	c.962C>A	p. S321*	Homozygous	Nonsense	rs147766868 (PMID 30255293) (17)
		6	P8, P16, P17, P18, P45, P33	Intron 15	c.1791+2T>G		Homozygous	Essential splicing site	rs554063682 (PMID 26976630) (17, 18)
		2	P36, P55	Exon 7	c.698_698delC	p.P233Lfs*9	Homozygous	Deletion	Novel
		1	P14	Exon 8	c.599del	p.L200Rfs*3	Homozygous	Deletion	Novel (rs1169002203)
		1	P49	Intron 14	c.1738+2 T>A		Homozygous	Essential splicing site	Novel
		1	P53	Exon 6	c.511C>T	p.Q171*	Homozygous	Nonsense	Novel
IFNGR1	Partial	4	P2, P11, P44, P47	Exon 6	c.818_821 delTTAA	p.N274Hfs*2	Heterozygous	Deletion	Novel
	Complete	1	P12	Exon 5	c.601_601delC	p.Q201Sfs*2	Homozygous	Deletion	Novel
		1	P1	Exon 6	c.838_839dupA	p.S280Kfs*3	Homozygous	Duplication	Novel
		1	P48	Exon 3	c.328G>T	p.E110*	Homozygous	Nonsense	Novel
		1	P21	Exon 4	c.389C>A	p.P130H	Homozygous	Missense	Novel
		1	P35	Exon 2	c.110 T>C	p.I37T	Homozygous	Missense	rs945137618 (PMID 28744922) (19)
		1	P31	Exon 5	c.653_655del	p.E218del	Homozygous	In-frame deletion	rs587776858 (PMID 10811850) (20)
		1	P54	Exon 7	c.1068delG	p.T357Lfs*15	Homozygous	Deletion	Novel
IFNGR2	Complete	1	P6	Exon 2	c.196C>T	p.Q66*	Homozygous	Nonsense	Novel
		1	P20	Intron 4	c.561+1G>A		Homozygous	Essential splicing site	Novel
		1	P24	Exon 4	c.540G>A	p.W180*	Homozygous	Nonsense	Novel
		1	P29	Exon 6	c.782_790del TGCTGGCAG	p.V261_A263del	Homozygous	In frame deletion	Novel
		1	P38	Exon 4	c.488T>C	p.F163S	Homozygous	Missense	Novel
		1	P43	Intron 3	c.412+2T>A		Homozygous	Essential splicing site	Novel
		2	P51, P52	Intron 2	c.207-1G>A		Homozygous	Essential splicing site	Novel
ISG15	Complete	1	P37	Exon 2	c.454_455delCT	p.L152Afs*?	Homozygous	Deletion	Novel
STAT1	Complete	2	P4, P5	Exon 9	c.769dup	p.D257Gfs*22	Homozygous	Duplication	Novel
	Partial	1	P30	Exon 9	c.749G>A	p.G250E	Heterozygous	Missense	Novel
		1	P42	Intron 20	c.1728-4C>T		Heterozygous	Essential splicing site	Novel (rs760805208)
IL12B		1	P3	Exon 4	c.429G>A	p.W143*	Homozygous	Nonsense	Novel (rs751288779)
RORC	Complete	1	P26	Exon 5	c.558T>G	p.Y186*	Homozygous	Nonsense	Novel

** means termination; ? means unknown.*

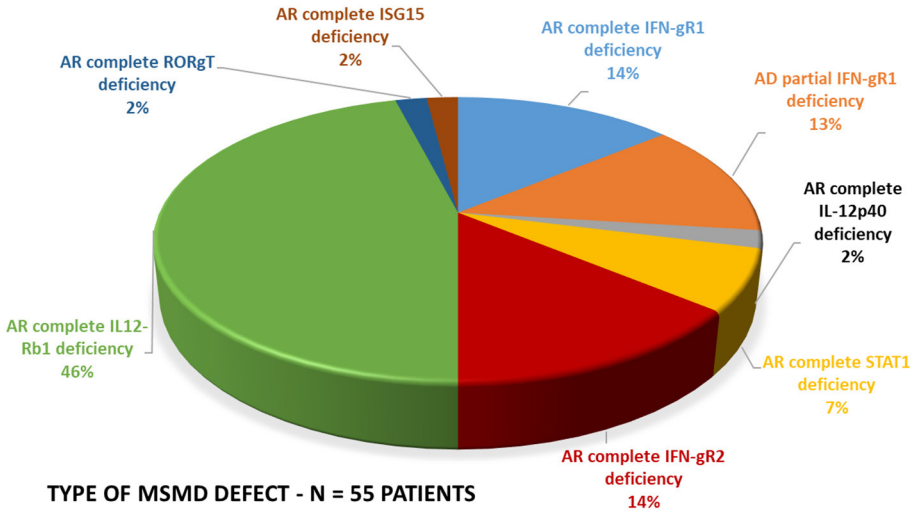

FIGURE 5 | Molecular defects. The molecular defects identified in our cohort were IL-12Rβ1 (46%) followed by complete IFN-γR1 (14%), complete IFN-γR2 (14%), PD-IFN-γR1 (13%), complete STAT1 defect (7%) and IL-12p40 defect (2%), RORC(2%), and ISG15(2%).

after birth recovered and had two episodes of infection with *Histoplasma*; right shoulder osteomyelitis and later disseminated histoplasmosis. The clue to suspecting MSMD comes from knowing the organism. Local endemicity pattern for a particular infection (e.g., *Leishmania donovani, Histoplasma capsulatum*) may provide a difference in the pattern of infections in MSMD in different parts of the world. In India, *Leishmania donovani* is endemic in states of Bihar and Uttar Pradesh, however, we did not see a single case of MSMD with *Leishmania donovani* in our cohort while we did have cases of MSMD presenting with Histoplasmosis which is endemic in India.

Presence of mycobacterial infection in all patients suggests that the burden of exposure to *Mycobacterium* is very high in India. Isolation of NTM at a site other than the cervical node or wound site especially in the scenario of multifocal bone tuberculosis should initiate work up for underlying MSMD (23, 24). NTM was present in only 5% of our patients which is much less than what is reported in literature (25).

There is now a greater emphasis of making a definitive diagnosis of *M. tb* in India and due to the availability of culture, GeneXpert, line probe assay, and NGS for mycobacterial genome, it is possible to make a correct microbiological diagnosis. In addition to accurately identifying the organism, the drug sensitivity pattern can help us treat our patients more appropriately and recognize multi-drug resistant (MDR) and extensively drug-resistant (XDR) tuberculosis.

Micro-organisms isolated can provide very important clues to underlying molecular defects. For instance, an association of Mycobacterial infection with CMV strongly suggests AR complete IFNγR1/R2 or AR complete STAT1 defect (26); with *candidiasis* suggests possibility of IL-12Rβ1 defect or RORγT deficiency (27, 28); with *Salmonella* or non-typhi *Salmonella* strongly suggests the possibility of complete IL-12Rβ1 or IL-12p40 defect (29). Brain calcification along with mycobacterial infection suggests the possibility of ISG15 deficiency (30). Multifocal bone osteomyelitis by *M. tb* or NTM strongly correlates with an underlying molecular defect of PD-IFNγR1 defect or PD-STAT1 defect (31). One can predict an underlying molecular defect based on the clinical presentation.

Complete IL-12Rβ1 Deficiency caused by bi-allelic mutations in the *IL12RB1* gene was the commonest underlying molecular defect identified in our cohort. Mutations resulting in premature stop codons, such as nonsense, and essential splicing-site mutations, represented the majority of IL-12Rβ1 deficiency causing mutations (92%). This defect is usually associated with a late presentation, however, in our cohort, the median age of presentation was earlier at 9 months due to mandatory BCG vaccination at birth. The high incidence of nonsense and essential splice site mutation could also contribute to earlier presentation. Almost 64% of patients had a single infection with MTBC, this finding is consistent with the finding reported previously in a survey of 141 IL-12Rβ1 patients (32). It reflects the protective role of the primary infection against the reactivation of a latent organism or secondary infection. When compared to IFNγR1 defects and STAT1 deficiency, IL-12Rβ1 defect had a milder course of the disease which was observed by lower mortality (16% in IL-12Rβ1 compared to 20% in IFNγR1, and 50% in STAT1 defects altogether), fewer patients suffering from infections with multiple organisms, fewer courses of ATT required during the course of disease (32, 33). Although, the commonest mutation reported in literature is intron 15 c.1791+2T>G (34), we found exon 9 c.962C>A mutation to be the commonest in our cohort, which was present in almost half the patients. Multifocal bone tuberculosis may be seen in as much as half of the patients of PD IFNγR1 deficiency (17). This is consistent with the finding in our cohort (57%) with three patients having multifocal bone tuberculosis and one patient having joint involvement.

FIGURE 6 | Clinical images of a case with partial dominant IFNγR1 deficiency. **(A–D)** Severe cutaneous and bony MAC infection. **(E,F)** Response to AKT. **(G)** Development of *Molluscum contagiosum*.

STAT1 defects may also present with multifocal bone tuberculosis although this was not observed in our cohort.

Identification of a molecular defect can help us to take therapeutic management decisions. Patients with complete IFN-γR1, IFN-γR2, and STAT1 deficiencies can be cured with HSCT (35). Majority of our patients were treated with 4 drug ATT as is the usual practice in our country, the duration of treatment ranging from 6 months to 2 years depending on the site of involvement and the clinical response to treatment. However, since most of the patients had BCG (Danish 1,331) disease, which is resistant to pyrazinamide, standard ATT was modified to include drugs according to the sensitivity pattern and more aggressive treatment was given for a longer duration. A few patients of IL-12Rβ1 had a recurrence of infections and worsening of symptoms despite being on treatment. Such patients may benefit from treatment with IFN-γ (36) which is currently not available in India. HSCT was performed in three patients (P33, P54, and P56) with IL-12Rβ1 defect who are currently doing well.

This study describes the clinical and molecular spectrum of a large cohort of MSMD patients from India. It highlights the importance of having a high index of suspicion in patients presenting with adverse effects to BCG vaccination and investigating the IFN-γ mediated immunity in all patients with a clinical suspicion of MSMD (21) irrespective of age. Flow cytometric evaluation is helpful in rapid diagnosis and provides important clues to the underlying genetic defect. It is also helpful for functional validation of novel genetic defects (37). With the increasing burden of MDR and XDR TB in India, awareness about MSMD will help clinicians to evaluate more patients for underlying genetic susceptibility to mycobacteria. Under the national TB program (RNTCP) microbiological confirmation is mandatory before initiation of ATT. With increasing awareness among physicians isolation of NTM/ EM while pursuing the microbiological diagnosis would prompt work up for underlying genetic defects. Knowledge of the underlying molecular defect is important not only in planning definitive therapy in the form of HSCT for severe forms of the disease but also providing genetic counseling to the affected families. Once a family member has been diagnosed with MSMD, BCG vaccination should be avoided in the next child until a genetic defect has been ruled out.

Tuberculosis still remains a major public health problem in India. With BCG vaccine given at birth and tuberculosis being endemic in India, diagnosing MSMD is critical not only for the appropriate management of the patient but also for the optimum control of tuberculosis. As seen in our cohort BCG-osis is the commonest presentation. We had high index of suspicion for BCG-osis in patients presenting with BCG adenitis, persistent AFB positivity, three episodes of MTB, strong family history of Koch, infections with Intracellular organisms like Non-typhi salmonella, Kala Azar, Histoplasma, and NTM. This has helped us in picking up cases of MSMD and instituting appropriate treatment including HSCT. This study also highlights the wide spectrum of micro-organisms seen with MSMD which is unique to our cohort. We also report higher prevalence of IFNγR2 defect in India. Autoimmunity in MSMD has not been reported any literature, presence of multiple autoimmunity in our cohort of IL-12Rβ1 requires further investigation and may indicate need for screening MSMD patients for autoimmune diseases. Flow cytometric analysis of MSMD is available at only two centers in our country this has limited functional validation in our cohort.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

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 from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the minor(s)' legal guardian/next of

kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PT, MD, and RY compiled the data, wrote, and edited the manuscript. VG, AAP, VVI, AC, ZG, SC, RA, PK, ND, BG, NF, EE, AA, AR, JD, VJ, RP, AJ, SuB, SaB, JU, NR, RR, RU, SP, HL, AA, MK, ZU, VB, and TK provided patient data and conducted clinical exploration and treatment of subjects. AD, UB, and PMK did the flow work up of MSMD cases. JB, JC, and AP provided the molecular analysis. MD, MM, SSP, and MB supervised the study, reviewed, and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Multicenter Outcome of Hematopoietic Stem Cell Transplantation for Primary Immune Deficiency Disorders in India

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Background: Hematopoietic stem cell transplantation (HSCT) is the curative option for many primary immune deficiency disorders (PID). In the last 5 years, increased awareness, availability of diagnostics based on flow cytometry, genetic testing, improved supportive care, use of reduced toxicity conditioning, and success of haploidentical donor HSCT have improved access to HSCT for children with PID in India. We present results on children with PID who underwent HSCT across India and the factors that influenced outcome.

Patients and Methods: We collected retrospective data on the outcome of HSCT for PID from seven centers. We analyzed the impact of the type of PID, conditioning regimen, time period of HSCT- before or after January 2016, graft versus host disease prophylaxis, cause of mortality and overall survival.

Results: A total of 228 children underwent HSCT for PID at a median age of 12 months (range, 1 to 220 months) with a median follow up of 14.4 months. Infants accounted for 51.3% of the cohort and the male female ratio was 3:1. SCID (25%) and HLH (25%) were the more frequent diagnoses. Matched family donor was available in 36.4% and 44.3% children had a haploidentical HSCT. Reduced and myeloablative conditioning regimens were used with 64% children receiving a treosulfan based conditioning regimen. Peripheral blood stem cells were the predominant graft source at 69.3%. The survival in infants (60.2%) was inferior to children aged over 1 year (75.7% p value = 0.01). Children with Wiskott Aldrich syndrome (74.3%) and chronic granulomatous disease (82.6%) had the best outcomes. The survival was superior in children receiving HSCT from a matched sibling (78%) versus an alternate donor HSCT (61% p value = 0.04). In the cohort

transplanted after January 2016 survival improved from 26.8% to 77.5% (p value = 0.00). Infection remains the main cause of mortality at in over 50% children. The 5-year overall survival rate was 68%.

Conclusion: Survival of children with PID undergoing HSCT in India has improved dramatically in last 5 years. Alternate donor HSCT is now feasible and has made a therapeutic option accessible to all children with PID.

Keywords: hematopoietic stem cell transplant, primary immune deficiency, conditioning, India, haploidentical

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is the curative option for many primary immune deficiency disorders (PID). Transplantation for PID requires early recognition and referral by the treating pediatrician. India has a population of over 500 million under the age of 18 years. Data from India is now available on the incidence of PID in India (1, 2). However, a significant number remain undiagnosed and would contribute to the infant and childhood mortality data. The Indian Society for Primary Immune Deficiency (ISPID) was established by a team of physicians involved in children's care in this field in March 2011 (3). Each year, awareness programs have been arranged systematically across the country through ISPID and International collaborators like Foundation for Primary Immunodeficiency Diseases (FPID) to train pediatricians to recognize PID's ten warning signs (4). In the last 5 years, centers of excellence for PID have been established to increase access to care across the country with diagnostic and management facilities at a subsidized cost. Access to technical information in this field, knowledge transfer from centers of excellence, and advances in molecular diagnosis has revolutionized care. Mutation screening is now possible for all children with PID. The first HSCT for PID in India was performed in 1998 at Vellore for a child with Wiskott Aldrich Syndrome (WAS). At present, there are over 75 centers in India performing HSCT with increasing numbers of pediatric transplant physicians leading the teams over the last 5 years, with over 1000 allogeneic transplant procedures performed each year. Advances in supportive care and access to generic drugs manufactured within the country have ensured that the programs are sustainable. Several governmental and non-governmental charitable organizations have joined hands to help children with rare diseases. Since January 2016, the number of HSCT for PID in India has increased due to high-resolution HLA typing, flow cytometry, molecular diagnosis, matched unrelated donor registries, haploidentical donors, and reduced toxicity conditioning medications being made available in the country. High-income countries report over 90% survival (5–7) in children undergoing HSCT and the survival % in India and other low to middle income countries ranges from 65% to 70 (8, 9).

We describe here, a multicenter study on the outcome of children with Primary immune deficiency that underwent hematopoietic stem cell transplantation across India.

PATIENTS AND METHODS

We retrospectively collected outcome data of children with PID undergoing HSCT at seven HSCT centers from Chennai, Vellore, Delhi, Gurgaon, and Bangalore. Retrospective data included the age at HSCT, sex of the patient, the type of PID, donor and stem cell source, conditioning regimen, and graft-versus-host disease (GVHD) prophylaxis, type of T cell depletion, cause of death and overall survival. The cause of death was analyzed with a focus on infection-related mortality. The overall survival time was defined as the time between transplantation and death from any cause. The overall survival was analyzed with regards to age, sex, diagnosis, and type of PID.

Statistical Analysis

All normally distributed continuous variables were represented as mean ± SD. Normality of data assessed by Shapiro-Wilk's test. Comparison of categorical variables was done by either Chi square test or Fisher's exact test based on the number of observations. An independent sample t- test was used to compare the continuous variables between two groups. Overall survival curve was drawn and estimates were calculated by Kaplan-Meier (KM) method. Log rank test was used to compare survival between factors. Data entry was done in Microsoft Excel 2007. Data analysis and validation was done by IBM SPSS Statistics for Windows Version 25.0, Armonk, NY: IBM corp. All 'p' values <0.05 was considered as statistically significant. Minimum outcome data form developed by ISCTR (Indian Stem Cell Registry) was used to collect data. The hospital ethics committee approved of the study and written informed consent was obtained from all patient families.

RESULTS

Demographic Data

The patient characteristics with regards to demographic data have been represented in **Table 1**. The median age of the children who underwent HSCT is 12 months with a range from 1 to 220 months. There were more boys than girls in the cohort and girls represented only a quarter of the cohort. Half the children transplanted were under the age of 1 year. The most common indications for HSCT were SCID (n = 55, 24.2%) and familial HLH (n = 61, 26.9%) which accounted for 50% of the children. The other indications were Wiskott Aldrich syndrome (n= 35, 15.4%), chronic granulomatous disease (n = 23, 10.1%),

TABLE 1 | Patient demographics.

Variable	Frequency
Age at HSCT	
Less than 1 year	117 (51.3%)
Over 1 year	111 (48.7%)
Sex	
Male	165 (72.4%)
Female	63 (27.6%)
Time period	
1998 to 2015	44 (19.3%)
2016 to 2019	184 (80.7%)
Diagnosis	
SCID	55 (24.1%)
HLH	61 (26.8%)
Others	112 (49.1%)
Donor type	
MSD	84 (36.8)
MUD	43 (18.9)
Haplo	101 (44.3)
Conditioning	
Nil	8(3.5%)
RIC	101 (44.3%)
MAC	119 (52.2%)
Chemotherapy	
Nil	8 (3.5%)
Treosulfan	146 (64%)
Other drugs	74 (32.5%)
Stem cell source	
PBSC	158 (69.3%)
Bone marrow	55 (24.1%)
Cord blood	15 (6.6%)
GvHD prophylaxis	
Calcineurin inhibitor	128 (56.1%)
T cell depletion	100 (43.9%)

leucocyte adhesion defect (n = 11, 4.5%), X linked agammaglobulinemia (n = 8, 3.5%), Mendelian susceptibility to mycobacterial disease (n = 7, 3.1%), Hyper IgM syndrome (n = 6, 2.6%), Hyper IgE syndrome (n = 5, 2.2%) and common variable immune deficiency (n = 2, 0.9%). Children with rare PIDs such as IPEX syndrome, heme oxygenase deficiency, LRBA deficiency and MHC Class II defects have also been transplanted in India (n = 15, 6.6%). Nearly 80% of the HSCT occurred in the period after January 2016 across all centers in India.

Details of HSCT

A fully matched family donor (n = 84, 36.8%) was available only for one third of the children and two thirds had an alternate donor source. Haploidentical family donor was the predominant donor source accounting for 101/228 (44.3%) of the transplantation. Despite access to matched unrelated donor registries, only 43 children (18.9%) had matched unrelated donor transplantation. Reduced intensity conditioning (n = 101, 43.3%) and myeloablative conditioning (n = 119, 52.2%) were equally represented and eight children had an unconditioned HSCT. Treosulfan based conditioning was used in 64% (n = 146) of the transplantation and in over 80% of the infants. Peripheral blood stem cells were used in over two thirds of the patients with a small cohort in the cord transplantation group. Notably, no cord blood transplantation has been performed after January 2016 in the country.

Graft versus host disease prophylaxis consisted predominantly of calcineurin inhibitors in 128 children (56.1%). Haploidentical HSCT was performed in 100 children (42.9%) with the use of post transplant cyclophosphamide in 57/100 (57%) and TCR alpha beta depletion in 43/100 (43%) children.

Variables Affecting HSCT Outcome

The impact of the demographics, donor source, conditioning regimen, and GvHD prophylaxis and have been represented in **Table 2**. The survival was superior in age over 1 year and the outcomes were similar in male and female children. Children with SCID had an inferior survival (50.9%) compared to children with Wiskott Aldrich syndrome, chronic granulomatous disease (CGD) and Hyper IgM (74.3%, 82.6% and 100% respectively). The most significant finding was the increase in the number of PID transplants from January 2016 in the country and the improved survival for children transplanted after January 2016 as shown in **Figure 1**.

The overall survival was superior for children undergoing HSCT from matched family donors at 78% compared to alternate donor

TABLE 2 | Transplant characteristics and impact on survival.

Variable	Mean survival time in months (95% CI)	Overall survival	P value (log rank test)
Age at HSCT			
Less than 1 year	95.2 (78.1–112.3)	60.2%	0.022
Over 1 year	77.8 (68.7–86.9)	75.7%	
Sex			
Male	102.4 (87.5–117.3)	64.6%	0.157
Female	86.9 (74.9–99)	77.0%	
Time period			
1998 to 2015	18.8 (3.6–33.9)	26.8%	0.0001
2016 to 2019	101.3 (4.6–92.1)	77.5%	
Diagnosis			
SCID	55.3 (40.2–70.4)	50.9%	0.009
HLH	65.8 (53.4–78.1)	68.9%	
Others	123.8 (108.1–139.5)	73%	
Donor type			
MSD	128.5 (111.4–145.8)	78%	0.023
MUD	55.7 (39–72.3)	60.5%	
Haplo	43.2 (36.3–50.1)	62.8%	
Conditioning			
Nil	41.9 (19.5–64.3)	62.5%	0.497
RIC	62.7 (52.6–72.9)	64%	
MAC	115.1 (98.4–131.8)	71.4%	
Chemotherapy			
Nil	37.4 (15.8–58.9)	55.6%	0.848
Treosulfan	69.6 (60.7–78.5)	69.2%	
Other drugs	106.4 (85.6–127.2)	65.7%	
Stem cell source			
PBSC	90 (78.8–101.3)	70.2%	0.038
Bone marrow	109.2 (82.2–136.3)	69.8%	
Cord blood	44.5 (17.6–71.3)	40.0%	
GvHD prophylaxis			
Calcineurin inhibitor	114.3 (98.8–129.7	70%	0.232
T cell depletion	44.2 (37.3–51.1)	64%	

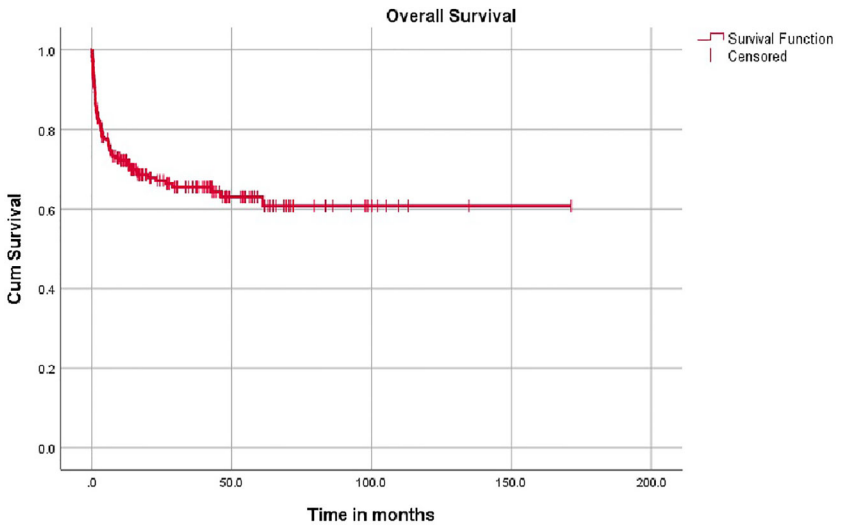

FIGURE 1 | Overall survival.

HSCT at 61% (**Figure 2**). In this mixed cohort, there was no difference in survival between myeloablative and reduced-intensity conditioning regimens. Cord as a source of stem cells resulted in poor survival of 40%. The survival in TCR alpha beta depletion 63.9% was equal to post-transplant cyclophosphamide at 64.9% (p value = 0.8) (**Figure 3**).

Infection was the leading cause of mortality at 53.9%, followed by graft versus host disease (GVHD) at 17%, graft rejection at 10.6%, and regimen related toxicity at 13.1%. A total of 11 children underwent a second HSCT for graft rejection and 8 children are alive following a second HSCT.

The most frequent presentation of disseminated BCG was a painful swelling or induration at the BCG vaccination site with axillary lymphadenitis. The other manifestations included intermittent fever, subcutaneous nodules, lesions in the liver and spleen, miliary involvement of the lung and osteomyelitis. These children were treated with a three drug regimen including isoniazid, ethambutol and levofloxacin during the transplant period. Calcineurin inhibitors interacted with rifampicin and this drug was introduced only when the immunosuppression was stopped. BCG infection is inherently resistant to pyrazinamide.

The overall mean survival time is 108 months (96.2–120.8). The 5 year overall survival rate is 68% (62%–74%) with a median follow up is 14.4 months (3.3–42.6) (IQR). Around 61% of patients had follow up of 2 years and 87.6% of patients had follow up of 5 years. Event free survival data was calculated on 118 patients with comprehensive data set was available and was 53.3%.

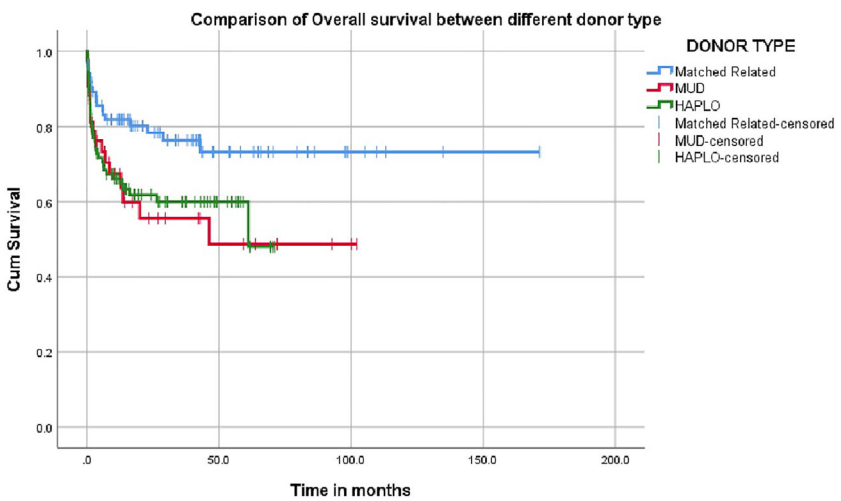

FIGURE 2 | Impact on donor source on survival.

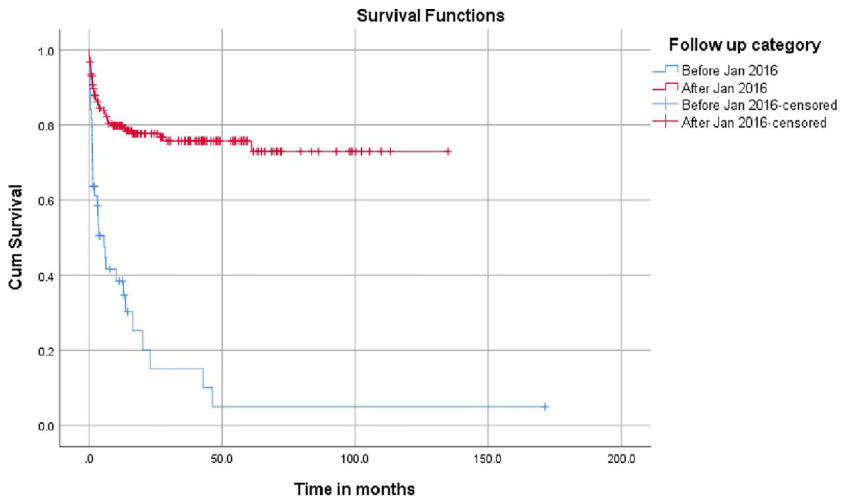

FIGURE 3 | Impact on time period on survival.

DISCUSSION

Advances in the field of HSCT have resulted in improved survival for children with PID in high-income countries (10, 11). The impact of working together as a team has been demonstrated by the Primary Immune Deficiency Treatment Consortium (PIDTC) in North America, which has supported the diagnosis and management and research in the field of PID (12–15). Similar results have been seen in the EBMT (16–18) and Latin American groups (19) with data from focused groups showing improvement in overall survival. Significant challenges in early diagnosis, supportive care and financial constraints need to be overcome in low and middle income countries as reported by groups from Brazil, Jordan, China, Iran and Turkey (20–26).

In India, the field of PID has come up in leaps and bound over the past 5 years. The formation of the Indian Society for Primary Immune Deficiency (ISPID) resulted in regular meetings conducted with the help of experts from around the world. Training of physicians involved in the diagnosis and management of PID and knowledge transfer have resulted in improved survival seen from January 2016 (27). The progress has been slow but steady, and the introduction of haploidentical HSCT allowed many children to be transplanted (28–31). HSCT in India is poised at a critical juncture where collaborative trails and research is feasible with the formal launch of ISBMT – Indian Society for Blood and Marrow Transplantation. New centers focused on HSCT for PID have been established and have reported outcomes on 21 procedures over the last 2 years with an overall survival of 60% in critically ill children requiring haploidentical HSCT (oral communication Battad et al.).

This is the first report of a multicenter data on HSCT for PID in India. All children with PID now have access to molecular diagnosis and treatment (32, 33). Although only a third of the children have a matched family donor and unrelated registries like DATRI have a donor database of over 600,000 volunteers in the country, and this has made MUD transplants a reality (34). Unrelated cord transplantation has now been phased out in all HSCT centers as delayed engraftment results in unacceptably high transplant related mortality due to bacterial infections (oral communication ISBMT). Haploidentical HSCT with PTCY and TCR alpha beta depletion show promising results although the cost of HSCT is high with TCR alpha beta depletion (35, 36). Haploidentical HSCT with TCR alpha beta depletion will continue to hold a place in HSCT performed for infants and children with significant comorbidity despite the high costs involved. These numbers would also increase once newborn screening programs for SCID are established in the country.

Primary immunodeficiencies (PID) are disorders resulting from mutations in genes involved in immune host defense and immunoregulation. Replacement of hematopoietic stem cells with full or partial ablation of the recipient's marrow with chemotherapy allows stable engraftment of donor-derived stem cell. The conditioning regimen is the key to reduce the risks of graft rejection and graft versus host disease (37–39). The use of treosulfan in 64% of the myeloablative conditioning has reduced mortality from sinusoidal obstruction syndrome. Nearly 70% of the infants undergoing HSCT were treated on the treosulfan. There are no laboratories to perform busulfan pharmacokinetics, and hence targeted busulfan therapy is not feasible and busulfan pharmacokinetics is unpredictable in infants. Generic forms of busulfan, melphalan, and thiotepa are now available in the country, which helps reduce the cost of conditioning drugs.

There has been a marked increase in the incidence of multidrug resistant bacterial infections since 2012 in the country. Multidrug resistant bacterial infections, late diagnosis and delayed referral contribute significantly to inferior outcomes in our country. Most infants have required intensive care support before transplantation due to infection as reported in published literature (40). Newer antifungal agents, such as anidulafungin and posaconazole, have helped combat fungal infections in this

cohort. Ganciclovir, valganciclovir, and cidofovir are available for the treatment of CMV infection. Disseminated BCG remains a challenge as all babies are vaccinated with BCG at birth. Over 80% of the children with PID required prolonged antitubercular drugs to eliminate BCG infection with steroid cover during immune reconstitution syndrome (IRIS).

Graft versus host disease resulted in mortality in 17% of the children and graft rejection in 10.6%. Newer therapies like ruxolitinib and extracorporeal photopheresis are now available and will help reduce mortality from graft versus host disease. Follow-up of these children requires optimal communication with the shared care pediatrician for vaccination, early treatment of infections, and evaluation for late effects. Graft rejection, the need for immunoglobulin replacement and chronic graft versus host disease and poor immune reconstitution resulted in poor quality of life in about 15% of our survivors.

Female children comprised only 27.6% of the cohort and this reflects gender inequality issues that have persisted for decades in the community where expensive therapy like HSCT are preferentially offered to a male child. The survival in infants with SCID has been poor due to late presentation due to delayed diagnosis and lack of financial support. HSCT for children with XLA and CVID is on the increase in the country as lifelong immunoglobulin replacement is a major therapeutic challenge in all low income countries (41, 42). Newer options like gene therapy require specialized infrastructure and funding. Several groups in India are now engaged in gene therapy research to provide a financially viable option for families with rare genetic disorders (43, 44).

CONCLUSION

The survival of children with PID undergoing HSCT in India has improved dramatically in last 5 years. Alternate donor HSCT has made HSCT accessible to children with PID. The overall survival of 68% is lower than centers in high-income countries. There is an urgent need to improve our services in early diagnosis, newborn screening, supportive care and the introduction of novel technologies like indigenous gene therapy and gene editing.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Apollo Speciality Hospital, Chennai, India. 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. BR helped with data analysis and all other authors delivered patient care and contributed data.

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