Progress in Rare Inflammatory Primary Immunodeficiency Diseases

Progressie in zeldzame inflammatoire primaire immuundeficiënties

Narissara Suratannon

PS Suratannon_BW V3 _stand.job_08/07/2023_1A

This PhD program was supported by the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

The studies described in the thesis were performed at the Laboratory Medical Immunology, Department of Immunology, University Medical Center Rotterdam, the Netherlands

The research for this thesis was achieved in the framework of the Erasmus MC Molecular Medicine Post Graduate School

The printing of this thesis was supported by:

Erasmus MC and Department of Internal Medicine.

ISBN/EAN: 978 90 361 0713 6

Illustrations: created with BioRender.com

Cover: N. Suratannon (partly created with BioRender.com)

Layout: N. Suratannon

Printing: Haveka BV Albasserdam

Progress in Rare Inflammatory Primary Immunodeficiency Diseases

Progressie in zeldzame inflammatoire primaire immuundeficiënties

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. A.L. Bredenoord

en volgens besluit van de College voor Promoties.

De openbare verdediging zal plaatsvinden op

dinsdag 12 september 2023 om 15.30 uur
door
Narissara Suratannon

geboren te Bangkok, Thailand

Erasmus University Rotterdam

PS Suratannon_BW V3 _stand.job_08/07/2023_2A

Promotiecommissie

Promotoren:	Prof.dr. P.M. van Hagen
	Prof.dr. P.J. van der Spek
Overige leden:	Prof.dr. A.M.C. van Rossum
	Prof.dr. F.J. Staal
	Dr. N. Mahlaoui
Copromotoren:	Dr. W.A. Dik
	Dr. V.A.S.H. Dalm

Table of Contents

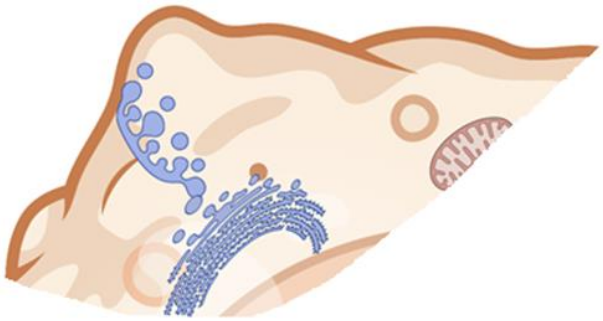
Chapter 1	General introduction	9
	1.1 Introduction to Inborn Errors of Immunity (IEI)	10
	1.2 The Diagnostic Work-Up for	12
	1.3 Outline and Aim of the Thesis	36
PART I	**Development of affordable techniques for the diagnosis of Inborn Errors of Immunity**	39
Chapter 2	Phenotypic Heterogeneity and Genotypic Spectrum of Inborn Errors of Immunity Identified through Whole Exome Sequencing in a Thai Patient Cohort	41
Chapter 3	Rapid Low-Cost Microarray-based Genotyping for Genetic Screening in Primary Immunodeficiency	61
Chapter 4	Microarray Bioinformatics	79
Chapter 5	Pediatric Prediction Model for Low Immunoglobulin G level based on Serum Globulin and Illness Status	93
Part II	**Description and functional analysis of monogenetic IEIs with immunological, including allergy and autoimmunity and non-immunological comorbidities**	111
Chapter 6	Hypomorphic Mutations in *DOCK8*: A Diagnostic Dilemma	113
Chapter 7	Recurrent Lymphomas in patients with a *DOCK8* Mutations	131
Chapter 8	"Neuroblastoma amplified sequence" (*NBAS*) gene mutations; a follow up of more than 30 years	141
Chapter 9	Adaptive immune defects in a patient with leukocyte adhesion deficiency type III with a novel mutation in *FERMT3*	155
Chapter 10	Delayed Graft Activity after Bone Marrow Transplantation in a Patient with *Phosphoglucomutase 3* mutation	163
Part III	**Inborn Errors of Immunity are frequently monogenic disorders, do allergies have a monogenic background?**	175
Chapter 11	A germline *STAT6* gain-of-function variant is associated with early-onset allergies	177
Part IV	**General discussion and Future Perspectives in Inborn Errors of Immunity**	195
	Inborn Errors of Immunity (IEI); genetic analysis in perspective	196
	Current and Future therapies in IEIs	200
Summary of the thesis		213
	English summary	214
	Nederlandse samenvatting	216

ADDENDUM 219
Acknowledgements 220
Awards and Honors 223
PhD Portfolio 224
Publications 230

APPENDIX
COVID-19 in Children: Heterogeneity within the Disease and Hypothetical Pathogenesis 235

PART I

Development of affordable techniques for the diagnosis of Inborn Errors of Immunity



Chapter 2

Phenotypic Heterogeneity and Genotypic Spectrum of Inborn Errors of Immunity Identified through Whole Exome Sequencing in a Thai Patient Cohort

Maliwan Tengsujaritkul, **Narissara Suratannon***, Chupong Ittiwut, Rungnapa Ittiwut, Pantipa Chatchatee, Kanya Suphapeetiporn, Vorasuk Shotelersuk*

**These authors contributed equally to this study*

Pediatr Allergy Immunol. 2022 Jan;33(1):e13701.

Abstract

Background: Inborn errors of immunity (IEI) comprise more than 400 rare diseases with potential life-threatening conditions. Clinical manifestations and genetic defects are heterogeneous and diverse among populations. Here, we aimed to characterize the clinical, immunological and genetic features of Thai pediatric patients with IEI. The use of whole exome sequencing (WES) in diagnosis and clinical decision making was also assessed.

Methods: 36 unrelated patients with clinical and laboratory findings consistent with IEI were recruited from January 2010 to December 2020. WES was performed to identify the underlying genetic defects.

Results: The median age of disease onset was 4 months (range; 1 month to 13 years) and 24 were male (66.7%). Recurrent sinopulmonary tract infection was the most common clinical presentation followed by septicemia, and severe pneumonia. Using WES, we successfully identified the underlying genetic defects in 18 patients (50%). Of the 20 variants identified, six have not been previously described (30%). According to the International Union of Immunological Societies (IUIS), 38.9% of these detected cases (7/18) were found to harbor variants associated with genes in combined immunodeficiencies with associated or syndromic features (Class II).

Conclusion: The diagnostic yield of WES in this patient cohort was 50%. Six novel genetic variants in IEI genes were identified. The clinical usefulness of WES in IEI was demonstrated, emphasizing it as an effective diagnostic strategy in these genetically heterogeneous disorders.

Key Message

Inborn errors of immunity (IEI) are a heterogeneous group of more than 400 monogenic disorders caused by defects in genes responsible for different components of the immune system. This study is the first and largest to investigate genetic causes in pediatric IEI cases in the Thai population. Exome sequencing was successfully performed to identify the genetic defects in 50% of cases. Our findings demonstrated the genetic and phenotypic heterogeneity of PIDs supporting the use of WES in diagnosis and clinical decision making. In addition, of all the 20 variants found to be associated with the diseases, six (30%) were novel expanding the genotypic spectrums of IEI.

INTRODUCTION

Inborn errors of immunity (IEI) are a heterogeneous group of more than 400 monogenic disorders caused by defects in genes responsible for different components of the immune system. IEI have phenotypic and genetic heterogeneity with varying degrees of immunodeficiency and immune dysregulation.[1,2] With the advent of next generation sequencing (NGS), the number of novel variants in known genes and newly identified genes responsible for IEI has been increasing rapidly.[3-5] This has expanded our understanding of genotype-phenotype correlations and provided better insights into the pathogenesis of IEI. Currently, these diseases are classified into 10 different categories by the International Union of Immunological Societies (IUIS).[6,7]

Genetic testing plays a vital role in the diagnosis and management of patients suspected with IEI. It facilitates rapid and timely diagnosis, making more precise treatment planning, leading to better patient outcomes.[4,8-10] In addition, knowing exact genetic defects can help determine the inheritance pattern and family members at risk. This genetic information could provide the basis for counselling on family planning.

The application of next generation sequencing (NGS) including whole exome sequencing (WES) and whole genome sequencing (WGS) has accelerated the discovery of novel disease-associated genes causing IEI and helped unravel disease-associated variants in several unresolved cases.[5,11,12] NGS has become a valid and cost-effective tool for diagnosis of IEI with diagnostic yield ranged from 15% to 79%.[13,14] It has been demonstrated that patients with IEI have a wide spectrum of clinical manifestations including atypical presentation and overlapping features. Therefore, WES could be used as a first-tier test for such cases[5,15] In this study, we aimed to characterize the clinical and genetic features of IEI in the Thai pediatric population. WES was performed in all cases. Our findings have expanded the phenotypic and genotypic spectrum of IEI.

METHODS

Patients

A total of 36 unrelated patients with clinical and laboratory findings suspected of IEI were recruited in the study. Most of the patients were evaluated at King Chulalongkorn Memorial Hospital from January 2010 to December 2020. Serum levels of immunoglobulins IgG, IgA, and IgM were measured using nephelometry. Serum IgE was measured using Elecsys® IgEII immunoassay (Roche, Basel, Switzerland). Flow cytometry for lymphocyte subpopulations was performed using BD Tritest™ CD3 FITC/CD4 PE/CD45PERCP, CD3 FITC/CD8 PE/CD45 PERCP, CD3 FITC/CD19 PE/CD45 PERCP, CD3 FITC/CD16, CD56 PE/CD45 PERCP (BD, California). T lymphocyte proliferation responses to phytohemagglutinin (PHA) were measured. The amount of 3H-thymidine detected in cells stimulated with PHA was divided by

3H-thymidine detected in unstimulated cells. The result was demonstrated as the stimulation index (SI). Dihydrorhodamine (DHR) flow cytometry test was used to evaluate granulocyte oxidative bursts. The SI was calculated as the ratio of geometric mean channel fluorescence intensity of phorbol myristate acetate-stimulated and unstimulated granulocytes. A healthy control was also used along with the patients' samples in the PHA stimulation test and DHR test.

Written informed consent was obtained from the patients and/or their parents. This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No.264/62) and conducted in accordance with the Declaration of Helsinki.

Whole exome sequencing and data analysis

After informed consent, three milliliters of peripheral blood were taken from the patients and their available parents. Genomic DNA was extracted from peripheral blood leukocytes using the Puregene blood kit (Qiagen, Hilden, Germany). Whole exome sequencing (WES) was performed by Macrogen, Inc (Seoul, Korea) as previously described.[16] In brief, DNA samples were prepared as an Illumina sequencing library, and in the exome capture step. The sequencing libraries were enriched by SureSelect Human All Exon V7 Kit. The captured libraries were sequenced using Illumina HiSeq 4000 Sequencer. Sequence reads were mapped against UCSC hg19 using Burrows-Wheeler Alignment (BWA) software (http://bio-bwa.source forge.net/). The single-nucleotide polymorphisms (SNPs) and Indels were detected by SAMTOOLS (http://samtools.source forge.net/) and annotated by dbSNP&1000G. A total of 1,648 genes listed in abnormality of the immune system (HP:0002715) were applied in the analysis. The variants were subsequently filtered out if they were present in our in-house database of 2,166 unrelated Thai exomes. The variants would be called novel if they were not listed in the ClinVar Miner database (https://clinvarminer.genetics.utah.edu/) and the Genome Aggregation Database (GnomAD) (https://gnomad.broadinstitute.org). Prediction software including PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (Sorting Intolerant From Tolerant; http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html), and MCAP (http://bejerano.stanford.edu/mcap/), was used to analyze the potential pathogenicity of the missense variants. In addition, for insertion and deletion variants, PROVEAN (Protein Variation Effect Analyzer; http://provean.jcvi.org) was used for protein function prediction. All novel potential causative variants were confirmed by PCR-Sanger sequencing.

RESULTS

A total of 36 patients clinically diagnosed with IEI were included and underwent WES. The median age of disease onset was 4 months (range; 1 month to 13 years) and 24 (66.7%) were male. The most frequent clinical presentation was recurrent sinopulmonary tract infections (33.3%), followed by septicemia (30.6%), and severe pneumonia (16.7%). The clinical manifestations and immunologic findings are summarized in Table 1. The clinical diagnosis distribution of all patients based on IUIS classification is shown in Figure 1A.

Whole exome sequencing (WES) was performed to investigate the genetic defects in all 36 cases. According to American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guideline for variant classification, 20 variants including pathogenic, and likely pathogenic explaining the patients' phenotypes were identified in 18 cases (50%) (Figure 1B, Table 2). Of the 20 variants identified, 6 of 20 (30%) were novel (Table 3). These variants included eight missense, six frameshift, three splicing, two nonsense and one in-frame deletion. According to IUIS classification, seven cases were found to harbor variants associated with genes in category II: combined immunodeficiencies with associated or syndromic features, four in category I: immunodeficiencies affecting cellular and humoral immunity, three in both category III: predominantly antibody deficiencies, and category IV: diseases of immune dysregulation, and one in category V: congenital defects of phagocyte number, function, or both. No variants were identified in genes classified in categories VI-X (Figure 1B). All of the patients harboring defects in genes associated with combined immunodeficiencies with associated or syndromic features, the molecular defects were able to identify by WES. (Figure 1B,1C). There were no significant differences in the number or percentage of children in other IUIS categories (Figure 1C).

Class I: Immunodeficiencies affecting cellular and humoral immunity

Four variants in different genes including *CD40LG, DOCK8*, *IL2RG,* and *RAG1* were identified in four unrelated patients with IEI (Table 2).

Patient 1 presented with disseminated cryptococcosis affecting skin, gastrointestinal tract, and blood. The leukocyte count and flow cytometry were in normal range while the immunoglobulin test showed an elevated IgM level (5.07 g/l). The diagnosis of hyper IgM syndrome was made by identifying a hemizygous variant, c.514T>C (p.Tyr172His) in *CD40LG*. This variant was inherited from the mother and previously reported in individuals with hyper IgM syndrome.[17]

Patient 2 had chronic eczema, recurrent bacterial skin infections, and recurrent pneumonia since the age of two years. He subsequently developed pulmonary tuberculosis at eight years old. His immunoglobulin test revealed an elevated IgE level (1,661 IU/ml). We identified a novel 1-bp insertion variant, c.3201dupT (p.Val1068Cysfs*3) in *DOCK8* (Figure 2a, Table 3). The valine residue 1068 is highly conserved among species, from zebrafish to humans

(Supplementary Figure 1S). Loss of function mutations in *DOCK8* are associated with autosomal recessive hyper IgE syndrome.

Patient 3 was found to have chronic diarrhea at the age of 3 months and subsequently developed severe pneumonia and sepsis. T- B+ NK+ severe combined immunodeficiency (SCID) was suspected. WES revealed a novel hemizygous variant, c.722G>A (p.Ser241Asn) in *IL2RG* which was inherited from the mother, confirming the diagnosis of X-linked severe combined immunodeficiency (X-SCID) (Figure 2b). The serine residue 241 is conserved (Supplementary Figure 1S). This variant was classified as likely pathogenic (Table 3).

Patient 4 developed pneumonia since the age of 4 months and later developed rotavirus gastroenteritis, fungal skin infections and BCGitis. The leukocyte count and immunoglobulin levels were extremely low (Table 1). T-B-NK+ SCID was suspected. WES revealed a homozygous variant, c.1871G>A (p.Arg624His) in *RAG1*. [18,19]

Class II: Combined immunodeficiencies with associated or syndromic features

Seven patients were found to carry variants in genes associated with combined deficiencies with associated or syndromic features. The variants were identified in KMT2D, PGM3, STAT3, and TTC37 (Table 2).

Patient 5 was found to have recurrent sinopulmonary tract infections and pancytopenia. She also had arched eyebrows, long palpebral fissures, depressed nasal tip, and short stature. WES revealed a de novo novel heterozygous 1-bp deletion, c.453delG (p.Gln152Argfs*56) in KMT2D that was classified as pathogenic from ACMG classification and associated with Kabuki syndrome (Figure 2C, Table 3). The glutamine residue 152 is also conserved (Supplementary Figure 1S).

Patient 6 developed recurrent sinopulmonary tract infections, severe atopic dermatitis, chronic diarrhea, and multiple food allergies since the age of two years. Low levels of CD3, CD4, CD8 and marked elevated IgE levels were observed. Using WES, two compound previously reported heterozygous variants, c.1527delC (p.Asn510Metfs*4) and c.1087A>G (p.Thr363Ala) in *PGM3* were identified, confirming the diagnosis of PGM3 deficiency.[20]

Patients 7, 8, 9, and 10 developed recurrent skin infections and recurrent pneumonia. They all had extremely elevated levels of IgE (more than 2,000 IU/ml) (Table 1). The known heterozygous variants, c.1110-2A>G, and c.1397A>G (p.Asn466Ser) in *STAT3* were found in patients 8 and 9, respectively. Two patients (patients 7 and 10) harbored the same variant (c.1909G>A; p.Val637Met).

Patient 11 had persistent diarrhea, failure to thrive with light-colored, brittle hair starting at the age of one month. While T lymphocyte numbers were normal, PHA stimulation test was markedly decreased (SI = 2, control SI = 533). WES revealed two compound heterozygous variants, c.2689delT (p.Cys897Alafs*27) and c.154G>T (p.Glu52Ter; PMID: 29527791) in TTC37. The c.2689delT (p.Cys897Alafs*27) inherited from the father has not

been previously described (Figure 2D, Table 3). The cysteine residue 897 is highly conserved (Supplementary Figure 1S). Trichohepatoenteric syndrome (THES) was diagnosed in this case.

Class III: Predominantly antibody deficiencies

Three patients (patients 12, 13, and 14) were found to harbor disease-associated variants in the *BTK* gene associated with predominantly antibody deficiencies. All were male with recurrent sinopulmonary tract infections and low levels of immunoglobulins requiring monthly intravenous immunoglobulin. One of them (patient 13) developed chronic bronchiectasis. Mutation analysis showed three hemizygous variants, c.974+5G>A, c.179_181del (p.Lys60del) and c.1635T>A (p.Tyr545*) in *BTK* in patients 12, 13 and 14, respectively. The c.1635T>A (p.Tyr545*) was novel and classified as pathogenic (Figure 2E , Table 3). The tyrosine 545 is highly conserved (Supplementary Figure 1S). The c.974+5G>A and c.179_181del (p.Lys60del) were previously described.

Class IV: Disease of immune dysregulation

There were three patients with PIDs carrying variants in the PRF1, RAB27 and UNC13D genes.

Patients 15 and 17 had sepsis with splenomegaly and cytopenia. Their clinical features were consistent with hemophagocytic lymphohistiocytosis (HLH). WES revealed a homozygous variant, c.658G>A, p.Gly220Ser in *PRF1* in patient 15 and compound heterozygous variants, c.446delG (p.Gly149Alafs*13) and c.2709+1G>A in *UNC13D* in patient 17. Both *PRF1* and *UNC13D* genes were known to be associated with familial hemophagocytic lymphohistiocytosis syndrome (Table 2).

Patient 16 was a 4-month-old boy born to consanguineous parents. He was found to have fever, progressive splenomegaly, pancytopenia, hyperferritinemia (1,856 µg/l), hypofibrinogenemia (<100 mg/dl) and hypertriglyceridemia (598 mg/dl). Bone marrow aspiration and biopsy revealed hemophagocytosis. He also had oculocutaneous albinism with silver-colored hair and eyebrows. The microscopic examination of his hair showed pigment clumps in the medullary area. A novel deletion variant, c.377delC (p.Pro126Glnfs*3) in *RAB27A* was identified by WES confirming the diagnosis of Griscelli syndrome type 2 (Figure 2f). The mother was heterozygous for this variant. The paternal DNA was unavailable. The proline residue 126 is highly conserved (Supplementary Figure S1). It is classified as pathogenic (Table 3).

Class V: Congenital defects of phagocytic number, function or both

One patient was found to carry a variant in the *G6PD* gene.

Patient 18 developed *Chromobacterium violaceum* skin infection and necrotizing pneumonia at the age of one year. An elevated white blood cell count with neutrophil predominance was seen (WBC: 46,000 cells/μL, neutrophil 80%). Dihydrorhodamine (DHR) test showed reduced oxidative burst (SI = 17.4, control SI = 110.7) with broad histograms. The DHR pattern from the mother showed bimodal distribution. WES identified a known hemizygous variant, c.496C>T, p.Arg166Cys in G6PD (Table 2) in the patient. His G6PD activity was 0 U/gHb (4.6-13.5). The variant was also identified in the mother.

DISCUSSION

We studied 36 pediatric patients with clinical and immunologic features consistent with IEI. With whole exome sequencing, as a first-tier diagnostic tool, we successfully identified pathogenic, likely pathogenic variants and variants of uncertain significance in 18 patients (50%). 30% of these identified variants have not been previously described. In addition, 38.9% of these detected variants were found in genes responsible for combined immunodeficiencies with associated or syndromic features (Class II). Our study is the first and largest to investigate the genetic defects underlying IEI using WES in the Thai population.

Due to the phenotypic and genetic heterogeneity of IEI, molecular diagnosis by NGS has become a crucial part for evaluating these patients with complex conditions. In addition, cases with atypical features or severe manifestations would require rapid and definitive diagnosis that could be possibly made by using NGS. These results can lead to appropriate decision and life-saving treatment in some patients. Patients 4 and 16 were found to carry a homozygous variant in *RAG1* and *RAB27A,* respectively. Patient 4 underwent hematopoietic stem cell transplantation (HSCT) shortly afterwards. Due to severe infection in patient 16, he has not received HSCT. Previous studies have demonstrated that overall survival rate of HLA-matched HSCT in both diseases are approximately 70%; however, poor T-cell engraftment and immune function could develop unless conditioning prior to cell infusion was given.[21-23]

We also identified a hemizygous variant in *IL2RG*, confirming the diagnosis of X-linked severe combined immunodeficiency (X-SCID) in patient 3 (Table 2). He was the second child with a healthy brother. He was expired due to severe infection before HSCT. The mother was found to be a carrier. Genetic counseling was then provided. Subsequently, the mother was pregnant for the third time. The baby boy was born at term after an uneventful pregnancy. He was found to harbor a similar variant. T-cell receptor excision circles (TRECs) obtained from peripheral blood at birth were undetectable. At age nine days, flow cytometry was performed and revealed lymphopenia with markedly low T-cell and NK cell numbers (lymphocyte 1,330 cells/μL), CD3 (0.009 x 10^9/L; normal range 2.50-5.50), CD4 (0.009 x 10^9/L; normal range 1.60-4.00), CD8 (0.009 x 10^9/L; normal range 0.56-1.70), CD19 (0.76 x 10^9/L; normal range 0.30-2.00),

and CD56 (0.08 x 10^9/L; normal range 0.17-1.10). The immunoglobulin testing also showed normal levels of serum IgG (7.68 g/l; normal range 6.31-14.31), and IgA (<0.05 g/l; normal range 0-0.08) with high IgM (0.25 g/l; normal range 0.01-0.21). Intravenous immunoglobulin (IVIG) and prophylactic medications including fluconazole, acyclovir, and co-trimoxazole were given starting at the age of 19 days. He received HSCT from his healthy 4-year-old brother at the age of two months and showed favorable outcomes. IVIG has been administered monthly and his laboratory findings at the age of one year and four months old revealed a normal absolute lymphocyte count (3,697 cells/µL), CD3 (2.96 x 10^9/L; normal range 1.46-5.44), CD4 (1.70 x 10^9/L; normal range 1.02-3.60), CD8 (0.89 x 10^9/L; normal range 0.57-2.23), CD19 (0.52 x 10^9/L; normal range 0.50-1.50), CD56 (0.15 x 10^9/L), and IgG (8.33 g/l; normal range 3.44-11.8). He is currently one year and five months old without a history of severe infection.

As shown in patient 18, patients with severe G6PD deficiency could present with recurrent infections mimicking the phenotype of chronic granulomatous disease.24,25 The reduction of granulocyte NADPH oxidase leads to the impairment of neutrophil extracellular trap (NET) formation, resulting in susceptibility to infections.26 Our patient currently receives co-trimoxazole prophylaxis with no episodes of severe infections.

There are six disease-associated variants that have not been previously described. The functional consequences of these newly identified variants require further studies. Functional validation of variants remains fundamental to attribute pathogenicity with certainty.[27]

There were 18 cases suspected of IEI with negative WES results (50%) (Figure 1B). There were no differences in severity between children who had a positive and negative WES. Of note, WES was able to identify molecular defects in all patients classified as class II and class IV. On the contrary, less than one-third of patients clinically presented with immunodeficiencies affecting cellular and humeral immunity (class I) the molecular defects were identified using WES (Figure 1). This may be due to the broad spectrum and varieties of clinical presentations in this group. WES and WGS have markedly increased the number of newly identified disease-associated genes, re-analysis of the exome data for those novel genes could lead to diagnosis in some patients.3,28,29 There is also a possibility that copy number or structural variants, variants located deep within introns, and repeat expansions could be missed by WES. It has been demonstrated that whole genome sequencing (WGS) could be used for further evaluation if the cases remained undiagnosed after WES. If potential new disease-causing genes could be identified, evaluating the validity, and performing functional studies to confirm disease-gene association and elucidate the pathophysiology underlying diseases are required. Discovering novel PID-associated genes could provide molecular insights into the pathway involved in the human immune system and expand our knowledge of the molecular mechanism underlying PIDs or immune-related disorders. This could bring new therapeutic opportunities leading to improved patient outcomes.

In conclusion, this study is the first and largest to investigate genetic causes in pediatric IEI cases in the Thai population. WES was successfully performed to identify the genetic defects in 50% of cases. Of all the 20 variants found to be associated with the diseases, six

(30%) were novel. Our findings also demonstrated the genetic and phenotypic heterogeneity of IEI supporting the use of WES in diagnosis and clinical decision making.

ACKNOWLEDGMENTS

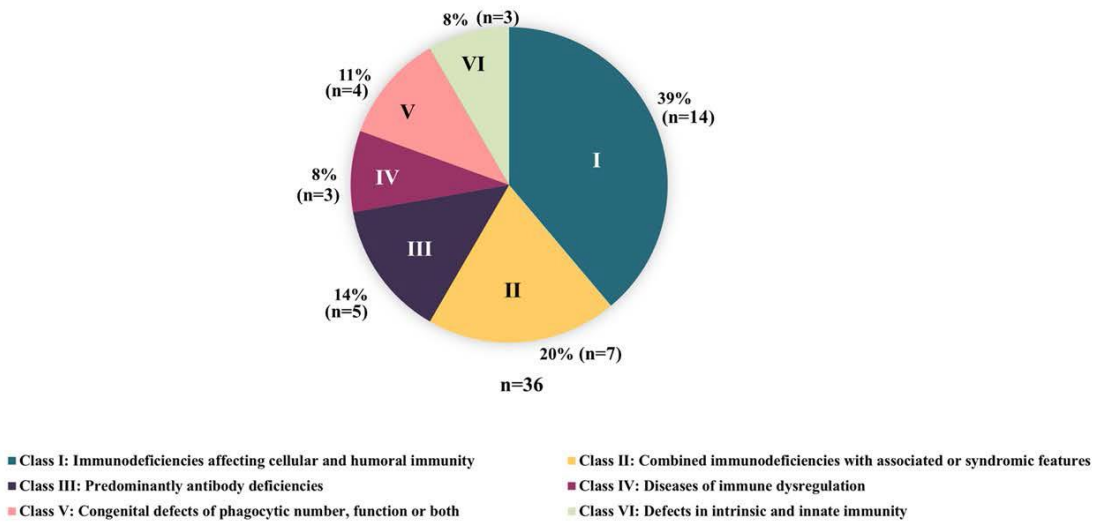
We would like to thank the patients and their families for participating in this study. This work was supported by Thailand Research Fund (BRG5980001, DPG6180001, MRG6080172), Health Systems Research Institute, TSRI Fund (CU_FRB640001_01_30_10), and Grants for Development of New Faculty Staff, Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University.

REFERENCES

1. Bousfiha A, Jeddane L, Picard C, et al. The 2017 IUIS Phenotypic Classification for Primary Immunodeficiencies. *J Clin Immunol.* 2018;38(1):129-143.
2. Notarangelo LD. Primary immunodeficiencies. *J Allergy Clin Immunol.* 2010;125(2 Suppl 2):S182-194.
3. Meyts I, Bosch B, Bolze A, et al. Exome and genome sequencing for inborn errors of immunity. *J Allergy Clin Immunol.* 2016;138(4):957-969.
4. Seleman M, Hoyos-Bachiloglu R, Geha RS, Chou J. Uses of Next-Generation Sequencing Technologies for the Diagnosis of Primary Immunodeficiencies. *Front Immunol.* 2017;8:847.
5. Stray-Pedersen A, Sorte HS, Samarakoon P, et al. Primary immunodeficiency diseases: Genomic approaches delineate heterogeneous Mendelian disorders. *J Allergy Clin Immunol.* 2017;139(1):232-245.
6. Bousfiha A, Jeddane L, Picard C, et al. Human Inborn Errors of Immunity: 2019 Update of the IUIS Phenotypical Classification. *J Clin Immunol.* 2020;40(1):66-81.
7. Tangye SG, Al-Herz W, Bousfiha A, et al. Human Inborn Errors of Immunity: 2019 Update on the Classification from the International Union of Immunological Societies Expert Committee. *J Clin Immunol.* 2020;40(1):24-64.
8. Notarangelo LD, Fleisher TA. Targeted strategies directed at the molecular defect: Toward precision medicine for select primary immunodeficiency disorders. *J Allergy Clin Immunol.* 2017;139(3):715-723.
9. Heimall JR, Hagin D, Hajjar J, et al. Use of Genetic Testing for Primary Immunodeficiency Patients. *J Clin Immunol.* 2018;38(3):320-329.
10. Castagnoli R, Delmonte OM, Calzoni E, Notarangelo LD. Hematopoietic Stem Cell Transplantation in Primary Immunodeficiency Diseases: Current Status and Future Perspectives. *Front Pediatr.* 2019;7:295.
11. Arts P, Simons A, AlZahrani MS, et al. Exome sequencing in routine diagnostics: a generic test for 254 patients with primary immunodeficiencies. *Genome Med.* 2019;11(1):38.
12. Rudilla F, Franco-Jarava C, Martinez-Gallo M, et al. Expanding the Clinical and Genetic Spectra of Primary Immunodeficiency-Related Disorders With Clinical Exome Sequencing: Expected and Unexpected Findings. *Front Immunol.* 2019;10:2325.
13. Yska HAF, Elsink K, Kuijpers TW, Frederix GWJ, van Gijn ME, van Montfrans JM. Diagnostic Yield of Next Generation Sequencing in Genetically Undiagnosed Patients with Primary Immunodeficiencies: a Systematic Review. *J Clin Immunol.* 2019;39(6):577-591.
14. Al-Herz W, Chou J, Delmonte OM, et al. Comprehensive Genetic Results for Primary Immunodeficiency Disorders in a Highly Consanguineous Population. *Front Immunol.* 2018;9:3146.
15. Platt C, Geha RS, Chou J. Gene hunting in the genomic era: approaches to diagnostic dilemmas in patients with primary immunodeficiencies. *J Allergy Clin Immunol.* 2014;134(2):262-268.
16. Ittiwut R, Sengpanich K, Lauhasurayotin S, et al. Clinical and molecular characteristics of Thai patients with ELANE-related neutropaenia. *J Clin Pathol.* 2020.
17. Athipongarporn A, Ittiwut C, Manuyakorn W, Assawawiroonhakarn S, Larbcharoensub N, Shotelersuk V. Diagnosis of Hyper IgM syndrome in a Previously Healthy Adolescent Boy Presented with Cutaneous and Cerebral Cryptococcosis. *Pediatr Infect Dis J.* 2021;40(1):e18-e20.

18. Cifaldi C, Scarselli A, Petricone D, et al. Agammaglobulinemia associated to nasal polyposis due to a hypomorphic RAG1 mutation in a 12 years old boy. *Clin Immunol.* 2016;173:121-123.
19. Alsmadi O, Al-Ghonaium A, Al-Muhsen S, et al. Molecular analysis of T-B-NK+ severe combined immunodeficiency and Omenn syndrome cases in Saudi Arabia. *BMC Med Genet.* 2009;10:116.
20. Ittiwut C, Manuyakorn W, Tongkobpetch S, et al. Compound Heterozygous PGM3 Mutations in a Thai Patient with a Specific Antibody Deficiency Requiring Monthly IVIG Infusions. *J Clin Immunol.* 2020;40(1):227-231.
21. Gennery AR, Lankester A, Inborn Errors Working Party of the European Society for B, Marrow T. Long Term Outcome and Immune Function After Hematopoietic Stem Cell Transplantation for Primary Immunodeficiency. *Frontiers in pediatrics.* 2019;7:381.
22. Greenberg-Kushnir N, Lee YN, Simon AJ, et al. A Large Cohort of RAG1/2-Deficient SCID Patients-Clinical, Immunological, and Prognostic Analysis. *Journal of clinical immunology.* 2020;40(1):211-222.
23. Al-Mofareh M, Ayas M, Al-Seraihy A, et al. Hematopoietic stem cell transplantation in children with Griscelli syndrome type 2: a single-center report on 35 patients. *Bone marrow transplantation.* 2020;55(10):2026-2034.
24. Agudelo-Flórez P, Costa-Carvalho BT, Alvaro López J, et al. Association of glucose-6-phosphate dehydrogenase deficiency and X-linked chronic granulomatous disease in a child with anemia and recurrent infections. *American journal of hematology.* 2004;75(3):151-156.
25. Tangye SG, Al-Herz W, Bousfiha A, et al. Human Inborn Errors of Immunity: 2019 Update on the Classification from the International Union of Immunological Societies Expert Committee. *J Clin Immunol.* 2020.
26. Siler U, Romao S, Tejera E, et al. Severe glucose-6-phosphate dehydrogenase deficiency leads to susceptibility to infection and absent NETosis. *J Allergy Clin Immunol.* 2017;139(1):212-219 e213.
27. Bucciol G, Van Nieuwenhove E, Moens L, Itan Y, Meyts I. Whole exome sequencing in inborn errors of immunity: use the power but mind the limits. *Curr Opin Allergy Clin Immunol.* 2017;17(6):421-430.
28. Thaventhiran JED, Lango Allen H, Burren OS, et al. Whole-genome sequencing of a sporadic primary immunodeficiency cohort. *Nature.* 2020;583(7814):90-95.
29. Sun Y, Liu F, Fan C, et al. Characterizing sensitivity and coverage of clinical WGS as a diagnostic test for genetic disorders. *BMC Med Genomics.* 2021;14(1):102.

A



B

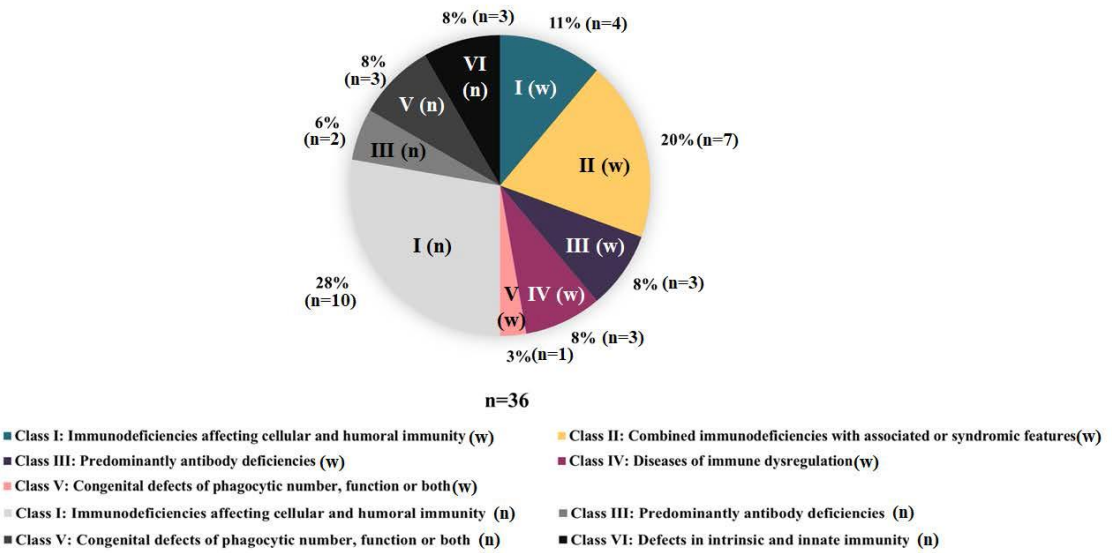


Figure 1. IEI categorized by IUIS classification**. (A)** Clinical diagnosis distribution of all patients**. (B)** Molecular findings in patients with IEI categorized by IUIS classification**.** IEI, Inborn errors of immunity; IUIS, International Union of Immunological Societies; n, negative WES; w, positive WES; WES, whole-exome sequencing

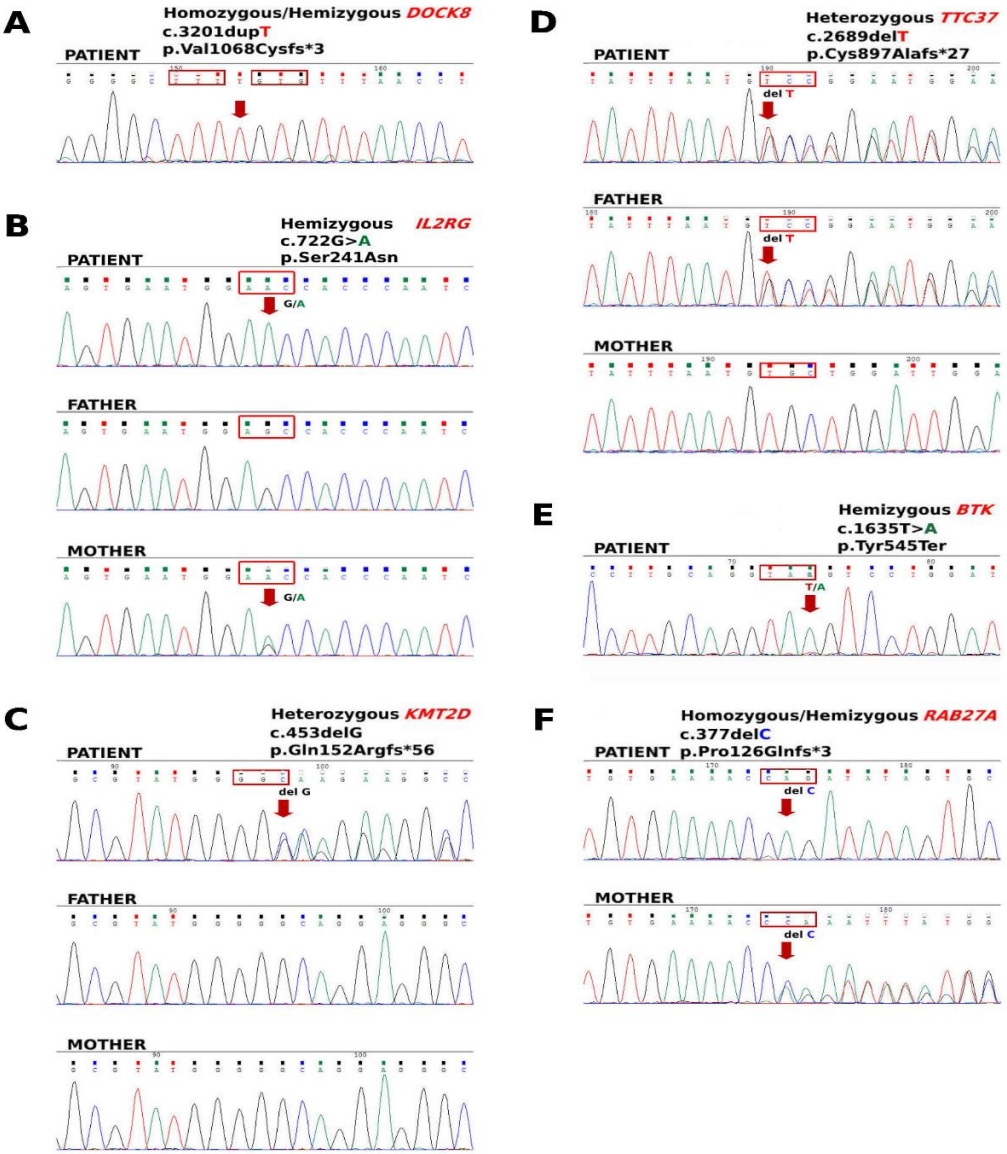


Figure 2 Electropherograms showing all six novel variants identified in the PID genes. The 1-bp insertion variant, c.3201dupT (p.Val1068Cysfs*3) in *DOCK8* was present in patient 2 **(a)**. The hemizygous variant, c.722G>A (p.Ser241Asn) in *IL2RG* was detected in patient 3 and his mother **(b)**. The *de novo* heterozygous 1-bp deletion, c.453delG (p.Gln152Argfs*56) in *KMT2D* was identified in patient 5 **(c)**. The c.2689delT (p.Cys897Alafs*27) in the *TTC37* gene was found in patient 11 and her father **(d)**. The c.1635T>A (p.Tyr545*) in *BTK* was identified in patient 14 **(e)**. The deletion variant, c.377delC (p.Pro126Glnfs*3) in *RAB27A* was present in patient 16 and his mother ***(f)***.

TABLE 1. CLINICAL FEATURES AND IMMUNOLOGIC FINDINGS IDENTIFIED IN PATIENTS WITH INBORN ERRORS OF IMMUNITY (IEI)

ID	SEX	Age at onset (Y)	Clinical manifestation	CD3 (10^9/L)	CD4 (10^9/L)	CD8 (10^9/L)	CD19 (10^9/L)	CD56 (10^9/L)	IgG (g/l)	IgM (g/l)	IgA (g/l)	IgE (IU/ml)	IUIS classification by clinical manifestation
1	M	13	Disseminated cryptococcosis	↑ 4.76 (1.40-2.00)	↑ 3.20 (0.70-1.10)	↓ 0.30 (0.60-0.90)	normal 0.30 (0.30-0.50)	normal 0.10 (0.07-0.48)	↓ 2.89 (6.98-11.94)	↑ 5.07 (0.59-0.99)	↓ 0.10 (0.22-2.74)	normal 100 (<200)	1
2	M	2	Recurrent skin and sinopulmonary tract infections	normal 1.80 (1.61-4.23)	↓ 0.77 (0.90-2.86)	normal 0.84 (0.63-1.91)	↑ 15.78 (0.70-1.30)	↑ 4.69 (0.18-0.92)	↑ 15.50 (3.44-11.80)	normal 0.67 (0.12-1.04)	↑ 1.90 (0.02-0.98)	↑↑ 1,661 (<60)	2*
3	M	3 months	Severe pneumonia and chronic diarrhea	↓ 0.26 (2.07-6.54)	↓ 0.10 (1.46-5.12)	↓ 0.06 (0.65-2.45)	↑ 9.93 (0.50-1.50)	normal 0.18 (0.17-0.83)	NA	NA	NA	NA	1
4	M	4 months	Recurrent pneumonia and fungal infections	↓↓ 0.01 (2.28-6.45)	↓↓ 0.01 (1.69-4.60)	↓↓ 0.01 (0.72-2.49)	↓↓ 0.01 (0.50-1.50)	↑ 1.86 (0.17-0.83)	↓↓ 0.04 (0.55-7.99)	↓↓ 0.01 (0.06-0.77)	↓↓ 0.01 (0-0.64)	Normal 0.2 (<15)	1
5	F	2 months	Recurrent sinopulmonary tract infections	↓ 2.02 (2.07-6.54)	↓ 1.45 (1.46-5.12)	normal 1.12 (0.65-2.45)	↑ 1.69 (0.50-1.50)	normal 0.36 (0.17-1.10)	↑ 12.60 (1.92-6.68)	↑ 1.29 (0.08-0.50)	↑ 0.76 (0-0.47)	↑ 184 (<15)	1*
6	M	2 months	Recurrent sinopulmonary tract, fungal infections, severe atopic dermatitis	↓ 0.73 (2.07-6.54)	↓ 0.30 (1.46-5.12)	↓ 0.43 (0.65-2.45)	↓ 0.30 (0.50-1.50)	normal 0.31 (0.17-1.10)	↑ 13.20 (1.92-6.68)	normal 0.50 (0.08-0.50)	normal 0.30 (0-0.47)	↑↑ 13,877 (<15)	2
7	M	1 month	Recurrent skin and sinopulmonary tract infections	↓ 0.98 (2.07-6.54)	↓ 0.40 (1.46-5.12)	↓ 0.56 (0.65-2.45)	↓ 0.01 (0.50-1.50)	normal 0.29 (0.17-1.10)	↑ 24.86 (1.92-6.68)	↑ 1.53 (0.08-0.50)	↑ 0.50 (0-0.47)	↑↑ 48,332 (<15)	2
8	M	4 months	Recurrent skin and sinopulmonary tract infections	normal 3.66 (2.28-6.45)	normal 2.08 (1.69-4.60)	normal 0.86 (0.72-2.49)	normal 1.02 (0.50-1.50)	normal 0.41 (0.17-0.83)	↑ 12.30 (0.55-7.99)	↑ 1.77 (0.06-0.77)	↑ 1.46 (0-0.64)	↑↑ 2,000 (<15)	2
9	M	1	Recurrent skin abscess	normal 4.92 (1.46-5.44)	normal 2.58 (1.02-3.60)	normal 1.94 (0.57-2.23)	↑ 7.03 (0.50-1.50)	↑ 1.30 (0.16-0.95)	↑ 20.00 (2.23-10.99)	↑ 1.62 (0.08-1.00)	↑ 1.39 (0.01-0.73)	↑↑ 38,500 (<60)	2

10	M	8	Recurrent skin and sinopulmonary tract infections	↓ 0.83 (1.40-2.00)	↓ 0.24 (0.70-1.10)	↑ 0.99 (0.60-0.90)	↑ 6.51 (0.30-0.50)	↑ 1.09 (0.10-0.48)	normal 14.19 (4.11-14.35)	↑ 1.26 (0.15-1.15)	normal 0.74 (0.34-2.14)	↑↑ 6,987 (<90)	2
11	F	1 month	Chronic diarrhea with brittle light-colored hair	normal 2.84 (2.07-6.54)	↓ 1.44 (1.46-5.12)	normal 1.22 (0.65-2.45)	↑ 2.01 (0.50-1.50)	↓ 0.07 (0.17-1.10)	normal 5.05 (1.92-6.68)	↑ 0.90 (0.08-0.50)	↑ 1.59 (0-0.47)	NA	2
12	M	1	Gram negative septicemia	↑ 7.41 (1.46-5.44)	↑ 5.21 (1.02-3.60)	↑ 5.96 (0.57-2.23)	↓↓ lower limit of detection	NA	↓ 0.07 (2.23-10.99)	normal 0.58 (0.08-1.00)	normal 0.27 (0.01-0.73)	normal 4.00 (<60)	3
13	M	4	Recurrent sinopulmonary tract infections and bronchiectasis	normal 2.30 (1.61-4.23)	normal 1.30 (0.90-2.86)	↓ 0.62 (0.63-1.91)	↓ 0.10 (0.70-1.30)	normal 0.67 (0.13-0.72)	↓ 0.07 (4.73-13.85)	↓ 0.18 (0.20-0.92)	↓ 0.06 (0.39-1.47)	normal 5.00 (<60)	3
14	M	7	Recurrent sinopulmonary tract infections	↓ 0.92* (1.40-2.00)	↓ 0.40* (0.70-1.10)	↓ 0.56* (0.60-0.90)	↓↓ 0.01* (0.30-0.50)	normal 0.35 (0.10-0.48)	↓ 1.29 (4.11-14.35)	normal 0.17 (0.15-1.15)	↓ 0.23 (0.34-2.14)	normal 18.50 (<90)	3
15	F	2 months	Hemophagocytic lymphohistiocytosis	NA	NA	NA	NA	NA	NA	NA	NA	NA	4
16	M	4 months	Hemophagocytic lymphohistiocytosis with silver-colored hair and eyebrows	↓ 1.13 (2.28-6.45)	↓ 0.66 (1.69-4.60)	↓ 0.45 (0.72-2.49)	NA	NA	↑ 9.09 (0.55-7.99)	↑ 1.51 (0.06-0.77)	↑ 1.12 (0-0.64)	↑ 32 (<15)	4
17	M	11 months	Hemophagocytic lymphohistiocytosis	NA	NA	NA	NA	NA	NA	NA	NA	NA	4
18	M	1	Necrotizing pneumonia	normal 1.79 (1.46-5.44)	↓ 0.95 (1.02-3.60)	normal 0.81 (0.57-2.23)	↑ 3.71 (0.50-1.50)	↑ 7.19 (0.18-0.92)	normal 10.90 (2.23-10.99)	↑ 1.09 (0.08-1.00)	normal 0.61 (0.01-0.73)	NA	5
19	F	1 month	Subdural empyema	↓ 0.25 (2.07-6.54)	↓ 1.19 (1.46-5.12)	normal 1.24 (0.65-2.45)	normal 0.51 (0.50-1.50)	normal 0.32 (0.17-1.10)	normal 3.97 (1.92-6.68)	↑ 0.55 (0.08-0.50)	normal 0.26 (0-0.47)	NA	1
20	F	3 months	Disseminated tuberculosis	normal 5.60	normal 1.93	↑ 3.17	normal 1.16	↑ 1.16	↑ 11.70	↑ 1.90	normal 0.41	↑ 53.30	1

				(2.07-6.54)	(1.46-5.12)	(0.65-2.45)	(0.50-1.50)	(0.17-1.10)	(1.92-6.68)	(0.08-0.50)	(0-0.47)	(<15)	
21	M	5	Disseminated varicella infection	normal 1.95 (1.61-4.23)	↓ 0.88 (0.90-2.86)	normal 0.91 (0.63-1.91)	↓ 0.54 (0.70-1.30)	↑ 2.70 (0.13-0.72)	normal 11.37 (4.73-13.85)	↓ 0.12 (0.20-0.92)	↑ 2.23 (0.39-1.47)	↑ 476 (<60)	1
22	F	8 months	Meningitis	normal 2.40 (2.28-6.45)	normal 1.99 (1.69-4.60)	↓ 0.38 (0.72-2.49)	normal 1.37 (0.50-1.50)	NA	↓ 0.22 (2.23-10.99)	normal 0.19 (0.08-1.00)	normal 0.24 (0.01-0.73)	NA	1
23	M	6 months	Recurrent sinopulmonary tract infections and bronchiectasis	↓ 0.37 (2.28-6.45)	↓ 0.15 (1.69-4.60)	↓ 0.19 (0.72-2.49)	↓ 0.36 (0.50-1.50)	↓ 0.16 (0.17-0.83)	↑ 15.50 (0.55-7.99)	↑ 0.78 (0.06-0.77)	↑ 0.76 (0-0.64)	↑ 12.50 (<15)	1
24	F	1	Severe pneumonia	NA	NA	NA	NA	NA	NA	NA	NA	NA	1
25	F	4 months	Pneumocystis pneumonia	↓ 1.96 (2.28-6.45)	↓ 1.12 (1.69-4.60)	normal 0.78 (0.72-2.49)	↑ 2.20 (0.50-1.50)	↑ 3.20 (0.17-0.83)	normal 7.22 (0.55-7.99)	normal 0.17 (0.06-0.77)	normal 0.06 (0-0.64)	↑ 39.90 (<15)	1
26	M	11 months	Recurrent sinopulmonary tract infections	↓ 0.70 (1.46-5.44)	↓ 0.54 (1.02-3.60)	↓ 0.14 (0.57-2.23)	normal 1.43 (0.50-1.50)	normal 0.60 (0.18-0.92)	↓ 2.10 (2.23-10.99)	normal 0.17 (0.08-1.00)	normal 0.10 (0.01-0.73)	normal 11.6 (<60)	1
27	F	1 month	Disseminated staphylococcal infection	↑ 8.68 (2.07-6.54)	↑ 6.21 (1.46-5.12)	normal 2.04 (0.65-2.45)	normal 0.86 (0.50-1.50)	normal 0.96 (0.17-1.10)	normal 3.22 (1.92-6.68)	normal 0.42 (0.08-0.50)	normal 0.06 (0-0.47)	↑↑ 32,100 (<15)	1
28	M	3 months	Severe pneumonia with psoriasis	↓ 0.91 (2.07-6.54)	↓ 0.67 (1.46-5.12)	↓ 0.44 (0.65-2.45)	↓ 0.13 (0.50-1.50)	NA	↓ 1.60 (1.92-6.68)	↑ 0.51 (0.08-0.50)	normal 0.26 (0-0.47)	↑ 284 (<15)	1
29	F	1 month	Recurrent skin abscess	↓ 0.89 (2.07-6.54)	↓ 0.75 (1.46-5.12)	↓ 0.14 (0.65-2.45)	normal 0.70 (0.50-1.50)	NA	normal 5.08 (1.92-6.68)	↓ 0.05 (0.08-0.50)	normal 0.05 (0-0.47)	NA	3
30	M	1	Recurrent sinopulmonary tract infections and bronchiectasis	normal 2.54 (1.46-5.44)	normal 1.53 (1.02-3.60)	normal 0.88 (0.57-2.23)	NA	NA	normal 3.05 (2.23-10.99)	↓↓ 0.01 (0.08-1.00)	normal 0.20 (0.01-0.73)	NA	3
31	M	1	Severe pneumonia	NA	NA	NA	NA	NA	NA	NA	NA	NA	5
32	F	1 month	Disseminated staphylococcal infection	↓↓	↓↓	↓↓	↓↓	↓↓ lower limit of	normal 6.14 (1.92-6.68)	normal 0.40 (0.08-0.50)	normal 0.26 (0-0.47)	↑ 90.2 (<15)	5

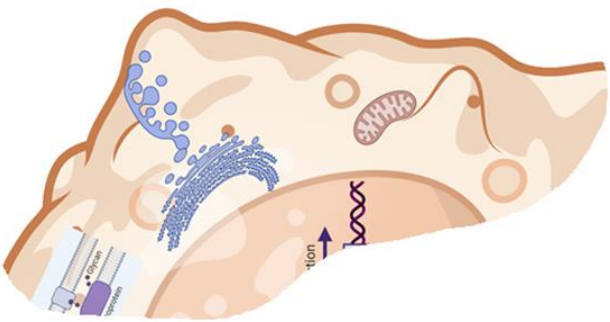
				lower limit of detection	lower limit of detection	lower limit of detection	lower limit of detection	detection					
33	F	6 months	Multiple liver and splenic abscesses secondary to Chromobacterium violaceum bacteremia	↑ 6.68 (2.28-6.45)	normal 3.34 (1.69-4.60)	↑ 3.09 (0.72-2.49)	↓ 0.34 (0.50-1.50)	↓ 0.12 (0.17-0.83)	↑ 10.09 (0.55-7.99)	↑ 1.04 (0.06-0.77)	↑ 1.74 (0-0.64)	NA	5
34	M	4 months	Disseminated tuberculosis and candidiasis	normal 2.81 (2.28-6.45)	↓ 1.54 (1.69-4.60)	normal 1.07 (0.72-2.49)	↓ 0.11 (0.50-1.50)	↓ 0.16 (0.17-0.83)	↑ 14.65 (0.55-7.99)	↑ 0.89 (0.06-0.77)	↑ 1.21 (0-0.64)	↑↑ 2,246 (<15)	6
35	F	13	Recurrent Salmonella septicemia with osteomyelitis	NA	NA	NA	NA	NA	NA	NA	NA	NA	6
36	M	1 month	Recurrent herpes infections	normal 2.61 (2.07-6.54)	↓ 1.36 (1.46-5.12)	normal 0.78 (0.65-2.45)	↑ 3.08 (0.50-1.50)	normal 0.23 (0.17-1.10)	↑ 18.26 (1.92-6.68)	↑ 1.95 (0.08-0.50)	↑ 2.44 (0-0.47)	↑↑ 1,297 (<15)	6

TABLE 2. DIAGNOSTIC VARIANTS IDENTIFIED IN PATIENTS WITH INBORN ERRORS OF IMMUNITY (IEI)

ID	Gene	Transcript ID	Variant (s)	Source	Inheritance	Type(s)	Reported in	ACMG	Molecular diagnosis	Zygosity	IUIS
1	*CD40LG*	NM_000074.2	c.514T>C (p.Tyr172His)	Maternal	XL	Missense	Athipongarporn A *et al.* 2021	LP	Hyper IgM syndrome	Hemi	1
2	*DOCK8*	NM_203447.3	c.3201dupT (p.Val1068Cysfs*3)	NA	AR	Frameshift	Novel	P	DOCK8 deficiency	Homo	1*
3	*IL2RG*	NM_000206.2	c.722G>A (p.Ser241Asn)	Maternal	XL	Missense	Novel	LP	Severe combined immunodeficiency	Hemi	1
4	*RAG1*	NM_000448.3	c.1871G>A (p.Arg624His)	Paternal Maternal	AR	Missense	Cifaldi C *et al.* 2016	LP	Severe combined immunodeficiency	Homo	1
5	*KMT2D*	NM_003482.3	c.453delG (p.Gln152Argfs*56)	*De novo*	AD	Frameshift	Novel	P	Kabuki syndrome	Het	2*
6	*PGM3*	NM_001199917.2	c.1527delC (p.Asn510Metfs*4) c.1087A>G (p.Thr363Ala)	Paternal Maternal	AR	Frameshift Missense	Ittiwut C *et al.* 2020 Ittiwut C *et al.* 2020	P P	PGM3 deficiency	Comp het	2
7	*STAT3*	NM_139276.3	c.1909G>A (p.Val637Met)	*De novo*	AD	Missense	Saikia B *et al.* 2021	P	Hyper IgE syndrome	Het	2
8	*STAT3*	NM_139276.3	c.1110-2A>G	*De novo*	AD	Splicing	Woellner C *et al.* 2010	P	Hyper IgE syndrome	Het	2
9	*STAT3*	NM_139276.3	c.1397A>G (p.Asn466Ser)	Paternal	AD	Missense	Minakawa S *et al.* 2016	P	Hyper IgE syndrome	Het	2
10	*STAT3*	NM_139276.3	c.1909G>A (p.Val637Met)	*De novo*	AD	Missense	Saikia B *et al.* 2021	P	Hyper IgE syndrome	Het	2
11	*TTC37*	NM_014639.4	c.2689delT (p.Cys897Alafs*27) c.154G>T (p.Glu52Ter)	Paternal Maternal	AR	Frameshift Nonsense	Novel Chong JH *et al.* 2015	P P	Trichohepatoenteric syndrome	Comp het	2
12	*BTK*	NM_000061.2	c.974+5G>A	Maternal	XL	Splicing	Conley ME *et al.* 2005	P	X-linked agammaglobulinemia	Hemi	3
13	*BTK*	NM_000061.2	c.179_181delAGA (p.Lys60del)	Maternal	XL	In-frame	Doğruel D *et al.* 2019	P	X-linked agammaglobulinemia	Hemi	3
14	*BTK*	NM_000061.2	c.1635T>A (p.Tyr545*)	NA	XL	Nonsense	Novel	P	X-linked agammaglobulinemia	Hemi	3
15	*PRF1*	NM_005041.4	c.658G>A (p.Gly220Ser)	Paternal Maternal	AR	Missense	Pronicka E *et al.* 2016	LP	Familial hemophagocytic lymphohistiocytosis	Homo	4
16	*RAB27A*	NM_004580.4	c.377delC (p.Pro126Glnfs*3)	Maternal	AR	Frameshift	Novel	P	Griscelli syndrome	Homo/hemi	4
17	*UNC13D*	NM_199242.2	c.2709+1G>A c.446delG (p.Gly149Alafs*13) (rs758813224)	Paternal Maternal	AR	Splicing Frameshift	Liu D *et al.* 2017 https://www.ncbi.nlm.nih.gov/snp/rs758813224?vertical_tab=true	P P	Familial hemophagocytic lymphohistiocytosis	Comp het	4
18	*G6PD*	NM_001042351.1	c.496C>T (p.Arg166Cys)	Maternal	XL	Missense	Moradkhani K *et al.* 2012	LP	G6PD deficiency	Hemi	5

TABLE 3. NOVEL VARIANTS IDENTIFIED IN PATIENTS WITH INBORN ERRORS OF IMMUNITY (IEI)

	Patient 2	**Patient 3**	**Patient 5**	**Patient 11**	**Patient 14**	**Patient 16**
Gene	*DOCK8*	*IL2RG*	*KMT2D*	*TTC37*	*BTK*	*RAB27A*
Chromosome location	chr9:399226dupT	chrX:70329113 C>T	chr12:49448147 delC	chr5:94850573 delA	chrX:100608973 A>T	chr15:55516177 delG
Variant	c.3201dupT (p.Val1068Cysfs*3)	c.722G>A (p.Ser241Asn)	c.453delG (p.Gln152Argfs*56)	c.2689delT (p.Cys897Alafs*27)	c.1635T>A (p.Tyr545*)	c.377delC (p.Pro126Glnfs*3)
SIFT[a]	NA	Damaging (0.00)	NA	NA	NA	NA
PROVEAN[b]	NA	Deleterious (-2.88)	NA	NA	NA	NA
Polyphen-2[c]	NA	Probably damaging (1.00)	NA	NA	NA	NA
M-CAP[d]	NA	Possibly pathogenic (0.818)	NA	NA	NA	NA
CADD[e]	33	26.4	23.2	33	35	33
GnomAD	None	None	None	None	None	None
In-house database	None	None	None	None	None	None
Classification†	Pathogenic	Likely pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic
Expected consequences	Frameshift leading to a valine to cysteine substitution at 1068 and a premature stop codon at position 3 of the new reading frame	A serine to asparagine substitution at 241 (fibronectrin III domain)	Frameshift leading to a glutamine to arginine substitution at 152 and premature stop codon at position 56 of the new reading frame	Frameshift leading to a cysteine to alanine substitution at 897 and premature stop codon at position 27 of the new reading frame	A tyrosine to stop codon change at position 545 predicted to result in truncated protein products	Frameshift leading to a proline to glutamine substitution at 126 and premature stop codon at position 3 of the new reading frame



Chapter 3

Rapid Low-Cost Microarray-based Genotyping for Genetic Screening in Primary Immunodeficiency

Narissara Suratannon*, Rogier T.A. van Wijck*, Linda Broer, Laixi Xue, Joyce B.J. van Meurs , Barbara H. Barendregt, Mirjam van der Burg, Willem A. Dik, Pantipa Chatchatee, Anton W. Langerak, Sigrid M.A. Swagemakers, Jacqueline A.C. Goos, Irene M.J. Mathijsse, Virgil A.S.H. Dalm, Kanya Suphapeetiporn, Kim C. Heezen, Jose Drabwell, André G. Uitterlinden, Peter J. van der Spek, P. Martin van Hagen and the South East Asia Primary Immunodeficiencies (SEAPID) Consortium*

**These authors contributed equally to this study*

Front Immunol. 2020 Apr 15;11:614. doi: 10.3389/fimmu.2020.00614. eCollection 2020.

Abstract

Background: Genetic tests for primary immunodeficiency disorders (PIDs) are expensive, time-consuming, and not easily accessible in developing countries. Therefore, we studied the feasibility of a customized single nucleotide variant (SNV) microarray that we developed to detect disease-causing variants and copy number variation (CNV) in patients with PIDs for only 40 Euros.

Methods: Probes were custom-designed to genotype 9,415 variants of 277 PID-related genes and were added to the genome-wide Illumina Global Screening Array (GSA). Data analysis of GSA was performed using Illumina GenomeStudio 2.0, Biodiscovery Nexus 10.0, and R-3.4.4 software. Validation of genotype calling was performed by comparing the GSA with whole-genome sequencing (WGS) data of 56 non-PID controls. DNA samples of 95 clinically diagnosed PID patients, of which 60 patients (63%) had a genetically established diagnosis (by Next-Generation Sequencing (NGS) PID panels or Sanger sequencing), were analyzed to test the performance of the GSA. The additional SNVs detected by GSA were validated by Sanger sequencing.

Results: Genotype calling of the customized array had an accuracy rate of 99.7%. The sensitivity for detecting rare PID variants was high (87%). The single sample replication in two runs was high (94.9%). The customized GSA was able to generate a genetic diagnosis in 37 out of 95 patients (39%). These 37 patients included 29 patients in whom the genetic variants were confirmed by conventional methods (26 patients by SNV and 3 by CNV analysis), while in 8 patients a new genetic diagnosis was established (6 patients by SNV and 2 patients suspected for leukemia by CNV analysis). Twenty-eight patients could not be detected due to the limited coverage of the custom probes. However, the diagnostic yield can potentially be increased when newly updated variants are added.

Conclusion: Our robust customized GSA seems to be a promising first-line rapid screening tool for PIDs at an affordable price, which opens opportunities for low-cost genetic testing in developing countries. The technique is scalable, allows numerous new genetic variants to be added, and offers the potential for genetic testing not only in PIDs, but also in many other genetic diseases.

Introduction

Primary immunodeficiency disorders (PIDs) are a heterogeneous group of diseases, including more than 400 distinct monogenic inherited disorders, that affect the development and function of the immune system (21, 22) (23). Obtaining a genetic diagnosis in PID patients is crucial for providing an optimal standard of care and personalized treatment tailored to specific molecular defects (24). Current genetic diagnostic approaches for PIDs are based on Sanger sequencing, next-generation sequencing (NGS), and copy number variant (CNV) analysis. However, these techniques are time-consuming, costly and involve complicated data interpretation. Due to high costs and resource limitations, not all genetic tests are available in developing countries; therefore, rapid, robust, and inexpensive molecular tools must be developed to meet this need.

Single nucleotide polymorphism (SNP) arrays are high-throughput DNA microarrays that originated from the early 2000s and are a powerful platform for simultaneously analyzing hundreds of thousands of SNPs and evaluating CNVs in a single experiment (25). Recently, the cost of SNP arrays has decreased substantially (from 300 Euro to 40 Euros per sample), driven by the very large sample sizes needed to perform genome-wide association studies (GWAS). Both Affymetrix/Thermofisher and Illumina have designed cost-effective arrays that contain ~800,000 variants, allowing a wide range of genetic variants to be assessed.

To our knowledge, this "proof of principle study" is the first to use the Illumina Global Screening Array (GSA; v1) to detect rare Mendelian mutations, consisting of single nucleotide variants (SNVs), small insertions and deletions (INDELs) and CNVs, rather than SNPs. To the multi-ethnic genome-wide GSA v1, we added 9,415 custom variants within the 277 validated PID genes listed in the International Union of Immunological Studies (IUIS) 2015 (Bousfiha et al., 2015) to capture pathogenic variants. We then analyzed 151 blood DNA samples derived from 95 patients clinically diagnosed with PIDs and 56 non-PID controls that had previously undergone whole-genome sequencing (WGS) at 80x coverage, enabling the GSA results to be technically validated by comparison with the WGS data.

Materials & Methods

Array design

The Illumina Custom GSA was produced as a research use-only tool in San Diego, USA, ~50,000 positions for a custom design. Our array contained custom content representing all variants and INDELs at the time of manufacture in the 277 PID-related genes described by IUIS 2015 (26), derived from the licensed human gene mutation database (HGMD) professional (27). Of the 10,250 variants selected, 9,415 (91.9%) were successfully placed on the array by Illumina. The aim of this custom part of the array was to identify the known PID variants by calling SNVs and CNVs in these PID genes. In addition, the Illumina GSA contains a multi-ethnic genome-wide backbone of SNPs with 696,375 probes, which enables genome-wide CNV calling.

Sample selection and DNA preparation

On the first run, a total of 95 patients clinically diagnosed with PIDs from the Erasmus Medical Center PID Biobank/Clinical Repository were randomly selected to test the performance of the array to diagnose PIDs. A detailed description of the PID patients is shown in Supplementary Table E1 and Table E2. All patients had undergone conventional genetic testing by either targeted NGS PID panel or Sanger sequencing based on their clinical phenotype. Seventy-one SNVs and/or small INDELs and four exon deletions including 3 homozygous exon deletions and 1 hemizygous deletion were discovered. A genetic diagnosis was established in 60 of the 95 patients (63%). The remaining 35 patients were unresolved, indicating the possibility of an uncharacterized genetic variant. In 7 of these 35 patients, some variants were discovered (patient no.1-7, Supplementary Table E2); however, these were not found to be sufficient enough to account for the clinical phenotype based on the inheritance pattern of the disease.

A single sample of a healthy individual that had undergone WGS sequencing was added on the first run for array validation (HapMap NA 19240). DNA was extracted from blood using a DNA Isolation Kit (Qiagen, Valencia, CA, USA).

On the second run of the array, DNA samples of 41 PID patients (that were also included in the first run) and 55 non-PID controls which had pre-existing WGS data were selected. All samples were registered by the biobanking and biomolecular resources research infrastructure (BBMRI) and/or consent for molecular diagnostic testing according to the Helsinki Guidelines.

Validation of array-based genotyping

For validation of the genotype calling of the customized array, we used one HapMap sample, which has been investigated to benchmark various WGS platforms at the Erasmus

University Medical Center (Stubbs et al., 2012) and 55 samples with pre-existing WGS data reaching an average depth of 80x. These samples were originated from unrelated parents of a genetic study of craniofacial malformations (Florisson et al., 2013). For these 56 non-PID samples that previously had undergone WGS, SNV position genotypes of the custom probes were called from the BAM files, using SAMtools/ BCFtools (Li, 2011), and were compared to the genotype called from the array for the same sample. Probes with different callings between the array and the WGS for these 56 control samples were excluded from analysis because of the possibility of probe malfunction.

Genetic Data Analysis

1. Array quality control

Two IDAT files (green, red) were generated per sample and uploaded on to Illumina GenomeStudio 2.0 software prior to quality control (QC) analysis using PLINK (v1.9) (28). QC analysis tested for genotyping efficiency per SNP at a threshold of 97.5%, with the sample call rate set at 97.5% and deviations from Hardy-Weinberg equilibrium defined as $P < 1x10^{-4}$. Additionally, zCall was performed to improve rare variant calling (29), after which a stringent SNP and sample call rate filter of 98% was applied. Ethnic origin and sex/gender were used to control for data integrity during sample processing and analysis.

2. SNV calling

Detailed variant analysis for SNV detection was performed on post-QC data using PLINK (v1.9), GenomeStudio 2.0, and R-3.4.4. Only disease-causing variants according to HGMD classifications were selected. Next, variants were filtered against the gnomAD database with a frequency threshold of below 0.5 %. Variants that occurred more than once in the dataset were excluded as controls for the effect of faulty probes, while all X-linked heterozygous calls in males were excluded, these would lead likely to false-positive results. Lastly, all remaining variants were manually checked using genotyping module SNP graphs generated by GenomeStudio 2.0 to determine whether the genotype call matched the expected genotype by their signal intensity, if not the variant was excluded.

3. CNV calling

CNV analysis was carried out using the SNP-FASST2 Segmentation Algorithm within BioDiscovery Nexus Copy Number Discovery Version 10.0 (El Segundo, USA). Large chromosomal aberrations were visually identified by making log-ratio and B-allele frequency plots in order to detect deletions, amplifications, polyploidy or long-contiguous stretch of homozygosity (LCSH). For small CNVs, the significance threshold for CNV calling within Nexus 10.0 was set at $5x10^{-6}$ requiring a minimum of 4 probes per segment and a maximum contiguous probe spacing of 1000 Kbp. Log_2 ratio values of -0.3 and -1.1 were used to detect single and more than two copy losses, respectively, while values of 0.2 and 0.7 were used to detect single and more than two copy gains. Only CNVs, that were located in the 277 PID

according to the IUIS classification genes were selected. Next, these CNVs were filtered against the Nexus database for known CNVs. For heterozygous CNVs, the overlap with known CNVs was set to 0%, whereas no threshold was used for homozygous CNVs. Lastly, a frequency filter was applied such that only CNVs occurring once in the dataset were selected to filter any CNVs that arose due to probe malfunction when capturing CNVs.

4. Validation by Sanger sequencing and calculation of diagnostic yields

Sanger sequencing was performed to validate all newly detected SNVs. The sensitivity (of previously known variants) and the overall sensitivity (including the addition of newly detected "Sanger confirmed" variants) was defined as the proportion of SNVs and CNVs that could be replicated in both techniques. The diagnostic yield was calculated at the level of patients when a genetic diagnosis could be made. Reaching a conclusive genetic diagnosis is based on identifying a genetic variant with an inheritance pattern that matches the inheritance pattern reported in the Online Mendelian Inheritance in Man (OMIM) database, on observing close correlation with the clinical PID phenotypes, and/or on mentioning of the variant in the IUIS database.

5. Assessment of the reproducibility

To assess the reproducibility of the array, we focused only on the custom content of the GSA and compared the post-QC data for the 41 overlapping PID samples in the two array runs. The genotypes acquired in the two runs were compared for the same sample.

Results

Overall technical array performance

We performed two array genotyping experiments, where DNA samples were on the array (run 1 and run 2) to allow inter-assay comparison. Of the 9,415 custom variants added to the standard GSA v1 content, 8,883 and 8,852 variants were included in the post-QC data of the custom content for the first and second runs, respectively. In the second run, 3 samples (2 PID and 1 non-PID) were excluded from further analysis as they failed to achieve the sample call rate threshold of 98%, leaving 39 PID samples for inter-assay validation and, 55 samples from non-PID healthy controls (in whom WGS was performed) for comparison of the array genotype calling with WGS genotype callings. The correlation between the genotype of the custom SNVs in the PID array and WGS data of the 55 non-PIDs was robust. Of the 8,852 post-QC variants in the second run, 1,902 probes captured small (INDELs) and 6,950 probes captured SNVs. These 6,950 variants were subsequentially checked against the WGS data (of all non-PID controls) and genotyping of 6,928 (99.7%) of the variants on the custom GSA v1 matched that acquired by WGS (at 80x coverage). Subsequently, we checked all the non-reference genotype SNV calls made by the GSA (n=2,626) as these would be identified as positive results and observed that only 11 (0.15%) did not match the genotype called by WGS. Moreover, these 2,626 calls were almost exclusively reported as benign variants/polymorphisms in HGMD.

SNV analysis in PID patients

We then analyzed the array-based genetic diagnosis in 95 patients with clinically diagnosed PIDs. Our customized GSA v1 detected 80 SNVs or small INDELs, of which 30 variants were originally detected by conventional methods (Figure 2 and Supplementary Table E3). The diagnosis was confirmed in 26 out of 60 patients who had a previously established genetic diagnosis (Supplementary Table E1). In 31 patients, the array could not replicate the genetic variants found by conventional diagnostics. Seven of these 31 patients had variants that could have been measured by the specific probes on the array, but the GSA failed to do so, because these probes did not pass the stringent QC after rare-variant calling performed with zCall. For 2 of these 31 patients, the GSA was only able to identify 1 of 2 variants as these patients were compound heterozygous. For the remaining 21 patients (29 variants as some patients had multiple variants), the GSA could not replicate the SNV and/or INDEL as the variants were not known at the time of GSA manufacturing process and therefore no probes were added to the array to investigate these variants.

Interestingly, in 35 patients whose conventional diagnostics did not find a causative genetic variant (Supplementary Table E2), our customized GSA could detect pathogenic variants and lead to a diagnosis in 6 patients. These variants included heterozygous variants in *signal transducer and activator of transcription 1 (STAT1), colony stimulation factor 3 receptor (CSF3R), tumor necrosis factor receptor superfamily member 13B (TNFRSF13B), inhibitor of nuclear factor-kappa B kinase subunit gamma (IKBKG), C-X-C chemokine receptor type 4 (CXCR-*

4), and a homozygous mutation in *vacuolar protein sorting 45 homolog (VPS45)*. These genes were not analyzed as the potential genetic cause during conventional diagnostics, possibly due to overlapping clinical phenotypes of the patients. In 7 patients some heterozygous variants were previously detected (patient no.1-7, Supplementary Table E2), the variants could be reproduced in 2 patients. For the other 5 patients, GSA could not replicate the results found from the conventional methods as the probes were not added on the array. A newly updated version of the GSA is expected to allow all these SNVs to be assessed in more detail and reveal how well they can be detected.

Next, we set out to validate the observed causal variants newly found by GSA with Sanger sequencing (n=46). For 5 variants in 3 samples, Sanger sequencing could not be performed because of lack of available DNA. For the remaining 41 variants, Sanger sequencing could confirm 38 out of 41 variants (92.7%) demonstrating a high validation rate between GSA and Sanger sequencing, although some variants yielded a different nucleotide change at a slightly different position from the investigated position with the array (n=8).

CNV analysis

The genome wide CNV analysis detected large inter- and intragenic regions of LCSH suggesting consanguinity in 39 PID samples (Supplementary Table E1 and Figure 1B). In 12 PID patients, CNV analysis based on the array genotyping data could reveal large chromosomal aberrations and microdeletions at the gene level. Three of the previously known exon deletions that were discovered during conventional diagnostics could be reproduced, including 2 patients with a homozygous *DNA cross-link repair protein 1C (DCLRE1C)* deletion **(**Figure 1A**)** and 1 patient with a hemizygous *x-linked inhibitor of apoptosis protein (XIAP)* deletion (patient no.3,6,27 in Supplementary Table E1). One patient with an established homozygous loss of i*mmunoglobin heavy constant mu (IGHM)* could not be replicated by the GSA during CNV analysis (patient no. 34 in Supplementary Table E1). Both SNV and CNV analysis detected genetic variants in one patient with a mutation in *SH2 Domain Containing 1A (SH2D1A)* (patient no.13 in Supplementary Table E1).

Interestingly, 2 PID patients without a previous genetic diagnosis were recognized as having monosomy 7, which is suspected of a hematologic malignancy rather than PID (patient no. 4,8 in Supplementary Table E2). In another patient, we identified (in a single GSA experiment) both trisomy 8 (by CNV analysis) as well as a *GATA-binding factor 2 (GATA2)* variant (by SNV calling; patient no. 26 in Supplementary Table E1 and Figure 1c, d), which is suggestive for a secondary hematologic malignancy in this patient.

Sensitivity based on variants and diagnostic yield at the patient level

In this proof of principle study, the customized GSA array could replicate 30 out of 71 previously detected SNVs. However, 34 variants could not be investigated due to limited coverage of probes at the time of GSA design; and 3 of the 4 previously detected CNVs could also be replicated. The sensitivity for identifying the known genetic variants (SNVs and CNVs) that the GSA can detect compared to conventional methods was 80% (33/41). Sanger

sequencing could confirm 38 out of 46 newly found SNVs by GSA, although 5 variants could not be investigated due to a lack of DNA in 3 patients. The overall sensitivity including the addition of these newly found variants that underwent Sanger sequencing was 71/82 (87%) (Supplementary Table E3).

At the patient level, we were able to establish a genetic diagnosis using the GSA technology in 37 out of 95 PID patients (39%). These 37 patients included 29 patients in whom conventional methods had previously detected genetic variants (26 patients by SNV and 3 by CNV analysis), but also 8 newly suspected patients (6 by SNV and 2 patients suspected from leukemia as detected by CNV analysis). In twenty-eight patients (that had established variants with conventional techniques) the variants could not be replicated by GSA due to the sparse coverage of the custom probes at the time that the array was designed and therefore these patients could not obtain a conclusive genetic diagnosis. In 20 PID patients, neither conventional nor GSA testing were able to obtain a conclusive genetic diagnosis. The detected variants leading to the diagnosis and numbers of patients in whom a diagnosis was made, comparing between conventional methods and GSA is shown in Figure 2 and Supplementary Table E3.

Reproducibility

As indicated above, 39 PID samples could be investigated for inter-assay validation in the second run. Our results demonstrated that for 37 out of 39 patients, the genetically causal variants from the first run could be replicated in the second run (94.9%) (Supplementary Table E1 and E2). Large CNVs such as large chromosomal aberrations were replicated; however, small heterozygous exon deletions did not replicate well (probably due to short primer length of the custom probes). Finally, we compared all post-QC data for the custom variants from the first run with the data generated from the second run for these 39 patients (8,883 and 8,852 variants respectively). There was overlap between 8,541 variants and we found only 13 (0.22%) differences between the genotype calls in the two runs.

Costs

The costs for NGS in one patient including analysis in a clinical diagnostic setting is about 1000 Euros. However, the costs depend strongly on the national economic system and the local health care infrastructure. The net price for WES without analysis and overhead varies from 350-600 Euros. GSA, however, can be performed for less than 10% of the WES price. With our effort, we performed the PID array test for the affordable price of about 40 Euros per sample. In this study, we were able to diagnose roughly 40% of patients by the array. Given a GSA cost of €40 per sample, initial costs for 100 patients is €4,000. The 60 remaining undiagnosed samples will undergo NGS or targeted gene panel sequencing assuming €1000 per sample. Total costs for this scenario will be roughly €64,000. On the other hand, if we use directly NGS or targeted gene panel sequencing, the costs will be €100,000. For both NGS and GSA techniques, we perform Sanger sequencing for confirmation. So, by using GSA as a screening tool in PID diagnostics we can save about 36,000 Euros per 100 patients. This makes

the diagnostic array an affordable promising candidate for initial screening analysis in the standard-of-care, in particular in developing countries, where genetic testing is not yet available.

Discussion

A *high-throughput*, *rapid* and *inexpensive* tool is required for identifying underlying genetic defects in a clinical care setting, especially in low-income countries. SNP array technology is a powerful genomic analysis tool which has been widely used at the population level, but not for detecting rare pathogenic SNV variants.

In this study, we present a comprehensive diagnostic SNP array that is able to screen at a genome-wide level, including rare PID gene variants. This method possesses potential advantages as compared to conventional targeted gene panel NGS diagnostics. As an initial screening test, the customized PID array had a high sensitivity (87%) for capturing rare mutations and had a strong reproducibility (0.22% difference in genotype callings between two runs). Moreover, we observed a high validation accuracy compared to NGS data (0.16% difference in genotype callings). This is different from a recent previous study, which found a very low association between array and NGS data. (Karczewski et al. 2019). However, there are important differences between the two studies. Firstly, their study used an Affymetrix/ Thermofisher product while ours used Illumina technology. SNP calling is based on different algorithms in these two platforms and thus might influence variant calling, particularly for rare variants. Secondly, we used the zCall algorithm to enhance rare variant calling, results in better genotype calling for this class of variants. A large number of newly found variants could also be reproduced by Sanger sequencing. However, there was some disagreement regarding the precise nucleotide change (n=8). Most likely, this is due to the hybridization of the probe when a variant (in the near vicinity) is present. This emphasizes the need for close investigation of the specific region with Sanger sequencing and further research on the quality of calling of individual probes.

Certainly, there is a clear trend towards implementing NGS in academic institutions for genetic diagnostics; however, there is also a trend towards array genotyping, particularly in direct-to-consumer companies. The basic cost price is 10-fold higher for NGS as compared to GSA arrays. NGS outputs a large quantity of data with concomitant much higher costs for storage and analysis. The overall price including analysis and overhead will be significantly higher for NGS than GSA. The price for NGS is expected to remain high over the next five years, therefore NGS will remain unaffordable in developing countries. Although NGS will still be necessary to detect novel variants, most NGS approaches for PID reported a diagnostic yield from 15-79% within a mixed PID population (Yska et al., 2019), with a diagnostic yield with our customized GSA of 39% falling within this range. A limitation of current NGS diagnostic approaches is that unless the DNA is sequenced with appropriate depth, NGS technology cannot accurately capture CNVs. Detection of CNVs increases the diagnostic yield by an average of 4.2% (Yska et al., 2019). Furthermore, there is no consensus about the interpretation of the variants revealed by NGS, whereas the interpretation of GSA output is simpler since the investigated variants are known beforehand.

The GSA has several clear advantages. The average turn-around-time for GSA is one week, therefore our economic and rapid customized PID array represents an ideal approach

to broadly detecting known pathogenic variants in a primary screen, which can be followed by further NGS analysis when no genetic diagnosis can be made. Since the GSA was designed to simultaneously capture both SNVs and CNVs in a single experiment, it may also indicate secondary malignancies in at-risk PID patients. This is illustrated in our study by the co-occurrence of a *GATA2* variant and a CNV aberration of chromosome 8, which is indicative for acute myeloblastic leukemia (Wlodarski et al., 2017). Due to the complexity and variety of clinical presentations, hematological malignancies can be misdiagnosed as PID. We also detected excessive LCSH in some of our patients, which clearly indicated consanguinity. Such LCSH areas are likely to reflect the origin of recessive diseases, such as imprinting disorders, triploidy and duplications in which the pathogenic mutations are located (24). Although not observed in this study, 22q11 microdeletion syndrome (24) and IL-25 hyperdiploidy can be discovered by CNV analysis (30). SNVs related to inflammatory and autoimmune complications in common variable immunodeficiency (CVID) were reported (31). SNV analysis is also capable of detecting a Uniparental Disomy as shown recently in a patient with *lipopolysaccharide (LPS)-responsive and beige-like anchor protein* (*LRBA*) Deficiency (32).

Finally, enriched GSA with hotspot mutations enable the screening for other immune-mediated diseases such as Blau syndrome (NOD2), mastocytosis (KIT), and as cancer driver mutations in proto-oncogenes such as *v-raf murine sarcoma viral oncogene homolog B1 (BRAF)* or tumor suppressor genes such as *tumour protein* 53 *(TP53).* Therefore, a similar approach of an enriched array with known pathogenic variants may be a promising screening tool for other genetic diseases. Although it is unlikely that the array will work better for one disease than another, trials should be performed to conclusively demonstrate the utility of arrays for detecting rare disease-causing genetic variants in a range of genetic disorders.

Despite its advantages, this customized array has some limitations. Firstly, it is unable to detect novel SNV, i.e., those that have not yet been reported in literature and/or databases, it can only detect variants that are included in the array. However, since GSA is scalable, it is possible to increase the number of variants in additional genes. The current version of our customized array contained only selected variants of 277 PID-causing genes derived from the IUIS 2015 classification (as published at the time the array was designed). The total number of known PID genes has now vastly increased, with 420 inborn errors of immunity as described in the updated IUIS 2019 classification (Tangye et al., 2020). By updating the GSA with newly found variants, the diagnostic yield is expected to improve significantly. This warrants further assessment of the GSA as a primary screening tool. Although we cannot test every probe by validation with known carriers and cannot guarantee for possible probe malfunction, we expect no technical reasons (given the high accuracy rate compared with NGS and the high validation rate compared with Sanger sequencing) why the sensitivity and diagnostic yield should not increase accordingly. Indeed, it should be possible to create a user-friendly, automated platform that analyzes data efficiently, thus benefiting medical decision making in patients with PIDs.

In conclusion, this robust customized array is a promising first-line rapid screening tool for PID and probably other genetic diseases at an affordable cost. This method provides new

perspectives for genetic testing in developing countries where PIDs are currently under-diagnosed.

Abbreviations: ***BRAF***: *v-raf murine sarcoma viral oncogene homolog B1*, ***BTK***: *bruton tyrosine kinase*, **CNV:** copy number variants, ***CSF3R***: *colony stimulation factor 3 receptor,* ***CVID***: *common variable immunodeficiency,* ***CXCR-4***: *C-X-C chemokine receptor type 4,* ***DCLRE1C***: *DNA cross-link repair protein 1C,* ***GATA2***: *gata-binding factor 2,* **GSA:** global screening array, **GWAS:** genome-wide association study, ***IKBKG***: *inhibitor of nuclear factor kappa B kinase subunit gamma,* **INDELs:** Insertions and Deletions, **IUIS**: the International Union of Immunological Studies, **LCSH:** long-contiguous stretch of homozygosity, ***LRBA***: *lipopolysaccharide (LPS)-responsive and beige-like anchor protein,* **NGS:** next-generation sequencing, **PIDs:** primary immunodeficiency disorders, **SNP:** single nucleotide polymorphism, **SNV:** single nucleotide variants, **STAT1:** signal transducer and activator of transcription 1, ***TNFRSF13B***: *tumor necrosis factor receptor superfamily member 13B,* ***TP53***: *tumor protein* 53, ***VPS45***: *vacuolar protein sorting 45 homolog,* ***WAS***; *wiskott–aldrich syndrome*, **WGS:** whole-genome sequencing, ***XIAP***: *x-linked inhibitor of apoptosis protein*

Acknowledgements & Funding: This study was supported by Internal funding of Department of Internal Medicine, Department of Pathology & Clinical Bioinformatics, Erasmus Medical Center, Rotterdam, the Netherlands and grant from H2020 ImmuneAID, Bigmedilytics, Moodstratification and TranSYS.

References

1. Bousfiha, A., Jeddane, L., Al-Herz, W., Ailal, F., Casanova, J.L., Chatila, T., et al.)2015(. The 2015 IUIS Phenotypic Classification for Primary Immunodeficiencies. *J Clin Immunol* 35)8(, 727-738. doi: 10.1007/s10875-015-0198-5.
2. Bousfiha, A., Jeddane, L., Picard, C., Ailal, F., Bobby Gaspar, H., Al-Herz, W., et al.)2018(. The 2017 IUIS Phenotypic Classification for Primary Immunodeficiencies. *J Clin Immunol* 38)1(, 129-143. doi: 10.1007/s10875-017-0465-8.
3. Goldstein, J.I., Crenshaw, A., Carey, J., Grant, G.B., Maguire, J., Fromer, M., et al.)2012(. zCall: a rare variant caller for array-based genotyping: genetics and population analysis. *Bioinformatics* 28)19(, 2543-2545. doi: 10.1093/bioinformatics/bts479.
4. Green, M., Camilleri, E., Gandhi, M.K., Peake, J., and Griffiths, L.R.)2011(. A novel immunodeficiency disorder characterized by genetic amplification of interleukin 25. *Genes and immunity* 12)8(, 663.
5. Heimall, J.R., Hagin, D., Hajjar, J., Henrickson, S.E., Hernandez-Trujillo, H.S., Tan, Y., et al.)2018(. Use of Genetic Testing for Primary Immunodeficiency Patients. *Journal of clinical immunology* 38)3(, 320-329.
6. Louhelainen, J.)2016(. SNP Arrays. *Microarrays (Basel)* 5)4(. doi:10.3390/microarrays 5040027.
7. Orange, J.S., Glessner, J.T., Resnick, E., Sullivan, K.E., Lucas, M., Ferry, B., et al.)2011(. Genome-wide association identifies diverse causes of common variable immunodeficiency. *Journal of Allergy and Clinical Immunology* 127)6(,1360-1367. e1366.
8. Picard, C., Gaspar, H.B., Al-Herz, W., Bousfiha, A., Casanova, J.-L., Chatila, T., et al.)2018(. International union of immunological societies: 2017 primary immunodeficiency diseases committee report on inborn errors of immunity. *Journal of clinical immunology* 38)1(, 96-128.
9. Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A., Bender, D., et al.)2007(. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81)3(, 559-575. doi: 10.1086/519795.
10. Soler-Palacin, P., Garcia-Prat, M., Martin-Nalda, A., Franco-Jarava, C., Riviere, J.G., Plaja, A., et al.)2018(. LRBA Deficiency in a Patient with a Novel Homozygous Mutation Due to Chromosome 4 Segmental Uniparental Isodisomy. *Front Immunol* 9, 2397. doi: 10.3389/fimmu.2018.02397.
11. Stenson, P.D., Mort, M., Ball, E.V., Evans, K., Hayden, M., Heywood, S., et al.)2017(. The Human Gene Mutation Database: towards a comprehensive repository of inherited mutation data for medical esearch, genetic diagnosis and next generation sequencing studies. *Human genetics* 136)6(, 665-677.
12. Tangye, S.G., Al-Herz, W., Bousfiha, A., Chatila, T., Cunningham-Rundles, C., Etzioni, A., et al.)2020(. Human Inborn Errors of Immunity: 2019 Update on the Classification from the International Union of Immunological Societies Expert Committee. *Journal of Clinical Immunology*, 1-41.

Supplementary material: The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00614/full#supplementary-material.

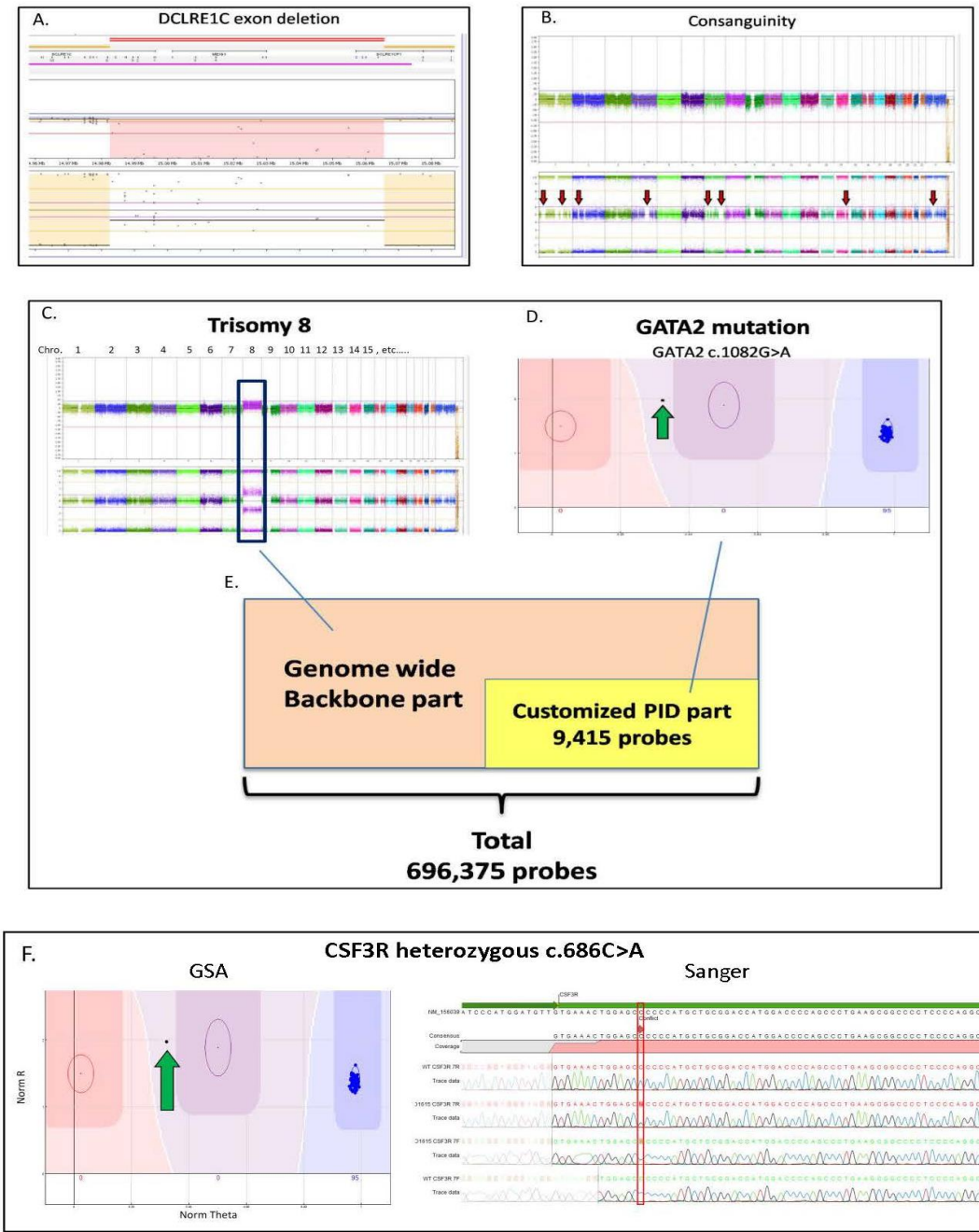


Figure 1. (A) Log-ratio plot, which shows the amount of DNA per probe (normal is two copies) and B-allele frequency plot that shows the genotype for a probe (normal is AA/AB/BB). The

log-ratio plot shows a homozygous deletion depicted by the light red area in *DCLRE1C* (*Artemis*) deletion detected by CNV analysis. The call was based on 40 consecutive probes having intensity values below the threshold.; (B) The presence of multiple regions of long-contiguous stretch of homozygosity (LCSH) in the B-allele frequency plot is suggesting consanguinity (red arrows); In a single experiment, the customized PID Global Screening Array (GSA) array identified (C) a trisomy 8 (blue box) seen in the log-ratio plot by amplification of DNA and seen in the B-allele frequency plot by polyploidy (AAA/AAB/ABB/BBB) by CNV calling along with (D) a *GATA2* mutation (green arrow) by SNV calling suggesting a secondary malignancy in the patient; (E) The customized GSA array comprising 9,415 PID related variants/ INDELs and a multi-ethnic genome-wide backbone on the entire array adds up to 696,375 probes; (F) A *CSF3R* mutation detected by SNV analysis (green arrow) was confirmed by Sanger sequencing.

Abbreviations: CNV: copy number variants, ***CSF3R****: colony stimulation factor 3 receptor,* ***DCLRE1C****:* DNA cross-link repair protein 1C, ***GATA2****: gata-binding factor 2,* ***GSA****: Global Screening Array, Sanger: Sanger sequencing,* **INDELs**: Insertions and Deletions, **PIDs**: primary immunodeficiency disorders, **SNV**: single nucleotide variants.

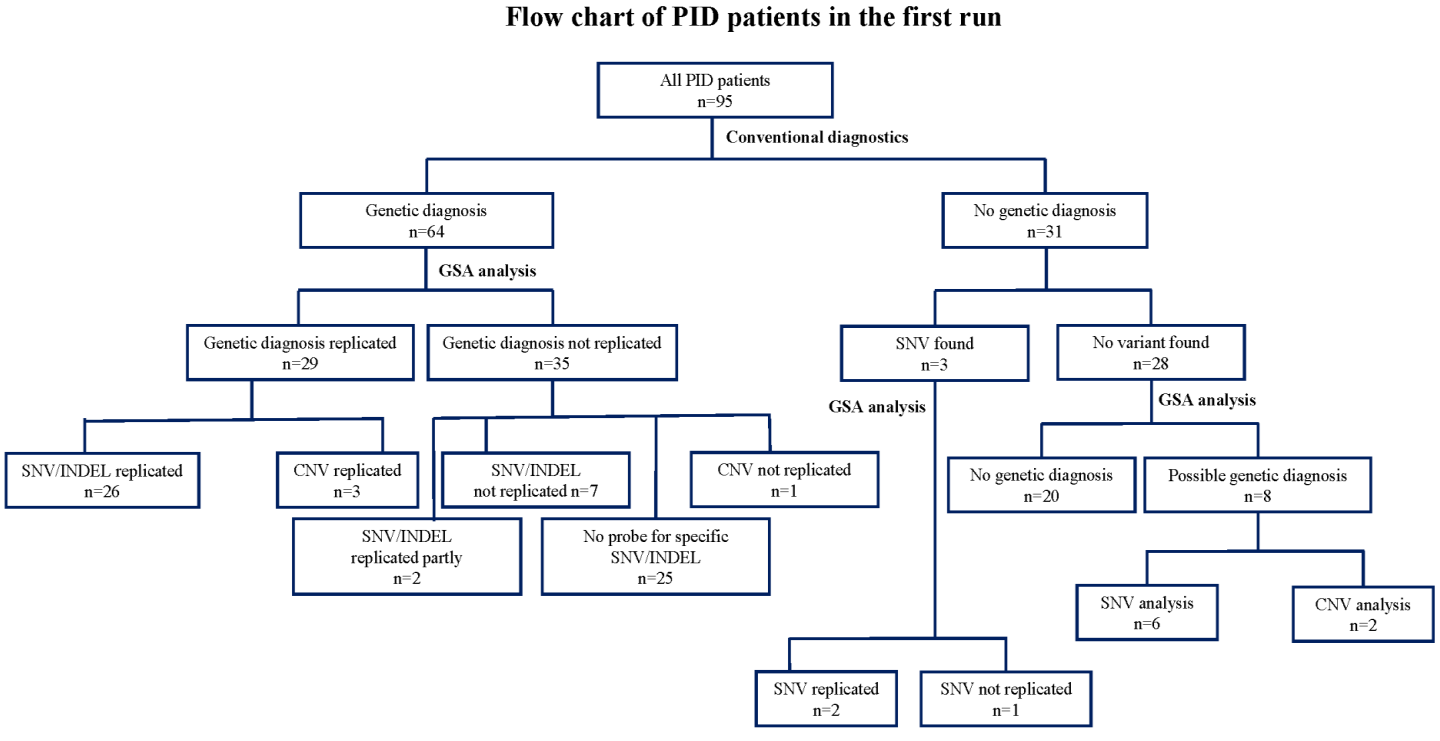
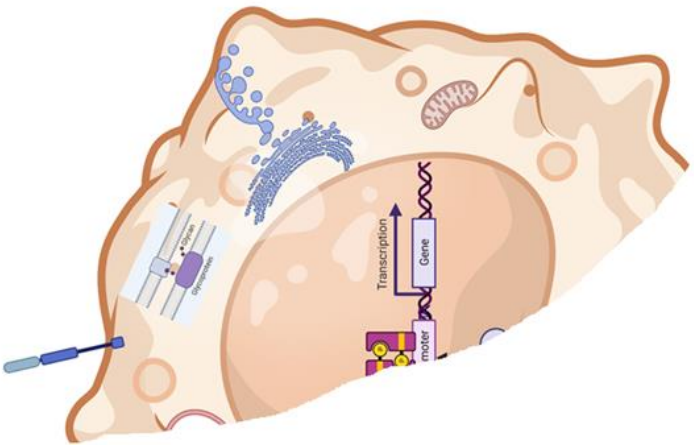


Figure 2. Flow chart describing the numbers of variants identified by GSA array compared to conventional methods in 95 clinically diagnosed PID patients in the first run**.** ***CNV:*** *copy number variants; DCLRE1C*: ***GSA:*** *Global Screening Array;* ***PCR****: Sanger sequencing;* ***NGS****: next-sequencing sequence;* ***PID****: Primary immunodeficiency disorder;* ***SNV****: single nucleotide variants.*



Chapter 5

Pediatric Prediction Model for Low Immunoglobulin G level based on Serum Globulin and Illness Status

Narissara Suratannon, *Phimphika Tantithummawong, Cameron Paul Hurst, Yuda Chongpison, Jongkonnee Wongpiyabovorn, P. Martin van Hagen, Willem A. Dik, Pantipa Chatchatee*

Front Immunol. 2022 Feb 21; 13:825867. doi: 10.3389/fimmu.2022.825867.

Abstract

Hypogammaglobulinemia is a condition that requires prompt diagnosis and treatment. Unfortunately, serum immunoglobulin (Ig) measurements are not widely accessible in numerous developing countries. Serum globulin is potentially the best candidate for screening of low IgG level (IgGLo) due to its high availability, low cost, and rapid turnover time. However, multiple factors may influence the probability of prediction. Our study aimed to establish a simple prediction model using serum globulin to predict the likelihood of IgGLo in children. For retrospective data of patients who were suspected of having IgGLo, both serum IgG and globulin were simultaneously collected and measured. Potential factors interfering with serum globulin and IgG levels were investigated for their impact using bivariate binary logistic regression. A multivariate binary logistic regression was used to generate a formula and score to predict IgGLo. We obtained 953 samples from 143 pediatric patients. A strong positive correlation between serum globulin and IgG levels was observed ($r=0.83$, $p<0.001$). A screening test model using serum globulin and illness status was constructed to predict IgGLo. The formula for predicting IgGLo was generated as follows; Predicted score = (2 x globulin (g/dl)) – illness condition score (well=0, sick=1). When the score was <4, the patient has the probability of having IgGLo with a sensitivity of 0.78 (0.71, 0.84), a specificity of 0.71 (0.68, 0.74), PPV of 0.34 (0.29, 0.40) and NPV of 0.94 (0.92, 0.96). This formula will be useful as rapid and inexpensive screening tool for early IgGLo detection, particularly in countries/ locations where serum IgG measurement is inaccessible.

Introduction

Hypogammaglobulinemia refers to a reduction in all types of immunoglobulins (Ig). This condition is strongly associated with recurrent serious infections with encapsulated pyogenic bacteria, including *Streptococcus pneumoniae* and *Haemophilus influenzae type b* (1). IgG is the most abundant idiotype in the circulation. Low IgG level (IgGLo) is defined as a decrease in IgG concentrations at least 2 standard deviations below the mean for the age group (2). Diseases underlying hypogammaglobulinemia and IgGLo may vary from inborn errors of immunity (IEI) (3) to acquired causes (malignancies, severe infections, malnutrition, excessive protein loss from the gastrointestinal tract, skin, and kidneys, or as a side effect of medications) (4, 5). Prompt diagnosis of IgGLo and subsequent treatment with IgG replacement therapy is crucial to prevent long-term morbidity and death (6, 7).

In case of suspected hypogammaglobulinemia, international consensus guidelines recommend prompt measurement of serum IgG (8-10). However, in numerous developing countries, such tests are only available in referral centers or tertiary care hospitals. Therefore, the availability of a simple and inexpensive tool that is widely accessible and applicable would be beneficial, especially for shortening the diagnostic delay of IgGLo. Serum globulin levels, routinely measured in liver function testing, typically yield information on three globulin fractions, namely alpha, beta, and gamma globulins. Serum Igs, in particular IgG, constitute a significant part of the gamma globulin fraction. Therefore, in developing countries, measurement of serum globulin levels is an attractive candidate to screen for IgGLo due to its high availability, low cost, and rapid turnover time.

A few studies have reported a positive correlation between serum globulin and IgG levels and demonstrated the feasibility of using serum globulin level as a screening test for hypogammaglobulinemia (8, 9, 11). However, these studies did not consider potential factors that might influence the accuracy of prediction. The main influential factor is serious infection accompanied by an increase in complement proteins, which in turn raises serum globulin levels (9). The simple formula that is suitable for clinical settings has never been established. Also, data on pediatric populations is limited (12). Moreover, a simple formula that can be applied in clinical settings has never been established. Our study aimed to establish a simple and rapid formula based on inexpensive serum globulin measurement that can be implemented in a clinical setting to predict the probability of IgGLo in patients <18 years of age.

Material & Methods

Patients

Medical records from the period of 2011-2021 of patients under 18 years of age (Department of Pediatrics, King Chulalongkorn Memorial Hospital, Bangkok, Thailand) with suspected hypogammaglobulinemia or IgGLo were reviewed. Patients were enrolled in this study when serum IgG and globulin levels measured from the same time point were available. Age, sex, causes of hypogammaglobulinemia, intravenous immunoglobulin (IVIG) replacement, and illness conditions from the time of blood sample collection were retrieved from the medical files. This study was approved by the Ethics Committee of King Chulalongkorn Memorial Hospital, Bangkok, Thailand (IRB No. 504/59).

Operating definition

Low serum IgG levels (IgGLo)

In general, IgGLo is defined as a decrease in IgG concentrations of at least 2 standard deviations (SDs) compared to mean age-specific IgG level. However, the risk of recurrent and severe infections generally occurs particularly when serum IgG levels are <500 mg/dl (13). Therefore, in our study, IgGLo was defined as a serum IgG level <500 mg/dl, regardless of age.

The causes of IgGLo

The causes of IgGLo were divided into main two categories: primary and secondary causes. Primary causes of IgGLo related to IEI, particularly B cell differentiation defects. Secondary or acquired causes were assigned to patients when diseases or other extrinsic factors related to IgGLo were identified. These categories are: (1) loss of IgG, including burns, congenital lymphangiectasia, and nephrotic syndrome; (2) drugs that inhibit IgG production or lead to increased IgG metabolization, such as corticosteroids, immune suppressants, and anticonvulsants; (3) malignancies or collagen vascular diseases; (4) viral infections, including Epstein-Barr virus, rubella, HIV, and cytomegalovirus; and (5) other causes related to IgGLo, such as severe malnutrition, severe infections, and prematurity (14).

Illness conditions

Two illness conditions were classified in our study. “Sick” referred to the condition in which the patient had a fever, was admitted to the hospital, or received antibiotics for a treatment at the time blood samples were drawn for IgG and globulin measurement. “Well”

referred to the condition in which the individual was in good health when blood samples were drawn.

Intravenous immunoglobulin (IVIG) replacement therapy

The half-life of plasma IgG from IVIG is approximately 26–41 days (15). It usually takes 4-5 half-lives to clear the majority of IVIG from the body. IVIG replacement affects vaccination responses for six months after the last dose (16). So, in our study, a sample was defined as receiving IVIG replacement when the time of blood sample collection was within six months after the last dose of IVIG.

Blood sample measurement

The globulin fraction (g/dl) was obtained as part of the liver function test, which was determined from the difference between serum total protein and albumin levels. Total protein and albumin levels were measured by the architect biuret method and colorimetric bromocresol green method, respectively. Serum IgG levels (mg/dl) were determined using nephelometry. Both serum IgG and globulin were measured at the same collection time.

Statistical analysis

The characteristics of the included patients were described using means and SDs for continuous variables, counts, and percentages for categorical characteristics. Pearson's correlation was used to measure the strength and direction of the association between serum globulin and IgG levels. Then we formulated the model for predicting IgGLo from serum globulin. Firstly, potential factors that might interfere with serum globulin and IgG levels were selected and investigated using bivariate binary logistic regression. Potential diagnostic factors with a p-value less than 0.2 in the bivariate analysis were then included in a multivariable binary logistic regression and were removed if they were not statistically significant. After identifying the significance of the diagnostic factors and generated models, their utility in diagnosing IgGLo was investigated using receiver operating characteristic (ROC) curves. We fitted the diagnostic model with the variables and removed individual variables to examine whether it reduced model accuracy. Once we decided on the final model, we simplified the model for practical use in clinical situations by rounding the coefficients and comparing the ROC curves of the original and simplified models. We generated the sensitivity, specificity, negative predictive values (NPV) and positive predictive values (PPV), and negative and positive likelihood ratios for the simplified model. Finally, we simplified the cut-off point and investigated whether the diagnostic accuracy of the model was changed. All analyses were conducted using the R statistical package (R core team, 2017); (17), (18) and ROC analysis using the R library pROC (19). Differences were considered statistically significant at $p<0.05$.

Results

Demographic data

Demographic data of the patients and samples collected are provided in **Table I** and **Table S1**. In this study, 953 samples from 143 patients were included. Sixty-nine percent of the participants were male. The mean age ± SD of patients having IEI was 7.8 ±5.1 years old, while the mean age ± SD of patients with acquired IgGLo was 4.76 (±5.0) years old. Seventy-six percent of the samples were collected from patients with IEIs, half of whom had predominantly antibody deficiencies. The leading cause of acquired IgGLo was sepsis (40.3%). Eighty percent of IEI samples were collected while the patients were in good health, while around sixty percent of samples with acquired IgGLo were collected when the patients were getting sick. Ninety percent of IEI samples and 75 percent of samples with acquired IgGLo were drawn when IVIG was given.

Correlation between serum globulin and IgG levels

The association between serum globulin and IgG levels is shown in **Figure 1**. Our results demonstrated a strong positive correlation between serum globulin and IgG levels ($r=0.83$, $p<0.001$). Subgroup analysis of 725 samples from patients with IEI and 228 samples from patients with acquired IgGLo also showed a strong correlation between serum globulin and IgG levels ($r=0.84$, $p<0.001$ and $r=0.82$, $p< 0.001$, respectively). However, among patients with IEI, patients who had immunodeficiencies affecting cellular and humoral immunity, and other IEIs including congenital defects of phagocyte and combined immunodeficiency (CID) with associated or syndromic features, a stronger correlation was observed ($r=0.89$, $p<0.001$ and $r= 0.86$, $p<0.001$, respectively), while a weaker between serum globulin and IgG levels correlation ($r=0.48$, $p <0.001$) was observed in patients with predominantly antibody deficiencies.

Prediction models for IgGLo

Considering that multiple factors can influence serum globulin and IgG levels, we gathered information regarding potential factors including patient age, sex, IVIG replacement, the causes of IgGLo and the illness conditions when serum IgG and globulin levels were determined. We found that patient age, illness conditions, and IVIG replacement were the significant factors that affected serum globulin and IgG levels ($p=0.01$, $p<0.001$, and $p <0.001$ respectively). Using ROC analysis, the area under the curve (AUC) of the model to predict IgGLo taking all significant factors into account was 0.8705(0.838, 0.903). When the IVIG replacement was removed, the AUC of the model was 0.8875 (0.827, 0.948). Although the AUC of both models were significantly different ($p=0.0046$), the magnitude of difference is quite small (0.02 or 2%). Therefore, IVIG replacement therapy was not included in the final model.

Different models from serum globulin, patient age and illness conditions were created and tested for accurate prediction of IgGLo. The models and performance characteristics, including sensitivity, specificity, NPV, and PPV, are provided in **Table II**. ROC curves illustrate the diagnostic ability of models (**Figure 2)**.

The first prediction model generated was **"Predictive score = (- 0.05 x age) + (- 3 x globulin (g/dl) + (1.3 x illness condition score); while well=0, sick=1)"** which we further simplified to **"Predictive score = age (years) + (60 x globulin (g/dl)) – (25 x illness condition score)**". When the predictive score was $\leq$ 127.6, the model could predict IgGLo with a sensitivity of 0.77 (0.69, 0.83), specificity of 0.79 (0.76, 0.81), PPV of 0.41 (0.35, 0.47) and NPV of 0.95 (0.93, 0.96). We observed that the removal of "age of patient" from the model did not affect the model performance, however, it provided more simplicity. Therefore, the new model generated without considering patient age was **"Predictive score = -2.85 x globulin (g/dl) + (1.62 x illness condition score)"**. With the predictive score of $\leq$ 5.6, the diagnostic performance of the later model was as follows; sensitivity of 0.77 (0.69, 0.83), specificity of 0.79 (0.76, 0.82), PPV of 0.42 (0.36, 0.48) and NPV of 0.95 (0.93, 0.96).

We again rounded up the coefficients of the model to **"Predictive score = 2 x globulin (g/dl) - illness condition score"**. With a predictive score of $\leq$ 3.9, the performance of the diagnostic model remained good, with a sensitivity of 0.75 (0.68, 0.82), specificity of 0.80 (0.77, 0.83), PPV of 0.42 (0.36, 0.48) and NPV of 0.94 (0.92, 0.96). Finally, we rounded up the predictive score to the "4" and tested whether the diagnostic value of the model was compromised. Our result showed that with the predictive score $\leq$ 4, the probability to have IgGLo was not compromised, with a sensitivity of 0.78 (0.71, 0.84), specificity of 0.71 (0.68, 0.74), PPV of 0.34 (0.29, 0.40) and NPV of 0.94 (0.92, 0.96).

Discussion

Delay in the recognition of IgGLo leads to diagnostic and therapy delay, resulting in devastating consequences. Limited access to IgG measurement is a major reason for delayed diagnosis of this condition in developing countries where measurement of serum IgG levels can be performed only in referral centers. The ultimate goal of this study was to establish a realistic model to predict IgGLo in a clinical setting. We carefully investigated factors potentially influencing IgGLo and generated different models to obtain the most appropriate model and cut-off score to predict IgGLo.

Serum IgG are the major constituents of the serum gamma globulin fraction. Therefore, serum globulin measurement could be an attractive candidate for screening IgGLo. Using serum globulin as a first screening tool is practical because of its high availability, low cost, and rapid turnover time. Previous studies have shown a strong correlation between serum globulin and IgG (8, 9). These studies also proposed serum globulin cut-off of levels for predicting IgGLo (8, 9). However, data on children is scarce (12). Importantly, we believe that factors other than serum globulin should also be taken into account for proper prediction of IgGLo. Our results demonstrate that illness condition is an important factor that influences the diagnostic model and performance in children. This is in line with the fact that acute phase proteins are increasing during acute infection and therefore have an impact on the serum globulin level measured under such condition (20). We found no relationship between the causes of IgGLo and the correlation between serum globulin and IgG, making the model more widely applicable.

Even though serum IgG levels in children under one year of age might be influenced by maternal IgG, the age of the patient at the moment of sample collection did not significantly affect our prediction model. In our study 12.3% of samples (117 out of 953 samples) were collected from patients under one year of age (33% of total numbers of patients included). Our analysis revealed that the predictive accuracy of the model was not compromised when the samples obtained at age < 1 year were included. We consider this important since certain patients with IEI with low IgG levels present at very early age (such as severe combined immunodeficiency or X-linked agammaglobulinemia). Therefore, the result of our study is beneficial in early detection of these populations, especially in places where measurement of serum IgG levels is not available.

The reason why the correlation between serum globulin and IgG levels was weaker in patients with predominant antibody deficiencies compared to the other IEI subgroups remains unclear. Additionally, although not proven in our study, some conditions can interfere with the components of serum globulin [e.g. hyperlipoproteinemia (increase alpha-1 globulin), metastatic malignancy (increase alpha-1 and alpha-2 globulin), alpha-1 antitrypsin deficiency (decrease alpha-1 globulin), hemoglobin-haptoglobin complexes secondary to hemolysis (increase alpha-2 globulin), and iron-deficiency anemia with high transferrin (increase beta globulin)] (21). Thus, they may compromise the predictive accuracy for low IgG levels.

In conclusion, early diagnosis of IgGLo is crucial. Serum globulin measurement is a rapid, simple, inexpensive, and widely available tool for predicting IgGLo. We constructed the following screening model/formula for predicting IgGLo in children (age <18 yrs): **"Predicted score = (2xglobulin (g/dl)) – illness condition score (well=0, sick=1)", with a score of ≤4 being predictive for IgGLo.** We propose that application of this simple and cheap model in developing countries where measurement of serum IgG is generally unavailable would be a valuable tool to reduce diagnostic delay and optimize the use of healthcare resources for the diagnosis of IgGLo in children (age <18 years).

Abbreviations: Ig: immunoglobulin, IgGLo; low IgG level, **IEI**: inborn errors of immunity, **ESID**: European Society for Immunodeficiencies, **IVIG**: intravenous immunoglobulin, **ROC**: receiver operating characteristic, **NPV**: negative predictive values, **PPV**: positive predictive values (PPV), **CID**: combined immunodeficiency, **AUC**: area under the curve.

Acknowledgements & Funding: This study was supported by Ratchadapiseksompotch Fund, Faculty of Medicine, Chulalongkorn University (RA58/35) and Ratchadaphiseksomphot Endowment Fund Part of the "Research Grant for New Scholar CU Researcher's Project (RGN_2558_016_08_30), Chulalongkorn University, Bangkok, Thailand

References

1. Fried AJ, Bonilla FA. Pathogenesis, Diagnosis, and Management of Primary Antibody Deficiencies and Infections. *Clinical microbiology reviews* (2009) 22(3):396-414.
2. Bertinchamp R, Gérard L, Boutboul D, Malphettes M, Fieschi C, Oksenhendler E. Exclusion of Patients with a Severe T-Cell Defect Improves the Definition of Common Variable Immunodeficiency. *The Journal of Allergy and Clinical Immunology: In Practice* (2016) 4(6):1147-57. doi: https://doi.org/10.1016/j.jaip.2016.07.002.
3. Bousfiha A, Jeddane L, Picard C, Ailal F, Bobby Gaspar H, Al-Herz W, et al. The 2017 Iuis Phenotypic Classification for Primary Immunodeficiencies. *J Clin Immunol* (2018) 38(1):129-43. doi: 10.1007/s10875-017-0465-8.
4. Jaffe EF, Lejtenyi MC, Noya FJD, Mazer BD. Secondary Hypogammaglobulinemia. *Immunology and Allergy Clinics* 21(1):141-63. doi: 10.1016/S0889-8561(05)70197-1.
5. Francisco A Bonilla M, PhD. Secondary Immunodeficiency Due to Underlying Disease States, Environmental Exposures, and Miscellaneous Causes (2017). Available from: https://www.uptodate.com/contents/secondary-immunodeficiency-due-to-underlying-disease-states-environmental-exposures-and-miscellaneous-causes?search=secondary%20immunodeficiency&source=search_result&selectedTitle=1~82&usage_type=default&display_rank=1.
6. Wood P, Stanworth S, Burton J, Jones A, Peckham DG, Green T, et al. Recognition, Clinical Diagnosis and Management of Patients with Primary Antibody Deficiencies: A Systematic Review. *Clinical and experimental immunology* (2007) 149(3):410-23. Epub 2007/06/15. doi: 10.1111/j.1365-2249.2007.03432.x.
7. Duraisingham SS, Buckland M, Dempster J, Lorenzo L, Grigoriadou S, Longhurst HJ. Primary Vs. Secondary Antibody Deficiency: Clinical Features and Infection Outcomes of Immunoglobulin Replacement. *PLoS One* (2014) 9(6):e100324. Epub 2014/06/28. doi: 10.1371/journal.pone.0100324.
8. Jolles S, Borrell R, Zouwail S, Heaps A, Sharp H, Moody M, et al. Calculated Globulin (Cg) as a Screening Test for Antibody Deficiency. *Clinical and experimental immunology* (2014) 177(3):671-8. doi: 10.1111/cei.12369.
9. Holding S, Khan S, Sewell WA, Jolles S, Dore PC. Using Calculated Globulin Fraction to Reduce Diagnostic Delay in Primary and Secondary Hypogammaglobulinaemias: Results of a Demonstration Project. *Ann Clin Biochem* (2015) 52(Pt 3):319-26. doi: 10.1177/0004563214545791.
10. Holding S, Jolles S. Current Screening Approaches for Antibody Deficiency. *Current opinion in allergy and clinical immunology* (2015) 15(6):547-55. doi: 10.1097/ACI.0000000000000222.
11. Hoo T, Lim EM, John M, D'Orsogna L, McLean-Tooke A. Calculated Globulin as a Screening Tool for Hypogammaglobulinaemia or Paraproteins in Hospitalized Patients. *Ann Clin Biochem* (2021) 58(3):236-43. Epub 2021/01/13. doi: 10.1177/0004563221989737.
12. Piza CFSdT, Aranda CS, Solé D, Jolles S, Condino-Neto A. Serum Protein Electrophoresis May Be Used as a Screening Tool for Antibody Deficiency in Children and Adolescents. *Frontiers in Immunology* (2021):3332.
13. European Society for Immunodeficiencies (ESID). *Esid Registry – Working Definitions for Clinical Diagnosis of Pid [Internet]. 2016 [Cited 2017 Dec 10]. Available From: https://Esid.Org/Content/Download/13053/372959/File/Esidregistry_Clinical%20criteria.Pdf*.

14. Huq M, Bhatnagar NK, Hostoffer RW. Hypogammaglobulinemia. *StatPearls [Internet]* (2021).
15. Mankarious S, Lee M, Fischer S, Pyun KH, Ochs HD, Oxelius VA, et al. The Half-Lives of Igg Subclasses and Specific Antibodies in Patients with Primary Immunodeficiency Who Are Receiving Intravenously Administered Immunoglobulin. *J Lab Clin Med* (1988) 112(5):634-40. Epub 1988/11/01.
16. Morikawa Y, Sakakibara H, Kimiya T, Obonai T, Miura M, Tokyo Pediatric Clinical Research N. Live Attenuated Vaccine Efficacy Six Months after Intravenous Immunoglobulin Therapy for Kawasaki Disease. *Vaccine* (2021) 39(39):5680-7. Epub 2021/08/29. doi: 10.1016/j.vaccine.2021.07.097.
17. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2017 [Cited 2018 Jan 10]. Available From: Https://Www.R-Project.Org/.
18. Bates D, Maechler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using Lme4. *J Stat Software* (2015) 67(1):1-48.
19. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. Proc: An Open-Source Package for R and S+ to Analyze and Compare Roc Curves. *BMC bioinformatics* (2011) 12:77. Epub 2011/03/19. doi: 10.1186/1471-2105-12-77.
20. Gabay C, Kushner I. Acute-Phase Proteins and Other Systemic Responses to Inflammation. *N Engl J Med* (1999) 340(6):448-54. Epub 1999/02/11. doi: 10.1056/NEJM199902113400607.
21. Murray DL. Laboratory Methods for Analyzing Monoclonal Proteins (October 9, 2021) [January 20, 2022]. Available from: www.uptodate.com.

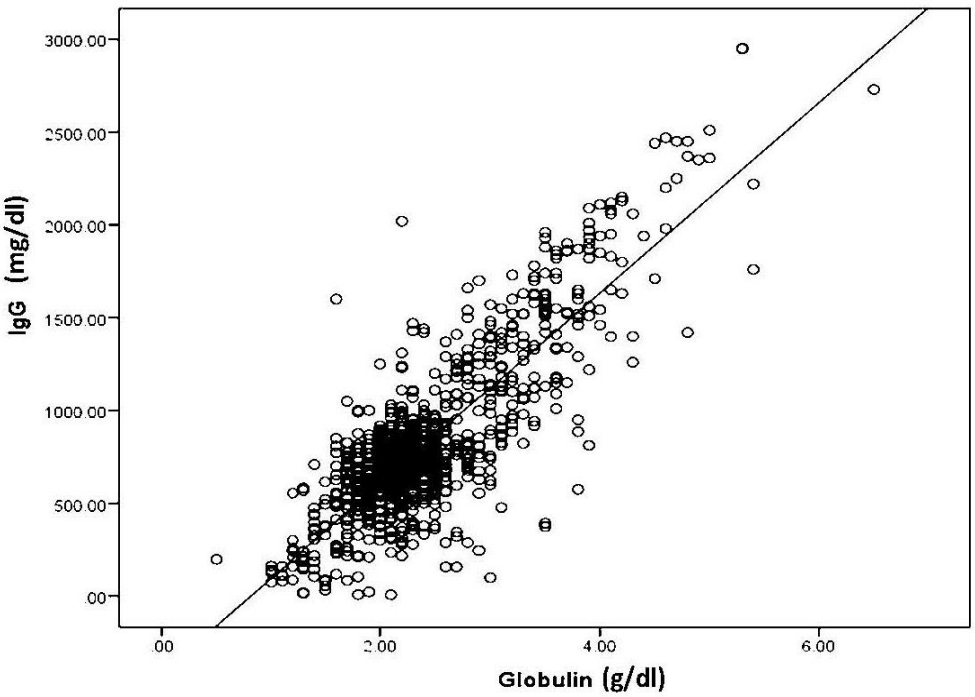


Figure 1: A scatter plot showing a strong positive correlation between serum globulin levels and serum immunoglobulin G levels in all 953 serum samples; r^2=0.83, p <0.001.

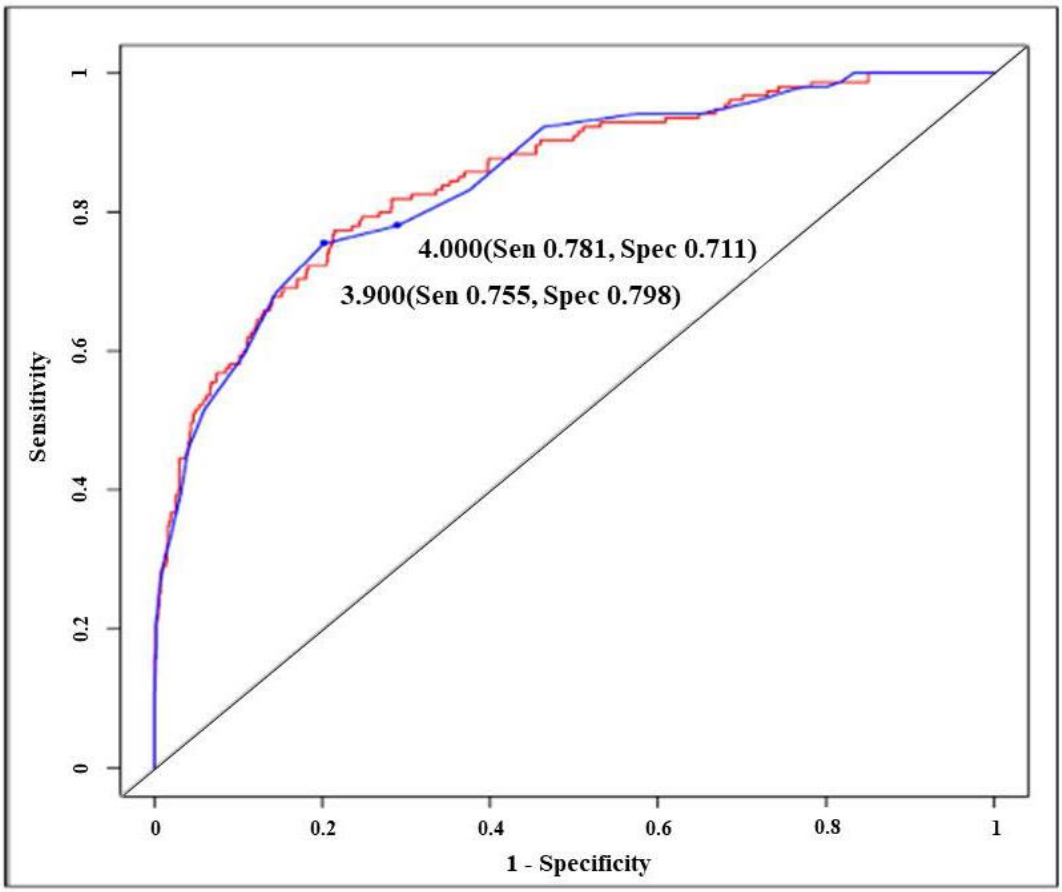


Figure 2: Receiver operating characteristic curves illustrating the diagnostic ability of an original model (red line) and a simplified model with two different cut-off predictive scores (blue line), Sen; sensitivity, Spec; specificity.

- Original model; Predictive score = -2.85 x globulin (g/dl) + (1.62 x illness condition score)
- Simplified model; Predictive score = 2 x globulin (g/dl) - illness condition score
- Illness condition score (well=0, sick=1)

Table 1. Demographic Characteristics of Patients and Samples

Characteristic	Inborn Errors of Immunity (IEI)				Secondary immunodeficiency				Total
	Ab def	Combined	Others	Total subgroup	Sepsis/ severe infections	Recurrent pneumonia	Hematologic disorders	Total subgroup	
Patients (Total numbers = 143)									
No. of patients; n (%)	16 (11.2)	13 (9.1)	5 (3.5)	34 (23.7)	43 (30.1)	27 (18.9)	39 (27.3)	109 (76.2)	**143 (100)**
Age of the patients; mean years (SD)	6.4 (5.2)	2.9 (4.2)	4.0 (4.1)	4.7 (4.8)	2.8 (4.2)	6.2 (4.1)	4.4 (4.6)	4.2 (4.5)	**4.3 (4.5)**
Male sex; n (%)	11 (44.0)	10 (40.0)	4 (16.0)	25 (73.5)	31 (41.9)	16 (21.6)	27 (36.5)	74 (67.9)	**99 (69.2)**
Patients receiving IVIG; n (%)	15 (93.8)	8 (61.5)	1 (20.0)	24 (79.4)	11 (25.6)	3 (11.1)	1 (2.6)	15 (13.8)	**39 (27.3)**
Samples (Total numbers = 953)									
No. of samples; n (%)	411 (43.1)	251 (26.3)	63 (6.6)	725 (76.1)	92 (9.7)	73 (5.2)	63 (6.6)	228 (23.9)	**953 (100)**
Sick condition; n (%)	42 (10.2)	84 (33.5)	8 (12.7)	134 (18.5)	75 (81.5)	42 (57.5)	23 (36.5)	140 (61.4)	**274 (28.8)**
Sample obtained during IVIG administration; n (%)	393 (96.6)	231 (94.7)	53 (100)	677 (93.4)	33 (80.5)	9 (60.0)	3 (23.1)	45 (19.7)	**722 (75.8)**

Serum globulin levels before IVIG administration (g/dl); mean (SD)	1.6 (0.5)	2.4 (0.9)	N/A	2.1 (0.8)	1.5 (0.5)	2.3 (0.8)	2.8 (-)	1.8 (0.7)	**1.9 (0.8)**
Serum globulin levels after IVIG administration (g/dl); mean (SD)	2.2 (0.4)	2.8 (0.9)	2.3 (0.3)	2.4 (0.7)	2.1 (0.7)	2.5 (0.7)	4.2 (0.5)	2.3 (0.9)	**2.4 (0.7)**
Serum IgG levels before IVIG administration (mg/dl); mean (SD)	122.9 (89.5)	453.1 (432.2)	N/A	333.0 (377.1)	324.7 (242.3)	1054.7 (673.0)	1330 (-)	591.0 (528.7)	**467.6 (470.9)**
Serum IgG levels after IVIG administration (g/dl); mean (SD)	686.2 (170.2)	1091.6 (565.0)	693.6 (109.2)	825.1 (404.0)	746.3 (524.2)	823.6 (507.9)	1943.3 (406.7)	841.6 (586.4)	**826.2 (417.1)**

IgG, immunoglobulin G; IVIG, intravenous immunoglobulin; N/A, not available; Ab def, predominantly antibody deficiencies; Combined immunodeficiencies affecting cellular and humoral immunity; Others, other IEIs including congenital defects of phagocyte and combined immunodeficiencies with associated or syndromic features

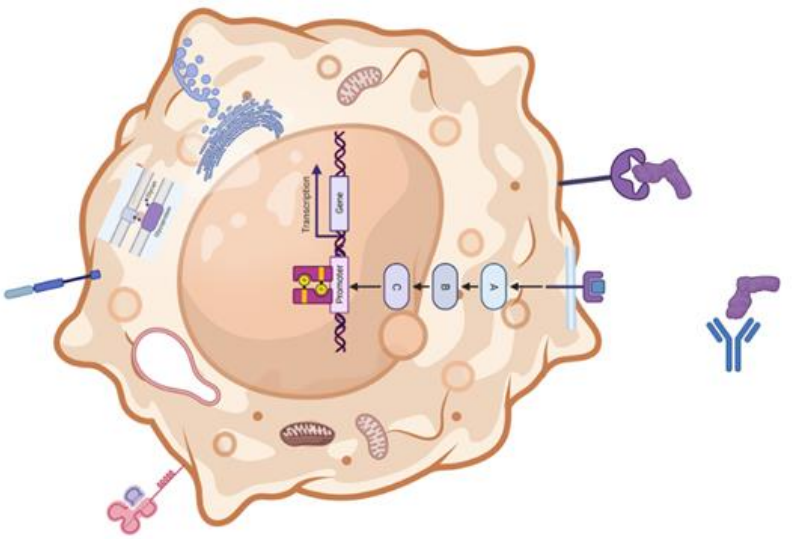
Table 2: Performance characteristics of the different models for diagnosing low IgG levels

Model for calculated predictive score (x)	Cutoff score	Sensitivity (95%CI)	Specificity (95%CI)	PPV	NPV
X = age + (60 x globulin) – (25 x I)	127.6	0.77 (0.69, 0.83)	0.79 (0.76, 0.81)	0.41	0.95
X = (-2.85 x globulin) + (1.62 x I)	5.6	0.77 (0.69,0.83)	0.79(0.76,0.82)	0.42	0.95
X = (2 x globulin) – I	3.9	0.75 (0.68, 0.82)	0.80 (0.77, 0.83)	0.42	0.94
X = (2 x globulin) – I	4.0	0.78 (0.71, 0.84)	0.71 (0.68, 0.74)	0.34	0.94

X, predictive score; I, illness condition score (well=0, sick=1); PPV, positive predictive value; NPV, negative predictive value. Age was described in years, the unit of globulin level was g/dl; IgG, immunoglobulin

Part II

Description and functional analysis of monogenetic IEIs with immunological, including allergy and autoimmunity and non-immunological comorbidities



Chapter 9

Adaptive immune defects in a patient with leukocyte adhesion deficiency type III with a novel mutation in *FERMT3*

Narissara Suratannon, *Patra Yeetong, Chalurmpon Srichomthong, Pramuk Amarinthnukrowh, Pantipa Chatchatee, Darintr Sosothikul, P. Martin van Hagen, Mirjam van der Burg, Marjolein Wentink, Gertjan J. Driessen, Kanya Suphapeetiporn, Vorasuk Shotelersuk*

Pediatr Allergy Immunol. 2016 Mar;27(2):214-7. doi: 10.1111/pai.12485.

Leukocyte adhesion deficiency (LAD) is a rare primary immunodeficiency disease characterized by impairment of phagocyte adhesion (1,3). Three subtypes have been classified by distinct phases of the adhesion cascade. LAD-III is caused by defects in signaling pathways used for integrin activation in all hematopoietic cell types leading to recurrent infections with poor platelet aggregation resembling Glanzmann's thrombasthenia (4). Mutations in *FERMT3* have been identified to underlie LAD-III (4,5). *FERMT3* encodes kindlin-3, one of the focal adhesion proteins which contain a FERM domain located at the carboxyl terminus binding to β-integrin cytoplasmic tails. This molecule cooperates with the cytoskeletal protein talin leading to integrin activation. It also stabilizes active conformations of the integrin subunits and the ligand binding (5,6). Evidently, integrins are widely expressed in many cell types including T and B lymphocytes. Defects in integrin function therefore could lead to both innate and adaptive immune dysfunctions. However, almost all reported cases of LAD-III only had innate immune defects. Here we describe a female Thai patient who was diagnosed with LAD-III, yet presenting with a mild atypical phenotype in which a humoral immune defect was detected.

Our patient was the second child of consanguineous parents who were first cousins. The pedigree of the family is shown in Fig. 1a. She presented with early-onset severe gram-negative infections, thrombasthenia, hepatosplenomegaly and defective wound healing. Between three and eight months old, she experienced four episodes of bacterial pneumonia with sepsis. Firstly, she had severe pneumonia with ARDS with sepsis. Tracheal suction culture revealed *Acinetobacter baumannii*. *Salmonella spp.* was also reported from stool causing diarrhea. In the second episode of pneumonia, *Acinetobacter baumannii* was reported again from tracheal suction. *Pseudomonas aeruginosa* was revealed from ear discharge. Thirdly, the patient had pneumonia with septic shock. Tracheal suction culture reveals *Streptococcus mitis* and *Escherichia coli*. Finally, necrotizing pneumonia with sepsis was reported. The patient's blood culture was positive for *Pseudomonas aeruginosa*. She had the ability to form pus, though minimal, and umbilical cord separation occurred at the age of nine days. After prolonged courses of antibiotics, the patient developed mucocutaneous candidiasis. She did not suffer from invasive fungal infections, as described in other patients with LAD-III (3,7). Her bleeding symptoms were mild and appeared only during episodes of infections, while spontaneous intracranial bleeding and/or massive pulmonary hemorrhage have been reported in patients with typical LAD-III (66, 70). Initial investigations and immunologic assessment at the age of five months revealed persistent leukocytosis with neutrophilia, anemia and thrombocytopenia. A complete blood count showed a hematocrit of 28 % (29-42), white blood cell count of 45,430 cells/mm^3 (6,000-17,500), neutrophils of 25,440 cells/mm^3 (4,000-12,000), lymphocytes of 10,903 cells/ mm^3 (2,000-17,000) and platelets of 109,000 cells/mm^3 (300,000-700,000). Flow cytometric analysis of lymphocyte populations demonstrated normal numbers of total T cells (CD3+), CD4+ T cells, CD8+ T cells, B cells (CD19+) and NK cells (CD16+56+). Analysis of lymphocyte subpopulations showed low percentages of class switched memory B cells (CD27+IgD-IgM-) 0.5% (0.9-29), marginal zone-like B cells (CD27+IgD+IgM+) 1.7 % (2-43), CD27- memory B cells (CD27-IgD-) 0.5% (1.6–3.6), and

plasmablasts (CD24-CD38hi) 0.1 % (0.1-4) (Fig.1b). The proportion of CD4+ terminally differentiated effector memory T cells (Temra) was also reduced to 4.5% (<9.5). The proportion of CD11b positive neutrophils was comparable to healthy controls; 30.2 versus 37.8 %. Immunoglobulin (Ig) levels were measured at the age of 5 months; IgG was in the lower normal range (2.85 g/l, normal range 2.41-6.13). Serum IgM/ IgA/ IgE (0.64, 0.1, <0.04 g/l) were within normal ranges. Lymphocyte proliferation stimulated by phytohemagglutinin, PPD antigen and tetanus antigen were normal compared with healthy controls. Anti-HBs after vaccination was low (28.7 mIU/mL) while the protective value was more than 10 mIU/mL). Rabies virus neutralizing antibody to a three-dose regimen (day 0, 3, 7) of purified vero-cell rabies vaccine was markedly reduced compared to controls measured a by rapid fluorescent focus inhibition test (1.75 vs 7.57 IU/ml). X-rays of the extremities showed an increased bone density (osteopetrosis). Platelet aggregation studies using G protein-coupled receptor (GPCR) agonists (ADP, collagen and arachidonic acid) showed completely absent aggregation. Notably, ristocetin stimulation revealed normal platelet aggregation.

Regular intravenous immunoglobulin (IVIG) therapy because of a suspected humoral immune defect started at the age of 11 months. Thereafter, she had only one urinary tract infection at the age of 15 months. IVIG was discontinued when she was 19 months old as she was in stable condition. Six weeks after IVIG discontinuation, she developed gross hematuria, urinary tract infection and severe sepsis requiring intensive care unit admission. Her IgG, IgM and IgA levels were low; 4.22 (5.53–9.71), 0.19 (0.35-0.81) and 0.10 g/L (0.26-0.74), respectively. IVIG was reintroduced from the age of 21 months onwards. No severe infections occurred up to her last follow-up at the age of 3 years.

After informed consent, the patient's and the parents' genomic DNA was extracted from peripheral blood leukocytes using AchivePure DNA Blood Kit**.** Whole exome sequencing was performed and revealed a homozygous novel missense mutation in *FERMT3* (11:63990633; hg19) at nucleotide position 1784, changing A to C (c.1784A>C). This mutation resulted in a change of codon 595 (p.Q595P) (Fig. 2a). Both parents were heterozygous for this mutation. Several lines of evidence indicate that the mutation is pathogenic. It changes the amino acid from hydrophilic neutral glutamine to a hydrophobic proline. Furthermore, it is in the F3 subdomain, which appears to be functionally important in molecular interactions of FERMT3 with integrin β subunit cytoplasmic tails (5). The mutation is located at an evolutionarily conserved residue found in other species using ClustalX program (Fig. 2b) and is predicted to be probably damaging by Polyphen-2 (score of 0.995). The mutation was absent in 50 unaffected Thai controls. In addition, immunoblot showed absent FERMT3 protein (Fig. 2c).

Our case represents the first patient with a homozygous missense mutation in *FERMT3*. So far, only one other LAD-III patient carrying a missense mutation in *FERMT3* has been reported (8). However, this case was compound heterozygous for a missense mutation (c.922G>A, p.Gly308Arg) and a frameshift deletion (c.1275delT, p.Glu426ArgfsX3). Most previously reported mutations were nonsense or frameshift mutations (71). This could explain the milder manifestations in our patient compared to previously reported cases. We expected

that our patient might have a small amount of FERMT3 protein with some remaining functions. However, the immunoblot using proteins extracted from her leukocytes showed absence of FERMT3 protein expression. The reason for the milder phenotype remains to be elucidated.

There has been only one report from Robert et al. describing adaptive immune defects in LAD-III (9). A decreased lymphocyte proliferation in response to anti-CD3 antibody and a low immunoglobulin production was demonstrated (9). The patients described in this report were homozygous for a splice site mutation, c.310-2A>C (located before exon 3) while our patient had the mutation in exon 14. Adaptive immune dysfunctions have not been described in other LAD-III patients with mutations in exon 14 (8).

We hypothesize that the adaptive immune defects in LAD-III patients could be caused by the impairment of immunological synapse formation between $CD4^+$ T cells and antigen-presenting cells as well as $CD4^+$T cells and B cells, not from intrinsic B cell defects. To prove this hypothesis, we performed *in vitro* stimulation of sorted naïve B cells from the patient and a healthy control using anti-IgM, anti-CD40 and IL-21 to evaluate immunoglobulin synthesis by ELISA and expression of surface beta-1 integrin in an active conformation *(*CD29*)* by flow cytometry. The results showed that the patient could produce IgG at 6.9 mg/ml/million cells *(*control 12.1 mg/ml/million cells*)* and IgA at 30.2 ng/ml/million cells *(*control 31.2 ng/ml/million cells*).* The surface expression of CD29 of the patient and control before and after stimulation was 9% vs 10% and 61% vs 27%, respectively, indicating a reduced CD29 expression on B cells.

When the B cell receptor engages, intracellular protein kindlin-3 is activated and stabilizes the active conformation of the integrin which is required to sustain contact between T and B cells (9). Mice lacking kindlin/integrin binding have suboptimal B cell numbers in lymph nodes and low antibody responses *in vivo* (10). B cells from our patient had an ability to produce antibodies *in vitro* but impaired antibody production *in vivo.* This may be explained by the fact that *FERMT3* mutations could cause a defect in integrin activation, leading to impairment of B cell homing to lymph nodes and B-T cell interaction.

Impairment of B and T interaction might account for the low proportion of class switched memory B-cells. Marginal zone-like B cells and CD27- memory B cells, predominantly developing in a T-cell independent way, were also low, suggesting that at least a part of these cells could originate from the germinal center. This has been shown in patients with the class switch recombination defect CD40L deficiency, who also had low numbers of class switching memory B cells and marginal zone-like B cells (11). However, why these features only occur in some patients with integrin defects remains elusive. We demonstrated that immunoglobulin therapy was able to reduce the severity and frequency of infections indicating that humoral immune defects could play a major role in the susceptibility to infection in this case. Whether IVIG therapy would benefit other LAD-III cases with normal immunoglobulin levels requires further studies.

In summary, we identified a LAD-III patient with a mild clinical phenotype, harboring a novel homozygous missense mutation in *FERMT3*. The patient had immunological defects involving both innate and adaptive immune responses. Our study emphasized the importance

of investigating adaptive immune function, particularly serum immunoglobulin levels, in addition to phagocytic functions in patients with LAD-III. The findings in our patient suggest that IVIG could be beneficial in LAD-III patients with low immunoglobulin levels. We propose that IVIG might be the treatment of choice in patients with a mild phenotype in the presence of a humoral immune defect or an adjunctive treatment in more severe cases, awaiting bone marrow transplantation as definitive therapy.

Acknowledgments

We would like to thank Dr. Jarungchit Ngamphaiboon for providing excellent patient care and helpful advice and Prof. Dr. Nattiya Hirankarn for providing useful advice. This study was supported by the Thailand Research Fund (RTA5680003, RSA5480022), and the Ratchadapiseksomphot Endowment Fund of Chulalongkorn University (RES560530177-HR).

References

1 Hanna S, Etzioni A. Leukocyte adhesion deficiencies. *Ann N Y Acad Sci*. 2012: **1250**: 50-5.

2 Etzioni A. Defects in the leukocyte adhesion cascade. *Clin Rev Allergy Immunol*. 2010: **38**: 54-60.

3 Kilic SS, Etzioni A. The clinical spectrum of leukocyte adhesion deficiency (LAD) III due to defective CalDAG-GEF1. *J Clin Immunol*. 2009: **29**: 117-22.

4 Svensson L, Howarth K, McDowall A*, et al.* Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation. *Nat Med*. 2009: **15**: 306-12.

5 Malinin NL, Zhang L, Choi J*, et al.* A point mutation in KINDLIN3 ablates activation of three integrin subfamilies in humans. *Nat Med*. 2009: **15**: 313-8.

6 Alon R, Etzioni A. LAD-III, a novel group of leukocyte integrin activation deficiencies. *Trends Immunol*. 2003: **24**: 561-6.

7 Kuijpers TW, van Bruggen R, Kamerbeek N*, et al.* Natural history and early diagnosis of LAD-1/variant syndrome. *Blood*. 2007: **109**: 3529-37.

8 van de Vijver E, Maddalena A, Sanal O*, et al.* Hematologically important mutations: leukocyte adhesion deficiency (first update). *Blood cells, molecules & diseases*. 2012: **48**: 53-61.

9 Arana E, Harwood NE, Batista FD. Regulation of integrin activation through the B-cell receptor. *J Cell Sci*. 2008: **121**: 2279-86.

10 Morrison VL, Uotila LM, Llort Asens M, Savinko T, Fagerholm SC. Optimal T Cell Activation and B Cell Antibody Responses In Vivo Require the Interaction between Leukocyte Function-Associated Antigen-1 and Kindlin-3. *J Immunol*. 2015: **195**: 105-15.

11 Berkowska MA, Driessen GJ, Bikos V*, et al.* Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood*. 2011: **118**: 2150-8.

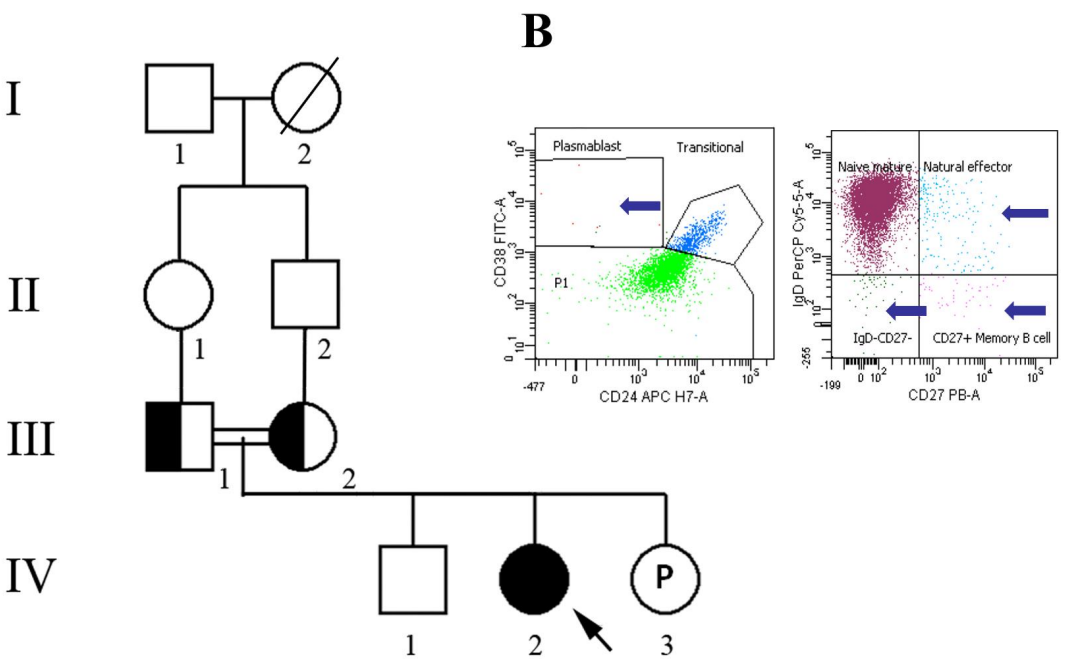


Figure 1. (a) Pedigree. (b) Flow cytometry revealed low proportions of class switching memory B cells(CD27+IgD-), marginal zone-like cells (CD27+IgD+IgM+), CD27- memory B cells (IgD-CD27-) and plasmablasts (CD24-CD38hi) (arrows), for the exact proportions see text.

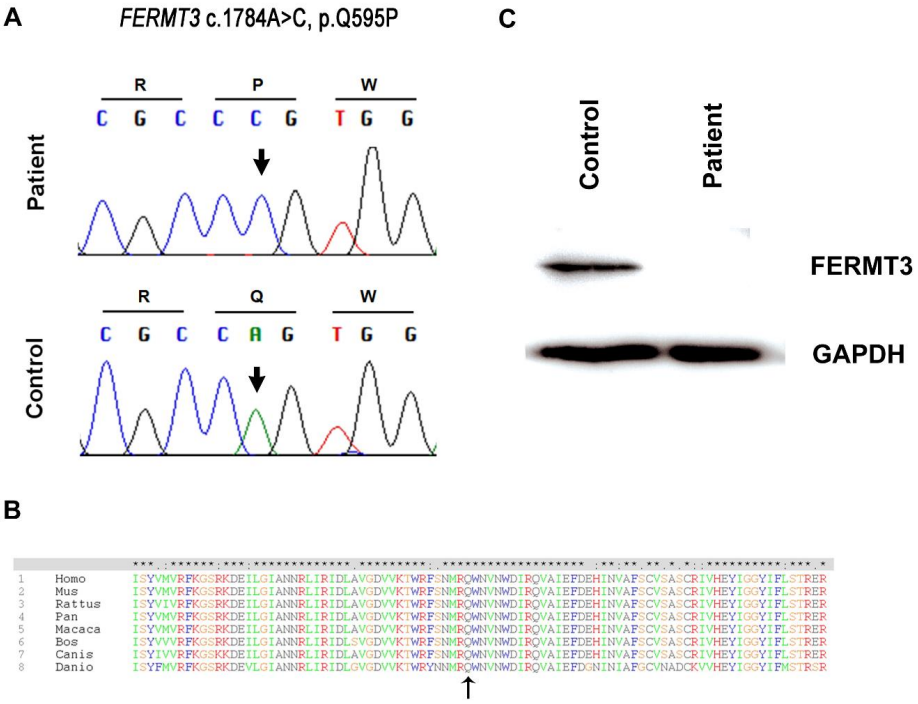


Figure 2. Mutation analysis of *FERMT3*. (a) Chromatogram of the patient (upper) and unaffected control (lower). (b) Multiple sequence alignment of *FERMT3* in different species. (c) Immunoblot showing that *FERMT3* protein was absent in our patient.

Part III

Inborn Errors of Immunity are frequently monogenic disorders, do allergies have a monogenic background?

Part IV

General discussion and Future Perspectives in Inborn Errors of Immunity

Inborn Errors of Immunity (IEI); genetic analysis in perspective

The immune system is a complex biological network composed of genes, (glyco)proteins, cells, tissues and organs that work together to protect the body against infections and cancer. Maintenance of homeostasis in the immune system is very important. A dysregulated immune response may lose the capacity to defend the organism against microbes while a dysregulated response against normal body cells and other constituents causes autoimmunity, diseases caused by this response are termed 'autoimmune diseases'.

Inborn errors of immunity (IEI) are a group of rare genetic disorders that are characterized by defects in (components of) the human immune system. IEIs are caused by defects in genes that are important for the proper function of the immune system. There are several different types of IEIs which can affect any part of the immune system resulting in various similar and dissimilar phenotypes. Depending on the genetic variant and consequent dysfunction of specific cells and/or proteins the clinical phenotypes may vary. Some IEIs affect the body's ability to produce complement factors, which are proteins that help to prevent or fight infections, such as meningococcal meningitis. Other IEIs may affect the ability to produce immune cells, including granulocytes/macrophages, which play an important role in the innate immune response, or T cells, B cells and antigen presenting cells which are all indispensable for the adaptive immune defense. The affected cell type(s) result(s) in a more or less typical phenotype. IEIs may have a high morbidity, because they might cause (recurrent) severe infections that can be life-threatening or lead to irreversible organ damage if not treated promptly. Besides, some IEIs may lead to other characteristic health issues including autoimmune diseases, autoinflammatory diseases, allergies, and cancer. Immune deficiencies with or without one or more of these typical immunological phenotypes are nowadays entitled ‘immune dysregulation syndromes’.

As described in the previous paragraph, IEIs are a heterogeneous group of diseases/disorders. Until now, almost 500 monogenetic disorders have been recognized. (1) Both gain-of-function (GOF) and loss-of-function (LOF) damaging variants in the same gene are described that result in an immune dysregulation syndrome with a distinct clinical phenotype.

Obtaining a confirmed genetic diagnosis in IEI patients is crucial for a better understanding of the underlying pathogenesis/pathways involved and subsequent clinical phenotype in the patient. Importantly, a genetic diagnosis may provide direction for more patient specific therapy that is tailored to the specific molecular defect. (2) Current genetic diagnostic approaches for IEIs are based on Sanger sequencing, next generation sequencing (NGS), whole exome sequencing (WES), whole genome sequencing (WGS) and copy number variant (CNV) analysis, however these techniques are time-consuming, costly, and involve complicated data interpretation. Due to high costs and resource limitations, these tests are not always available in low income/developing countries. Therefore, rapid, robust, and inexpensive molecular/genetic tools are needed and must be developed. As mentioned in

chapter 3 we developed a cheaper method for genetic diagnosis of IEI. This can be a cheap first-line screening technique for known variants, a step before the use of the more expensive sequencing procedures. This technique is based on a single nucleotide polymorphism (SNP) array, these are high-throughput DNA microarrays that originated from the early 2000s and are powerful platforms for simultaneously analyzing hundreds of thousands of SNPs and evaluating CNVs in a single experiment. (3) The costs of SNP arrays have dropped substantially (from 300 to 30 Euros per sample), this was driven by the very large sample sizes needed to perform genome-wide association studies (GWAS). Both Affymetrix/Thermofisher and Illumina have designed cost-effective arrays that contain about 800,000 variants, allowing a wide range of genetic variants to be assessed. In the customized array additional known damaging variants were added. Availability of such an array does not replace WES or WGS, and the limitation is that it will not discover unknown variants and novel disease-causing genes. Interestingly, certain characteristically damaging variants, are very frequently described (hotspots) in certain IEIs as for example in Familial Mediterranean Fever (FMF) or a syndrome which is composed of retinal dystrophy, optic nerve edema, splenomegaly, anhidrosis and headache (ROSAH syndrome). Additionally, copy number variations (CNVs), the difference in the genomic segments resulted from deletions, duplications, insertions, unbalanced translocations and inversions (4), can be assessed using SNP array platform.

The importance of defining IEIs is to establish or confirm a genetic diagnosis, but also to understand immunological (and non-immunological) comorbidities in the patients. A single genetic defect may explain the various immunological comorbidities including autoimmunity, autoinflammation, allergy, and malignancy. As an example, allergies are frequent comorbidities in IEIs as described in the patients' examples in this thesis, in particular patients withdamaging variants in *DOCK8* (chapter 6), and *PGM3* (chapter 10). Monogenic disorders that cause atopic disorders are not as thoroughly described. Recently, we described for the first time a GOF variant in *STAT6* causing early-onset severe food allergies in a Thai family (chapter 11). Since then, several other reports describing the association between *STAT6* GOF variants and early onset severe food allergies have been published. (5,6,7) Whether these *STAT6* variants have broader immune effects is currently under investigation. Of interest, in the study described in this thesis the father with the same *STAT6* variant as the index patient developed kidney fibrosis, albeit with unknown cause. This was also observed in a conditional *STAT6* GOF variant mouse model. STAT6 signaling is primarily activated by Th2 cytokines such as IL-4 and IL-13 and is associated with the pathogenesis of lung (8,9), liver (10), and renal fibrosis. (11,12)

Genetic defects in IEIs are typically germline variants; however, somatic mutations have also been described in patients with an immune dysregulation phenotype. (13) This indicates that cell type-dependent sequencing may be indicated in certain circumstances in order to detect somatic mutations.

Besides organ-directed autoimmunity, such as autoimmune hypothyroidism, and granuloma formation, which is considered as an autoinflammatory reaction, germline variants may have an intrinsic biochemical effect in non-immune organs. Particular germline damaging

variants potentially have the capability to affect the function of cells and organs in which the gene has an important function. This may lead to characteristic organ involvement or multi-organ diseases. So, beyond immune dysregulation, other comorbidities might become part of the phenotype. These include, for example, growth delay, specific cutaneous features, psychiatric disorders, and ocular disorders. (14-16) (postulated in Figure 1). Bone formation is disturbed in a significant number of IEIs, resulting in growth delay and/or dysmorphia. Striking examples of disturbed bone formation were reported, such as the SOPH syndrome (described in chapter 8), which stands for short stature, optic nerve atrophy, and Pelger-Huet anomaly (SOPH). This syndrome also includes antibody deficiency and recurrent viral infections with CMV and EBV, which suggest an accompanying impaired T cell function. SOPH is caused by homozygous or compound heterozygous loss-of-function (LOF) variants in the *neuroblastoma amplified sequence* (*NBAS*). Skeletal dysplasia in the reported patients showed thin, gracile bones with stenotic diaphysis and flared metaphysis, and mild generalized epiphyseal dysplasia. These findings suggest a defective bone mineralization pattern.

On the other hand, the patient with *FERMT3* variants presented with osteopetrosis (chapter 9), characterized by abnormal bone growth and overly dense bones throughout the body. These examples clearly illustrate that the action of IEI genes involved in bone formation requires a precise balanced regulation of bone formation and degradation. In addition, both loss of function as well as gain of function of a particular transcription factor may result in differential immune and bone formation/growth defects. Loss-of-function genetic defects in the "*signal transducer and activator of transcription 3*" (*STAT3*) result in osteoporosis. (17) STAT3 plays a role in complex network related to bone remodeling and the development of osteoporosis. This transcription factor is obligatory for the differentiation of IL-17 producing cells. This cytokine activates granulocytes directly and indirectly in order to phagocytize bacteria and fungi. So, *STAT3* LOF variants result in recurrent staphylococcal and fungal infections but also osteoporosis, through currently not well-understood mechanisms. (18,19) On the other hand, GOF variants in *STAT3* may result in growth delay. This is caused at another level through the inhibition of STAT5 action by an overactive STAT3, resulting in a defective (post-) growth hormone receptor (GH-R) signaling. (20)

The comparison of known IEI genes and the transcriptome involved in bone formation reflects interesting similarities. Out of 379 IEI genes, 64 overlaps with genes involved in bone development (non-published data). The terminology "immunodeficiency" and even "IEI" frequently describes the clinical phenotypes incompletely. In-depth clinical phenotyping is essential to determine and compare the extent of disease (affected organs) in cohorts of patients with IEIs and gene-expression profiles of the affected organ systems may clarify the underlying pathophysiology.

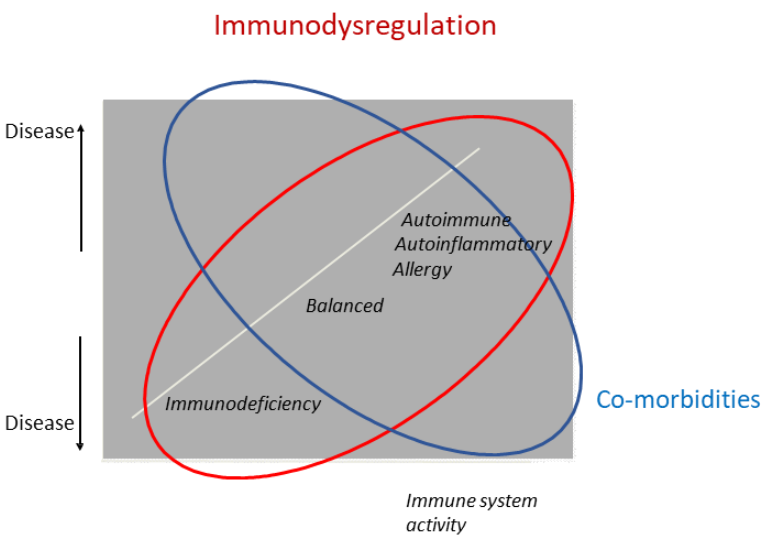


Figure 1. Inborn errors of immunity present with immune dysregulation and other non-immune co-morbidities.

Whether the protein domain location of a particular gene variant influences the clinical phenotype is probably gene dependent. In a recent clinical study on 191 patients with *STAT3* GOF variants (21), most patients had damaging variants located in the coiled-coil, DNA-binding domain (DBD), SH2, and transactivation domain of STAT3. Comparison between the various domains did not reveal clear phenotypic differences. On the other hand, patients with *NBAS* variants showed a clear relationship between mutated domain location and phenotype (chapter 8). Severe hepatic involvement was associated with variants affecting the NBAS-Nter and Sec39 domains, whereas milder liver involvement and immunodeficiency were generally associated with variants located at the N-terminus and C-terminus of the protein. (22)

Current and Future therapies in IEIs

IEIs have heterogeneous phenotypes, as mentioned above. In the treatment of IEIs with a significant immunodeficiency (PID) component, general therapeutic approaches for PID patients are applied.

- **Antibiotic prophylaxis:** Depending on the background of the genetic defect and susceptibility to specific microorganisms, different antibiotic prophylaxis regimens are utilized. (23) For example, in case of hypogammaglobulinemia, azithromycin or low dose trimethoprim-sulfamethoxazole is frequently chosen, while typical prophylaxis against *Pneumocystis jerovecii* involves mostly oral trimethoprim- sulfamethoxazole or pentamidine via inhalation.
- **Immunoglobulin Replacement Therapy (IGRT):** IGRT, administered either subcutaneously or intravenously, is considered in patients with antibody deficiencies. Candidates for IGRT include those with severe and/or recurrent infections, bronchiectasis, and patients with an impaired vaccination response to polysaccharide and/or polysaccharide-protein conjugate vaccines, which is important for the defense against encapsulated bacteria.
- **Cytokine therapy:** In certain IEIs, characterized by defects in cytokine production or inappropriate cytokine production, cytokine therapy can be applied. Well-known examples include the use of G-CSF in neutropenia (ELA2 gene-encoding neutrophil elastase) and interferon-gamma prevention therapy in chronic granulomatous disease (CGD).
- **Immunizations** with killed/inactive vaccines can be safely administered and may introduce specific immunoglobulins and/or T cell memory.
- **Hygienic prevention:** Depending on the severity of the IEI, safe food intake and avoiding potentially infected water is of importance.

IEIs interfere with normal cell function through several mechanisms, particularly by impairing one or more biochemical pathways, which can result in either hyper- or hypocellular activity depending on the type of DNA variant. This alteration in biochemical activity can disrupt cell function, leading to cellular exhaustion, senescence, and apoptosis. When aiming for biochemical correction of a specific pathway, the goal is to normalize its activity rather than completely blocking or overstimulating it. One notable example is activated PI3kinase delta syndrome (APDS), an immune dysregulation syndrome with primary immunodeficiency and autoimmunity. Overactivation of the PI3K pathway in these patients leads to senescence and exhaustion in CD8+ T cells, resulting in an impaired defense against viral infections such as CMV and EBV. The impaired B cell function results in hypogammaglobulinemia and the immune dysregulation results in autoimmunity. (24) With the developments in biomedical research, pathway analysis resulted in significant progress. While complete suppression of a

pathway is of particular interest in malignancies, pathway correction holds great significance in the context of IEIs. For instance, the newly developed PI3K inhibitor leniolisib (Figure 2) has shown remarkable success in treating APDS patients. Biochemical evidence, as well as cellular functional changes, have demonstrated the correction of hyperactive PI3K action. (25) Interestingly, B cell recovery, accompanied by successful differentiation into plasma cells and restoration of IgG production, allows for discontinuation of IgG therapy in these patients. Figure 3 illustrates the effect of the treatment of an APDS patient with leniolisib.

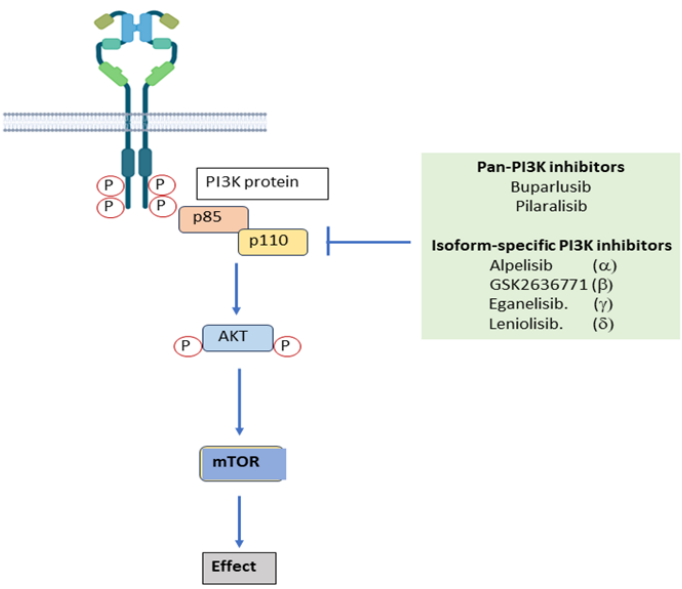


Figure 2. PI3-kinase pathway, interaction with the diverse inhibitors of this pathway including leniolisib recover/inhibit the PI3K-mTOR pathway activity. (partly created with BioRender.com)

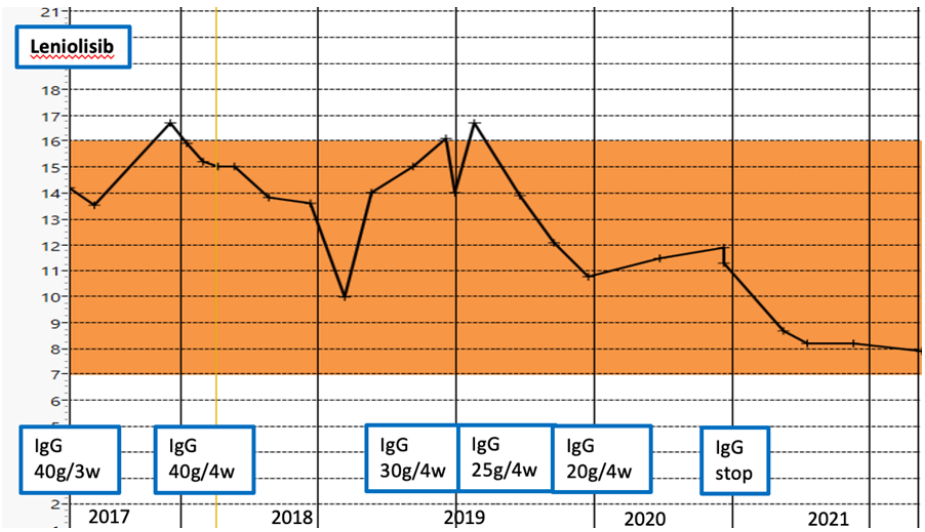


Figure 3. Treatment of a patient with APDS with leniolisib leads to discontinuation of IgG substitution therapy because of recovery of the patient's own IgG synthesis.

In this thesis we have described the newly discovered *STAT6* GOF variant in chapter 11. GOF variant in *STAT6* leads to the overactivation of the STAT6-IL4/IL13 pathway, resulting in increased production of cytokines in the allergic cascade including CCL2 and CCL24 causing an allergic phenotype. (26) Excitingly, new biologics such as dupilumab, an IL4/IL13 receptor blocker, have shown effectiveness in treating allergic patients with *STAT6* GOF variants. (5)
In recent years, the development of drugs targeting diverse pathways has led to an expanding list of immune dysregulation disorders that can be treated with these innovative chemical drugs or biologics. Table 1 provides examples of several well-known and new targeting drugs, their molecular targets, and their off-label use in IEIs.

Table 1. Targeted therapies for non-infectious inflammation in IEIs. [adapted from Leiding et al. 2019 (27), with permission]

Drug	Molecular target	Licenced, off-label use and potential IEI disease candidates
Sirolimus	mTOR	NLCR4-GOF, POMP deficiency, CTLA-4 haploinsufficiency, APDS
Abatacept	B7-1, B7-2	CTLA-4 haploinsufficiency, LRBA deficiency
Belatacept	B7-1, B7-2	CTLA-4 haploinsufficiency
Anakinra	IL-1R	CAPS, FCAS, MWS, FMF, DIRA
Canakinumab	IL-1β	CAPS, FCAS, MWS, FMF, DIRA
Rilonacept	IL-1β	CAPS, FCAS, MWS, FMF, DIRA
Tocilizumab	IL-6R	*STAT3*-GOF Blau syndrome (uveitis, lymphadenopathy, hepatosplenomegaly)
Etanercept	TNF-α	SAVI, CANDLE syndrome, POMP deficiency, PAPA syndrome, Blau syndrome
Infliximab	TNF-α	SAVI, CANDLE syndrome, POMP deficiency, PAPA syndrome, Blau syndrome
Adalimumab	TNF-α	SAVI, CANDLE syndrome, POMP deficiency, PAPA syndrome, Blau syndrome
Emapalumab	Anti-IFN- γ	Hemophagocytic lymphohistiocytosis (HLH)
Tadekinig-alfa	IL-18	*NLCR4* GOF XIAP deficiency
Ustekinumab	P40 subunits of IL-12 and IL-23	LAD, CGD-colitis
Ruxolitinib	JAK1 and JAK 2	*STAT3* GOF, *STAT1* GOF, CANDLE syndrome
Tofacitinib	JAK1 and JAK 3	STAT3 GOF, STAT1 GOF, CANDLE syndrome
Baricitinib	JAK1 and JAK 2	*STAT1* GOF, CANDLE syndrome, Aicardi-Goutières syndrome, *SOCS1* haploinsufficiency
Rituximab	CD20	Granulomatous CVID
PEG-ADA	ADA	*Adenosine deaminase* deficiency
Leniolisib	PI3Kδ	APDS
Plerixafor	CXCR4	WHIM syndrome
Dupilumab	STAT6	Allergy

The therapeutic implications of these advancements necessitate genetic analysis whenever possible and available. However, the extent of genetic analysis performed depends on the healthcare system in each country. This issue is not limited to the diagnosis of immune dysregulation disorders but also applies to other conditions such as inborn errors of metabolism and cancer.

An important consequence of increased genetic analysis in patients suspected of having immune dysregulation disorders is the identification of variants of undetermined significance (VUS). Determining the functional significance of a genetic defect can be challenging considering the vast number of variants revealed by whole exome sequencing (WES) and whole genome sequencing (WGS). The American College of Medical Genetics and

Genomics and the Association for Molecular Pathology (ACMG-AMP) system have established a classification system for variants, consisting of five categories: benign, likely benign, variant of unknown significance (VUS), likely pathogenic, and pathogenic. A VUS classification indicates that there is insufficient or conflicting evidence regarding the role of a molecular alteration in the disease. Variants are suspected to be damaging if they are located in evolutionarily conserved regions of DNA or if they are believed to be involved in important mechanisms such as RNA splicing.

The risk associated with a damaging variant can be assessed using the Combined Annotation Dependent Depletion (CADD) score, which represents a ranking rather than a prediction. There is no defined threshold for a specific purpose, but higher CADD scores indicate a higher likelihood of being deleterious. Scores are calculated as 10 times the negative logarithm of the rank, so variants with scores above 20 are predicted to be among the top 1.0% most deleterious substitutions in the human genome. However, a high CADD score alone is not definitive evidence of a damaging variant. To confirm functionality, appropriate cell systems must be studied through transfection, transduction, or gene editing of the particular mutant, and the results compared with the function of the wild-type (WT) variant. This is considered the most convincing approach. It is important to note that studying genetic variants in artificial cell systems does not always reflect the behavior of the original affected cells, tissues, or organs of the patient. Factors such as endogenous production of the protein of interest by the cell line used can interfere with the outcome. Additional procedures, such as pre-transfection/transduction knockout may be necessary but may induceadditional cell toxicity. Furthermore, functionality studies are often very labor-intensive and need a considerable amount of time to complete. (28)

Based on the affected pathways confirmed by these analyses, a functional pathway-based categorization can be hypothesized, as depicted in Figure 4.

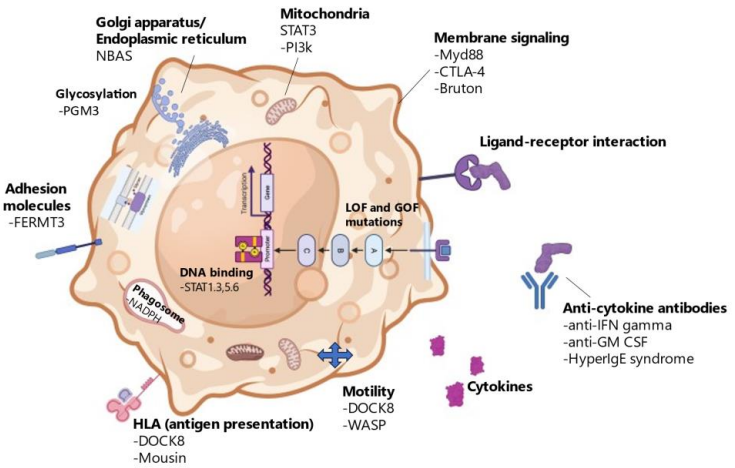


Figure 4. Proposed functional pathway-based categorization for IEIs. (*created with BioRender.com*)

Hematopoietic stem cell transplantation, gene therapy and gene editing

Haematopoietic stem cell transplant (HSCT) or bone marrow transplant is a specialized medical procedure that involves the transplantation of blood-forming stem cells to treat various life-threatening diseases and disorders. The use of allogeneic HSCT as a treatment for IEIs has a long history dating back over 50 years. The first successful transplant for a patient with severe combined immunodeficiency (SCID) was reported in 1968. (29) HSCT has emerged as a potentially curative treatment option for selected patients with IEIs, offering the possibility of restoring a functional immune system. This therapy is considered for patients with severe forms of primary immunodeficiency disorders who have a high risk of life-threatening infections, despite optimal medical management. Selection criteria typically include age, disease severity, current infections, availability of a suitable donor, and the presence of other complications related to the underlying IEI. Genetically well-characterized IEIs for which HSCT should be considered as an established therapy are summarized by Pai in 2019. (30) In this thesis, we presented a successful HSCT case of an adult woman with compound heterozygous damaging variants in the *phosphoglucomutase 3* (*PGM3*) (chapter 10). During the course of her disease, the patient developed a malignant EBV-negative non-Hodgkin's lymphoma, which prompted us to treat her with chemotherapy and a haploidentical HSCT, resulting in a successful outcome. Interestingly, it took one year for full recovery of PGM3 activity to be measured in peripheral blood graft T cells. The reason for this delayed expression remains unclear, but we speculate that the lack of adequate glycosylation in the bone marrow microenvironment may have influenced T cell development and reconstitution.

The role of recipient T cells is crucial in allogeneic HSCT. In severe combined immunodefiency (SCID) patients, the lack of functional T cells renders them unable to reject allogeneic grafts, which has led to the development of adjusted lower-intensity conditioning regimens. (31) The primary goals of conditioning regimens for patients with IEIs undergoing HSCT are to eliminate recipient T cells and natural killer (NK) cells, preventing rejection of donor-derived hematopoietic stem cells (HSCs), and to create sufficient space in the recipient's bone marrow for the engraftment of the donor stem cells. Adjusted lower-intensity conditioning regimens have resulted in lower side effects and improved outcomes in certain IEIs. For example, in chronic granulomatous disease (CGD), more recent studies on HCT outcomes have shown improvement. A multi-institutional study of HCT in 56 patients with CGD using matched sibling donor or well-matched unrelated donor HSCT with a uniform reduced-intensity conditioning regimen demonstrated successful outcomes. Among the surviving patients, 93% achieved myeloid donor chimerism of 90%, and only 5% experienced graft failure. (31)

HSCT offers potential treatments for patients with certain IEIs, however, there are limitations and challenges associated with these approaches. HSCT relies on finding a suitable donor, typically a matched sibling donor or a well-matched unrelated donor. Access to potential donors is obligatory, lack of these will limit the feasibility of HSCT as a treatment option. Regional genetic diversity may decrease the likelihood of finding a compatible donor. HSCT requires matching of human leukocyte antigens (HLAs) between the donor and recipient to minimize the risk of graft rejection and graft-versus-host disease (GVHD). GVHD occurs when the donor's immune cells recognize the recipient's tissues as foreign and attack them. Despite efforts to match HLAs, there can still be variations that increase the risk of GVHD. Additionally, even with a matched donor, there is still a risk of immune incompatibility due to minor histocompatibility antigens and other factors, which can impact the success of HSCT. A striking example of a patient who developed lethal GVHD (HSCT because of homozygous *DOCK8* deficiency and malignancy) was described in chapter 7. Adjustment of conditioning regimens used in HSCT involves the use of chemotherapy and/or radiation to eliminate the recipient's immune cells and to create space in the bone marrow for donor HSCs engraftment. While these regimens are essential for successful transplantation, they can have significant side effects, including increased risk of infections, organ toxicity, and long-term complications such as infertility and secondary malignancies. Adjusting the intensity of conditioning regimens has been explored to reduce side effects, but finding the right balance between efficacy and toxicity remains a challenge. As our understanding of IEIs and the intricacies of HSCT continues to advance, ongoing research aims to improve patient outcomes further.

Novel approaches, such as gene therapy and advanced cellular therapies, may offer additional treatment options for patients with an IEI, potentially reducing the risks associated with HSCT and enhancing its efficacy. The novel alternative option of *ex vivo* CD34+ stem cell gene therapy aims to correct genetic defects by genetically modifying a patient's own hematopoietic stem cells. *Ex vivo* gene therapy for IEIs has shown significant clinical benefit in multiple Phase I/II clinical trials. (32) By using retroviral or lentiviral transduction of autologous

CD34+ hematopoietic stem and progenitor cells, only a portion of the hematopoietic stem cell compartment is genetically modified. (33) Successful gene therapy approaches have been reported, such as in ADA-deficient SCID (ADA-SCID) patients. Stem cell gene therapy for ADA-SCID has received endorsement and approval for use in children without a matching bone marrow donor in April 2016 by the Committee for Medicinal Products for Human Use of the European Medicines Agency. (34) Treatment of ADA-SCID with ex vivo lentiviral HSPC gene therapy resulted in high overall and event-free survival with sustained ADA expression, metabolic correction, and functional immune reconstitution. (35) A recent comprehensive overview of 55 gene therapy trials in 14 diseases between 1995 to 2020 was reported. (36) These included 406 patients with IEIs (55.2%), metabolic diseases (17.0%), haemoglobinopathies (24.4%) and bone marrow failures (3.4%). The patients were treated with gammaretroviral vector (γRV) (29.1%), self-inactivating γRV (2.2%) or lentiviral vectors (LV) (68.7%). (36) There were 21 genotoxic (genotoxic side effects) events out of 1504.02 person-years of observation (PYO), which occurred in γRV trials (0.99 events per 100 PYO, 95% CI = 0.18–5.43) for IEIs. Stable reconstitution of hematopoiesis in most recipients with superior engraftment and safer profile was observed in patients receiving LV-transduced HSCs. (36)

However, this treatment approach can have potential severe side-effects. In addition to myelodysplastic events as recently reported in LV-treated cerebral adrenoleukodystrophy patients, (37), aberrant vector-gene fusion transcripts may appear causing clonal expansions in LV X-SCID and beta-thalassemia patients. (38-40) The long-term impact of random vector insertion and potential for oncogenesis remains a long-term concern.

In addition, there are limitations associated with this approach. These include the efficiency of gene delivery, potential off-target effects of gene editing tools, limited long- term follow-up data, and challenges in achieving stable and sufficient transgene expression in the modified cells. Furthermore, immune responses to the viral vectors used for gene delivery can impact the effectiveness of the therapy. It is important for doctors and researchers to carefully consider these limitations and challenges when evaluating the suitability of HSCT or gene therapy as treatment options for patients with IEIs. Registration outcomes, contineous research and advancements are needed to improve the efficacy, safety, and accessibility of these therapies.

The rapid development of genome editing technologies to more accurately correct the mutations underlying the onset of genetic disorders has provided a new alternative, yet promising platform for the treatment of such diseases. (41,42) The genotoxicities due to random vector integrations in HSCs, are a potential subject of targeted correction with CRISPR-Cas9- based genome editing and offer improved precision for functional correction for various IEIs. Preclinical studies demonstrated the safety of this approach and provided for example the information and rationale for the translation of a CRISPR-Cas9-targeted IL2RG insertion approach to treat X-SCID patients. (43) This improved both efficiency and safety of IL2RG correction via the CRISPR-Cas9-based thermodynamic integration (TI) approach and provided a strong rationale for a clinical trial for treatment of X-SCID patients.

For gene correction in hematopoietic stem cells, promising results have been shown in pre-clinical studies for X-SCID, ADA-SCID, X-CGD, and X-HIM syndrome. (44) The rapid development of targeted therapies that correct cell function in IEIs includes both; molecular targeted drug therapies, that correct defective pathways with current and newly developed targeted drugs and, permanent genetic corrections. The basis for these tailored therapies is the detection of damaging variants in IEIs in general, utilizing appropriate diagnostic methods as described in this book. However, in case of targeted therapies, clinical trials are often unavailable, but clinicians may conclude that a specific compound could be of additional value to a patient based on the genetic defects and underlying pathways. For example, in patients with CTLA4-insufficiency, abatacept would be considered, while baricitinib/ruxolitinib would be considered for those with *STAT1* GOF variants, based on their modes of action. Unfortunately, these therapies are generally not reimbursed by insurance companies due to a perceived lack of evidence. Cooperative international studies are necessary for the registration of these rare disorders in order to compare the outcomes of the newly developed promising therapies. Collaborative studies in IEIs during the recent COVID19 pandemic have shown to be of great value in clinical and vaccination outcomes, including genetic susceptibility studies. (45,46) For example, new IEIs were found in the regulation of type I interferon responses and RNA degradation causing “Multisystem Inflammatory Syndrome in children” (MISc) after SARS-CoV2 infection. (47) A detailed description of MISc is added in the appendix.

The cooperation between IEI interest groups, such as specialized pediatricians and internists, researchers and patient organizations are warranted to understand the pathophysiology and the clinics of IEIs and are essential for including novel targeted therapies in IEI guidelines and to ensure successful clinical implementation in the near future.

References

1. Bousfiha A, Moundir A, Tangye SG, Picard C, Jeddane L, Al-Herz W, et al. The 2022 update of IUIS phenotypical classification for human inborn errors of immunity. Journal of Clinical Immunology. 2022;42(7):1508-20.
2. Heimall JR, Hagin D, Hajjar J, Henrickson SE, Hernandez-Trujillo HS, Tan Y, et al. Use of genetic testing for primary immunodeficiency patients. Journal of clinical immunology. 2018;38:320-9.
3. Louhelainen J. SNP arrays. MDPI; 2016. p. 27.
4. Shaikh TH. Copy number variation disorders. Current genetic medicine reports. 2017;5:183-90.
5. Sharma M, Leung D, Momenilandi M, Jones LC, Pacillo L, James AE, et al. Human germline heterozygous gain-of-function STAT6 variants cause severe allergic disease. Journal of Experimental Medicine. 2023;220(5):e20221755.
6. Takeuchi I, Yanagi K, Takada S, Uchiyama T, Igarashi A, Motomura K, et al. STAT6 gain-of-function variant exacerbates multiple allergic symptoms. Journal of Allergy and Clinical Immunology. 2023;151(5):1402-9. e6.
7. Baris S, Benamar M, Chen Q, Catak MC, Martínez-Blanco M, Wang M, et al. Severe allergic dysregulation due to a gain of function mutation in the transcription factor STAT6. Journal of Allergy and Clinical Immunology. 2023.
8. Walford HH, Doherty TA. STAT6 and lung inflammation. Jak-stat. 2013;2(4):e25301.
9. Kim M-J, Lee Y-J, Yoon Y-S, Lim JH, Park E-M, Chong YH, et al. A STAT6 inhibitor AS1517499 reduces preventive effects of apoptotic cell instillation on bleomycin- induced lung fibrosis by suppressing PPARγ. Cellular Physiology and Biochemistry. 2018;45(5):1863-77.
10. Du P, Ma Q, Zhu Z-D, Li G, Wang Y, Li Q-Q, et al. Mechanism of Corilagin interference with IL-13/STAT6 signaling pathways in hepatic alternative activation macrophages in schistosomiasis-induced liver fibrosis in mouse model. European journal of pharmacology. 2016;793:119-26.
11. Yan J, Zhang Z, Yang J, Mitch WE, Wang Y. JAK3/STAT6 stimulates bone marrow– derived fibroblast activation in renal fibrosis. Journal of the American Society of Nephrology. 2015;26(12):3060-71.
12. Yukawa K, Kishino M, Goda M, Liang X-M, Kimura A, Tanaka T, et al. STAT6 deficiency inhibits tubulointerstitial fibrosis in obstructive nephropathy. International journal of molecular medicine. 2005;15(2):225-30.
13. Solís-Moruno M, Batlle-Masó L, Bonet N, Aróstegui JI, Casals F. Somatic genetic variation in healthy tissue and non-cancer diseases. European Journal of Human Genetics. 2023;31(1):48-54.
14. Thanveer F. Cutaneous manifestations in primary immunodeficiency diseases. Journal of Skin and Sexually Transmitted Diseases. 2021;3(2):143-50.
15. Isung J, Williams K, Isomura K, Gromark C, Hesselmark E, Lichtenstein P, et al. Association of primary humoral immunodeficiencies with psychiatric disorders and suicidal behavior and the role of autoimmune diseases. JAMA psychiatry. 2020;77(11):1147-54.
16. Pham MN, Fuleihan RL, Sullivan KE, Cunningham-Rundles C. Ocular Manifestations in Primary Immunodeficiency Disorders: A Report From the United States Immunodeficiency Network (USIDNET) Registry. The Journal of Allergy and Clinical Immunology: In Practice. 2022;10(7):1788-96.
17. Grimbacher B, Holland SM, Gallin JI, Greenberg F, Hill SC, Malech HL, et al. Hyper-IgE syndrome with recurrent infections—an autosomal dominant multisystem disorder. New England Journal of Medicine. 1999;340(9):692-702.
18. Zhou S, Dai Q, Huang X, Jin A, Yang Y, Gong X, et al. STAT3 is critical for skeletal development and bone homeostasis by regulating osteogenesis. Nature Communications. 2021;12(1):6891.
19. Hou X, Tian F. STAT3-mediated osteogenesis and osteoclastogenesis in osteoporosis. Cell Communication and Signaling. 2022;20(1):1-17.

20. Sediva H, Dusatkova P, Kanderova V, Obermannova B, Kayserova J, Sramkova L, et al. Short stature in a boy with multiple early-onset autoimmune conditions due to a STAT3 activating mutation: could intracellular growth hormone signalling be compromised? Hormone research in paediatrics. 2017;88(2):160-6.
21. Leiding JW, Vogel TP, Santarlas VG, Mhaskar R, Smith MR, Carisey A, et al. Monogenic early-onset lymphoproliferation and autoimmunity: natural history of STAT3 gain-of-function syndrome. Journal of Allergy and Clinical Immunology. 2023;151(4):1081-95.
22. Staufner C, Peters B, Wagner M, Alameer S, Barić I, Broué P, et al. Defining clinical subgroups and genotype–phenotype correlations in NBAS-associated disease across 110 patients. Genetics in Medicine. 2020;22(3):610-21.
23. Kuruvilla M, De la Morena MT. Antibiotic prophylaxis in primary immune deficiency disorders. The Journal of Allergy and Clinical Immunology: In Practice. 2013;1(6):573- 82.
24. Lucas CL, Chandra A, Nejentsev S, Condliffe AM, Okkenhaug K. PI3Kδ and primary immunodeficiencies. Nature Reviews Immunology. 2016;16(11):702-14.
25. Rao VK, Webster S, Šedivá A, Plebani A, Schuetz C, Shcherbina A, et al. A randomized, placebo-controlled phase 3 trial of the PI3Kδ inhibitor leniolisib for activated PI3Kδ syndrome. Blood, The Journal of the American Society of Hematology. 2023;141(9):971-83.
26. Suratannon N, Ittiwut C, Dik WA, Ittiwut R, Meesilpavikkai K, Israsena N, et al. A germline STAT6 gain-of-function variant is associated with early-onset allergies. Journal of Allergy and Clinical Immunology. 2023;151(2):565-71. e9.
27. Jennifer W Leiding, Lisa R Forbes. Mechanism-Based Precision Therapy for the Treatment of Primary Immunodeficiency and Primary Immunodysregulatory Diseases. J Allergy Clin Immunol Pract . 2019 Mar;7(3):761-773. doi: 10.1016/j.jaip.2018.12.017.
28. Kim TK, Eberwine JH. Mammalian cell transfection: the present and the future. Analytical and bioanalytical chemistry. 2010;397:3173-8.
29. Gatti R, Meuwissen H, Allen H, Hong R, Good R. Immunological reconstitution of sex- linked lymphopenic immunological deficiency. The Lancet. 1968;292(7583):1366-9.
30. Pai S-Y. Treatment of primary immunodeficiency with allogeneic transplant and gene therapy. Hematology 2014, the American Society of Hematology Education Program Book. 2019;2019(1):457-65.
31. Güngör T, Teira P, Slatter M, Stussi G, Stepensky P, Moshous D, et al. Reduced- intensity conditioning and HLA-matched haemopoietic stem-cell transplantation in patients with chronic granulomatous disease: a prospective multicentre study. The Lancet. 2014;383(9915):436-48.
32. Arlabosse T, Booth C, Candotti F. Gene therapy for inborn errors of immunity. The Journal of Allergy and Clinical Immunology: In Practice. 2023 Jun;11(6):1592-1601. doi: 10.1016/j.jaip.2023.04.001. Epub 2023 Apr 20. DOI: 10.1016/j.jaip.2023.04.001
33. Cavazzana-Calvo M, Hacein-Bey S, Basile GvdS, Gross F, Yvon E, Nusbaum P, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science. 2000;288(5466):669-72.
34. Aiuti A, Roncarolo MG, Naldini L. Gene therapy for ADA-SCID, the first marketing approval of an ex vivo gene therapy in Europe: paving the road for the next generation of advanced therapy medicinal products. EMBO molecular medicine. 2017;9(6):737-40.
35. Kohn DB, Booth C, Shaw KL, Xu-Bayford J, Garabedian E, Trevisan V, et al. Autologous ex vivo lentiviral gene therapy for adenosine deaminase deficiency. New England Journal of Medicine. 2021;384(21):2002-13.
36. Tucci F, Galimberti S, Naldini L, Valsecchi MG, Aiuti A. A systematic review and meta- analysis of gene therapy with hematopoietic stem and progenitor cells for monogenic disorders. Nature communications. 2022;13(1):1315.

37. Lauer A, Speroni SL, Choi M, Da X, Duncan C, McCarthy S, et al. Hematopoietic stem- cell gene therapy is associated with restored white matter microvascular function in cerebral adrenoleukodystrophy. Nature Communications. 2023;14(1):1900.
38. Cesana D, Sgualdino J, Rudilosso L, Merella S, Naldini L, Montini E. Whole transcriptome characterization of aberrant splicing events induced by lentiviral vector integrations. The Journal of clinical investigation. 2012;122(5):1667-76.
39. Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, Fusil F, et al. Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. Nature. 2010;467(7313):318-22.
40. De Ravin SS, Liu S, Sweeney CL, Brault J, Whiting-Theobald N, Ma M, et al. Lentivector cryptic splicing mediates increase in CD34+ clones expressing truncated HMGA2 in human X-linked severe combined immunodeficiency. Nature Communications. 2022;13(1):3710.
41. Rai R, Thrasher AJ, Cavazza A. Gene editing for the treatment of primary immunodeficiency diseases. Human gene therapy. 2021;32(1-2):43-51.
42. Li T, Yang Y, Qi H, Cui W, Zhang L, Fu X, He X, Liu M, Li P,Yu T. CRISPR/Cas9 therapeutics: progress and prospects. Signal Transduction and Targeted Therapy. 2023;8(36).
43. Brault J, Liu T, Liu S, Lawson A, Choi U, Kozhushko N, et al. CRISPR-Cas9-AAV versus lentivector transduction for genome modification of X-linked severe combined immunodeficiency hematopoietic stem cells. Frontiers in Immunology. 2022;13:1-15.
44. Kohn DB, Kuo CY. New frontiers in the therapy of primary immunodeficiency: from gene addition to gene editing. Journal of Allergy and Clinical Immunology. 2017;139(3):726-32.
45. Delmonte OM, Castagnoli R, Notarangelo LD. COVID-19 and Inborn Errors of Immunity. Physiology.2022;37(6):290-301
46. Casanova JL, Su HC, COVID Human Genetic Effort. Cell.2020;181(6):1194-1199
47. Buciol G, COVID Human Genetic Effort; Meyts I. Inherited and acquired errors of type I interferon immunity govern susceptibility to COVID-19 and multisystem inflammatory syndrome in children. The Journal of Allergy and Clinical Immunology.2023;151(4):832-840

Summary of the thesis

English summary

The field of IEI is rapidly evolving after the introduction of next-generation sequencing (NGS) platforms, such as whole exome sequencing (WES) and whole genome sequencing (WGS). In recent years, the application of these tools has significantly increased the identification of genetic defects causing IEI. This expansion has enhanced our understanding of genotype-phenotype correlation and provided valuable pathogenetic and pathophysiological insights into IEI. However, further studies are needed to complete our understanding, especially in specific geographical populations where studies are conducted rarely such as in South-East Asia region. Furthermore, most modern genetic approaches, such as WES and WGS, are not affordable or available to a large portion of the global population, particularly in low-income developing countries. Even simple initial immunological tests such as immunoglobulin measurement, may not be accessible in such countries. Therefore, there is a need to develop or optimize accessible and affordable tests.

Part I of this thesis describes the development of affordable techniques for the diagnosis of IEI. **Chapter 2** represents the first study in which WES was applied for genetic testing in the Thai pediatric population suspected of IEI. Importantly, the study revealed novel variants associated with IEIs, which guided to a definite diagnosis and changed the treatment approaches for the tested patients, including the decision to perform the allogeneic stem cell transplantation. The results from this study expand our knowledge of the genotype-phenotype spectrum of IEI.

However, utilizing WES and WGS for genetic analysis in a clinical setting has limitations. These techniques are time-consuming, costly, and involve complex data analysis and interpretation. Due to high costs and limited resources, NGS is not widely available in many developing countries. Therefore, there is a need to explore rapid, robust, and inexpensive molecular tools to address this gap. **Chapter 3** describes the development of a rapid, low-cost customized microarray for genetic diagnosis in IEI. This technique demonstrated its promising potential as a first-line screening tool in developing countries. Further, the methods for microarray bioinformatics are reviewed in **Chapter 4**.

While modern genetic testing methods provides important insights into the pathophysiology of IEI and guides to the therapeutic approach, the analysis starts with simple tests ("basic to the basics") in order to investigate immunological changes in patients according to their phenotype. International consensus guidelines recommend prompt measurement of serum immunoglobulin (Ig)G in patients suspected of having an antibody deficiency. However, in many developing countries, even such tests are only available in referral centers and not easily accessible to a substantial number of people. Measurement of total serum globulin levels, routinely assessed in liver biochemistry testing, can be an attractive candidate for screening hypogammaglobulinemia in developing countries. **Chapter 5** describes the development of a model that predicts immunoglobulin G levels in children using serum globulin levels combined with the patient's disease status. This model facilitates timely

decision-making for initiating immunoglobulin therapy in pediatric patients suspected of hypogammaglobulinemia in developing countries.

The presentation of IEI patients can be very diverse with atypical immunological manifestations and non-immunological comorbidities. **Part II** of the thesis describes different intriguing cases, demonstrating the diverse clinical spectrum of IEI patients. **Chapter 6** describes a patient with hypomorphic *DOCK8* variants who exhibited a milder phenotype compared to the typical *DOCK8*-deficient patients. Making a definite diagnosis is a challenge for such a patient. In comparison, *DOCK8* deficiency was diagnosed in another more typical patient **(chapter 7)** who was experiencing recurrent episodes of different types of hematological malignancies, highlighting the role of DOCK8 in immune surveillance of malignancies. **Chapter 8** describes the lifelong clinical course of two sisters with identical damaging *NBAS* variants, who suffered from combined immunodeficiency, autoimmunity, allergy and malignancy. **Chapter 9** describes a patient in whom the innate immune system is affected. Genetic analysis revealed damaging *FERMT3* variants. The patient was then diagnosed with leukocyte adhesion deficiency (LAD) type III. However, unexpectedly an impairment of the antibody production was found as well in this patient. This was never reported and indicated a defect in the adaptive immune system. Immunoglobulin replacement therapy led to a dramatic clinical improvement in this patient. **Chapter 10** provides insights into a patient with congenital glycosylation disorder and a severe immune deficiency. Novel compound heterozygous variants in *PGM3* were found. The longstanding history of this patient with immunodeficiency as well as the recovery time of PGM3 activity after a matched related donor stem cell transplantation is discussed.

Traditionally, allergy has been regarded as a polygenic disease, influenced by genetic susceptibility in the form of single nucleotide polymorphisms (SNPs) that interact with immunomodulating factors in the environment. However, allergy has been recognized as a frequent immunological comorbidity in IEIs. Recently, a new subcategory of IEIs known as "primary atopic disorders" has been proposed, mainly referring to allergic patients who possess genomic variants contributing to their allergic manifestations. **Part III Chapter 11** presents, for the first time, a family with a gain-of-function (GOF) variant in *STAT6*, causing an early onset severe allergic phenotype. This study provided important pathophysiological insights in patients with monogenic severe allergies early in life and sheds light on the role of STAT6 in allergic diseases.

Finally, in **Part IV**, the general discussion of the entire thesis covers the current perspectives in IEIs. This knowledge expands further the recognition of the clinical spectrum of IEIs and emphasizes the importance of diagnosing IEI in patients who present with unusual clinical symptoms, enabling the implementation of mechanism-based therapeutic approaches.

Nederlandse samenvatting

Genetische defecten die leiden tot stoornissen in het immuunsysteem worden “Inborn Errors of Immunity” (IEI) genoemd. Deze omvatten een diverse groep immunologische aandoeningen die de werking van het immuunsysteem beïnvloeden, en welke weer kunnen leiden tot een breed scala aan klinische manifestaties. Deze omvatten immuundeficiënties met een verhoogde gevoeligheid voor infecties, de ontwikkeling van autoimmuunziekten en autoinflammatie, allergieën en zelfs maligniteiten. Het ziektebeeld bij de patiënt kan een of meerdere immunologische componenten bevatten. Bovendien lijden patiënten met IEIs ook frequent aan niet-immunologische ziekten, zoals bijvoorbeeld groeistoornissen.

Door de recente biotechnologische ontwikkelingen worden steeds meer varianten in genen gevonden die geassocieerd zijn met IEIs. Met name sinds de beschikbaarheid en invoering van “next generation sequencing” (NGS) is het begrip over de relatie tussen het genotype (genetische afwijking) en het fenotype (klinisch beeld) sterk vergroot. Deze verbetering van inzicht in de onderliggende pathofysiologie heeft geleid tot betere en meer op de patiënt gerichte behandelingsmogelijkheden.

De beschikbaarheid van geavanceerde diagnostische hulpmiddelen zoals NGS en bepaalde immunologische tests, waaronder serumimmunoglobuline-analyse, is in veel ontwikkelingslanden beperkt vanwege de kosten. Hierdoor bestaat de behoefte aan kosteneffectieve laboratoriumtesten om deze kloof te overbruggen. Dit proefschrift introduceert goedkope innovatieve en betaalbare diagnostische tests; een op serumglobuline gebaseerd model voor het voorspellen van hypogammaglobulinemie en op maat gemaakte microarrays voor het stellen van een genetische diagnose. Deze heel goedkope methode draagt bij om een IgG deficiëntie op te sporen als de IgG bepaling niet beschikbaar is. Er werd verder een goedkope techniek ontwikkeld, op basis van een zogenaamde SNP-array, om genetisch diagnostische analyse te verrichten. Er werden meer dan 9000 bekende genetische varianten die een IEI veroorzaken toegevoegd aan de SNP-array. In een “proof of principle” onderzoek met betrekking tot de werkzaamheid van deze array konden vele varianten teruggevonden worden en ook deleties in het DNA aangetoond worden. Dit maakt deze procedure veelbelovend als een goedkope screeningsmethode in de diagnostische fase voor de veel duurdere NGS-methode. Echter de laatste is superieur omdat alle varianten gevonden kunnen worden terwijl met de array methode alleen de toegevoegde bekende varianten gevonden kunnen worden.

In dit proefschrift werd verder het gebruik van genetische analyse, in het bijzonder exome-sequencing, in Thaise kinderen en Nederlandse volwassen patiënten beschreven. Hierdoor werden verschillende opmerkelijke IEIs werden opgespoord en beschreven met ter illustratie het brede klinische spectrum. Bovendien werd door ons team voor het eerst een nieuwe IEI ontdekt in een familie met primaire atopische stoornissen inclusief een ernstige voedingsallergie, deze bleek te berusten op een gain of function (GOF) mutation in *STAT6*. Deze transcriptiefactor speelt een essentiële rol bij allergische reacties en bleek overactief in de bestudeerde familie.

Als laatste werd in dit proefschrift beschreven hoe COVID-19 bij kinderen een uitgebreid immunologisch ontstekingsbeeld veroorzaakt, waarin meerdere organen aangedaan zijn. Deze immunologische reactie, “Multisystemic Inflammatory Syndrome in children” (MISc) genaamd ontstaat gewoonlijk zo’n 2 tot 6 weken na een SARS-CoV2 infectie. De bechrijving van MISc werd aan de appendix toegevoegd.

De verschillende onderzoeken die in dit proefschrift worden gepresenteerd, dragen bij tot een beter begrip van het klinische spectrum van IEIs en onderstrepen het belang van een tijdige diagnose bij patiënten met (a)typische klinische symptomen. Hierdoor kan de implementatie van een gerichte therapeutische strategie op basis van de onderliggende genetische afwijking zonder vertraging plaatsvinden.

ADDENDUM

Acknowledgements
Curriculum vitae
Awards and Honors
PhD portfolio
Publications

Acknowledgements

“THE DOCTORAL JOURNEY”

“But as it is written, Eye hath not seen, nor ear heard, neither have entered into the heart of man, the things which God hath prepared for them that love him.” (Bible; 1 Corinthians 2:9)

It’s been a journey to earn a Ph.D. at Erasmus MC than just a feeling of graduation. At the beginning, it seemed tough, but looking back at challenges, hopes and achievements, I appreciate my Ph.D. experience more than ever. I would like to extend my gratitude and appreciation to all those who have contributed to the completion of this thesis. Their support, encouragement, and assistance have been invaluable throughout this journey.

First and foremost, my deepest thanks to my promoter, Martin. You have been my hero, providing constant support, guidance, and encouragement. You are a great, smart, incredibly kind person and have supported me from day one. Thank you also Peter for your great guidance. You have always surprised me with a great idea and opened my perspective about how to integrate bioinformatics into medical knowledge. Wim and Virgil, you both are amazing supervisors. Your insightful feedback and unwavering support play a pivotal role in shaping this thesis. Thank you also Hanna who always takes time to give invaluable comments on my work. I hope to continue working with all of you in the future. In addition, I am grateful to the doctoral committee and the PhD examining committee for generously dedicating their time to offer valuable advice throughout this process.

A special thanks to Pantipa, my mentor, who helped me grow both personally and professionally. You have been more than just a mentor; you've been a role model. On my darkest days, you showed me how to stay positive and inspired me to excel in all aspects of life. Nattiya, you were the one who introduced me to immunology and made it possible for me to study Ph.D. at Erasmus MC. Without both of you, I wouldn't be where I am today. Thank you for being there for me through thick and thin.

I am incredibly grateful to Chulalongkorn University, my beloved workplace, for providing me with the necessary resources and facilities for my research. I sincerely thank all my friends and colleagues at Chulalongkorn and Erasmus MC for their support during difficult times, especially Prof. Jarungchit, Thaneeya our nurse, and everyone from Department of Pediatrics, Faculty of Medicine, Chulalongkorn University. Special thanks to Dr. Supranee, Prof. Vorasuk, Prof. Tanapat, Prof. Kanya, Dr. Nipan, Namthip, Namkang, Arm, Dr. Mirjam, Sandra, Barbara, Ingrid, Conny, Nicole, Benjamin, Rina, Prayer, Kornvalee, and Verna – each of you played a role in completing this thesis. On top of that, your involvement made this journey more meaningful, and I couldn't have done it without you. Special thanks to my beloved sisters and

brother; Olivia, Kiki, Sigrid, and Rogier, for your scientific support and being there for me throughout my journey. I feel so warm having you guys around. Thanks also to Ning, for being a great supporter during my time in the Netherlands and helping me stay motivated.

Furthermore, I would like to acknowledge the valuable contributions of Judy. You are a wonderful lady, supporting me and other Ph.D. students with your heart. I am also thankful to the IPOPI team, especially my dear friends Jose, Johan, Nizar, and Martin. You are all wonderful people! Collaborating with you has been an inspiring experience that has contributed significantly to certain aspects of my thesis.

Lastly, my heartfelt thanks go to my family for their unconditional love. Without you, it would not have been as significant or fulfilling. Thank you for being an essential part of this journey. I am forever grateful for your enduring support.

Curriculum vitae

Name	Narissara Suratannon, MD
Mailing address	Center of Excellence for Allergy & Clinical Immunology, Division of Allergy, Immunology and Rheumatology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, The Thai Red Cross Society, Rama IV Road, Pathumwan, Bangkok 10330, Thailand
Telephone	(662) 2564933, (6681) 2950190
Email	narissara.su@chula.ac.th, mayzped@gmail.com,
Fax	(662) 2564933
Current Position	***Assistant Professor***, Division of Allergy, Immunology and Rheumatology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand ***Head*****,** Center of Excellence for Allergy & Clinical Immunology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand ***Group Leader*****,** Clinical Immunology Interest Group, Allergy, Asthma and Immunology Society of Thailand ***Chair*****,** Education and Training Working Party, Asia Pacific Society for Immunodeficiencies ***Member***, Southeast Asia Primary Immunodeficiencies (SEAPID) Consortium

Education

1997-2003:	***Medical Degree (First class honor)***, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
2004-2007:	***Pediatric Residency Training***, Department of Pediatrics, Chonburi Hospital, Chonburi, Thailand
2008-2010:	***Pediatric Allergy and Immunology Clinical Fellowship*** Department of Pediatrics, Chulalongkorn University, Bangkok, Thailand
2012-2013:	***Research Fellowship in Immunology*** Department of Immunology, Erasmus Medical Center, Rotterdam, the Netherlands
2016-2022:	***PhD candidate in Immunology*** Department of Immunology, Erasmus Medical Center, Rotterdam, the Netherlands

Experiences & grants

2007-present	Reviewer, Asian Pacific Journal of Allergy and Immunology
2014-present	Medical Liaison Officer, ThaiPOPI (Thai patient organization of primary immunodeficiency disorders), member of IPOPI (the International Patient Organization for Primary Immunodeficiency)
2017	New Researcher Grant from Thailand Research Fund (TRF) on the topic of "Functional characterization of the STAT6 in allergy"
2018	The National Science and Technology Development Agency (NSTDA) on the topic of "The development of early life gut microbiome and microbial proteomics and association with allergic diseases: A longitudinal cohort study of Thai infants"
2022	Scientific committee, International Primary Immunodeficiencies Congress (IPIC) 2022
2023	Scientific committee, 2023 HAEi Regional Conference APAC

Awards and Honors

2012	Research Fellowship award from European Academy of Allergy, Asthma & Immunology (EAACI)
2017	New Researcher Grant from Thailand Research Fund (TRF)
2018	Nestle Young Investigator Award, Thailand

PhD Portfolio

ACTIVITIES	YEAR	WORKLOAD (ECTS)
Courses		
Basic Introduction Course on SPSS	2017	0.7
Advance Immunology Course	2016	4.5
Summer School: Translational research for rare diseases	2017	0.5
Presentations and (inter)national conferences		
Presentation in the international congress, MSAI-APSID, Kualalumpur, Malaysia	2022	2.0
Presentation in the international congress, 3rd Care-for-Rare- IPOPI-Erasmus University Medical Center-Chulalongkorn University, Bangkok, Thailand	2022	1.5
Presentation in the international congress, 59th JSPACI & APAPARI meeting, Okinawa, Japan	2022	1.0
Presentation in the international congress, IPACI, Indonesia (virtual meeting)	2022	0.7
Presentation in the international congress, MSAI-IPOPI; Regional Asian PID Meeting, Malaysia (virtual meeting)	2022	0.7
Presentation in the international congress; 5th IPIC 2022, Vilamoura, Portugal	2022	0.7
Presentation in AAIAT annual meeting, Bangkok, Thailand, topic “Diagnostic Test in Food Allergy: Current knowledge and application” and “How device and formulation contribute to successful treatment”	2022	1.0
Presentation in Thai Rhinologic Society, Bangkok, Thailand, topic “Gut microbiome in allergic rhinitis”	2022	0.7
Presentation in short course meeting 2022, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, topic “Recurrent infections: allergy or immunodeficiency” and “Anaphylaxis: an update”	2022	1.7
Presentation in Fellow refresh course; AAIAT meeting, Bangkok, Thailand, topic “Oral immunotherapy”	2022	0.7
Presentation in AAIAT mid-annual meeting, Bangkok, Thailand, topic “Food Allergy: Year-in-Review from GI & Allergy perspectives: and “Tips and Tricks in Allergy Practice 2: Food Allergy; From Lab to Clinic”	2022	1.0
Presentation in AAIAT virtual meeting, topic “HAE: Patient journey in Thailand”	2022	0.5
Presentation in the annual meeting of Thai Pediatric Nutrition Society, Bangkok, Thailand, topic “soy milk and gut microbiome”	2022	0.7
Presentation in AAIAT virtual meeting, topic “Asthma diagnosis of general practice”	2022	0.5
Presentation in AAIAT virtual meeting, topic “Update in cow’s milk protein allergy”	2022	0.5
Presentation in AAIAT virtual meeting, topic “HAE: Diagnosis and long-term treatment”	2022	0.5
Presentation in AAIAT virtual meeting; Biologics in asthma and chronic urticaria”	2022	0.5
Presentation in the international congress, Australian Food Allergy Working Group (virtual meeting), topic “Food Allergy in Asian Developing Countries: Data from Birth Cohort Study”	2021	0.5

Presentation in the international congress, Regional Asian PID ThaiPOPI-IPOPI Meeting, Thailand (virtual meeting), topic “Monogenic disorders in Allergy”	2021	0.7
Presentation in the international congress MSAI meeting, Malaysia (virtual meeting); topic “Component-resolved diagnostics in food allergy”	2021	0.7
Presentation in the international congress, IPOPI-Indonesia (virtual meeting), topic “Genetic diagnosis in PIDs”	2021	0.5
Presentation in AAIAT HAE Raising awareness series (virtual meeting), topic “HAE: Long-term prophylaxis”	2021	0.5
Presentation in short course meeting 2021, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand	2021	1.5
Presentation in AAIAT virtual meeting, topic “EAACI 2021: highlight on Food and Drug allergy”	2021	0.5
Presentation in AAIAT HAE Raising awareness series, Chonburi hospital, Chonburi, Thailand, topic “HAE: Diagnosis and Principle of Treatment”	2021	0.5
Presentation in AAIAT annual meeting, Bangkok, Thailand, topic “Primary Immunodeficiency disorders in COVID-19 Era”	2021	0.7
Presentation in AAIAT virtual meeting, topic “Asthma treatment: Role of ICS+LABA”	2021	0.5
Presentation in AAIAT virtual meeting, topic “Soy allergy: Health Benefit and Role to Gut microbiome”	2021	0.5
Presentation in AAIAT HAE Raising awareness series, Pramongkutklao Hospital, Bangkok, Thailand, topic “HAE: Diagnosis and Principle of Treatment”	2021	0.5
Presentation in Biomed Annual meeting, Bangkok, Thailand, topic “Role of specific IgE testing in Allergy Diagnosis ”	2021	0.5
Presentation in AAIAT Fellow refresh course 2021, topic “Component-resolved diagnosis: role for allergy management”	2021	0.5
Presentation in Pediatric nurse workshop, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, topic “Oral food challenge & drug provocation test”	2021	0.5
Presentation in AAIAT virtual meeting; topic “HAE: Patient journey in Thailand”	2021	0.5
Presentation in Pediatric Research Day, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, topic “PID: From Care to Cure”	2021	0.5
Presentation in AAIAT virtual meeting; topic “Food Allergy Treatment”	2021	0.5
Presentation in Thai Asthma Council annual meeting, Bangkok, Thailand, topic “Immunologic diagnosis in allergic diseases: Roles of phadiatop”	2021	1.0
Presentation in AAIAT virtual meeting, topic “Practicing OIT for Food Allergy: Office base”	2021	0.5
Presentation in AAIAT virtual meeting, topic “Update in Food allergy”	2021	0.5
Presentation in the international congress, Regional Asian Doctors & PID Patients Meeting (virtual meeting), topic “Regional priorities – SEAPID perspectives”	2020	0.5
Presentation in the international congress, 3rd APSID 2020 congress, Chandigarh, India, topic “Rapid tools on genetic testing for PID”	2020	0.7
Presentation in AAIAT virtual meeting, topic “Highlights from FAAM-EUROBAT DIGITAL 2020”	2020	0.5

Presentation in AAIAT virtual meeting, topic “Highlights from ESID 2020”	2020	0.5
Presentation in AAIAT annual meeting, Bangkok, Thailand, topic “Update in Food Allergy: Allergy Perspective, Investigations in Food allergy: From Bench to Bedside”	2020	1.0
Presentation in short course meeting 2020, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, topic “Food allergy: a multidisciplinary approach”	2020	1.5
Presentation in AAIAT virtual meeting, topic “Highlight from FAAM FAAM-EUROBAT Digital 2020 congress”	2020	0.5
Presentation in AAIAT virtual meeting, topic “Component-resolved diagnostics for food allergy”	2020	0.5
Presentation in AAIAT virtual meeting, topic “Practical points for AR management”	2020	0.5
Presentation in 56th Annual congress meeting 2020, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, topic “New trends in Pediatric Allergy”	2020	0.5
Presentation in AAIAT virtual meeting, topic “Treatment for Cow's milk allergy: an Update”	2020	0.5
Presentation in Allergy summit 2020, topic “Primary Immunodeficiency disorders” and “Early nutrition and gut microbiome”	2020	0.7
Presentation in AAIAT Allergy Focus Group, topic “EAACI 2020: what's new?”	2020	0.5
Presentation in the international congress, APSID school 2019, Manado, Indonesia, topic “General tests for diagnosis of PID”	2019	1.0
Presentation in the international congress, 15th congress of ASPR, Manado, Indonesia, topic “Approach to bacterial infection”	2019	1.0
Presentation in the international congress, APAPARI 2019, Bali, Indonesia, topic “Development of PID diagnostic network” and “Update treatment of allergic rhinitis”	2019	1.5
Presentation in the international congress, IPIC 2019, Madrid, Spain, topic “Genetic testing: democratizing access”	2019	1.0
Presentation in the international congress, IPOPI Vietnam meeting, Ho chi min, Vietnam, topic “Rapid Genetic testing for PID” & “Functional testing for PID (flow cytometry)” & “HyperIgM syndrome”	2019	1.0
Presentation in AAIAT annual meeting 2019, topic “Update on Food Allergy Treatment”	2019	0.7
Presentation in the international congress, 11th NACLIS meeting, Kualalumpur, Malaysia, topic “PID Molecular Testing in Southeast Asia”	2019	0.7
Presentation in DAAD Summer School, Munich, Germany, topic “Rapid Genetic testing for PID”	2019	1.3
Presentation in 2nd Bangkok International Pediatrics Update or BIPU 2018, topic “Management in Anaphylaxis”	2018	1.0
Presentation in the 2018 KAAACI-EAAS-SERIN ASIA Joint Congress in conjunction with APAAACI, Seoul, South Korea, topic “Alterations of gut microbiota associated with distinct allergic phenotypes: Data from an Asian longitudinal birth cohort study”	2018	1.0

Presentation in the 6th Thai society of Hematology International Symposium, Bangkok, Thailand, topic “Phagocytic defect”	2018	1.5
Presentation in IPOPI Regional Patients & Doctors Meeting, Bangkok, Thailand	2018	0.7
Presentation in APAAACI & APAPARI Congress, Bangkok, Thailand, topic “Gut microbiome in atopic dermatitis” and “Genetic testing in Primary Immunodeficiencies”	2018	1.7
Presentation in Erasmus-Chula joint meeting, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, topic “STAT3 gain of function mutation”	2017	0.5
Presentation in the international congress, Vietnamese PID patients-doctor meeting, Hanoi, Vietnam, topic “SEAPID and ThaiPOPI- what this means to you?”	2017	0.5
Presentation in the international congress, Junior molecular diagnostic workshop, Stockholm, Sweden, topic “CRD in Shrimp allergy: Molecular diagnosis for clinical use”	2017	0.7
Presentation in the international congress, Japanese society allergy Meeting Nara, Japan, topic “Shrimp tropomyosin in the diagnosis of shrimp allergy”	2017	1.0
Presentation in AAIAT annual meeting 2017, Bangkok, Thailand, topic “Hot topics in AAAAI annual meeting 2016”	2017	1.0
Presentation in Erasmus-Khonkan MOU meeting, Khonkan, Thailand, topic “Primary Immunodeficiency disorders”	2017	0.5
Presentation in the international congress, APSID congress 2017, Hongkong, topic “SEAPID network for PID”	2017	1.0
Presentation in the international congress, APSID Spring school 2017, Hongkong, topic “Interesting cases in PID”	2017	0.7
Presentation in the international congress, IPOPI Asian Meeting 2017, Hongkong, topic “PID network; what this means to you?”	2017	0.5
Presentation in AAIAT annual meeting, Bangkok, Thailand, topic “Food allergy” and “Classic Cases of Asthma and Allergic Rhinitis Failure in control: Reasons and Consequences”	2017	1.0
Presentation in IPOPI 2016 Asian Regional Patient Meeting, Hongkong, topic “SEAPID update: what has been done so far and what is coming ahead?”	2016	0.6
Presentation in APSID congress 2016, topic “SEAPID Network – History Development”	2016	1.5
Oral and poster presentation at the international congress		
Poster presentation international congress, ESID 2018	2018	1.5
Oral presentation in 2018 NNI Young Researcher’s Award, Singapore	2018	0.5
Poster presentation international congress, EAACI 2016	2016	1.5
Poster presentation in the 17th biennial meeting of the European Society for Immunodeficiencies (ESID), Barcelona, Spain	2016	1.0
Attending congress		
Attending AAAAI 2022 congress (virtual meeting)	2022	1.0
Attending EAACI 2022 congress	2022	0.7
Attending AAAAI 2021 congress (virtual meeting)	2021	1.0
Attending EAACI 2021 congress (virtual meeting)	2021	0.7
Attending AAAAI 2020 congress (virtual meeting)	2020	1.0

Attending EAACI 2020 congress (virtual meeting)	2020	0.7
Attending FAAM FAAM-EUROBAT Digital 2020 congress (virtual meeting)	2020	0.5
Attending AAAAI 2019 congress	2019	1
Attending PAM 2019 congress	2019	0.7
Attending EAACI 2019 congress	2019	0.7
Attending AAAAI 2018 congress	2018	1.0
Attending EAACI 2018 congress	2018	1.3
Attending SEA advocacy and media training workshop	2018	0.3
Attending FAAM 2018 congress	2018	0.7
Attending HAEi Global Conference 2016	2016	1.0
Attending FAAM 2016 congress	2016	0.7
Attending NNI Regional Advanced Pediatric Nutrition Course	2016	0.7
Teaching		
Teaching Allergy and Immunology resident rotations (36 hours)	2022	1.5
Teaching Allergy and Immunology resident rotations (36 hours)	2021	1.5
Teaching Allergy and Immunology resident rotations (36 hours)	2020	1.5
Teaching Allergy and Immunology resident rotations (36 hours)	2019	1.5
Teaching Allergy and Immunology resident rotations (36 hours)	2018	1.5
Teaching Allergy and Immunology resident rotations (36 hours)	2017	1.5
Teaching Allergy and Immunology resident rotations (36 hours)	2016	1.5
Others		
Supervisor for Journal club and Topic presentation for fellows	2016-2022	4.0
Supervisor for collective review of fellows	2016-2022	1.5
Supervisor for poster and oral presentation at AAAAI congress and EAACI congress	2016-2022	2.0
Supervisors for fellow oral presentation in APSID autumn school, Bangkok, Thailand	2018	0.5
Supervisors of fellows to do the oral presentation in APSID school, Chongqing, China	2018	0.5
Total ECTS		**102.4**

MANUSCRIPTS THAT FORM THE BASIS OF THIS THESIS

- Tengsujaritkul M*, *Suratannon N*,* Ittiwut C, Ittiwut R, Chatchatee P, Suphapeetiporn K, Shotelersuk V. Phenotypic heterogeneity and genotypic spectrum of inborn errors of immunity identified through whole exome sequencing in a Thai patient cohort.
  Pediatr Allergy Immunol. 2022 Jan;33(1):e13701.

- *Suratannon N**, van Wijck RTA*, Broer L, Xue L, van Meurs JBJ, Barendregt BH, van der Burg M, Dik WA, Chatchatee P, Langerak AW, Swagemakers SMA, Goos JAC, Mathijssen IMJ, Dalm VASH, Suphapeetiporn K, Heezen KC, Drabwell J, Uitterlinden AG, van der Spek PJ, van Hagen PM; South East Asia Primary Immunodeficiencies (SEAPID) Consortium. Rapid Low-Cost Microarray-Based Genotyping for Genetic Screening in Primary Immunodeficiency.
  Front Immunol. 2020 Apr 15;11:614. doi: 10.3389/fimmu.2020.00614. eCollection 2020.

- *Suratannon N*, Tantithummawong P, Hurst CP, Chongpison Y, Wongpiyabovorn J, van Hagen PM, Dik WA, Chatchatee P. Pediatric Prediction Model for Low Immunoglobulin G Level Based on Serum Globulin and Illness Status.
  Front Immunol. 2022 Feb 21;13:825867. doi: 10.3389/fimmu.2022.825867.

- *Suratannon N*, van der Spek PJ, van Hagen PM. Microarray Bioinformatics 2018.
  In eLS, John Wiley & Sons, Ltd (Ed.). https://doi.org/10.1002/9780470015902.a0005957.pub3

- *Suratannon N*, Yeetong P, Srichomthong C, Amarinthnukrowh P, Chatchatee P, Sosothikul D, van Hagen PM, van der Burg M, Wentink M, Driessen GJ, Suphapeetiporn K, Shotelersuk V.Adaptive immune defects in a patient with leukocyte adhesion deficiency type III with a novel mutation in *FERMT3*. Pediatr Allergy Immunol. 2016 Mar;27(2):214-7. doi: 10.1111/pai.12485.

- *Suratannon N*, Ittiwut C, Dik WA, Ittiwut R, Meesilpavikkai K, Israsena N, Ingrungruanglert P, Dalm VASH, van Daele PLA, Sanpavat A, Chaijitraruch N, Schrijver B, Buranapraditkun S, Porntaveetus T, Swagemakers SMA, IJspeert H, Palaga T, Suphapeetiporn K, van der Spek PJ, Hirankarn N, Chatchatee P, Martin van Hagen P, Shotelersuk V. A germline STAT6 gain-of-function variant is associated with early-onset allergies.
  J Allergy Clin Immunol. 2023 Feb;151(2):565-571.e9.

- *Suratannon N*, Dik WA, Chatchatee P, Hagen PMV. COVID-19 in children: Heterogeneity within the disease and hypothetical pathogenesis.
  Asian Pac J Allergy Immunol. 2020 Sep;38(3):170-177. doi: 10.12932/AP-170720-0920.

*Denotes equal contribution

Publications

1. Chan CM, Abdul Latiff AH, Noh LM, Ismail IH, Abd Hamid IJ, Liew WK, Zhong Y, **Suratannon N**, Nantanee R, Santos-Ocampo FJ, Castor MAR, Nguyen-Ngoc-Quynh L, Van Nguyen AT, Thuc HT, Tuan NM, Muktiarti D, Amalia R, Chean S, Try L and Ali A. Transition practice for Primary immunodeficiency diseases in Southeast Asia: a regional survey. Front. Immunol. 2023; 14:1209315.
2. A germline *STAT6* gain-of-function variant is associated with early-onset allergies. **Suratannon N**, Ittiwut C, Dik WA, Ittiwut R, Meesilpavikkai K, Israsena N, Ingrungruanglert P, Dalm VASH, van Daele PLA, Sanpavat A, Chaijitraruch N, Schrijver B, Buranapraditkun S, Porntaveetus T, Swagemakers SMA, IJspeert H, Palaga T, Suphapeetiporn K, van der Spek PJ, Hirankarn N, Chatchatee P, Martin van Hagen P, Shotelersuk V.J Allergy Clin Immunol. 2022 Oct 7:S0091-6749(22)01334-3.
3. Monogenic early-onset lymphoproliferation and autoimmunity: Natural history of STAT3 gain-of-function syndrome. Leiding JW, Vogel TP, Santarlas VGJ, Mhaskar R, Smith MR, Carisey A, Vargas-Hernández A, Silva-Carmona M, Heeg M, Rensing-Ehl A, Neven B, Hadjadj J, Hambleton S, Ronan Leahy T, Meesilpavikai K, Cunningham-Rundles C,; STAT3 GOF Working Group members; **Suratannon N**, Seppänen MRJ, Torgerson TR, Sogkas G, Ehl S, Tangye SG, Cooper MA, Milner JD, Forbes Satter LR.J Allergy Clin Immunol. 2022 Oct 11:S0091-6749(22)01182-4.
4. Effects of Maternal Exclusion Diet for Infants Suspected Food Allergy on Fatty Acid Composition in Breast Milk. Furangseroj T, Suteerojntrakool O, Hongvisitagul P, Chatchatee P, **Suratannon N**, Sapwarobol S, Techasukthavorn V, Dahlan W, Chomtho S. Breastfeed Med. 2022 May 11. doi: 10.1089/bfm.2021.0352. Online ahead of print.PMID: 35544150
5. Did variants in inborn errors of immunity genes contribute to the extinction of Neanderthals? Zhou Z, M A Swagemakers S, S Lourens M, **Suratannon N**, J van der Spek P, Dalm VA, A Dik W, IJspeert H, van Hagen PM. Asian Pac J Allergy Immunol. 2022 Dec; 40(4):422- 434.
6. Targeted Gene Sanger Sequencing Should Remain the First-Tier Genetic Test for Children Suspected to Have the Five Common X-Linked Inborn Errors of Immunity. Chan KW, Wong CY, Leung D, Yang X, Fok SFS, Mak PHS, Yao L, Ma W, Mao H, Zhao X, Liang W, Singh S, Barbouche MR, He JX, Jiang LP, Liew WK, Le MHT, Muktiarti D, Santos-Ocampo FJ, Djidjik R, Belaid B, Ismail IH, Abdul Latiff AH, Lee WS, Chen TX, Liu J, Jin R, Wang X, Chien YH, Yu HH, Raj D, Raj R, Vaughan J, Urban M, van den Berg S, Eley B, Lee AC, Isa MS, Ang EY, Lee BW, Yeoh AEJ, Shek LP, Quynh Le NN, Nguyen VAT, Phan Nguyen Lien A, Capulong RD, Mallillin JM, Villanueva JCMM, Camonayan KAB, Vera M, Casis-Hao RJ, Lobo RCM, Foronda R, Binas VWE, Boushaki S, Kechout N, Phongsamart G, Wongwaree S, Jiratchaya C, Lao-Araya M, Trakultivakorn M, **Suratannon N**, Jirapongsananuruk O, Chantveerawong T, Kamchaisatian W, Chan LL, Koh MT, Wong KJ, Fong SM, Thong MK, Latiff ZA, Noh LM, de Silva R, Jouhadi Z, Al-Saad K, Vignesh P, Jindal AK, Rawat A, Gupta A, Suri D, Yang J, Au EY, Kwok JS, Chan SY, Hui WY, Chua GT, Duque JR, Cheong KN, Chong PCY, Ho MHK, Lee TL, Wong WH, Yang W, Lee PP, Tu W, Yang XQ, Lau YL. Front Immunol. 2022 Jul 8;13:883446.
7. Pediatric Prediction Model for Low Immunoglobulin G Level Based on Serum Globulin and Illness Status. **Suratannon N**, Tantithummawong P, Hurst CP, Chongpison Y, Wongpiyabovorn J, van Hagen PM, Dik WA, Chatchatee P. Front Immunol. 2022 Feb 21;13:825867.
8. Novel CD55 Mutation Associated With Severe Small Bowel Ulceration Mimicking Inflammatory Bowel Disease in a Pair of Siblings. Chongsrisawat V, **Suratannon N**, Chatchatee

P, Ittiwut R, Ittiwut C, Weerapakorn W, Theamboonlers A, Rohlfs M, Klein C, Kotlarz D, Suphapeetiporn K. Inflamm Bowel Dis. 2022 Feb 4:izac001.

9. Characteristics and Laboratory Findings of Food-Induced Anaphylaxis in Children: Study in an Asian Developing Country. Nantanee R, **Suratannon N**, Chatchatee P. Int Arch Allergy Immunol. 2021 Sep 7:1-9.
10. Phenotypic Heterogeneity and Genotypic Spectrum of Inborn Errors of Immunity Identified through Whole Exome Sequencing in a Thai Patient Cohort. Tengsujaritkul M, **Suratannon N**, Ittiwut C, Ittiwut R, Chatchatee P, Suphapeetiporn K, Shotelersuk V. Pediatr Allergy Immunol. 2022 Jan;33(1):e13701.
11. A randomized trial to evaluate the impact of copra meal hydrolysate on gastrointestinal symptoms and gut m9icrobiome. Sathitkowitchai W, **Suratannon N**, Keawsompong S, Weerapakorn W, Patumcharoenpol P, Nitisinprasert S, Nakphaichit M. PeerJ. 2021 15;9:e12158.
12. ITS2 Sequencing and Targeted Meta-Proteomics of Infant Gut Mycobiome Reveal the Functional Role of Rhodotorula sp. during Atopic Dermatitis Manifestation. Mok K, **Suratanon N**, Roytrakul S, Charoenlappanit S, Patumcharoenpol P, Chatchatee P, Vongsangnak W, Nakphaichit M. J. Fungi 2021, 7, 748.
13. Reduced ELANE and SLPI expression compromises dental pulp cell activity. Sriwattanapong K, Sa-Ard-Iam N, Boonprakong L, Subbalekha K, Trachoo V, **Suratannon N**, Porntaveetus T, Shotelersuk V. Cell Prolif. 2021 Nov;54(11):e13132.
14. MetGEMs Toolbox: Metagenome-scale models as integrative toolbox for uncovering Metabolic functions and routes of human gut microbiome. Patumcharoenpol P, Nakphaichit M, Panagiotou G, Senavonge A, **Suratannon N**, Vongsangnak W. PLoS Comput Biol. 2021; 6;17(1): e1008487.
15. IL-10-producing innate lymphoid cells increased in house-dust-mite allergic rhinitis patients following immunotherapy. Boonpiyathad T, Tantilipikorn P, Ruxrungtham K, Pradubpongsa P, Mitthamsiri W, Piedvache A, Thantiworasit P, Sirivichayakul S, Jacquet A, **Suratannon N**, Chatchatee P, Morisaki N, Saito H, Sangasapaviriya A, Matsumoto K, Morita H. J Allergy Clin Immunol. 2021 Apr;147(4):1507-1510.
16. Analysis of Human Gut Microbiome: Taxonomy and Metabolic Functions in Thai Adults. Raethong N, Nakphaichit M, **Suratannon N**, Sathitkowitchai W, Weerapakorn W, Keawsompong S, Vongsangnak W. Genes (Basel). 2021 Feb 25;12(3):331.
17. Rapid Low-Cost Microarray-Based Genotyping for Genetic Screening in Primary Immunodeficiency. **Suratannon N**, van Wijck RTA, Broer L, Xue L, van Meurs JBJ, Barendregt BH, van der Burg M, Dik WA, Chatchatee P, Langerak AW, Swagemakers SMA, Goos JAC, Mathijssen IMJ, Dalm VASH, Suphapeetiporn K, Heezen KC, Drabwell J, Uitterlinden AG, van der Spek PJ, van Hagen PM; South East Asia Primary Immunodeficiencies (SEAPID) Consortium. Front Immunol. 2020 Apr 15;11:614.
18. Analysis of the infant gut microbiome reveals metabolic functional roles associated with healthy infants and infants with atopic dermatitis using metaproteomics. Kingkaw A, Nakphaichit M, **Suratannon N**, Nitisinprasert S, Wongoutong C, Chatchatee P, Krobthong S, Charoenlappanit S, Roytrakul S, Vongsangnak W. PeerJ. 2020; 25;8:e9988.
19. COVID-19 in children: Heterogeneity within the disease and hypothetical pathogenesis. **Suratannon N**, Dik WA, Chatchatee P, Hagen PMV. Asian Pac J Allergy Immunol. 2020 Sep;38(3):170-177.

20. Outcomes of young children hospitalized with acute wheezing. Chinratanapisit S, Sritipsukho P, Satdhabudha A, Matchimmadamrong K, Deerojanawong J, **Suratannon N**, Chatchatee P. Asian Pac J Allergy Immunol. 2020 May 17.
21. Current Perspectives and Unmet Needs of Primary Immunodeficiency Care in Asia Pacific. Leung D, Chua GT, Mondragon AV, Zhong Y, Nguyen-Ngoc-Quynh L, Imai K, Vignesh P, **Suratannon N**, Mao H, Lee WI, Kim YJ, Chan GCF, Liew WK, Huong LTM, Kanegane H, Muktiarti D, Zhao X, Santos-Ocampo FJ, Latiff AHA, Seger R, Ochs HD, Singh S, Lee PP, Lau YL. Front Immunol. 2020 Aug 13;11:1605.
22. Unremitting diarrhoea in a girl diagnosed anti-N-methyl-D-aspartate-receptor encephalitis: A case report. Onpoaree N, Veeravigrom M, Sanpavat A, **Suratannon N**, Sintusek P. World J Clin Cases. 2020 Oct 26;8(20):4866-4875.
23. Anaphylaxis - Lessons learnt when East meets West. Tham EH, Leung ASY, Pacharn P, Lee S, Ebisawa M, Lee BW, Wong GWK; APAPARI Anaphylaxis Study Group. Pediatr Allergy Immunol. 2019 Nov;30(7):681-688.
24. Prevalence and risk factors of allergic rhinitis in children in Bangkok area. Chinratanapisit S, **Suratannon N**, Pacharn P, Sritipsukho P, Vichyanond P. Asian Pac J Allergy Immunol. 2019;37(4):232-239.
25. Prevalence and severity of asthma, rhinoconjunctivitis and eczema in children from the Bangkok area: The Global Asthma Network (GAN) Phase I. Chinratanapisit S, **Suratannon N**, Pacharn P, Sritipsukho P, Vichyanond P. Asian Pac J Allergy Immunol. 2019; 37(4):226- 231.
26. Assessing the reactogenicity of Tdap vaccine administered during pregnancy and antibodies to Bordetella pertussis antigens in maternal and cord sera of Thai women. Wanlapakorn N, Maertens K, Chaithongwongwatthana S, Srimuan D, **Suratannon N**, Vongpunsawad S, Tran TMP, Hens N, Van Damme P, Locht C, Poovorawan Y, Leuridan E. Vaccine. 2018. 7;36(11):1453-1459.
27. Detecting Allergens From Black Tiger Shrimp Penaeus monodon That Can Bind and Cross- link IgE by ELISA, Western Blot, and a Humanized Rat Basophilic Leukemia Reporter Cell Line RS-ATL8. Jarupalee T, Chatchatee P, Komolpis K, **Suratannon N**, Roytrakul S, Yingchutrakul Y, Yimchuen W, Butta P, Jacquet A, Palaga T. Allergy Asthma Immunol Res. 2018;10(1):62-76.
28. Comparison of specific IgE detection by immunoblotting and fluorescence enzyme assay with in vivo skin prick test. Wongpiyabovorn J, **Suratannon N**, Boonmee S, Chatchatee P. Asian Pac J Allergy Immunol. 2018;36(3):159-165.
29. Mumps antibody in the Thai population 17 years after the universal measles mumps rubella vaccination program. Ngaovithunvong V, Wanlapakorn N, Tesapirat L, **Suratannon N**, Poovorawan Y. J Infect Dev Ctries. 2016 Aug 2;10(7):735-40.
30. The minor house dust mite allergen Der p 13 is a fatty acid-binding protein and an activator of a TLR2-mediated innate immune response. Satitsuksanoa P, Kennedy M, Gilis D, Le Mignon M, **Suratannon N**, Soh WT, Wongpiyabovorn J, Chatchatee P, Vangveravong M, Rerkpattanapipat T, Sangasapaviliya A, Piboonpocanun S, Nony E, Ruxrungtham K, Jacquet A; Mite Allergy Research Cohort (MARC) study team. Allergy. 2016;71(10):1425-34.
31. The House Dust Mite Major Allergen Der p 23 Displays O-Glycan-Independent IgE Reactivities but No Chitin-Binding Activity. Soh WT, Le Mignon M, **Suratannon N**, Satitsuksanoa P, Chatchatee P, Wongpiyaboron J, Vangveravong M, Rerkpattanapipat T, Sangasapaviliya A, Nony E, Piboonpocanun S, Ruxrungtham K, Jacquet A; Mite Allergy Research Cohort (MARC) Study Team.Int Arch Allergy Immunol. 2015;168(3):150-60.

32. Adaptive immune defects in a patient with leukocyte adhesion deficiency type III with a novel mutation in *FERMT3*. **Suratannon N**, Yeetong P, Srichomthong C, Amarinthnukrowh P, Chatchatee P, Sosothikul D, van Hagen PM, van der Burg M, Wentink M, Driessen GJ, Suphapeetiporn K, Shotelersuk V. Pediatr Allergy Immunol. 2016;27(2):214-7.
33. Dust mite infestation in cooking flour: experimental observations and practical recommendations. Suesirisawad S, Malainual N, Tungtrongchitr A, Chatchatee P, **Suratannon N**, Ngamphaiboon J. Asian Pac J Allergy Immunol. 2015;33(2):123-8.
34. Component-resolved diagnostics for the evaluation of peanut allergy in a low-prevalence area. **Suratannon N**, Ngamphaiboon J, Wongpiyabovorn J, Puripokai P, Chatchatee P. Pediatr Allergy Immunol. 2013;24(7):665-70. Erratum in: Pediatr Allergy Immunol. 2014;25(2):204.

APPENDIX

COVID-19 in Children: Heterogeneity within the Disease and Hypothetical Pathogenesis

REVIEW ARTICLE

Asian Pacific Journal of
Allergy and Immunology

APJAI

COVID-19 in children: Heterogeneity within the disease and hypothetical pathogenesis

Narissara Suratannon,[1,2] Willem A. Dik,[2,3] Pantipa Chatchatee,[1] P. Martin van Hagen[1,2,3]

Abstract

The disease course of coronavirus disease 2019 (COVID-19) is usually mild and self-limiting in previously healthy children, but they may also develop severe disease. Severe COVID-19 infection is especially observed in very young children or those with underlying comorbidities. Moreover, a multisystem inflammatory syndrome that mimics the Kawasaki disease shock syndrome can develop in children that are genetically predisposed to displaying an overactive immune response to SARS-CoV-2 infection. In this review, we describe the clinical phenotypes of mild and severe COVID-19 and multisystem inflammatory syndrome in children (MIS-C). We also discuss the possible immunobiological mechanisms that may be involved in the protection of children against COVID-19 and the development of multisystem inflammatory syndrome.

Key words: SARS-CoV-2; COVID-19; pediatric multisystem inflammatory disease, COVID-19 related; immunity, innate; T-lymphocytes; immunosenescence, immunity.

From:
[1] Pediatric Allergy & Clinical Immunology Research Unit, Division of Allergy and Immunology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, the Thai Red Cross Society, Bangkok, Thailand
[2] Department of Immunology, Laboratory Medical Immunology, Erasmus Medical Center, Rotterdam, the Netherlands
[3] Department of Internal Medicine, Division of Clinical Immunology, Erasmus Medical Center, Rotterdam, the Netherlands

Corresponding author:
Pantipa Chatchatee
E-mail: pantipa.c@chula.ac.th

Abbreviations:

SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
COVID-19	corona virus disease 2019
ACE2	angiotensin-converting enzyme 2
MIS-C	multisystem inflammatory syndrome in children
KDSS	Kawasaki disease shock syndrome
KD	Kawasaki disease
NK	natural killer

Introduction

Currently, the world is experiencing a coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In adults, the intensity of COVID-19 infection can differ substantially between individuals, ranging from mild to severe disease.[1] Numerous studies have highlighted the low incidence of SARS-CoV-2 infection in children, who account for only 0–7.4% of the COVID-19 cases identified worldwide.[1-5] Approximately 20% of the affected children are asymptomatic.[1,6] A recent systematic review of 11 studies[7] and a multinational, multicenter cohort study in Europe[6] revealed that only 4–8% of children infected with COVID-19 were admitted to the intensive care unit (ICU). These incidences are substantially lower than those observed in adults; ~16% of the adult study population was severely affected.[6] Moreover, the fatality rate of children with COVID-19 is extremely low (0–0.69%)[1,6,7] compared to that of the adult population (8–14.8%).[8]

Clinical disease spectrum of COVID-19 in children

The clinical spectrum of COVID-19 in children is summarized in **Table 1**. As indicated above, most children with COVID-19 experience mild disease. Similar to the clinical spectrum observed in adults, the most common presentations were fever and respiratory symptoms. About 17% of the children suffered from gastrointestinal (GI) symptoms such as abdominal pain, vomiting, or diarrhea. The incidence of GI symptoms was even higher in a recent Chinese study on 14 hospitalized boys and 20 girls, among them nearly 27% had diarrhea and about 25% had nausea or vomiting.[9] Nearly half of the children in this study were also infected with other respiratory pathogens such as influenza A virus, influenza B virus, or *Mycoplasma pneumoniae*. Interestingly, 7% of the patients reported isolated GI symptoms without typical respiratory tract symptoms.[6] There was no predominant male pattern similar to that published for adult populations.[6,7,8]

Table 1. Clinical spectrum of COVID-19 infection in children

Characteristic	Mild to moderate COVID-19 infection[7]	Severe COVID-19 infection[11]	COVID-19 infection related to MIS-C[21]
Numbers of patients	211	48	21
Age, median (IQR), year	6.5 (0–12)	13 (4.2–16.6)	7.9 (3.7–16.6)
Ethnicity, no (%)	not mentioned	not mentioned	24 (57) were from Sub-Saharan, African origin
Health conditions	all were previously healthy	83% with pre-existing comorbidities	all were previously healthy, none had underlying cardiac disease
Presenting symptoms, no (%)	122 (49) cough, 118 (47) fever	respiratory symptoms 35 (73)	all had high fever, mucous membrane involvement, rash, lymphadenopathy, fits with criteria of Kawasaki diseases
Male gender, no (%)	147 (59)	25 (52)	9 (43)
Respiratory symptoms, no (%)	151 (60) had mild pneumonia	35 (73)	mild or absent respiratory symptoms (numbers did not show)
Gastrointestinal symptoms, no (%)	42 (17)	1 (2)	21 (100)
Cardiovascular symptoms, no (%)	not mentioned	2 (4)	16 (76) myocarditis, 5 (24) moderate coronary artery dilations
Skin	not mentioned	not mentioned	16 (76) had polymorphous skin rash and changes of the lips and oral cavity; 17 (81) had bilateral bulbar conjunctival injection
Neurological symptoms	1 (0.5) had encephalopathy	2 (4)	6 (29) had headache, confusion, meningeal irritation
Signs of inflammation & inflammatory markers	22 (28) had high CRP and procalcitonin	not mentioned	12 (57) had serous effusion, all had high CRP/procalcitonin/serum IL-6, 19 (95) had high D-dimers
Obesity, no (%)	not mentioned	7 (15)	16 (76)
SARS-CoV-2 detection	100% RT-PCR positive from nasopharyngeal/ throat swabs	100% RT-PCR positive from nasopharyngeal/throat swabs	90% had evidence of recent SARS-CoV-2 infection (positive RT-PCR result in 38%, positive IgG antibody detection in 90%)
Treatment, no (%)	not mentioned	28 (61) receiving specific drugs (hydroxychloroquine, azithromycin, remdesivir, tocilizumab); 12 (25) required vasoactive drugs; 18 (38) on ventilator	All received intravenous immunoglobulin and low dose aspirin; 10 (48%) also received corticosteroids; 11 (52) on ventilator
Fatality rate, no (%)	0	2 (4.2)	0

MIS-C: multisystem inflammatory syndrome in children; CRP: C-reactive protein (CRP); SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; RT-PCR: Reverse transcription polymerase chain reaction; Ig: immunoglobulin; IL: interleukin

Symptomatic treatment alone could resolve the symptoms of most children.[10]

Recently, in a study on a small group of children with severe or even fatal COVID-19, the most common initial presentations were respiratory symptoms requiring respiratory support (81%), with 13% requiring extracorporeal supportive therapies. Eighty-seven percent of the subjects had at least one organ failure. Interestingly, in this group, only 2% showed gastrointestinal symptoms, and this value was significantly lower than that of children with mild diseases.[11] Sixty-one percent of the children received COVID-19 related pharmacotherapies such as hydroxychloroquine, azithromycin, remdesivir, and tocilizumab, either as a single agent or in combination. The fatality rate in this study was 4.2%.[11] So far, two factors have been consistently associated with severe COVID-19 in children: preexisting medical conditions and a very young age. Eighty percent of the children admitted to the ICU due to COVID-19 had preexisting or therapy-related comorbidities that compromised their defenses, such as long-term ventilation support due to developmental delay (e.g., prolonged tracheostomy), malignancies, or immunosuppressive drug therapy (**Table 1**).[11] These comorbidities were also observed in a recent large multinational study.[6] As already mentioned, very young age is a contributing factor to severe COVID-19.[6] Approximately 10% of infants less than 1-year-old have suffered from severe COVID-19 disease as compared to 7.3% and 3.7% children in the age groups of 1–5 years and > 5 years old, respectively.[12] Another study showed that an age of less than 1 month increased the risk of severe COVID-19 by 2.5-fold.[6] These observations emphasize the need for close

follow-up and prompt treatment of children with comorbidities and very young infants that are suspected to be infected with SARS-CoV-2. A recent National Health System study in the UK revealed data for the comparison of the primary care records of 17,278,392 adults who were pseudonymously linked to 10,926 COVID-19-related deaths. These data revealed various determinants related to COVID-19 mortality, including age.[13] Conditions that contribute to the development of severe COVID-19 in adults include diabetes, chronic renal and liver disease, previous malignancies, dementia and chronic use of immunosuppressive drugs are rare or absent in pediatric populations.

The pathophysiology of both subclinical and severe COVID-19 in children remains unclear. Critically ill adult patients have been reported to demonstrate impaired or delayed anti-viral interferon (IFN) responses against SARS-CoV-2 infection.[14,15] On the contrary, the consequences of an uncontrolled overwhelming viral load include the overproduction of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), inducing a so-called cytokine storm. Impaired anti-viral responses were also observed in immunocompromised patients and infants under the age of 6 months.[16] An age-associated increase in the capacity to produce IFN-γ and TNF-α after the first year of life was recently reported. This may explain, in part, the severity of COVID-19 infection at an age of less than one year.[17] Immunological reactions that activate the clotting system may result in thrombosis. This is actually a major risk factor for mortality in adults, and is not taking place in children in whom thrombosis has a low prevalence in general. A genetic factor was also found to contribute to severity of COVID-19. Recently, rare putative *loss-of-function* variants of *TLR7* were identified in 4 young male patients who suffered from severe COVID-19.[18] These variants were functionally associated with impaired type I and II IFN responses.[18] However, all these factors are probably not the only ones contributing to the severity of COVID-19 in children and hence need further exploration.

The "multisystem inflammatory syndrome in children" (MIS-C) or "pediatric inflammatory multisystemic syndrome" has recently been reported. The term is used to refer to the development of severe inflammation involving multiple organs in healthy children, probably due to SARS-CoV-2 infection. Although there is a strong association between MIS-C and SARS-CoV-2, it is unclear whether it is a post-viral complication or a primary effect of SARS-CoV-2 infection. Clinical reports of MIS-C have recently been published in various countries, including China,[9] the United States,[19] Italy,[20] the United Kingdom,[21] France,[22] and Switzerland.[23] The median duration between symptoms of COVID-19 and the onset of MIS-C was 42 days (range 18–79 days). In some of the affected children, the symptoms started at the earliest 48 hours after the acute infection period (16), indicating that MIS-C might even occur during the late phase of infection. RT-PCR results for SARS-CoV-2 were positive for 38% of children with MIS-C, although almost nobody showed any symptoms of acute SARS-CoV-2 infection. IgG antibodies against SARS-CoV-2, on the other hand, were detected in 90% of the patients.

The presentation of MIS-C was similar to Kawasaki disease shock syndrome (KDSS), including myocarditis with a shock-like state combined with typical signs of Kawasaki disease (KD).[24] From previous literature, KDSS was reported in older children compared to classic KD.[24] KDSS was suspected to be related to viral triggers, including seasonal coronaviruses in some studies.[24] All patients with MIS-C also had cardiovascular involvement. Many of them suffered from impaired myocardial function, which is not commonly seen in typical KD whom the pathophysiology mainly involves the coronary arteries. This feature is the major difference between MIS-C and severe acute COVID-19 infection, in which only 4% of cases showed cardiovascular symptoms.[11] Additionally, in MIS-C, significant gastrointestinal symptoms were present, while only mild respiratory symptoms were reported. Skin rash, red eyes, and oral mucous membrane involvement were observed in about 76-81% cases. MIS-C was more prevalent in older individuals and in non-Asian children unlike that seen in typical KD. Obesity, an important risk factor for severity and mortality in adult patients with COVID-19, was also prominent in some MIS-C cohorts, as found in a recent French study.[22,25]

Mechanisms protecting children from SARS-CoV -2 infection and severe COVID-19

The mechanisms underlying the relative resistance of children to SARS-CoV-2 infection and clinical disease are still unclear and probably have a multifactorial background. The lining of the respiratory and gastro-intestinal system, including the local environment and secretions, is the first defense against viral entrance, followed by the innate immune response. However, the innate immune response to COVID-19 is not well understood. Binding of the virus to pattern recognition receptors (PRRs)/ toll-like receptors (TLRs) can activate downstream pathways, resulting in the secretion of various cytokines, particularly type I and III IFNs and the pro-inflammatory cytokines, interleukin (IL)-1, IL-6, IL-8, IL-18, and TNF-α.[26] SARS-CoV-2 proteins, however, could inhibit type-1 IFN responses, resulting in incomplete type-1 IFN signatures.[26] Various immune cell types are involved in early immune defense, including myeloid cells such as granulocytes and innate lymphoid cells, including natural killer cells (NK), which can produce cytokines or are cytotoxic (excellent review by Vabret N. et al., 2020).[26] These immune cells are part of the defense system but are also associated with severe complications including the acute respiratory distress syndrome (ARDS), when they are over-activated. Local tissue damage exaggerates this initial immune process. The innate antiviral response activates and potentiates the subsequent adaptive immune responses against the virus, particularly CD8 responses.

Besides the above mechanisms, we will discuss additional hypothesis that fill the knowledge gap of SARS-CoV-2 defense in the pathobiology of COVID-19 in children, specifically involving age-related angiotensin-converting enzyme 2 (ACE2) receptor expression, trained immunity, immunosenescence and exhaustion.

Angiotensin-converting enzyme 2 receptor

Several studies indicate that the expression of the ACE2 receptor, which is recognized as a receptor by SARS-CoV-2 for host entry,[27] differs between children and adults. The nasal epithelium is the first interaction site between SARS-CoV-2 and the host. Recently, Bunyavanich et al.[28] demonstrated age-dependent ACE2 receptor gene expression in the nasal epithelium, with significantly lower ACE2 receptor gene expression in young children than in older children, adolescents, and adults. Therefore, it is likely that the relatively low nasal epithelial ACE2 receptor expression confers protection against SARS-CoV-2 infection in children. In contrast, the ACE2 receptor is expressed at a higher level in the lower respiratory tract of children aged < 10 years compared to older subjects (19–71 years).[29] Under physiological conditions, ACE2 acts as a protease for the conversion of angiotensin II to its anti-inflammatory metabolites, angiotensin-1 to 7.[30] In the lower respiratory tract, decreased ACE2 expression can be a sign of severe acute respiratory distress and lung injury, suggesting a protective role of ACE2 in severe lung injury by limiting angiotensin 2-mediated pulmonary capillary leak and inflammation.[31] This information highlights the dual role of ACE2 as a viral receptor and a protective agent in acute lung injury. Although future in-depth studies exploring the regulation of ACE2-ACE-2 receptor-angiotensin-II system are required, anatomical and regulatory differences of this system between children and adults may represent one of the mechanisms providing relative protection to children against infection with SARS-CoV-2.

Trained immunity

The innate immune system is crucially important in young children, as adaptive immunity has not yet been fully developed. In other words, young children (< 2 years of age) are highly dependent on a rapid innate immune response to control disturbances in homeostasis, including infections. Trained immunity is the long-term epigenetic reprogramming of innate immune cells, such as myeloid cells and natural killer (NK) cells, and occurs upon exposure to homeostasis perturbance by exogenous or endogenous molecules

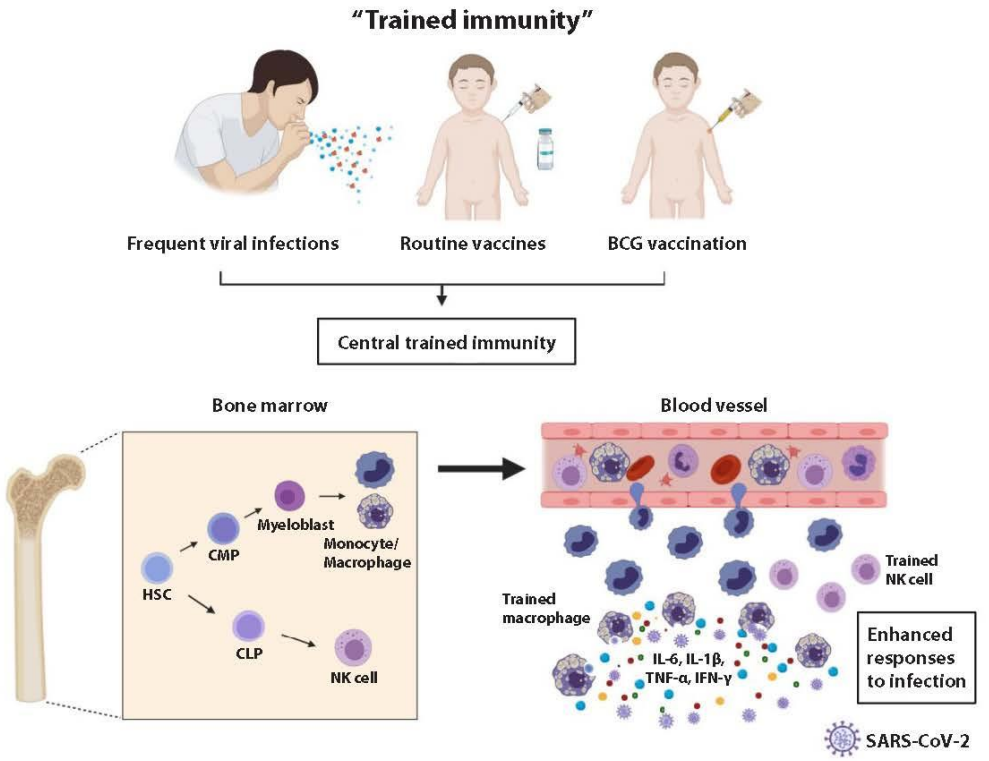


Figure 1. Trained immunity. Trained immunity resulting from frequent viral respiratory tract infections, routine vaccinations, and BCG vaccination shapes and enhances the innate immune response to SARS-CoV-2 in children at the level of myeloid progenitors in the bone marrow, and is called "central trained immunity". BCG, Bacillus Calmette-Guérin; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; HSC, hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; IL, interleukin; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; NK, natural killer.

(e.g., pathogens) (**Figure 1**). Trained immunity leads to increased immune response upon secondary stimulation with, for instance, the same or even unrelated pathogens.[32] Such secondary stimulation is typically associated with increased production of cytokines such as IFN-γ, IL-1β, IL-6, and TNF-α, which are key cytokines in the immune response to many pathogens.[32,33] From *in vitro* and *in vivo* studies, Bacillus Calmette- Guérin (BCG) vaccination, live vaccines (against measles, mumps, rubella, influenza, smallpox, and oral polio)[34,35] and frequent viral infections were found to induce trained immunity. This defense mechanism cross-protects against infection with *Candida albicans*, *Schistosoma mansoni*, *Mycobacterium tuberculosis*, and various respiratory viruses such as respiratory syncytial virus, Influenza A, and Influenza B viruses. Trained immunity, thus, represents a very important protective mechanism against (re)infection, and likely is of great importance especially in early life. Although the protective effect of trained immunity is unlikely to last beyond 1–2 years, it may very well be that it contributes to the relative protection of children against SARS-CoV-2 infection. It might be worth evaluating the impact of trained immunity as a therapeutic target to protect against SARS-CoV-2 infection in the future.

Immunosenescence and exhaustion

T-lymphocytes are central in defense during viral infections. $CD4^+$ T-lymphocytes are potent cytokine producers that further activate the immune response and help B-lymphocyte in antibody production and Ig class switching; whereas, cytotoxic $CD8^+$ T cells destroy virus-infected cells in order to reduce the viral load and inhibit further the spread of the virus. SARS-CoV-2 infection causes peripheral blood ($CD4^+$ and $CD8^+$) lymphopenia, a process which is probably driven by the induction of homing factors and extensive apoptosis/cell death through the effects of IL-6 and Fas-FasL interactions. $CD8^+$ T lymphocytes are predominantly affected more than $CD4^+$ T lymphocytes, as their peripheral blood levels correlated well with disease activity and mortality.[36] In contrast, patients with mild disease, however, have normal or slightly higher T lymphocyte counts.[37,38] T lymphocytes in severe COVID-19 are more activated and may exhibit a trend toward exhaustion as measured by the expression of programmed cell death protein 1 (PD-1) and T-cell immunoglobulin mucin-3 (TIM-3), indicating an impaired function.[39]

Another contributing risk factor for severity and mortality in COVID-19 in adult compared to children may relate to the process called immunosenescence. This term refers to a gradual deterioration of the immune system as a result of aging. The adaptive immune system is more affected than the innate immune system.[40] One of the most characteristic of immunosenescence is the loss of a functional thymus from a subsequent decline in naïve T lymphocyte production (excellent review by van den Broek T. et al., 2018).[41,42] The progressive decline in thymus output has been deduced from an output of ~16 million T lymphocytes per day in young adults to < 1 million T lymphocytes per day in adults older than 65 years of age.[42] The frequencies of naïve CD4 T lymphocytes only moderately decline with age, while the naïve CD8 T lymphocytes compartment clearly shrinks. Although the repertoire diversity (i.e., the number of different T lymphocyte clones) remains very high for both naïve CD4 and CD8 T lymphocytes subsets, infant and older adult T lymphocytes undergo differential changes in their DNA, including changes in telomere length, amount of DNA damage, and epigenetic modifications.[43] These processes may lead to functional impairments resulting in defective immune responses to infections and impaired vaccination responses in the elderly. The immune system of an older individual has a reduced response to both known antigens and neo-antigens. Older adults also have a significant higher risk of mortality from vaccine-preventable diseases than children.[44] For example, despite annual vaccination, influenza infections continue to be associated with high morbidity and mortality rates. The first vaccinations with live viruses, such as yellow fever virus, are associated with increased morbidity and even mortality in older adults.[43] Therefore, impaired immune response to neo-antigens may contribute significantly to the course of COVID-19 in adults.

Remarkably, obesity, another risk factor for severe disease and mortality in COVID-19 patients, is associated with low-grade chronic inflammation and accelerated immunosenescence. Epigenetic changes and aberrant numbers of T lymphocytes were found in patients with morbid obesity (BMI > 40).[46] Obesity affects antiviral defense and are at risk to generate a poor vaccine-induced immune response.[45] Moreover, obesity has been identified as a risk factor for increased disease severity and mortality in individuals infected with influenza. Obese hosts have delayed and impaired antiviral responses to influenza virus infection, and they experience also a poor recovery from the disease.[47] The combination of aging and obesity can result in a severely impaired immune response to SARS-COV-2. While the prevalence of obesity is rapidly increasing in children, it has not reached the prevalence of obesity in adults. This explanation may be another influencing factor for the difference of COVID-19 disease course in children and adults.

To conclude, current evidence suggests that several factors, including differences in ACE2 receptor expression, trained innate immunity, and a young and fit immune system contribute to the protection of children from severe SARS-CoV -2 infections. The magnitude of the contribution of each of the indicated mechanisms should be further investigated.

Hypothetical pathogenesis of MIS-C

The pathophysiology of MIS-C is currently not well understood and is under active investigation. It is suggested that this syndrome results from an abnormal immune response to the virus, with some similarities/overlaps with KD, macrophage activation syndrome/hemophagocytic lymphohistiocytosis, and cytokine release syndrome. Although causality has not been established, the temporal relationship between the MIS-C and the COVID-19 pandemic raised the suggestion that the mechanism of molecular mimicry, where a foreign antigen shares sequence or structural similarities with self-antigens, might be involved.[49]

Autoimmunity related to molecular mimicry can occur both at the cellular and humoral immune levels and has been described following vaccination or infection, as previously

reported in Guillain-Barré syndrome, multiple sclerosis, and KD, but does require a susceptible genetic background to occur.[48] So far, studies exploring the contribution of molecular mimicry between SARS-CoV-2 antigens and self-antigens, specifically in relation to MIS-C, have not been conducted. However, *in vitro* experiments confirmed homology between the spike and nuclear proteins of SARS-CoV-2 and human tissue antigens; cross-reactions between SARS-CoV-2 IgM/IgG might occur with transglutaminase 3 (tTG3), transglutaminase 2 (tTG2), extractable nuclear antigens (ENA), myelin basic protein, mitochondria, myosin, thyroid peroxidase, collagen, Claudin 5+6, and S100B.[49] Antigen-autoantibody complexes can precipitate inside tissues and, in particular, in blood vessels, and induce an inflammatory reaction through the activation of the complement system, in which complement anaphylatoxins (C3a and C5a) recruit neutrophils, leading to inflammation, including vasculitis, in the affected organ.[50]

Moreover, autoreactive T helper 1 (Th1) and Th17 lymphocytes release proinflammatory cytokines that contribute to the cytokine storm, recruiting more (cytokine-producing) macrophages, neutrophils, and monocytes, and resulting in progressive tissue damage. The clinical spectrum of disease depends on the affected organs, which may include; neurological symptoms (headache, irritability, and encephalopathy), impaired cardiac ventricular function, gastrointestinal symptoms (vomiting, abdominal pain, and/or diarrhea), mucocutaneous symptoms mimicking KD (conjunctivitis, rash), and acute kidney injury. Hematologic abnormalities, including lymphopenia and low platelets, were also reported. The hypothetical pathogenesis of MIS-C is demonstrated in **Figure 2**.

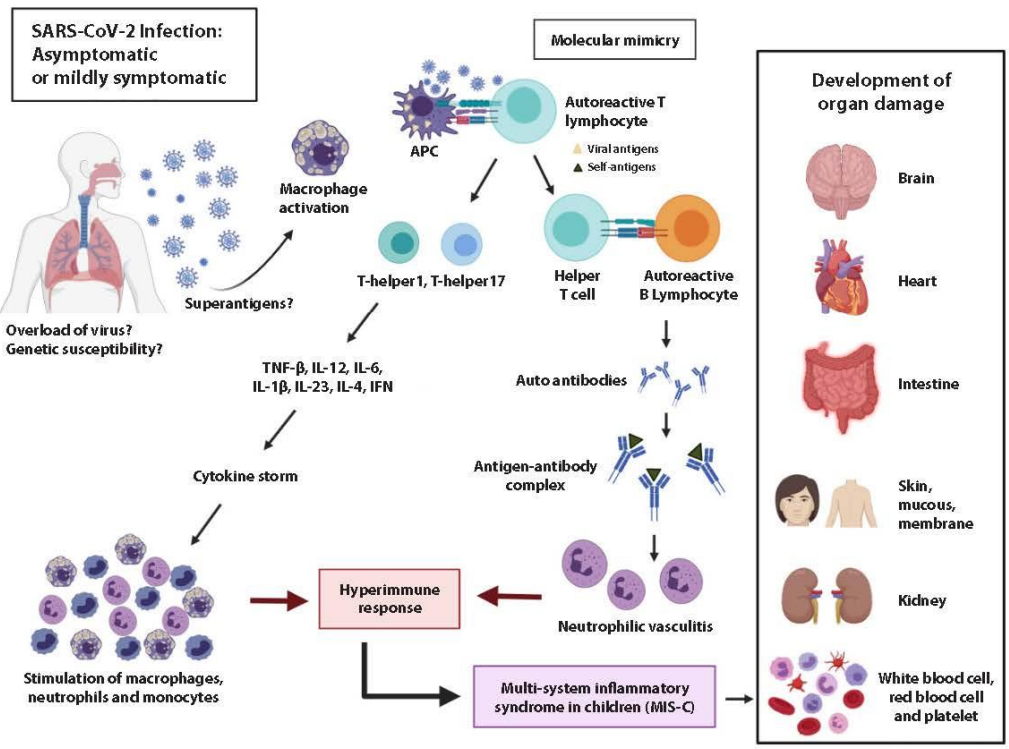


Figure 2. Hypothetical pathogenesis of MIS-C. Early infection with SARS-CoV-2 is likely to be asymptomatic or mildly symptomatic in children. The early infection appears to trigger macrophage activation. SARS-CoV-2, where some antigens are similar to self-antigens, is captured by antigen presenting cells and stimulates autoreactive T lymphocytes. This in turn leads to cytokine release and stimulation of macrophages, neutrophils, and monocytes, along with B lymphocyte activation and subsequent production of autoreactive antibodies, leading to a hyperimmune response. This results in the damage of tissues such as the brain, heart, intestines, skin, mucous membranes, kidneys, and blood cells and presents as the clinical manifestation of MIS-C. MIS-C, multisystem inflammatory syndrome in children; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; APC, antigen presenting cell; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.

Conclusion

SARS-CoV-2 infection in children has a more benign disease course compared to that in adults. The potential causality of mild SARS-CoV-2 infection in healthy children is based on many factors, including the lack of preexisting comorbidities, low obesity prevalence, low thrombosis rate, different ACE-2 receptor expression, trained immunity, and general immune fitness. Prognostic factors to identify children who may develop the new hyperinflammatory condition, MIS-C, need to be determined. It has been speculated that autoreactive B- and T- lymphocytes generating from the cross-reactivity between viral and host antigens, together with macrophage activation play a role in generating a cytokine storm which leads to specific organ tissue inflammation.

Conflict of interests

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to thank Professor Kiat Ruxrungtham for his invaluable scientific inputs and critical reading of the manuscript, and Ms. Natsaran Meprayooonwong for her support in figure drawing.

References

1. Wu Z, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. JAMA. 2020;323(13):1239-42.
2. Omori R, Mizumoto K, Nishiura H. Ascertainment rate of novel coronavirus disease (COVID-19) in Japan. Int J Infect Dis. 2020;96:673-5.
3. Gudbjartsson DF, Helgason A, Jonsson H, Magnusson OT, Melsted P, Norddahl GL, et al. Spread of SARS-CoV-2 in the Icelandic Population. N Engl J Med. 2020;382(24):2302-15.
4. Bi Q, Wu Y, Mei S, Ye C, Zou X, Zhang Z, et al. Epidemiology and transmission of COVID-19 in 391 cases and 1286 of their close contacts in Shenzhen, China: a retrospective cohort study. Lancet Infect Dis. 2020; 20(8):911-9.
5. Ludvigsson JF. Systematic review of COVID-19 in children shows milder cases and a better prognosis than adults. Acta Paediatr. 2020;109(6): 1088-95.
6. Gotzinger F, Santiago-Garcia B, Noguera-Julian A, Lanaspa M, Lancella L, Calo Carducci FI, et al. COVID-19 in children and adolescents in Europe: a multinational, multicentre cohort study. Lancet Child Adolesc Health. 2020. S2352-4642(20)30177-2.
7. Mustafa NM, L AS. Characterisation of COVID-19 Pandemic in Paediatric Age Group: A Systematic Review and Meta-Analysis. J Clin Virol. 2020; 128:104395.
8. Guan WJ, Liang WH, Zhao Y, Liang HR, Chen ZS, Li YM, et al. Comorbidity and its impact on 1590 patients with COVID-19 in China: a nationwide analysis. Eur Respir J. 2020;55(5):2000547.
9. Zhang C, Gu J, Chen Q, Deng N, Li J, Huang L, et al. Clinical and epidemiological characteristics of pediatric SARS-CoV-2 infections in China: A multicenter case series. PLoS medicine. 2020;17(6):e1003130.
10. Gudbjartsson DF, Helgason A, Jonsson H, Magnusson OT, Melsted P, Norddahl GL, et al. Spread of SARS-CoV-2 in the Icelandic Population. N Engl J Med. 2020;382(24)2302-2315.
11. Shekerdemian LS, Mahmood NR, Wolfe KK, Riggs BJ, Ross CE, McKiernan CA, et al. Characteristics and outcomes of children with coronavirus disease 2019 (COVID-19) infection admitted to US and Canadian Pediatric Intensive Care Units. JAMA pediatrics. 2020. May 11. doi:10.1001/jamapediatrics. 2020. 1948.[Epub ahead of print]
12. Dong Y, Mo X, Hu Y, Qi X, Jiang F, Jiang Z, et al. Epidemiological characteristics of 2143 pediatric patients with 2019 coronavirus disease in China. Pediatrics. 2020;145(6):e20200702.
13. Williamson EJ, Walker AJ, Bhaskaran K, Bacon S, Bates C, Morton CE, et al. OpenSAFELY: factors associated with COVID-19 death in 17 million patients. Nature. 2020:1-11.
14. Hadjadj J, Yatim N, Barnabei L, Corneau A, Boussier J, Pere H, et al. Impaired type I interferon activity and exacerbated inflammatory responses in severe Covid-19 patients. Science. 2020;eabc6027. MedRxiv. 2020.
15. Blanco-Melo D, Nilsson-Payant BE, Liu W-C, Uhl S, Hoagland D, Møller R, et al. Imbalanced host response to SARS-CoV-2 drives development of COVID-19. Cell. 2020;181(5):1036-45.e9. Cell. 2020.
16. Wilson C, Westall J, Johnston L, Lewis D, Dower S, Alpert A. Decreased production of interferon-gamma by human neonatal cells. Intrinsic and regulatory deficiencies. The Journal of clinical investigation. 1986;77(3): 860-7.
17. Simon AK, Hollander GA, McMichael A. Evolution of the immune system in humans from infancy to old age. Proceedings of the Royal Society B: Biological Sciences. 2015;282(1821):20143085.
18. van der Made CI, Simons A, Schuurs-Hoeijmakers J, van den Heuvel G, Mantere T, Kersten S, et al. Presence of Genetic Variants Among Young Men With Severe COVID-19. JAMA 2020;24;e2013719. JAMA. 2020.
19. DeBiasi RL, Song X, Delaney M, Bell M, Smith K, Pershad J, et al. Severe COVID-19 in children and young adults in the Washington, DC metropolitan region. J Pediatr. 2020;223:199-203.e1. The Journal of Pediatrics. 2020.
20. Verdoni L, Mazza A, Gervasoni A, Martelli L, Ruggeri M, Ciuffreda M, et al. An outbreak of severe Kawasaki-like disease at the Italian epicentre of the SARS-CoV-2 epidemic: an observational cohort study. The Lancet. 2020;395(10239):1771-8.2020.
21. Riphagen S, Gomez X, Gonzalez-Martinez C, Wilkinson N, Theocharis P. Hyperinflammatory shock in children during COVID-19 pandemic. The Lancet. 2020;395(10237):1607-8.
22. Toubiana J, Poirault C, Corsia A, Bajolle F, Fourgeaud J, Angoulvant F, et al. Kawasaki-like multisystem inflammatory syndrome in children during the covid-19 pandemic in Paris, France: prospective observational study. BMJ. 2020 ;369:m2094.bmj. 2020;369.
23. Belhadjer Z, Méot M, Bajolle F, Khraiche D, Legendre A, Abakka S, et al. Acute heart failure in multisystem inflammatory syndrome in children (MIS-C) in the context of global SARS-CoV-2 pandemic. Circulation. 2020; 120.048360. Circulation. 2020.
24. Li Y, Zheng Q, Zou L, Wu J, Guo L, Teng L, et al. Kawasaki disease shock syndrome: clinical characteristics and possible use of IL-6, IL-10 and IFN-γ as biomarkers for early recognition. Pediatric Rheumatology. 2019;17(1): 1-9.
25. Hennon TR, Penque MD, Abdul-Aziz R, Alibrahim OS, McGreevy MB, Prout AJ, et al. COVID-19 associated Multisystem Inflammatory Syndrome in Children (MIS-C) guidelines; a Western New York approach. Progress in Pediatric Cardiology. 2020;101232.
26. Vabret N, Britton GJ, Gruber C, Hegde S, Kim J, Kuksin M, et al. Immunology of COVID-19: current state of the science. Immunity. 2020; 52(6):910-941.
27. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 2020;181(2): 271-280.e8.
28. Bunyavanich S, Do A, Vicencio A. Nasal Gene Expression of Angiotensin -Converting Enzyme 2 in Children and Adults. JAMA. 2020;323(23): 2427-2429.
29. Bezara MEO, Thurman A, Pezzulo A, Leidinger MR, Klesney-Tait JA, Karp PH, et al. Heterogeneous expression of the SARS-Coronavirus-2 receptor ACE2 in the human respiratory tract. bioRxiv. 2020. Available on line at https://www.biorxiv.org/content/10.1101/2020.04.22.056127v1
30. Sriram K, Insel PA. A hypothesis for pathobiology and treatment of COVID-19: The centrality of ACE1/ACE2 imbalance. British Journal of Pharmacology. 2020.; Apr 24. doi: 10.1111/bph.15082 [Epub Ahead of Print]
31. Gemmati D, Bramanti B, Serino ML, Secchiero P, Zauli G, Tisato V. COVID-19 and Individual Genetic Susceptibility/Receptivity: Role of ACE1/ACE2 Genes, Immunity, Inflammation and Coagulation. Might the Double X-Chromosome in Females Be Protective against SARS-CoV-2 Compared to the Single X-Chromosome in Males? International Journal of Molecular Sciences. 2020;21(10):3474.
32. Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. Nature Reviews Immunology. 2020:1-14.

33. O'Neill LA, Netea MG. BCG-induced trained immunity: can it offer protection against COVID-19? Nature Reviews Immunology. 2020;20(6): 335-7.
34. Benn CS, Netea MG, Selin LK, Aaby P. A small jab–a big effect: nonspecific immunomodulation by vaccines. Trends in immunology. 2013;34(9):431-9.
35. Myśliwska J, Trzonkowski P, Szmit E, Brydak L, Machała M, Myśliwski A. Immunomodulating effect of influenza vaccination in the elderly differing in health status. Experimental gerontology. 2004;39(10):1447-58.
36. Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, et al. Reduction and functional exhaustion of T cells in patients with coronavirus disease 2019 (COVID-19). Frontiers in Immunology. 2020;11:827.
37. Thevarajan I, Nguyen TH, Koutsakos M, Druce J, Caly L, van de Sandt CE, et al. Breadth of concomitant immune responses prior to patient recovery: a case report of non-severe COVID-19. Nature medicine. 2020;26(4):453-5.
38. Thevarajan I, Buising KL, Cowie BC. Clinical presentation and management of COVID-19. Med J Aust. 2020:10.5694/mja2.50698. The Medical Journal of Australia. 2020:1.
39. Zheng H-Y, Zhang M, Yang C-X, Zhang N, Wang X-C, Yang X-P, et al. Elevated exhaustion levels and reduced functional diversity of T cells in peripheral blood may predict severe progression in COVID-19 patients. Cellular & molecular immunology. 2020;17(5):541-3.
40. Fulop T, Larbi A, Dupuis G, Le Page A, Frost EH, Cohen AA, et al. Immunosenescence and inflamm-aging as two sides of the same coin: friends or foes? Frontiers in immunology. 2018;8:1960.
41. van den Broek T, Borghans JA, van Wijk F. The full spectrum of human naive T cells. Nature Reviews Immunology. 2018;18(6):363-73.
42. Tang F, Quan Y, Xin Z-T, Wrammert J, Ma M-J, Lv H, et al. Lack of peripheral memory B cell responses in recovered patients with severe acute respiratory syndrome: a six-year follow-up study. The Journal of Immunology. 2011;186(12):7264-8.
43. Westera L, van Hoeven V, Drylewicz J, Spierenburg G, van Velzen JF, de Boer RJ, et al. Lymphocyte maintenance during healthy aging requires no substantial alterations in cellular turnover. Aging cell. 2015;14(2):219-27.
44. Rich R, Fleisher T, Shearer W, Schroeder H, Frew A, Weyand C. Clinical Immunology: Principles and Practice. 5th ed. New York, US: Elsevier; 2018.
45. Gardner P, Pabbatireddy S. Vaccines for women age 50 and older. Emerging infectious diseases. 2004;10(11):1990-5.
46. van der Weerd K, Dik WA, Schrijver B, Schweitzer DH, Langerak AW, Drexhage HA, et al. Morbidly obese human subjects have increased peripheral blood CD4+ T cells with skewing toward a Treg-and Th2-dominated phenotype. Diabetes. 2012;61(2):401-8.
47. Honce R, Schultz-Cherry S. Impact of obesity on influenza A virus pathogenesis, immune response, and evolution. Front Immunol. 2019; 10:1071. 2019
48. Segal Y, Shoenfeld Y. Vaccine-induced autoimmunity: the role of molecular mimicry and immune crossreaction. Cellular & molecular immunology. 2018;15(6):586-94.
49. Vojdani A, Kharrazian D. Potential antigenic cross-reactivity between SARS-CoV-2 and human tissue with a possible link to an increase in autoimmune diseases. Clinical Immunology (Orlando, Fla). 2020;217: 108480.
50. Roncati L, Ligabue G, Fabbiani L, Malagoli C, Gallo G, Lusenti B, et al. Type 3 hypersensitivity in COVID-19 vasculitis. Clinical Immunology. 2020:108487.