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MULTIOMICS STRATEGY IN CLINICAL IMMUNOLOGY AIDING UNSOLVED ANTIBODY DEFICIENCIES

Hassan Abolhassani, M.D., MPH, Ph.D.

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Multiomics Strategy in Clinical Immunology Aiding Unsolved Antibody Deficiencies

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By

Hassan Abolhassani, M.D., MPH, Ph.D.

Principal Supervisor:
Professor Lennart Hammarström
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Immunology

Co-supervisor(s):
Professor Asghar Aghamohammadi
Tehran Univ. of Medical Sciences
Department of Pediatrics
Division of Clinical Immunology

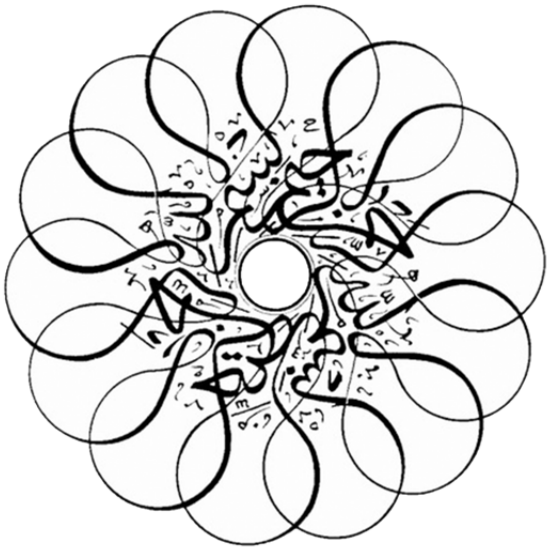
Professor Qiang Pan-Hammarström
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Immunology

Opponent:
Professor Thomas Fleisher
NIH Clinical Center
Department of Laboratory Medicine
Bethesda, MD, USA

Examination Board:
Professor Anders Örn
Karlolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Professor Ola Winqvist
Karlolinska Institutet
Department of Medicine

Associate Professor Karlis Pauksens
Uppsala University
Department of Medical Sciences



In the name of God, the Most Compassionate, the Most Merciful

To my family

& all my teachers, masters and colleagues

& respectfully dedicated to my patients and their families

ABSTRACT

The etiology of 80% of patients with primary antibody deficiency (PAD), the second most common type of human immune system disorders after human immunodeficiency virus infection, is yet unknown. A correct molecular diagnosis in patients with primary immunodeficiencies is crucial for the classification of these disorders with a heterogeneous clinical/immunological phenotype and subsequent therapeutic management. Primary antibody deficiency is a group of immune diseases with highly diverse clinical and immunologic features. Next generation sequencing and hightrouput multiomics studies has facilitated genetic investigations of rare inherited diseases during the past decade, thus allowing an appropriate diagnosis and treatment in these patients.

Clinical and immunological phenotyping of a cohort of consecutive dysgammaglobulinemic patients with unknown genetic defects underwent genomic (e.g. whole-exome sequencing) and other relative multiomics (e.g. transcriptomics, proteomics, epigenomics and immunomics) investigations after having been subjected to classical targeted sequencing.

Exome sequencing analysis was performed on 126 PAD probands (55.5% male, 95.2% childhood onset) born to predominantly consanguineous parents (82.5%) and thus expected to carry homozygous mutations with an autosomal recessive pattern of inheritance. This genomic approach and subsequent immunological investigations identified potential disease-causing variants in 86 patients (68.2%), however, 27 of these patients (31.4%) carried autosomal dominant (7%) and X-linked (24.4%) gene defects. Using this advanced method and multiomics confirmatory studies, we described new phenotypes of known genes (**Paper I, III**), new inheritance pattern of known genes (**Paper II**) and discovered a new gene in human disorder (**Paper IV**). Clinical and immunologic phenotypes of the remaining unsolved 40 patients were compared with patients with identified molecular defects. Medical implications of the definite molecular diagnosis were reported in ~50% of the patients, including hematopoietic stem cell transplantation, follow-up visits schedule and tertiary preventive screening tests (such as reducing radiation exposure for radiosensitive patients), targeted medication and prenatal diagnosis. Finally, we propose a clinical/immunologic workup followed by a standard genomic and multiomics analysis for an approach to PAD patients (**Paper V**).

Due to misclassification of the conventional clinical and immunological phenotyping for targeted sequencing, employing next generation sequencing as a preliminary step of molecular diagnosis approach to patients with dysgammaglobulinemia is essential and could help in many facets of management and treatment of the patients and their family members. This study also illustrates the power of exome sequencing in the identification of novel and candidate genes underlying primary antibody deficiency and nesciciate confirmatory multiomics functional assays. The findings of this study demonstrate a new workup and clinical guideline to approach patients with different types of dysgammaglobulinemia and highlight the importance of multiomics approach in the filed of clinical immunology.

POPULAR SCIENCE SUMMARY

Just or False; How may the B cells orchestra be tuned?

As a part of our well programmed and designed immune system, B cells work with high-quality sound instruments to bring our body into a state of internal and external harmony. Think of B cell as an orchestra, which has many different instruments with different expert performers making up its whole with different sounds that suit different audiences. They follow the music notes generated in the brain of the conductor (DNA) and delivered as a note page (RNA) to make beautiful melodies of peace (proteins of antibodies) against external sorrows (pathogens and allergens) and internal griefs (aging and intolerance).

If a part of this concert is not “in tune” and vibrating at the correct frequency, or if the musician is emotionally under par or mentally overburdened, the result is a stressed, under functioning music that feels as if being everything from unwell down to quite ill (infections, allergies, autoimmunity and cancers). This “dis-ease” of B cells should be detected and can be aided by the application of sound to re-tune, and start to bring about internal and external harmony if we understand which stage has the issue.

Best songs needed for the beginning of a harmonic life is delivered by our mothers love (IgG from umbilical cords and IgA from breast milk). However, after 6 months of age all children should orchestrate their own bands of B cells to practice and generate their own life songs. Unfortunately, the conductor (DNA) is not always in a good mood in all humans to act onetime or functional. Without proper conductor for the orchestra there will sometimes be no products (agammaglobulinemia) or low dysfunctional products (hypogammaglobulinemia). Without the optimal achievement of the top-level goal of harmony, which is the responsibility of the conductor, the body will be faced with infections, allergies, autoimmunity and cancers from early years of life.

Although the B cells orchestra contains many other components, including the players and their instruments (IgA, IgG, IgM, IgE and IgD producers), and although each of these components can affect the goal-oriented function if they misbehave, it is logical to start to investigate the conductor first in the case of chaos (genomic investigation). The reason being that only the conductor is in a position to oversee the achievement of the top-level goal. According to the high reflective B cells orchestra, the impact of the failed conductor can be found systematically in the other functions of the group (in a different level of epigenome, transcriptome, proteome, and immunome).

Just because we do not yet know the meaning of every note of B cells orchestra’s conductor in the human, it does not mean we cannot find the reason for 80% of dysfunction of this orchestra. Much progress in immune system knowledge consists of more and more detailed understanding of how each note is represented in particular B cells and in particular subclasses of B cells. There is no reason to suppose that this progress will not continue, until such point where we can account for the representation of those meanings which correspond to our "folk antibodies music" intuitions about the central control mechanisms of the orchestra. This study took some of the wrong notes into account, quog (DNA mutations), which affects the emotional experience of listening to a sensational music in some of my patients, that impeded them from undergoing a whole range of emotions that they deserved to experience from their B cells orchestra.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Rimligt eller Falskt; Hur kan B-cellens orkester stämmas?
Som en del av vårt välprogrammerade och utformade immunsystem arbetar B-celler med högkvalitativa ljudinstrument för att få vår kropp i ett tillstånd av intern och extern harmoni. Tänk på B-cellen som en orkester, som har många olika instrument med olika expertartister som gör det hela med olika ljud som passar olika publik. De följer de musikanteckningar som genereras i ledaren (DNA) och levereras som en anteckningssida (RNA) för att skapa vackra melodier av fred (proteiner av antikroppar) mot externa sorger (patogener och allergener) och inre sorger (åldrande och intolerans).

Om en del av den här konserten inte "stämmer" och vibrerar med rätt frekvens, alternativt om dirigenten är emotionellt nedstämd eller psykiskt överbelastad, är resultatet en stressig, undermålig musik som förmedlar otrevliga förnimmelser i alltifrån obehag till sjukdomskänsla (infektioner, allergier, autoimmunitet och cancer). Detta tillstånd med B-cellerna bör detekteras och kan, om vi förstår vilket stadium i processerna som har problemet, mildras genom att använda ljudet för återjustering och införande av intern och extern harmoni.

Bästa sångerna som behövs i början av ett harmoniskt liv levereras av våra mödrars kärlek (IgG från navelsträngar och IgA från bröstmjölk), men efter 6 månaders ålder borde alla barn organisera sina egna band av B-celler för att träna och generera sina egna livssånger. Tyvärr är ledaren (DNA) inte alltid i gott humör hos alla människor för att förmå agera funktionsenligt. Utan korrekt ledare för orkestern kan produkterna utebli (agammaglobulinemi) eller så blir resultatet dysfunktionella produkter (hypogammaglobulinemi). Med avsaknad av uppnått, överordnat mål av harmoni - vilket är ledarens ansvar - kommer kroppen att möta infektioner, allergier, autoimmunitet och cancer redan i början av livet.

Även om B-cellerna innehåller många andra komponenter - inklusive spelarna och deras instrument (IgA-, IgG-, IgM-, IgE- och IgD-tillverkare) och även om underfunktion i en enda komponent av dessa kan inverka på den målorienterade funktionen - är det logiskt att börja undersöka ledaren först gällande dessa tillstånd (genomisk utredning). Detta eftersom endast ledaren är i position att övervaka det överordnade målet att allt fungerar som det skall. Enligt den högreflekterande B-cellorkestern kan effekten av en dysfunktionell - ”misslyckad” - ledare hittas systematiskt i gruppens övriga funktioner (i olika nivåer av epigenom, transkriptom, proteom och immunom).

Bara för att vi ännu inte känner till betydelsen av varje anteckning från B-cellorkesterens dirigent i människan, innebär inte detta att vi inte kan hitta orsaken till 80% av alla kända dysfunktioner i denna orkester. Mycket av framstegen i kunskapen om immunsystemet utgörs av ökad detaljerad förståelse av hur varje ”nota” representeras i specifika B-celler samt inom specifika underklasser av B-celler. Det finns ingen anledning att anta att denna utveckling inte kommer att fortsätta, tills en sådan punkt där vi kan förklara representationen av den innebörd som motsvarar vår ”antikroppsmusik”-metafor om orkesterns centrala kontrollmekanismer. Denna studie fastslog några av de felaktiga ”musikanteckningarna” - DNA-mutationerna - vilka påverkar den hos mina patienter känslomässiga erfarenheten av att lyssna på sensationell musik. Det hindrar dem från att uppleva en rad känslor som de förtjänar att erfara från deras B-cellorkester.

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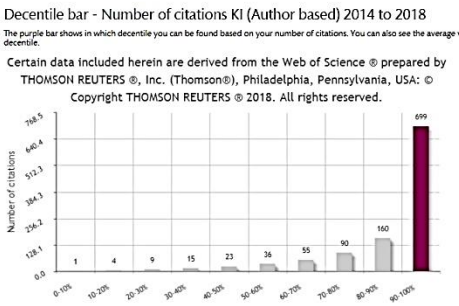

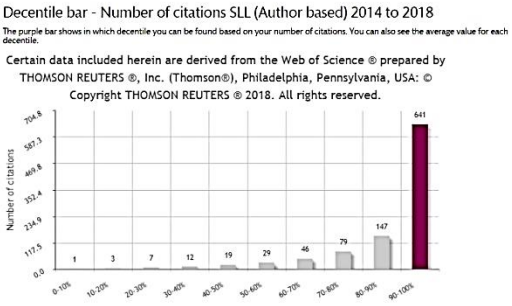

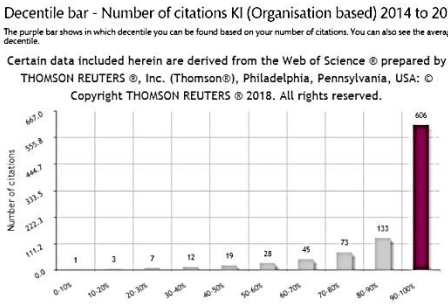

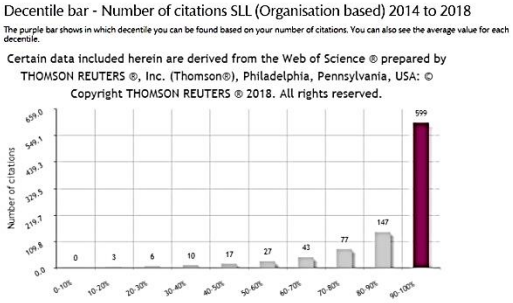

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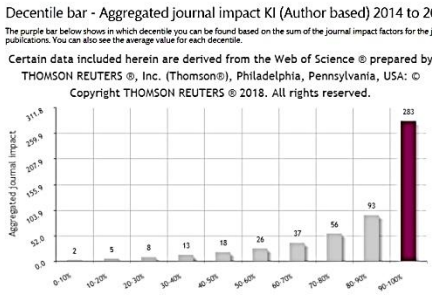

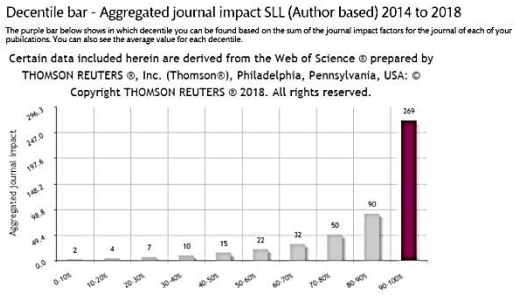

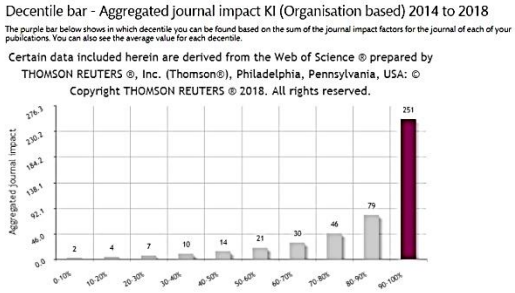

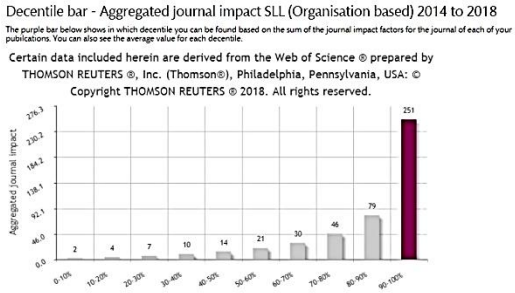

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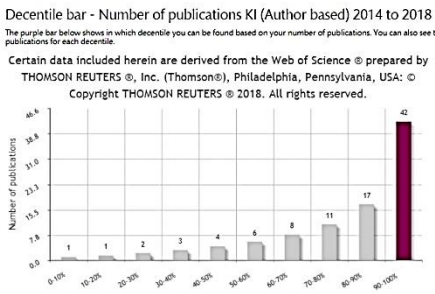

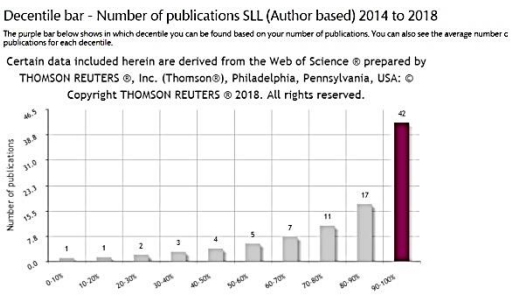

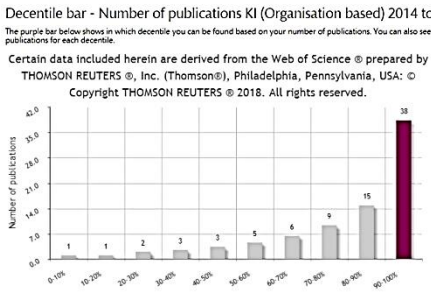

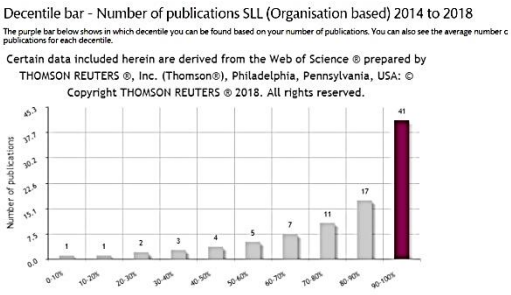

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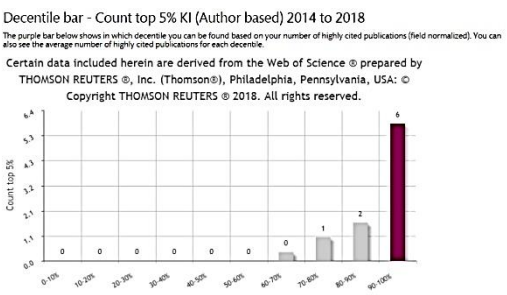

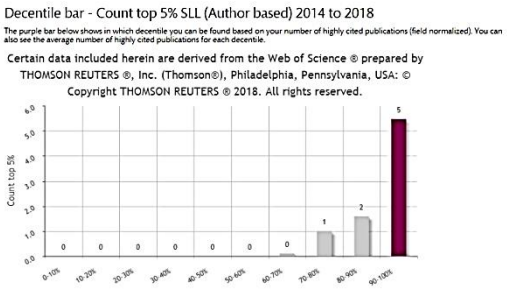

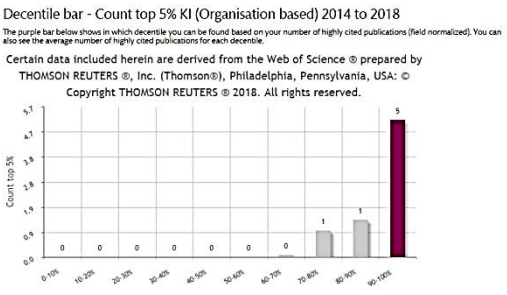

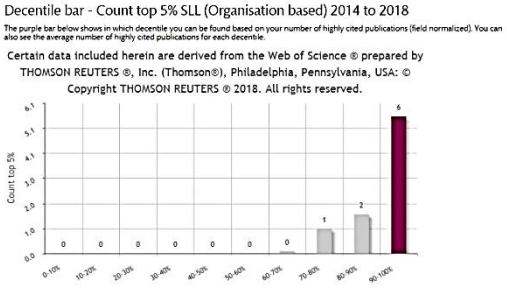

CONTENTS

LIST OF ABBREVIATIONS

AAAAI	American Academy of Allergy, Asthma and Immunology
ACMG	American College of Medical Genetics and Genomics
AGG	Agammaglobulinemia
AH50	Alternative haemolytic complement activity, 50%
BCR	B cell receptors
BCA	Bi cinchoninic Acid
Breg	Regulatory B cells
CARD	Caspase recruitment domain
CD	Cluster of differentiation
cDNA	Complementary DNA
CH50	Complement haemolytic activity, 50%
CLP	Common lymphoid progenitor
CNV	Copy number variation
CSR	Class-switch recombination
CVID	Common variable immunodeficiency
DCs	Dendritic cells
EBV	Epstein–Barr virus
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ESID	European Society for Immunodeficiencies
Fab	Fragment antigen-binding of antibody
Fc	Fragment crystallizable region of antibody
FFP	Fresh frozen plasma
GATK	Genome analysis toolkit
GCB	Germinal center B cells
G-CSF	Granulocyte-colony stimulating factor
HGG	Hypogammaglobulinemia
HIgM	Hyper immunoglobulin M syndrome
HRP	Horseradish peroxidase-conjugated
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IB	Immature B cells
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgAD	Immunoglobulin A deficiency

IgR	Immunoglobulin replacement therapy
IL	Interleukin
Indel	Insertions/deletions
iNKT	Invariant NK-T cells
ISD	Immunoglobulin G subclass deficiency
κREC	Kappa-deleting recombination excision circle
LPD	Lymphoproliferative disorder
MB	Memory B cells
MHC	Major histocompatibility complex
MZB	Marginal zone B cells
NB	Naïve B cells
NGS	Next generation sequencing
NK cells	Natural killer cells
PAD	Primary antibody deficiencies
PbB	Plasmabalst B cells
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PcB	Plasmacells Bcells
PCR	Polymerase chain reaction
PID	Primary immunodeficiency disorders
PI3K	Phosphatidyl inositide 3 kinases
RAC2	Ras-related C3 botulinum toxin substrate 2
RAG	Recombination-activating gene
RIPA	Radio immuno precipitation assay
SAD	Specific antibody deficiency
SDS	Sodium dodecyl sulfate
SHM	Somatic hypermutation
SNP	Single nucleotide polymorphisms
SOAP	Short oligonucleotide analysis package
TMB	Tetra methyl benzidine
THI	Transient form of a humoral immunodeficiency
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TREC	T cell receptor excision circle
WES	Whole exome sequencing
WGS	Whole genome sequencing

1 INTRODUCTION

1.1 PRIMARY ANTIBODY DEFICIENCIES

Primary antibody deficiency (PAD) is the most common form of primary immunodeficiency (PID), with a prevalence of about 1 in 600 in the general population [1, 2]. The clinical picture is highly variable (ranging from asymptomatic to severe) and includes infection, autoimmunity, lymphoproliferation, enteropathy and malignancy [3-5]. The wide spectrum of immunological presentations of PAD constitutes B-cell lymphopenia, agammaglobulinemia, hyper-IgM syndrome, hypogammaglobulinemia, isotype deficiencies as well as a transient form of a humoral immunodeficiency (**Figure 1**). Several mutations in PAD patients that play key roles in B-cell activation, proliferation, differentiation, class-switch recombination, somatic hypermutation and apoptosis have been identified; however, the etiology remains unknown in a majority of the patients [4, 6-10].

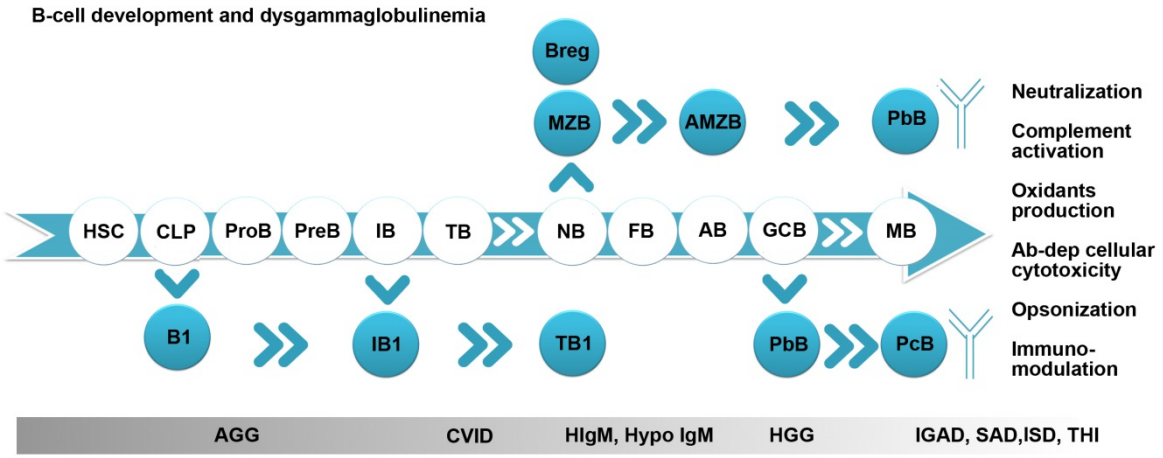

Figure 1- Model of pathogenesis in dysgammaglobulinemia representing different immunologic phenotypes based on the maturational arrest in different steps of B-cell development. HSC: hematopoietic stem cell CLP: common lymphoid progenitor, IB: immature B cells, TB: transitional B cells, NB: naïve B cells, MZB: marginal zone B cells, AMZB: activated marginal zone B cells, Breg: regulatory B cells, FB: Follicular B cells, AB: activated B cells, Ab-dep: antibody dependent, GCB: germinal center B cells, MB: memory B cells, PbB: Plasmabalst B cells, PcB: Plasmacells Bcells, AGG: agammaglobulinemia, CVID: common variable immunodeficiency, HIgM: hyper-IgM syndrome, HGG: hypogammaglobulinemia, IgAD: IgA deficiency, SAD: specific antibody deficiency, ISD: IgG subclass deficiency, THI: transient form of a humoral immunodeficiency.

According to non-redundant functions of different types of antibodies, several immune functions (Fab-mediated and Fc-mediated) may be disturbed as a consequence of PAD. Fab-mediated functions includes antigen recognition (against all types of molecules including carbohydrates, nucleic acids and phospholipids but are best suited to bind a polypeptide pathogens and allergens), neutralization of surface proteins and toxins (prevents the access of the pathogen to the host receptors and prevents destruction of host cells by dimeric mucosal IgA) and providing first line of systemic defense (binding to multivalent antigens by pentameric IgM antibodies rapidly generated in blood) and targeted defense (by diffusing IgG into the tissues). Fc-mediated effector functions of antibodies

provide activation of effector cells (including phagocytic cells like macrophages and neutrophils, T cells like natural killer cells, eosinophils and mast cells using receptor for the Fc fragment), complement binding (activates complement cascade and release of chemical mediators from mast cells, reinforce phagocytosis and cell lysis) and opsonization (coated antibodies at surface of the pathogen allow binding of Fc domains to Fc receptors present on scavenger cells) [11-14].

1.2 GENETIC DEFECTS CAUSING PRIMARY ANTIBODY DEFICIENCIES

B cells development and its cellular interactions have a central role in the humoral immune response and antibody production /secretion. B cell development begins in bone marrow and continues in secondary lymphoid organs. Expression of different lineage-specific markers on B cell precursors indicates different stages of B cell development.

Several gene products are responsible for early B cell development in bone marrow. The classical gene from this group is known as Bruton tyrosine kinase (*BTK*). **Figure 2** represents other involved genes in early B cell development and B cell receptor signaling. Mutations in genes involved in this step of development result in severe PAD, which is characterized by blockade of B cell differentiation before the production of surface Ig, markedly reduced mature B cell counts in the peripheral circulation, profound hypogammaglobulinemia or agammaglobulinemia, and early onset of recurrent bacterial infections in affected children [15].

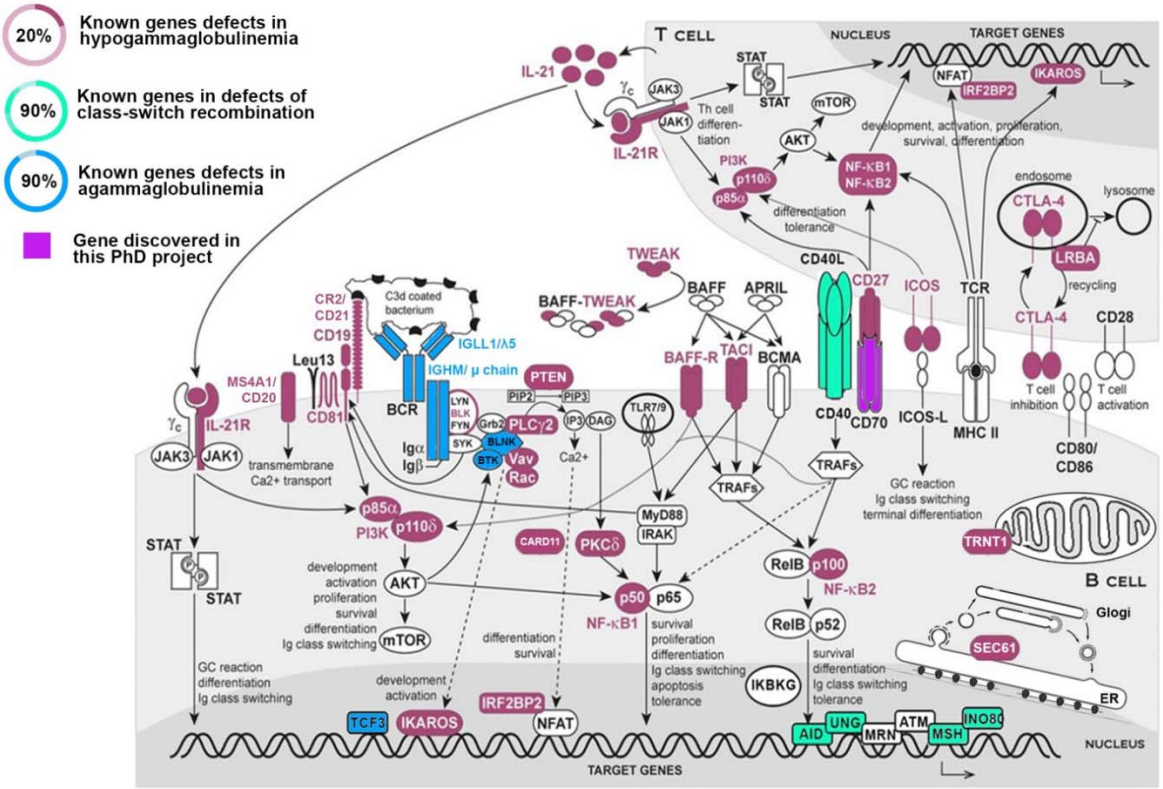

Figure 2- Discovered cytoplasmic and membrane molecules encoded by genes involved in B-cell development and known to be involved in primary antibody deficiencies due to their role mainly in early development (in bone marrow depicted by blue), class switching recombination (in the secondary lymphoid organs depicted by green) and terminal maturations of B cells (depicted by red) [16-19, 7, 10].

In secondary lymphoid organs, class-switch recombination (CSR) and somatic hypermutation (SHM) are the mechanisms necessary for the generation of effector plasma cells secreting high-affinity IgG, IgA, and IgE antibodies. The classical gene that plays a key role in CSR and SHM is CD40 ligand (*CD40L*) and **Figure 2** depicts the remaining involved known genes in this process. Defects in CSR are characterized by low serum levels of IgG, IgA, and IgE leading to recurrent bacterial infections associated with normal or elevated serum IgM levels [20, 21].

The terminal stages of B-cell development are also controlled by different genetic signatures including TNF receptor superfamily members, CD19–B cell receptor (BCR) complex, the B-cell differentiation cytokines and apoptosis/autophagy pathways (**Figure 2**) [22].

Approximately 30 genes causing B cell developmental defects in humans have been described since 1952 to 2013 prior to starting of this Ph.D. study. Furthermore, the largest gap between clinical and molecular diagnosis in the field of PID is seen in patients with PAD (**Table 1**) [23]. Close to 90% of patients with late B cell development disorders, 10% of cases with a defect in early B cell development and 10% of those with a CSR defect have no definite causative gene mutation.

Table 1. Frequency of patients of each category of primary immunodeficiencies in Iran.

Category	Probable prevalence	Expected frequency	Clinically diagnosed (n; %)	Definitely diagnosed (n; %)
Combined immunodeficiencies	1/50,000	1528	265 (17.3)	21 (7.9)
Syndromic immunodeficiency	1/100,000	764	271 (35.5)	80 (29.6)
Predominantly antibody deficiencies	1/650	117,394	545 (0.5)	77 (14.4)
Diseases of immune dysregulation	<1/1,000,000	76	80 (105.3)	52 (65)
Congenital defects of phagocytes	1/250,000	306	390 (127.4)	95 (24.4)
Defects in innate immunity	<1/1,000,000	76	36 (47.4)	6 (16.6)
Autoinflammatory disorders	1/10,000	7642	580 (7.59)	490 (84.5)
Complement deficiencies	1/50,000	1528	32 (2.1)	0 (0)
Total	≥1/600	≥127,300	2199 (1.76)	790 (35.2)

1.3 CLINICAL CONCEPT OF MOLECULAR DIAGNOSIS OF PRIMARY ANTIBODY DEFICIENCIES

Positive predictive values of clinical algorithms for identifying PAD range from 19.1% in patients with hypogammaglobulinemia to 33.3% in patients with a CSR defect presenting hyper-IgM syndrome in the Medicaid database [24], indicating the necessity of a correct genetic diagnosis. Besides confirming the clinical diagnosis, molecular diagnosis also plays a pivotal in the identification of new genetic defects, pre-symptomatic diagnosis, treatment decisions, prognosis prediction and family counseling [25]. Moreover, the clinical presentation of mutations in known PID genes varies due to the type of mutation (missense, nonsense, insertion/deletion), the protein domain involved and the presence of modifying genes or environmental factors. Recent reports on atypical manifestations of known primary immunodefciencies have revealed a role for molecular diagnosis in defining a new phenotype for a known monogenic disorder [26].

Advances in next generation sequencing (NGS) methods allow an unbiased approach to PAD patients in order to obtain a correct diagnosis and subsequently adjust the clinical management and treatment. Targeted NGS panels with several hundreds of known PID genes may provide a first screening step, thus improving the classical approach of the sequencing of selected candidate genes [27]. Nevertheless, this method is not sufficiently efficient due to the heterogeneous nature of these diseases, resulting in a clinical sensitivity of 15-40% in PID patients [28-31]. For the time being, whole exome sequencing (WES) seems to be the most cost effective approach for genomic evaluation of PAD patients with an unknown etiology due to our current cognition about monogenic disorders (enriched in coding exome the most conserved region of the genome across metazoans), and less analysis complexity and risk of incidental findings [32]. Moreover, the majority of previous studies have only focused on patients with hypogammaglobulinemia, and the molecular basis of a considerable proportion of patients with other forms of PAD remains unknown, particularly in high-frequency disorders such as selective IgA deficiency and IgG subclass deficiency [18, 33].

2 AIMS

2.1 GENERAL RESEARCH AIM

The aim of this study was to perform WES of a substantial number of PAD patients with unidentified genetic defects and to investigate its impact on the clinical diagnosis and subsequent clinical management.

2.2 SPECIFIC SCIENTIFIC AIMS

2.2.1 To functionally characterize the genes involved in patients with early B cells defects without identified genetic causes and to provide a clinically and immunologically relevant summary.

2.2.2 To functionally characterize the genes involved in patients with CSR defects without identified genetic causes and to provide a clinically and immunologically relevant summary.

2.2.3 To functionally characterize the genes involved in patients with terminal B cell defects without identified genetic causes and to provide a clinically and immunologically relevant summary.

2.2.4 To design, develop and benchmark a complete cost-effective multiomics approach to PAD patients starting with genomic step and followed by relevant confirmatory functional assays.

2.2.5 To evaluate the feasibility and usefulness of current genomic approach in the field of clinical immunology, and to solve pitfalls in the analysis steps and pipelines designed for molecular diagnosis using next generation sequencing.

2.2.6 To improve the performance of *in-silico* analysis after identification of candidate genetic defect by integration of computational models and predicative software.

2.3 TRANSLATIONAL AIM

The translational aim of this thesis was to provide clinicians treating patients suffering from PADs with updated clinical, laboratory and mutation analyses for selected cases with early onset of the disease and a high consanguinity rate. Moreover, we have tried to suggest new modalities for targeted treatment of the patients according to the discovered gene and consult patient and patients' family with appropriate carrier detection and genetic counseling. Finally, we propose a clinical/immunologic workup followed by a standard genetic analysis for an approach to PAD patients.

3 MATERIALS AND METHODS

3.1 PATIENTS

Among all registered symptomatic PAD patients in the Iranian national PID registry [34], available individuals who were referred to Children's Medical Center (Pediatrics Center of Excellence affiliated to Tehran University of Medical Sciences, Tehran, Iran) were consecutively recruited into this study. Informed consent (including explanations about the risks and benefits of research-based NGS) for the performed studies was obtained from all patients and their relatives, according to the principles of the ethics committee of the Tehran University of Medical Sciences and the Karolinska Institutet. An evaluation document was used to summarize the demographic information of the patients, including gender, date of birth, clinical parameters and previous medical history, family history, and laboratory and molecular data. Consanguinity of parents was evaluated by interview. All patients were diagnosed as PAD based on the updated diagnostic criteria of ESID (the European Society for Immunodeficiencies, *http://esid.org/WorkingParties/Registry/Diagnosis-criteria*) and AAAAI (The American Academy of Allergy, Asthma & Immunology) practice parameter for the diagnosis and management of PID [35], including agammaglobulinemia, hyper-IgM syndrome (HIgM), common variable immunodeficiency (CVID), IgA deficiency (IgAD), IgG subclass deficiency, specific antibody deficiency and transient hypogammaglobulinemia. All patients were re-evaluated for fulfilling either the probable or possible diagnostic criteria. Secondary causes of dysgammaglobulinemia were ruled out. A computerized database program (new registry section at *http://rcid.tums.ac.ir/*) was designed for final data collection and direct summarizing/analysis of data.

3.2 MULTIOMICS STRATEGY

3.2.1 Genomic Approach

WES was performed on PAD patients without any known underlying genetic defects. To classify a patient as undefined PAD, a classic genetic analysis was performed for patients with agammaglobulinemia (mutations in the *BTK, BLNK, CD79A, CD79B, IGLL1* and *IGMH* genes) and HIgM syndrome (mutations in the *CD40L, CD40, AICDA* and *UNG* genes) using targeted sequencing [36-38], whereas other forms of dysgammaglobulinemia (including CVID and IgAD) were considered as undefined forms of PAD at baseline and directly recruited for WES.

For patients with undefined PAD, 6μg extracted genomic DNA was randomly fragmented, amplified by ligation-mediated polymerase chain reaction (PCR) and captured and sequenced according to the protocol described previously [39]. After raw image file processing, sequences were generated and aligned to the human genome reference (UCSC hg 19 version; build 37.1) using the SOAP aligner software (Short Oligonucleotide Analysis Package, v.2.21). Duplicated reads were filtered out and only uniquely mapped reads were kept for subsequent analyses. The SOAPsnp software (v.1.03) was subsequently used with default parameters to assemble the consensus sequence and call genotypes in target regions. Low-quality single nucleotide polymorphisms (SNP) that met one of the four following criteria were filtered out: a genotype quality of less than 20 bp; a sequencing depth of less than 4; an estimated copy number of more than 2 and a distance from the adjacent SNPs of less than 5 bp. Small insertions/deletions (Indels) were detected using the Unified Genotype tool from GATK (Genome Analysis Toolkit ,v.1.0.4705) following the alignment of quality reads to the human reference genome using BWA (Burrows-Wheeler Aligner, v.0.5.9-r16). For analysis of WES, we followed the protocol described previously for prioritizing candidate variants,

predicting their effect on protein, homozygosity mapping, large deletion and copy number variation (CNV) detection [39].

The pathogenicity of all disease attributable gene variants was re-evaluated using the updated guideline for interpretation of molecular sequencing by the American College of Medical Genetics and Genomics (ACMG), considering allele frequency in the population database, computational data, immunological/functional data, familial segregation, parental data and clinical phenotyping [40].

To validate the mutations observed and complete familial segregation, Sanger sequencing was performed. Specific primers for each mutation were designed using PRIMER3 Input version (0.4.0) software (*bioinfo.ut.ee*). Standard PCR was performed in a final volume of 15 µl containing 10 ng of genomic DNA. Amplified products were resolved in 1% agarose gel and purified using the NucleoSpin kit (Macherey-Nagel GmbH). The resulting PCR products were sequenced at the Macrogen Company, South Korea. The sequences were analyzed using the Lasergene software package (DNAStar, Madison).

The observed mutations were classified as a novel (newly identified in PAD patients), new phenotype (PID gene known to be disease causing with a new presentation) or known phenotype (PAD gene with a typical presentation) [41, 42]. Genotype and phenotype correlations were integrated and clinical implications of the molecular diagnoses were evaluated.

3.2.2 Transcriptomic Approach

Total RNA was isolated from peripheral blood mononuclear cells (PBMC, extracted by standard Hypaque-Ficoll, GE Healthcare) using the RNeasy Mini kit (Qiagen). Complementary DNA (cDNA) was synthesized using the RevertAid first strand cDNA synthesis kit (Fermentas). Mutant genes mRNA levels were quantified by quantitative real-time PCR using the $2e^{-\Delta\Delta Ct}$ relative method and a known gene was used as a housekeeping gene for normalization. The PCRs were run in a StepOne Instrument (StepOne Software v.2.3, Applied Biosystems, Life Technologies Corporation).

3.2.3 Proteomic Approach

PBMCs were isolated from whole blood based on the abovementioned density gradient centrifugation, cryopreserved in freezing medium (Synth-a-freeze CTS, Life Technologies) for further proteome. Before the proteomic investigation, PBMCs were thawed and washed twice in R10 (RPMI-1640 Medium AQmedia, Sigma Aldrich) containing 10 % fetal bovine serum, 50 IU/mL penicillin and 50 µg/mL streptomycin.

For direct Western Blotting, PBMC extracts were boiled for 5 minutes in Radio Immuno-Precipitation Assay lysis buffer (RIPA buffer). Protein concentrations were measured by the Pierce bicinchoninic acid (BCA) protein assay (ThermoFisher Scientific). Equal amounts of protein were separated on a 10% Sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto a nitrocellulose membrane (Hybond-ECL, GE healthcare, Little Chalfont, Buckinghamshire). The membrane was blocked with PBS-T (Phosphate buffered saline, 0.05% Tween 20, 5% milk powder). Membranes were subsequently incubated with polyclonal antibodies against either the studied gene's product or a housekeeping protein. They were then incubated with horseradish peroxidase-conjugated (HRP) labeled secondary antibodies in appropriate dilution. The signal was detected by chemiluminescence using the

enhanced chemiluminiscence (ECL) Plus™ Western Blotting detection system (GE Healthcare) with the GeneSys software (v.1.4.1.0, Syngene) and evaluated by densitometry (GeneTools software v.4.03, Syngene).

The protocol for extracellular and intracellular staining of flowcytometry evaluation of lymphocytes and the related functional markers has previously been described elsewhere [43]. Briefly, cells were counted on a Nucleocounter (ChemoMetec A/S), resuspended in R10 containing 10 U/mL DNase I (Roche Diagnostics) and rested for 7 hours at 37 °C. U-bottom plates were plated with 5-10 × 10^5 PBMCs/well. Regarding functional analysis, cells were also supplemented together with designed stimuli at the start of the stimulation protocol at 37°C 5% CO_2 incubation [44]. Cells were stimulated for 10 hours. The cells were transferred to V-bottom plates, washed in fluorescence-activated cell sorting (FACS) buffer and stained with a LIVE/DEAD Aqua amine dye solution containing the extracellular antibodies for 30 minutes. PBMCs were washed in FACS buffer and fixed/permeabilized using the forkhead box P3 transcription factor buffer kit (eBioscience, San Diego, CA). In case of required evaluation, monoclonal antibodies against intracellular markers were incubated with the cells for 1 hour. After further washing with Perm Wash solution (eBioscience), the cells were resuspended in PBS containing 1 % paraformaldehyde. PBMCs were analyzed on a 4 laser LSR Fortessa (BD Biosciences), where minimally 150,000 events were collected per run. Antibody capture beads (BD Biosciences) were stained individually with all antibodies used in the experiments for compensation setup. Gating analysis was performed using FlowJo 8.8.7 (Treestar). Manual gates were based on unstained cells or fluorescence minus one (FMO) gating strategies as previously described [45, 46].

Regarding evaluation of proteome in the serum samples, the Sandwich enzyme-linked immunosorbent assay (ELISA) measures the amount of protein of interest between two layers of antibodies (i.e. capture and detection antibody). Briefly, 96-wells ELISA microtiter plate was coated with the capture antibody in PBS and was incubated overnight at 4°C. The remaining protein-binding sites of plates were blocked using a blocking buffer, and then diluted samples and standards (triplicates) were applied for accurate quantitative results and to compare the signal of unknown samples against those of a standard curve. After washing steps, secondary antibody biotin-conjugated, diluted at the optimal concentration in blocking buffer, were added. The substrate for HRP was coupled to oxidation of a hydrogen donor which changes color during the reaction and TMB (3,3',5,5'-tetramethylbenzidine) solution was used for detection. After implication of stopping solution (1M H_2SO_4) plate were read the optical density at 450 nm using ELISA reader (Varioskan™ LUX, Thermo Fisher).

In selected patients, specific methods for functional tests of the candidate gene were implemented including transduced cell line using vectors carrying mutant versus wild type gene, cytotoxic T and NK cell killing assays, and virus-specific cytotoxic T cell activation [47-49].

3.2.4 Immunomic Approach

Complete blood count, lymphocyte subpopulations, serum immunoglobulin (Ig) levels and specific antibody response were measured as previously described [47, 50-56]. We classified our patients based on two main classifications for B-cell subsets including the EUROclass classification [57] and the B-cell pattern classification [58].

Genomic DNA and cDNA synthetized from RNA extracted from PBMCs were subsequently used for a nested PCR assay to amplify the recombined fragments from *in vivo* switched B-

cells (to evaluated switch recombination junctions) and somatic hypermutation rate as previously described [59].

In patients with a progressive decreased levels of lymphocytes, T-cell recombination excision circles (TREC) and kappa-deleting element recombination circle (κREC) analysis were performed on genomic DNA samples according to the previously published protocol [60].

3.3 STATISTICAL ANALYSES AND BIOINFORMATICS

Statistical analysis was performed using a commercially available software package (SPSS Statistics v.17.0.0, SPSS, and R statistical systems v.3.4.1., R Foundation for Statistical Computing). One-sample Kolmogorov-Smirnov test was applied to estimate whether data distribution is normal. Parametric and nonparametric analyses were performed based on the finding of this evaluation. A *p*-value of 0.05 or less was considered statistically significant.

Several software was used for *In-silco* analysis regarding positive selection detection, population structure detection, population structure inference, haplotype phase inference, familial aggregation analysis, cluster analysis, pedigree drawing and reconstruction, motif search, secondary structure prediction and connectome/pathway analysis [47-49, 61].

3.4 REFLECTIONS ON ETHICAL CONSIDERATIONS IN THE PROJECT

The basis for this project was an exploration of the genome of patients affected by a group of inherited disorders of the immune system, entitled PAD. Most of the patients in this category of disorders are children with chronic complications. Based on the purpose of this project, we needed to collect and record all medical data, family history, complete clinical examination and immunological investigation of each individual as well as the results of WES and the multiomics functional assays. In the last stage of the project, we also performed carrier detection in the siblings and first-degree relatives of the index case.

In this study, according to the methods of research, we followed the ethical guideline concerning following issues dealing with human subjects, children, mental incomplete persons, principles for using human tissues and samples and genetic study. For all patients in the PAD registry, participation in genetic screening and definite molecular diagnosis were voluntary (autonomy) and in the informed consent form, we have declared that the results will not be disclosed to employers, insurers, or others without the individual's consent (if the patient needs the certificate for completion of the insurance), in order to avoid possible discrimination.

We have declared to the PAD patients that we are responsible for research information to provide the patients with the outcome of the project unless s/he refrains from receiving the information. We have also guaranteed that all information will be considered as confidential. For both adult and pediatric patients informed consent has been given under the complete freedom and with a reasonable amount of time for decision without any force, threat, allure or temptation. All registered individuals have been treated based on the updated standard treatment, even if they declined enrollment in the study. We respected the patients' privacy and kept their private information confidential as well as respecting their right to change their mind, to decide to withdraw early or late the project without any consequences. By the outcome of the study and information that might emerge in the course of research, we have committed to updating patients and their legal guardian about the outcome even if it might change their assessment of the risks and benefits of participating as well as what were learned

from the research. Within the informed consent, we depicted for all registered PAD patients the aims, methods, anticipated benefits (as mentioned above), the duration of the investigation and potential risk (PAD patients require monthly Ig troughlevel test, therefore we merged our sampling with this patients'routine lab test to not increase the risk of needling).

Since the majority of the current PAD registry consists the children due to the nature of these inherited disorders, special consideration was taken to account for following ethical guidelines for research on pediatric cases. The study has conducted only on the child if we could not conduct it on adults (in families with multiple cases and with aggregation of patients with different ages the adult cases have been prioritized). Although the genetic investigations in the PAD children have both benefits and detrimental effects, it has been proven that beneficial effects for the next generations are not ignorable. However, in a patient with severe medical conditions, risk evaluation to patient's participation in our research project has been done by a committee including parents or legal guardian, investigators, professionals, the ethics committee, and even children.

In the current study to obtain consent from children, we have divided them into two groups: under 7 years and 7-15 years old. In patients <7 years, the consent is obtained from a legal guardian and every effort is made to satisfy the child, while if the child was in the age range of 7-15 year both patient and the legal guardian have been asked to fill in the consent form and in cases where the decisions differed, the ethics committee was involved. As the investigator of this study are aware that children should be involved in decision making for their health and safety, we give them the right to receive useful information as much as they can understand, to express their opinion and to decide. The method of giving information and obtaining consent in the second group of PAD has been adopted for their age and their level of understanding.

Of note, some PAD patients with probable defects in genes involving in the process of DNA repair have syndromic features accompanied by neurological symptoms and mental incompetence of decision, therefore, if for any reason the adults and/or child patient cannot make a decision, the patient's legal guardian has been asked to contribute to the consent form. For assessing the patient's capacity for decision making about participation in the study, the patient's family physician, who was not part of the research team could be requested to judge, otherwise, an unrelated physician had to mediate. In this group of patients if the medical evaluation revealed an improvement in the mental condition further tries have been done to receive the patients' consent as well. Furthermore, there are several PAD cases with these syndromic diseases that they cannot decide for themselves, but they can introduce another person into decision making, so far we have considered this request for substituting the person by informing the ethical committee. By the way, the investigators excluded the patient from the project if s/he had severe emotional problems such as suicide attempts and supportive care and patient was referred to the psychiatrists.

For all, three categories of the patients including adults, children, and mentally retarded patients genetic results have been followed by genetic counseling, family planning particularly when they indicate the direct association or causality effect on the clinical condition. As it happened often during the process of our study and as we had planned based on the finding of the genetic test, the therapeutic modalities have been revised by the panel of experts and if treatment or prevention was available, this has been prescribed as soon as possible. For example, the treatment of several cases should change from intravenous replacement therapy to the hematopoietic stem cell therapy and bone marrow transplantation.

Finally, the team of the project attempt to inform patients and their families, at regular intervals, of new developments in testing and treatment.

4 RESULTS AND DISCUSSION

4.1 WHOLE EXOME SEQUENCING IN DYSGAMMAGLOBULINEMIA

A correct molecular diagnosis in patients with PID is crucial for the classification of disorders with a heterogeneous clinical/immunological phenotype and subsequent therapeutic management. As the above mentioned PAD have highly diverse clinical features and, in most cases, an as yet unknown etiology. NGS has facilitated genetic investigations of rare inherited diseases during the past decade, thus allowing an appropriate molecular diagnosis in these patients. Clinical and immunological phenotyping were documented for a cohort of consecutive dysgammaglobulinemic patients with unknown genetic defects after having been subjected to classical targeted sequencing and they underwent WES analysis using a previously described filtering pipeline [39].

4.1.1 Demographic Features of Undefined Primary Antibody Deficiencies

Among all registered PAD patients (n=545, 27.2% of all patients in our PID registry), molecular defects were found in 49 individuals with agammaglobulinemia and 28 patients with HIgM syndrome using conventional genetic methods and 342 were deceased or unavailable for molecular investigation.

The 126 remaining available patients (70 males, 56 female) from 109 unrelated kindreds were classified as undefined PAD and enrolled for WES (**Table 2**). Although the national registry encompasses all age ranges of PAD patients, most patients were children and adolescents at the time of the study (52.3% were less than 18 years old) and parental consanguinity was recorded in 82.5 %. The median age of the patients at the onset of symptoms was 2 years (range 0.5–36 years; early onset manifestation in 95.2%) and the median diagnostic delay (the gap between the onset of the symptoms and diagnosis of PAD) was 4 years (range 0.25–39 years).

Based on the immunologic profile of the remaining undefined patients they were classified as CVID in 81, HIgM in 14, agammaglobulinemia in 11, IgAD in 11, specific antibody deficiency in 5, IgG subclass deficiency in 3 and 1 patient was diagnosed with IgM deficiency. Of note, 10 patients (7.9%) progressed to a more severe form of PAD during the course of the disease.

4.1.2 Molecular Diagnosis Outcome

WES analysis and subsequent confirmatory assays resulted in a genetic diagnosis in 86 of the 126 probands (68.2 %), where 2 patients with variants newly implicated in disease were identified (*CD70*), as well as 37 patients with known PID genes with newly identified phenotypes (19 unique genes), and 47 patients with mutations leading to the expected phenotypes (15 unique genes). Experimental data and the results of functional assays on a selected group of this genetically diagnosed cohort have been published in details [47, 48, 19, 62, 61, 49, 39, 63].

The majority of our patients were born in consanguineous marriages and they would thus be expected to demonstrate an autosomal (homozygous) recessive defect. However, the mode of inheritance was judged to be recessive in only 23 of the 35 genes (65.7 %) accounting for genetic inheritance in 58 patients (67.4 %). Three genes were found to be X-linked (8.5 %) and 9 genes were assigned as autosomal dominant due to loss-of-function (6 genes; 17.1 %) or gain-of-function (3 genes; 8.5 %).

Table 2. Clinical characteristics of 126 primary antibody deficient patients	
Characteristics	**Value**
Sex	
Male (%)	70 (55.5)
Female (%)	56 (44.5)
Age	
Age at the time of study, year (range)	18 (4-44)
Age of onset, year (range)	2 (0.5-36)
Age of clinical diagnosis, year (range)	8 (0.5-40)
Family structure	
Non consanguineous kindress	
One affected child (%)	18 (16.5)
Two or more affected children (%)	2 (1.8)
Consanguineous kindress	
One affected child (%)	77 (70.6)
Two or more affected children (%)	12 (11.1)
Ethnicity based on Greater Middle Eastern genetic variation	
Persian Peninsula (%)	106 (84.1)
Turkish Peninsula (%)	11 (8.7)
Arabian Peninsula (%)	9 (7.2)
Clinical phenotype	
Infections only (%)	40 (31.7)
Autoimmunity (%)	45 (35.7)
Lymphoproliferation (%)	47 (37.3)
Enteropathy (%)	34 (26.9)
Malignancy (%)	7 (5.5)
Allergy (%)	15 (11.9)
Overlap phenotype	48 (38.0)
Immunodeficiency diagnosis	
Common variable immune deficiency	81 (64.3)
Unsolved agammaglobulinemia	11 (8.7)
Unsolved hyper IgM syndrome	14 (11.1)
IgA deficiency	11 (8.7)
Other types of primary antibody deficiency	9 (7.2)

All disease causing variants were pathogenic or likely pathogenic based on the ACMG standards and were private or rare as compared to our in-house database (<1% of more than 300 unrelated individuals sequenced), the Greater Middle Eastern variation database (<1% of 1,111 unrelated individuals, including 168 persons from the Persian peninsula, 214 from the Arabian peninsula and 140 from Turkish peninsula), the Exome Aggregation Consortium database (<0.01% of 60,706 unrelated individuals, http://exac.broadinstitute.org/) and the genome AD database (<0.01% of 123,136 WES and 15,496 whole-genome sequences from unrelated individuals, http://gnomad.broadinstitute.org/). Of note, a large deletion of coding regions was identified in 4 patients carrying genetic defects within the *LRBA* and *DOCK8* genes. Detail of the genetic diagnosis of the different types of PAD patients is summarized in **Figure 3**.

4.1.3 Genotypic and Phenotypic Correlation

We decided to evaluate the genotype–clinical phenotype correlation in our cohort of PAD patients especially for affected individuals within the same family, using a standard method of phenotype subdivision which has been shown to correlate with the quality of life and morbidity among patients with infections only, autoimmunity, lymphoproliferation and enteropathy [64]. However, no significant correlation was observed, indicating an effect of environmental factors and/or other modifier genes on the medical complications of the patients.

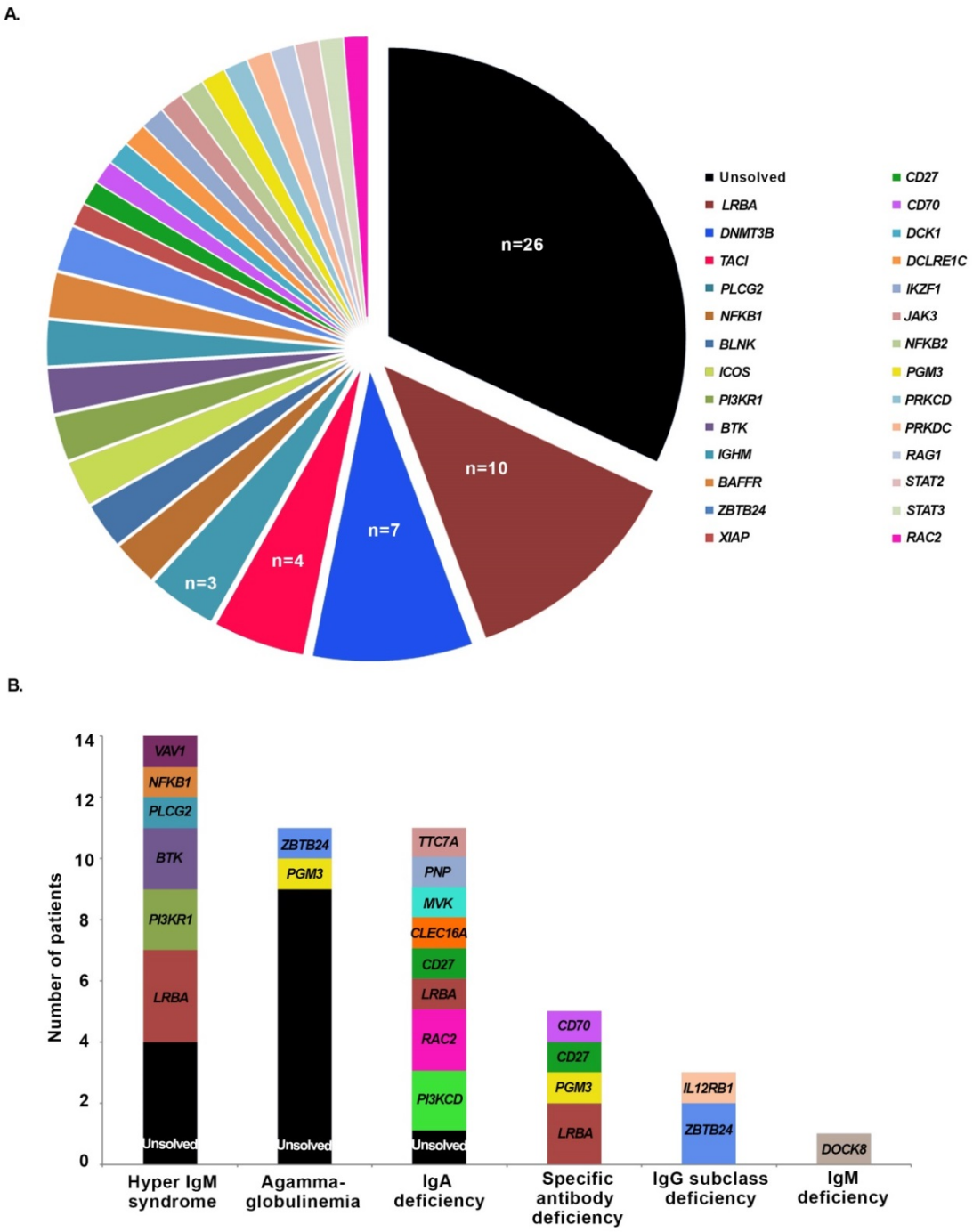

Figure 3- Frequency of different monogenic defects among patients with different types of primary antibody deficiency as well as unsolved patients (**A**) among 81 patients with a common variable immune deficiency like phenotype and (**B**) 45 patients with an immunologic phenotype suggesting a hyper IgM syndrome (n=14) agammaglobulinemia (n=11), IgA deficiency (n=11), IgG subclass deficiency (n=3), specific antibody deficiency (n=5) and hypo IgM syndrome (n=1).

Given the immunologic heterogeneity of undefined PAD patients, we used the identified underlying gene defect to correlate potential immunopathologic mechanisms with the point of arrest in B-cell development using a B-cell subset analysis. An association between the affected gene and the pattern of abnormalities in the size of the B cell subsets were identified, mirroring the respective pathologic mechanism of the damaged molecule. Based on the observed five distinct B-cell patterns, we could demonstrate that combined B cell production and germinal center defects (low numbers of transitional B cells and memory B cells) often represent DNA repair/recombination gene defects, being associated with an increased radiosensitivity and a mild form combined immunodeficiency, involving the *RAG1, DCLRE1C, PRKDC, DNMT3B and ZBTB24* genes. Early peripheral B cell maturation or survival arrest (loss of naive mature, marginal zone–like, and memory B cells) is associated with mutations in *TNFRSF13B* and *TNFRSF13C*, which is in line with impaired baseline constitutive activation and subsequently impaired anti-apoptotic signaling. Mutations in BCR associated genes (e.g. *BTK, BLNK* and *IGMH*) show a phenotype of both B cell activation and proliferation defects (combined reduction of the marginal zone–like and memory B cells). Isolated germinal center blockage (exclusive decrease in the number of memory B cells associated with a normal or high level of IgM) suggests gene defects in costimulatory molecules for T-dependent immunity (e.g. *CD27*, *CD70* and *ICOS*) which modify signaling for class-switch recombination and somatic hypermutation. Finally, the pattern of post-germinal center impairment (defects in terminal plasma-cell maturation, survival or homing), leading to an isolated reduction of long-term plasma cells, might be compatible with LRBA and XIAP deficiencies (**Figure 4**).

4.1.4 Clinical and Immunological Features of Solved versus Unsolved Patients

In the 86 PAD patients with a genetic diagnosis, defects in genes that encode proteins involved in the post-germinal center survival pathway (LRBA and XIAP) accounted for 19.7% of the total disease-causing etiologies while proteins involved in DNA repair and recombination pathways defects (*RAG1, DLCRE1C, PRKDC, DNMT3B* and *ZBTB24*) accounted for 17.4% of genetic defects. Defects in BCR signaling (in 13.9% of patients) and in the PI3K signaling pathway (phosphatidylinositide 3-kinases signaling affected in 11.6% of patients) were also the other frequently observed defects in our PAD cohort.

The lowest diagnostic yield was obtained in patients with agammaglobulinemia (2 of 11 tested; 18.1%). We performed stratification on the patients who underwent sequencing to determine the parameters associated with a diagnosis. Of note, consanguinity and the severity of clinical presentation were similar between those who had a molecular defect identified (n=86) and those who did not (n=40). However the clinical diagnosis of agammaglobulinemia ($p<0.001$), a late age of presentation (onset of disease > 10y; $p=0.03$), and the absence of multiple affected family members ($p=0.01$) were significantly more frequent in the patients who had no genetic defect identified. Genetic defect was identified in 90% of patients with a progressive form of PAD suggesting a higher rate of diagnostic yield in this subgroup of patients compared to other patients ($p=0.01$). Among the 40 patients with non-definitive pathogenic genetic variants, immunologic phenotypes were mainly compatible with a pattern of post-germinal center impairment ($p=0.03$).

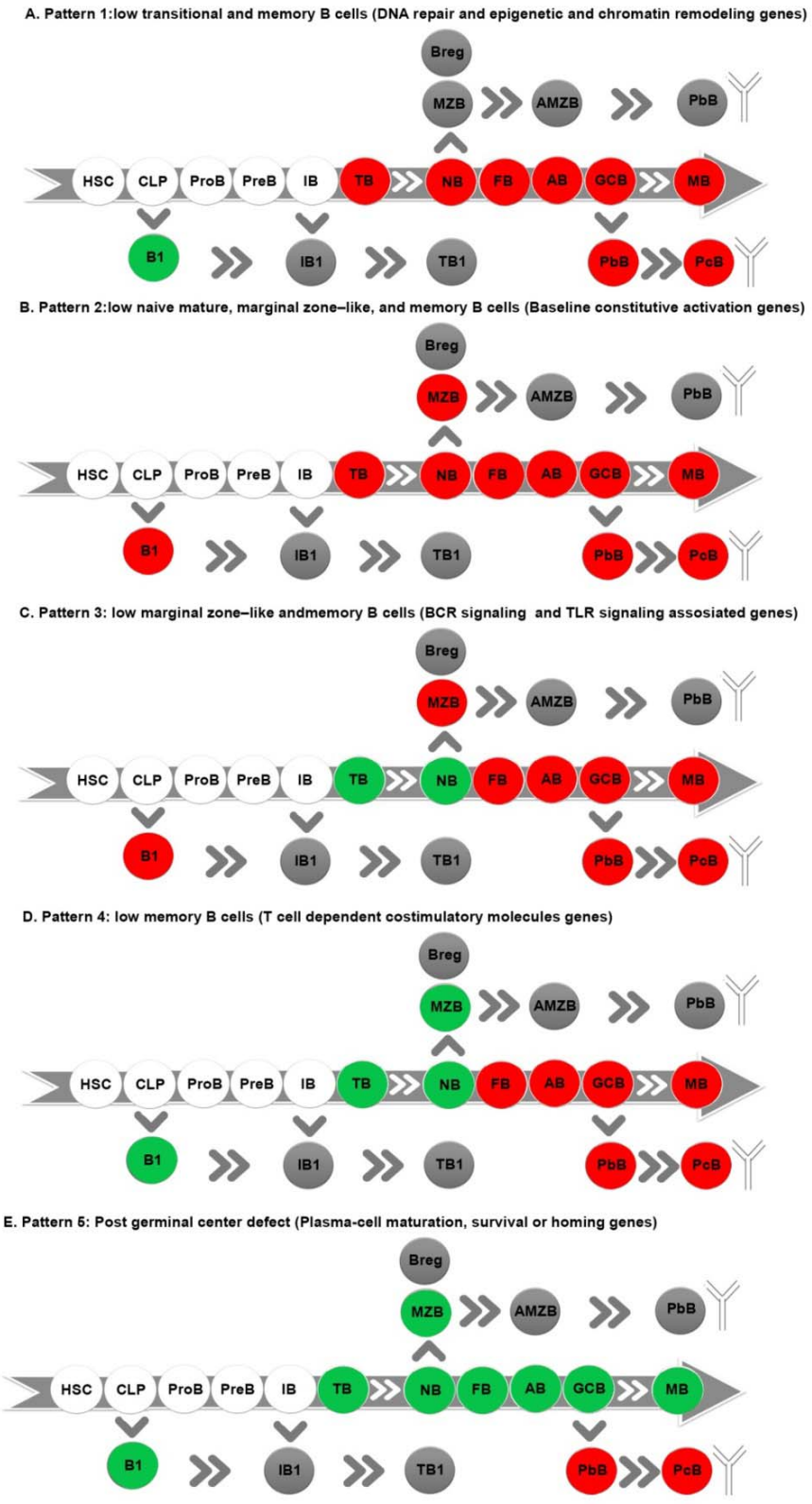

Figure 4- Model of the pathophysiological background of five B-cell patterns in antibody deficient patients based on the level of maturational arrest level. Green circles mean normal maturation and peripheral cell counts and red circles depict interrupted maturational step with decreased proliferation. HSC: hematopoietic stem cell, CLP: common lymphoid progenitor, ProB: Pro B-cell, PreB: Pre B-cell, IB: immature B-cell, TB: transitional B-cell, NB: naïve B-cell, MZB: marginal zone B-cell, Breg: regulatory B-cell, AMZB: activated marginal zone B-cell , FB: follicular B-cell, AB: activated B-cell, GCB: germinal center B-cell, PbB: plasmablast B-cell, PcB: plasma cell B-cell, MB: memory B-cell.

4.1.5 Clinical Implications of Molecular Diagnoses

Our therapeutic approach was changed in 26 patients (20.6 %) from Ig replacement to hematopoietic stem cell transplantation (HSCT) in a selected group with atypical combined immunodeficiency (*LRBA*, *DCLRE1C*, *RAG1, PRKCD, JAK3, PNP*, *CD27* and *CD70* mutations). Regular screening for cancer and avoidance of malignancy triggers were added to a routine management of 15 patients (11.9 %) with defects in their DNA repair system. More mechanistically precise treatment, such as supplementation of rapamycin in patients with PI3KR1, PI3KCD and LRBA deficiencies, was initiated in 22 patients. The results of WES aided 49 families (38.8%) in family counseling, leading to the performance of the prenatal diagnosis in 25 families (19.8 %). In total, a correct genetic diagnosis affected the clinical treatment and management of 48.4% of probands in whom a pathogenic or probably pathogenic variant was identified.

4.2 NEW PHENOTYPE OF A KNOWN GENE IN DYSGAMMAGLOBULINEMIA

Recombination-activating gene 1 (RAG1) deficiency presents with a varied spectrum of combined immunodeficiency, ranging from a $T^-B^-NK^+$ type of disease to a $T^+B^+NK^+$ phenotype. We sought to assess the genetic background of patients with PAD according to the above-mentioned method. In **paper I**, one of these 126 patients given an immunodeficiency diagnosis resembling CVID, who was born to a consanguineous family and thus would be expected to show an autosomal recessive inheritance, was subjected to clinical evaluation, immunologic assays, homozygosity gene mapping, exome sequencing, Sanger sequencing, and functional analysis. This 14-year-old patient had liver granuloma, extranodal marginal zone B-cell lymphoma, and autoimmune neutropenia. Genetic analysis of this patient showed a homozygous hypomorphic RAG1 mutation (p.C358Y) with a residual functional capacity of 48% of wild-type protein. Our finding broadens the range of known clinical phnotypes associated with *RAG* mutations and highlights that timely molecular diagnosis might have important therapeutic implications.

4.3 NEW INHERITANCE PATTERN OF A KNOWN GENE IN DYSGAMMAGLOBULINEMIA

In **paper II,** using WES analysis on a female patient born to consanguineous parent, we identified a novel homozygous nonsense variant in codon 56 (p.W56X) of Ras-Related C3 Botulinum toxin substrate 2 (RAC2) protein. The same homozygous mutation was identified in her brother, and in a heterozygous form in their mother. Both siblings suffered from a CVID-like immunodeficiency in addition to hormone deficiencies, and a coagulation defect (factor XI deficiency). Western blot analysis showed absence of the RAC2 protein in designed transfection assay and functional analysis of patients'neutrophils showed decreased chemotaxis. We performed transmission electron microscopy and identified abnormalities of the neutrophil granules in milder form as described in the previously reported patients with missense mutations.

Prior to our publication, only *de novo* dominant negative mutations affecting RAC2 (p.D57N) had been reported in 2 male infants with phagocytosis disorder. The first case presented with a complex neutrophil dysfunction disease, which was characterized by multiple and progressive soft-tissue infections during the first few weeks of life, neutrophilia, and a neutrophil chemotaxis defect. The second case exhibited reduced numbers of Trec in the newborn screening, and later developed omphalitis, paratracheal abscess, and a neutrophil chemotaxis defect. Both infants underwent successful HSCT, therefore their humoral immune status were not defined. Our results illustrate that different types of mutations in a given gene might be associated with vastly different clinical phenotypes.

4.4 NEW GENE DISCOVERY IN DYSGAMMAGLOBULINEMIA

CD27 is a member of the tumor necrosis factor receptor superfamily and is involved in a pathway (with its unique ligand CD70) regulating immunity versus tolerance by several immune mechanism including T cell expansion and survival, co-stimulation of antigen presentation, germinal center formation, B cell activation and antibody production. The clinical and immunologic features of CD27 deficiency remain obscure because only a few patients had been identified before this study. In **paper III**, we have identified novel mutations in *CD27* (homozygous p.C96Y in two patients and homozygous p.R78W in one patient) during the process of the project and we sought to provide an overview of clinical, immunologic, and laboratory phenotypes in patients with CD27 deficiency. Review of the medical records and molecular, genetic, and flow cytometric analyses of the patients and family members were performed. Treatment outcomes of previously described patients and another joint cohort of 4 new patients were followed up. Epstein–Barr virus (EBV)-associated lymphoproliferative disease, hemophagocytic lymphohistiocytosis, Hodgkin lymphoma, uveitis, and recurrent infections were the predominant clinical features. Expression of cell-surface and soluble CD27 was significantly reduced in patients and heterozygous family members. Immunoglobulin substitution therapy was administered in 5 of the newly diagnosed cases due to primary or secondary antibody deficiency. CD27 deficiency is potentially fatal and should be excluded in all cases of severe EBV infections to minimize diagnostic delay. Flow cytometric immunophenotyping offers a reliable initial test for CD27 deficiency. Determining the precise role of CD27 in immunity against EBV might provide a framework for new therapeutic concepts.

CD70 is also a co-stimulatory molecule expressed on several immune cells including T cells, B cells and dendritic (DC) cells, and its interaction with CD27 leads to the activation of CD70-positive cells via anti-apoptotic kinases. Aligned with the process of the main project and using a power of WES for detection of novel gene defects, in **paper IV** we also identified first human CD70 deficiency in two patients from our cohort (homozygous p.S84Pfs27X) and in two other patients from other ethinicity, predominantly manifesting as susceptibility to EBV-related diseases. Three patients presented with EBV-associated Hodgkin's lymphoma and hypogammaglobulinemia mimicking a CVID phenotype; one also had severe varicella infection. The fourth had suspected viral encephalitis during infancy and EBV infection. Homozygous frameshift or in-frame deletions in CD70 in these patients abolished either CD70 surface expression or binding to its cognate receptor CD27. Lymphocyte numbers were normal, but the proportions of memory B cells and EBV-specific effector memory $CD8^+$ T cells were reduced. Furthermore, although T cell proliferation was normal, in vitro-generated EBV-specific cytotoxic T cell activity was reduced. This reflected impaired activation by, rather than effects during killing of EBV-transformed B cells. Thus, autosomal recessive CD70 deficiency is a novel cause of PID and EBV-associated diseases, reminiscent of CD27 deficiency. CD70-CD27 interactions therefore play a non-redundant role in T- and B- cells mediated immunity, especially for protection against EBV and humoral immunity.

4.5 DISCUSSION AND NEW GUIDELINE IN GENETIC STUDY OF DYSGAMMAGLOBULINEMIA

Our findings reflect that PAD is a group of clinically and genetically heterogeneous disorders, necessitating a wide molecular approach for a definitive diagnosis [65]. Although it is not yet a consensus, recent genetic diagnostic studies on undefined dysgammaglobulinemic patients have tried to include all known PID genes in this subgroup of PID patients using NGS.

The success rate of this approach was reported to be 23.5% in 34 UK CVID patients[17], 30% in a US CVID cohort of 50 patients [16], and 41.6% in 36 patients in a multinational antibody deficiency cohort [66]. The diagnostic yield in these three NGS investigations are close to the results of targeted gene panel testing (15-40%, with a lower time consumption and cost for the latter) due to inadequate computational analysis for CNV, lack of utilization of the gene discovery power of NGS and lack of functional assays for confirmation of the pathogenicity of the observed variants [28-31] . Although the genetic defect was identified in 68.2% of our patients, we were not able to find the disease-causing variant in 40% of currently solved patients if we had used targeted sequencing for a known list of genes of PAD (based on the latest classification of International Union of Immunological Societies [18]). However, our conclusion is that a high-throughput genomic approach should be performed as a first screening step for patients with dysgammaglobulinemia due to the overlap of clinical phenotypes derived from distinct genotypes. In line with this notion, our investigation resulted in an expansion of the clinical spectrum of mutations in 19 genes.

Of note, BTK, BLNK and μ heavy chain deficient patients with atypical presentation of the hypogammaglobulinemia and normal peripheral B cell counts, mimicking a CVID-like phenotype, indicates the potential problem using a conventional genetic approach, which is based on the immunoglobulin profile and lymphocyte counts. Although several efforts have been made during the past decade to classify dysgammaglobulinemia based on different clinical phenotypes and Ig profiles, our results complement other studies suggesting an early and comprehensive genetic strategy (e.g. WES). Reduced penetrance or variable expressivity was observed in our patients with LRBA deficiency (presenting as agammaglobulinemia, HIgM and CVID-like phenotypes) and PI3KR1 deficiency (presenting as HIgM, CVID and IgAD), indicating an extension of the PID phenotype spectrum. As there is considerable immunologic heterogeneity in individuals with exactly the same gene defect (within families), this highlights the potential role of as yet unidentified modifier genes. This finding is also consistent with progression of different forms of dysgammaglobulinemia. Nonetheless, in contrast to the Ig profile, the B cell developmental pattern showed a robust association with the diseases associated gene (**Figure 4**). This general observation emphasizes the importance of filtering variants in collaboration with the treating clinicians and immunologists.

On the other hand, patients with hypomorphic mutation in severe combined immunodeficiency associated genes (e.g. *RAG1, JAK3, PRKDC* and *DCLRE1C*) and incomplete and atypical presentation of syndromic disorders (e.g. associated with *ZBTB24, DNMT3B, PGM3, DKC1, PNP* and *TTC7A* mutations) illustrates the need for careful assessment of PAD patients in order to provide a reliable prognosis and to initiate appropriate treatment. This approach may be of considerable value in these patients, where the establishment of diagnosis on a molecular level would suggest a different type of therapy, shifting from Ig replacement to HSCT or gene therapy. Establishing a correct differential diagnosis list for PAD including a more broad panel of PID genes is also suggested by our results, as mutations in *RAC2* (congenital defects of phagocyte genes with defects of neutrophils motility), *NLRP12* and *MVK* (autoinflammatory disorders genes affecting the inflammasome), *IL12RB1* and *STAT2* (genes involved in intrinsic and innate immunity) and *STAT3* (known gene involved in immune dysregulation) could manifest with an aberrant Ig profile. These findings and previous genetic causes reported to underlie of antibody deficiency in other studies, urge us to expand the expected genetic candidate of PAD.

In **paper V,** we suggested, as demonstrated in our workup chart (**Figure 5**), a stepwise clinical and molecular diagnosis in patients with different types of PAD. In a mixed group of antibody deficient patients, patients with gene defects that are associated with other forms of PID should be detected by NGS, including those with defects associated with syndromes or combined immunodeficiencies since they would be at risk for a high morbidity and mortality. However, such patients are likely to exhibit several abnormal immune parameters in addition to a perturbed Ig profile (**Figure 6**). In our suggested decision tree for the diagnostic workup, probable pathogenesis, and appropriate treatment choices, it is recommended to start with clinical/immune parameters (particularly the B cell subset pattern) whereupon it is possible to distinguish other or additional defects and, in some cases, a genetically defined defect. What is left from the cohort of antibody deficient patients will be a group of idiopathic PAD patients that should be investigated for other probable etiologies, including modifier genes, defects in enhancer, promoter and intronic regions or other structural abnormalities, low-grade mosaicism, epigenetic markers and environmental susceptibility factors. Another potential factor that should be considered is inadequate coverage of the gene of interest (variants that have low quality or reads support less than 4 is usually filtered during analysis process) in some known or potential genes.

The main pathways involved in our PAD patients were the post-germinal center survival pathway and DNA repair signaling. We calculated and plotted the network of all 189 known PID-causing genes related to PAD using the human genome connectome-predicted direct biological distance between human genes. We found that PAD related genes tend to be the central hub of the following signaling pathways: B-cell activation (p=2.9×10^{-29}), antigen receptor-mediated signaling pathway (p=9.3×10^{-24}), activating cell surface receptor signaling pathway (p=1.1×10^{-21}), and lymphocyte differentiation (p=2.0×10^{-21}).

Additional capabilities of NGS on *In silco* analysis of CNV and detection of new candidate genes provide further support for this approach as the first step in the genetic diagnosis of PAD. Lack of identification of disease-causing CNVs, particularly in compound heterozygous forms in cohorts with a large proportion of non-consanguineous patients, could be a potential explanation for differences in diagnostic yield between our survey and other studies [16, 17]. Although we found that 4.6% of the solved cases harbored large homozygous deletions (mainly within the *LRBA* and *DOCK8* genes with a high level content of transposable elements [67]), it supports the notion of a potential presence of a selected group of antibody deficient patients with biallelic defects due to an allele with large heterozygous deletion from one parent together with an allele defective due to a mutation from another parent. Similar findings have been shown in a recent study investigating different categories of PID, revealing an approximately 10% mutational burden of CNV in diseases pathogenesis [66]. Although different methods could ultimately be integrated into the diagnostic process, a computational algorithm for NGS analysis might be an appropriate and cost effective method for CNV detection as previously described [39, 68].

Despite advances in CNV analysis and novel gene hunting algorithms in NGS, we also evaluated other potential factors that may affect the diagnostic yield rates in our study as compared to other reports. The effect of severe clinical phenotype, use of homozygosity mapping, and even parental consanguinity could not explain this level of difference (comparing 40 unsolved to 86 solved patients). However, familial segregation analysis including a healthy sibling or another first-degree relative using NGS in multiple case families could increase the discovery rate, being more efficient than trios, a fact which seems to be consistent among different studies with different settings [16, 17, 66].

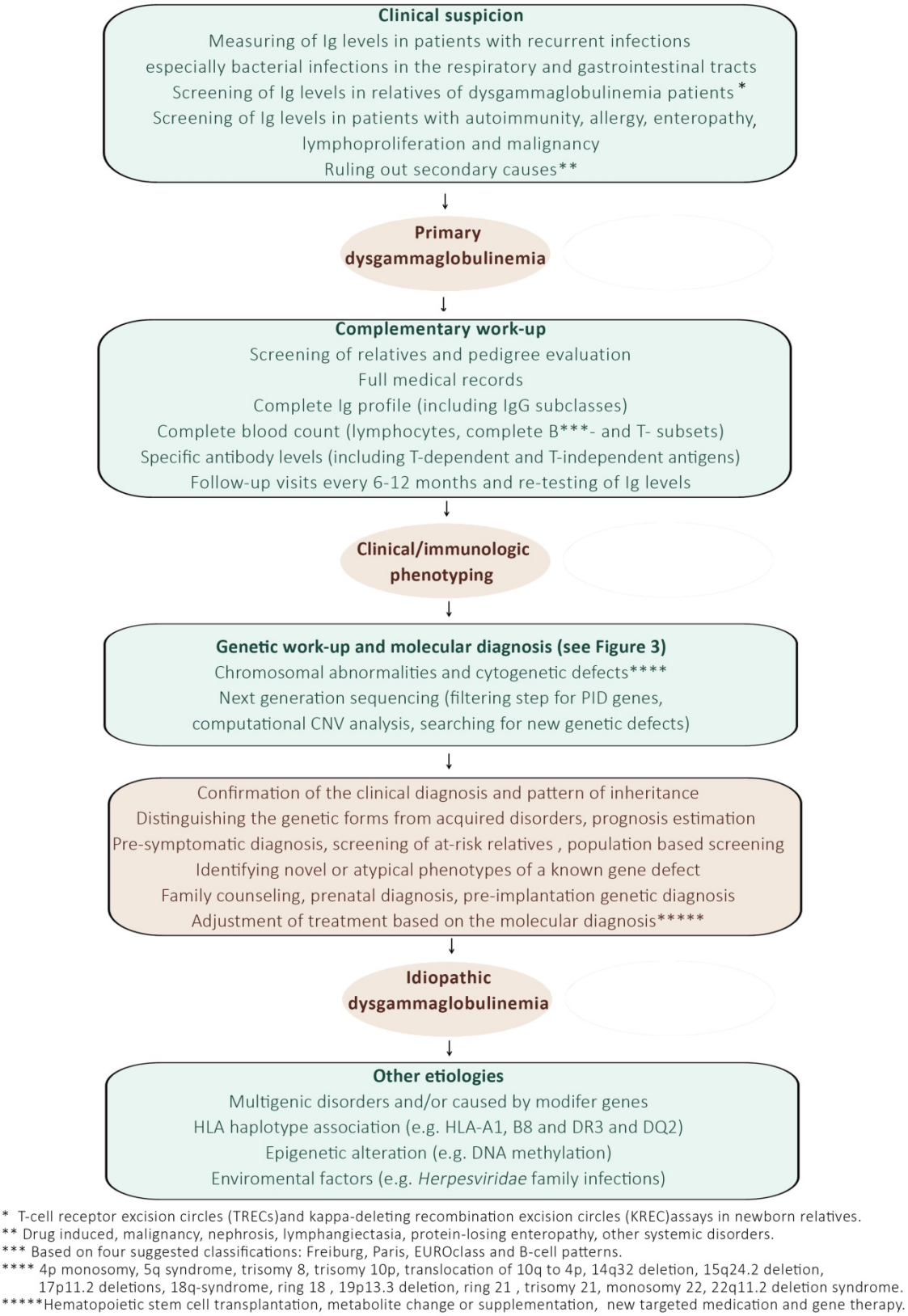

Figure 5- A Clinical, immunological and genetic approach for a molecular diagnosis of primary antibody deficiency

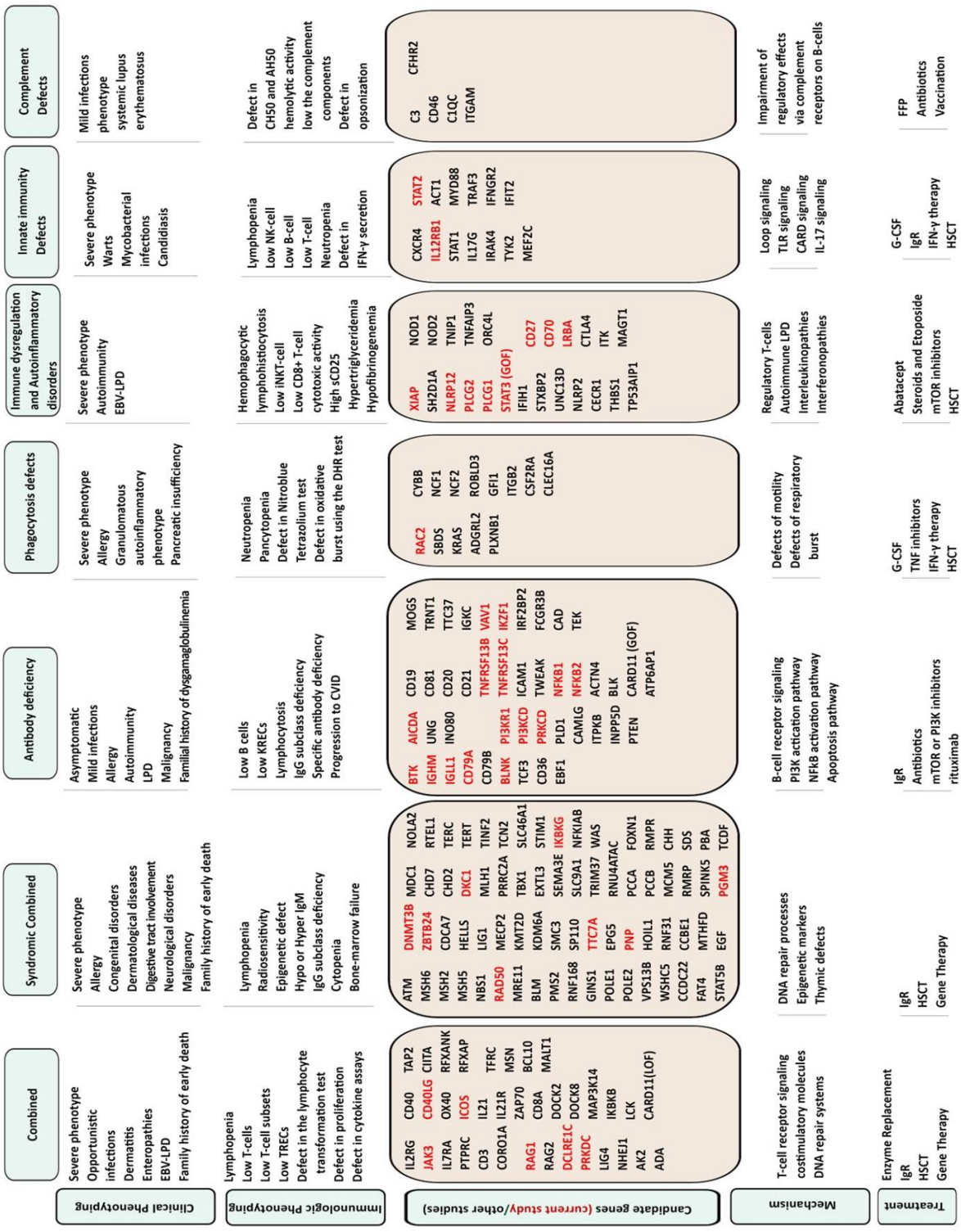

Figure 6- Candidate gene defects and pathological mechanisms in patients with primary antibody deficiency based on clinical and immunological phenotyping and their appropriate treatment modalities. AH50: 50% alternative haemolytic complement activity, KREC: kappa-deleting recombination excision circle, CARD: Caspase recruitment domain, EBV: Epstein–Barr virus, CH50: 50% haemolytic complement activity, FFP: fresh frozen plasma, G-CSF: granulocyte-colony stimulating factor, HSCT: Hematopoietic stem cell transplantation, iNKT: invariant NK-T cells, IgR: immunoglobulin replacement therapy, IFN-g: interferon gamma, LPD: lymphoproliferative disorder, TLR: Toll-like receptors, TNF: tumor necrosis factor; TREC: T-cell receptor excision circle. References reviewed for compiling the gene list underlying dysgammaglobulinemia were [16-19, 7, 10].

Whole genome sequencing (WGS) enables an advanced CNV detection (in the case of PCR free library preparation), and targeting all arbitrary exome, deep intronic, regulatory domains and structural intragenic regions (due to the absence of capture step and reference biases). For the time being, however, WES seems to be the most cost effective approach for PAD patients with unknown etiology [32].

Our follow-up on patients with a molecular diagnosis using NGS provided valuable guidance for the treating physicians towards appropriate clinical management, prenatal diagnosis, and targeted therapy (where recent studies have been provided evidence for utilization of rapamycin, abatacept and tocilizumab as new modalities in the treatment of PAD [69-71]). Prenatal diagnosis in childhood-onset patients has an important significance apart from decreasing the burden of diseases, since the parents and also the first relatives of patients are still in childbearing ages and may thus need genetic counseling for their next pregnancy. Furthermore, a selected group of patients with symptoms suggesting a combined immunodeficiency could potentially benefit from HSCT, including those with mutations in *RAG1, CD27* and *CD70*.

5 CONCLUSION AND FUTURE PRESPECTIVES

Although our gene hunting was conducted on a national patient cohort from the Middle East region, the mutated genes from this region have also been documented in western non-consanguineous populations where they have been reported in compound heterozygous forms. We suggest that NGS could replace the conventional multistep genetic approach because it can expanded to cover all known PID associated genes and potentially detect CNV and new genes associated with primary immunodeficiency. More efforts should be spent on improving the NGS processing timeframe and gene-capture coverage to integrate it in a rapid molecular diagnostic pipeline, including the confirmation of a positive newborn screening test. Despite a substantial decrease in the cost for NGS in recent years, it still somewhat hampers its large scale implementation although this is expected to change within the foreseeable future.

Dysgammaglobulinemia, particularly CVID, is likely to be a collection of several genetically distinct disorders. Although all the patients in our cohort could not be shown to suffer from a monogenic disorder, these patients should be further investigated for additional, non-genetic susceptibility factors.

In parallel, regarding patients without identified gene even after WES, we will continue to investigate the "genome" of the patients with WGS. In a same time we will try to improve our computational pipeline analysis on NGS. However, my future projects will involve mainly on establishing single cell methods for "transcriptome" and "proteome" analysis of patients whom we have discovered novel gene defect to empower our functional assays and to assess the subsequent molecular events downstream of defect in specific cells.

For PAD patients with gene defects in epigenetic controlling pathways whom evaluated currently by DNA methylome profile (data not shown in this summary), we will continue to expand the level of "epigenome" investigation to find alteration in histone modification and miRNA profile. The auto-antibodies against major cytokines for development of B cells and high-throughput major histocompatibility typing are other potential factors that will be investigated in the unsolved patients.

Genes affecting the mucosal immunity as well as Ig production will be subjected for performing "microbiome", particularly in patients with enteropathies. Patients with defect in genes involved in DNA repair system will be subjected for "immunome" profile using high throughput techniques for investigation of total B- and T-cell receptor repertoire and rearrangement composition, switch junctions, double strand breakage point and somatic hyper mutation profile.

Moreover we will continue preforming WES on additional probands with primary antibody deficiency especially with a diagnosis of IgAD, IgG subclass deficiency, specific antibody deficiency patient and transient hypogammaglobulinemia of infancy.

The output of this doctoral thesis will add more information about the genetic association of PAD, enabling earlier diagnosis and management of patients and their relatives. As above-mentioned, molecular diagnosis plays a pivotal in PAD diagnosis (e.g. identification of new genetic defects, distinguishing genetic form from an acquiring disorder, confirming the clinical diagnosis, positive newborn screening test and pattern of inheritance, pre-symptomatic diagnosis, cascade screening of at-risk relatives and population based screening, identifying novel or atypical phenotype of a known gene defect), assisting treatment decisions (e.g. hematopoietic stem cell transplantation, metabolite change or supplementation, new targeted medication and gene therapy), prognosis estimation (e.g. long-term prospective of treatment) and possibility of family counseling (e.g. prenatal diagnosis and pre-implantation

genetic diagnosis). The therapeutic approach was changed from Ig replacement to HSCT in a selected group of patients with atypical combined immunodeficiency. Regular screening for cancer and avoidance of malignancy triggers were added to a routine management of patients with deficiencies in DNA repair system. More precise mechanistically and targeted treatment was initiated and the results of this study aided several families for family consulting, particularly for prenatal diagnosis. Generally multomics approach is the future diagnostic and therapeutic horizon in patients with PAD, PID and other type of rare inherited disorders.

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