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Novel Genetic Associations with Common Variable Immunodeficiency

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Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by E-Print AB 2016

ISBN 978-91-7676-304-9

Novel Genetic Associations with Common Variable Immunodeficiency

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

In the name of Allah, the Entirely Merciful, the Especially Merciful

To my family

&

For the welfare of mankind

ABSTRACT

Common variable immunodeficiency (CVID) is the most frequently encountered primary immunodeficiency disorder in clinical practice and is a cause of significant morbidity and mortality for patients. The main clinical features are hypogammaglobulinemia, recurrent infections and autoimmune disorders. Unlike the majority of primary immunodeficiency disorders, pinpointing a specific genetic association has been challenging.

The objective of this work was to use next generation sequencing (NGS) technology in the form of whole exome sequencing (WES) to identify genetic associations with CVID.

Candidate patients were selected based on parental consanguinity and availability of clinical specimens from them and their family members. Whole exome sequencing was performed and identified potential mutations which were confirmed by Sanger sequencing, followed by characterization of the novel mutations by immunological techniques.

In **Paper I**, genetic analysis identified four novel *CD27* mutations: homozygous missense mutations C96Y and R78W; heterozygous nonsense C10X; and compound heterozygous W8X-R107C resulting in a clinical phenotype of CD27 deficiency.

In **Paper II**, whole exome sequencing revealed a novel W56X mutation in the *RAC2* gene resulting in a CVID-like phenotype with prominent autoimmune disease. Newly identified abnormalities of neutrophil granules were identified by transmission electron microscopy.

In **Paper III**, five novel mutations in the *LRBA* gene resulting in varied clinical phenotypes were presented in the context of a review of all published cases, thus providing a clinical summary.

In conclusion, this work has shown the validity of employing whole exome sequencing in identifying genetic associations with CVID and CVID-like disease; and has provided a better overview of the diverse clinical phenotypes associated with mutations in *CD27*, *RAC2* and *LRBA* and their resultant protein deficiencies.

LIST OF SCIENTIFIC PAPERS

I. *Novel Mutations in TNFRSF7/CD27: Clinical, Immunologic, and Genetic Characterization of Human CD27 Deficiency.*

Alkhairy OK, Perez-Becker R, Driessen GJ, Abolhassani H, van Montfrans J, Borte S, Choo S, Wang N, Tesselaar K, Fang M, Bienemann K, Boztug K, Daneva A, Mechinaud F, Wiesel T, Becker C, Dückers G, Siepermann K, van Zelm MC, Rezaei N, van der Burg M, Aghamohammadi A, Seidel MG, Niehues T, Hammarström L.
J Allergy Clin Immunol. 2015 Sep; 136(3):703-712.

II. *RAC2 Loss-of-function Mutation in 2 Siblings With Characteristics of Common Variable Immunodeficiency.*

Alkhairy OK, Rezaei N, Graham RR, Abolhassani H, Borte S, Hultenby K, Wu C, Aghamohammadi A, Williams DA, Behrens TW, Hammarström L, Pan-Hammarström Q.
J Allergy Clin Immunol. 2015 May; 135(5):1380-4.

III. *Spectrum of Phenotypes Associated with Mutations in LRBA.*

Alkhairy OK, Abolhassani H, Rezaei N, Fang M, Andersen KK, Chavoshzadeh Z, Mohammadzadeh I, El-Rajab MA, Massaad M, Chou J, Aghamohammadi A, Geha RS, Hammarström L.
J Clin Immunol. 2016 Jan; 36(1):33-45.

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

I. *Genome-wide Association Study of Late-Onset Myasthenia Gravis: Confirmation of TNFRSF11A, and Identification of ZBTB10 and Three Distinct HLA Associations.*

Seldin MF, **Alkhairy OK**, Lee AT, Lamb JA, Sussman J, Pirskanen-Matell R, Piehl F, Verschuuren JJ, Kostera-Pruszczyk A, Szczudlik P, Mckee D, Maniaol AH, Harbo HF, Lie BA, Melms A, Garchon HJ, Willcox N, Gregersen PK, Hammarström L.

Mol Med. 2015 Oct 10. doi: 10.2119/molmed.2015.00232.

CONTENTS

LIST OF ABBREVIATIONS

AID	Autoimmune disease
AIHA	Autoimmune hemolytic anemia
BCR	B cell receptor
CD	Chronic diarrhea
CSR	Class-switch recombination
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CVID	Common variable immunodeficiency
DCs	Dendritic cells
DHR	Dihydrorhodamine
ESID	European Society for Immunodeficiencies
FISH	Fluorescence in situ hybridization
GH	Growth hormone
GWAS	Genome-wide association study
HGG	Hypogammaglobulinemia
IBD	Inflammatory bowel disease
IFNγ	Interferon-gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-2	Interleukin-2
IL-21	Interleukin-21
IL-7	Interleukin-7
Indels	Insertion/deletion
IVIG	Intravenous immunoglobulin
JIA	Juvenile idiopathic arthritis
KO	Knockout
KREK	Kappa-deleting element recombination circle
LFA-1	Leukocyte function-associated antigen-1
LFTs	Liver function tests

LRBA	Lipopolysaccharide responsive beige-like anchor protein
MHC	Major histocompatibility complex
MMCP-7	Mouse mast cell protease 7
MZ	Marginal zone
NA	Not applicable
NETs	Neutrophil extracellular traps
NHEJ	Non-homologous end joining
NK	Natural killer
NLH	Nodular lymphoid hyperplasia
NS/SS	Non-synonymous/splice site
OM	Organomegaly
PADs	Primary antibody deficiencies
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PID	Primary immunodeficiency disorders
PTH	Parathyroid hormone
Rac2	Ras-Related C3 Botulinum Toxin Substrate 2 (murine)
RAC2	Ras-Related C3 Botulinum Toxin Substrate 2 (human)
RICD	Restimulation-induced cell death
RTI	Respiratory tract infections
RT-PCR	Real-time polymerase chain reaction
SCID	Severe combined immunodeficiency
SD	Standard deviation
TBST	Tris-buffered saline containing 0.1% Tween-20
TNFRSF	Tumor necrosis factor receptor super family
TREC	T-cell recombination excision circles
WES	Whole exome sequencing

1 INTRODUCTION

1.1 PRIMARY ANTIBODY DEFICIENCY

The spectrum of the disease phenotypes related to disorders of immunity encountered in clinical practice are numerous and lead to much suffering for patients [1-4]. Primary immunodeficiency disorders (PIDs) occur as a result of mutations affecting genes related to the function of proteins and cells of the immune system [5-9]. Primary antibody deficiencies (PADs) represent the most common forms of PIDs according to the majority of studies and national registries [2, 3, 10-38]. Alternatively, some studies suggest that PADs are less frequent than other PIDs in those populations [39-45].

Among PADs, the most common clinical phenotype is selective IgA deficiency (IgAD), followed by common variable immunodeficiency (CVID), a disorder characterized by hypogammaglobulinema, a varied phenotype and an onset that might range from early childhood to late adulthood [2, 46-49].

Common variable immunodeficiency comprises a heterogeneous group of disorders with a wide spectrum of immunological and clinical features [2, 50, 51]. Common clinical and laboratory characteristics include hypogammaglobulinemia, inadequate response to antigen challenge and recurrent infections (mostly of the respiratory tract) [52-59].

However, other clinical features also occur, including autoimmune disorders (e.g., autoimmune cytopenias, autoimmune hepatitis, primary biliary cirrhosis), inflammatory bowel disease (IBD), IBD-like disease, nodular lymphoid hyperplasia (NLH) and liver disease/abnormal liver function tests (LFTs) [2, 53, 58, 60, 61].

1.2 PREVALENCE, DIAGNOSIS AND ETIOLOGY/GENETICS OF CVID

Precise data on the prevalence of CVID is lacking, but estimates are between 1:10,000 and 1:100,000 of the population worldwide suffers from the disease, with no predilection for race [62-64], making it the most common symptomatic primary immunodeficiency encountered in clinical practice [22, 65]. Most cases of CVID are sporadic, but approximately ten percent demonstrate familial clustering [66].

Establishing a diagnosis of CVID (and subsequent treatment plans) is not always a simple task [2, 67-69], as reflected by the latest Working Definitions for Clinical Diagnosis of PID from the European Society for Immunodeficiencies (ESID) [70].

The current diagnostic criteria are as follows:

1. At least one of the following:
   - Increased susceptibility to infection
   - Manifestations of autoimmune disease
   - Granulomatous disease
   - Unexplained polyclonal lymphoproliferation
   - Affected family member with antibody deficiency
2. AND marked reduction of IgG and IgA levels with or without low IgM levels (measured at least twice; less than 2 standard deviations of the normal levels for age);
3. AND at least one of the following:
   - Poor antibody response to vaccines (and/or absent isohaemagglutinins);

• Low proportions of switched memory B cells (<70% of age-related reference value)

4. AND secondary causes of hypogammaglobulinaemia have been excluded
5. AND a diagnosis should be established after the 4th year of life (however, symptoms may occur before the age of 4 years)
6. AND the absence of profound T cell deficiency, which is defined as the presence of two out of the following three parameters (y=year of life):

   • $CD4^+$ T cell count/microliter:

   - 2y-6y <300,
   - 6y-12y <250,
   - Above 12y <200

   • Proportion of naive $CD4^+$ T cells:

   - 2y-6y <25%,
   - 6y-16y <20%,
   - Above 16y <10%

   • Absent T cell proliferation

The significant variation in clinical features, challenges in establishing/unifying laboratory cut-off values across different populations and the considerable overlap that exists between other conditions (e.g., class-switch recombination (CSR) defects) and CVID, has led to the designation "CVID-like disease" for cases that are initially labelled as CVID or that do not fulfill the 'official' diagnostic criteria [71, 72].

Investigations into the underlying etiology of CVID have led to the identification of several genes associated with CVID and CVID-like disease.

Many studies conducted in the search for genetic causes of CVID have been performed on mixed cohorts of IgAD and CVID patients, as both disorders may occur in the same kindred or progress from IgAD to CVID [66, 73-75].

Genetic defects identified thus far include mutations in the genes encoding ICOS (inducible costimulator) [76-81], TNFRSF13B (tumor necrosis factor receptor superfamily member 13B), also known as TACI (transmembrane activator and CAML interactor) [82-84], BAFFR (B-cell activating factor receptor) [85], IL-21 (interleukin-21) [86], MSH5 [87], CD20 [88], LRBA (lipopolysaccharide responsive beige-like anchor protein) [51], IRF2BP2 (Homo sapiens interferon regulatory factor 2 binding protein 2) [89], CTLA4 (cytotoxic T-lymphocyte-associated protein 4) [90], CLEC16A (C-type lectin domain family 16, member A) [91], CD27 [92, 93], RAG1 (recombination-activating gene 1) [94], JAK3 [95], ITPKB (inositol 1,3,4, trisphosphate kinase β) [96], CD19 [97], CD21 [98], PI3KR1 [99] and CD81 [100].

However these mutations only account for the etiology of approximately 10% of CVID patients, and the potential genetic associations remain largely unknown in the vast majority of cases [50, 51, 101, 102].

Conventional approaches for determining the potential genetic etiologies for PIDs have included the candidate gene approach, conventional Sanger sequencing, polymorphism analysis, positional cloning, somatic cell-fusion experiments, fluorescence in situ hybridization (FISH), genetic linkage and/or homozygosity mapping [92, 98, 103-116]. Genome-wide association studies (GWAS) have also been employed [91, 117, 118].

The advent of more advanced technologies, such as next-generation sequencing (NGS), coupled with enhanced algorithms for bioinformatics have enabled a much wider approach for genetic analysis and has been successful in the search for genetic associations with PIDs [119-128]. The process has subsequently become less labor-intensive, more cost-effective

and, from a clinical standpoint, offers shorter turn-around times for reaching definitive or tentative genetic diagnoses in patients with immunodeficiencies by using whole exome sequencing (WES) or whole genome sequencing (WGS) alone, or combined with conventional methods [99, 118, 121, 122, 125, 126, 129-158].

1.3 GENETIC LINKAGE ANALYSIS

Genetic linkage studies have been conducted in the context of CVID/IgA deficiency with earlier studies focusing on the major histocompatibility complex (MHC) region on chromosome 6 and mostly show linkage without pinpointing the exact location of the putative disease-related gene in the MHC region [159-161]. The MHC susceptibility locus was designated IGAD1 [162, 163].

Following these studies, genome-wide linkage studies were performed. An analysis of 210 IgA deficiency/CVID families identified HLA-DQ/DR as the major IGAD1 locus [164]. Further genome-wide linkage analysis of 101 families with 383 marker loci showed the highest linkage scores at the short arm of chromosome 6, and these scores were not obtained in any other location of the genome [164]. Subsequent re-analysis specifically searching for CVID-associated loci (40 families with a minimum of one member affected by CVID) showed a susceptibility locus on the long arm of chromosome 16 and the *WWOX* (WW domain-containing oxidoreductase) as a putative CVID-associated gene, but no mutations were detected upon sequencing [73].

Another study identified genetic linkage of autosomal dominant CVID to chromosome 4q in a genome-wide scan of a five-generation family with five

cases of IgAD, six cases of CVID, and three cases of dysgammaglobulinaemia [74]. Further extension of the study to include 32 families (with at least one case of CVID and another case with CVID or IgAD) supported the linkage to chromosome 4q. Potential candidate genes in the region (*NFκB1, SCYE, CASP6, DAPP1, BANK1*) were sequenced in a single individual from the large family but no mutations were identified.

1.4 GWAS AND NGS IN CVID

CVID shows heterogeneity in both clinical features and in immunological phenotype which would point to a high probability of a complex polygenic etiology. Genetic mutations have been identified in a small proportion of CVID cases.

Most studies have focused on monogenic defects causing or contributing to a CVID or CVID-like phenotype, as well as a limited number of genetic linkage studies. Advances in technology have allowed high-throughput genome-wide single nucleotide polymorphism (SNP) genotyping.

Recently, Abolhassani et al, using a combination of homozygosity mapping and whole exome sequencing, identified a hypomorphic recombination-activating gene 1 (*RAG1*) mutation in a patient with CVID-like disease [94].

Mutations in *LRBA* were identified in five cases of early-onset hypogammaglobulinemia with autoimmunity and IBD-like disease using genome-wide SNP typing, DNA sequencing and genetic linkage analysis [51]. Additional novel *LRBA* mutations were identified using WES and confirmatory sequencing in CVID-like disease and IBD-like disease [142, 149].

Li et al. compared 778 CVID cases with approximately 11,000 controls across 123,127 SNPs using the Immunochip (a genotyping array with dense

coverage of SNPs across 186 known disease loci identified in 12 immune-mediated diseases), thus identifying a novel CVID susceptibility locus harboring *CLEC16A* (C-Type Lectin Domain Family 16, Member A) [91].

van Schouwenburg et al. used whole genome sequencing and RNA sequencing (RNAseq) on 34 patients of Caucasian origin with sporadic CVID who fulfilled the ESID diagnostic criteria at the time of enrollment [165]. Their cohort included 29 patients who were analyzed in the study by Orange et al [117]. The study identified 43 potentially pathogenic rare variants of which 39 were located in the MHC region. Their results corroborated earlier findings of variation in *TNFRSF13B*, *TNFRSF13C* and *NLRP12* [82, 166-169]; and identified variants that would potentially produce a CVID-like phenotype by affecting B-cell receptor (BCR) function, including *IKBKB*, *CD79a*, *BTK*, *KRAS*, *ARID3A*, *INPP5D*, *BANK1*, *BLK*, *GAB2*, *BTK*, *BLK* and *ARID3A*.

Additional potential pathogenic pathways leading to the development of CVID were identified. Among these, the death receptor pathway was identified by both whole genome sequencing and RNA-seq analyses. This finding, coupled with the increased expression of the apoptosis inducing receptor gene *FAS*, and variations in other apoptosis related genes (*TNFAIP3* and *TNIP1*), suggest dysregulated apoptosis in patients with CVID [165, 170].

A role for defects of the non-homologous end joining (NHEJ) pathway (an essential pathways V(D)J recombination and class–switch recombination) in the pathogenesis of CVID-like disorders has been hypothesized based on observations of an incomplete developmental block in B cell ontogeny in the bone marrow of CVID patients compared to healthy subjects [171-173]. van Schouwenburg et al. also detected variants in *DCLRE1C*, *PRKDC*, *RAG2*, *NHEJ1*, *MRE11A*, *ATM* and *NLRP2* which are all important in the NHEJ pathway in B cells [165].

Overall, these studies illustrate the utility of GWAS, NGS and RNAseq analyses in investigating the genetic etiology/associations with CVID and CVID-like disease pointing to a polygenic etiology of these immunodeficiencies.

2 AIMS

2.1 GENERAL AIM

The general aim of the work presented in this thesis was to identify a genetic association with common variable immunodeficiency by screening probands from consanguineous families to confirm any genetic associations and to perform follow up analyses.

2.2 SPECIFIC AIMS

2.2.1 Paper I

To functionally characterize the novel *CD27* mutations identified and to provide a clinically relevant summary of all cases of CD27 deficiency reported.

2.2.2 Paper II

To functionally characterize the novel loss-of-function mutation identified in the *RAC2* gene leading to a CVID-like phenotype.

2.2.3 Paper III

To provide a clinically relevant summary of all reported cases of LRBA deficiency together with five novel mutations leading to the LRBA deficiency phenotype.

2.3 TRANSLATIONAL AIM

The translational aim of this thesis was to provide clinicians treating patients suffering from PIDs with the following:

1. Updated clinical, laboratory and mutation analysis for selected cases of common variable immunodeficiency.
2. Provide awareness of the feasibility of using whole exome sequencing to identify a genetic etiology for PIDs.

3 MATERIALS AND METHODS

3.1 SELECTION OF PROBANDS

Medical records and family pedigrees were evaluated for patients and family members (n =130 families) from multiple medical centers including follow-up of previously reported patients. Informed consent was obtained from all patients and/or their legal guardians. Ethical permits were obtained from the ethics committees of all participating centers. Candidate probands for whole exome sequencing were selected based on the clinical history, family history of parental consanguinity and availability of clinical samples for DNA extraction from the probands and family members.

3.2 WHOLE EXOME SEQUENCING

3.2.1 DNA extraction, library preparation and exome sequencing

Genomic DNA was extracted using the salting-out method on peripheral blood samples collected from the probands and their family members [174]. Three micrograms of genomic DNA was randomly fragmented using the Covaris Acoustic System. Adapters were then ligated to both ends of the fragments. The adaptor-ligated DNA templates were purified with Agencourt AMPure SPRI beads where fragments with an insert size of about 200 bp were excised. Extracted DNA was then amplified by the ligation-mediated PCR (LM-PCR), purified and hybridized to the Agilent SureSelect Human All Exon 50 Mb kit for enrichment. The hybridized fragments were subsequently bound to streptavidin beads and non-hybridized fragments were washed off after 24 hours. Captured LM-PCR products were subjected to quantitative PCR by an Agilent 2100 Bioanalyzer to measure the magnitude of the enrichment. Each captured library was then loaded on a Hiseq2000 Illumina sequencer according

to the protocol of the manufacturer and high-throughput sequencing was performed to obtain the desired sequencing depth.

3.2.2 Read mapping and variant analysis

Processing of raw image files was done using the Consensus Assessment of Sequence and Variation software (CASAVA, version 1.7) for base calling using default parameters. Then sequences for each subject were generated as 90-bp paired-end reads. The sequenced reads were aligned to the human genome reference (UCSC hg 19 version; build 37.1) with SOAP aligner (soap2.21) software [175]. This was followed by filtering out duplicated reads and only uniquely mapped reads were kept for further analysis. SOAPsnp (version 1.03) software [176] was then used with the default parameters to assemble the consensus sequence and call genotypes in target regions. For single nucleotide polymorphism (SNP) quality control, low-quality SNPs that fulfilled one of the following four criteria were filtered out:

(1) Quailty of genotype < 20
(2) A sequencing depth < 4
(3) Estimated copy number > 2
(4) Distance from the adjacent SNPs < 5 base pairs.

The Unified Genotype tool from GATK (version v1.0.4705) [177] was employed to detect small insertions/deletions after alignment of the high-quality reads to the human reference genome by using the Burrows-Wheeler transformation (version 0.5.9-r16) [178].

3.2.3 Analysis protocol for exome sequencing results

Synonymous mutations were filtered out and then common variants (frequency > 1%) were eliminated. The prioritization of non-synonymous/splice site

(NS/SS) changes and insertion/deletions (Indels) was performed according to the following criteria:

1. The presence of homozygous or compound heterozygous variants;
2. Primary immunodeficiency disease associated genes based on the RAPID website (http://rapid.rcai.riken.jp/RAPID/index_html) and in-house database;
3. The core obtained by predictive software scoring the probability of pathogenicity (Polyphen-2: http://genetics.bwh.harvard.edu/pph2 and SIFT: http://sift.bii.a-star.edu.sg).
4. Searching genes in the regions previously highlighted by homozygosity mapping analysis.

3.3 CONFIRMATORY SANGER SEQUENCING

Confirmatory sequencing for mutations was performed using sequence-specific primers using standardized protocols as previously described for *CD27* [179], *RAC2* [94, 180] and *LRBA* [51].

3.4 TRANSMISSION ELECTRON MICROSCOPY

Neutrophil transmission electron microscopy was performed according to the previously published method [51].

3.5 SOLUBLE CD27 ENZYME-LINKED IMMUNOSORBENT ASSAY

The concentration of soluble-CD27 (sCD27) was determined by enzyme-linked immunosorbent assay (ELISA) in stored serum samples using the PeliKine

Compact™ human soluble CD27 ELISA kit (Sanquin, Amsterdam, The Netherlands) following the manufacturer's instructions.

3.6 B CELL ACTIVATING FACTOR (BAFF) ELISA

The concentration of soluble BAFF was measured using the Human BAFF SimpleStep ELISA™ kit (Abcam, Cambridge, United Kingdom) following the manufacturer's instructions.

3.7 STATISTICAL ANALYSES

Statistical analysis was performed using the SPSS software package v21. One-sample Kolmogrov-Smirnov test was used to estimate the type of distribution of the ELISA data. Parametric and nonparametric analyses were subsequently performed based on this evaluation and a *p* value of less than 0.05 was considered statistically significant

3.8 RAC2 WESTERN BLOTTING

Wild type and RAC2 mutant expressing constructs were generated by previously described methods [181]. The expressing constructs were transfected into human fibroblast cell lines using Turbofect according to the manufacturer's protocol (Fermentas, Berlington, Ontario, Canada).

Protein samples were diluted in Laemmli's sample buffer (Bio-Rad, CA, USA), and 30 µg of total cell lysates/lane was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred onto Amersham Hybond electrogenerated chemiluminescence (ECL) Nitrocellulose membranes (Amersham, GE Healthcare, United

Kingdom) using a mini-Trans-Blot cell system (Bio-Rad). Following transfer, membranes were washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) and then blocked in Blocking buffer (5% skim milk in TBST) for one hour at room temperature. The membranes were then incubated overnight with anti-RAC2 rabbit polyclonal carboxy-terminus antibody at a dilution of 1:5000 (07-604; EMD Millipore, MA, USA) in TBST containing 5% skim milk at 4ºC. After washing five times for 5 min in TBST, membranes were incubated with anti-Rabbit immunoglobulin conjugated with horseradish peroxidase (HRP) at a dilution of 1:50000 (P0399; Dako Agilent Technologies, CA, USA) for one hour at room temperature. The membranes were washed four times (10 min per wash) with TBST. Finally, proteins were visualized with ECL Plus Western Blotting Detection Reagent (Amersham, GE Healthcare, United Kingdom), and exposed to Hyperfilm ECL (Amersham, GE Healthcare, United Kingdom).

3.9 LRBA WESTERN BLOTTING

Peripheral blood mononuclear cell (PBMC) extracts were obtained by standard techniques and protein levels were measured using the Micro BCA™ Protein Assay Kit according to the manufacturer's instructions (Thermo Scientific, MA, USA). Western blotting was performed according to standard techniques and anti-LRBA antibody as previously described [51] with the substitution of the in-house gel with pre-cast Mini-PROTEAN® TGX™ 4%-20 gradient gel (Bio-Rad, CA, USA).

3.10 TREC AND KREC ANALYSIS

T-cell recombination excision circles (TREC) and kappa-deleting element recombination circle (KREC) analysis was performed according to the previously published protocol [182] on DNA samples obtained for whole exome sequencing by the salting out method.

4 RESULTS AND DISCUSSION

This research project focused on searching for genetic alterations potentially contributing to the pathogenesis of primary antibody deficiency by employing whole exome sequencing as the primary investigative tool in selected candidate probands.

The search culminated in the identification of ten novel mutations associated with CVID, CVID-like disease, lymphoproliferative disease and inflammatory bowel disease.

4.1 PAPER I

Novel Mutations in TNFRSF7/CD27: Clinical, Immunologic, and Genetic Characterization of Human CD27 Deficiency

4.1.1 Novel mutations resulting in CD27 deficiency

Four novel mutations that resulted in CD27 deficiency were identified:

- Homozygous missense c.G287A (p.C96Y)
- Homozygous missense c.C232T (p.R78W)
- Heterozygous nonsense c.C30A (p.C10X)
- Compound heterozygous c.G24A - c.C319T (p.W8X - p.R107C)

Two homozygous mutations (p.W8X and p.C53Y) resulting in a CD27 deficiency phenotype had been described previously [92, 93, 146]. The identification of these novel mutations correlated with the finding in the

previously published patients and also suggested that a compound heterozygous mutation can also lead to a CD27 deficiency phenotype. The location of the novel mutations and previously identified mutations are illustrated in **Figure 1**.

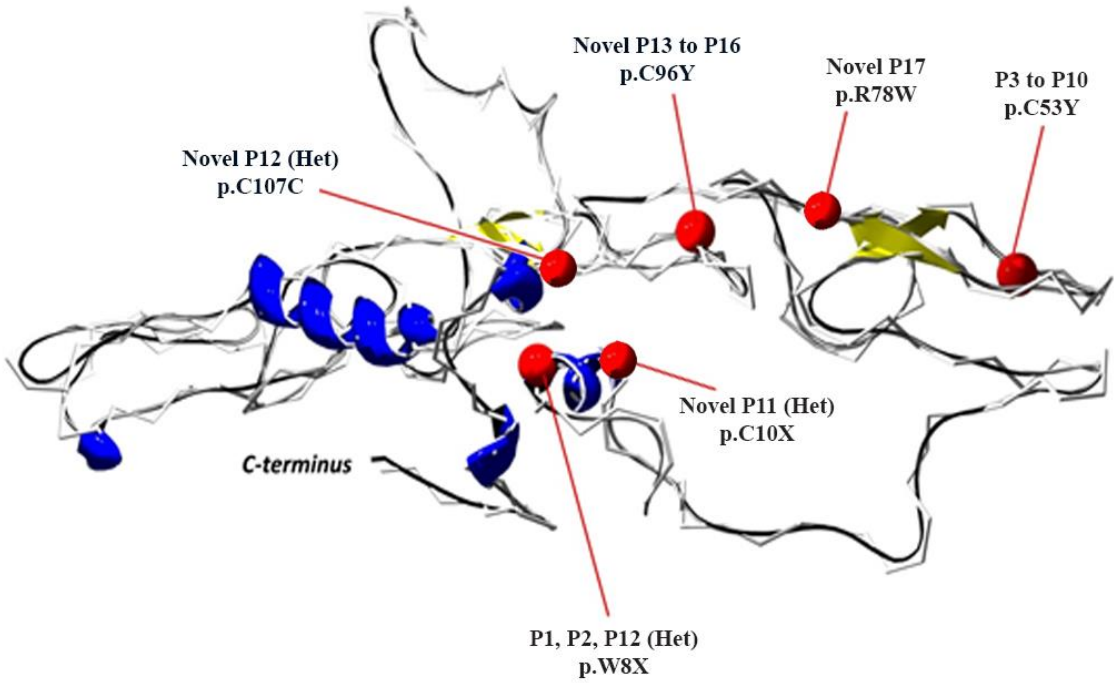

Figure 1. Three-dimensional structure of human CD27 showing the locations of the identified mutations. [179] (Paper I)

Analysis of the c.C319T/p.R107C mutation *in silico* using the Scratch Protein Predictor [183] showed that it would induce a disulphide bond to form between Cysteine residue 107 and Cysteine residue 112, resulting in replacement of the bond between Cysteine residue 106 and Cysteine residue 112, potentially resulting in the formation of a mis-folded protein.

The SIFT (Sorting Intolerant From Tolerant) and PolyPhen-2 (Polymorphism Phenotyping v2) software programs were used to predict the effect of the substitution of an amino acid on protein structure and function.

The SIFT (0.04) and PolyPhen-2 (1.000) scores for p.C96Y predicted a damaging effect on protein function. The p.C96Y mutation would probably abolish the formation of a disulphide bond involved in protein folding [184].

The SIFT (0.01) and PolyPhen-2 (1.000) scores for mutation p.R78W also predicted a damaging effect on protein function.

For the p.R107C mutation, a damaging effect was predicted by the SIFT score (0.02), and a possibly damaging effect was predicted the by PolyPhen-2 score (0.767).

4.1.2 Variable phenotype of CD27 deficiency

Although Epstein-Barr virus (EBV) infection, Epstein-Barr virus-associated lymphoproliferative disease (EBV-LPD), hemophagocytic lymphohistiocytosis (HLH) and malignancy appear to predominate in CD27 deficiency, a single genotype appears to result in different phenotypes. Primary antibody deficiency was not the dominant feature overall as it occurred in a minority of patients (P1, P15 and P17), all of whom had EBV-related clinical features. As with other primary immunodeficiencies, environmental factors and modifier genes might impact the clinical phenotype. This was striking in the case of the p.C96Y mutation in members of two unrelated kindreds (kindreds G and H, Table I in Paper I) where siblings P13 and P14 had a milder clinical phenotype compared with P15 and P16, despite all patients having the same genotype. These variable clinical phenotypes have been observed in other primary immunodeficiencies [185-187].

4.1.3 Reduced expression of CD27

A marked reduction of cell surface expression of CD27 was observed in both T and B cells (**Figures 2** and **3**), and decreased soluble CD27 (sCD27) in members of kindred H (P15, P16 and family members) (**Figure 4**). The switched memory B lymphocyte panel shows absent expression of surface CD27 for patient P16. Other family members (heterozygous) had expression levels similar to the father.

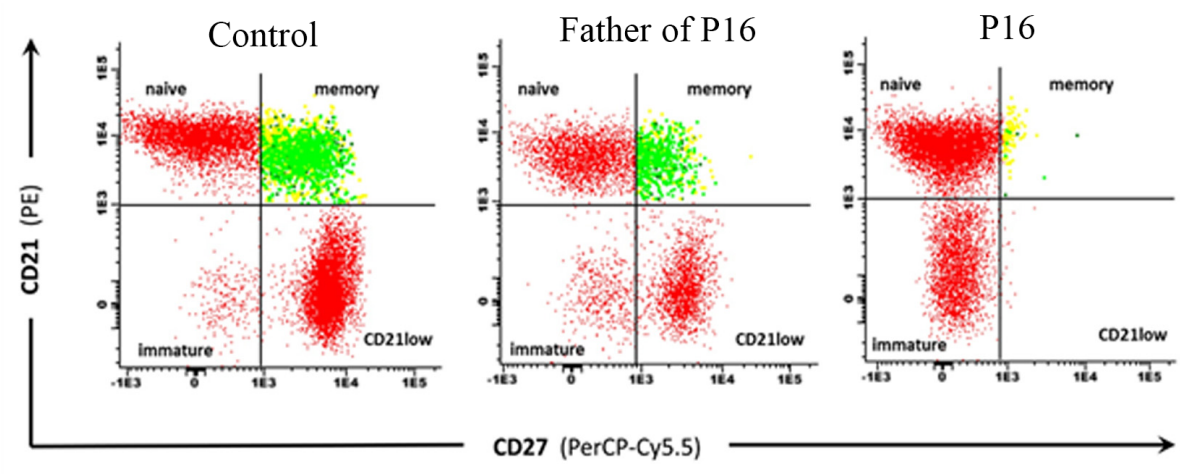

Figure 2: Flow cytometric lymphocyte immunophenotyping showing the proportion of $CD21^+$ and $CD27^+$ B cells in the $CD19^+$ B-cell fraction was evaluated in P16 (homozygous C96Y), the father of P16 (heterozygous C96Y), and in a healthy control. PerCP, peridinin-chlorophyll-protein complex; PE, Phycoerythrin (Paper I).

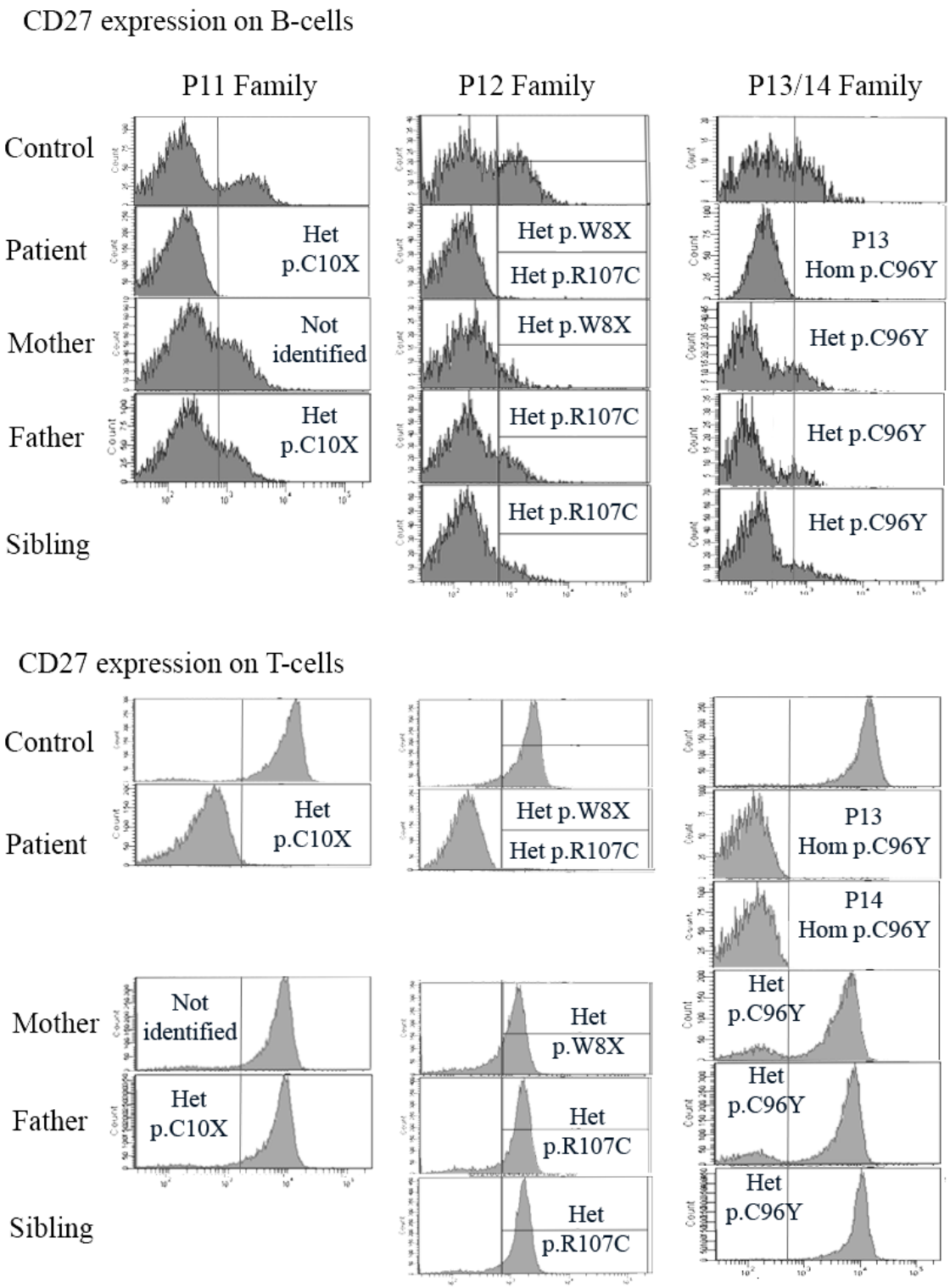

Figure 3: Flow cytometric lymphocyte immunophenotyping of patients P11, P12, P13, P14 and their parents compared to healthy controls. Hom, homozygous; Het, heterozygous (Paper I).

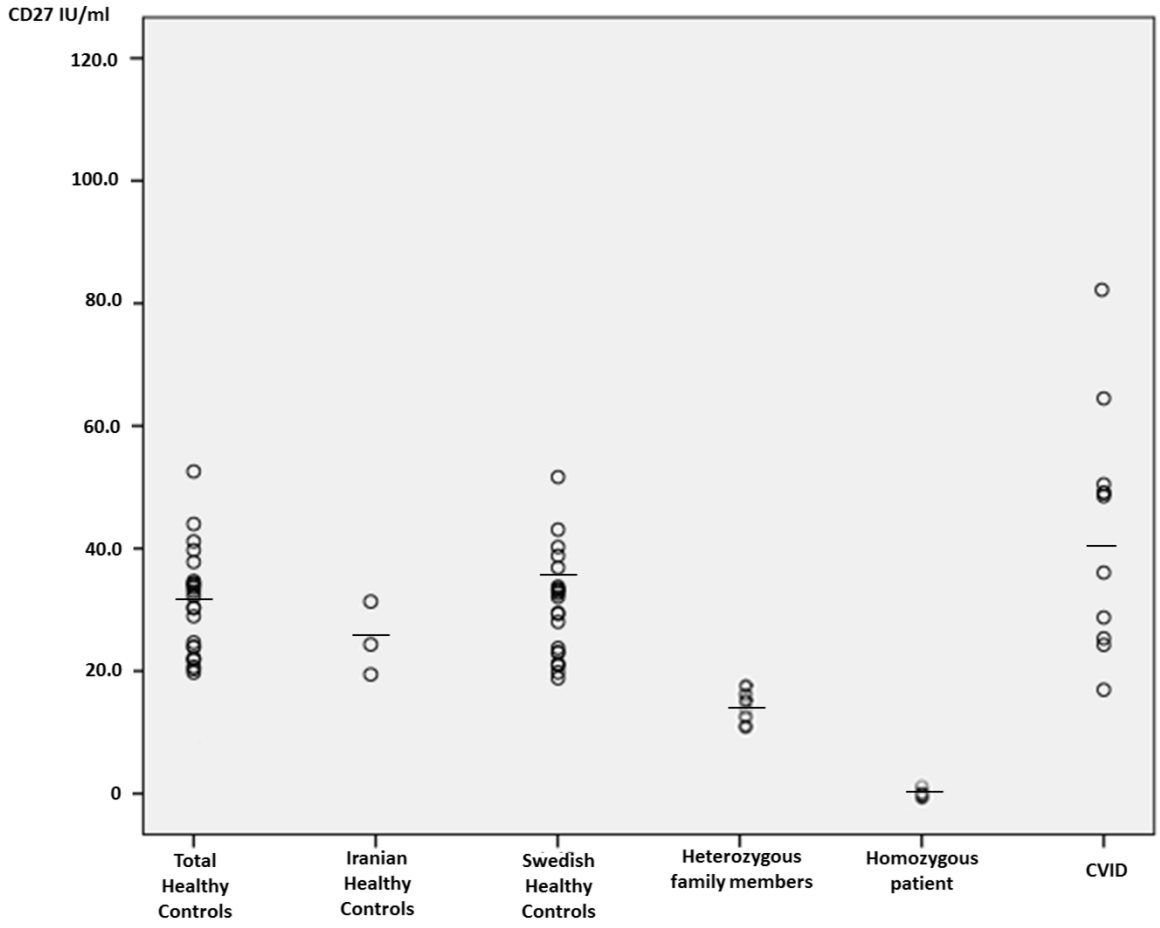

Figure 4 : Comparison of ELISA results of soluble-CD27 between 25 anonymized healthy controls and 10 CVID patients without CD27 deficiency, 4 individuals with a heterozygous C96Y mutation (measured 4 times) and patient P16 with the homozygous C96Y mutation (measured 4 times).

4.1.4 Increased susceptibility to EBV infection and cellular features

The increased susceptibility to EBV infection in patients with CD27 deficiency manifests as a spectrum of severity including a relatively benign course, recovery after therapy or hematopoietic stem cell transplantation (HSCT), severe morbidity, and death due to various complications.

This finding is significant in view of epidemiologic studies indicating that more than 95% of adults worldwide have been infected with EBV [188]; and that there is a robust life-long immunologic control of EBV infection in

immunocompetent individuals dependent on priming of T cells [189, 190]. Indeed, studies on murine lymphocytes indicate that there is a significant defect of $CD8^+$ T cell priming as a result of CD27 deficiency, which will be discussed.

In the T cell compartment, $CD4^+$ and $CD8^+$ T cell subset numbers were normal in the majority of cases. This finding is consistent with findings in the murine CD27 knockout (CD27 KO) model, where proportions were similar to WT (wild type) mice [191]. However, as human beings are the only known natural host of EBV [192], drawing conclusions from murine studies with regards to this particular susceptibility is extremely challenging.

Naive $CD4^+$ and $CD8^+$ T cells constitutively express CD27 and expression is upregulated upon activation [193-195]. The difficulty in interpreting the consequences of the presence or lack of CD27 stimulation *in vivo*, is CD27's relationship with its only natural ligand, CD70, which also functions as a receptor for signal transduction in T and B lymphocytes, dendritic cells (DCs) and macrophages [195-199]. *In vitro* studies using lymphocytes transfected with either CD27 or CD70 show that both molecules have important roles in interaction between T cells and between T cells and B cells, as well as autocrine functions [191, 200-203].

The absolute counts of $CD8^+$ T cells were high in four patients, which might be due to the timing of analysis during active infection. A reduced proportion of $CD8^+CCR7^+CD45RA^-CD45RO^+$ T cells was observed in one patient (P16) which might be a result of impaired generation and maintenance of EBV-specific $CD8^+$ T cells. Murine studies show impaired secondary cytotoxic $CD8^+$ T cell expansion in CD27 KO mice exposed to lymphocytic choriomeningitis virus [204].

In vitro analysis of murine $CD8^+$ T cells shows that stimulation via the CD27/TCR pathway in the absence of signaling via the interleukin-2 receptor results in $CD8^+$ T cell proliferation without differentiation into effector cells

[201]. The enhanced cell survival apparently results from CD27-mediated facilitation of interleukin-7α (IL-7α) expression [201]. *In vivo*, IL-7 is essential for the survival of peripheral murine $CD8^+$ T cells [205].

Murine studies also indicate that signaling via CD27 is important for T cell survival, particularly by inducing the production of interleukin-2 (IL-2) that stimulates $CD8^+$ T cells in an autocrine manner enhancing anti-viral and anti-tumor immunity as a result of increased survival and accumulation in tissue and draining lymph nodes [202]. CD27 KO mice had significantly lower numbers of $IL\text{-}2^+$ interferon-γ^+ $(IFN\gamma)^+$ $CD8^+$ T cells in splenic tissue, pulmonary tissue, and draining lymph nodes after influenza virus infection [202]. A similar mechanism might be involved in human CD27 deficiency, leading to the deficiency of an EBV-specific subset of $CD8^+$ T cells (both memory and effector cells).

CD27 KO C57BL/6 mice were found to have decreased accumulation of $CD8^+$ and $CD4^+$ effector T cells in the lungs, which was in contrast to the normal immune response to infection with the influenza virus [203]. The decrease in influenza virus-specific $CD8^+$ T cells in the lungs was a further indicator that CD27 was a major determining factor in the cellular response at the infection site.

Murine studies have also shown that the *in vivo* role of the CD27-CD70 costimulatory pathway is non-redundant for the activation of naive $CD8^+$ T cells [206]. CD27 promotes gene expression toward a Th1 phenotype and facilitates the accumulation of antigen-specific $CD4^+$ T helper cells at tissue sites and priming sites, thus also facilitating an improved supportive capacity for a primary cytotoxic $CD8^+$ T cell response in a setting of antigen fragment cross-presentation [207].

Other indirect *in vivo* observations in murine studies using blockade of CD70, indicate that the loss of CD27 costimulation results in inhibition of $CD8^+$ T cell

priming during viral infections (vesicular stomatitis virus and vaccinia virus) and bacterial infection (Listeria monocytogenes) [206]. A similar defect might occur in humans where $CD8^+$ T cell priming in EBV infection is affected.

Regarding the B lymphocyte compartment, cell-surface expression of CD27 is used in flow cytometry-based lymphocyte subset immunophenotyping of memory B cells in order to classify primary immunodeficiencies, including CVID [208, 209]. Stimulation of CD27 on human B lymphocytes has been linked to the promotion of immunoglobulin production in a coordinated pattern related to other surface receptors including CD40 (and its ligand CD154) and CD134 (and its ligand OX40) [210, 211].

$CD27^+$ B lymphocytes appear morphologically and functionally distinct from $CD27^-$ B lymphocytes. $CD27^+$ B lymphocytes are larger and contain more abundant cytoplasm, and are present in different stages of the cell cycle; whereas $CD27^-$ B lymphocytes are smaller and contain scant cytoplasm, and are in a single stage of the cell cycle [212, 213].

Murine B lymphocytes do not express CD27 at the naive and transitional stages, and only begin to express CD27 during the germinal center reaction (centroblasts). Somatically mutated B cells do not express CD27 and only a minority of memory B cells express CD27 [214]. This is in contrast to human B cells, where CD27 is expressed on a larger proportion of germinal center B cells, is maintained during differentiation and is expressed on the majority of memory B cells [209, 215, 216]. This difference in expression pattern would expectedly result in a dramatically different outcome with regards to immune responses and subsequent immunoglobulin production. Although hypogammaglobulinemia has not been reported in the CD27 KO murine model, delayed IgG responses were observed despite influenza virus-specific IgG antibody responses [214]. Additionally, the smaller germinal centers observed in both human CD27 deficiency [92] and murine CD27 KO lymph nodes [214] might indicate a yet unidentified cellular immune defect.

Jacquot et al. observed that CVID patients with an extremely low percentage of $CD27^+$ memory B cells had more severe clinical phenotypes, profound hypogammaglobulinemia and reduced peripheral B cells compared to patients with a higher percentage of $CD27^+$ memory B cells. Additionally, the kinetic B cell profiles of two patients failed to upregulate to the levels observed in other patients, despite both having a normal percentage of T cells [217].

Studies on human cell lines have shown that EBV-induced transformation of B lymphocytes is prevented by the EBV-specific antibodies in human intravenous immunoglobulin (IVIG) [218]. Although the CD27 deficiency patients did produce EBV-specific antibodies, the total amount of antibodies required to prevent transformation of all transforming B cells might have been reduced due to the primary or secondary hypogammaglobulinemia.

The absence of CD27 does not solely explain the primary hypogammaglobulinemia in some patients, who nevertheless mounted a specific antibody response to EBV. In certain cases CD27 deficiency might be a progressive disease with more severe manifestations occurring as a result of deleterious pathophysiological mechanisms secondary to severe EBV infection.

Regarding NK cells, it has been observed that impairment of NK cell function increases an individual's susceptibility to EBV infection and EBV-associated malignancy [189, 219]. CD27 appears to contribute to NK cell physiology at multiple levels and, thus impairment at the CD27-CD70 interface would result in perturbed function.

Peripheral blood NK cell function was found to be reduced in the majority of patients tested (5/7) and all had EBV-associated clinical manifestations. The impaired function might be a direct result of CD27 deficiency or secondary to infection with EBV. In addition, impaired function was measured in peripheral blood NK cells, which only represents a subset of NK cells.

Based on the expression of CD27, two distinct subsets of NK cells exist in both murine [220] and human [220-222] lymphocyte populations. In humans, approximately 6% of peripheral blood $CD3^{-}CD56^{+}$ NK cells express CD27 [222]; and $CD56^{high}$ NK cells express higher levels of CD27 [223]. The surface expression pattern of CD27 appears to be an ontological marker of maturity. Stage I to stage III NK cells are $CD27^{-}$, stage IV NK cells are $CD27^{+}$, and stage V NK cells are $CD27^{-}$ [222].

Activation of human NK cells using recombinant IL-2 results in an upregulated expression of CD27, with the highly enriched NK cell population showing less upregulation compared to the less enriched population, suggesting that interaction with other PBMCs might be required for full NK cell activation [223]. $CD27^{+}$ and $CD27^{-}$ NK cells express different levels of NK receptors (both inhibitory and activating), IFNγ and cytolytic proteins [222].

The intensity of surface expression of CD56 is related to that of CD27. $CD56^{high}$ $CD27^{high}$ NK cells are the dominant population in tonsils and lymph nodes; and $CD56^{low}$ $CD27^{low}$ NK cells predominate in peripheral blood [224, 225]. The distinct tissue distribution of a given lymphocyte subset might provide a tissue-specific immune response. Murine NK cells constitutively express CD27 [226]. Murine $CD27^{low}$ cells are the dominant NK cell population in peripheral blood; form a mixed population with $CD27^{high}$ NK cells in bone marrow; are abundant in pulmonary tissue and relatively scarce in lymph nodes [226].

Human $CD56^{high}$ $CD27^{high}$ NK cells are poorly cytotoxic [227] which is in contrast to murine $CD27^{high}$ NK cells, despite similar expression of NK cell receptors on both cells [221]. Murine $CD27^{low}$ NK cells have low proliferative capacity [220]. Human and murine $CD27^{high}$ NK cell have been shown to be potent producers of IFNγ [220]. *In vitro*, IFNγ production after ligation of CD27 on $CD56^{high}$ NK cells is enhanced [228]. A reduction in IFNγ levels in secondary lymphoid tissues (particularly tonsillar tissue) as a result of CD27

deficiency might contribute to predisposition to increased severity of EBV infection and induction of EBV-associated malignancy.

Moreover, both human and murine studies show that the CD27-CD70 interaction is an important mechanism in immunity to tumors [229-235]. *In vivo*, murine NK cells reject tumor cells that express CD70 [233]. One hundred percent of all subtypes of Hodgkin lymphoma have been shown to express CD70 [236]. The loss of CD27 expression in patients with CD27 deficiency, might thus deprive them of vital immune responses against tumors cells, especially those that express CD70.

Determining the exact role of the loss of CD27 expression will depend on analyzing lymphocytes (T, B and NK cells) and other cells at specific anatomical sites related to ongoing infection, as well as investigating the interaction between lymphocyte subsets that are not conventionally thought to contribute to antibody responses (e.g., B cells and NK cells) and which have not been extensively studied [237, 238]. The oropharyngeal lymphoid tissue (to investigate EBV infection) and bone marrow and mucosal lymphoid tissue (to investigate antibody deficiency) should be the primary focus.

In conclusion, CD27 deficiency results in immunodeficiency with varied clinical features. Although our patients represent a relative "knockout model" of disease, CD27 deficiency cannot strictly be considered a monogenic cause of the clinical phenotype(s).

4.2 PAPER II

RAC2 Loss-of-function Mutation in 2 Siblings with Characteristics of Common Variable Immunodeficiency

4.2.1 Identification of a novel mutation in the *RAC2* gene

4.2.1.1 W56X mutation

Whole-exome sequencing analysis of DNA from the proband revealed a novel homozygous nonsense mutation in codon 56 (W56X) of the *RAC2* (Ras-Related C3 Botulinum Toxin Substrate 2) gene. This mutation was validated by confirmatory Sanger sequencing and was found to be in a homozygous form in her brother. The mother carried a heterozygous form of the mutation. DNA from their father was not available to confirm if he was a heterozygous carrier, however, he would most probably be a carrier given the parental consanguinity.

4.2.1.2 Immunodeficiency phenotype of RAC2 deficiency

The clinical phenotype of the proband and her sibling (case histories in Paper II) contrasted significantly with the primary neutrophil dysfunction phenotype observed in the two previously reported patients with the D57N mutation ([239-241] and Table E4 in Paper II).

The first reported case was a male infant born at term with delayed separation of the umbilical cord who subsequently presented with peri-rectal abscess, and peri-umbilical infections from which *Escherichia coli* and *Enterococcus* species were cultured. The absence of granulation tissue in the wound was noted with

subsequent recurrence of the peri-rectal abscess. Complete wound healing was only achieved after granulocyte transfusions [240].

The second reported patient was an apparently healthy two week old full-term male infant, who was found to have reduced TREC counts in the Wisconsin statewide Newborn Screening for T cell lymphopenia program [241, 242]. Further clinical evaluation revealed leukocytosis, increased neutrophils, and $CD4^+$ T cell lymphopenia with an increased percentage of memory $CD4^+CD45RO^+$ T cells. However, the magnitude of reduction in lymphocyte subset counts was not as low as those typically observed in infants suffering from severe combined immunodeficiency (SCID). At 26 days of life, the infant presented with fever and omphalitis, requiring surgical debridement. At day 56 he developed a paratracheal abscess requiring drainage. *Stenotrophomonas* and *Prevotella* species were subsequently grown from the cultures of the abscess. Neutrophil chemotaxis was severely reduced in the patient [241].

The proband and her brother were born to consanguinous parents and were apparently healthy at birth and did not present with soft tissue infections that were described in the two unrelated previously reported patients born to non-consanguinous parents [239-241]. The proband and her brother later manifested with recurrent infections, hypogammaglobulinemia, post-streptococcal glomerulonephritis, autoimmune hypothyroidism and endocrine hormone abnormalities (for details see Paper II).

In our patients with the novel W56X mutation, the increased levels of parathyroid hormone (PTH) in the proband might be linked to RAC2 deficiency, as human RAC2 (and murine Rac2) expression is mainly hematopoietic cell/tissue-specific [243-246]. Osteoclasts are cells of hematopoietic origin and are the only cells that possess resorptive function for bone modelling [247-250]. Several abnormalities related to osteoclasts occur in Rac2 KO mice [251]. These abnormalities include increased trabecular bone mass in male mice, increased numbers of osteoclasts per total bone area,

increased expression of Rac1 (a separate Rac family protein), an abnormal actin cytoskeleton, reduced chemotaxis and an impairment of resorptive capacity. Similar defects might have affected osteoclasts in our patients resulting in impaired calcium homeostasis. The proband had a slightly reduced serum calcium level of 8.1 mg/dL (normal range: 8.5-10.2 mg/dL), a finding that might correspond to the impairment of resorptive capacity present in Rac2 KO mice. Bone resorption is an essential mechanism for the maintenance of normal calcium levels in blood [252]. Though serial measurements of serial calcium were not performed, and more importantly ionized calcium levels were not documented, hypocalcemia might have been a trigger for the secretion of increasing amounts of PTH through the feedback mechanism that regulates blood PTH levels, thus causing secondary hyperparathyroidism [253, 254].

Rac2 KO mice have an augmented anabolic response to treatment with exogenous PTH, as evident from increased cortical bone thickness and increased bone mass density [255]. This increased anabolic response might also occur in human oseteoclasts, and might lead to certain pathologic changes.

The deficiency of growth hormone in the sibling is harder to explain in relation to RAC2 deficiency. Growth hormone is secreted by the anterior pituitary gland (adenohypophysis) and the ‘classic’ model of the embryonic development of the adenohypophysis states a non-neural origin [256]. However, an alternative neural origin has been suggested [257]. Another RAC protein, RAC3 is the predominant RAC protein expressed in brain tissue, whereas RAC2 is not [245]. Investigating the expression pattern of Rac2 in animal models might offer some insights into the role Rac2 might play in pituitary function and hormone secretion/regulation.

4.2.2 Loss of RAC2 protein expression

There was a complete loss of expression of $RAC2^{W56X}$ in transfected human fibroblasts (Figure 1C in Paper III). As W56X is a missense mutation the loss of expression is expected. This finding is in contrast to the finding of reduced expression of $RAC2^{D57N}$ mutant protein that had a dominant negative effect, resulting in reductions of neutrophil oxidative metabolism [239, 240].

4.2.3 Neutrophil features in RAC2 deficiency

4.2.3.1 Normal cell size

The total volume of cytoplasm (measured in two dimensions) was similar in both the healthy control and in the proband's neutrophils (10.1 ± 1.7 μm^2 and 10.2 ± 1.4 μm^2, respectively) indicating no differences in cell size.

4.2.3.2 Numerical abnormalities of neutrophil granules

By comparing the number of primary (azurophilic) granules and secondary (specific) granules, a significant reduction in the number of granules was observed in proband neutrophils compared to control neutrophils (n = 15 for each).

The average counts of specific and secondary granules were 3.8 ± 0.7/μm^2 in the proband's neutrophils compared to 5.9 ± 1.3/μm^2 in the healthy control's neutrophils ($P = 2.3 \times 10^{-5}$, Student *t* test).

Neutrophil granules are classified as primary (azurophilic, peroxidase-positive) and secondary (peroxidase-negative) granules [258]. Neutrophils from Rac2

KO mice have been observed to show the complete loss of ability to release primary granules in response to both physiologic and artificial priming [259]. Subsequent murine studies supported this finding, while showing that murine secondary granule release was independent of Rac2 function [260, 261]. This functional abnormality of absence of primary granule release was also observed in the first human case [239], and a later study supported a role for human RAC2 in primary granule release [262].

A link apparently exists between these (numerical and functional) abnormalities of neutrophil granules and impaired cytoskeleton function, which is strongly suggested to exist in Rac2/RAC2 deficiency. Human neutrophil phagocytosis and (primary and secondary) granule exocytosis are regulated by the actin cytoskeleton [263-266]. Murine Rac2 regulates chemotaxis (in both neutrophils and macrophages) and appears to be essential for formation of neutrophil extracellular traps (NETs), both of which are processes that are thought to be dependent of cytoskeletal function [243, 267-269]. NETs formation is also a feature of human neutrophils [265, 270]. In addition, studies on murine macrophages expressing the dominant negative $Rac2^{D57N}$ mutant protein show decreased micropinocytosis, another process dependent on a functional cytoskeleton [271].

Murine Rac2, in contrast to RAC2 in human neutrophils, is not the predominant Rac protein in murine neutrophils and is expressed at similar levels to its isoform Rac1 [272]. Thus, it is plausible that a compensatory role for Rac1 diminishes the effects of Rac2 deficiency on cytoskeletal function relating to granule regulation in murine neutrophils, but which does not occur in human neutrophils, thus resulting in the abnormalities observed in our patient.

4.2.3.3 Abnormal secondary granule morphology

The shape of the secondary granules in the proband's neutrophils was often more elongated or collapsed compared to the control (**Figure 5c-d**).

4.2.3.4 Increased cytoplasmic inclusions

Dense multi-membrane cytoplasmic inclusions were observed in the proband's neutrophils (**Figure 5a and e**) which were not present in control neutrophils (**Figure 5b and f).** These inclusions were interpreted to be autophagosomes.

Rac2 (and RAC2) protein has not been linked to altered autophagy. However, a decreased level of autophagy has been observed in cells of a human HeLa cell line transfected to overexpress RAC3 [273]. Perturbed RAC3 activity has been linked to breast cancer [274] and prostate cancer [275].

4.2.3.5 Abnormal neutrophil chemotaxis

Neutrophil chemotaxis of the sibling was mildly reduced when compared to reference ranges (Table 1 in Paper II). This is in contrast to the marked reduction in neutrophil chemotaxis observed in the patients with the $RAC2^{D57N}$ dominant negative mutant protein [239-241].

4.2.4 Increased serum BAFF levels

Serum levels of BAFF in two independent samples from the living sibling were analyzed and a 5 to15 fold higher level of BAFF was detected in the patient as compared to controls (6.9 ng/mL and 2.3 ng/mL; controls 0.41-0.47 ng/mL).

Elevation of serum BAFF has been observed in autoimmune thyroid disease [276] and in chronic urticaria [277].

4.2.5 RAC2 deficiency and other leukocytes

4.2.5.1 Lymphocyte abnormalities

The decreased recent thymic emigrants, relative B lymphopenia and the impaired antibody production in our patients support a role of RAC2 in T- and B-cell development and function, as evidenced by murine studies [278-280] as well as some T- and B-lymphopenias observed in the second reported patient carrying the D57N mutation [241].

Murine studies indicate that both Rac1 and Rac2 have redundant, but critical, parts to play in T cell development [281]. Rac2 deficiency has been linked to defective immune tolerance in mice due to impairment of the restimulation-induced cell death (RICD) mechanism in T cells [282]. Potential impairment of peripheral immune tolerance in our patients with the W56X mutation could account for the autoimmune hypothyroidism common to both siblings. *RAC2* has been suggested to be a susceptibility gene for the development of autoimmune diseases based on studies of haplotype analysis of patients with Crohn's disease, multiple sclerosis and age matched healthy controls who were all of (Italian) Caucasian ethnicity [283].

Rac1 and Rac2 are required for adequate generation of the common lymphocyte progenitor in murine bone marrow [281]. However, in contrast to our patients, Rac2 KO C57BL/6 mice showed a 40% increase in the proportions of peripheral blood B cells [280]. Additionally, these mice had reduced bone marrow B cell counts, due to reduced recirculating $B220^{high}sIgM^{+}$ B cells, and absent splenic marginal zone (MZ) B cells; and reduced $CD5^{+}sIgM^{+}$ peritoneal

B-1a lymphocytes [280]. These mice also showed 2-8 fold elevations of IgG1 and IgG2b, which also contrasted with the normal levels in the proband and reduced levels observed in the sibling.

Murine Rac2 is a key protein in the downstream signaling cascade after B cell receptor (BCR) antigen recognition [284]. The activation of Rac2 and Rap1 connects BCR-proximal signaling with activation of leukocyte function-associated antigen-1 (LFA-1)-mediated adhesion facilitating the formation of immunological synapses [284]. Human RAC2 might also have a similar downstream role in BCR signaling and immunological synapse formation, and thus a deficiency might directly contribute antibody deficiency.

4.2.5.2 Mast cells

The urticaria may be related to the significant impairment of mast cell functions observed in murine studies. Rac2 is abundantly expressed in murine mast cells and is required for exocytosis in these cells [285]. Murine Rac2 KO bone marrow-derived mast cells have reduced expression of the *mouse mast cell protease 7* (*MMCP-7*) gene, a major component of the secretory granules of mature mast cells. The impaired mast cell tryptases, especially MMCP-7, are responsible for airway smooth muscle hyper responsiveness and other allergic reactions, as well as regulate fibrin-platelet clot formation [286]. However, pinpointing a specific mechanism leading to urticaria will require additional investigation.

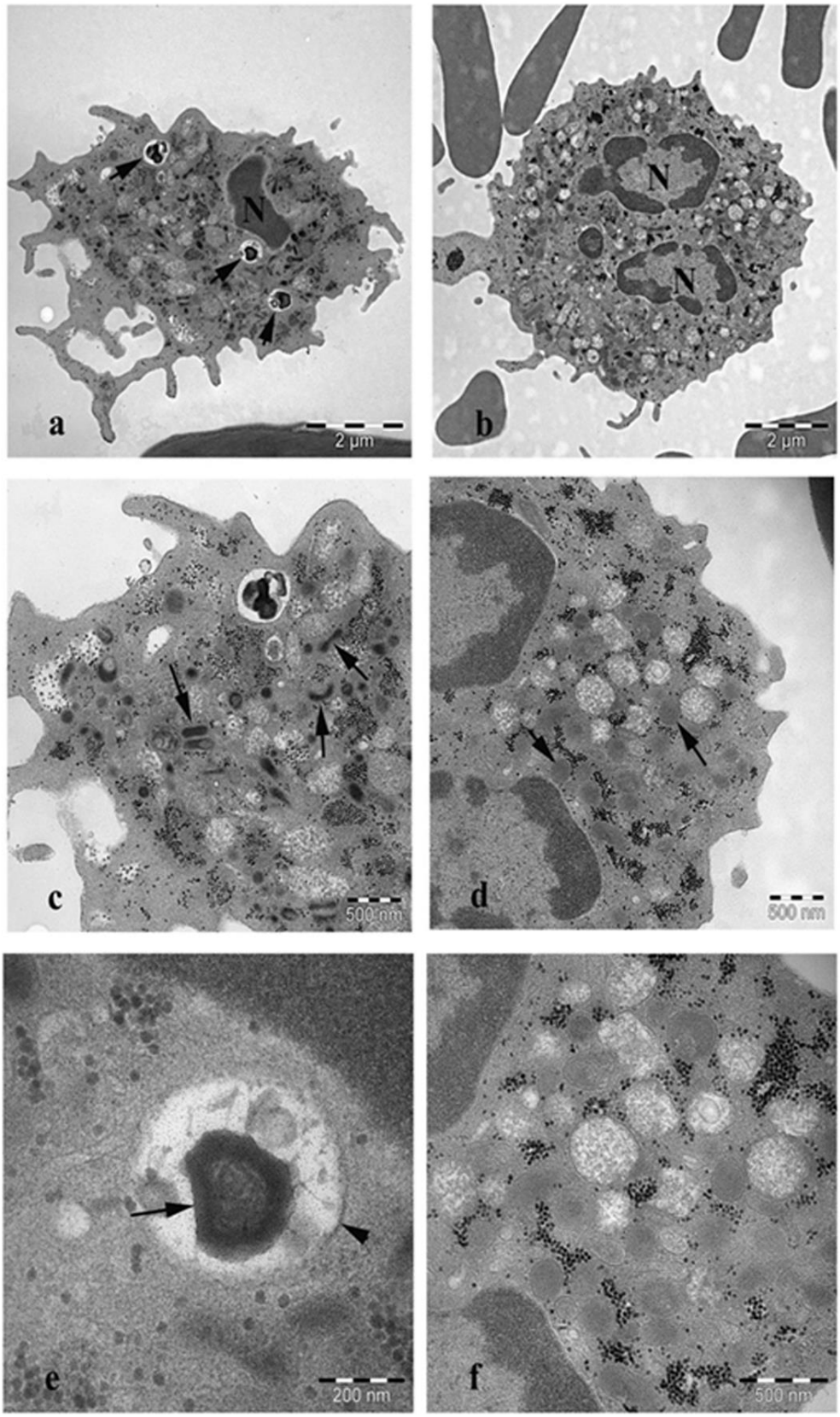

Figure 5. Transmission electron micrographs of neutrophils: a) proband neutrophil: cytoplasmic inclusions (arrow). b) control neutrophil, normal ultrastructure. c) proband neutrophil: fewer cytoplasmic granules, with the majority secondary granules displaying an abnormal shape (arrow). d) control neutrophil: normal, rounded secondary granules (arrow).

e) proband neutrophil: cytoplasmic inclusion, showing multi membrane structure (arrow) surrounded by a double membrane (arrowhead). f) control neutrophil: normal ultrastructure. N, nucleus. (Paper II).

PAPER III

Spectrum of Phenotypes Associated with Mutations in LRBA

This review provided a summary of previously reported cases of LRBA deficiency [51, 142, 149, 287-293] in addition to case histories and mutation analysis of five novel *LRBA* mutations.

4.2.6 Clinical features of LRBA deficiency

The clinical features of patients in this review correlate with those recently found by Gámez-Díaz et al [294] and Lo et al [295]. Although Gámez-Díaz et al. grouped enteropathy and autoimmunity together; the most common clinical features (chronic diarrhea, autoimmune disease, hypogammaglobulinemia, respiratory tract infections) were in line with our findings.

4.2.6.1 LRBA deficiency present in early childhood

This study shows that LRBA deficiency is a disorder that presents in childhood, an observation that has been confirmed in other studies [294, 295].

The presenting features are most commonly gastrointestinal, autoimmunity-related or infectious, with significant overlapping of clinical manifestations (**Figure 6**).

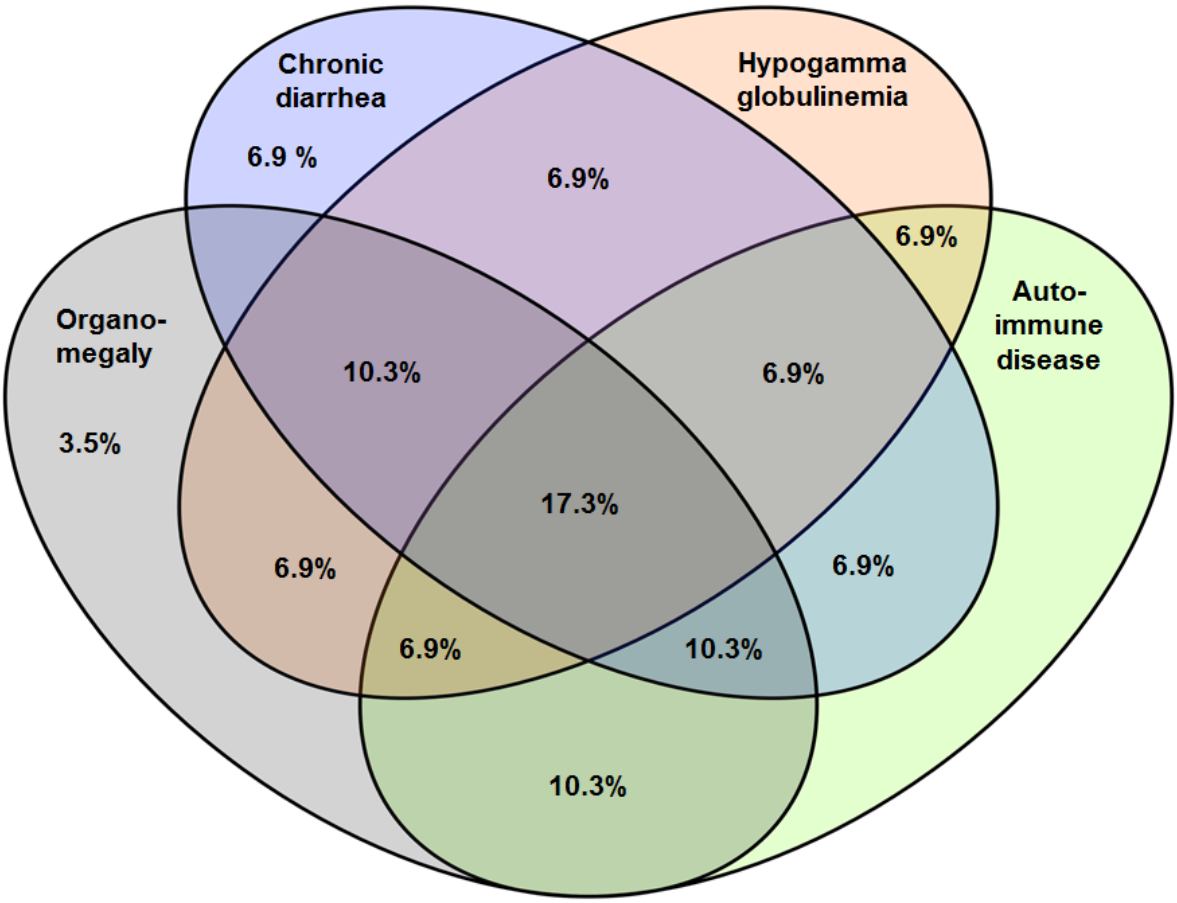

Figure 6. Venn diagram illustrating the percentages of overlap of clinical phenotypes among patients with LRBA deficiency (Paper III).

4.2.6.2 'Asymptomatic' phenotype of LRBA deficiency

One of the unexpected findings of this review was that two patients (P22 and P26), both from different kindreds and with symptomatic siblings carrying the homozygous mutations p.S2713fs*13 and p.S1605X, respectively, were asymptomatic at the time of analysis (P22 had been completely asymptomatic despite lymphopenia [288], and P26 was a clinically well adult with the exception of some respiratory infections during childhood) and neither expressed the LRBA protein on Western blot analysis ([288] and Figure 1G in Paper III). This finding shows that, like other PIDs, LRBA deficiency in individuals with the same genotype might have a relatively 'benign' phenotype.

4.2.6.3 Neurologic complications

A striking feature of LRBA deficiency is that more than a fifth of the patients had neurological complications. Despite being from different kindreds and having distinct genotypes, the similarity of neurologic complications warrants special attention for clinicians.

Radiologic evaluation of patients P1 and P3 showed cerebral granulomas (P3 also suffered seizures); patients P28 and P31 both suffered unilateral optic nerve atrophy; and patient P29 developed a lesion in the parietal lobe. Myasthenia gravis in patient P5 might be categorized as an autoimmune disease; however, notwithstanding that, approximately 20% of the LRBA deficiency patients did develop neurologic conditions. In their cohort of 22 LRBA deficiency patients, Gámez-Díaz et al mention only one patient with neurologic/psychiatric features and one patient with deafness [294]; and Lo at el, mention one patient with seizures [295].

Histologic analysis of human neuronal tissue (including glial and neuronal cells) reveals that LRBA is expressed in cerebral, cerebellar and hippocampal tissue [296]. The role of the LRBA protein deficiency has been thought to be limited to immune dysfunction in lymphoid tissues [51], however, in view of these neurologic features, the role of LRBA in neuronal tissue should be investigated.

4.2.7 Novel LRBA mutations

Five novel mutations were identified using WES and presented with the clinical and laboratory findings form previously published cases. The novel mutations are presented in Table 1.

All patients with novel mutations (P24-P31) were born to consanguinous parents.

Table 1. Summary of clinical data in patients with novel mutations in LRBA deficiency.

Patient	Sex	Ethnicity	Age of onset (years)	Mutation	Main phenotype
P24	F	Iranian	3	c.1014+1 C>T	HGG, RTI, AID, OM
P25	M	Iranian	2	p.S1605X	HGG, RTI, CD, OM
P26	M	Iranian	NA	p.S1605X	Asymptomatic (past RTI)
P27	M	Iranian	2	p.R182X	HGG, CD, AID, OM
P28	M	Iranian	0.2	detected None	HGG, RTI, CD, OM
P29	F	Lebanese	3	p.N988fs*7	HGG,RTI, OM
P30	F	Iranian	6	c.4729+2insA	HGG, RTI, AID, OM
P31	F	Iranian	13	c.4729+2insA	AID, OM, RTI

M, male; F, female; HGG, hypogammaglobulinemia; RTI, respiratory tract infections; AID, autoimmune disease; OM, organomegaly; CD, chronic diarrhea; NA, not applicable.

4.2.7.1 Novel mutations: Loss of LRBA protein expression

Western blot analyses from patients P25-P28 and P30-31 showed complete loss of expression (Figure 1G in Paper III). These findings are in line with the recent findings of Gámez-Díaz et al [294] who identified both homozygous and compound heterozygous mutations leading to loss of expression of LRBA.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 OVERALL CONCLUSIONS

- The spectrum of disorders encompassing PADs, represented by CVID appear to be ever-expanding, particularly as the definition of CVID remains fluid.

- The novel mutations identified in this work indicate the viariable genotype-phenotype relation, as observed in other PIDs.

- WES is an effective method to identify mutations resulting in immunodeficiencies and other disorders.

5.2 CONCLUSIONS FROM INDIVIDUAL PAPERS

- **Paper I**

  Homozygous and heterozygous mutations affecting the CD27 gene predispose to the occurrence of EBV infections together with other infections in individuals with or without hypogammaglobulinemia causing significant morbidity and high mortality. CD27 deficiency should be considered in the differential diagnosis of all cases regardless of consanguinity. CD27 expression on lymphocytes should be implemented as a diagnostic screening test for all patients with a history of clinically severe EBV infection, lymphomas, or both, and in all cases of atypical EBV infection. Comprehensive immunologic, serologic and molecular testing for EBV infection and primary immunodeficiency

should be performed, in addition to a complete physical examination, with particular emphasis on considering atypical features of EBV infection.

- **Paper II**

  The W56X nonsense mutation is a novel mutation affecting the *RAC2* gene associated with a CVID-like disorder, autoimmunity and neutrophil granule abnormalities.

- **Paper III**

  LRBA deficiency might result in a variable clinical phenotype and should be considered in the clinical evaluation of CVID and CVID-like disorders.

5.3 FUTURE PRERSPECTIVES

As new patients are being diagnosed with mutations in *CD27*, *RAC2* and *LRBA*, compiling the information will allow for pinpointing the precise defect(s) by genetic, biochemical and cellular analyses. We are now in the process of proposing a registry on CD27 deficiency to increase awareness among clinicians for finding and collecting patient samples and data. The proposal will be presented at the upcoming meetings of the German, Austrian and Swiss Pediatric Immunologists (API) and the European Society for Immunodeficiencies (ESID).

Both human and animal studies are required to identify novel pathways/defects that CD27 (and CD70), RAC2 and LRBA are involved in and the subsequent function/dysfunction at the cellular and/or systemic level. *Ex vivo* studies might offer particular insights into these aspects, given that murine models may "sub-optimally" reflect their roles in humans.

The characterization of distinct genes and mutations contributing to the pathogenesis of primary antibody deficiency and other primary immunodeficiencies will prompt both the designing of more accurate diagnostic tools and the targeted therapeutic correction of genetic errors at the molecular level before or during the early stages of clinical symptoms and signs, thus preventing their effects from progressing to systemic complications. Indeed, mutation-targeted therapies are now being developed that mitigate the pathological mutation at the nuclear level, based on the accurate characterization of mutations.

Examples of such targeted therapies include read-through compounds and chimeric RNA/DNA oligonucleotides (chimeraplasts) that are already being developed for the treatment of genetic diseases other than primary immunodeficiencies [297].

The conventional difficulty encountered in clinical practice in screening for mutations in multiple PID genes can now be overcome in a cost-effective manner using NGS, either employing WES, WGS or a targeted “chip” sequencing of PID-associated genes. This analytic approach has proved valuable in cases with informative pedigrees (parental consanguinity) as well as those with other cases.

6 LAY SUMMARY

Let's think of each cell in a living creature as a computer; and remember that a computer can only function properly as long as it has a good (software) operating program.

All living creatures possess a master software program molecule called deoxyribonucleic acid (DNA) within each cell, based on which all the functions of the cell are orchestrated and organized. Cells specialize and multiply forming bodily systems that must work in harmony for the creature to function normally in its environment.

If we think of the human body as a nation, then like any nation composed of billions of inhabitants (cells), the body must be protected by an efficient combined defense and fighting force equipped with deterrent barriers and weapons. The immune system represents this system in the human body. It is composed of what you might think of as an administrative command, military academies, armed forces, weapons factories and support structures, the quality of which are directly or indirectly dependent on that master program coded in DNA.

Enemies that attack the body might be foreign (bacteria, viruses and parasites), domestic (cancer) or a combination of both. Sometimes, a native inhabitant (a cell) will join forces with a foreign agent (a virus), transforming the former into a cancer cell. An example of such a foreign agent is the Epstein-Barr virus (EBV). Most of us have been infected with it and don't realize it or just had a brief illness that we've long forgot about. It is the virus that causes the disease nicknamed "mono", "kissing disease" or "glandular fever". Once we have had it, we develop life-long protective immunity to it (if we have healthy immune systems). Sometimes EBV joins forces with a cell, turning that cell into a cancer cell. Hodgkin lymphoma is one example of this phenomenon.

If the immune system is defective due to a problem relating to DNA, then the defect is called ‘primary immunodeficiency’. That problem is often related to the order of the ‘code’ of DNA. Think of DNA as the very paragraph you are reading right now. A change or addition of a single letter can change the meaning of the word, and then also change the meaning of the sentence. For example, ‘fat’ becomes ‘fit’, ‘cat’ becomes ‘bat’, and ‘slim’ becomes ‘slime’. This defect is known as a ‘mutation’.

A healthy person generally recovers from infections because the immune system fights back, eliminating or destroying the harmful agents. If a person has a primary immunodeficiency caused by a mutation, then the story of the infection changes. For example, instead of recovering from EBV infection, the infection becomes really severe, turns body cells into cancer cells, causes medical complications or even leads to the person’s death.

In order to identify the defects within the immune system, we need special tools. The most modern tool we have today is next generation sequencing (NGS). This basically gives us the ability to sequence (read) the DNA code just as a computer reads zeros and ones. In the past, sequencing a tiny fragment of DNA took months or years to do. NGS technology has now helped us sequence much more DNA in a much shorter period (days or weeks).

In this study, my colleagues and I, gathered medical records, blood samples and DNA specimens from hundreds of patients and members of their families. Using NGS, we were able to identify several mutations in DNA that we think contributed to our patients developing the diseases that they suffered from. We then performed additional medical tests to determine the impact these mutations might have had on their immune systems. We identified mutations in *CD27*, *RAC2* and *LRBA*, all of which are fragments of DNA (genes) that encode molecules that function in cells of the immune system. The patients with *CD27* mutations, suffered from severe EBV infections, Hodgkin lymphoma and other diseases resembling lymphoma (**Paper I**). The sister and brother with the *RAC2*

mutation suffered from multiple infections, kidney disease and thyroid disease (**Paper II**). Finally, the patients with *LRBA* mutations suffered from multiple health problems which included infections and chronic diarrhea (**Paper III**).

We hope that these discoveries will help health care professionals and scientists all over the world to quickly diagnose new patients with primary immunodeficiency, develop new therapies, and perhaps even prevent potential patients from getting disease by pre-emptively treating them before they develop symptoms.

7 LAY SUMMARY IN SWEDISH ‘SAMMANFATTNING FÖR LEKMÄN’

A translation of the lay summary by Gustav Östervall

Låt oss tänka på varje enskild cell i en levande varelse som en dator; och komma ihåg att en dator endast kan fungera ordentligt om den har en mjukvara av hög kvalitet.

Alla levande varelser har deoxyribonukleinsyra (DNA), en molekyl av mjukvara inuti varje enskild cell som ligger till grund för hur samtliga av cellens funktioner är organiserade. Genom att specialisera sig och föröka sig formar celler kroppsliga system och dessa system måste fungera i harmoni för att varelsen skall fungera normalt i sin miljö.

Om vi föreställer oss den mänskliga kroppen som en nation, så måste kroppen liksom vilken annan nation som helst, bestående av flera miljarder invånare (celler i detta fall), beskyddas med hjälp av en militärstyrka utrustat med vapen och avskräckande barriärer. Det immunologiska systemet är den mänskliga kroppens motsvarighet till en sådan militärstyrka. Immunsystemet utgörs av vad du kan föreställa dig motsvara en administrativ myndighet, beväpnade styrkor, vapenfabriker och stödstrukturer, vilkas kvalitet är direkt eller indirekt beroende av mjukvaran DNA.

Fienderna som angriper kroppen kan vara främlingar (bakterier, virus och parasiter), inhemska (cancer) eller en kombination av båda. Ibland kan en invånare (en cell) förena sig med ett för kroppen främmande agens (t. ex. ett virus) och omvandlas till en cancercell. Ett exempel på ett sådant främmande agens är Epstein-Barr-virus (EBV). Merparten av oss har varit infekterade med detta virus utan att ha märkt det eller med en övergående allmän sjukdomskänsla som följd. Det är detta virus som orsakar sjukdomen känt som “körtelfeber” i dagligt tal. Efter att vi drabbats av infektionen utvecklar vi en

livslång immunitet mot EBV (förutsatt att vårt immunförsvar är friskt). Ibland kan detta virus alliera sig med en cell och omvandla denna cell till en cancercell. Hodgkins lymfom är ett av dessa sjukdomar.

Om immunsystemets funktion felar och problemet har sin grund i DNA kallas tillståndet för ‘primär immunbrist’. Detta problem är oftast relaterat till den ordning med vilket DNA kodas. Föreställ dig att det textstycke du nu läser motsvarar DNA. Att så lite som en enda bokstav ersätts av en annan eller att ytterligare en bokstav läggs till kan förändra betydelsen för hela ordet och därmed hela frasen. Till exempel att ordet “fet” blir “fat”, “katt” till ordet “hatt” och “mat” blir “matt”. Denna defekt benämns mutation.

En frisk individ återhämtar sig vanligtvis från infektioner tack vare att immunsystemet slåss tillbaks, förstör och eliminerar skadliga agens. Förutsättningarna förändras dock om individen ifråga är drabbad av primär immunbrist orsakat av mutationer. Istället för att tillfriskna från en infektion med EBV så blir infektionen extremt allvarlig med omvandling av kroppsegna celler till cancerceller och kan framkalla medicinska komplikationer eller till och med resultera i dödsfall.

I syfte att kunna identifiera defekter i immunsystemet fordras speciella verktyg. Det mest moderna verktyget vi idag använder oss av är “massiv parallell sekvensering” (NGS; “next generation sequencing”) av DNA. Detta ger oss möjligheten att sekvensera (avläsa) DNA-koden på samma sätt som en dator avläser ettor och nollor. Förr i tiden tog det månader eller åratal att sekvensera ett litet DNA-fragment. Ny teknologi har numera gjort det möjligt för oss att sekvensera betydligt större mängder DNA inom kortare tidsramar (dagar eller veckor).

Jag och mina kolleger samlade i denna studie ihop patientjournaler, blodprover och DNA-exemplar från hundratals patienter samt deras familjemedlemmar. Med hjälp av NGS-teknik identifierade vi flertalet mutationer i DNA som vi

anser bidrog till patienternas sjukdomsutveckling. Därefter utförde vi ytteligare medicinska tester i syfte att utröna den grad av påverkan mutationerna ifråga hade på immunsystemet. Vi identifierade mutationer i *CD27*, *RAC2* och *LRBA*, av vilka samtliga är DNA-fragment (gener) som kodar för de molekyler som uppfyller funktioner i immuncellerna. Patienterna med mutationer i *CD27* led av extrema EBV-infektioner, Hodgkins lymfom och sjukdomar som liknar lymfom **(Papper I)**. Syskonparet, en syster och en bror, med mutation i *RAC2* hade multipla infektioner och njursjukdom **(Papper II)**. De patienter med mutationer i *LRBA* led av multipla hälsoproblem, inklusive infektioner och kronisk diarré **(Papper III)**.

Vi hoppas att dessa upptäckter skall underlätta för vårdgivare och forskare att i tidigt skede diagnosticera nya patienter med primär immunbrist, att utveckla nya behandlingar och förhoppningsvis till och med förhindra att högriskindivider drabbas av sjukdom genom att behandla dem innan de utvecklar symtom.

8 A TRANSLATION OF THE TITLE(S) INTO ARABIC (THESIS AND PAPERS)

الورقة البحثية الأولى:

إكتشاف طفرات جديدة في مورثة CD27:
الأعراض السريرية والتوصيف الوراثي لمرض
عوز بروتين CD27

الورقة البحثية الثانية:

إكتشاف طفرة جديدة في مورثة RAC2 في شقيقين
مصابين بأعراض مشابهة لمرض CVID

الورقة البحثية الثالثة:

التوصيف النوعي للأعراض السريرية و المخبرية لدى
المرضى الذين يحملون طفرات في مورثة LRBA

أطروحة مقدمة لنيل درجة الدكتوراه البحثية

بعنوان

إكتشاف طفرات جينية (موروثة) جديدة مساهمة في تطور أمراض عوز المناعة الناتجة عن نقص الأجسام المضادة
Common Variable Immunodeficiency

إعداد الدكتور

عمر بن خالد بن شريف الخيري

بكالوريا س الطب و الجراحة

إشراف

الأستاذ الدكتور/ لينارت هامارشتروم

الأستاذة الدكتورة /شانغ بان هامارشتروم

الأستاذ الدكتور/ علي حجير

9 ACKNOWLEDGEMENTS

This thesis was accomplished at the Division of Clinical Immunology and Transfusion Medicine, Department of Laboratory Medicine, Karolinska Institutet at the Karolinska University Hospital Huddinge.

First, ‘Alhamdu li Allah’. All praise be to **Allah** (God) for easing the path to completing this PhD degree.

Second, my sincere thanks to everyone who supported and guided me during the PhD journey, with special thanks to:

Professor **Lennart Hammarström**, my principal supervisor, for mentoring and guiding me through this wonderful journey in the realm of immunodeficiency; for believing in me when I doubted my own abilities; for supporting me both professionally and personally.

Professor **Qiang Pan-Hammarström,** my co-supervisor, for her mentoring, teaching and instruction. I am especially grateful for teaching me how to reply to reviewer comments.

Professor **Ali Hajeer**, for the support he has given me and for which my words cannot do him justice.

Dr **Abdulaziz Al-Ajlan**, for believing in my abilities, supporting me, facilitating my pursuit of a career in the wonderful world of Immunology.

Dr **Hanaa Bamefleh**, for her guidance, support, jovial attitude and great care.

Dr **Khalid Al-Saad**, for his support, valuable advice, care and nice attitude.

Dr **Mohammad Ashraf Ali** for his support, guidance and mentoring.

Dr **Sulaiman Al-Ajaji** for his support, mentoring, and for his wonderful attitude and valuable professional and personal advice.

Dr **Abd'Allah Al-Qouzi** for his friendship and great collegiality.

Dr **Hassan Abolhassani,** for so much that it bewilders me where to begin; for his wonderful attitude to life, his warm daily greetings, his wonderful company and conversations, for teaching me, for reassuring me when I was in doubt, for spending long hours working in the lab with me on weekdays and weekends, for reciting wonderful poetry and for 'being there' whenever I was in need.

Dr **Stephan Borte** for his friendship, teaching professional advice and extensive collaboration on our projects.

Dr **Chenglin Wu** for his friendship, teaching and collaboration.

Dr **Jovanka King** for her warm daily greetings, ever-present smiles, wonderful conversations and for proofreading this thesis.

Dr **Harold Marcotte** for his friendly attitude and help these past years, and for being Defence Chairman during my public defense of this PhD.

Naradja Wissmar, Pernilla Kylve Busa and **Moa Thorin** for their wonderful friendship, administrative support, teaching me Swedish and wonderful conversations.

Professor **Andrej Weintraub, Marita Ward** and **Arja Kramsu** for their valuable academic guidance, advice and jovial attitudes.

Kerstin Bergman and **Ingegerd Löfving-Arvholm** for their wonderful instruction, teaching and support for completing my projects; and for setting me off on my first steps to learning the beautiful Swedish language. *Tack så mycket!*

My dear office mate friends, **Yin Lin, Gökce Gunaydin, Che Kang Lim, Sonal Pendharkar, Marcel Frankowiack, Ning (Nina) Wang, Konstantinos Georgiou, Likun Du, Andrea Björkman, Luke O'Brien,**

Chloé Maurizi, Margarita Bartish and **Jezabel Varadé** for their wonderful company, warm attitude and their continuous sharing of nice treats.

Kasper Krogh-Andersen, for his very welcoming attitude, friendship, great teaching, valuable advice and for proofreading the introduction to this thesis.

All my dear friends (past and present) at the Division of Immunology**, Noel de Miranda, Ann Gardulf, Apostolos Zaravinos, Renée Enqvist, Annica Lindkvist, Kyriaki (Kiki) Liadaki, Jinqiao Sun, Rosa Romano, Mia Olsson, Fanglei Zuo, Georgia Kokaraki, Xiaogan Wang, Radhika Pankaj Kamdar, Rozina Caridha, Nina Wang, Mahya Dezfouli, Weicheng Ren,** and **Sofia Appelberg.**

Gustav Östervall for his wonderful friendship, lively conversations and great personality. I also thank him for providing the wonderful translation into Swedish of the lay summary into the Swedish "Sammanfattning för lekmän".

All my friends and colleagues at the Department of Pathology and Laboratory Medicine at King Abdulaziz Medical City, Saudi Arabian National Guard Health Affairs, Riyadh, Saudi Arabia.

All our collaborators, without whom these wonderful projects could not be accomplished. I mention specifically **Professor Tim Niehues, Professor Asghar Aghamohammadi, Dr Ruy Perez-Becker, Dr Mingyan Fang, Dr Nima Rezaei, Dr Markus Seidel, Dr Javad Mohammadi, Dr Kjell Hultenby, Dr Sharon Choo, Professor David Williams** and **Professor Raif Geha.**

All my friends at the Department of Laboratory Medicine at the Karolinska Institute, **Salah Shanan, Waleed Mazi, Mohammed Almuhayawi, Amir Saeed, Salah Zenganah, Karnam Guruswamy, Hadi Abd, Krister Landin, Bala Siddaiah Anangi, Markus Krantz, Fredrik Tingstedt, Amare Worku Kalu, Nigus Fikrie Telele, Mamun Ur-Rashid and Abu Bakar Siddik.**

Mr **AbduAl-Rahmaan Al-Mazhoud** and Mr **Ahmad Al-Abnawi** for being my mentors during my studies in Stockholm.

Dr **Zeyd Merenkoz** for being a wonderful a wonderful friend, teacher, advisor and for introducing me to a very special and wonderful genre of literature.

Dr **Mohammad Al-Balwi**, Dr **Isam Amir** and **Lulwa Al-Hammad** for their friendship and for introducing to the fundamentals of molecular pathology and cytogenetics.

My dear friends and colleagues in lands near and far**, Walid Salih Ridwan, Muayyad Al-Hilali, Sami Sulaiman Almuhaimeed, Ryan Abd'Arrahman Abd'Al-Jawad, Nayef Moshawwah Al-Anazi, Alhosain Sagar Al-Hazmi, Abd'Allah Al-Saraan, Abd'Allah Al-Shibaili, Omar Al-Hazza, Naved Abd'Al-Rahman, Saeed Al-Qarni, Iryna Olegivna Baranova, Amin (Carlos) Fernández Moro, Andra Busa, Jesse Gill, Awwad Ahmad, Mohammad Al-Housani, Ahmad Abbad, Christine & Ray Czajko, Hikmat Gerges, Zeyad Othman, Marion & David Nield, Kahlid Batarfi, Fahad Mayuf Al-Sufyani, Khalid Al-Madi, Hosam Mukhtaar Fallatah, Nasrin Perskvist, Bilal Al-Doghaiman, Khalid Al-Zahrani, Aamir Al-Homaidan, Abd'Al-Aziz Al-Dahi, Marylin & Vin Clark, Abd'Assamad, Sheree Poulton, Ghazi Al –Dahi, Rachelle Farah, Dr Ali Al-Somily, Dr Fayhan Al-Rougi, Dr Abdullah Al-Suwaidan, Alwaleed Al-Hammad, Majid Al-Shaibani, Simone West, Anne Gordon, Mohannad Mukhtaar Fallatah, Asha Wallace, Abdul Kadir Mukarram, Ahmed Abd'Al-Wahid Saeed, and Nayef Bejad Al-Otaibi.**

Dr Abdullah Obaid Al-Anazi, his wife Umm Muteb, and their beautiful children Muteb, Aryaf and Atyaf, for their friendship and company during my stay in Stockholm.

Audrey Joan Larsen, for being a wonderful friend and neighbour; and for being such an inspiration by sharing the story how you started university at

the age of eighty years and completed your Master's degree at the age of eighty eight years.

Many others remain whom I wish to acknowledge here by name, but have not been able to. Thank you all, for enriching my life with your affection and your words!

Last, the most important people in my life:

My parents, for more than I can ever acknowledge, from before I was born to this day, and, Allah-willing, in the days to come. O Allah! I pray and beseech You to actuate me with the feeling of gratitude and gratefulness for Your grace; guide me and grant me success in being dutiful to my parents. Ameen!

My dearest sister, **Aisha**, for supporting me and for more than I can acknowledge in mere words.

My sisters, **Maryam**, **Abeer** and **Saleha**, for all the joy they bring to my life.

My beloved **Khala**, and my beloved cousins **Adher, Moeen, Sulayman, Faisal and Zenaat,** for all their love and affection; Zenaat's wonderful husband, **Faisal,** for his great friendship; **Umm Mohammad** and **Umm Osama** for always making family gatherings joyful.

All my beloved nephews and nieces, especially **Yusuf, Mohammad, Hassan and Abd'Allah**, who would so lovingly pick me up at the airport and drop me off, during my visits to Riyadh.

I bear witness that no one has the right to be worshipped except Allah, and I bear witness that Muhammad is His servant and messenger.

Omar Khalid Sharif Alkhairy

19th Rajab 1437 A.H.

26th April 2016 C.E.

Stockholm, Sweden

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