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UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Mario Šestan

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DISSERTATION



Zagreb, 2022.

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This dissertation was made at the Division of Clinical Immunology, Respiratory and Allergic Diseases, and Rheumatology, Department for Paediatrics, University Hospital Centre Zagreb; Division for Cytogenetics, Department of Laboratory Diagnostics, University Hospital Centre Zagreb, and the Centre for Personalised Immunology and China-Australia Centre for Personalised Immunology at the John Curtin School of Medical Research, The Australian National University (Canberra, Australia).

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LIST OF ABBREVIATIONS

ACE – Angiotensin-converting enzyme

ACMG – American College of Medical Genetics and Genomics and the Association for Molecular Pathology

aCL – Anti-cardiolipin antibody

ACR – American College of Rheumatology

ADAR – Adenosine deaminase acting on RNA

AECA – Anti-endothelial cell antibodies

AGLM – Anti-smooth muscle antibody

ALT – Alanine aminotransferase

ANA – Antinuclear antibody

ANCA – Anti-neutrophil cytoplasmic antibody

Anti-dsDNA – Anti-double-stranded DNA

Anti-Sm – Anti-Smith antibody

Anti-U1-RNP – anti-U1-Ribonucleoprotein autoantibodies

AST – Aspartate aminotransferase

Atg5 – Autophagy related 5

ATG16L2 – Autophagy Related 16 Like 2

AZA – Azathioprine

BAD – B-cell lymphoma 2 associated agonist of cell death

BAFF – B-cell activating factor

BANK1 – B cell scaffolding protein with ankyrin repeats 1

BCL10 – B-cell lymphoma/leukemia 10 protein

Beta-2-GPI – Beta-2-Glycoprotein I

BLK – B lymphocyte kinase

C3 – complement component 3

C4 – complement component 4

CADD – Combined annotation dependent depletion

CARD15 – Caspase recruitment domain family member 15

CD – Cluster of differentiation

Cdkn1b – Cyclin-dependent kinase inhibitor 1b

cDNA – Complementary DNA

CFD – Complement factor D

C1GALT1 – Core 1 synthase glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1

CH50 – total complement activity

Clec16a – C-type lectin domain family 16 member a

CNS – Central nervous system

CNV – Copy number variation

CR – Complement receptor

CVID – Common variable immunodeficiency

CYC – Cyclophosphamide

dbSNP – Single Nucleotide Polymorphism Database

DXH58 – DExH-box helicase 58

DMARD – Disease-modifying antirheumatic drug

DNASE1 – Deoxyribonuclease 1

DNASE1L3 – Deoxyribonuclease 1 like 3

DRAM1 – DNA damage regulated autophagy modulator 1

dU – Daily urine sample

cSLE – Childhood-onset systemic lupus erythematosus

EULAR – European Ligue Against Rheumatism

ExAC – Exome Aggregation Consortium

GalNAc – Galactose to N-acetylgalactosamine

GC – Glucocorticoid

Gd-IgA$_1$ – Galactose deficient IgA$_1$

gDNA – Genomic DNA

GERP – Genomic Evolutionary Rate Profiling

GWAS – Genome wide association study

HCQ – Hydroxychloroquine

HLA – Human lymphocyte antigen

HSP – Henoch-Schönlein purpura

IBD – Inflammatory bowel disease

IFIH1 – Interferon-induced helicase-1

IFN – Interferon

IgAV – Immunoglobulin A vasculitis

IgAVN – Immunoglobulin A vasculitis nephritis

IKZF – Ikaros family of zinc finger protein

IL – interleukin

IRF – interferon regulatory factor

ISKDC – International Study of Kidney Disease in Children classification

ITGAM – Integrin alpha M

IVIG – Intravenous immunoglobulin

JAK – Janus kinase

KDM6A – Lysine-specific demethylase 6A

KMT2D – Histone-lysine N-methyltransferase 2D

LAC – Lupus anticoagulant

LIFR – Receptor for leukemia inhibitory factor

LKM-1 – Liver kidney microsome type 1 antibodies

NFκB – Nuclear factor kappa-light-chain-enhancer of activated B cells

MAF – Minor allelic frequency

MAPK – Mitogen activated protein kinase

MBL – Mannose-binding lectin

MCP1 – Monocyte chemoattractant protein 1

MEFV – Mediterranean fever

MEST-C score – M: mesangial hypercellularity, E: endocapillary hypercellularity, S: segmental sclerosis, T: interstitial fibrosis/tubular atrophy, C: crescents

MHC – Major histocompatibility complex

MMF – Mycophenolate mofetil

mRNA – Messenger ribonucleic acid

MTX – Methotrexate

NOS – Nitric oxide synthase

NSAID – Non-steroidal anti-inflammatory drug

OR – Odds ratio

PAMP – Pathogen-associated molecular patterns

PBMC – Peripheral blood mononuclear cell

Polyphen2 – Polymorphism Phenotyping2

PON1 – Serum paraoxonase and arylesterase 1

PRES – Paediatric Rheumatology European Society

PRINTO – Paediatric Rheumatology International Trials Organization

Prkcb – Protein kinase C beta type

PROVEAN – Protein Variation Effect Analyzer

Pt – Patient

PTPN22 – Tyrosine phosphatase nonreceptor type 22

PVAS – Paediatric Vasculitis Activity Score

PVDI – Paediatric Vasculitis Damage Index

qPCR – Quantitative real-time polymerase chain reaction

RAG2 – Recombination-activating gene 2

Ras – Rat sarcoma virus

RF – Rheumatoid factor

RNA – Ribonucleic acid

RNaseH2 – Ribonuclease H2 complex

RTX – Rituximab

SAMHD1 – Sterile Alpha Motif domain and Histidine-Aspartic domain-containing protein 1

SHARE – Single Hub and Access point for paediatric Rheumatology in Europe

SH2B3 – Src homology 2-B adaptor protein 3

SIFT – Sorting intolerant from tolerant

SiPhy – SIte-specific PHYlogenetic analysis

SLE – Systemic lupus erythematosus

SLEDAI – Systemic Lupus Erythematosus Disease Activity Index

SLEDAI-2K – modified Systemic Lupus Erythematosus Disease Activity Index

SLICC – Systemic lupus erythematosus International Collaborating Clinics

SNP – Single nucleotide polymorpisms

SNV – Single nucleotide variation

SSZ – Sulfasalazine

TCR – T cell receptor

Tfh – T follicular helper cell

TGF-β1 –Transforming growth factor beta 1

TLR – Toll like receptor

TNF – Tissue necrosis factor

TNFAIP3 – Tumor necrosis factor alpha-induced protein 3

Tnfsf4 – Tumor necrosis factor ligand superfamily member 4

TNIP1 – Tumor necrosis factor alpha-induced protein 3-interacting protein 1

TREX1 – Three Prime Repair Exonuclease 1

TYK2 – Tyrosine-protein kinase 2

Ube2l3 – Ubiquitin-conjugating enzyme E2 L3

ULN – Upper limits of normal

VUS – Variant of uncertain significance

WES – Whole exome sequencing

WGS – Whole genome sequencing

1. INTRODUCTION AND BACKGROUND FOR THE PROPOSED RESEARCH

This research is focused on possible monogenic forms of two autoimmune diseases with complex and not fully understood etiopathogenesis: childhood-onset systemic lupus erythematosus (cSLE) and IgA vasculitis (IgAV) or Henoch-Schönlein purpura (HSP). The thesis describes the use of whole-exome sequencing (WES) to identify genetic variants that are associated with the two diseases.

1.1. CHILDHOOD-ONSET SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that may involve any organ system, and is characterised by systemic inflammation of blood vessels and connective tissue due to the production of antibodies directed against nuclear and cytoplasmic antigens, complement activation and deposition of immune complexes (1, 2). Given the possibility of the involvement of all organs and a wide range of clinical manifestations, from subclinical to very severe clinical pictures, including lethal outcome, it is often referred to as "the disease with a thousand faces" (3). The inflammatory process most often affects the skin, kidneys, brain, lungs and heart (4).

In up to 15-20% of SLE patients, disease begins during childhood and can be diagnosed under the age of 18 years (5, 6). It is commonly referred to as cSLE. Although there are similarities in the clinical picture of SLE and immunological findings in children and adults, due to a number of particularities, it is necessary to single out cSLE as a separate clinical entity. There are numerous differences in disease manifestations between these two age groups (5, 7, 8).

First of all, in childhood, at the outset of the disease, i.e. at the time of diagnosis, it usually has a more severe clinical picture (proteinuria, hemolytic anemia, leukopenia and rash in the zygomatic region). Furthermore, in childhood, the disease usually affects a large number of organs and organ systems, with the kidneys dominating. The range of renal involvement according to the literature is 60-80% in children and 35-50% in adults. There is also an evident difference in central nervous system involvement: 20-50% in children and 10-25% in adults. On the other hand, in adult patients, the lungs (20-90% in adults and 15-40% in children) and joints (80-95% in adults, and 60-70% in children) are more often affected (9, 10). In cSLE, renal biopsy, dialysis and transplantation are more common, convulsions are more frequent,

and there is a higher risk of myocardial infarction. cSLE generally has a more aggressive clinical course, so over time, permanent damage to organs and organ systems is more likely to occur. In view of all of these considerations, children receive much more intensive therapy, where treatment with glucocorticoids and immunosuppressants is used more frequently. As a result, compared to adults, children incur significantly more damage associated with corticosteroid use, such as cataracts and avascular bone necrosis (11).

Unlike SLE in adults, where the disease occurs nine times more often in females, in children this difference is still significantly less pronounced and the ratio of girls and boys with cSLE is 4-5:1. In addition to the above, it should be noted that cSLE is associated with primary immunodeficiencies, primarily the lack of components of complement, and shows a higher disease activity index. Last, but not least, genetic factors may play a more important role than environmental and hormonal factors in early-onset SLE, as opposed to adult-onset SLE (12). Therefore, in childhood SLE, rare monogenic forms of disease resulting from mutation in a specific gene occur more frequently. Such monogenic forms are inherited according to Mendel's laws, and have completely altered the previously formed paradigm of SLE as one disease with an exclusively polygenic basis (13).

1.1.1. Epidemiology of cSLE

In recent decades the incidence of SLE has been increasing. The incidence of the disease in adult patients, depending on various data from the literature, is between 1 and 10 per 100,000 person-years, and the prevalence is 5.8 to 130 per 100,000 population (14). The prevalence range of cSLE ranges from 1.9 to 25.7 per 100,000 children, with an incidence of between 0.3 and 0.9 per 100,000 children, where differences are dependent on gender and geographical distribution (1, 5). The incidence is highest in female black children of African descent and lowest in male white children. According to a large study conducted in the Republic of Croatia (Figure 1), the annual incidence rate of cSLE is from 1 to 15 per million children, i.e. among girls from 2 to 23, and among boys from 0 to 7 per million children, with a female-to-male ratio of about 5:1, which is slightly above the world average (15). In the largest number of affected children, cSLE is diagnosed between 11 and 15 years, while the disease is rare in those under five years. The median age at which the disease occurs in our country is 13.4 years (15).

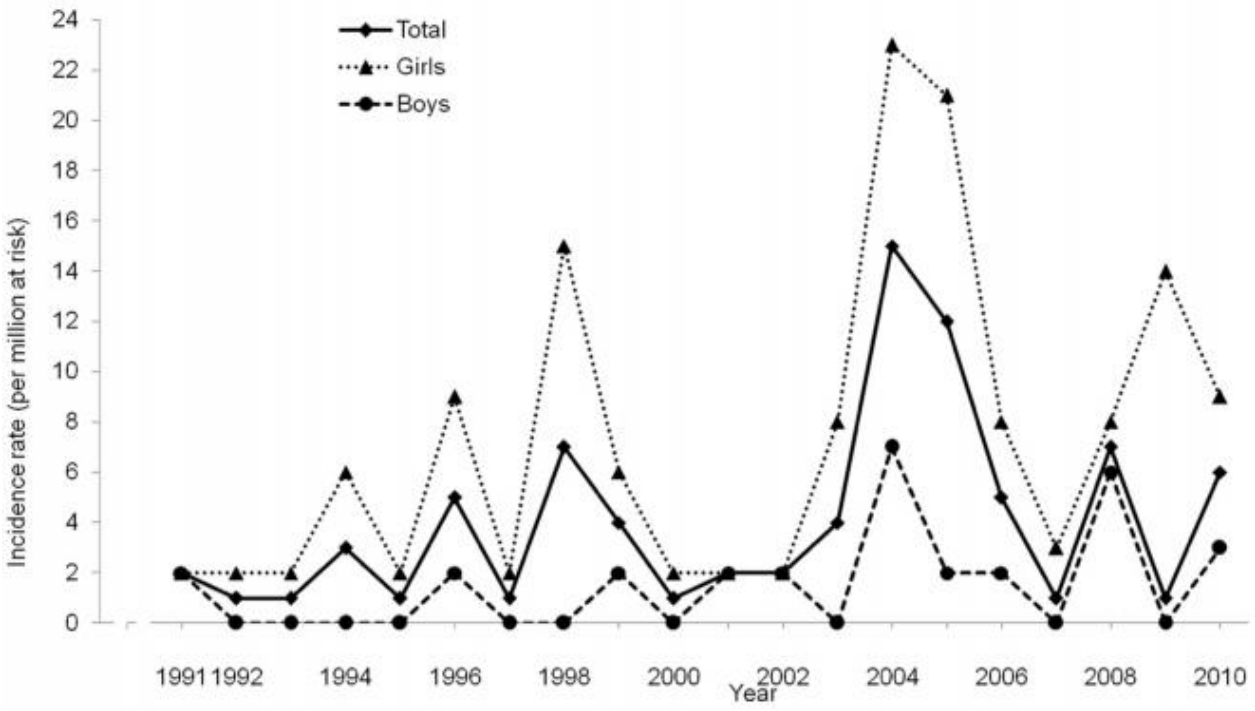


Figure 1. Annual incidence rates of cSLE in the Republic of Croatia in the period 1991-2010. Reproduced from the reference 15.

1.1.2. Etiopathogenesis of cSLE

The ethiopathogenesis of cSLE is complex and not fully elucidated. There are three key factors involved in the etiology of cSLE: genetic risk factors, epigenetic mechanisms and environmental triggers (16).

1.1.2.1. Genetic risk factors

Genetic factors play an important role in the development of the disease. The risk of developing SLE is approximately 0.1% in the population and 0.2% in females respectively. Thus, on average, about 7% of patients with SLE have first-degree relatives with the same disease (17). The risk is 4 to 8% in first-degree relatives (18), however, it can be higher, with up to 10% for sisters of SLE patients (19) and in countries where consanguinity is more prevalent, this percentage is much higher (for example, in Oman it is as high as 48%). Siblings of patients with SLE have an 8 to 20 times higher risk of developing the same disease compared to the general population (20, 21, 22). The strong genetic influence is perhaps best illustrated

by the fact that there is a 10-fold increase in SLE risk among monozygotic as compared to dizygotic twins (21, 23). The estimated heritability of SLE is 44-66% with a concordance of about 24-56% in monozygotic twins as opposed to dizygotic ones where it is only 2-5% (24, 25, 26, 27, 28).

The occurrence of autoimmune diseases in the family is also a risk factor for the occurrence of SLE, and this risk increases with the number of relatives suffering from autoimmune diseases. Genome-wide association studies (GWAS) have so far identified more than 100 gene loci associated with SLE susceptibility, but these loci may also contribute to the development of other autoimmune diseases (29, 30). Therefore, a family history of autoimmune disease is associated with an elevated risk of SLE by a factor of 4.1, and this risk increases with the number of relatives with autoimmune disease by up to 11.3 times (31). According to the data provided by the Centre of Reference for Paediatric and Adolescent Rheumatology Republic of Croatia, in the period from 1991 to 2016, 8.5% of patients with SLE had first-degree relatives with the same disease, and 20% had family history of another autoimmune disease, with autoimmune thyroiditis being the most prevalent among these diseases, followed by rheumatoid arthritis, ulcerative colitis and diabetes type I (32).

The first described gene association with SLE was the major histocompatibility complex (MHC) on chromosome 6, which contains the human lymphocyte antigens (HLA) (33). According to current knowledge, the SLE susceptibility genes can be divided into four groups (29) (Figure 2).

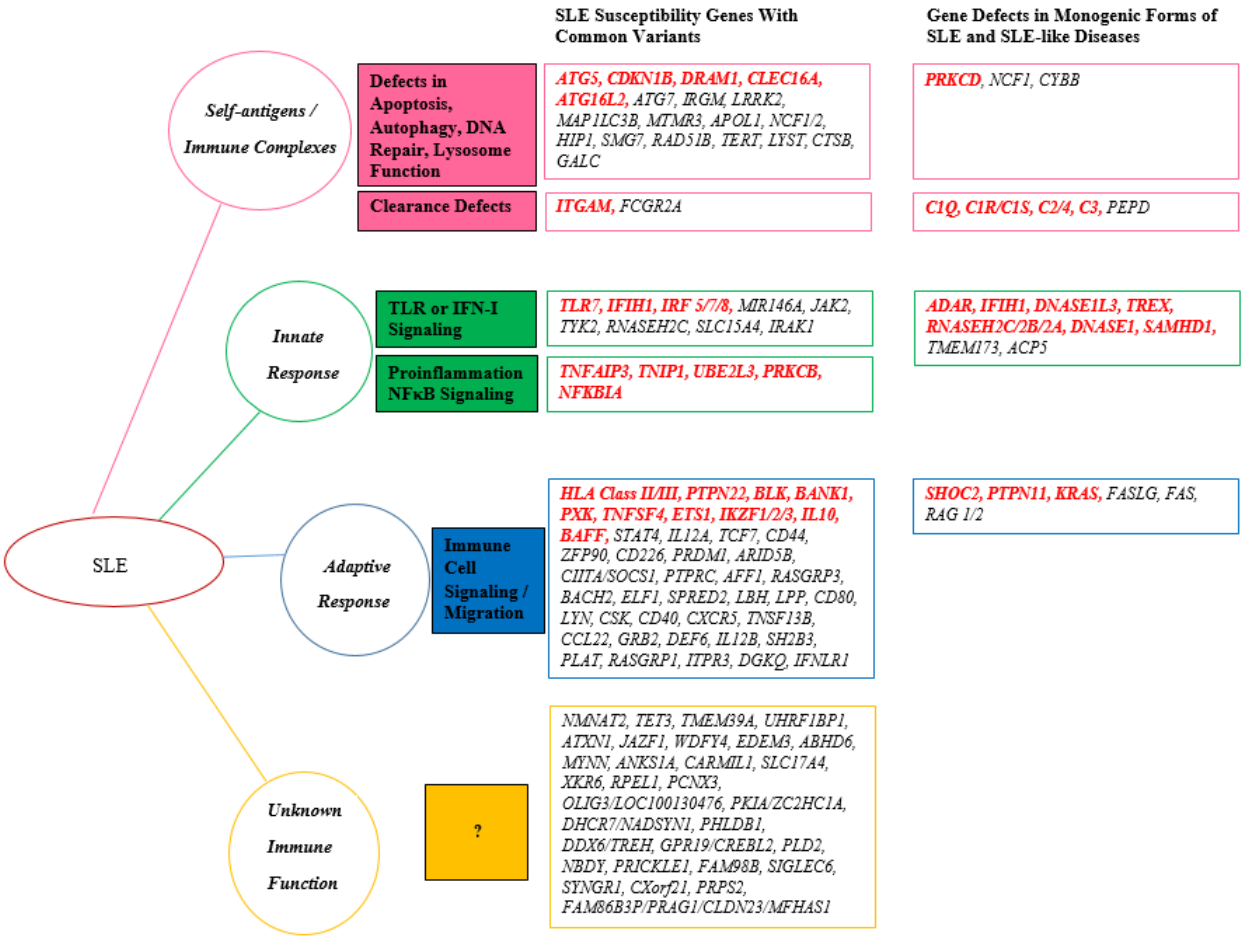


Figure 2. Overview of the important genes involved in SLE pathogenesis. The most important genes are marked in red. Modified according to reference 29. DNA: deoxyribonucleic acid; IFN-I: type I interferon; NFκB: nuclear factor κB; SLE: systemic lupus erythematosus; TLR: Toll-like receptor.

The first group includes genes related to apoptosis, autophagy, DNA repair, lysosome function and clearance of immune complexes. These genes are classified in the same group because their dysfunction, related to the aforementioned processes, may result in increased exposure of nuclear autoantigens to the immune system and deposition of immune complexes. These processes are involved in initiating and perpetuating the autoimmune response in lupus. Autophagy is the cellular "self-digestion" process which enables the degradation of long-lived proteins and cytoplasmic organelles (34). Autophagy-related processes can regulate many immune responses including the delivery of antigens to MHC compartments, lymphocyte survival/homeostasis regulation and cytokine production. GWAS have identified several autophagy-related genes which can be associated with susceptibility to SLE and five that have the strongest association include *ATG5*, *CDKN1B*, *DRAM1*, *CLEC16A*, and *ATG16L2* (34). A

number of other susceptibility genes, such as *ATG7*, *IRGM*, *LRRK2*, *MAP1LC3B*, *MTMR3*, and *APOL1* are also known to play key roles in this signaling pathway (29, 34).

Autophagy related 5 (Atg5) is a protein that, in humans, is encoded by the *ATG5* gene. It is indispensable for autophagic vesicle formation and has a central role in autophagy but has many other functions including mitochondrial quality control after oxidative damage, negative regulation of the innate antiviral immune response, lymphocyte development and proliferation, MHC II antigen presentation, adipocyte differentiation and apoptosis (35). Although it is known that both common and rare variants of *ATG5* are associated with susceptibility to SLE, the mechanism by which *ATG5* triggers lupus is not yet known (34). There are clues that *ATG5* initiates the development of SLE by promoting a cytokine imbalance or disrupting antigen presentation.

Cyclin-dependent kinase inhibitor 1b (Cdkn1b) is an enzyme inhibitor that is encoded by the *CDKN1B* gene. It is an atypical tumor suppressor with many functions such as cell cycle regulation, cell proliferation and differentiation (36). Its role in T lymphocyte development is especially important and it has been found that it is required for the induction of T cell tolerance and anergy. Mice with a cyclin-dependent kinase inhibitor p27 deficiency show mild lupus-like abnormalities, with reduced number and activity of regulatory T cells (T_{reg} cells) (36).

The DNA damage regulated autophagy modulator 1 (*DRAM1*) gene encodes a lysosomal membrane protein that is required for the induction of autophagy (34). It was found that *DRAM1* expression is induced after induction of DNA damage by UV irradiation, which is a possible explanation for how *DRAM1* may be involved in the development of SLE (34, 37), by playing the role of a link between genetic factors of autophagy and environmental stimulators

The C-type lectin domain family 16 member a (Clec16a) protein regulates the selective degradation of mitochondria by autophagy and modulates T-cell selection and reactivity in the thymic epithelium (34). *CLEC16A* was found to be genetically associated with multiple autoimmune disorders including multiple sclerosis, rheumatoid arthritis, Crohn's disease and SLE. The mechanism of how *CLEC16A* participates in the development of SLE remains elusive. Perhaps the reduced expression of *CLEC16A* isoforms observed in SLE may cause enhanced autophagic activities (38).

ATG16L2 (Autophagy Related 16 Like 2) is a protein-coding autophagy-related gene proposed as a SLE risk locus and is associated with multiple sclerosis and Crohn's disease.

ATG16L2 might play an important role in autophagy, specifically in T-cells (39), however, this role remains unknown.

In the first group of genes whose rare deficiency and common variants involved in immune complexes clearance contribute to SLE susceptibility, the *ITGAM* gene should certainly be mentioned. The Integrin alpha M (*ITGAM*) gene encodes the integrin alpha M chain which combines with the beta 2 chain to form a macrophage receptor 1 (Mac-1) or complement receptor 3 (CR3) which is involved in the adherence of neutrophils and monocytes to stimulated endothelium and also plays a role in the phagocytosis of complement-coated particles and immune complexes, as well as regulation of leukocyte apoptosis (40). It was shown that missense variants in *ITGAM* have an impaired phagocytosis function in monocytes, neutrophils and macrophages, which results in altered clearance of immune complexes, deposition, tissue damage and elevated type I interferon (IFN-I) levels (41).

The second group consists of genes of innate immunity involved in the signaling pathways of IFN-I, Toll-like receptors (TLR) and nuclear factor κB (NFκB) (29). They are classified in the same group because all these genes are associated with innate immunity.

IFN-I plays a key role in SLE pathogenesis and an increased expression of IFN-I-inducible genes can be demonstrated in peripheral blood cells of the majority of SLE patients (42). It can be said that IFN-I related genes are the most important SLE susceptibility genes since more than half of the identified SLE susceptibility genes encode proteins with functions directly or indirectly linked to IFN-I production or responses (29). IFN-I has many functions and multiple immune effects, including promotion of monocytes and plasmacytoid dendritic cell differentiation, activation of autoreactive T/B cells, stimulation of autoantibody production and induction of pro-inflammatory cytokines and chemokines (29). Different triggers may induce production of IFN-I in SLE; such as increased exposure of nucleic acids, which are part of immune complexes, necrotic debris, endosomal receptors (e.g., TLR7) or cytosolic sensors (e.g., IFIH1) (43).

TLR7 is localised inside intracellular endosomes, recognises single-stranded RNA and plays a central role in the defense against invading viruses. TLR7 activation can result in both IFN-I production and NFκB activation in different cell populations, such as dendritic cells, monocytes, macrophages and B cells. IFN-I can promote SLE development (44). It was demonstrated that sera from SLE patients contain TLR7 ligands in the form of immune complexes that may activate plasmacytoid dendritic cells and induce IFN-I secretion. Also, SLE

sera can induce TLR7 expression in neutrophils and these are then prepared for NETosis, which is also increased in SLE (45). NETosis is a form of neutrophil cell death during which neutrophils release neutrophil extracellular traps (NETs), which can capture pathogens to prevent them from spreading.

The interferon-induced helicase-1 (*IFIH1*) gene encodes the melanoma differentiation associated protein 5 (Mda5), an intracellular pattern recognition receptor involved in the recognition of double-stranded RNA. During the viral replication cycle, double-stranded RNA molecules are formed and then bind to Mda5, which initiates a series of events leading to the production of type I and III interferons (IFN-I and IFN-III) (46). The gain of function mutation in *IFIH1* gene results in activation of dendritic cells and macrophages, and production of IFN-alpha in response to nucleic acid which leads to the activation of T cells and production of autoantibodies (47).

Different interferon regulatory factor (*IRF*) genes encode proteins which regulate transcription of interferons. Genetic variation in three genes of the IRF family has been linked to SLE susceptibility: *IRF5*, *IRF7* and *IRF8* (48). IRF5 and IRF7 are downstream IRF proteins. They interact with the MyD88 adaptor protein following TLRs engagement. Both IRF5 and IRF7 can induce transcription of IFN-α mRNA. IRF8 does not interact with MyD88, and seems to play a role in the production of inflammatory cytokines in dendritic cells in response to the TLR9 ligand (29, 48). Genetic variants in the *IRF5* and *IRF7* genes which have been associated with SLE susceptibility, seem to be gain-of-function variants related to increased serum IFN-alpha in SLE patients with anti-double stranded DNA (anti-dsDNA) and anti-RNA-binding protein autoantibodies (29, 48). In SLE patients who lacked anti-dsDNA and anti-RNA-binding protein autoantibodies, no relationship was found between IRF5 and/or IRF7 and serum IFN-alpha levels (29, 48).

In the second group of SLE susceptibility genes, genes associated with NFκB pathway should also be listed: *TNFAIP3*, *TNIP1*, *UBE2L3*, *PRKCB*, and *NFKBIA* (29). The NFκB pathway activates many important cytokines and the NFκB target genes are involved in different immune functions including the development, activation, and differentiation of lymphocytes, the maturation and inflammatory functions of innate immune cells (49). Abnormal NFκB signaling lead to the production of auto-reactive T-cells, which have an important role in SLE and promotes plasma cell development.

The tumor necrosis factor alpha-induced protein 3 (*TNFAIP3*) gene encodes enzyme A20 which has been shown to inhibit NFκB activation, TNF-mediated apoptosis and NLRP3 inflammasome (50). The risk *TNFAIP3* alleles are linked to the decreased expression of A20 in patients with SLE, leading to enhanced NFκB signaling.

Tumor necrosis factor alpha-induced protein 3-interacting protein 1 is a protein encoded by the *TNIP1* gene. This is an A20-interacting protein and functions as a physiological inhibitor of NFκB. *TNIP1* variants with loss inhibitor function contribute to the development of SLE via enhanced activation of NFκB (51).

The ubiquitin-conjugating enzyme E2 L3 (Ube2l3) participates in ubiquitination of an NFκB precursor to target proteins for degradation and may play a role in B cell proliferation and differentiation (49). The risk allele is associated with increased *UBE2L3* expression, which results in enhanced NFκB activation and increased circulating plasma cell numbers in SLE patients (52).

The protein kinase C beta type (Prkcb) is an enzyme which is involved in B-cell receptor-mediated NFκB activation (49). Variants of *PRKCB* were found to be associated with SLE by increased NFκB activation and B-cell hyperactivity (53).

NFκB Inhibitor Alpha (*NFKBIA*) is a transcription factor gene that participates in the activation of genes involved in immune responses (54). Its association with SLE susceptibility is probably the result of enhanced NFκB activation.

The third group consists of genes of adaptive immunity involved in the signaling and migration of immune cells, which can be divided into HLA genes and genes outside the HLA system, including various kinases, cytokines and transcription factors related to signal transduction within lymphocytes, activation, proliferation and interaction of immune cells (29). Variants in these genes may result in a loss of immune cell tolerance and sustained autoantibody production.

Major histocompatibility complex (MHC), termed human leukocyte antigen (HLA) in humans, is located on chromosomal region 6p21.3 and contains more than 200 genes coding for leukocyte antigens, complement factors and other non-HLA genes which are also involved in immune functions (36). The HLA region is subdivided into class I, II and III regions. Class I and II contain genes encoding glycoproteins that process and present peptides for T-cell recognition. Class I is responsible for presentation of peptide from inside the cell, such as viral

fragments, and inducing CD8+ cytotoxic immune responses. Class II contains genes for presenting peptides from outside the cell to CD4+ T-lymphocytes for eliciting helper T responses and B-cell antibody responses. Class III encodes complement components (C2, C4A, C4B, and CFB), TNF and other immune genes. Meta-analysis, using the results of GWAS, has shown that the strongest and most potent genetic risk factors for SLE reside in the HLA region (55, 56). Although due to the enormous complexity of this region, the detailed nature of the susceptibility variants remains elusive, the most consistent HLA associations with SLE can be found in the class II alleles *HLA-DR3* (*DRB1*0301*) and *HLA-DR2* (*DRB1*1501*) in white populations (57). Large GWAS found that the best association was a combination of HLA alleles including *B*08:01* and *B*18:01* in class I, *DQB1*02:01*, *DRB3*02:00*, and *DQA*01:02* in class II and SNP (rs74290525) located in *SLC44A4*, class III (58). Several mechanisms have been proposed to explain the association of *DR* and *DQ* alleles with autoimmunity. Variation in binding regions of DR or DQ molecules could lead to the presentation of limited, specific sets of self-peptide, allowing autoreactive T cells to escape tolerance and enter the periphery (59). Another theory is that DR or DQ molecules could select autoreactive T cells or fail to select appropriate regulatory T cells populations (60). Misfolded class II genes may inappropriately present an endogenous antigen (61).

Genes outside the HLA system include genes involved in T and B cell signaling (the most important *PTPN22*, *BLK*, *BANK1*, *PXK*, *TNFSF4*), transcription factors (the most important *ETS1*, *IKZF1*, *IKZF2*, and *IKZF3*) and cytokines (the most important *IL10* and *BAFF*) (29, 62).

The *PTPN22* gene encodes tyrosine phosphatase nonreceptor type 22, which is expressed primarily in lymphoid tissues. This enzyme is involved in several signaling pathways associated with the immune response: inhibits T-cell activation, it is involved in the central and peripheral tolerance of B cells and in multiple B cell developmental stages (29, 62, 63). A gain-of-function variant that encodes a more active phosphatase, reduces the threshold for T cell receptor signaling, alters B-cell receptor signaling with enhanced B cell autoreactivity, affects the removal of developing autoreactive B cells in humans and is involved in the central and peripheral tolerance of B cells, thus promoting autoimmunity (64).

B-lymphoid tyrosine kinase (Blk) has many functions in intracellular signaling and in the regulation of B cell proliferation, differentiation and tolerance (29). It is encoded by the *BLK* gene. *BLK* variants associated with SLE susceptibility result in decreased *BLK* expression that may affect development and functional responses in B cells.

BANK1 encodes an adaptor protein, named the B cell scaffold protein, with ankyrin repeats 1 that facilitates intracellular calcium release and alters the B cell activation threshold (29). *BANK1* variants, linked with SLE, decrease B cell signaling and enhance the expansion of memory B cells (65). The rare variants found in patients impair suppression of IFN-I in human B cell lines and increase pathogenic lymphocytes in lupus-prone mice (66).

A phox domain (PX) containing protein is included in the regulation of synaptic transmission (29). It is encoded by the *PXK* gene. Risk variants in the *PXK* gene which contribute to SLE lead to reduced B cell receptor internalisation. This may have an effect on the regulation of B cell signaling, but the genetic mechanism behind this alteration in B cell receptor internalisation is still not understood (67).

The tumor necrosis factor ligand superfamily member 4 (Tnfsf4) is an inflammatory factor that has been associated with different inflammatory diseases and cancers. Tnfsf4 is primarily expressed on activated CD4+ T cells. It is hypothesised that increased expression of the *TNFSF4* predisposes to SLE by increasing T cell and antigen presenting cell interaction or by destabilising peripheral tolerance through inhibiting IL-10-producing CD4+ type 1 regulatory T cells (68).

ETS1 encodes protein C-ets-1 that belongs to the Erythroblast Transformation Specific family of transcription factors (ETS). Ets1 is primarily expressed in lymphocytes and is present at reduced levels in peripheral blood mononuclear cells from SLE patients (69). It plays an important role in maintaining B cell tolerance.

Ikzf1, Ikzf2 and Ikzf3 are three members of the Ikaros family of zinc finger proteins. They are transcription factors that regulate lymphocyte differentiation, proliferation, and self-tolerance through regulation of B-cell, T-cell, and dendritic cell signaling (70). The functional mechanism by which causative variants in *IKZF1*, *IKZF2*, and *IKZF3* are linked to SLE remains unknown (29).

Interleukin 10 (IL-10) is an immunoregulatory cytokine with both immunosuppressive and immunostimulatory properties. It is mainly produced by B cells, which use it for their proliferation, and by myeloid cells, which use it to reduce proinflammatory responses. *IL10* risk alleles in SLE patients result in increased IL-10 production by the peripheral blood B cells and monocyte and elevated serum IL-10 levels correlate with disease activity (71). High levels of IL-10 contribute to SLE susceptibility and severity by promoting B-cell proliferation (72).

B cell-activating factor (BAFF) is a cytokine that promotes the survival, proliferation and maturation of B lymphocytes and is encoded by the *TNFSF13B* gene. Risk variants increase BAFF expression and are related to active disease, renal and hematological involvement (73). Excessive BAFF expression is associated with increased survival and expansion of autoreactive B cells.

Finally, **the fourth group** contains genes important for immune functions but whose role has not yet been elucidated. Some of these genes encode for membrane proteins (e.g. *C3orf21*, *DHCR7*, *PLD2*), but most encode gene products with unknown immune functions (29).

These genetic variants or single nucleotide polymorpisms (SNPs) detected within the designated loci are common but have a small effect on disease susceptibility, i.e. carry a small relative risk of disease. They explain 30-50% of SLE heritability, which means that other factors are more important in SLE susceptibility, such as rare genetic variants, epigenetic effects and epistasis (gene interactions) (74, 75).

Apart from SNPs, other genetic diversities participate in SLE susceptibility, such as copy number variations (CNVs) that include deletion, insertion, and duplication of genomic regions (76). Thus, for example, *NCF1* encodes neutrophil cytosol factor 1, which acts as a cytosolic subunit of neutrophil NADPH oxidase. Some SNPs in the *NCF1* gene result in reduced oxidative burst and low production of reactive oxygen species, which lead to an increased expression of IFN-I-regulated genes, and is associated with SLE (77). Decreased CNVs (0 and 1 copy) of *NCF1* also predispose to SLE, but increased CNVs of *NCF1* ($\geq$ 3 copies) have a protective effect (78).

Overall, gene loci with the highest risk of developing SLE are complement components *C1q*, *C4A*, *C4B* and *C2B*, followed by the genes involved in the signaling pathway of IFN-I (*IRF5*, *ITGAM*), and genes involved in the signaling of B lymphocytes (*BANK1* and *BLK*).

SLE and other autoimmune diseases share many susceptibility loci, but the role of a particular locus in predisposition is not always the same in different diseases. Sometimes the same variant has the opposite effect (e.g. some *PTPN22* variants predispose to SLE but are protective against inflammatory bowel diseases) or different degree of effect (e.g. some *NCF1* variants have a strong link with SLE, but a modest link with Sjögren's syndrome and only mild link with rheumatoid arthritis) (29).

In most cases, SLE is a polygenic disease and both genetic predisposition and environmental factors are necessary to the development of the disease. These gene variants are rarely located within the coding segments. To detect more common variants that are unlikely to reach genome-wide significance, it is necessary to focus on multiple independent variants in each locus, meta-analysis of existing data, and to develop international collaborations to further increase the power of association studies, with integration of information from gene expression profiles, protein complexes, and signal transduction pathways and regulatory networks (36).

1.1.2.2. Monogenic SLE

SLE has long been thought to be an exclusively polygenic disease associated with gene polymorphisms, but in rare cases, which account for only between 1 and 3% of the total number of SLE patients, the disease can result from a single gene mutation and this form of lupus represents monogenic SLE (13, 79). Although these forms of the disease are rare, they are important because they provide insight into the mechanism of the disease, better understanding of the pathogenesis of SLE and the molecular mechanisms of immune tolerance and allow the development of new, targeted treatment strategies (79). The discovery of monogenic forms of lupus has supported the concept that SLE is not a single disease but a heterogeneous collection of individually rare, genetically distinct disorders (lupus-like diseases or lupus subtypes) (36).

The main features that raise the suspicion on a monogenic form of SLE include early onset of the disease (especially before the fifth year of life), evidence of a Mendelian type of inheritance or a strong, positive family history of the same disease, an atypical clinical picture (e.g., severe forms of cutaneous, neurological or joint manifestations), disease refractory to standard therapy, male gender and consanguinity, even in the absence of a positive family history (13, 80).

Due to GWAS, several groups of genes involved in different physiological pathways have been discovered, the mutation of which causes monogenic forms of SLE (Figure 2). In contrast to polygenic SLE, in monogenic forms of SLE almost 30 genes related to single mutations in DNA-coding regions of the genome have been identified (81). Similar to susceptibility genes in polygenic forms of the disease, genes that cause monogenic forms of SLE can be divided into several groups. These are genes related to the complement system (deficiencies of complement components and its disorders) which are important for the clearance of immune complexes, followed by a group of genes involved in lymphocyte signaling (within T and B

lymphocytes) and IFN signaling pathways involving nucleic acid recognition or interferon production (29, 82).

It has been shown that an hereditary deficiency of certain components of complement (C1q, C1r, C1s, C2, C3 C4A, C4B) is linked with SLE susceptibility (83). The highest prevalence of SLE, up to 90%, is related to the homozygous form of deficiency of the C1q component of complement, while the risk of developing SLE due to the lack of other components is lower. C1q is encoded by three genes (*C1QA*, *C1QB*, and *C1QC*) on chromosome 1 (84). Deficiency of C1q results in inadequate clearance of apoptotic debris, which may stimulate the presentation of self-antigen and subsequent loss of tolerance. Characteristics of these forms of monogenic SLE include early onset of the disease, recurrent pyogenic infections, or infections caused by *Neisseria meningitidis*, frequent photosensitive skin rash, nephritis, oral ulceration, arthritis and often a presence of negative antinuclear antibodies (ANA) (85, 86). C1r and C1s deficiencies are rare and these patients usually die at a young age due to severe infections (86). SLE develops in only 10% of patients with C2 deficiency due to the fact that C2 can be bypassed by the alternative complement pathway. These patients have similar characteristics to other SLE patients besides frequent infections. C4 is encoded by two genes, *C4A* and *C4B*, located on chromosome 6. C4 deficiency causes an increase in the number of self-reactive B cells and alters B cell tolerance and is associated with glomerulonephritis development and high levels of autoantibodies (86). Complement deficiencies are inherited as autosomal recessive disorders (86).

The next group of monogenic forms of SLE is those caused by mutations in genes associated with IFN signaling pathways, the so-called interferonopathies. Examples are pathogenic variants in *TREX1*, *IFIH1*, *SAMHD1*, *RNASEH2A*, *RNASEH2B* and *RNASEH2C*.

TREX1 is a gene involved in the repair of DNA damage and is responsible for degradation of genomic DNA in response to DNA damage. The mutation in *TREX1* leads to the overproduction of IFN-I due to an accumulation of self DNA because of inadequate clearance of extracellular, endosomal, and cytosolic DNA, which constitutes a damage-associated molecular pattern, inappropriately stimulates the activation of intracellular nucleic acid sensing pathways, activates IFN-I response and systemic inflammation (86, 87). Patients with *TREX1* mutations may develop a variety of autoimmune diseases, including familial chilblain lupus, Aicardi-Goutières syndrome, retinal vasculopathy with cerebral leukodystrophy and cerebral SLE. Familial chilblain lupus is characterised by painful, sometimes ulcerative, skin lesions

similar to frostbite that appear in early childhood. Aicardi-Goutières syndrome is marked by severe encephalopathy, progressive neurological damage, basal ganglia calcifications and white matter abnormalities of the brain with, in some instances, the occurrence of skin changes resembling frostbite (88, 89). Most patients show biallelic variants within *TREX1* with autosomal recessive inheritance but, in some of them, heterozygous mutations have been identified with autosomal dominant inheritance (86).

Patients with *IFIH1* mutations can develop early-onset SLE and Aicardi-Goutières syndrome-like disease, including musculoskeletal involvement (86). *IFIH1* mutations are inherited as autosomal dominant disorders (86).

The *SAMHD1* gene encodes Sterile Alpha Motif (SAM) domain and Histidine-Aspartic (HD) domain-containing protein 1, included in cell stability and preventing reverse transcription of retroviruses (86). *SAMHD1* mutations cause an increase in DNA damage, which results in upregulation in IFN-stimulated genes. Patients can develop SLE, Aicardi-Goutières syndrome and chilblain lupus. *SAMHD1* mutations are inherited as autosomal recessive disorders (86).

RNASEH2A, *RNASEH2B* and *RNASEH2C* are three genes that encode for the three protein components of the RNaseH2 (Ribonuclease H2) complex, enzyme involved in breaking down RNA-DNA hybrids formed during DNA replication, when these molecules are no longer needed. Mutations in these genes result in accumulation of ribonucleotides in genomic DNA during replication which results in chronic DNA damage and IFN-I production (86). The patients have cutaneous changes, photosensitivity, arthritis, lymphopenia, and autoantibody formation. *RNASEH2A*, *RNASEH2B* and *RNASEH2C* mutations are recessively inherited (86).

From the third group of genes, those involved in lymphocyte signaling (within T and B lymphocytes), whose mutations can cause monogenic forms of SLE, it is important to emphasise genes in the Ras/MAPK signaling pathway and *PRKCD*. The rat sarcoma virus (Ras)/mitogen activated protein kinase (MAPK) pathway is included in the number of cellular processes, such as proliferation, differentiation, apoptosis, and are important for the maturation of T lymphocytes in the immune system (86). Examples of genes associated with the Ras/MAPK pathway, whose mutations are associated with the clinical picture of so-called "RASopathies" include *PTPN11*, *KRAS*, *NRAS*, *SOS1*, *SHOC2* and *SHP2* and are inherited in an autosomal dominant manner (86). Mutations in the gene system mentioned are linked with Noonan's syndrome, which is characterised by facial dysmorphia, short stature, congenital heart

defects, hemorrhagic diathesis and increased risk of malignant diseases. Patients who developed SLE have also been described in the literature and hepatosplenomegaly, lymphadenopathy, increased frequency of pericarditis and autoimmune cytopenias are some of the frequent features described in their clinical picture (90). Another example are mutations in the *PRKCD* gene, which encodes the protein kinase C delta (PKCδ), which is involved in regulating B cell development, proliferation and apoptosis. Monogenic forms of SLE associated with a mutation in *PRKCD*, which causes dysregulated B cell proliferation and loss of B cell tolerance, are characterised by hepatosplenomegaly, lymphadenopathy and susceptibility to infections, especially chronic Epstein-Barr virus (EBV) and cytomegalovirus (CMV) infections, with typical features of SLE including autoantibody production as well as an increased incidence of glomerulonephritis (87, 91). *PRKCD* mutations are inherited in an autosomal recessive manner (86).

Other examples of Mendelian inheritance in SLE include mutations in *DNASE1* (deoxyribonuclease 1) and *DNASE1L3* (deoxyribonuclease 1 like 3) genes, that encode proteins involved in the nucleic acid degradation pathway. The two enzymes digest extracellular DNA from apoptotic cells. In the event of dysfunction of these enzymes, circulating microparticles from apoptotic cells may activate plasmacytoid and myeloid dendritic cells, resulting in the production of IFN-alpha (86). *DNASE1* mutations are dominantly inherited, while *DNASE1L3* mutations are recessively inherited (86).

FASL gene encodes Fas ligand involved in programmed cell death (apoptosis) (92). Mutations in the *FASL* gene, inherited in an autosomal dominant manner, result in failure to remove autoreactive cells and lead to an autoimmune lymphoproliferative syndrome but these were also described in patients with SLE (93).

Recently, mutations in the *LRBA* gene were associated with cSLE (94). The *LRBA* gene encodes lipopolysaccharide-responsive and beige-like anchor protein. It is an intracellular protein involed in the regulation of trafficking intracellular vesicles. LRBA promotes the expression of cytotoxic T lymphocyte-associated protein 4 (CTLA4). LRBA deficiency, inherited in an autosomal recessive manner, is associated with intense autoantibody production (95) and may be associated with clinical manifestations of SLE (94).

1.1.2.3. Epigenetics in SLE

Epigenetic dysregulation has been described as having a very important role in the pathogenesis of SLE (96). Epigenetic changes are functionally relevant changes to the genome that do not involve a change in the nucleotide sequence, but affect gene activity and expression and may cause heritable phenotypic changes. The three most important epigenetic mechanisms included in SLE pathogenesis are altered patterns of DNA methylation, histone modifications and noncoding RNAs (ncRNA) in autoreactive T cells and B cells (97).

DNA methylation is a process by which methyl groups are added to the DNA molecule (on CG dinucleotides) and typically acts to suppress gene transcription. In SLE patients, global T-cell hypomethylation is described, which results in overexpression of the number of autoimmune-related genes (98). A genome-wide DNA methylation study has found that in CD4+ T cells of SLE patients compared to healthy controls, there are 105 hypermethylated and 236 hypomethylated CG sites in the 27,578 CG sites located within the promoter regions of 14,495 genes (99). The extent of hypomethylation of CG dinucleotides correlates with autoantibody production, anti-ds DNA level and disease activity (97). Demethylation of X chromosome in women may be the reason for the predominance of SLE in females (97).

Epigenetic modifications of histones, proteins wrapping the DNA to form the chromosomal structure nucleosome, important for SLE, include acetylation and methylation. Acetylation is characterised by the addition of an acetyl group to histone proteins, which leads to a transcriptionally active chromatin structure (euchromatin) and increases gene expression. Deacetylation by contrast leads to an inactive, condensed chromatin structure (heterochromatin). Histone methylation includes the transfer of methyl groups to histone with variable effects on gene expression: in some cases it leads to transcription activation, while in another, gene expression is reduced. Histones form octamers composed of a central part of two copies of both histones H3 and H4, flanked by two copies of histones H2A and H2B. Histones of the histone octamer contain tail residues, lysine (K) and arginine (R), which may be methylated at varying degrees. It was found that in CD4+ T cells of SLE patients histones H3 and H4 are hypoacetylated and histone H3K9 is hypomethylated (100). It is also known that NETs from SLE patients contain more acetylated H4-K8,12,16 and H2B-K12. Hyperacetylated chromatin of NETs may activate myeloid and plasmacytoid dendritic cells and cause activation of autoreactive T and B cells (100).

A microRNA (miRNA) is a small single-stranded ncRNA molecule that plays important roles in RNA silencing and post-transcriptional regulation of gene expression. It inhibits translational activity of target genes and/or reduces messenger RNA (mRNA) stability. It was shown that one of the epigenetic mechanisms included in SLE pathogenesis involves some ncRNAs. For example, in the peripheral blood mononuclear cells (PBMCs) from SLE patients, reduced expression of microRNA-146a (mir-146a) was revealed (101). It is a negative regulator whose function is to prevent overactivation of inflammatory responses in multiple immunological pathways including the IFN-I pathway. Decreased mir-146a expression results in upregulated expression of IFN response genes in SLE patients (101).

1.1.2.4. Environmental triggers

Different environmental factors, such as ultraviolet light, especially ultraviolet B, infections and toxins are suspected of triggering the onset and exacerbation of SLE (16). Some environmental triggers may influence through epigenetics. Exposure to ultraviolet light is believed to stimulate apoptosis of cells (keratinocytes), which leads to exposure of the degradation products of DNA on the cell surface, acting as a trigger in the production of antibodies directed against components of the nucleus (16). In addition, there are hypotheses that infections, especially herpesvirus viruses (particularly EBV), can stimulate the autoimmune process by activating the innate immune system and B lymphocyte differentiation, stimulating autoantibody production, although the details of these complex mechanisms are not yet known. Some drugs (e.g., minocycline, procainamide, chlorpromazine and interferon alpha etc.) may induce the SLE by altered patterns of DNA methylation. Smoking, which may induce an inflammatory reaction, is also described as one of the risk factors. Possible early-life risk factors include low birthweight (< 2500 g), preterm birth (≥1 month early) and exposure to agricultural pesticides (102).

1.1.2.5. Hypothetical model of SLE development

According to some hypotheses, the disease develops in three phases (103). In the first, asymptomatic phase, with interaction still not fully clarified regarding environmental, genetic and epigenetic factors, immune tolerance to nuclear self-antigens is interrupted. In the second phase, there is further dysregulation and enhancement of the impaired immune response, which

can be demonstrated by detection and laboratory measurements of different antibodies, for example, ANA. In the third, final phase, the target organs are damaged (skin, kidneys, blood vessels, joints and brain etc.) by an inflammatory reaction and there is a clinical manifestation of the disease (16).

Immune system disorders in SLE affect both innate and adaptive immunity (104, 105). Innate immune disorders contribute to increased exposure to self-antigens, and include decreased clearance of apoptotic cells, decreased phagocytosis and increased NETosis. NETosis is a form of cell death of neutrophils in which they produce NETs, which facilitate the extracellular destruction of microorganisms. Since NETs consist of neutrophilic DNA, RNA and histones, they are immunogenic. In the serum of patients with SLE, these extracellular traps cannot be effectively degraded. The action of these factors results in the release of IFN-I, mostly from plasmacytoid dendritic cells. Oxidized mitochondrial DNA released by SLE neutrophils can also stimulate plasmacytoid dendritic cells to produce IFN-I. IFN-I propagates the maturation of monocytes into myeloid dendritic cells and then activates antigen-presenting dendritic cells increasing their ability to present antigens, including autoantigens, to T lymphocytes. T lymphocites produce various cytokines and molecules on their surface, thereby enhancing the immune response directed to self-antigens as well as the inflammatory response. Finally, loss of immune tolerance results in an increase in the number of autoreactive effector B lymphocytes. B lymphocytes are stimulated by T lymphocytes through their interactions which are mediated by CD40 on B lymphocytes and CD40 ligand on T lymphocytes. TNF produced by dendritic cells, BAFF (binding on receptors TACI, BCMA and BAFF-R) secreted by myeloid cells, APRIL (a proliferation-inducing ligand) expressed by T cells, dendritic cells, monocytes, and macrophages, exposed self-antigens, T-cell cytokines, and many others have an important role in stimulating B lymphocytes to generate autoantibodies. The signal transducer and activator of transcription 1 and T-box transcription factor contribute to pathogenic autoantibody production. Follicular dendritic cells are also important for the activation and selection of B cells within germinal centres in secondary lymphoid organs. Ultimately, B lymphocytes produce antibodies directed against self-antigens with which they form immune complexes that are deposited in tissues, activate complement, recruit myeloid cells (especially neutrophils) and stimulate the release of enzymes from neutrophil granules and reactive oxygen radicals from macrophages, finally causing inflammation and damage to target organs. Immune complexes can be taken up through the B-cell antigen receptor on B cells or Fc receptor-γ on dendritic cells. This process can activate intracellular innate receptors,

including TLR7 and TLR9, which leads to the production of inflammatory cytokines, including IFN-I (Figure 3).

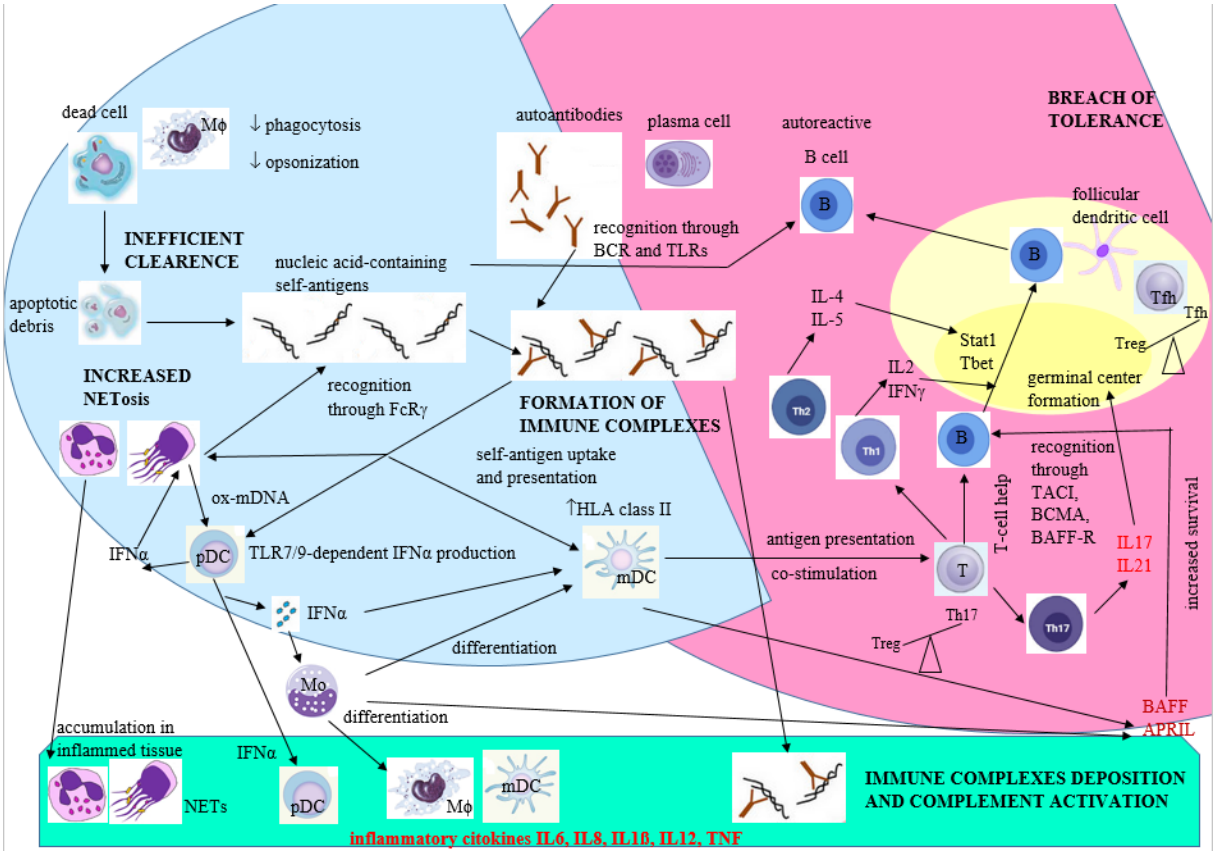


Figure 3. Hypothetical model of SLE development. Modified according to reference 105. APRIL: a proliferation-inducing ligand; B: B cell; BAFF: B-cell-activating factor; BAFF-R: B-cell-activating factor receptor; BCMA: B-cell maturation antigen; BCR: B-cell antigen receptor; FcRγ: Fc receptor-γ; fDC: follicular dendritic cell; HLA class II: human leucocyte antigen class II; mDC: myeloid dendritic cell; MΦ: macrophage; NET: neutrophil extracellular trap; ox-mDNA: oxidized mitochondrial DNA; pDC: plasmacytoid dendritic cell; Stat1: signal transducer and activator of transcription (a transcription factor); T: T cell; TACI: transmembrane activator, calcium modulator and cyclophilin ligand interactor; T-bet: a T-box transcription factor; Tfh: T follicular helper; TLR7/9: Toll-like receptors 7 and 9.

In conclusion, it can be said that adaptive immune disorders result in the formation of autoantibodies, i.e. antibodies to self-antigens, which continuously accumulate due to the innate immune disorder.

1.1.3. Clinical presentation of cSLE

SLE is a multisystem inflammatory disease which affects many organs. The clinical manifestations of SLE are very heterogeneous, thus patients may have different combinations of symptoms, organ involvement and different laboratory findings and autoantibodies. The clinical picture can vary from very mild disease to severe life-threatening conditions. Initial presentations of cSLE are often nonspecific and highly variable, which results in delays in diagnosis ranging from 1 month to 3.3 years (106). One third of children may have atypical symptoms, most often gastrointestinal (107). At the onset of the disease, 40-90% of children have general symptoms, such as fever, weakness and weight loss. There is often a combination of fever, weight loss, arthralgia or arthritis, a photosensitive and/or malar rash, and renal disease (108).

According to the results of our national study, the most common initial symptoms of cSLE were musculoskeletal (among them, arthropathy and arthritis dominated), followed by skin and mucosal involvement (especially malar rash) and general symptoms (fatigue, weakness, fever and weight loss). The following were renal-related symptoms (primarily hematuria and proteinuria). Less common were the initial symptoms related to the involvement of other abdominal organs (such as abdominal pain), cardiovascular and nervous systems as well as immune disorders such as splenomegaly, lymphadenopathy, or antiphospholipid syndrome, and it was found that the disease hardly ever began with respiratory symptoms (15). In the laboratory findings of our pediatric patients, the most prominent were an increased erythrocyte sedimentation rate, followed by hypocomplementemia (more often decreased C3 than C4), hematuria, proteinuria and anemia. Thrombocytopenia, leukopenia or pancytopenia, and increased serum creatinine were somewhat less common. The most common ANA in our patients were anti-dsDNA and antibodies to histones.

1.1.4. Classification criteria used in cSLE

There are no specific clinical or laboratory diagnostic criteria for cSLE. Although intended for use in research studies on adults, classification criteria for SLE in the adult population are often used to aid the diagnosis of cSLE (16). In 1982 the American College of Rheumatology (ACR) proposed, and then in 1997, revised the classification criteria for the assessment of adult patients with SLE (ACR-97) (109) (Table 1). As it is estimated that the presence of four criteria from the above 11 allows the classification of cSLE with very high sensitivity (96%) and

specificity (100%), they are also applied as classification criteria for cSLE. The criteria are primarily used for classification, not for the diagnosis of SLE, so it is possible that patients diagnosed as SLE do not meet the four criteria, or that those with other connective tissue diseases meet four or more classification criteria. Also, there are parameters that are important in clinical work and are not included in the classification criteria.

Table 1. 1997 Update of the 1982 American College of Rheumatology revised criteria for classification SLE. Modified according to reference 109.

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, sparing the nasolabial folds
2. Photosensitivity	Unusual reaction to sunlight resulting in a skin rash, by patient history or clinician observation
3. Discoid rash	Erythematosus raised patches with adherent keratotic scaling and follicular plugging; in older lesions atrophic scarring may occur
4. Oral ulcers	Usually painless oral or nasopharyngeal ulceration observed by a clinician
5. Arthritis	Nonerosive arthritis that involves 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	Pleuritis – pleuritic pain or rubbing heard by a clinician or evidence of pleural effusion OR Pericarditis – documented by electrocardiogram, rub, or evidence of pericardial effusion
7. Renal disorder	Persistent proteinuria greater than 500 mg/24 hours or, if quantification is not performed, greater than 3+ OR Cellular casts – red cell, hemoglobin, granular, tubular, or mixed
8. Neurologic disorder	Seizures OR psychosis – in the absence of offending drugs or known metabolic derangements (uremia, ketoacidosis, or electrolyte imbalance)
9. Hematologic disorder	Hemolytic anemia – with reticulocytosis OR Leukopenia – <4000/mm^3 total on 2 or more occasions OR Lymphopenia – <1500/mm^3 on 2 or more occasions OR Thrombocytopenia – <100,000/mm3 (in the absence of offending drugs)

Criterion	Definition
10. Immunologic disorders	Anti-dsDNA – antibody to native DNA in abnormal titer OR Anti-Sm – presence of antibody to Sm nuclear antigen OR Positive antiphospholipid antibody: - an abnormal serum level of IgG or IgM anticardiolipin antibodies OR - a positive test result for lupus anticoagulant using a standard method OR - a false-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilisation or fluorescent treponemal antibody absorption test
11. Positive antinuclear antibodies	An abnormal titer of ANA by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome

Anti-dsDNA: anti-double-stranded DNA; Anti-Sm: anti-Smith antibody; IgG: immunoglobulin G; IgM: immunoglobulin M; ANA: antinuclear antibodies

Therefore, in 2012, the international group of authors behind the research of SLE, the Systemic Lupus International Collaborating Clinics (SLICC), modified the classification criteria (110). The key difference from the ACR criteria is that despite the same number of required classification criteria, both clinical and serological criteria must be present, and patients without positive autoantibodies, or have a low complement, cannot be classified as having SLE. In addition, the SLICC criteria allow that patients with biopsy-proven lupus nephritis, according to the histological classification of the International Society of Nephrology/Renal Pathology Society, in the presence of ANA or anti-dsDNA, are classified as SLE, without other criteria being confirmed (Table 2).

Table 2. SLICC criteria for classification SLE. Modified according to reference 110.

Clinical criteria	Immunological criteria
Acute cutaneous lupus - lupus malar rash; maculopapular lupus rash; photosensitive lupus rash; subacute cutaneous lupus, etc.	**ANA** level above laboratory reference range
Chronic cutaneous lupus - discoid rash; lupus panniculitis (profundus); mucosal lupus; chilblains lupus, etc.	**Anti-dsDNA antibody** level above laboratory reference range
Oral or nasal ulcers	Presence of **antibody to Sm nuclear antigen**
Non-scarring alopecia	**Antiphospholipid antibody** positivity
Joint disease - synovitis involving 2 or more joints or tenderness in 2 or more joints and at least 30 minutes of morning stiffness	Low **C3**; low **C4**; or low **CH50**
Serositis	**Direct Coombs test** in the absence of hemolytic anemia
Renal - proteinuria representing 500 mg protein/24 hours or red blood cell casts	Must have **4 of 17 criteria**, including **at least 1 clinical criterion** and **1 immunologic** criterion OR **biopsy-proven lupus nephritis** with the presence of **ANAs** or **anti-dsDNA** antibodies
Neurologic - seizures; psychosis; mononeuritis multiplex; myelitis; peripheral or cranial neuropathy or acute confusional state	
Hemolytic anemia (in the absence of other known causes)	
Leukopenia or lymphopenia (in the absence of other known causes)	
Thrombocytopenia (in the absence of other known causes)	

ANA: antinuclear antibodies; Anti-dsDNA: anti-double-stranded DNA; Anti-Sm: anti-Smith antibody; C3: complement component 3, C4: complement component 4; CH50: total complement activity

Although the SLICC criteria are more sensitive, but less specific, the ACR-97 criteria are still the "gold standard" in the classification of patients with cSLE due to their high specificity.

In addition to the ACR and SLICC criteria, new classification criteria were published in 2019, supported by the European League Against Rheumatism (EULAR) and ACR (111). According to these criteria, a patient is classified as SLE in cases where there is a positive ANA with an accumulation of 10 or more points in 10 different domains which are grouped into 7 clinical (constitutional, hematologic, neuropsychiatric, mucocutaneous, serosal, musculoskeletal, renal) and 3 immunological domains (antiphospholipid antibodies, complement proteins, SLE-specific antibodies). Compared to the ACR-97 criteria, they showed higher sensitivity, while compared to the SLICC criteria they showed greater specificity.

Further development of classification criteria specific for cSLE is still expected, which would take into account all the particularities of cSLE.

1.1.5. Assessment of disease activity and damage in cSLE

Disease activity represents the reversible manifestations of the underlying inflammatory disease process. Increase in disease activity or a disease flare implies a quantifiable exacerbation of SLE activity in at least one organ system, which result in new or deteriorating clinical signs (112). Depending on the severity of a flare, more intensive therapy may be needed. In assessing disease activity in children, scoring systems are used that assess disease activity in adults. The severity of the symptoms of the affected organs and changes in laboratory parameters are assessed by assigning a certain score to each parameter. In the end, these scores are added up to provide a final figure. The higher the final figure, the more severe or active the disease is.

The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) is a global index that was developed and introduced in early 1985 as a clinical index to assess overall disease activity. SLEDAI is commonly used to measure the activity of cSLE. SLEDAI-2K is a more recent and modified version of SLEDAI, which allows the recording of activity during the course of the disease, as well as the appearance of any new activity or deterioation of the disease (113). The SLEDAI-2K is based on the presence/absence of 24 symptoms and laboratory parameters that represent specific manifestations in 9 organ systems. The final scores range from 0 to 105. The higher the score, the more significant the degree of disease activity. The clinical activity of the disease based on the SLEDAI-2K score is divided into 5 ranks: no activity (SLEDAI-2K = 0); mild activity (SLEDAI-2K = 1-5); moderate activity (SLEDAI-2K = 6-10); high activity (SLEDAI-2K = 11-19) and very high activity (SLEDAI-2K> 20) (Table 3).

Table 3. SLEDAI-2K score. Modified according to reference 113.

Parameter/manifestation	Description	SLEDAI-2K score
Seizure	Recent onset seizure after exclusion of metabolic, infectious or drug related causes	8
Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality after exclusion of uremia and drug causes	8
Organic brain syndrome	Altered mental function with impaired orientation, memory, or other intellectual function after exclusion of metabolic, infectious or drug related causes	8
Visual disturbance	Retinal changes after exclusion of hypertensive, infectious or drug related causes	8
Cranial nerve disorder	New onset sensory or motor neuropathy involving cranial nerves	8
Lupus headache	Severe, persistent headache which may be migrainous but must be nonresponsive to narcotic analgesia	8
Cerebrovascular accident	New onset stroke after exclusion of arteriosclerosis	8
Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages or biopsy, and angiogram proof of vasculitis	8
Arthritis	≥2 joints with pain and signs of inflammation (i.e., tenderness, swelling, or effusion)	4
Myositis	Proximal muscle aching/weakness associated with elevated creatinine phospnokinase/aldolase, electromyogram changes or a biopsy showing myositis	4
Urinary casts	Heme granular or red blood cell urinary casts	4

Parameter/manifestation	Description	SLEDAI-2K score
Hematuria	>5 red blood cells/high-power field after exclusion of stone, infection or other cause	4
Proteinuria	>0.5 g/24 hours	4
Pyuria	>5 white blood cells/high-power field after exclusion of infection	4
Rash	Inflammatory-type rash	2
Alopecia	Abnormal, patchy or diffuse loss of hair	2
Mucosal ulcers	Oral or nasal mucosal ulcers	2
Pleurisy	Pleuritic chest pain with pleural rub/effusion or pleural thickening	2
Pericarditis	Pericardial pain with ≥1 of the following: rub, effusion, or electrocardiogram/echocardiogram confirmation	2
Low complement	CH50, C3, or C4 decreased below lower limit of normal for lab	2
High DNA binding	Increased DNA binding by Farr assay above normal range for lab	2
Fever	Temperature >100.4 °F (38°C) after exclusion of infectious causes	1
Thrombocytopenia	Platelets <100 x 10^9/L after exclusion of drug related causes	1
Leukopenia	White blood cells <3 x 10^9/L after exclusion of drug related causes	1

Damage is defined as an irreversible change in an organ or organ system, which must be present for at least 6 months in order to avoid confusion between active inflammation and damage. The Systemic Lupus International Collaborating Clinics/American College of Rheumatology-Damage Index (the SLICC/ACR Damage Index) was developed in 1996 by an international group of authors to assess damage in patients with SLE that has occurred since the onset of the disease (114). The SLICC/ACR Damage Index was developed primarily for adult patients with SLE, but is also used in children. The index is based on the presence/absence of

42 elements, which include an assessment of 12 organ systems with a maximum score of 46. The values of the index increase with the progression of the disease, and at the same time the index is a good tool for assessing the prognosis and mortality. Since the SLICC/ACR Damage Index does not cover all forms of possible pediatric damage, a pediatric version of the SLICC/ACR Damage Index was developed. There is still no consensus on whether growth retardation and delayed puberty, which are included in that index, should be considered as damage given that they may be reversible (115).

1.1.6. Treatment of cSLE

In 2017, Single Hub and Access Point for Pediatric Rheumatology in Europe (SHARE) recommendations for the treatment of cSLE and lupus nephritis were published (116, 117). According to the recommendations, hydroxychloroquine is the basic drug used in the treatment of cSLE and all children with cSLE should be on hydroxychloroquine as routine. Hydroxychloroquine has a beneficial effect on skin and musculoskeletal disease, with higher rates of remission, lower rates of relapse and organ damage. Because hydroxychloroquine improves the lipid profile and, consequently, reduces the accelerated atherosclerosis of patients with cSLE, its importance as a basic drug used in the treatment of cSLE is even greater.

Systemic glucocorticoids remain the mainstay in the treatment of cSLE (118). Given significant iatrogenic complications associated with chronic glucocorticoid treatment, the goal in cSLE management is to minimise glucocorticoids to the absolute lowest necessary dose to control cSLE activity. If gradual dose reduction leads to exacerbation of the disease with oral glucocorticoids, a disease-modifying antirheumatic drug (DMARD), such as mycophenolate mofetil, azathioprine, methotrexate or cyclophosphamide, in severe cases, should be introduced (116).

Biologic therapy, primarily rituximab, is administered in cases of acute, life-threatening conditions or refractory cSLE to standard treatment (116). Rituximab is particularly effective in treating cSLE-associated cytopenias, lupus nephritis, refractory cSLE manifestations and neuropsychiatric manifestations of cSLE (16).

Belimumab is a human immunoglobulin (Ig)G_1 monoclonal antibody which targets soluble BAFF, which is elevated in SLE patients promoting abnormal B cell activation and differentiation, and antagonises its biological activity. Since 2011, belimumab has been

approved for the treatment of adults with active SLE and with autoantibody-positive SLE, and recent randomised, placebo-controlled and tested belimumab has proved to be well tolerated by paediatric patients. Safety profiles proved to be similar to those of adults and response to belimumab, compared with placebo, was consistent with the results in adults with SLE (119).

In the literature, there are cases of refractory thrombocytopenia, pancytopenia, central nervous system involvement, secondary antiphospholipid syndrome, and lupus nephritis being successfully treated with intravenous immunoglobulins (120). Therapeutic plasma exchange offers an alternative therapeutic modality for patients with SLE-related catastrophic antiphospholipid syndrome, as well as for patients resistant to other agents and where leucopenia is present (121). In patients with cSLE and antiphospholipid syndrome, antiplatelet agents should be considered for primary prevention of thrombosis, while after a thrombotic event, long-term anticoagulation therapy is indicated (122).

Guidelines for personalised cSLE treatment have not yet been defined. The treatment of monogenic SLE is different in relation to polygenic forms because it depends on the pathogenic mechanism, i.e. the molecular pathway involved in gene mutation. Thus, the choice of therapy for complement deficiencies is intravenous immunoglobulins, plasmapheresis and antibiotic prophylaxis due to the tendency of infections. Monogenic SLE linked with defective apoptosis is treated with rituximab, hydroxychloroquine and hematopoietic stem cell transplantation, and cSLE associated with overproduction of INF-alpha with anti-INF alpha monoclonal antibodies (123). Recently, the Food and Drug Administration (FDA) approved IFN-I receptor antagonist anifrolumab for the treatment of adult patients with moderate to severe SLE along with standard therapy (124). It is a fully human monoclonal antibody that binds to subunit 1 of the IFN-I receptor, blocking the activity of IFN-I.

1.2. IMMUNOGLOBULIN A VASCULITIS

IgAV, formerly known as HSP, is the non-granulomatous systemic vasculitis, histologically characterised by infiltration of the walls of the blood vessels, mainly arterioles, capillaries and venules, by neutrophils with deposits of immune complexes containing predominantly IgA in the endothelium of small blood vessels in the skin, synovial membrane, intestines and urinary system (125). It is the most common systemic vasculitis in childhood, clinically manifested as purpuric rash accompanied by either gastrointestinal symptoms, arthritis, and/or nephritis (126). The main attribute of the disease is palpable purpura which affects the lower extremities and

buttocks (125). The term HSP was replaced with IgAV by the 2012 revised Chapel Hill International Consensus Conference for Nomenclature of Vasculitides (127).

Although the disease is most often self-limiting and lasts an average of up to four weeks, various acute and chronic complications are possible (128, 129). Among the acute complications of the disease, the most frequent are those related to the gastrointestinal system, including intussusception, bowel perforation, and massive bleeding as the most serious ones (130). However, the renal aspect of the disease is the most important chronic complication and the main cause of morbidity and mortality among children suffering from IgAV, and thus the main prognostic factor (131).

IgAV can also infrequently occur in adulthood, but the clinical manifestations are different: adult patients with IgAV have a higher frequency of joint involvement while abdominal pain is rarely present and some reports suggest that in adults there is a higher rate of progression to end-stage renal disease (132).

Even though IgAV is the most common form of vasculitis in childhood, many questions remain unanswered and there are still dilemmas concerning IgAV etiopathogenesis, diagnosis and determining disease severity, duration of the autoimmune process, optimal treatment choices and prognosis, the most important of which are: which patients will develop more severe forms of gastrointestinal complications, which patients will develop IgAV nephritis (IgAVN) that will progress to kidney failure and how to treat patients with IgAVN (133).

1.2.1. Epidemiology of IgAV

The incidence of IgAV varies worldwide ranging from 3 to 27 cases per 100,000 children, while the prevalence varies between 6.1 and 20.4 per 100,000 children (125, 134, 135, 136). A recent study published by our research group showed the most accurate estimate of IgAV incidence in Croatia as being 6.79 per 100,000 children, which is similar to other European countries (137) (Figure 4).

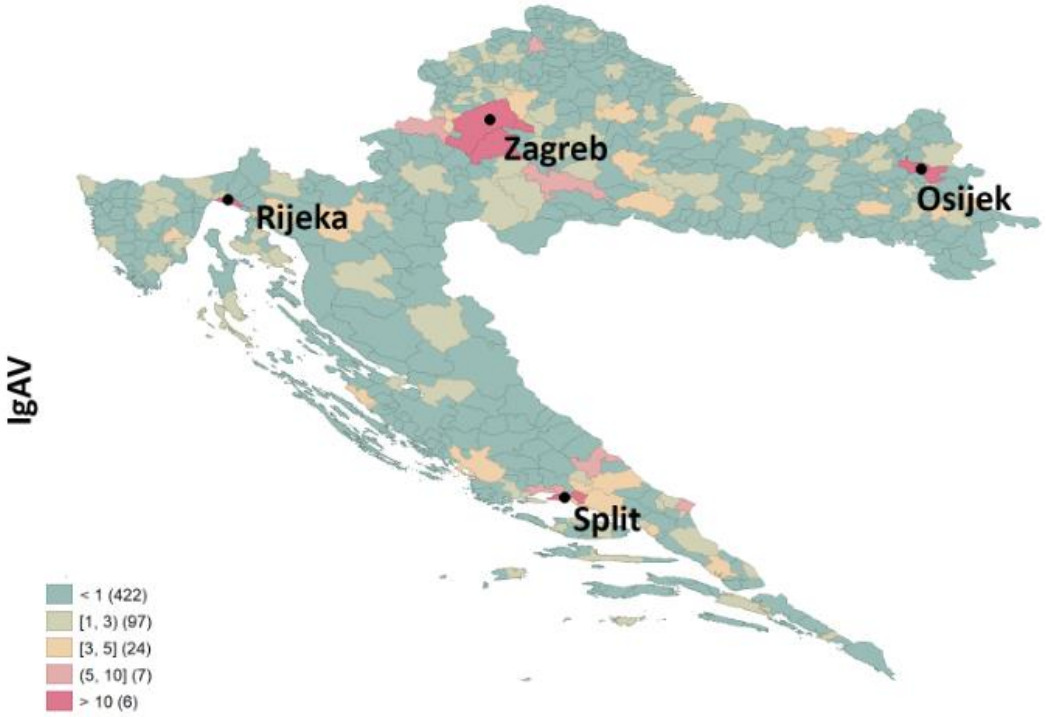


Figure 4. Raw count of IgAV average annual incidences per 100,000 children in different part of the Republic of Croatia. Numbers in parentheses represent the number of administrative units belonging to each category. Reproduced from reference 137.

The highest occurrence of IgAV is found in East Asians, intermediate in Europeans, and the lowest in individuals of African ancestry. Furthermore, we have demonstrated that both IgAV and IgAVN may not be randomly distributed in an area, but clustered more around cities, while IgAVN showed linear clustering in the eastern part of Croatia, which follows the course of the Drava and Danube rivers as described (137). The reasons for these geospatial variations of IgAV and IgAVN are not known, but it can be hypothesised that hotspot clusters appear where genetic and environmental factors substantially overlap. The median age of onset is around 6 years and 90% of patients are younger than 10 years, with males being more frequently affected than females at a ratio of 1.5:1 (126). The prevalence of IgAVN in Croatia is 19.6%, with the average annual incidence rate at 1.33 per 100,000 children, which corresponds to the range described in the literature, according to which IgAVN occurs in 20-60% of children suffering from IgAV (138, 139).

1.2.2. Etiopathogenesis of IgAV

Similar to SLE, the complexity of the etiopathogenesis of IgAV is reflected in the interaction of genetic and environmental factors, with special emphasis on infections (135, 140).

1.2.2.1. Genetic risk factors

Three groups of evidence support the genetic basis of the disease (201). First, the incidence and geospatial distribution of IgAV and IgAVN differ around the world and between different ethnicities (126). Second, some studies have indicated that the incidence of IgAV has a tendency for familial aggregation (142, 143, 144). Third, GWAS highlights the significance of common gene variants in the pathogenesis of this disorder (145, 146). Nevertheless, unlike SLE, the overall heritability of IgAV cannot be estimated, mostly due to the lack of large family-based studies or twin studies (141).

Studies investigating the genetic background of the disease were expected to identify candidate genes associated with IgAV susceptibility, particularly IgAVN (133). However, GWAS have indicated so far the significance of the HLA class II genes (145, 146). It is important to emphasise that there are currently no large GWAS for pediatric IgAV and IgAVN, and only two small studies have been published to date. The results summarise the complex pathogenesis of IgAV caused primarily by interactions among a number of different genes, with a limited role played by each individual gene in disease development.

The first GWAS on IgAV susceptibility included 285 patients diagnosed with IgAV and 1,006 healthy controls from Spain, who were genotyped by Illumina HumanCore BeadChips (145). The study highlighted the significance of the HLA class II genes, particularly the amino acid positions 13 and 11 in the *HLA-DRB1* gene, although the small sample size did not allow analysis to further dissect this signal and reported no significant associations outside the HLA region. The polymorphisms with the highest risk of developing disease were detected in the *HLA-DQA1* and *DQB1* intergenic zone and at the *HLA-DRB1*11* and *B1*13* loci, and a slightly lower risk was associated with HLA class I alleles. The top SNP proved to be rs9275260 in the region between *HLA-DQA1* and *DQB1* genes, with a relatively large effect size (OR = 1.79, 95%, CI = 1.47–2.17) and attained genome-wide significance.

The second GWAS study included a well-characterised study group of 46 patients with childhood-onset IgAV (146). Almost all patients with IgAV included in the study developed

IgAVN, hence the results are referred to as IgAVN. The authors also assessed the results of 49 children with biopsy-proven Crohn's disease and/or orofacial granulomatosis and a large reference population consisting of 18,757 Finnish bone marrow and blood donors, representing the Finnish population. The most important result was that haplotype *DQA1*01:01/DQB1*05:01/DRB1*01:01* was associated with susceptibility to IgAV but not with other autoimmune diseases, while these alleles were protective in patients with inflammatory bowel disease. The authors observed that two of these three alleles (*DQA1*01:01*, *DQB1*05:01*) have been linked to an increased risk for IgA nephropathy (IgAN) in previous research. IgAN is the clinical entity, around which there was a certain amount of controversy over whether the disease is a form of IgAV limited to the kidney in the absence of extrarenal clinical signs (147, 148).

The results of the GWAS to date classify IgAV as a prototype of a disease related to HLA class II loci, therefore sharing some features with giant cell arteritis and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). However, unlike AAV, in which GWAS showed that different ANCA specificities within the AAV group have a different genetic background, no similar data were obtained in IgAV, nor potential susceptibility loci to IgAV were associated with different phenotypes, i.e., IgAVN or severe gastrointestinal complications (149, 150).

The role of the HLA class II region has also been studied in other genetic studies, identifying *HLA-DRB1*01* as a susceptibility allele in Spanish and Italian patients, and *HLA-DRB1*11* in patients from Italy, India and Turkey (151, 152, 153, 154). The same studies suggested that *HLA-DRB1*07, DRB1*10* and *DRB1*03* alleles may have a protective role.

Although GWAS of IgAV have not detected potential susceptibility loci to IgAV outside HLA class II genes which attained a genome-wide level of significance, previous studies have shown that variants in various non-HLA genes associated with immune and inflammatory response (such as genes for cytokines, chemokines, adhesion molecules, T lymphocytes and nitric oxide production), and in particular SNPs, may also have significance in the etiopathogenesis of IgAV (155). Probably the reason for such GWAS results is that the studies conducted are underpowered to detect smaller allelic effects outside the HLA region, so numerous other non-HLA risk alleles escaped statistical detection (141). In contrast to GWAS, other studies showed that some of non-HLA genes could be associated, not only with disease susceptibility, but also with different disease phenotypes and IgAV severity.

The most important non-HLA genes associated with IgAV susceptibility include cytokines genes (*IL18, TGFB1*), chemokines genes (*MCP1*), adhesion molecules genes (*SELP*), renin-angiotensin system (RAS) genes (*Agt, ACE*) and other different genes (*C1GALT1*, *NOS2A*, *eNOS*, *PON1*, *MEFV*) (155).

It is hypothesised that genetic predisposition and/or mucosal infection and concomitant interleukin-6 production cause aberrant glycosylation by altering the glycosylation machinery (156). Two potential genetic loci may be responsible for aberrant glycosylation: *C1GALT1* and *C1GALT1C1*. They were identified in a GWAS in adult patients with IgAN and increased serum levels of galactose deficient IgA_1 (Gd-IgA_1) (157). Aberrantly glycosylated IgA_1 plays a key role in the pathogenesis of IgAV (158). Core 1 synthase glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (C1GALT1) is an enzyme that catalyses the process of the attachment of galactose to N-acetylgalactosamine (GalNAc). Some *C1GALT1* polymorphisms result in reduced activity of this molecule, which could lead to aberrant glycosylation (159). Five *C1GALT1* polymorphisms were evaluated in patients with IgAV, but only one was proposed as a genetic risk factor for developing IgAV (160). The variants are inherited in an autosomal dominant manner.

The *C1GALT1C1* gene on the X chromosome encodes a type II transmembrane protein that acts as a molecular chaperone important for the folding, stability and full activity of the core 1 synthase glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (156, 157). *C1GALT1C1* variants are inherited in an X-linked recessive manner.

Interleukin-18 (IL-18) is a proinflammatory cytokine, also known as an interferon-gamma inducing factor, which is encoded by the *IL18* gene. This protein induces IFN-gamma and other T-helper (Th)1 cytokines. It plays an important role in the differentiation of naive T cells into Th2 cells. IL-18 is also able to incite severe inflammatory reactions, which could explain its role in certain inflammatory disorders such as chronic inflammation and autoimmune disorders (161). Genetic variants located at the promoter of the *IL18* gene may be associated with a predisposition to IgAV, but, interestingly, other variants are linked with SLE susceptibility (161, 162). *IL18* rs187238 –137 G allele frequency was increased in patients with IgAV compared to controls. Explicit information on the mode of inheritance cannot be found in the literature.

Transforming growth factor beta 1 (TGF-β1) is a multifunctional cytokine included in the control of cell growth, cell proliferation, cell differentiation and apoptosis. It can inhibit the

actions of other T cells (interleukin-1- and interleukin-2-dependent activated T cells, quiescent helper T cells and cytotoxic T cells). A genetic variant at the promoter of *TGFB1* gene was found to increase IgAV susceptibility, which is explained with the finding of an increased number of TGF-β-secreting T cells in children with acute IgAV (163). Carriers of *TGFB1* rs1800469 –509 TT genotype may have an increased risk for IgAV. Explicit information on the mode of inheritance cannot be found in the literature.

Monocyte chemoattractant protein 1 (MCP1) is a chemokine that is a strong chemoattractant for monocytes, memory T cells and dendritic cells to the sites of inflammation as a result of tissue damage or infection. Some *MCP1* variants showed to be susceptibility factors for IgAV (164). *MCP1* –2518 TT genotype and the *MCP1* –2518 T allele are considered susceptibility factors for IgAV. Explicit information on the mode of inheritance cannot be found in the literature.

P-selectin, a cell adhesion molecule, is involved in the recruitment of leukocytes on the vascular surface at inflammatory foci, their extravasation and the adhesion of platelets to the endothelium. Genetic variants located at the promoter of P-selectin gene (*SELP*) were found to increase IgAV predisposition (165). The *SELP* -2123 GG genotype and *SELP* -2123 G allele were linked to an increased IgAV predisposition. There is no explicit information on the mode of inheritance in the literature.

Angiotensin (Agt) II is a peptide hormone that causes vasoconstriction of the venous and arterial smooth muscle and an increase in blood pressure. Some *Agt* polymorphisms were described as a susceptibility factor in IgAV (166). *Agt* rs4762 T174M-T, rs699 M235T-MT, rs699 M235T-TT and rs699 M235T-T were associated with IgAV susceptibility with no clear information on the mode of inheritance.

The angiotensin-converting enzyme (ACE) converts the angiotensin I to the active vasoconstrictor angiotensin II. Several authors found that some *ACE* variants were linked with increased IgAV susceptibility (166, 167). *ACE* I6D-D, I6D-DD, I6D-ID/DD and I6D-ID+DD were proposed as susceptibility variants in the context of IgAV without information on the mode of inheritance.

Nitric oxide synthases (NOS) are enzymes that synthesise nitric oxide (NO). Endothelial NOS (eNOS), encoded by *eNOS*, is primarily responsible for the generation of NO in the vascular endothelium, while inducible NOS2A, encoded by *NOS2A*, is expressed in hepatocytes. eNOS in the vascular endothelium plays a crucial role in regulating vascular tone,

cellular proliferation, leukocyte adhesion and platelet aggregation. *NOS2A* and *eNOS* polymorphisms are found to be associated with disease predisposition and increased levels of NO were detected in children with acute phase IgAV (168, 169). The information about the mode of inheritance is not available.

Serum paraoxonase and arylesterase 1 (PON1) is a glycoprotein which associates with high-density lipoprotein in the circulation and is involved in the prevention of lipid peroxidation, reducing atherosclerosis. Decreased activity of PON1 was described in patients diagnosed with vasculitis, by influencing the development and progression of arterial damage (170). *PON1* polymorphisms located at coding regions were found to increase the frequency of IgAV (171). There is no data about the mode of inheritance of these variants.

MEFV (Mediterranean fever) is a gene that encodes a protein called pyrin (also known as marenostrin), which is produced in some white blood cells (neutrophils, eosinophils and monocytes) and plays a role in inflammation and in fighting infection. Some authors found an association with *MEFV* variants and IgAV susceptibility (172). Most *MEFV* variants are inherited in an autosomal recessive fashion.

Although the results of these studies provide more insight into the various molecular pathways, they are not sufficiently large, and the potential association with IgAV is not as strong as is the case with HLA genes. The list of the most important genes is summarised in Table 4.

Table 4. Summary of the most important genes associated with IgAV known to date. Modified according to reference 145.

List of the most important IgAV susceptibility genes with common variants	**List of the most important IgAV protective genes with common variants**
*HLA-B*15*	*HLA-B*7*
*HLA-B*35*	*HLA-B*40*
*HLA-B*4102*	*HLA-B*49*
*HLA-B*52*	*HLA-B*50*
*HLA-A*2*	*HLA-A*1*
*HLA-A*11*	*HLA-DRB1*3*
*HLA-A*26*	*HLA-DRB1*7*
*HLA-DRB1*0103*	*Agtrs699M235T*
*HLA-DRB1*11*	*MEFV*[1]
*HLA-DQA1*0301*	*PON*
HSPA21267GG	
IL1815187238-137G	
MCP1-2518TT	
MCP1-2518T	
TGF beta rs1800469-509TT	
Agt	
ACE	
C1GALT1rs	
NOS2A	
eNOS	
PONI192QQ	
MEFV[1]	

[1]conflicting results from literature regarding the association of *MEFV* variants M694V and E148Q with IgAV susceptibility

1.2.2.2. Epigenetics in IgAV

Similar to the SLE patients, it was shown that epigenetic changes are involved in the pathogenesis of IgAV. In the PBMC of patients with IgAVN a genome-wide excessive H3 acetylation and H3K4 methylation have been demonstrated, accompanied by a positive correlation with disease activity (173). In CD4+ T cells of patients with IgAV, in promoter and enhancer regions of interleukin 4, which is a Th2 cytokine, H3 acetylation and H3K4 methylation were increased. It was concluded that Th2 cells play a role in the pathogenesis of IgAV (173).

1.2.2.3. Environmental triggers

Although the exact etiology of the disease is not known, various infectious agents, drugs, vaccines, food allergens and insect bites may be precipitants triggering the onset of this disease in genetically predisposed individuals (130, 140, 141). In more than 75% of patients, upper respiratory tract or gastrointestinal infections precede the onset of disease (174). Multiple bacterial and viral infections have been described as triggers of IgAV: group A streptococcal infection (most common), infectious mononucleosis, subacute bacterial endocarditis, hepatitis, *Mycoplasma pneumoniae* infection, *Campylobacter* enteritis, *Helicobacter pylori* infection, *Yersinia* infection, *Shigella* infection, *Salmonella* infection, *Brucellosis*, *Legionella* species, Parvovirus, Adenovirus, Varicella-zoster virus infection and Rotavirus etc. (175, 176). The role of infections in etiology and pathogenesis is further confirmed by observations of seasonal tendencies with fewer cases seen during the summer months and showing a peak in autumn and winter.

Different non-infective triggers, such as drugs (e.g., ampicillin, erythromycin, penicillin, quinidine, quinine, losartan and cytarabine), vaccines, foods, cold temperatures and insect bites have been described to potentially be associated with a higher risk of developing IgAV (177).

1.2.2.4. Hypothetical model of IgAV development

Acording to some authors, two multi-hit pathogenesis models for IgAV and IgAVN are described (156, 178) (Figure 5).

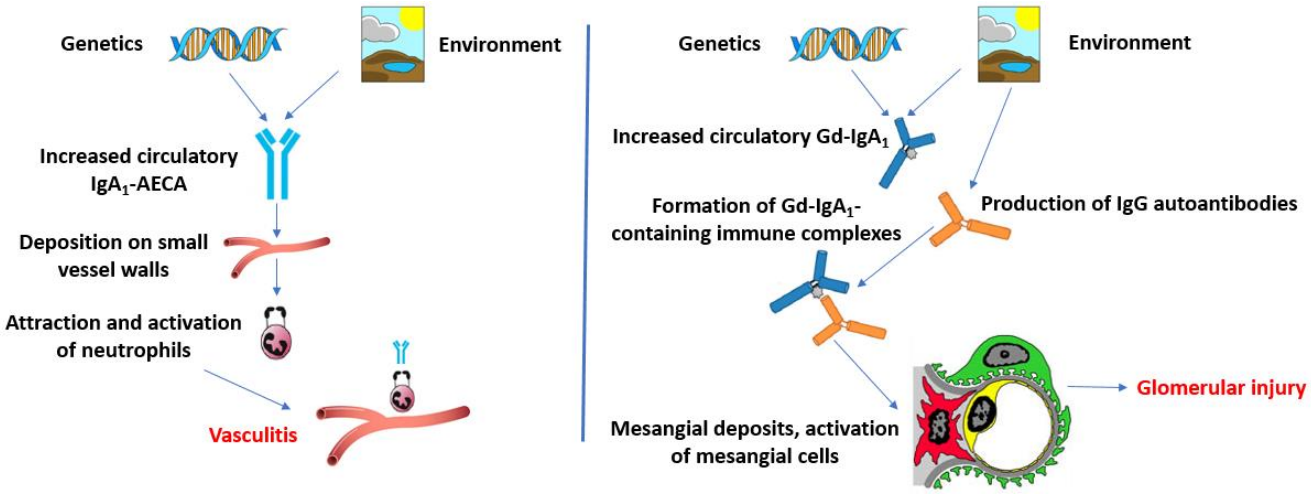


Figure 5. Two hypothetical models of IgAV and IgAVN pathogenesis. Modified according to references 156 and 178. AECA: anti-endothelial cell antibodies; Gd-IgA$_1$:galactose deficient IgA$_1$.

In patients with IgAV, and especially in those patients who developed renal involvement, it seems that the aberrantly glycosylated IgA$_1$ plays a key role in the pathogenesis. Namely, IgA$_1$ from most patients with IgAV lack galactose residues (Gd-IgA$_1$). Aberrant glycosylation occurs predominantly due to decreased galactosyltransferase activity in the Golgi apparatus of IgA$_1$-producing immune cells. Gd-IgA$_1$ might be recognised as an autoantigen by IgA, IgG antibodies, leading to the formation of polymeric immune complexes (Gd-IgA$_1$-IgA, Gd-IgA$_1$-IgG and Gd-IgA$_1$-sCD89, where sCD89 is a soluble IgA Fc alpha receptor). Circulating immune complexes may accumulate in the blood serum, resulting in their deposition in the endothelium of small blood vessels in the skin, synovial membrane, intestines and urinary system. It has been shown that the serum level of Gd-IgA$_1$ is higher in IgAVN patients compared to IgAV patients without nephritis (179). In IgAV patients who developed IgAVN, some of these complexes, Gd-IgA$_1$-IgG, deposit in the kidneys, resulting in mesangial cell activation, release of inflammatory mediators and glomerular injury (141).

Nonetheless, there is a second multi-hit hypothesis to explain the systemic symptoms of IgAV and IgAVN (178). In this model it is proposed that infection with microorganisms that have similar antigenic structures, as human vessel walls or genetic influences, could lead to the production of cross-reactive anti-endothelial cell antibodies (IgA$_1$-AECA). IgA$_1$-AECA bind to small vessels and induce the production of interleukin-8, which is a potent chemoattractant for neutrophils. Neutrophils become activated and cause damage to the vascular endothelial cells.

However, none of the proposed models can explain the IgAV pathogenesis in the proportion of IgAV patients (<10%) who do not have elevated Gd-IgA_1 in serum or in biopsy specimens, nor can they explain the observation that the disease occurs only in some people with a IgA_1 glycosylation defect, while in others with elevated Gd-IgA_1 the disease does not occur (139, 180).

1.2.3. Clinical presentation of IgAV

Cutaneous manifestations in the form of nonthrombocytopenic purpura or petechiae with lower limb predominance are the most common and are a characteristic sign of the disease (125). Alterations to the skin have proved to be a symptom in approximately 75% of patients, usually preceding other symptoms. They are present in all patients, but atypical distributions of skin changes are also possible, affecting the head and neck area, involving the upper extremities more than the lower extremities, sparing the lower extremities totally or otherwise leaving diffusely distributed lesions (181). In the most severe cases, bullae, ulcerations and necrotic lesions can be seen. Recently we have demonstrated that such skin alterations developed in 2.45% of our patients and that with increasing severity and duration of cutaneous manifestations in IgAV, the risk of developing IgAVN increases, making the prognosis worse with a greater likelihood to have required more aggressive treatment (182). Furthermore, we have found that the presence of ulcerations and necroses, persistent purpura (≥ 1 month) and age were significant predictors of IgAVN, while persistent purpura, male gender and age were predictors of persistent IgAVN (hematuria and/or proteinuria > 3 months).

Musculoskeletal manifestations are the second most common feature and up to 70-90% of patients will have arthralgia or arthritis (181). The affected joints have painful ranges of movement due to periarticular swelling but the characteristic findings of warmth, erythema and effusion are often absent.

Gastrointestinal manifestations are present in more than 50% of children with IgAV and about 10-20% of patients with gastrointestinal involvement may develop serious complications such as intussusception, bowel perforation and massive bleeding (130). The abdominal pain is the most frequent sign of gastrointestinal involvement, it is characteristically colicky and localised to the periumbilical and epigastric regions. According to our study published recently the patients with IgAV started with gastrointestinal symptoms, older children with severe

gastrointestinal symptoms (severe abdominal pain, intussusception, hematochezia and/or massive gastrointestinal bleeding) of IgAV were a particularly high-risk group for developing IgAVN (183).

Renal involvement ranges from urinary abnormalities (including hematuria or/and proteinuria) through nephritic and nephrotic syndrome to chronic renal failure. It is typically mild and manifested only by pathological urine findings. However, chronic renal failure has been reported in 1 to 15% of children with IgAV who develop nephritis, and in the vast majority it is diagnosed within 6 months of disease onset (137, 184, 185).

In 10-20% of boys, orchitis may develop. Other rare manifestations include lung involvement, neurological manifestations and multiple organ involvement (186).

1.2.4. Classification criteria used in IgAV

Classification of IgAV is based on the criteria defined by the European League Against Rheumatism (EULAR), Paediatric Rheumatology International Trials Organization (PRINTO) and Paediatric Rheumatology European Society (PRES) (125). The criteria provide sensitivity and specificity in the classification of IgAV (using other forms of vasculitis as controls) at 100% and 87%, respectively. The mandatory criterion is palpable purpura in association with at least one of the following: diffuse abdominal pain, arthritis or arthralgia, renal involvement (hematuria and/or proteinuria), and IgA deposition in biopsy specimens (skin, intestinal tract and kidney) (Table 5). A skin biopsy is not obligatory, but it is required in the event of atypically distributed skin change and is recommended if the rash is severe, in order to exclude other forms of vasculitis.

Table 5. European League Against Rheumatism/Paediatric Rheumatology International Trials Organization/Paediatric Rheumatology European Society classification criteria for IgAV. Modified according to reference 125.

Criterion	Description
Mandatory - skin changes	Purpura or petechiae with lower limb predominance not related to thrombocytopenia
and at least one of the four following criteria	
Abdominal pain	Diffuse abdominal colicky pain with acute onset assessed by history and physical examination. May include intussusception and gastrointestinal bleeding
Biopsy proven IgA deposits	Typically leucocytoclastic vasculitis with predominant IgA deposit or proliferative glomerulonephritis with predominant IgA deposit
Arthritis or arthralgia	Acute onset joint swelling or joint pain with limitation on motion
Renal involvement	Proteinuria >0.3 g/24 h or >30 mmol/mg of urine albumin/creatinine ratio on a spot morning sample and/or >5 red blood cells/high power field or red blood cells casts in the urinary sediment or ≥2+ on dipstick

1.2.5. Assessment of disease activity and damage in IgAV

There are limited data for vasculitis activity and damage assessment in children with IgAV. Since disease activity and damage have been recognised as the key components of outcome measures in patients with various forms of vasculitis for a standardised comparison of patient cohorts for clinical trials, as well for observing the course of an individual patient's disease, it is important to validate the available assessment tools adjusted for the pediatric population (187). Therefore, for the purpose of determining the disease activity and degree of kidney damage in patients with IgAV/IgAVN, two clinical questionares are used: the Paediatric Vasculitis Activity Score (PVAS) and Paediatric Vasculitis Damage Index (PVDI).

PVAS is a set of 64 clinical variables (symptoms or signs of disease) which are divided into 9 organ systems and it is assessed on which of them are new or have deterioated in the previous 4 weeks or have persisted, but for no longer than a period of 3 months. The presence of each of the variables is evaluated with a certain number of points which are then added together (Table

6). The total number of points is in the range from 0 to 63 points and represents the activity of the disease at the time of scoring (187).

Table 6. The Paediatric Vasculitis Activity Score. Modified according to reference 187.

	PVAS persistent	**PVAS new/ worse**		**PVAS persistent**	**PVAS new/ worse**
1. General (maximum scores)	**2**	**3**	**6. Cardiovascular (maximum scores)**	**3**	**6**
Myalgia	1	1	Loss of pulses	1	4
Arthralgia or arthritis	1	1	Bruits over accessible arteries	1	2
Fever ≥ 38.0 ºC	2	2	Blood pressure discrepancy	1	2
Weight Loss ≥ 5% body weight	2	2	Claudication of extremities	1	2
2. Cutaneous (maximum scores)	**3**	**6**	Ischaemic cardiac pain	2	4
Polymorphous exanthema	1	1	Cardiomyopathy	3	6
Livedo	1	1	Congestive cardiac failure	3	6
Panniculitis	1	1	Valvular heart disease	2	4
Purpura	1	2	Pericarditis	1	3
Skin nodules	1	1	**7. Abdominal (maximum scores)**	**5**	**9**
Infarct	1	1	Abdominal pain	2	4
Ulcer	1	4	Peritonitis	3	9
Gangrene	2	6	Blood in stools or bloody diarrhea	2	6
Other skin vasculıtıs	1	1	Bowel ischaemia	3	9
3. Mucous membranes/eyes (maximum scores)	**3**	**6**	**8. Renal (maximum scores)**	**6**	**12**
Mouth ulcers/granulomata	1	2	Hypertension >95th centile (for height)	1	4
Genital ulcers	1	1	Proteinuria >0.3 g/24h, > 20mg/mmol creatinine	2	4
Adnexal inflammation	2	4	Haematuria ≥2+ or 5 RBC/HPF or red cell casts	3	6
Significant proptosis	2	4	GFR 50-80 ml/min/1.73 m^2	2	4

	PVAS persistent	**PVAS new/ worse**		**PVAS persistent**	**PVAS new/ worse**
Red eye (Epi)scleritis	1	2	GFR 15-49 ml/min/1.73 m^2	3	6
Red eye conjunctivitis Blepharitis Keratitis	1	1	GFR <15 ml/min/1.73m^2	4	8
Blurred vision	2	3	Rise in creatinine > 10% or Creatinine clearance (GFR) fall > 25%		6
Sudden visual loss		6	**9. Nervous system (maximum scores)**	**6**	**9**
Uveitis	2	6	Headache	1	1
Retinal vasculitis/retinal vessel thrombosis/retinal exudates/haemorrhages	2	6	Meningitis/encephalitis	1	3
4. ENT	**3**	**6**	Organic confusion/cognitive dysfunction	1	3
Nasal discharge/crusts/ulcers/granuloma	2	4	Seizures (not hypertensive)	3	9
Paranasal sinus involvement	1	2	Stroke	3	9
Subglottic stenosis/ hoarseness /stridor	3	6	Cord lesion	3	9
Conductive hearing loss	1	3	Cranial nerve palsy	3	6
Sensorineural hearing loss	2	6	Sensory peripheral neuropathy	3	6
5. Chest	**3**	**6**	Motor mononeuritis multiplex	3	9
Wheeze or expiratory dyspnea	1	2	**10. Other**		
Endobronchial/endotracheal involvement		3			
Nodules or cavities	2	4			
Pleural effusion/pleurisy	2	4			
Infiltrate	2	4			
Massive hemoptysis/Alveolar haemorrhage	4	6			

	PVAS persistent	PVAS new/ worse		PVAS persistent	PVAS new/ worse
Respiratory failure	4	6			

ENT: ear, nose and throat; GFR: glomerular filtration rate; HPF: high-power field; RBC: red blood cells.

PVDI represents a set of 72 clinical variables which are divided into 9 organ systems and an "other" section. Damage is defined by the duration of symptoms or signs lasting a period of at least 3 months, which have occurred at any time since the onset of the disease (187).

1.2.6. Treatment of IgAV

In the vast majority of patients with IgAV, during the self-limited nature of the disease, specific treatment is not required. The optimal way to treat patients with severe skin manifestation is not known, although the majority of these patients are treated with systemic glucocorticoids, sometimes in combination with dapsone or azathioprine (182). Musculoskeletal involvement is usually treated with rest and analgesia, while other treatment options are rarely indicated. However, the total opposite can be recommended in patients with severe gastrointestinal manifestations, renal involvement or those who develop other complications such as lung involvement, neurological manifestations and multiple organ involvement. Recently, the SHARE initiative developed European consensus-based recommendations for diagnosis and treatment of immunoglobulin A vasculitis (188).

In patients with severe abdominal pain or gastrointestinal hemorrhage, glucocorticoids should be considered: orally, or with pulsed glucocorticoids if the oral route is not an option or if they fail to respond. Second-line treatments may include mycophenolate mofetil, cyclophosphamide, intravenous immunoglobulin, rituximab, methotrexate, colchicine and hydroxychloroquine (181). Other supportive treatment may be required: nasogastric decompression, parenteral nutrition and antibiotics.

According to the SHARE management algorithm, IgAVN is divided into three categories, using three parameters: proteinuria, estimated glomerular filtration rate and percentage of crescents on a renal biopsy (188). Children without renal dysfunction or proteinuria usually do

not require any specific therapeutic intervention. First-line therapy in patients with mild forms of IgAVN, defined as <1 g/day of proteinuria in 24 hr urine collection, are oral glucocorticoids which are usually sufficient, and in the event of persistence of proteinuria, second-line drugs may be used: azathioprine, mycophenolate mofetil or glucocorticoid pulses. In the treatment of moderate IgAVN, defined as <50% crescents on a renal biopsy and an impaired estimated glomerular filtration rate (<80 ml/min/1.73 m^2) or severe persistent proteinuria (1-2.5 g/day of proteinuria in a 24 hr urine sample for more than 4 weeks), the preferred option is to use glucocorticoids, which usually need to be administered parenterally and in pulsed doses, and in the absence of any effect, second-line drugs are added: azathioprine, mycophenolate mofetil or cyclophosphamide parenterally. Treatment of the most severe forms of IgAVN consists of induction using pulsed doses of glucocorticoids in combination with intravenous cyclophosphamide pulses, and continues as a maintenance therapy with lower doses of glucocorticoids in combination with immunomodulators: azathioprine or mycophenolate mofetil. Angiotensin converting enzyme inhibitors or angiotensin receptor blockers are recommended to prevent or limit secondary glomerular damage in patients with IgAVN who have persistent proteinuria (lasting a period of more than 3 months).

For the most severe, unresponsive cases there is an option of plasma exchange which demonstrated an efficacy in one study, while there is not enough evidence regarding the use of rituximab (although it is used in severe cases, and there are case reports and case series concerning its usage) or intravenous immunoglobulins (181, 189).

1.3. WHOLE-EXOME SEQUENCING

Based on the coverage of the genome, genomic tests which use next-generation sequencing (NGS) technologies can be classified as 1. target region sequencing, 2. whole-exome sequencing (WES) and 3. whole-genome sequencing (WGS). These tests allow for the discovery of new genetic variants either associated with, or causing, various diseases in human pathology.

1.3.1. The genome

The human genome comprises roughly 3 billion nucleotide base pairs and contains approximately 20,000 genes (190). Each gene contains both protein-coding (exons) and non-

coding regions (introns). The exons comprise only about 1.5% of the genome, with the majority of the human genome consisting of intronic and non-protein coding sequences (191). Exons include information determining the amino-acid sequence of protein products and the sequence of the structural or regulatory RNA molecules. The non-protein coding DNA regions contain regulatory sequences, although still poorly understood, which include not only gene promoters, enhancing and silencing regions, but also small and long non-coding RNA molecules - which control multiple aspects of gene expression and function (192). Though protein-coding genes comprise only a small part of the genome, it is estimated that approximately 85% of disease-causing mutations reside in the exome.

1.3.2. Genetic variation

Genetic variation is a term used to describe differences in both the coding and noncoding DNA sequences between two genomes and it exists in a spectrum from individual single nucleotide variants (SNVs) to larger-scale chromosomal variants (193).

SNV are single base-pair changes in the DNA sequence that can be found within introns, exons and regulatory elements. Sometimes SNV are referred to as single nucleotide polymorphisms (SNP) if they are present in at least 1% of the population. SNV in exons can have a significant effect on protein structure and function. Exonic SNV are divided into synonymous variants, where the SNV does not change the encoded amino acid, implying no functional consequence, and non-synonymous variants which change the encoded amino acid and can potentially alter protein function, from loss of residues with critical functions (such as tyrosines or serines), to changes in the secondary and higher structures of the protein from alterations in the electropotential charge (194). Non-sense variants and out-of-frame insertions and deletions introduce a premature stop codon in the sequence leading to a complete loss of production of a specific protein (null alleles – typically leading to nonsense-mediated RNA decay). The different variants that exist for a specific gene are called alleles. Within a given population there are typically two allele possibilities at a particular nucleotide position. The frequency of the SNV is given in terms of allele frequencies, typically the minor allele frequency (MAF), or the frequency of the less common allele. Intronic SNV can also be a significant consequence due to changes in gene splicing, resulting in a loss of segments of the translated protein, or due to changes to gene activators or repressors resulting in altered gene expression.

Copy number variation (CNV) is defined as any insertion or deletion of genomic material, typically greater than 1,000 base pairs (large DNA gains or losses), and can be inherited, de novo or of somatic origin. The somatic genetic variants lead to somatic mosacism and result in different organs and tissues of the same individual having variations in the copy number; this is a mechanism by which identical twins may have different CNVs (195). Increased or decreased CNVs may predispose to a number of complex diseases, such as psoriasis, Crohn disease, glomerulonephritis or SLE (78, 196, 197, 198). However, there are some technical difficulties in detecting CNVs adequately (especially larger ones) using genomic technologies.

Chromosomal variation includes the addition of a whole or part of a chromosome (aneuploidy), loss of an entire chromosome set (monoploidy) or the gain of one or more complete sets of chromosomes (triploidy, polyploidy), resulting in a different effect on phenotypic expression (199). Cases of autoimmune diseases, including SLE, due to a duplication of part or whole chromosomes have been described in the literature, both in animals and humans (200, 201).

1.3.3. Genome-wide association studies (GWAS)

Genetic testing for classical and well-characterised non-syndromic SLE is not required for establishing the diagnosis, as monogneic forms of SLE are quite rare. In fact, most cases of SLE and IgAV are genetically complex autoimmune disorders that can be attributed to synergistic effects of common variants (population allele frequencies >5%) and appropriate environmental factors. These susceptibility variants are identified by GWAS in large cohorts of unrelated patients and healthy controls (202). GWAS use capture chips or beads conjugated with small DNA sequences. Alleles are detected by differential hybridisation with the sample DNA, permitting rapid simultaneous detection of over a million SNVs.

When a large number of variants are analysed in a large cohort, the likelihood that any difference detected due to random chance is high, so the risk of false positive associations is increased (203). To overcome this problem, various rigorous statistical tests are used, and the established probability cut-offs for significance are very restrictive (p values in the order of x 10^{-8}). As a result of rigorous statistical methods, rare variants will occur at rates too low to power an association adequately.

1.3.4. Mode of inheritance of monogenic forms of SLE and IgAV susceptibility variants

Most monogenic diseases are inherited by one of the classical modes of inheritance, autosomal recessive or autosomal dominant (202). An individual with an autosomal recessive genetic disorder must have biallelic pathogenic variants in the same gene, either in a homozygous or in a compound heterozygous state. Each pathogenic genetic variant must be transmitted from each unaffected parent (in *trans*) unless one mutation occurs as a *de novo* in the patient. The pathophysiology of recessively inherited diseases can usually be explained by a loss-of-function mechanism, which is when the loss of protein expression and/or function from both alleles causes the disease. An example of recessive hereditary forms of monogenic SLE are complement deficiencies caused by biallelic mutations in *C1QA*, *C1QB*, *C1QC*, *C1R*, *C1S*, *C4A* or *C4B* gene or PKCδ deficiency caused by biallelic mutations in *PRKCD* (86).

Dominantly inherited disorders result from a single pathogenic variant that either occurs *de novo* during gametogenesis or is otherwise inherited from an affected parent. However, there are examples of reduced penetrance alleles where a causal mutation is inherited from an unaffected parent or is present in other unaffected family members. Examples of dominantly inherited forms of monogenic SLE are early-onset familial chilblain lupus caused by heterozygous mutations in *TREX1* or SLE associated with a heterozygous null allele in *DNASE1* (86). Most dominantly inherited pathogenic variants are novel, but some are reported at a very low frequency in large public databases of human genetic variation (202).

In most patients with IgAV, an inherited disorder of posttranslational modification of the IgA_1 binding region, which results in aberrant O-linked glycosylation, i.e. pronounced production of Gd-IgA_1, has been demonstrated (203). The mode of inheritance is most often autosomal dominant with a penetration of about 80% (e.g., *C1GALT1*), and in a smaller proportion of patients it is X-linked (e.g., *C1GALT1C1*).

1.3.5. Approaches in genetic testing for autoimmune diseases

Genetic testing can be organised at the level of individual genes, gene panels or at the level of the whole exome or genome. Target sequencing of known genes involved in autoimmunity in disease cohorts is suitable for identification of rare variants at known genes (204). Unlike monogenic autoinflammatory diseases, in SLE and IgAV the sequencing of individual genes and the use of gene panels have a limited role due to a significant clinical overlap between

different monogenic forms of SLE or IgAV, so a differential diagnosis might require the sequential or simultaneous analysis of multiple genes. From a research perspective, WES and WGS allow us to discover novel variants (primarily SNVs) in genes that are already known to cause disease, but also to identify novel genes not yet associated with human disease (205).

The general sequencing process encompasses the same steps regardless of whether it is sequencing using gene panels, WES or WGS. The first step includes fragmentation of DNA in millions of DNA fragments in lengths of 25-1,000 bases. After the amplification process, these fragments are assembled into large contiguous segments that can be aligned to a reference genome to re-construct the individual genes, the exome or the genome. Finally, variant-calling algorithms are used to compare mapped reads to the reference sequence and to identify potential novel and rare genetic variants (206).

WES captures all coding exons that make up 1-2% of the human genome and has the ability to identify genetic variants in the known SLE genes, or potentially identify new genetic contributors to SLE (particularly when using trio exome sequencing which allows identification of *de novo* variants). As 85-90% of the risk loci associated with the polygenic forms of SLE are located outside the coding regions, it is obvious that using WGS has the advantage of being able to capture noncoding regions that might be missing when performing WES (74). In addition, WGS has the advantage of sequencing the introns and other regulatory sequences such as promoters, enhancers and silencers, further increasing the chances of identifying likely monogenic causes of SLE. The technological superiority of WGS also affords more uniform sequencing depth across the genomce and higher coverage compared to WES. However, the higher costs of sequencing, the far greater amount of data requiring storage space and analyses using bioinformatic systems consume a lot of time and still limit the applicability of WGS (207). Since the genetic variants of most monogenic forms of disease are located within the coding gene segments, they can be captured by WES.

1.3.6. The basic principles of WES

The first step of WES involves the acquisition of high-quality genomic DNA (gDNA), which is most commonly extracted from peripheral blood leukocytes using commercial extraction kits or manual DNA extraction procedures, such as the salting-out method, followed by assessing the DNA quality and quantity (208).

The next step is fragmentation of gDNA into random fragments either mechanically by ultrasonication methods, or biologically by enzymatic digestion. The third step in the exome sequencing workflow is target enrichment. Target enrichment is the process of isolating and separating relevant regions of the genome for focused analysis (208). Double-stranded DNA probes designed to bind complementary DNA to the exome DNA sequences are mixed with the genomic sample. The probes include a biotin molecule on one end. After adding magnetic beads coated in streptavidin, biotin, in the ends of the probes that were bound to complementary exome DNA sequences, binds to a streptavidin. Finally, a magnet is used to pull the bound exome DNA out of the solution. A collection of exonic DNA fragments is called an exome library. Exonic fragments are then amplified and sequenced, which results in producing millions of sequenced reads. The data are aligned to the human reference genome (209). Reference genome is a digital nucleic acid sequence database, derived from the sequencing of DNA from a number of individual donors. The first human genome reference was set up by The Human Genome Project in 2001. The Genome Reference Consortium (GRC) assembled the human reference genome version GRCh37 in 2009. It is also often referred to as HG19 because it was the 19th release. GRCh37 is still being used to some extent. In 2013 the human reference genome GRCh38 was released. GRCh38 reference contains more complete human reference genome information, especially regarding the centromere regions (210).

After sequencing, it is important to identify the genetic variants from the sequencing data. This process is denoted by the term 'variant calling'. Two widely used programs (*in silico* tools) for processing and analysing high-throughput sequencing data are SAMtools and GATK (208). The challenge for these programs is differentiate a true genetic variant from a sequencing error, particularly given the high error per base rate of the sequencing process.

The next step includes annotation of called variants (208). This is a process of assigning information to DNA variants and typically includes (211): a definition of the variant, measure of likelihood, genotype; identification of the gene that overlaps with the variant; determination of whether the variant is in an exon; determination of whether it is in a coding region; if a SNV, a determination of whether the encoded amino acid is misense; location determination - is it before or after an exon/intron boundary; if the variant adds or deletes nucleotides, it is annotated as an insertion or deletion.

Exome data are reduced by primary and secondary filtering (208). The main objective of primary filtering is the exclusion of benign variants which often includes: the removal of

synonymous variants, filtering by MAF, variant exclusion from control group databases (created from in-house sequencing data) and filtering by segregation. MAFs are available from publicly available databases, such as the Exome Aggregation Consortium (ExAC) and the Single Nucleotide Polymorphism Database (dbSNP), and can be used to differentiate rare variants from SNPs. A MAF cut-off that can range between 0.005 and 0.03, depending on the expected mode of inheritance (208). Filtering by segregation is a technique that attempts to determine if the pattern of phenotypes within families is consistent with the transmission of a major gene for that phenotype.

The aim of secondary filtering is to prioritise remaining candidate variants. Different computational programs have been created to predict whether the identified genetic variants affect protein function. Commonly employed programs for non-synonymous SNV prediction are the Sorting Intolerant from Tolerant (SIFT), Polymorphism Phenotyping2 (Polyphen2; PPN), and MutationTaster (208). The Combined Annotation Dependent Depletion (CADD) tool is used for scoring the deleteriousness of SNVs but also insertion/deletions variants (208). Frameshift, nonsense and canonical splice site variants have greater pathogenic potential and are considered most likely to disrupt gene function. PROVEAN (Protein Variation Effect Analyzer) is a software tool useful for filtering nonsynonymous or small insertions and deletions (indels) variants (212).

All computational programs used as prediction tools have limitations. Their sensitivity and specificity are poor and do not meet diagnostic standards. Most of them ignore gene-specific domains and multivariant interactions. It is recommended to combine different prediction tools to limit the risk of missing potentially-damaging variants.

1.3.7. Reporting on prioritised variants

The accurate interpretation of sequence variants is of crucial importance since this influences clinical outcomes. There is a standardised recommendation for classification of variant pathogenicity established by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG) in 2015 (213). According to this classification system, a variant can be classified as pathogenic, likely pathogenic, a variant of uncertain significance (VUS), likely benign, or benign. ACMG suggests using the term "variant" for both mutations (permanent change in the nucleotide sequence with a frequency below 1%) and polymorphisms permanent change in the nucleotide sequence with a frequency above 1%).

There are two sets of criteria: one for classification of pathogenic or likely pathogenic variants and one for classification of benign or likely benign variants (Table 7).

Table 7. Summary of criteria for the classification of benign or likely benign variants (left) and pathogenic or likely pathogenic variants (right). Modified from reference 213.

Evidence of benign impact
A. STAND-ALONE (SA)
BA1. Frequency is >5% healthy databases
B. STRONG (S)
BS1. Frequency greater than expected for disorder
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age
BS3. Established function test - no damaging effect
BS4. Lack of segregation
C. SUPPORTING (SP)
BP1. Missense variant where truncating variant cause a disease
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern
BP2. In-frame deletions/insertions in a repetitive region without a known function
BP3. In silico no impact, multiple lines
BP4. Found in a case with an alternate molecular basis for disease
BP5. Reputable source recently reports variant as benign
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved

Pathogenicity Criteria
A. VERY STRONG
PVS1. Null variant
B. STRONG (SP)
PS1. Same aa change previously pathogenic
PS2. De novo (confirmed parentity)
PS3. Established function test - damaging
PS4. Prevalence in patients > controls
C. MODERATE (MP)
PM1. Variant hotspot, functional domain
PM2. Absent, rare in healthy databases
PM3. For recessive, phase trans
PM4. Change in protein length
PM5. Same residue, different aa change pathogenic
PM6. Assumed de novo (w/o parentity)
D. SUPPORTING (SP)
PP1. Co-segregation in the family
PP2. Low benign variant frequency
PP3. In silico damaging, multiple lines
PP4. Phenotype specific for disease
PP5. Reported pathogenic, w/o evidence provided

The classification system is designed for Mendelian disorders. Each pathogenic criterion is weighted as very strong, strong, moderate or supporting. A pathogenic variant is a variant that is certain to disrupt gene function or cause disease. A likely pathogenic variant is a DNA change that is most likely to cause a deleterious effect on an encoded protein product that accounts for the observed symptoms. If a variant does not fulfill criteria using both sets (pathogenic and benign), or the evidence for benign and pathogenic is conflicting, the variant defaults to uncertain significance. VUS is a change in the DNA sequence whose association with disease is unknown. Benign variants are not considered to be the cause of the disease tested.

2. HYPOTHESIS

Using WES in carefully selected patients with cSLE and IgAV and their family members, it is possible to identify novel, rare gene variants that may be contributing to etiopathogenesis of these diseases and expand existing genetic databases, which will represent a small but important step towards understanding the complex pathophysiology of two diseases.

3. AIMS AND PURPOSE OF THE RESEARCH

3.1. General aim:

- to identify novel genes and variants involved in cSLE and IgAV.

3.2. Specific aims:

- to identify individuals and families with a likely monogenic cSLE and IgAV according to inclusion criteria and to obtain appropriate DNA samples from patients and consenting family members;
- to perform WES in selected individuals;
- to analyse exome sequencing data to identify genetic variants in all individuals sequenced;
- to predict the pathogenicity of putative variants by comparing exome sequencing data through the use of prediction tools;
- to characterise and define possible modes of inheritance of the variants.

4. MATERIALS AND METHODS

4.1. Subjects

This is a multiple case study in which two paediatric patient groups were investigated: patients with cSLE and patients with IgAV. The patients were recruited from two large databases created by the research team which include patients with cSLE and IgAV and contain data on clinical, laboratory and histological findings.

The first database comprises patients diagnosed with cSLE revised classification criteria by the ACR-97 (109) and the SLICC criteria (110) in the period from 1991 to 2019 at the University Hospital Centre Zagreb, Department for Paediatrics, Division of Clinical Immunology, Respiratory and Allergic Diseases, and Rheumatology. In order to increase the likelihood of identifying new genes and rare variants involved in this condition, only patients with cSLE that met one or more of the following criteria were selected:

- early onset of the disease (before the age of 5 years),

- cases with confirmed family history of the same disease within the family (particularly in first degree relatives) or other autoimmune conditions (e.g. autoimmune thyroiditis, diabetes mellitus, juvenile idiopathic arthritis and rheumatoid arthritis etc.),

- extremely severe clinical presentation (defined as SLEDAI-2K score of ≥ 20, organ or life-threatening, reflects the most serious form of systemic disease that requires potent immunosuppression; lupus nephritis: classes III, IV and VI; neuropsychiatric lupus, cardio-pulmonary involvement) and/or resistance to standard treatment (requiring systemic high dose oral glucocorticoids equivalent to prednisolone > 1 mg/kg/day, intravenous pulse glucocorticoids equivalent to pulse methylprednisolone (30 mg/kg), systemic immunomodulators (include biologicals, immunoglobulins and plasmapheresis) and/or therapeutic high dose anticoagulation in the presence of high dose steroids or immunomodulators),

- consanguinity,

- patients with syndromic cSLE, defined as the presence of at least one of the following: growth failure in height and weight not explained by drug exposures; intellectual deficiency; birth defects involving the heart, lungs, or kidneys; chilblains; dysmorphic features; or intracerebral calcifications,

- male cSLE patients.

The second database includes patients with IgAV and IgAVN reviewed in five Croatian University Centers for pediatric rheumatology and nephrology care in the period 2009 to 2019: Univeristy Hospital Centre Zagreb, Children's Hospital Zagreb, University Hospital Centre Split, Osijek and Rijeka. Diagnosis of IgAV and IgAVN was based on the criteria defined by EULAR/PRINTO/PRES (125). Paediatric patients with IgAV that met one or more of the following criteria were selected:

- early onset of the disease (before the age of 2 years),

- cases with confirmed family history of the same disease within the family (particularly in first degree relatives) or other autoimmune conditions (e.g. vasculitis, autoimmune thyroiditis, diabetes mellitus, juvenile idiopathic arthritis and rheumatoid arthritis etc.),

- extremely severe clinical presentation (severe IgAVN: proteinuria > 1 g per day (or > 40 mg/h/m^2), albumin/urine creatinine ratio ≥ 200 mmol and/or hypertension, and estimated glomerular filtration rate (eGFR) > 60 mL/min/1.73 m^2 or eGFR < 60 mL/min/1.73 m^2 or end stage renal disease (eGFR < 15 mL/min/1.73 m^2) or death; severe digestive IgAV: intussusception, massive gastrointestinal haemorrhage (requiring transfusion), intestinal ischemia, perforation; central nervous system effects, cardiac manifestations, and pulmonary haemorrhage and/or resistance to usual treatment (requiring systemic high dose oral glucocorticoids equivalent to prednisolone > 1 mg/kg/day, intravenous pulse glucocorticoids equivalent to pulse methylprednisolone (30 mg/kg), systemic immunomodulators (include biologicals, immunoglobulins and plasmapheresis)),

- consanguinity,

- patients with syndromic IgAV defined as the presence of at least one of the following: growth failure in height and weight not explained by drug exposures; intellectual deficiency; birth defects involving the heart, lungs or kidneys; dysmorphic features.

Both parents and family members or relatives suffering from the same illness were included.

Prior to enrollment in the research, parents or guardians of all children, as well as all children over the age of 8 years and all informative family members involved, provided signed informed consent.

4.2. Methods

4.2.1. Research plan

Study participants were identified and consented to the participation in the study. The ethical approval for the study was granted by the University of Zagreb School of Medicine. The clinical evaluation, including routine laboratory and imaging testing was performed and clinical/working diagnosis was established for each proband. Patients were assessed against existing classification criteria: the ACR-97 and the SLICC criteria were used for cSLE and EULAR/PRINTO/PRES criteria for IgAV. In addition, a detailed personal and family history was obtained, including the drawing up of 3-generation pedigrees. The clinical material for genetic testing included peripheral blood samples from probands, both parents and other informative family members. High quality high molecular weight DNA and peripheral blood mononuclear cells (PBMCs) from all probands were prepared, as well as DNA from the parents and informative family members. The clinical part of the research was carried out at the University Hospital Centre Zagreb, Department for Paediatrics, Division of Clinical Immunology, Respiratory and Allergic Diseases, and Rheumatology. The research plan is listed in Figure 6.

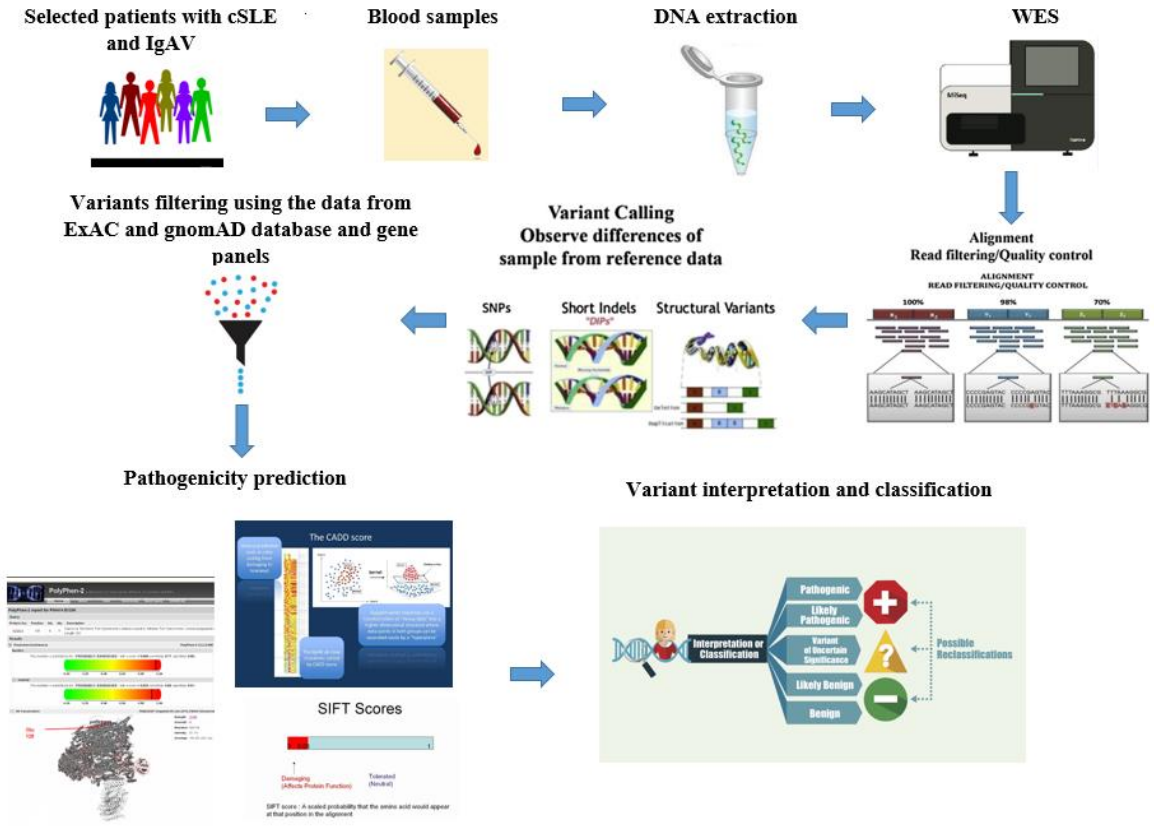


Figure 6. Schematic representation of the research plan

Two blood samples (5 ml) were collected in EDTA tubes and one blood sample (9 ml) in a citrate vacutainer. Genomic DNA was extracted from EDTA blood using a commercial DNA extraction kit and stored at -20 °C until analysis. The citrate blood sample was used to extract peripheral blood mononuclear cells (PBMCs) using the Ficoll density gradient method and stored at -80 °C until analysis. These procedures were performed at the Department of Laboratory Diagnostics, University Hospital Centre Zagreb, Division for Cytogenetics.

Frozen samples (DNA, PBMCs) were then transported on dry ice to the Centre for Personalised Immunology and the China-Australia Centre for Personalised Immunology at the John Curtin School of Medical Research, The Australian National University (Canberra, Australia), where WES and bioinformatics analyses were performed.

After completion of WES, data analysis and the identification of all genetic variants of interest, we proceeded on one patient with functional validation of the identified variant by examining the presence of an IFN gene expression signature in the PBMC sample which was investigated (consistent with the literature data supporting specific IFN signatures in patients with molecular changes in the relevant signaling pathways). Functional analyses were not performed on the other patients, either because the genetic variants were not assessed to be promising enough for further analysis or because, at present, there are no avaliable/known functional tests, or because functional analyses had already been performed in other studies.

4.2.2. DNA extraction

Genomic DNA was extracted from whole blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the standard manufacturer's protocol. In the first step, 20 µl QIAGEN Protease (proteinase K) was placed in the bottom of a 1.5 ml microcentrifuge tube and then 200 µl whole blood and 200 µl Buffer AL were added. The sample and Buffer AL were mixed by pulse-vortexing for 15 secs. to yield a homogeneous solution and then incubated at 56 °C for 10 min. After that, the 1.5 ml microcentrifuge tube was centrifuged briefly to remove droplets from the inside of the lid. Afterwards, 200 µl ethanol (96%) was added to the sample, and mixed by pulse-vortexing for 15 secs. After mixing, it was again briefly centrifuged to remove droplets from the inside of the lid. The mixture was then applied to the QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. In the QIAamp Mini spin column, 500

µl Buffer AW1 was added and the sample was centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded. In the QIAamp Mini spin column 500 µl Buffer AW2 was added and the sample was centrifuged at 20000 x g (14000 rpm) for 3 min. The QIAamp Mini spin column was again placed in a new 2 ml collection tube and the old collection tube with the filtrate was discarded. The sample was again centrifuged at 20000 x g (14000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. Afterwards, in the QIAamp Mini spin column, 200 µl Buffer AE was added and incubated at 25 °C for 1 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min. The DNA, thus isolated, was stored in AE Buffer at -20 °C.

4.2.3. WES and data analysis

DNA samples for WES were enriched with the Human SureSelect XT2 All Exon V4 Kit and sequenced by Illumina HiSeq 2000 (Illumina, inc). Variant calling was performed by the bioinformatics team and pipeline at the Australian National University, and uploaded on to the Centre for Personalised Immunology's Variant Database: https://database.cpi.org.au/cpi30. In brief, raw sequence reads were aligned to the reference genome (Hg19). SAMTools were used for removing duplicated reads, realignment, recalibration and variant identification. SNVs and small insertions and deletions (indels) were called from the aligned data using SAMtools. All *de novo* sequence variants and *de novo* indels were prioritised for closer investigation which also included all missense variants in a gene panel, as well as rare and nonsense variants with minor allele frequencies below 0.005 that were identified by filtering using the data from Exome Aggregation Consortium (ExAC) and Genome Aggregation Database (gnomAD). The first *in silico* gene panel included genes known to cause monogenic SLE (13, 58, 214), interferonopathies (215) or found within GWAS SLE loci (216) as well a list of genes generated from Jiang et al. which were used to investigate the prevalence of rare variants in lupus-risk genes (66). The second *in silico* gene panel included the Vasculitis and Inflammation Panel associated with vasculitis and autoinflammatory diseases (217, 218). The list of genes is presented in the Supplement.

To reduce the risk of missing potentially-deleterious variants, three prediction tools/programs were used to predict whether SNVs may affect protein function: Sorting Intolerant from Tolerant (SIFT), Polymorphism Phenotyping2 (Polyphen2) and Combined

Annotation Dependent Depletion (CADD). The SIFT program predicts whether amino acid replacement affects protein function based on physical properties and the conservation of amino acids. It is based on the assumption that important amino acids in the protein family are conserved, so changing at well-preserved positions will tend to be pathogenic (219). Variants are scored in the range 0 to 1, with a score of less than 0.05 indicating a deleterious mutation. The Polyphen2 program predicts the possible effect of amino acid replacement on protein structure and function by examining whether changes within the protein affect the ability to bind other molecules to form the secondary and tertiary structure of the protein (220). Variants are scored in the range of 0.0 (benign) to 1.0 (pathogenic). CADD evaluates the pathogenicity of SNVs and insertions/deletions in the human genome by integrating multiple annotations (221). The scoring range is from 1 to 99 and, the higher the score, the greater the deleteriousness of SNV-Indels. Using a bioinformatics pipeline and predictive tools, the list of potential candidate genes which could be causing the diseases was narrowed down.

Two additional annotations were used to assist with variant prioritisation as a measure of variant conservation: GERP scores and Siphy. GERP (Genomic Evolutionary Rate Profiling) is a method for the identification of slowly evolving regions in a multiple sequence alignment, defined as "constrained elements" (222). It is a score used to calculate the conservation of each nucleotide in multi-species alignment with ranges from -12.3 to 6.17, with 6.17 being the most conserved. Positive scores indicate that a site is under evolutionary constraint. Negative scores may provide weak evidence of accelerated rates of evolution. SiPhy (SIte-specific PHYlogenetic analysis) is another conservation score that takes into account the type of mutation (223). SiPhy scores are from dbNSFP, and are on the log odds scale, with most scores ranging between 0 and 20. Higher scores indicate higher conservation.

Familial relationships were checked by peddy and they all corresponded with what was expected.

Applying WES analysis, the mode of inheritance of the variants was analysed. The variants were classified according to the ACMG classification guidelines (213). Although the ACMG criteria are not strictly designed to be used in a research setting, they were applied as an exercise to potentially identify clinically relevant findings in this research which may be clinically reportable/of benefit for the patients and their families.

Additionally, HLA class I (*A, B, C*) and class II (*DRB1, DQA1, DQB1*) alleles were analysed in patients in whom novel and/or rare gene variants were detected while searching for SLE and IgAV susceptibility alleles (57, 58, 146).

4.2.4. PBMC isolation

PBMCs were isolated from a tube with the addition of anticoagulants (K3-EDTA) by the density gradient separation method. In short, the total amount of blood pre-diluted with phosphate buffered saline (PBS) (in the ratio 1:1) was slowly overlaid with a solution of Ficoll (Ficoll-Paque, GE Healthcare, Uppsala, Sweden) of a volume corresponding to the volume of undiluted blood. After centrifugation at 900 x g for 30 minutes the three phases separated. PBMCs presented in the so-called Ficoll ring were carefully pipetted into a new tube. The cells were twice washed in PBS and their precipitate was stored at -80 °C until further processing.

4.2.5. IFN gene expression signature in a PBMC sample

A RNA was purified from PBMC using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the standard manufacturer's protocol. In the first step, the cells were disrupted by adding Buffer RLT supplemented with β-mercaptoethanol. In the second step, the lysate was homogenised by pipetting into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at full speed. In the third step, One volume of 70% ethanol was added to the homogenised lysate, and mixed well by pipetting. In the forth step, 700 µl of the sample was transfered to an RNeasy MinElute® spin column placed in a 2 ml collection tube and centrifuged for 15 secs at 8000 x g (10000 rpm). The flow-through was discarded. In the fifth step, 350 µl Buffer RW1 was added to the RNeasy MinElute spin column and centrifuged for 15 secs at 8000 x g (10000 rpm) to wash the spin column membrane. The flow-through was discarded. In the sixth step, 10 µl DNase I stock solution was added to 70 µl Buffer RDD, mixed and centrifuged briefly to collect residual liquid from the sides of the tube. In the seventh step, the DNase I incubation mix (80 µl) was added to the RNeasy MinElute spin column and left at room temperature (25 °C) for 15 min. In the eighth step, 350 µl Buffer RW1 was added to the RNeasy MinElute spin column and centrifuged for 15 secs at 8000 x g (10000 rpm). The flow-through was discarded. In the ninth step, 500 µl Buffer RPE was added to the RNeasy MinElute spin column and centrifuged for 15 secs at 8000 x g (10000 rpm) to wash the spin column

membrane. The flow-through was discarded. In the tenth step, 500 µl 80% ethanol was added to the RNeasy MinElute spin column and centrifuged for 2 min at 8000 x g (10000 rpm) to wash the spin column membrane. In the eleventh step, the RNeasy MinElute spin column was placed in a new 2 ml collection tube and the old collection tube was discarded with the flowthrough. It was then centrifuged at full speed for 5 min. In the final step, the RNeasy MinElute spin column was placed in a new 1.5 ml collection tube and 14 µl RNase-free water was added directly to the spin column membrane and after that centrifuged for 1 min at 8000 x g (10000 rpm) to elute the RNA.

From the RNA samples extracted from the PBMCs, 500 ng of RNA was reverse transcribed into cDNA, following the protocols accompanying the ThermoFisher High-Capacity cDNA Reverse Transcription Kit. The cDNA was quantitatively analysed using an Applied Biosystems Taqman 7900HT Fast Real-Time PCR System. The outcome measure consisted of the cycle threshold (Ct) value which, when related to a housekeeping gene, could be used to quantify the presence of the transcripts and enable multiple calculations related to the IFN signature. An approximation method was used to determine relative gene expression with quantitative real-time PCR using the Delta-Delta-Ct (ddCt) Algorithm. Values have been normalised to the housekeeper gene RPL13a, and then with the mean of the controls. A subset of the following IFN-related genes was used to be able to detect the IFN signature: *ISG15*, *IF144*, *IFI44L*, *IFI6*, *MxA* and *OASL*. The genes were selected after literature research (224, 225).

5. RESULTS

5.1. Overview of basic data about the subjects

5.1.1. cSLE

100 patients (78 females and 22 males) with cSLE in the period from 1991 to 2019 were diagnosed and followed up at the University Hospital Centre Zagreb, Department for Paediatrics, Division of Clinical Immunology, Respiratory and Allergic Diseases, and Rheumatology. The median (range) age at diagnosis was 14 (6-19) years, the median (range) follow-up time was 8 (0.5-35) years, and 78% of patients were observed after the age of 18. Two patients died during the period studied. Median (range) SLEDAI-2K was 16.5 (6-47) at diagnosis.

Seven patients (7%) had cSLE onset before puberty, 30 (31%) during the peripubertal stage and 60 (62%) during the post-pubertal stage. Gender distribution was not equal in all three groups. The usual pattern of female predominance was found in groups with a later disease onset. In the peripubertal group, the girls to boys ratio was 2.7:1, and in the post-pubertal group it was 5:1. However, the prepubertal group showed a more homogeneous pattern, with the girls to boys ratio of only 1.3:1. When exploring values of the SLEDAI-2K scores within these groups, it was found that the SLEDAI-2K calculated at disease onset was significantly higher in the prepubertal group ($p = 0.019$).

After applying the inclusion criteria, 19 patients were identified who could be included in the study, but ultimately, WES was performed on 17 groups of three or "trios", each containing a proband case with cSLE and parents (including other informative family members), since by just using 2 patients it would not have been possible to assemble a "trio". The basic characteristics of the selected patients, with regard to the inclusion criteria, are shown in Table 8.

Table 8. Basic characteristic of included participants with cSLE with regard to the inclusion criteria. The red font colour in each patient highlightes the criteria that the patient met for inclusion in the study.

				Severity of SLE manifestations						
Pt	**Age at disease onset (years)**	**Family history of autoimmune diseases in first degree relatives**	**Family history of autoimmune diseases in second degree relatives**	**SLEDAI-2K score ≥20 in the disease course**	**ACR-97 and/or SLICC criteria present in the patient**	**Additional manifestations**	**Treatment**	**Consanguinity**	**Syndromic features**	**Gender**
1	15	negative	grandmother on father's side suffered from rheumatoid arthritis	23	1. oral ulcers 2. non-scarring alopecia 3. arthritis 4. hemolytic anemia, leukopenia, lymphopenia 5. lupus nephritis class V 6. seizures 7. ANA 1:200 8. aCL +, beta-2-GPI +; low CH50, C3, C4	1. interstitial lung disease 2. secondary hypogammaglobulinemia	CYC - 4 pulses, GC pulses, GCs, HCQ, MMF, IVIG	no	Kabuki syndrome	F
2	15	negative	negative	31	1. non-scarring alopecia 2. arthritis 3. proteinuria up to 0.64 g/dU, biopsy was not performed 4. seizures 5. hemolytic anemia, leukopenia, lymphopenia, thrombocytopenia 6. ANA 1:51200 7. anti-dsDNA +; aCL +, LAC +; low CH50, C3, C4	1. cutaneous vasculitis 2. secondary hypogammaglobulinemia	GC pulses, GCs, HCQ, AZA, IVIG	no	yes	F

				Severity of SLE manifestations						
Pt	**Age at disease onset (years)**	**Family history of autoimmune diseases in first degree relatives**	**Family history of autoimmune diseases in second degree relatives**	**SLEDAI-2K score ≥20 in the disease course**	**ACR-97 and/or SLICC criteria present in the patient**	**Additional manifestations**	**Treatment**	**Consanguinity**	**Syndromic features**	**Gender**
3	17	mother suffers from SLE and secondary antiphospho-lipid syndrome	cousin suffers from juvenile localized scleroderma and autoimmune thyroiditis	no	1. arthritis 2. pericardial effusion/pericarditis 3. lupus nephritis class II 4. ANA 1:640 5. anti-Sm +, anti-U1-RNP +, anti-histones +; LAC +, aCL +, beta-2-GPI +; low CH50, C4	1. myocarditis 2. pancreatitis	GC pulses, GCs, HCQ, AZA	no	no	F
4	13	mother suffers from ulcerative colitis, sister has autoimmune thyroiditis	father's sister suffers from ulcerative colitis, mother's brother suffers from spondylo-arthritis	no	1. malar rash 2. photosensitivity 3. ANA 1:5210 4. anti-dsDNA +; aCL +; low C3		GC, SZS, HCQ	no	no	F
5	9	negative	negative	23	1. malar rash 2. non-scarring alopecia 3. lupus nephritis class III 4. hemolytic anemia 5. ANA 1:640 6. low CH50, C3, C4; beta-2-GPI +	1. secondary hypogammaglobulinemia accompanied with numerous sepsis, septic shock, multiorgan failure	CYC - 1 pulse, GC pulses, GCs, HCQ, MMF, IVIG	no	no	F

				Severity of SLE manifestations						
Pt	**Age at disease onset (years)**	**Family history of autoimmune diseases in first degree relatives**	**Family history of autoimmune diseases in second degree relatives**	**SLEDAI-2K score ≥20 in the disease course**	**ACR-97 and/or SLICC criteria present in the patient**	**Additional manifestations**	**Treatment**	**Consanguinity**	**Syndromic features**	**Gender**
6	14	mother suffers from autoimmune thyroiditis	father's sister died due to rapidly progressive glomerulonephritis with renal failure, grandfather on mother's side suffered from type 1 diabetes	no	1. malar rash 2. photosensitivity 3. non-scarring alopecia 4. leukopenia, thrombocytopenia 5. ANA 1:640 6. SS-A+, SS-B +; low C4		GC, HCQ, AZA	no	no	F
7	16	mother suffers from autoimmune thyroiditis	negative	no	1. malar rash 2. non-scarring alopecia 3. arthritis 4. leukopenia 5. ANA 1:25600 6. anti-dsDNA +, anti-Sm +, anti-histones +, anti-U1-RNP +; low CH50, C4, C3	1. persistent AST/ALT elevation 4 x ULN	GC pulses, GCs, HCQ, AZA	no	no	F

				Severity of SLE manifestations						
Pt	**Age at disease onset (years)**	**Family history of autoimmune diseases in first degree relatives**	**Family history of autoimmune diseases in second degree relatives**	**SLEDAI-2K score ≥20 in the disease course**	**ACR-97 and/or SLICC criteria present in the patient**	**Additional manifestations**	**Treatment**	**Consanguinity**	**Syndromic features**	**Gender**
8	13	negative	mother's cousin suffers from SLE	28	1. malar rash 2. oral ulcers 3. lupus nephritis class V 4. hemolytic anemia, leukopenia, thrombocytopenia 5. lupus headache 6. ANA 1:3200 7. anti-dsDNA +, anti-Sm +, anti-histones +, anti-SS-A +, anti-SS-B +, anti-U1-RNP +, aCL +, beta-2-GPI + ; low C3, C4 and CH50	1. secondary antiphospholipid syndrome 2. cutaneous vasculitis	CYC - 6 pulses, RTX, GC pulses, GCs, MMF, HCQ	no	no	M
9	13	mother suffers from autoimmune thyroiditis	mother's aunt suffers from autoimmune thyroiditis and vitiligo	no	1. malar rash 2. photosensitivity 3. ANA 1:3200 4. anti-histones +; beta-2-GPI +	1. autoimmune cholangitis (AST/ALT/GGT elevation 6-8x ULN; histological and MR confirmation)	GCs, HCQ, AZA	no	no	F

				Severity of SLE manifestations						
Pt	**Age at disease onset (years)**	**Family history of autoimmune diseases in first degree relatives**	**Family history of autoimmune diseases in second degree relatives**	**SLEDAI-2K score ≥20 in the disease course**	**ACR-97 and/or SLICC criteria present in the patient**	**Additional manifestations**	**Treatment**	**Consanguinity**	**Syndromic features**	**Gender**
10	14	sister suffers from JIA (ERA)	negative	no	1. arthritis 2. lupus headache, CNS vasculitis 3. hemolytic anemia 4. ANA 1:10240 5. anti-dsDNA +, anti-histone +; aCL +	1. secondary Raynaud's phenomenon	GC pulses, GCs, HCQ, AZA, SZS	no	no	F
11	13	negative	father's aunt suffers from SLE	no	1. malar rash 2. pericarditis, pericardial effusion 3. lupus nephritis class IV 4. leukopenia 5. ANA 1:12800 6. anti-dsDNA +; aCL +; low C3; secondary immunodeficiency	1. secondary hypogammaglobulinemia	CYC - 5 pulses, GC pulses, GC, HCQ, MMF, RTX, IVIG	no	no	M
12	8	brother suffers from JIA (oligoarticular type), father suffers from MS	negative	no	1. malar rash 2. arthritis 3. leukopenia, hemolytic anemia 4. lupus nephritis class II/III 5. ANA 1:1280 6. anti-dsDNA +, anti-Sm +, anti-U1-RNP +; low CH50, C3, C4	1. secondary Raynaud's phenomenon 2. autoimmune hepatitis (AGLM +, not evaluated with biopsy)	GCs, HCQ, AZA, MTX	no	no	F

				Severity of SLE manifestations						
Pt	**Age at disease onset (years)**	**Family history of autoimmune diseases in first degree relatives**	**Family history of autoimmune diseases in second degree relatives**	**SLEDAI-2K score ≥20 in the disease course**	**ACR-97 and/or SLICC criteria present in the patient**	**Additional manifestations**	**Treatment**	**Consanguinity**	**Syndromic features**	**Gender**
13	11	brother and mother suffer from autoimmune thyroiditis	mother's grandmother suffer from autoimmune thyroiditis	no	1. malar rash 2. non-scarring alopecia 3. leukopenia 4. ANA 1:800 5. anti-dsDNA +, anti-histones +; low CH50, C3, C4	1. autoimmune hepatitis (AST/ALT elevation 4 x ULN, LKM-1 +, not evaluated with biopsy)	GCs, HCQ	no	no	F
14	15	mother suffers from autoimmune hemolytic anemia	negative	no	1. malar rash 2. photosensitivity 3. leukopenia, thrombocytopenia 4. ANA 1:1600 5. SS-A +, SS-B +	1. AST/ALT elevation 3 x ULN	GCs, AZA	no	no	M

				Severity of SLE manifestations						
Pt	**Age at disease onset (years)**	**Family history of autoimmune diseases in first degree relatives**	**Family history of autoimmune diseases in second degree relatives**	**SLEDAI-2K score ≥20 in the disease course**	**ACR-97 and/or SLICC criteria present in the patient**	**Additional manifestations**	**Treatment**	**Consanguinity**	**Syndromic features**	**Gender**
15	4	sister suffers from autoimmune hemolytic anemia	negative	no	1. rash 2. oral ulcers 3. lupus nephritis class IV 4. leukopenia, hemolytic anemia 5. ANA 1:3200 6. anti-dsDNA +, anti-Sm +, SS-A +, anti-histones +; low CH50, C3, C4	1. secondary hypogammaglobulinemia	CYC - 5 pulses, IVIG, GC pulses, GCs, MMF, HCQ,	no	no	M
16	13	negative	negative	30	1. malar rash 2. photosensitivity 3. seizures, acute confusion, altered perception, lupus headache, CNS vasculitis 4. renal - proteinuria up to 0.6 g/dU, biopsy was not performed 5. ANA 1:6400 6. anti-dsDNA +, anti-Sm +, SS-A +, anti-U1-RNP +, anti-ribosomal +; aCL +; low CH50, C3, C4;	1. AST/ALT elevation 3 x ULN	GC pulses, GCs, HCQ, AZA	no	no	M

				Severity of SLE manifestations						
Pt	**Age at disease onset (years)**	**Family history of autoimmune diseases in first degree relatives**	**Family history of autoimmune diseases in second degree relatives**	**SLEDAI-2K score ≥20 in the disease course**	**ACR-97 and/or SLICC criteria present in the patient**	**Additional manifestations**	**Treatment**	**Consanguinity**	**Syndromic features**	**Gender**
17	12	negative	negative	21	1. malar rash 2. non-scarring alopecia 3. oral ulcers 4. arthritis 5. persistent severe headache resistent to the therapy 6. hemolytic anemia, leukopenia 6. ANA 1:640 7. anti-Sm +, anti-U1-RNP +; low CH50, C3, C4	1. secondary Raynaud's phenomenon	GCs, HCQ, MTX	no	no	F

aCL: anti-cardiolipin antibody; AGLM: anti-smooth muscle antibody; ALT: alanine aminotransferase; ANA: antinuclear antibodies; anti-dsDNA: anti-double stranded DNA; anti-Sm: anti-Smith antibodies; anti-U1-RNP: anti-U1-Ribonucleoprotein autoantibodies; AST: aspartate aminotransferase; AZA: azathioprine; beta-2-GPI: beta-2-Glycoprotein I; C3: complement component 3; C4: complement component 4; CH50: total complement activity; CNS: central nervous system; CYC: cyclophosphamide; dU: daily urine sample; GC: glucocortiocid, HCQ: hydroxychloroquine; IVIG: intravenous immunoglobulin, LAC: lupus anticoagulant; LKM-1: liver kidney microsome type 1 antibodies; MMF: mycophenolate mofetil; MTX: methotrexate; Pt: patient; RF: rheumatoid factor; RTX: rituximab; SLEDAI-2K: modified Systemic Lupus Erythematosus Disease Activity Index; SSZ: sulfasalazine ULN: upper limits of normal.

It can be seen that cSLE did not begin in any of the patients included before the age of 5 and there was no consanguinity in any family. The most common inclusion criterion met by cSLE patients was severe clinical presentation (88.2%) followed by a positive family history of other autoimmune conditions in first degree relatives (58.8%), the most common of which were autoimmune thyroiditis (in five families), followed by juvenile idiopathic arthritis (2 families) and autoimmune hemolytic anemia (2 families). There were 5 males (29.4%) while only 2 patients had syndromic features (11.8%).

5.1.2. IgAV

Between 2009 and 2019, a total number of 611 children with IgAV, of which 320 boys and 291 girls with a median age of 6.33 (4.5 - 8.92) years, were referred to five tertiary teaching hospitals in Croatia. Statistically, there was no significant difference between gender proportions. The median (range) follow-up time was 49 (10-120) months. All patients had skin involvement. Out of 611 children, 475 (77.7%) had joint involvement, 281 (46%) had gastrointestinal manifestations, 130 (21.3%) had IgAVN, 29 (9.1% of male patients) had orchitis and 3 (0.5%) had central nervous system involvement. Among patients with IgAVN, 109 (17.8%) had nephritis at the time of diagnosis of IgAV or within one month of the first symptoms of IgAV, and in 21 patients (3.4%) IgAVN developed more than a month after the onset of the first symptoms of IgAV. The median (range) time for IgAVN development was 4 (0-390) days. Persistent IgAVN, defined as hematuria (>5 red blood cells/high power field or red blood cells casts or ≥2+ on dipstick) and/or proteinuria (>0.3 g/24 h or >30 mmol/mg of urine albumin/creatinine ratio) in a sample of the first morning urine that was present for more than 3 months, and was found in 48 (7.9%) children. Regarding gastrointestinal manifestations, 143 (50.9%) patients had mild gastrointestinal involvement, defined as abdominal pain with or without vomiting and diarrhea or positive fecal occult blood test without other gastrointestinal symptoms, 96 (34.1%) had moderate gastrointestinal manifestations, defined as abdominal pain with positive fecal occult blood test, while 42 out of 281 children (15%) had severe gastrointestinal manifestations including intussusception, hematochezia and/or massive gastrointestinal bleeding. Fifteen patients (2.5%) had the most severe skin symptoms which included bullae, ulcerations and necrotic lesions.

After applying the inclusion criteria, 5 patients were identified who could be included in the study, but ultimately, WES was performed on 3 of the "trios" containing a proband case with

IgAV and parents, since, as stated, with 2 patients it would not have been possible to assemble a "trio". The basic characteristics of the selected patients, with regard to the inclusion criteria, are shown in the Table 9.

Table 9. Basic characteristic of included participants with IgAV with regard to the inclusion criteria. The red font colour in each patient highlights the criteria that the patient met for inclusion in the study.

			Severity of clinical manifestations				
Pt	**Age at disease onset (years)**	**Family history of autoimmune diseases in first degree relatives**	**PVAS (the highest in the disease course)**	**Type of organ involvement**	**Treatment**	**Consanguinity**	**Syndromic features**
4*	5*	mother suffers from ulcerative colitis, sister has autoimmune thyroiditis	3	1. cutaneous - palpable purpura in the lower extremities with normal platelet count 2. joint - bilateral ankle arthralgia	NSAID	no	no
18	12	negative	27	1. cutaneous - palpable purpura in the lower extremities with normal platelet count 2. joint - pain in the wrists, knees, and ankles 3. renal - IgAVN class II according to ISKDC classification and M1E1S0T0-C0 acording to updated Oxford classification - proteinuria 1.34 g/dU, edema, hypertension 4. gastrointestinal - severe abdominal pain, diarrhea, positive occult bleeding 5. pulmonal - RDS 6. scrotum - scrotal edema	GC pulses, GCs, MMF	no	no
19	5	mother suffered from IgAV in childhood	6	1. cutaneous - palpable purpura starting on lower extremities and spreading on the trunk, upper extremities and face with normal platelet count 2. joint - arthritis of radiocarpal and talocrural joints 3. IgA deposits - confirmed with the skin biopsy	GCs, NSAID	no	no

4*: patient 4 fulfilled both criteria for cSLE and IgAV, since she was diagnosed with both diseases, in different period; dU: daily urine sample; GC: glucocorticoid; IgAV: IgA vasculitis; IgAVN: IgA vasculitis nephritis; ISKDC: International Study of Kidney Disease in Children classification; MEST-C score (M: mesangial hypercellularity, E: endocapillary hypercellularity, S: segmental sclerosis, T: interstitial fibrosis/tubular atrophy, C: crescents); MMF: mycofenolate mofetil; NSAID: non-steroidal anti-inflammatory drug; Pt: patient; PVAS: Paediatric Vasculitis Activity Score; RDS: respiratory distress syndrome.

The most common inclusion criterion met by IgAV patients was a positive family history of autoimmune conditions (66.7%), followed by severe clinical presentation (33.3%). None of the patients had early-onset disease, syndromic features or consaguinity. One patient (patient 4) fulfilled both criteria for cSLE and IgAV, since she was diagnosed with both diseases in a different period of time.

5.1.3. WES

After performing WES and analysis of gene variants, novel and/or rare variants were detected in 8 patients. Among these, 7 patients were diagnosed with cSLE and 1 patient with IgAV, while 1 patient was diagnosed with cSLE and IgAV in a different period of time. A total number of 20 variants were prioritised for more detailed analysis (Table 10).

Table 10. Novel and/or rare gene variants detected in patients with cSLE and IgAV.

Pt	Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
1	*KMT2D*	g.49432513delC (GRCh37)	NM_003482.3	c.8626delC	p.Gln2876Serfs*34	FS	34	0	N/A	N/A	N/A	2.27	pathogenic
	C1S	g.7173893G>A (GRCh37)	NM_001734.3	c.943G>A	p.Asp315Asn	MS	11	0.003	Del (N/A)	Prob Dam (0.976)	25	2.14	benign
	C1R	g.7242740G>C (GRCh37)	NM_001733.4	c.336G>C	p.Met112Ile	MS	3	0.004	Del (0.04)	Benign (0.053)	22.2	1.49	benign
2	*ADAR*	g.154561097A>G (GRCh37)	NM_001111.3	c.2815A>G	p.Ile939Val	MS	10	<0.0001	Del (0.01)	Prob Dam (0.97)	25.4	2.27	likely pathogenic
	SH2B3	g.111856347G>A (GRCh37)	NM_005475.2	c.398G>A	p.Cys133Thr	MS	2	<0.0001	Benign (0.81)	N/A	11.86	0.207	likely benign
3	*CR1*	g.207741268dup (GRCh37)	NM_000651.4	c.4052dup	p.Asp1351Glufs*23	FS	25	<0.0001	N/A	N/A	N/A	-1.38	likely pathogenic
	BLK	g.11405576G>A (GRCh37)	NM_001715.2	c.211G>A	p.Ala71Thr	MS	4	0.01	Del (0.01)	Benign (0.286)	5.856	1.71	likely pathogenic
4	*IRF5*	g.128587524C>T (GRCh37)	NM_001098629.1	c.722C>T	p.Ala241Val	MS	6	0.0005	Tol (0.22)	Benign N/A	14.76	-3	likely benign
	IRF3	g.50165280A>G (GRCh37)	NM_001197122.1	c.907A>G	p.Ser303Gly	MS	6	<0.0001	Tol (0.14)	Benign (0.007)	12.97	-2.97	likely benign
	LIFR	g.38489213A>G (GRCh37)	NM_001127671.1	c.2302A>G	p.Arg768Gly	MS	16	0.0009	Del (0.02)	Benign (0.054)	22.7	2.16	VUS
5	*RAG2*	g. 36592776A>G (GRCh38)	NM_000536.3	c.1393A>G	p.Thr465Ala	MS	3	0	Tol (0.07)	Benign (0.138)	22.9	3.98	VUS

Pt	Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
6	*TYK2*	g.10468477A>T (GRCh37)	NM_003331.4	c.2429A>T	p.Asp810Val	MS	17	<0.0001	Del (N/A)	Pos Dam (0.905)	24.8	2.6	VUS
	SAMHD1	g.35569456G>A (GRCh37)	NM_015474.3	c.334G>A	p.Val112Ile	MS	3	0.0003	Tol (0.45)	Benign (N/A)	0.002	-4.07	likely benign
	DNASE1	g.3707191G>A (GRCh37)	NM_005223.3	c.553G>A	p.Val185Ille	MS	7	0.003	Tol (1)	Benign (N/A)	12.75	-3.98	likely benign
	BCL10	g. 85733632A>T (GRCh37)	NM_003921.4	c.380A>T	p.Asp127Val	MS	3	0	Tol (0.4)	Benign (0.003)	19.32	-4.3	likely benign
7	*CFD*	g.861903G>A (GRCh37)	NM_001928.2	c.562G>A	p.Ala188Thr	MS	4	<0.0001	Tol (0.86)	Benign (0.001)	15.67	-2.11	likely benign
	PTPN13	g.87656789G>A (GRCh37)	NM_006264.2	c.2194G>A	p.Ala732Thr	MS	15	0.0004	Del (0.04)	Pos Dam (0.777)	24.8	1.75	VUS
18	*BAD*	g.64270254G>C (GRCh38)	NM_004322.3	c.462G>C	p.Trp154Cys	MS	3	0.0005	N/A	Prob Dam (0.999)	34	2.22	VUS
	MSH2	g. 47470984G>A (GRCh38)	NM_000251.2	c.1681G>A	p.Glu561Lys	MS	11	<0.0001	Tol (0.07)	Benign (0.367)	24.5	2.68	likely benign
	DHX58	g. 42110724A>G (GRCh38)	NM_024119.2	c.560A>G	p.Gln187Arg	MS	5	<0.0001	Tol (0.23)	Pos Dam (0.904)	27.8	2.54	VUS

A: adenine; AF: allele frequency; Ala: alanine; Arg: arginine; Asn: asparagine; Asp: aspartate; C: cytosine; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; Cys: cysteine; Del: deleterious; del: deletion; dup: duplication; FS: frame-shift; G: guanine; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; Gln: glutamine; Glu: glutamate; Gly: glycine; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; GRCh38: Genome Reference Consortium Human genome build 38; Ile: isoleucine; Lys: lysine; Met: methionine; MS: missense; N/A: not available; p.: protein protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; Prob Dam: probably damaging; Pos Dam: possible damaging; Pt: patient; Ser: serine; SIFT: Sorting Intolerant from Tolerant prediction tool; T: thymine; Thr: threonine; Tol: tolerant; Trp: tryptophan; Val: valine; VUS: variant of uncertain significance.

Since it is impossible to interpret the variants without a clinical context, the results obtained for each individual proband or "trio" are presented below, by first providing a brief summary of the participants involved in WES, then a detailed description of the clinical picture, including criteria based on the diagnosis made and inclusive criteria that had to be met by each individual subject, followed by the results of the genetic analysis undertaken.

5.2. Family 1

Case summary

The first proband (patient 1) is a 21-year-old Caucasian female with syndromic features of clinically unrecognised Kabuki syndrome, who developed cSLE at the age of 15, complicated with lupus nephritis, neurolupus, immunodeficiency and intestitial lung disease. WES was performed on five members of the family 1 (proband case, two healthy parents and two healthy siblings).

Clinical report

The patient was the first child of non-consanguineous parents. Her grandmother on her father's side suffered from rheumatoid arthritis. There was no other significant family history. She has two healthy parents and two healthy siblings.

During infancy, she sought medical attention due to bilateral mixed hearing loss and developmental delay. At the age of 14 she had an isolated episode of grand mal seizures, followed by painful swelling of the right knee which regressed spontaneously after a few days. Seven months later, she developed bilateral leg and face edema and painful swelling of the right knee and both ankles, oral ulcers accompanied by an elevated erythrocyte sedimentation rate.

At the age of 15, the girl was referred to the University Hospital Centre Zagreb, Department for Paediatrics, Division of Clinical Immunology, Respiratory and Allergic Diseases, and Rheumatology, with bilateral leg edema, swelling of the knees, ankles and face, developmental delay and dysmorphic features (Figure 7).



Figure 7. Phenotypic features of patient 1. The top left photo shows a round face, hypertelorism, large protruding earlobes, a flat, broadened tip of the nose, elevated eyebrows with symmetrically interrupted growth and flat philtrum. The top right photo shows deviation of the fourth finger and persistent fetal peds. The bottom photo: limb anomalies including a shorter second toe with the fifth toe in medial deviation on both feet. Author's own archive presented with permission of the parents and patient.

In a physical examination, to be highlighted are short stature (height below the 1st percentile), round face, hypertelorism, a flat, broadened tip of the nose, elevated eyebrows with

symmetrically interrupted growth, high arched palate with aphthous ulcers along the hard palate and somewhat shorter neck. Her fingers were spindly with a deviation of the fourth finger on both hands, a shorter second toe with the fifth toe in medial deviation on both feet. There was diffuse distal extremity swelling with pitting edema. Both knees and ankles were swollen.

Significant proteinuria (5 g in a 24 hour urine sample), highly elevated erythrocyte sedimentation rate, anemia, leucopenia, hypoproteinemia, low immunoglobulin levels with positive ANA and aPL (anti-cardiolipin (aCL) and anti-β_2-glycoprotein I) were the most prominent laboratory parameters, while C3, C4 and CH50 were normal. Due to severe proteinuria, a renal biopsy was performed and showed class V membranous lupus nephritis (ISN/RPS 2004 classification) with abundant deposits of C1q and C3 and moderate granular deposits of IgG and IgM.

The patient satisfied 7 out of 11 ACR-97 classification criteria for SLE (oral ulcers, arthritis, hematologic disorders, renal, neurologic and immunologic disorder, and positive ANA) as well as 10 of 17 SLICC criteria (7 clinical criteria: oral ulcers, non-scarring alopecia, arthritis, renal, neurologic, hemolytic anemia, leukopenia and 3 immunologic criteria: positive ANA and aPL, and, later on, low complement levels were detected).

Subsequently, the pediatric geneticist scored the phenotypic characteristics of the patient and established the diagnosis of Kabuki syndrome clinically, using the five cardinal manifestations. The patient had 5 out of a total of 10 points on the scoring system proposed by Makrythanasis et al. supporting the clinical diagnosis of Kabuki syndrome (226). The typical facial features were present, although somewhat obscured by the edematous features due to secondary glucocortiod use. The patient also had brachydactily affecting the second toe bilaterally, although brachydactily and syndactyly typically affecting the fifth digit have been described in Kabuki syndrome.

Initial satisfactory cSLE control was established with glucocorticoid treatment (pulse therapy for three consecutive days, followed by oral glucocorticoids) and pulse cyclophosphamide therapy (4 cycles). Later, the cyclophosphamide was replaced with mycophenolate mofetil due to repeated sepsis and the dose of glucocorticoids was slowly reduced. The persistently low immunoglobulin level was treated with intravenous gamma globulins and frequent albumin replacements were also required.

Two years after the onset of the disease, the patient's condition began to worsen. She experienced bilateral pneumonia several times, multiple vertebral fractures, for which she was

treated with bisphosphonates and multiple episodes of vomiting accompanied by a refusal to eat solid food and weight loss. Examination of the gastrointestinal tract revealed slower esophageal peristalsis, intestinal malrotation and edema of the upper gastrointestinal mucosa.

Five years on from the onset of the disease, a deterioration in renal function was observed. However, the planned rebiopsy of the kidney could not be completed due to the extremely poor visualisation of the urinary tract with an abnormally placed right kidney, conditioned by the constitution of the patient and also by the side effects of previous treatment.

During the follow-up, significant spirometrically-defined restrictive ventilatory defects were noticed with a mosaicism of attenuation of the lung parenchyma in terms of air-trapping and small airway disease. Furthermore, the existing immunodeficiency deterioated (extremly low CD4+ and CD19+ account), which required prophylaxis of opportunistic infections. At the age of 20 she had cataract surgery on both eyes.

At the last evaluation at the Department at the age of 21, an impaired general condition was observed, primarily in the form of physical deterioration - cachexia (she lost more than 20 kilograms of body weight), and cognitive regression with multiple hematomas on the lower extremities (Figure 8).



Figure 8. Photos taken during the last evaluation showing physical deterioration (left) and multiple hematomas on the lower extremities (right). Author's own archive presented with the permission of the parents and patient.

An extensive diagnostic assessment revealed multiple calcified micronodules in the lung parenchyma of all lobes, primarily sequelae of granulomatous inflammation with a ground glass pattern in the left upper lobe (Figure 9). Tuberculosis as a possible cause of the patient's condition was excluded.

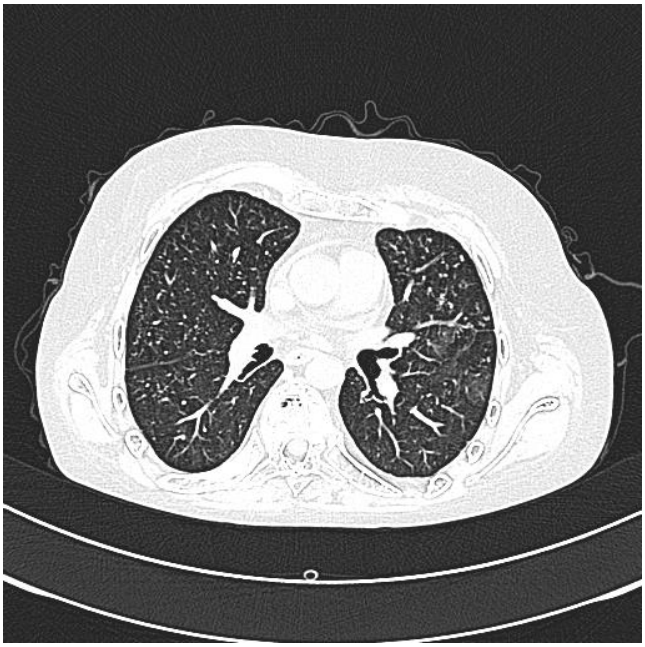


Figure 9. High-resolution computed tomography showing multiple calcified micronodules in the lung parenchyma of all lobes with a ground glass patern. Presented with the permission of the parents and patient.

Neverheless, a clear substrate that would explain repeated vomiting and cachexia was not revealed.

Patient 1 satisfied two inclusion criteria: severe clinical presentation (patient required intravenous pulses of glucocorticoids and cyclophosphamide for achieving remission at disease onset, developed lupus nephritis, neuropsychiatric lupus and later pulmonary involvement) and syndromic cSLE defined as the presence of growth failure in height and weight not explained by drug exposures, intellectual deficiency, as well as dysmorphic features. She had a confirmed family history of autoimmune conditions (rheumatoid arthritis), but not found in the first degree relatives.

Genetic analysis

Patient's 1 exome was found to contain a total number of 31,306 variants, including 12,042 homozygous, 18,610 heterozygous, 28,853 SNVs, 941 insertion and 1,003 deletion mutations. The list was first narrowed down to 12,255 variants, including only coding SNVs and indels and excluding synonymous and splicing mutations. By filtering, using the data from ExAC and gnomAD, variants with minor allele frequencies below 0.005 were identified and included in further analysis, which narrowed the candidate list to 2,208. After application of two *in silico* gene panels known to cause monogenic SLE, interferonopathies or found within GWAS SLE loci and the Vasculitis and Inflammation Panel, combined with a Phenotype to Genotype-based variant searching filter which uses the phenotypes collected from specified patients, a search was made for gene relationships using the Online Mendelian Inheritance in Man (OMIM), the online catalogue of human genes and genetic disorders, and resulted in 29 variants being prioritised for closer investigation. Among these variants using segregation analysis in the family, computational prediction tools SIFT, Polyphen2 and CADD, as well as the GERP score and the SiPhy evaluation of variant conservation, and a search in the literature regarding the possible association of the filtered variants with the fenotype, the analysis yielded three variants of particular interest (Figure 10).

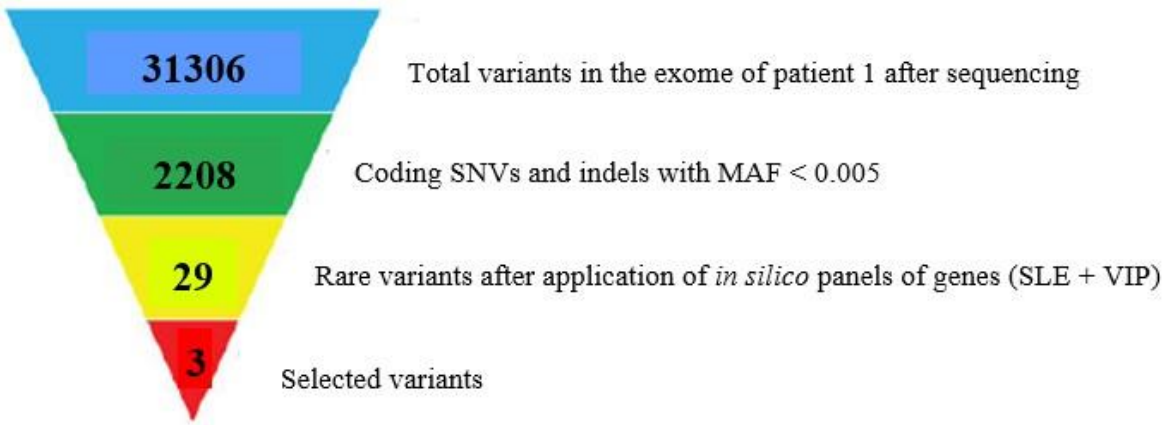


Figure 10. Schematic representation of the exome data-filtering approach used to identify variants in patient 1. MAF: minor allele frequency; SLE: systemic lupus erythematosus; SNVs: single nucleotide variants; VIP: Vasculitis and Inflammation Panel.

The variant that attained the highest score was a structural, frame-shift variant in exon 34 of the *KMT2D* gene as a consequence of 1bp deletion (NM_003482.3:c.8626delC; 55 reads C,

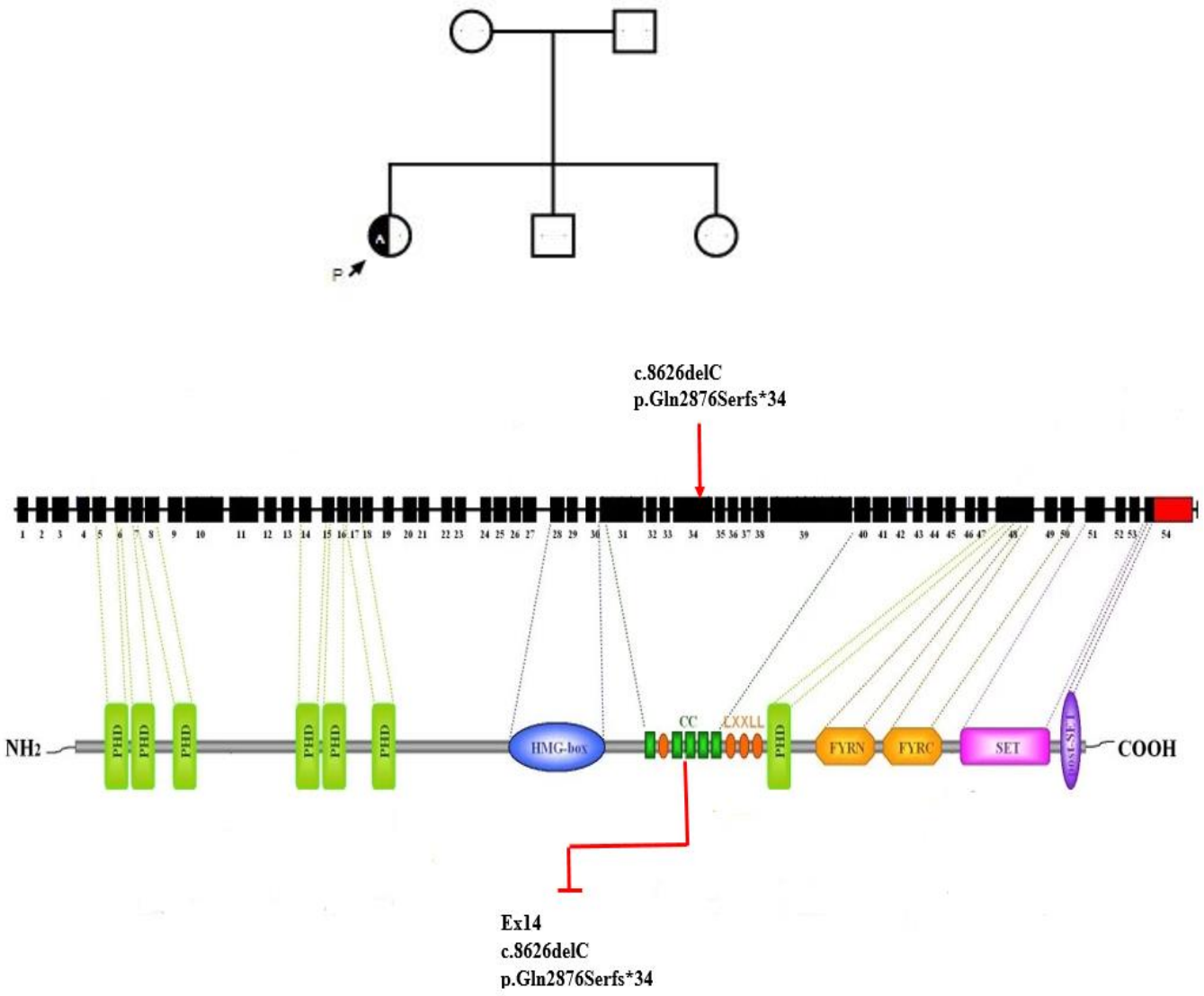
56 reads delC), on chromosome 12, at chromosome position 49432513 (GRCh37), predicted to truncate the protein (p.Gln2876Serfs*34) resulting in *KMT2D* loss of function. It was confirmed by Sanger sequencing. This *KMT2D* variant occurred "*de novo*" (not present in parents and siblings) and has not been described previously in the population databases. The variant was classified as pathogenic since it fulfilled one very strong, one strong, two moderate and one supporting criteria according to the ACMG classification guidelines (213) and submitted in ClinVar (submission number SUB4319245) (Table 11).

Table 11. Scoring *KMT2D* variant according to ACMG classification criteria.

Pathogenicity Criterion	***KMT2D*, (p.Gln2876Serfs*34)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	**Yes**	Frame-shift, truncating mutation in a gene where LOF is a known mechanism of disease
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	**Yes**	Paternity and maternity confirmed through analysis of rare variants and Mendelian conflicts in WES data
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	**Yes**	Absent
PM3. For recessive, phase trans	No	
PM4. Change in protein length	**Yes**	In-frame deletion variant
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	**Yes**	Kabuki syndrome
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 VSP + 1 SP + 2 MP + 1 SP	
INTERPRETATION	PATHOGENIC	

The KMT2D protein contains a number of domains. Two parts of the protein have a crucial role for protein function: plant homeotic domains (PHDs) in the N-terminus region (three PHDs per cluster) and an enzymatically active C-terminal SET domain (227). The PHDs in the second cluster (PHD4-6) recognise H4 tails on nucleosomes and could be critical for KMT2D-catalysed nucleosome methylation. SET domain is responsible for both enzymatic activity and

maintaining protein stability of KMT2D in cells. Near the SET domain are PHD and FY-rich N/C-terminal (FYRN and FYRC) domains. In addition, a high mobility group (HMG-I) and nine nuclear receptor interacting motifs (LXXLLs) are found within the protein. The mutation occurred at 34 exon (Figure 11), in the second half of the gene, which contains 54 exons and distant from the 3'-end of the KMT2D, which is important to emphasise since the position of mutation is associated with phenotype specificities (228, 229).



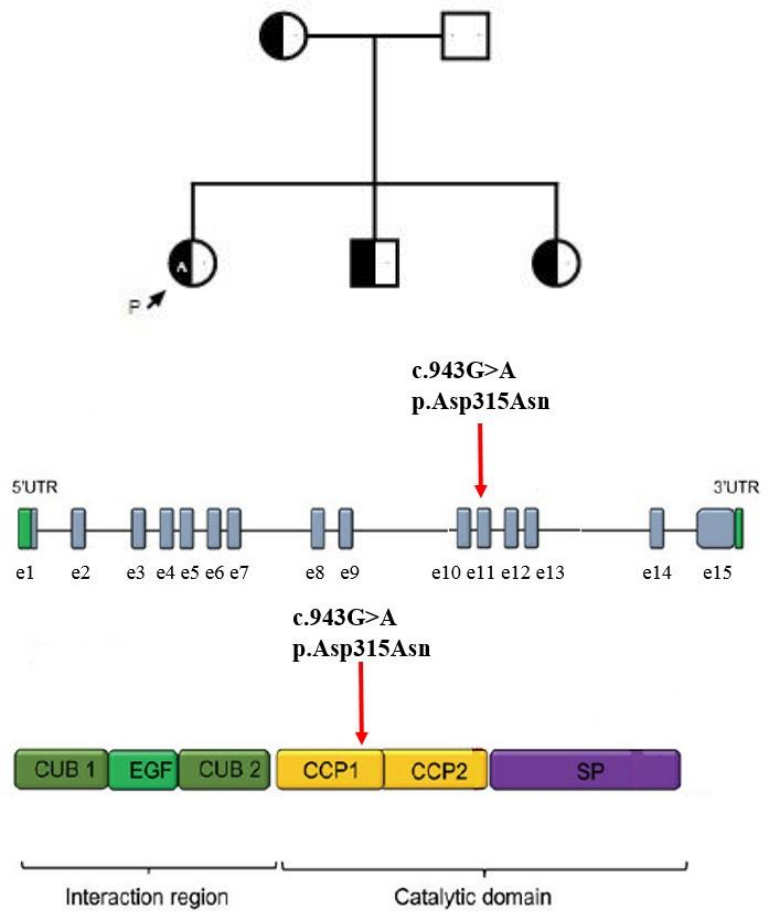
Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
KMT2D	g.49432513delC (GRCh37)	NM_003482.3	c.8626delC	p.Gln2876Serfs*34	FS	34	0	N/A	N/A	N/A	2.27	pathogenic

Figure 11. Pedigree of family 1 (top). Schematic representations of the *KMT2D* gene and KMT2D protein domains show the position of novel c.8626delC, (p.Gln2876Serfs*34) mutation (bottom). Modified according to the reference number 230. AF: allele frequency; C: cytosine; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; Del: deleterious; del: deletion; FS: frame-shift; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; Gln: glutamine; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; N/A: not available; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; Ser: serine; SIFT: Sorting Intolerant from Tolerant prediction tool.

Because this is a truncating frame-shift mutation, regardless of whether it occurred upstream of the SET domain, it will predictably affect it as well due to the frame-shift effect, which should jeopardise both enzymatic activity and protein stability.

Search of pathogenic *KMT2D* variants identified at least 88 pathogenic frameshift variants (54 nonsense mutations, 23 small deletions and 11 small indels) which truncate the *KMT2D* protein distal to the mutation identified in the patient, confirming its pathogenicity (231). To date, there are no functional tests to check the impact of the mutation *in vitro*, so the diagnosis was based on a confirmed pathogenic variant and compatible clinical features. The gene is linked with Kabuki syndrome, which is a rare multiple malformation disorder associated with immunodeficiency and often accompanied with autoimmunity, and is assessed to be consistent with patient clinical presentation and disease course. The results were published in 2019 (232).

Interestingly, this patient has two additional variants which are of interest for further analysis. The first is a rare (minor allele frequency (MAF) = 0.003) heterozygous missense mutation in *C1S* gene (NM_001734.3:c.943G>A; 20 reads G, 27 reads A) on chromosome 12, at chromosome position 7173893 (GRCh37), resulting in a substitution of aspartic acid with asparagine at amino acid position 315 (p.Asp315Asn), predicted to be probably damaging (Polyphen2 = 0.976). When the variant was analysed using SIFT, it was predicted to be deleterious, with a high CADD score (CADD = 25). Measuring variant conservation with a GERP score = 2.14 and Siphy Logodds = 13.8749, it was found to be in line with the observation that nucleotide substitution occurred at the site that is under evolutionary constraint, predicting that it may cause protein disruption. This is further supported by the fact that amino acid substitution occurred at position 315, which is located within CCP1 domain of the C1S protein. Specifically, the protein C1S consists of six domains: CUB1, EGF, CUB2, CCP1, CCP2 and SP domain. The SP domain is the most important one, since it has the catalytic activity (233), while CCP1 and CCP2 are involved in substrate recognition or dimerisation of the molecule and the first three domains are important for protein interactions. Thus, the change of amino acid change affected CCP1 domain, and one could expect this may alter with the protein function (Figure 12).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
C1S	g.7173893G>A (GRCh37)	NM_001734.3	c.943G>A	p.Asp315Asn	MS	11	0.003	Del (N/A)	Prob Dam (0.976)	25	2.14	benign

Figure 12. Pedigree of family 1 (top). Schematic representations of the *C1S* gene and C1S protein domains show the position of c.943G>A, (p.Asp315Asn) mutation. Modified according to the reference number 233. A: adenine; AF: allele frequency; Asn: asparagine; Asp: aspartate; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; Del: deleterious; G: guanine; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; MS: missense; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; Prob Dam: probably damaging; SIFT: Sorting Intolerant from Tolerant prediction tool.

Although aspartic acid and asparagine share some similar properties since they are in the same class and the physicochemical distance is not large, the substitution may influence the protein function according to the similar amino acid change in the phosphoglycerate kinase variant associated with red cell enzyme deficiency and heat instability, in hereditary deficiency

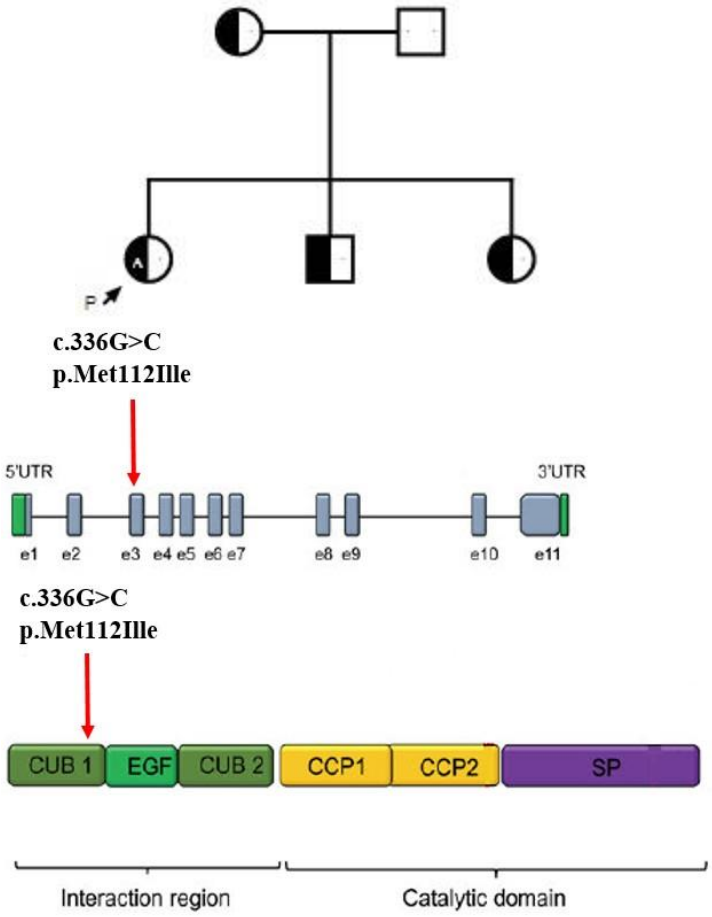
of the phosphoglycerate kinase which is linked with hemolytic anemia (234). Thus, the reported amino acid substitution may represent a change in the chemical composition and properties of the protein and an influence on protein function could be expected. The gene is associated with monogenic forms of SLE (with estimated penetrance of around 65%), which is in concordance with the disease of the patient (86). Mutation is inherited from the clinically unaffected mother and shared with the two clinically unaffected siblings. It unlikely causes SLE in the patient under the established autosomal recessive mode of inheritance of C1S deficiency (86). Using the criteria of the ACMG classification guidelines, the variant satisfied two strong criteria for benign impact, since there is a lack of segregation with the disease and presence of the same variant in an adult individual (mother) knowing that the monogenic forms of SLE caused by C1S deficiency occur in early childhood (Table 12).

Table 12. Classification of the *C1S* variant according to the ACMG criteria.

Pathogenicity Criterion	*C1S*, (p.Asp315Asn)	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Rare allele, MAF = 0.003
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	Yes	Polyphen2 = 0.976, SIFT = deleterious, CADD = 25
PP4. Phenotype specific for disease	Yes	SLE
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP + 2 SP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	*C1S*, (p.Asp315Asn)	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	Yes	Observed in healthy mother, the monogenic forms of SLE caused by C1S deficiency occur in early childhood
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in unaffected mother and two healthy siblings
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	No	
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	2 S	
INTERPRETATION	BENIGN	

The second variant is a rare (MAF = 0.004) heterozygous mutation on chromosome 12, at chromosome position 7242740 (GRCh37) of the *C1R* gene (NM_001733.4:c.336G>C). This mutation resulted in an amino acid change at position 112, within the CUB1 domain, which is the interaction domain of the C1R protein (233), and was predicted to be deleterious by SIFT = 0.04 and damaging according to high CADD scores (CADD = 22.2) (Figure 13).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
C1R	g.7242740G>C (GRCh37)	NM_001733.4	c.336G>C	p.Met112Ile	MS	3	0.004	Del (0.04)	Benign (0.053)	22.2	1.49	benign

Figure 13. Schematic representations of the *C1R* gene and C1R protein domains show the position of c.336G>C, (p.Met112Ile) mutation. Modified according to the reference number 233. AF: allele frequency; C: cytosine; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; Del: deleterious; G: guanine; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; Ile: isoleucine; Met: methionine; MS: missense; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; SIFT: Sorting Intolerant from Tolerant prediction tool.

However, *in silico* tools were not uniform in the prediction of the protein disruption, since according to Polyphen2 = 0.053 it was predicted to be tolerated. The substitution occurred in the semiconservative region (GERP score = 1.49), which indicates moderate impact on protein function. Similar to the previous variant, C1R deficiency is inherited in autosomal recessive mode and it is expected to be manifest in early childhood, so the presence of the same variant

in the healthy mother and both siblings suggest a lack of segregation, and fits in with the definition of a benign variant according to ACMG criteria (Table 13).

Table 13. Classification of the *C1R* variant according to the ACMG criteria.

Pathogenicity Criterion	***C1R*, (p.Met112Ile)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	**Yes**	Rare alle, MAF = 0.004
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	**Yes**	SLE
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP + 1 SP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***C1R*, (p.Met112Ile)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	**Yes**	Observed in healthy mother, the monogenic forms of SLE caused by C1S deficiency occur in early childhood
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	**Yes**	Present in unaffected mother and two healthy siblings
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	No	
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	2 S	
INTERPRETATION	BENIGN	

Finally, knowing that one of the strongest and most potent genetic risk factors for SLE resides in the HLA region, the HLA haplotypes in patient 1 were analysed in detail. The analysis revealed that the patient has *HLA-B*08:01, HLA-DQA1*01:02, HLA-DQB1*02:01* and *HLA-DRB1*03:01*, all proved to be SLE susceptibility alleles (57, 58).

5.3. Family 2

Case summary

WES was performed on three members of family 2 (proband case with cSLE and two unaffected parents). The proband is a 19-year-old Caucasian female (patient 2) with syndromic features of a clinically unrecognised syndrome, who developed cSLE at the age of 15, complicated with lupus nephritis, CNS involvement, immunodeficiency and coagulopathy.

Clinical report

The patient was the second child of non-consanguineous parents, with significant family history. Her brother suffers from epilepsy. Her mother has hyperpigmented changes on the forehead, chin and cheeks, as well as on the trunk and dorsum of the hands. The mother's aunt had dysmorphic features, including skeletal limb abnormalities, structural alterations in the abdominal wall and proptosis with normal psychomotor development. The father's grandmother gave birth to five children, three of whom died in childbirth (premature birth). On the paternal side of the family there is a relative with trisomy 21, who suffers from cerebral palsy. There is also a male child on the father's side of the family, with severe developmental delay, who died at the age of 17.

Poor weight gain, slow feeding habits, and physical delays were detected soon after delivery and during the first 3 months of life, the patient required medical attention due to dysphagia, dysmorphic features and failure to thrive. Diagnostic investigations were initiated on suspicion of a metabolic disease, primarily mucopolysaccharidosis type 1, which has not been proven. The diagnosis of Noonan syndrome was also considered but the child did not come for further check-ups until the age of 13. From early infancy to the age of 7, she was fed through a nasogastric tube due to food rejection. In the third year of her life she began to sit, walked at the age of 4, began to use words with meaning when she was 2 years old. At 14 years of age

she was hospitalised with a perityphlitic abscess, and also a year after due to clinical signs of bleeding with spontaneous hematomas on the extremities and pathological findings of coagulation parameters (prolonged values of prothrombin time, activated partial thromboplastin time, elevated fibrinogen, D-dimer and lupus anticoagulant). Persistent moderate proteinuria was noticed and other findings include splenomegaly and an atrial septal defect. The etiology of primary and consequent pathological events has not been revealed.

She was reffered to the University Hospital Centre Zagreb, Department for Paediatrics, Division of Clinical Immunology, Respiratory and Allergic Diseases, and Rheumatology when she was 15 years old with fever, numerous hematomas and petechiae, swelling of the knees, polyarthralgias, frequent diarrheal stools accompanied by abdominal pain, inappetence and weight loss (Figure 14).



Figure 14. Phenotypic features of patient 2: short stature, conspicuously dark pigmented skin, hypertelorism, coarse facial features, exophthalmos and strabismus of the left eye present and ptosis of the left eyelid. Archive of Professor Marija Jelusic, presented with the permission of the parents and patient.

Physical examination revealed short stature (height below the 1st percentile), weight below the 1st percentile, no signs of pubertal characteristics, conspicuously dark pigmented skin with areas of hyper- and hypopigmentation on the face, trunk and dorsal surfaces of the hands and feets, and with numerous hematomas on the limbs and trunk, prominent neurocranium, facial dysmorphia - hypertelorism, coarse facial features, exophthalmos and strabismus of the left eye present, ptosis of the left eyelid, pronounced philtrum, low-set ears wide neck with pterygium, systolic heart murmur, abdomen above chest level with palpable spleen, flexion contractures of both elbows and hypotrophy of limb musculature.

Laboratory findings showed highly elevated erythrocyte sedimentation rate, pancytopenia, coagulation disorder (prolonged prothrombin and activated partial thromboplastin time), proteinuria (0.64 g in 24 hour urine sample), positive antinuclear antibodies (ANA) in high titer (1:51200), positive anti-dsDNA, anti-histone and antiphospholipid antibodies (aPL) (anti-cardiolipin (aCL) and lupus anticoagulant (LAC)) and low C3, C4 and CH50. Other laboratory findings included CD4+ lymphopenia, hypovitaminosis D3 and B12 and a high titer of antibodies to tissue transglutaminase.

Among the other initial extensive work to be highlighted are the ultrasound findings of an enlarged liver with signs of diffuse lesion and splenomegaly, hypertrophic synovium of the elbows and knees with increased Doppler flow (synovitis), densitometry results indicating an extremely reduced bone mineral density and retinal detachment of the left eye with maculopathy (dystrophy) of the right eye.

The patient fulfilled 6 out of 11 ACR-97 classification criteria for SLE (arthritis, hematologic disorders, immunologic disorder and positive ANA at disease onset and later she developed grand mal epileptic seizures and renal disorder) as well as 11 of the 17 SLICC criteria (7 clinical criteria: non-scaring alopecia, arthritis, renal and neurologic disorder, hemolytic anemia, leukopenia, lymphopenia, thrombocytopenia and 4 immunologic criteria: positive ANA, anti-ds DNA and aPL with low complement). She was initially treated with glucocorticoids (she received 2 x three pulses of glucocorticoids at one week intervals, followed by oral glucocorticoids) with hydroxychloroquine and azathioprine.

She soon developed hypogammaglobulinemia, which required replacement therapy with intravenous gamma globulins and severe CD4+ lymphopenia, so prophylaxis for opportunistic infections was started.

As the disease progressed, grand mal type epileptic seizures occured repeatedly with electroencephalography-detected low-voltage dysrhythmic changes and superposition of beta activity, which required the use of intravenous pulses of glucocorticoids and antiepileptic drugs to establish satisfactory seizure control. Magnetic resonance imaging and cerebral angiography revealed moderate reduction of cerebral white matter, with consequently dilated lateral ventricles, hypotrophic chiasm and prehyasmatic segments of the optic nerves and enlarged right eye, without any signs typical for vasculitis of the central nervous system (Figure 15).

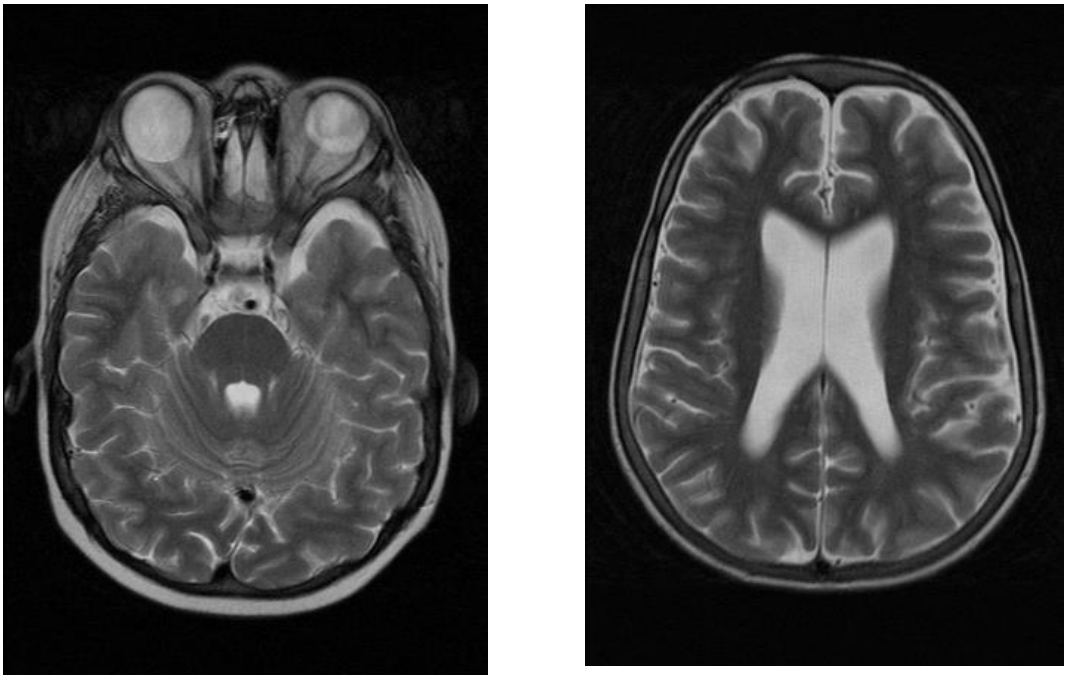


Figure 15. Magnetic resonance showing hypotrophic chiasm and prehyasmatic segments of the optic nerves and enlarged right eye (left) and moderate reduction of cerebral white matter, with midly dilated lateral ventricles (right). There are no calcifications. Presented with the permission of the parents and patient.

She also experienced osteoporosis with multiple vertebral fractures, for which she was treated with bisphosphonates, arterial hypertension and recurrent episodes of gastroenterocolitis accompanied by dehydration and metabolic acidosis.

The last evaluation at the Department at the age of 19 revealed a relapse of the disease (elevated erythrocyte sedimentation rate, leukopenia, hyperfibrinogenemia, decreased complement components C3 and C4 and CH50), apparently due to discontinuation of the recommended immunosuppressive therapy on her own initiative.

Patient 2 satisfied two inclusion criteria: severe clinical presentation (initial SLEDAI-2K score of 31, required six intravenous pulses of glucocorticoids for achieving remission at disease onset, later developed neuropsychiatric lupus) and syndromic cSLE defined as the presence of growth failure in height and weight not explained by drug exposures, intellectual deficiency, birth defects involving the heart as well as dysmorphic features.

Genetic analysis

Patient's 2 exome was found to contain a total number of 31,114 variants, including 11,689 homozygous, 18,439 heterozygous, 28,354 SNVs, 939 insertion and 951 deletion mutations. After excluding synonymous and splicing mutations, the list was narrowed down to 11,925 coding SNVs and indels that were further filtered using the data from ExAC and gnomAD to select only variants with MAF below 0.005, which resulted in a list of 2,063 candidates. Due to two *in silico* gene panels known to cause monogenic SLE, interferonopathies were found within GWAS SLE loci and the Vasculitis and Inflammation Panel, in combination with Phenotype to Genotype based variant searching filter, 26 variants were prioritised for closer investigation. Finally, after segregation analysis of the family as well as the assistance of SIFT, Polyphen2, CADD prediction tools, and using the GERP score and SiPhy combined with a search of the literature regarding the possible association of the filtered variants with the fenotype, two gene variants were identified (Figure 16).

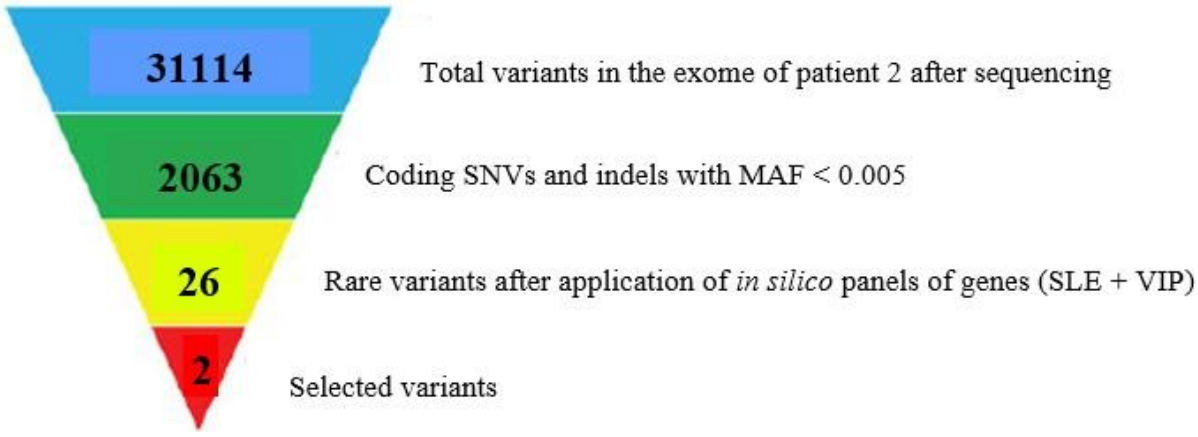
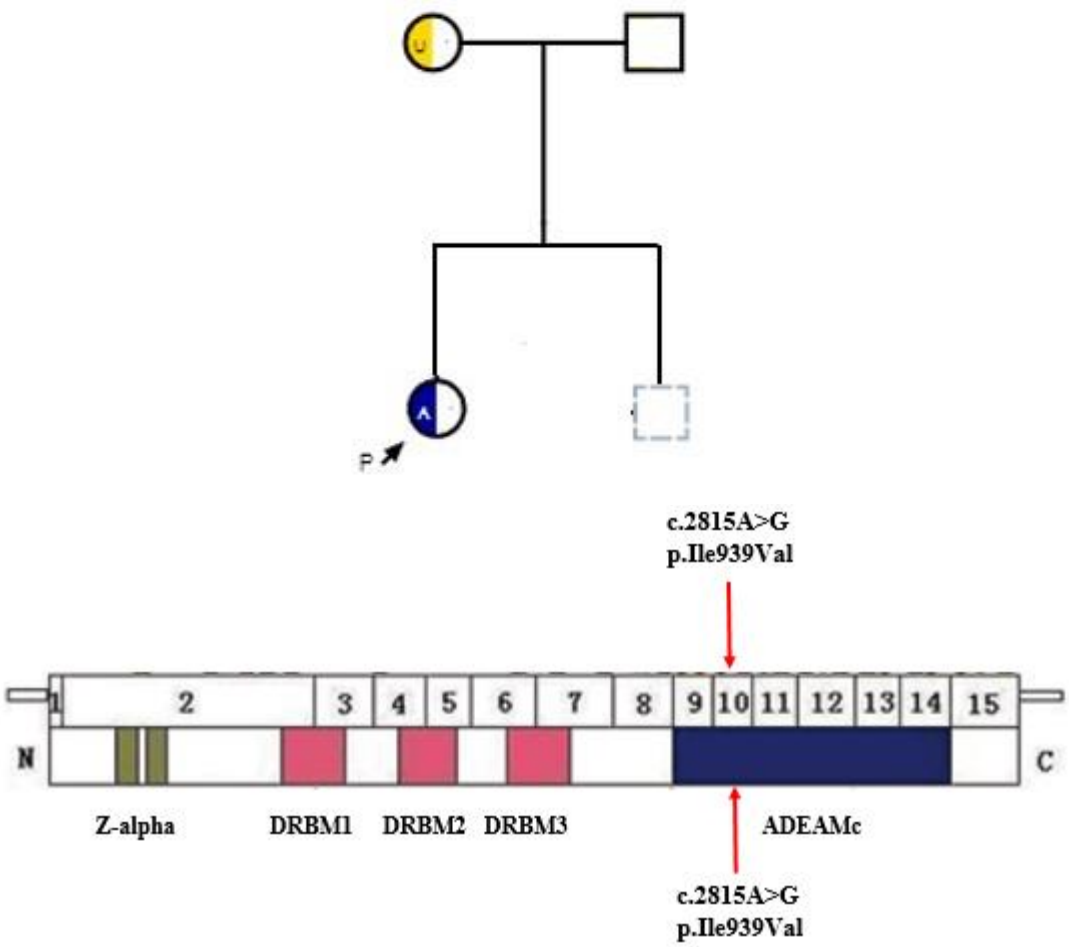


Figure 16. Schematic representation of the exome data-filtering approach used to identify variants in patient 2. MAF: minor allele frequency; SLE: systemic lupus erythematosus; SNVs: single nucleotide variants; VIP: Vasculitis and Inflammation Panel.

The variant which achieved the highest score was a rare (MAF <0.0001) heterozygous mutation in the *ADAR* gene (NM_001111.3:c.2815A>G; 13 reads A, 15 reads G) predicting to encode the protein (p.Ile939Val). As a result of missense mutation, aliphatic valine, branched hydrophobe, it replaces the isoleucin at position 939. The two amino acids are physicochemicaly very close, implicating possible low influence of the substitution on protein function. However, all three prediction tools were uniformly predicting that the mutation is deleterious (Polyphen2 = 0.97, SIFT = 0.01, CADD = 25.4).

Furthermore, from the liteature, it is known that the ADAR gene spans 30 kb and contains 15 exons with two Z-alpha motifs and three dsRNA-binding motif (DRBM) repeats extend over exon 2 and exons 2-7, respectively. The protein domain ADEAMc, essential for the enzyme function, i.e. deaminase activity, is positioned from exon 9 to exon 15 (235, 236). The mutation in patient 2 was found in exon 10, which contains the most important part for protein function. Measuring variant conservation with GERP score = 2.27 and Siphy Loggods = 15.2 was in line with the observation that nucleotide substitution occurred at the site that is evolutionary highly conserved. This is in concordance with the prediction that the mutation may lead to the disruption of protein function (Figure 17).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
ADAR	g.154561097A>G (GRCh37)	NM_001111.3	c.2815A>G	p.Ile939Val	MS	10	<0.0001	Del (0.01)	Prob Dam (0.97)	25.4	2.27	likely pathogenic

Figure 17. Pedigree of family 2 (top). Dashed lines in the pedigree indicate that in this family member WES was not performed. Schematic representations of the *ADAR* gene and ADAR protein domains show the position of c.2815A>G, (p.Ile939Val) mutation (bottom). Modified according to the reference number 237. A: adenine; AF: allele frequency; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; Del: deleterious; G: guanine; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; Ile: isoleucine; MS: missense; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; Prob Dam: probably damaging; SIFT: Sorting Intolerant from Tolerant prediction tool, Val: valine.

After researching the literature, it was found that *ADAR* mutations may be associated with the patient's phenotype in the form of either monogenic SLE (interferonopathy) or genodermatosis, which both correspond to the clinical picture (235, 238). Segregation analysis showed that the variant is inherited from the mother, who has skin changes somewhat compatible with the autosomal dominant genodermatosis caused by heterozygous *ADAR*

mutations. Applying the ACMG classification criteria the variant can still be classified as likely pathogenic since it satisfied two moderate and three supporting criteria (Table 14).

Table 14. Classification of the *ADAR* variant according to the ACMG criteria.

Pathogenicity Criterion		***ADAR*, (p.Ile939Val)**
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	Yes	Protein domain ADEAMc
PM2. Absent, rare in healthy databases	Yes	Rare alelle, MAF = 0.00001
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	Yes	Polyphen2 = 0.97, SIFT = 0.01, CADD = 25.4
PP4. Phenotype specific for disease	Yes	SLE, dyschromatosis symmetrica hereditaria
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	2 MP + 2 SP	
INTERPRETATION	LIKELY PATHOGENIC	

To evaluate potential effects of systemic autoimmunity in the patient on IFN-I responses, the presence of an IFN gene expression signature in the PBMC sample from the patient was investigated and a control group comprised a patient with a homozygous *TREX1* mutation (n = 1), and other SLE patients (n = 4).

Expression of specific IFN related genes are elevated in the blood from patients with type I interferonopathies (such as Aicardi-Goutières syndrome linked with *ADAR*) and correlate with disease severity in these conditions (239), therefore, the expression of a panel of these IFN related genes was investigated (*ISG15*, *IF144*, *IFI44L*, *IFI6*, *MxA* and *OASL*). These genes are upregulated in the presence of IFN-I and are often accepted as surrogate markers of IFN-I activity which can be difficult to detect. They are continuously found to be disregulated in transcriptomic analysis of known type I interferonopathies and are therefore commonly used to investigate the transcriptional activity of IFN-I in such diseases.

No significant difference in PBMC IFN related gene expression was noted between patient 2 and disease controls (other SLE patients), whereas samples collected from a patient with known type I interferonopathy, showed notably increased expression across all the genes measured. Results confirm that there is no elevation in the IFN transcriptional signature in the PBMC sample from the patient (Figure 18).

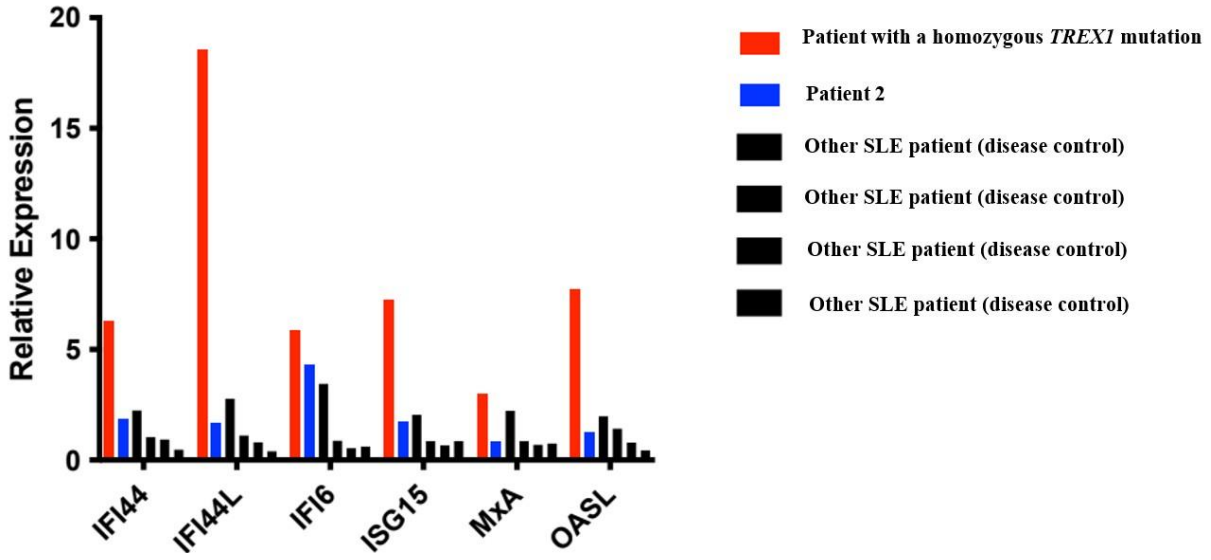


Figure 18. PBMC IFN related gene expression in patient 2, patient with a homozygous *TREX1* mutation and other SLE patients (disease controls). *ISG15, IF144, IFI44L, IFI6, MxA* and *OASL* are INF related genes which have been chosen after research on the literature.

The second variant which was investigated in more depth was the heterozygous mutation in the *SH2B3* gene (NM_005475.2:c.398G>A; 7 reads G, 6 reads A), with extremely low frequency in population databases (MAF <0.0001) encoding a cysteine to threonine substitution at amino acid position 133, predicted not to be damaging (SIFT = 0.81, CADD = 11.86), which is also in concordance with low GERP and Siphy scores (GERP score = 0.207, Siphy Logodds = 3.3504). The gene was implicated in the SLE pathogenesis (240). The variant is inhereted from an unaffected mother which, combined with the fact that according to several matching predictions using *in silico* tools in terms not affecting protein function, is sufficient to categorise it as a likely benign variant according to ACMG (Table 15).

Table 15. Classification of the *SH2B3* variant according to the ACMG criteria.

Pathogenicity Criterion	***SH2B3*, (p.Cys133Thr)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Rare alle, MAF < 0.0001
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	Yes	SLE
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP + 1 SP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***SH2B3*, (p.Cys133Thr)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in unaffected mother
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	Yes	SIFT = 0.81, CADD = 11.86, Polyhen2 = benign
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S + 1 SP	
INTERPRETATION	LIKELY BENIGN	

Separated analysis regarding HLA alleles showed that patient 2 has three alleles confirmed to be associated with SLE risk: *HLA-B*08:01* (homozygous), *HLA-DQB1*02:01* (homozygous) and *HLA-DRB1*03:01* (57, 58).

5.4. Family 3

Case summary

The third proband (patient 3) is a 21-year-old female with cSLE diagnosed at the age of 17, complicated with myocarditis, nephritis and pancreatitis. WES was preformed on the six members of family 3 (proband case, affected mother, healthy father, healthy aunt on the mother's side of the family, cousin with juvenile localised scleroderma and healthy grandfather on the mother's side).

Clinical report

The patient is the only child of non-consanguineous parents with confirmed family history of the same disease in a first degree relative. Namely, the patient's mother suffers from SLE and antiphospholipid syndrome. So far, the mother has had cerebrovascular insults on three occasions with consequent partial paresis of the right arm and memory impairment. Furthermore, the mother's sister has a child with juvenile localised scleroderma, treated with methotrexate, polyarthralgias and autoimmune thyroiditis.

At the age of 17, she first noticed swelling and redness of the fingertips of her left hand and after that, the same changes appeared on her right hand. They regressed after taking acetylsalicylic acid. She soon sought medical attention in another clinical hospital due to swelling and pain in both ankles. From the laboratory findings made at that time, to be highlighted are a positive anti-dsDNA, anti-Sm, anti-ribosomal P, anti-U1-ribonucleoprotein (anti-U1-RNP), anti-histone and aPL antibodies (LAC and aCL). No further assessment was performed and acetylsalicylic acid therapy was recommended.

After five months, the patient began vomiting profusely daily with pain in the epigastrium and heartburn. She lost 7 kilograms in ten days and was admitted to another hospital where vomiting continued; she could not tolerate oral intake. An abdominal ultrasound showed a delay in the passage of the contents through the stomach. A nasogastric tube was placed to evacuate a large amount of retained clear contents combined with metoclopramide and parenteral rehydration. A Colour Doppler of splanchnic circulation was performed and significant stenosis of the celiac trunk was found. This finding was confirmed by a CT angiography. An endoscopy of the gastrointestinal tract revealed a prominent area of the Vateri papilla with inflammatory changes. A control abdominal ultrasound was performed to show a thickened and

hypervascularised wall of the small intestine. Then she was referred to the University Hospital Centre Zagreb, Department for Paediatrics, Division of Clinical Immunology, Respiratory and Allergic Diseases, and Rheumatology.

On admission, the patient was malaised with an impaired general condition, the tip of the fifth and fourth fingers of the right hand were hyperemic, swollen and painful, there was scarce peristalsis and pretibial edema with poorly palpable peripheral arterial pulsations in the lower extremities.

Extensive diagnostic assessment revealed distinct concentric left ventricular hypertrophy, the myocardium of the left ventricle was asymmetrically thickened, predominantly in the area of the inferior part of the interventricular septum, with inflammatory changes and pericardial effusion. The reevaluation of the CT angiography determined a reduction in the width of the celiac trunk, which corresponded to 45% stenosis which, according to morphological criteria, was not hemodynamically significant, and the findings were consistent with an extravascular compression of the celiac trunk - medial cruciate ligament syndrome (Figure 19).



Figure 19. CT angiography showing stenosis of the celiac trunk.

Elevated amylase and lipase levels were noticed in the laboratory findings. It was concluded that gastrointestinal symptoms might be due to transient ischemia (as part of medial cruciate ligament syndrome), as all the affected organs are supplied by the truncus celiacus, but it was difficult to distinguish whether these changes might be of other etiologies (e.g. because of ischemia, cholecystitis, serositis or hypoproteinemia within SLE). Due to significant proteinuria (0.84 g in a 24 hour urine sample), a renal biopsy was performed and a patohistologic diagnosis of lupus nephritis class II was established. In repeated laboratory findings, positive ANA, anti-

Sm, anti-U1-RNP and aPL (LAC, aCL and anti-β2-glycoprotein I) antibodies with low C4 and CH50 were observed.

The patient fulfilled 5 out of the 11 ACR-97 classification criteria for SLE (renal and immunologic disorder, arthritis, serositis and positive ANA) as well as 7 of the 17 SLICC criteria (3 clinical criteria: renal disorder, synovitis, serositis, and 4 immunologic criteria: positive ANA, anti-Sm and aPL with low complement).

Treatment with pulsed doses of glucocorticoids was initiated and continued with gradually decreasing doses with the introduction of hydroxychloroquine and azathioprin. The symptoms of pancreatitis subsided and there was a significant improvement in the laboratory findings. Because of positive antiphospholipid antibodies, acetylsalicylic acid was also added in the therapy.

During the follow-up, a generally satisfactory control of the disease was established. However, aPL are still positive, alanine transaminase and aspartate transaminase levels are moderatley elevated, but autoimmune hepatitis was ruled out.

Patient 3 completed two inclusion criteria: confirmed family history of the same disease in the family (mother suffers from SLE) and other autoimmune conditions (cousin suffers from juvenile localised scleroderma and autoimmune thyroiditis) as well as severe clinical presentation (cardiac involvement, intravenous pulse glucocorticoids required).

Genetic analysis

The exome of patient 3 contains 59,442 variants, which is significantly higher than in previous probands. Among that number, there are 55,445 SNVs, 22,362 homozygous, 36,337 heterozygous, 1,741 insertion and 1,757 deletion mutations. The first aim was to exclude synonymous and splicing mutations, which successfully reduced the initial number to 23,430 and after exclusion of all the variants with MAF above 0.005, the number was further narrowed down to 1,992. With *in silico* gene panels for SLE and vasculitis, the number was reduced to 50 variants which were all analysed in more detail, one by one. Two variants were selected after using segregation analysis, prediction tools (SIFT, Polyphen2, CADD, GERP score and SiPhy) and consultation with the literature (Figure 20).

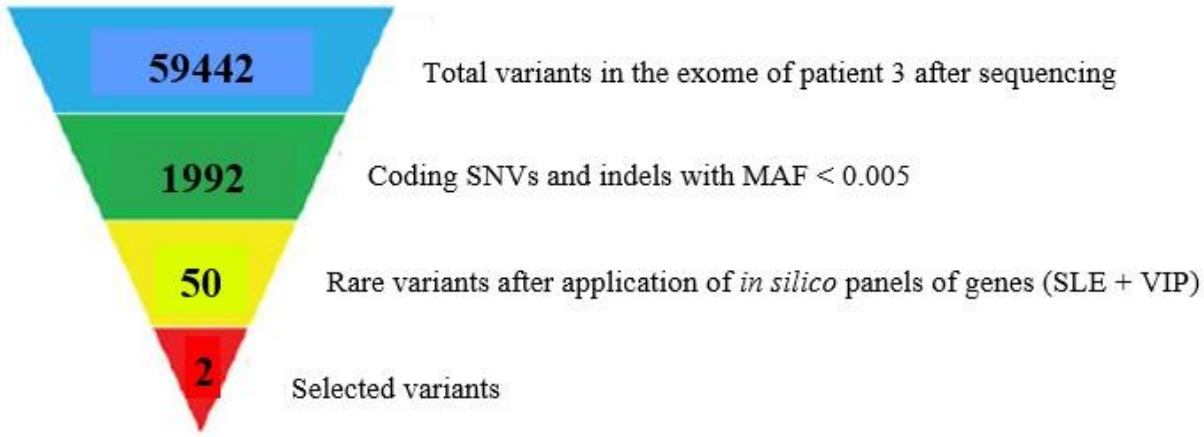
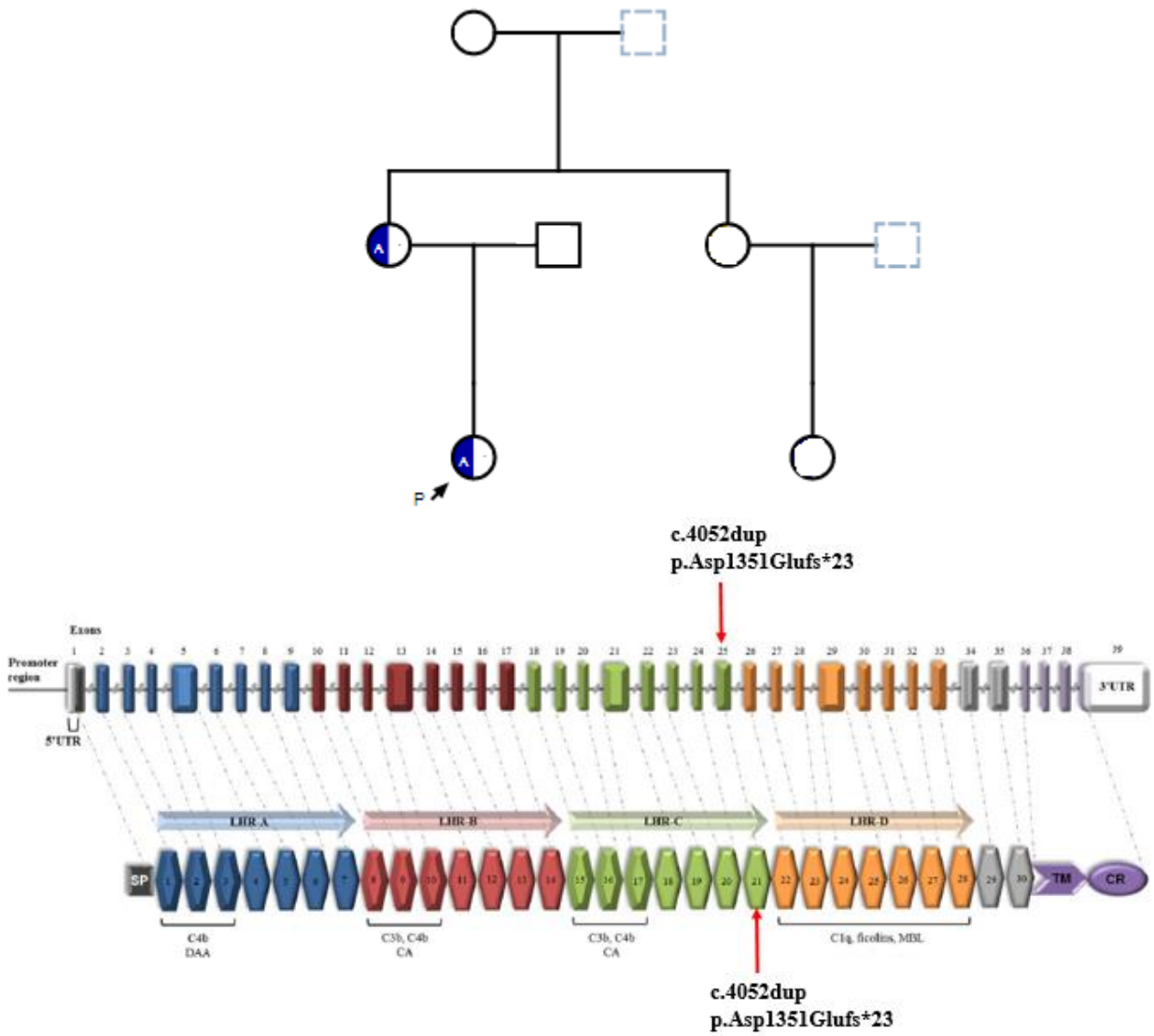


Figure 20. Schematic representation of the exome data-filtering approach used to identify variants in patient 3. MAF: minor allele frequency; SLE: systemic lupus erythematosus; SNVs: single nucleotide variants; VIP: Vasculitis and Inflammation Panel.

The first variant is the heterozigous insertion of C nucleotide in exon 25 at chromosome position 207741268 (GRCh37) of the *CR1* gene (NM_000651.4:c.4052dup). This very rare (MAF <0.0001) frameshift variant predicted to encode the protein p.Asp1351Glufs*23. The new reading frame resulted with the termination codon and the protein is expected to be of altered length. Since this is not a missense mutation, using SIFT, Polyphen2, CADD was not possible to predict the consequence on protein function, so the PROVEAN software tool was used – yielding no pathogenicity prediction. A negative GERP score (GERP = -1.38) indicated the exon does not exhibit a pattern of conservation typical of a protein-coding exon. Indeed, it is known that the CR1 protein is composed of 30 short consensus repeats (SCR) encoded by the exons 2-35. SCRs important for protein functions, i.e. those included in binding C4b opsonins, C3b, C1q, mannose-binding lectin and ficolins are encoded by the exons 2-5, 10-13 and 18-21 respectively. Another important part of the CR1 are SCRs 22, 24 and 25 that carry York and McCoy blood group antigens frequent in Africans, encoded by the exons 26 and 29, as well as the amino terminal region encoded by exon 1, transmembrane domain encoded by exons 36-37 and the intracytoplasmic carboxyl-terminal domain encoded by exons 38-39. The exon 25, where there is mutation in patient 3, encodes for SRP 21, which does not perform an important function (241). However, given the fact that this is a frameshift mutation, it is likely to lead to large-scale changes in the polypeptide length and chemical composition, resulting in a non-functional protein (Figure 21).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
CR1	g.207741268dup (GRCh37)	NM_000651.4	c.4052dup	p.Asp1351Glufs*23	FS	25	<0.0001	N/A	N/A	N/A	-1.38	likely pathogenic

Figure 21. Pedigree of family 3 (top). Dashed lines in the pedigree indicate that in this family member, WES was not performed. Schematic representations of the *CR1* gene and CR1 protein domains show the position of c.4052dup, (p.Asp1351Glufs*23) mutation (bottom). Modified according to the reference number 241. AF: allele frequency; Asp: aspartate; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; FS: frame-shift; g.: genomic sequence; dup: duplication; GERP: Genomic Evolutionary Rate Profiling score; Glu: glutamate; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; N/A: not available; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; SIFT: Sorting Intolerant from Tolerant prediction tool.

Literature data suggest that this gene could be associated with SLE (242), which is consistent with the phenotype of the patient. According to the AMCG criteria, the variant is a likely pathogenic since it is very rare, predicted to change the length of protein, there is co-segregation in the family (mother has SLE and carries the same variant) and the association with the phenotype is possible (Table 16). However, it unlikely causes SLE in the patient since only homozygous mutants of CR1 are associated with susceptibility to SLE (243).

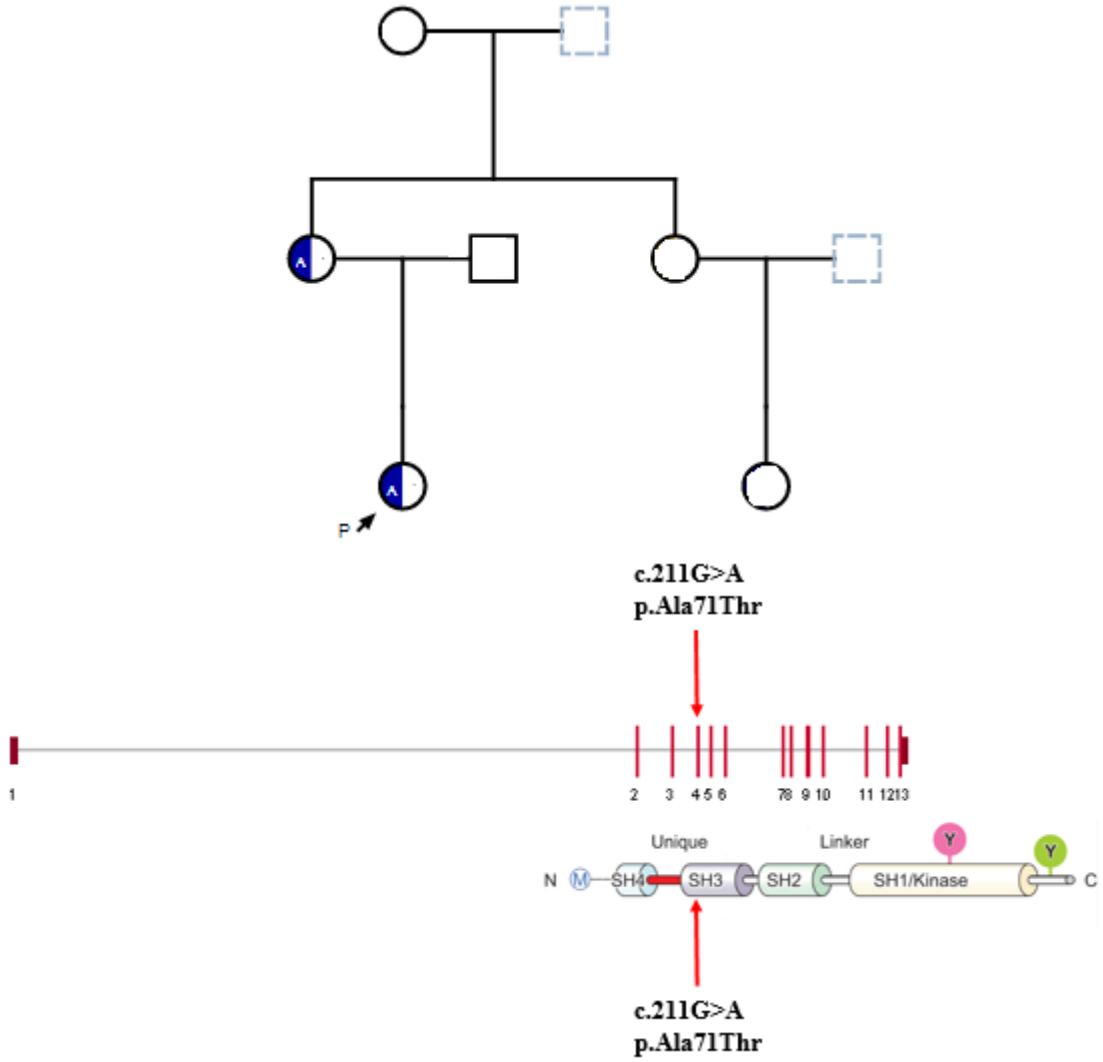
Table 16. Classification of the *CR1* variant according to the ACMG criteria.

Pathogenicity Criterion	***CR1*, (p.Asp1351Glufs*23)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Rare alle, MAF < 0.0001
PM3. For recessive, phase trans	No	
PM4. Change in protein length	Yes	Frame-shift mutation
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	Yes	Present in affected mother and proband
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	Yes	SLE
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	2 MP + 2 SP	
INTERPRETATION	LIKELY PATHOGENIC	

The second variant to be present is heterozygous G>A mutation at chromosome position 11405576 (GRCh37) of the *BLK* gene (NM_001715.2:c.211G>A). This mutation resulted in a p.71Ala >Thr amino acid change and was predicted to be deleterious by SIFT, with a score of 0.01. When the variant was analysed using Polyphen2 it was predicted to be benign, with a Polyphen2 score of 0.286 and low CADD score = 5.856, while GERP score = 1.71 and Siphy Logodds = 4.7253 indicated that this region at exon 4 is semi-conserved, so the amino acid substitution may cause a moderate effect on the protein function.

Nevertheless, it was demonstrated earlier that this variant, BLK^{A71T} (A indicates alanine, T indicates threonine and 71 indicates amino acid position where the substitution of amino acids occured in this variant) is associated with SLE (244). The BLK protein architecture consists of

four domains: the unique region, which varies among family members, followed by the SH3, SH2, and tyrosine kinase domains (245). Patient 3 has mutation in exon 4, which encodes for the SH3 domain (Figure 22).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
BLK	g.11405576G>A (GRCh37)	NM_001715.2	c.211G>A	p.Ala71Thr	MS	4	0.01	Del (0.01)	Benign (0.286)	5.856	1.71	likely pathogenic

Figure 22. Pedigree of family 3 (top). Dashed lines in the pedigree indicate that in this family member WES was not performed. Schematic representations of the *BLK* gene and BLK protein domains show the position of c.211G>A (p.Ala71Thr) mutation (bottom). Modified according to the reference number 246. A: adenine; AF: allele frequency; Ala: alanine; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; Del: deleterious; G: guanine; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; MS: missense; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; SIFT: Sorting Intolerant from Tolerant prediction tool; Thr: threonine.

The SH3 domain contributes to substrate recruitment by binding adaptor proteins/substrates, and regulation of kinase activity through intramolecular interaction, so the mutation could alter the interactions of BLK with other proteins, as well as those interactions taking place intramolecularly through the SH3 domain (244) (Figure 23).

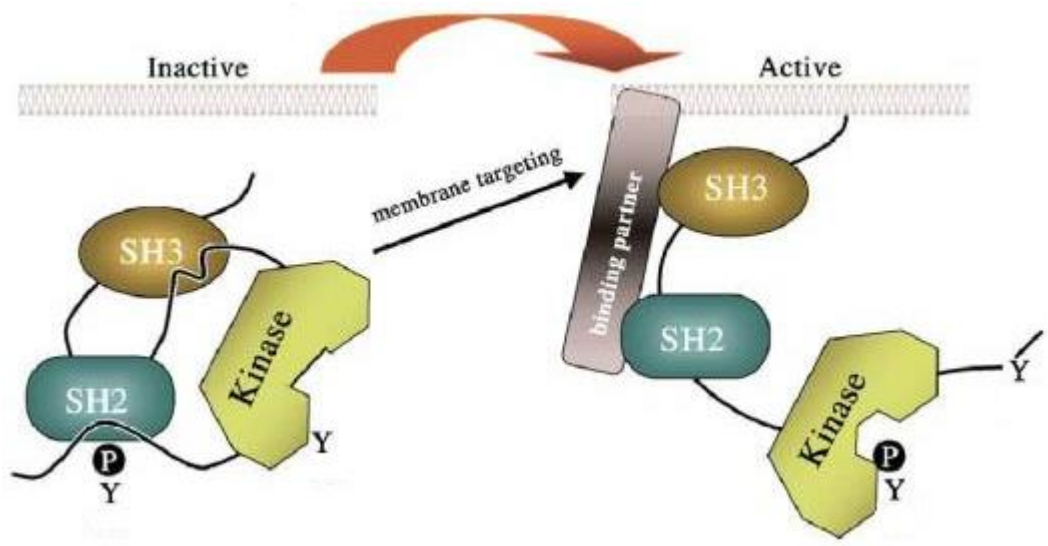


Figure 23. SH3 domain of BLK protein contributes to substrate recruitment by binding adaptor proteins. Modified from reference number 247.

Díaz-Barreiro et al. performed an investigation into the effects of this mutation on the amount of BLK in B cells, on the *BLK* mRNA expression, regulation and binding function (244). First, it was shown that homozygous cell lines for the BLK^{A71T} showed an extremely low level of BLK protein when compared with the cell line homozygous for the wild-type allele. After that, they demonstrated significantly reduced BLK levels even in the heterozygous cell lines for the BLK^{A71T}. Third, they showed that BLK half-life was shortened from 12 h to 6 h in cell lines homozygous for BLK^{A71T}. Fourth, the presence of the BLK^{A71T} variant did not affect the BLK mRNA suggesting other mechanisms then decreased BLK synthesis which may account for the extremely low protein level in homozygous cell lines. Fifth, they revealed that the amino acid substituion in the BLK^{A71T} variant resulted in conformational changes of the protein and increased activation, following increased degradation as a possible mechanism of decreased BLK levels in patients with the BLK^{A71T} variant. Sixth, the amino acid substitution

in the *BLK*^{A71T} variant resulted in impaired binding of BLK to the adaptor molecule BANK1 in B cells.

Taking into account the results of the functional studies, according to which, this mutation is important for protein function with low prevalence of the variant in population databases (MAF = 0.01) and an association with the risk of developing SLE from GWAS as well as co-segregation in the family (variant present in two family members with SLE), the variant was classified as likely to be pathogenic (Table 17).

Table 17. Classification of the *BLK* variant according to the ACMG criteria.

Pathogenicity Criterion	***BLK*, (p.Ala71Thr)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	Yes	Mutation in SH3 domain
PM2. Absent, rare in healthy databases	Yes	Rare alle, MAF = 0.01
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	Yes	Present in affected mother and proband
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	Yes	SLE
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	2 MP + 2 SP	
INTERPRETATION	LIKELY PATHOGENIC	

Analysis of HLA genes highlighted *HLA-DQA1*01:02* and *HLA-DRB1*15:01* as risk alleles for SLE (57, 58).

5.5. Family 4

Case summary

Patient 4 is a 20-year-old female with overlap syndrome involving cSLE and ulcerative colitis but she also had IgA vasculitis. WES was performed on the ten members of family 4 including a proband case, her sister with autoimmune thyroiditis, mother with ulcerative colitis, a healthy father, an uncle on mother's side with spondyloarthritis, one aunt on the father's side with ulcerative colitis, two healthy aunts on the father's side, a healthy grandfather on the mother's side and a healthy grandmother on the father's side.

Clinical report

The proband is the first child of non-consanguineous parents with a positive family history of different autoimmune diseases.

From infancy, she was sensitised to a number of nutritional and then inhaled antigens (cow's milk, egg white, feathers, animal hair, grass pollen, weeds and trees, dust and mites). The patient was hospitalised for the first time at the age of 4 due to recurrent bloody diarrhea. Endoscopy of the gastrointestinal system was performed and crypt abscesses were observed. Ulcerative colitis was diagnosed and treatment commenced with mesalazine and metronidazole. She was hospitalised several times due to a relapse of the disease and had regular check-ups and was treated with mesalazine, metronidazole and glucocorticoids. Azathioprine therapy was also tried but the patient developed pancreatitis, so it was discontinued. Satisfactory disease control was achieved with sulfasalazine.

At the age of 5, she had transient arthritis of her left knee that receded spontaneously. Two years later she had IgA vasculitis. A diagnosis was made based on the findings of palpable purpura in the lower extremities with normal platelet counts and the presence of bilateral ankle arthralgia. The disease was self-limited, without renal involvement. Due to an eating disorder at the age of 13, percutaneous endoscopic gastrostomy was performed.

Soon after, she developed vasculitic changes on the skin of the dorsum of the feet and ankles and a rheumatological assessment was initiated. In another clinical hospital, laboratory findings showed a positive ANA (1:5120), anti-dsDNA, perinuclear anti-neutrophil cytoplasmic

antibodies (p-ANCA) 1:2560 and antibodies against proteinase 3 (anti-PR3). She was treated for inflammation and abscesses of the skin and received percutaneous endoscopic gastrostomy.

The patient was referred to the University Hospital Centre Zagreb, Department for Paediatrics, Division of Clinical Immunology, Respiratory and Allergic Diseases, and Rheumatology when she was thirteen and a half years old, due to persistent facial erythema and with pronounced photosensitivity. On admission, malar rash was present while another clinical examination proved unremarkable. Repeated laboratory tests showed positive ANA (1:1600), anti-dsDNA, ANCA (1:1280), anti-PR3, aCL and low C3.

Four out of 11 ACR-97 classification criteria for SLE (malar rash, photosensitivity, immunologic disorder and positive ANA) as well as 5 of 17 SLICC criteria (1 clinical criteria: acute cutaneous lupus and 4 immunologic criteria: positive ANA, anti-dsDNA and aCL with low complement) were reached. Low-dose oral glucocorticoid and hydroxychloroquine were added to the therapy.

Satisfactory control of both diseases was achieved and the glucocorticoids were discontinued. At the age of 18 years she was diagnosed with allergic asthma. In further controls on two occasions she had a vasculitic rash on her lower legs, ANA was still positive as well as anti-ds DNA with persistence of ANCA and anti-PR3 with low C3.

Patient 4 fulfilled one inclusion criterion: confirmed family history of autoimmune diseases (ulcerative colitis, spondiloarthritis and autoimmune thyroiditis).

Genetic analysis

The initial number of 31,899 variants, including 29,441 SNVs, 12,191 homozygous, 19,286 heterozygous, 1,014 insertion and 1,139 deletion mutations were straightened in a stepwise manner. A total number of 12,443 coding SNVs and indels was reduced to 2,332 variants with MAF below 0.005 acording to the data from ExAC and gnomAD. After the application of gene panels for SLE and vasculitis used for performing targeted, outcome-driven analyses of WES data, 28 variants were chosen for final analysis. After segregation analysis, SIFT, Polyphen2, and CADD prediction, combined with the GERP score and SiPhy, and a literature search, three gene variants were identified as being of interest (Figure 24).

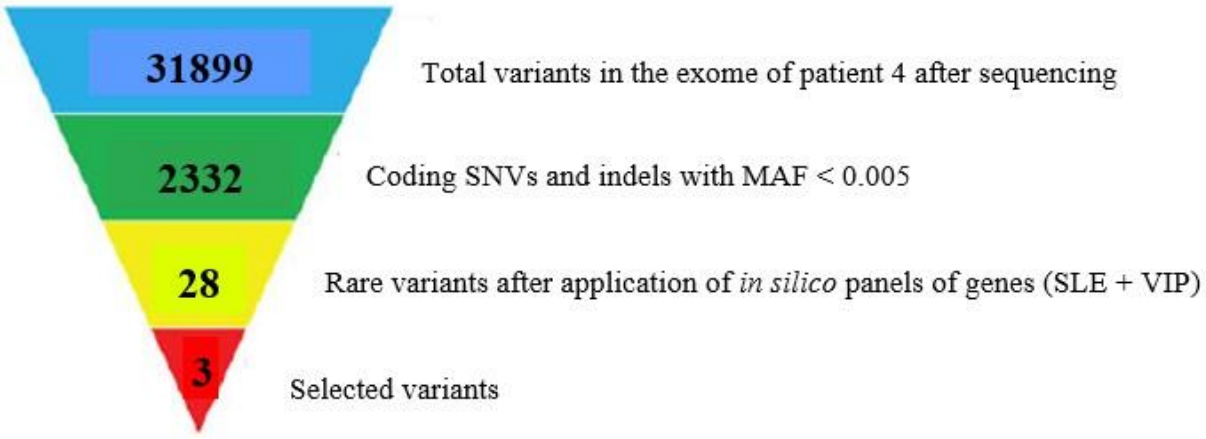
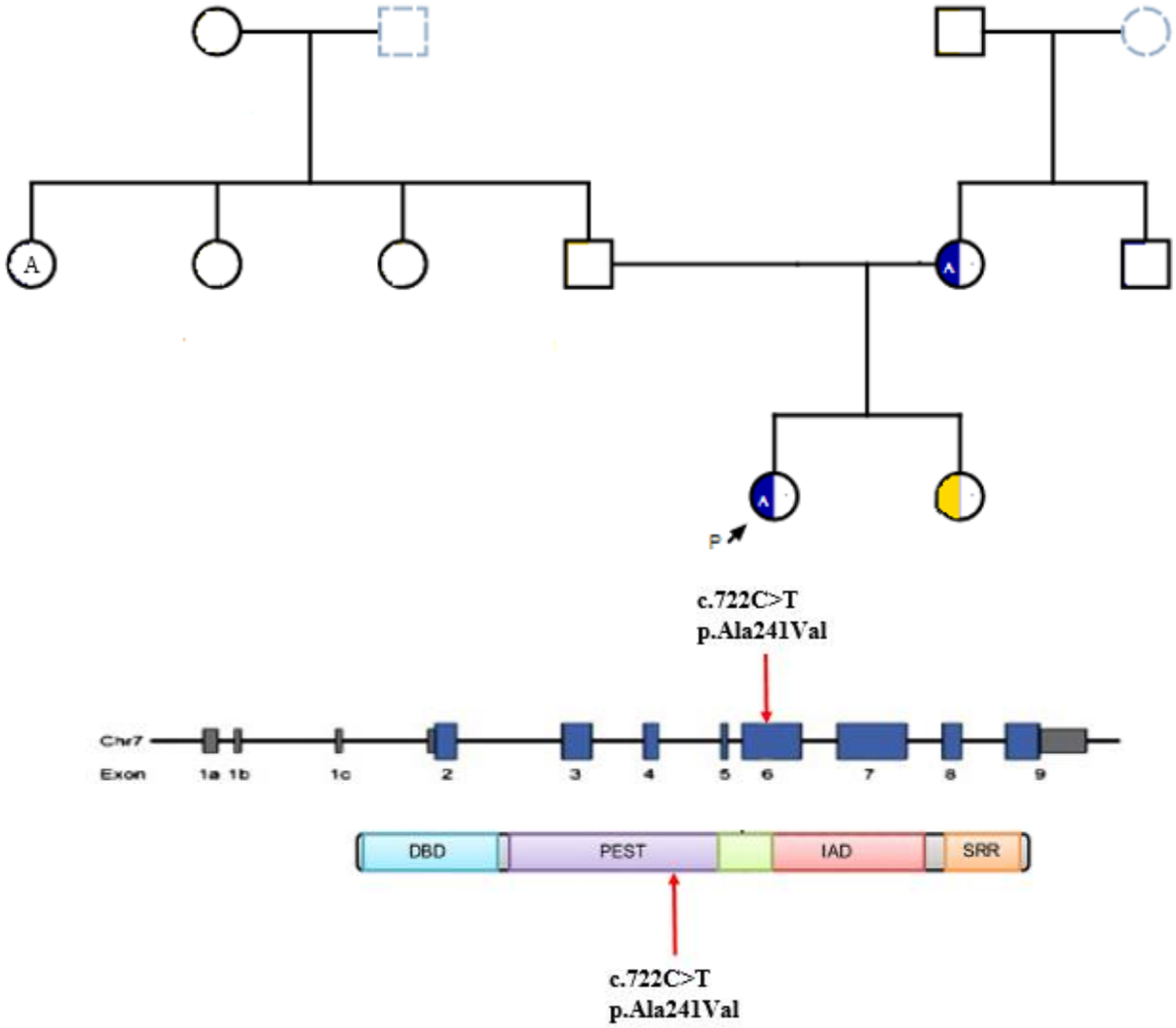


Figure 24. Schematic representation of the exome data-filtering approach used to identify variants in patient 4. MAF: minor allele frequency; SLE: systemic lupus erythematosus; SNVs: single nucleotide variants; VIP: Vasculitis and Inflammation Panel.

The first two variants are a rare (MAF = 0.0005) heterozygous missense mutation in *IRF5* (NM_001098629.1:c.722C>T) encoding an alanine to valine substitution at amino acid position 241 predicted to be benign and tolerant (SIFT = 0.22, CADD = 14.76) and a rare (MAF <0.0001) heterozygous missense mutation in *IRF3* (NM_001197122.1:c.907A>G) encoding a serine to glycine change at amino acid position 302. Both genes were implicated in SLE pathogenesis while *IRF5* was also linked with ulcerative colitis (248, 249).

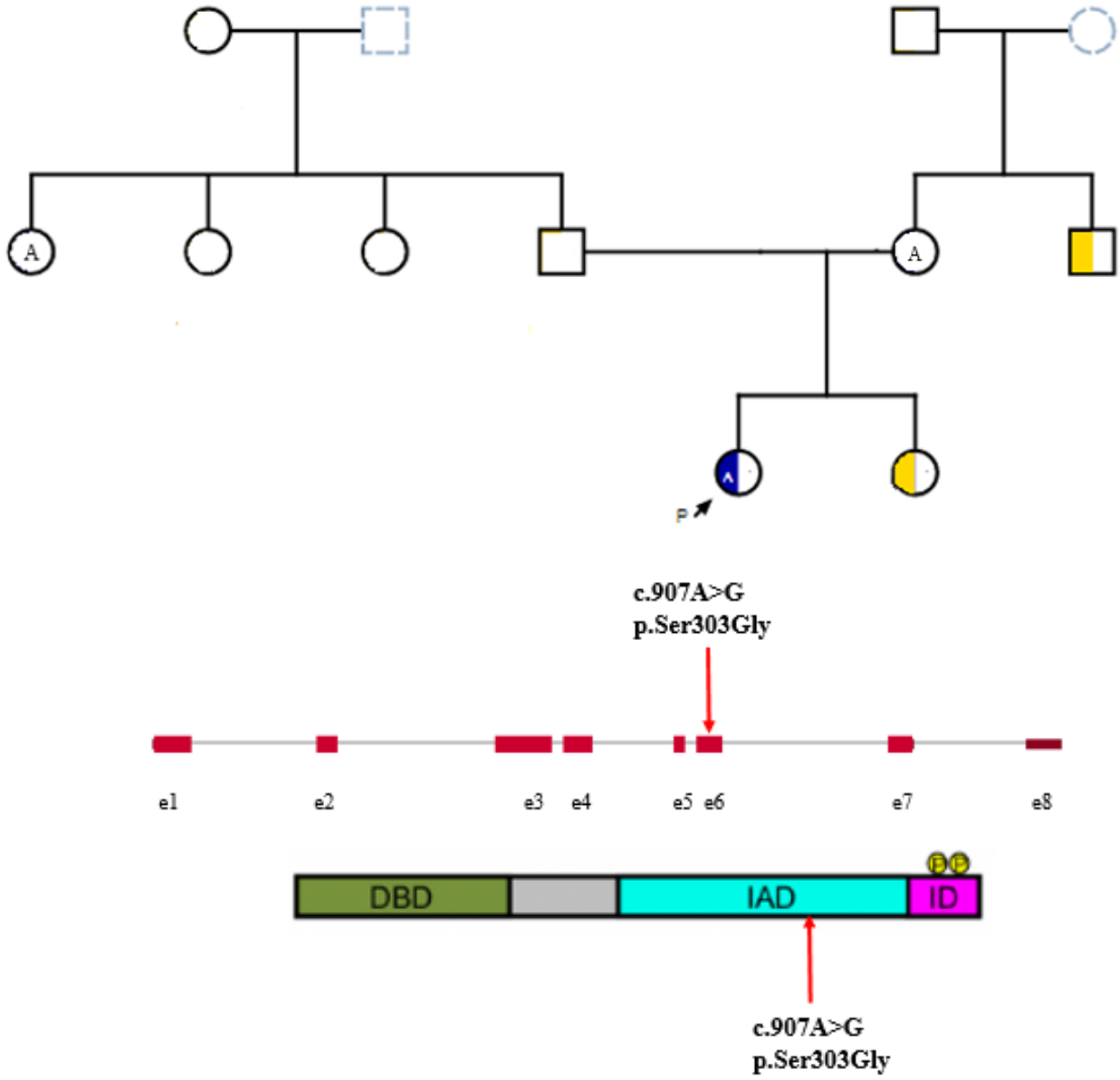
Mutation in the *IRF5* occured in exon 6, which encodes for a proline-, glutamic acid-, serine- and threonine-rich (PEST) domain of the IRF5 (250). Specifically, IRF5 and IRF3 both contain a DNA-binding domain (DBD), PEST domain, an IRF-association domain (IAD), and a serine-rich region (SRR) at the C terminus. The N-terminal DBD recognises a core DNA sequence within IRF-stimulated response elements and the C-terminal IAD mediates protein-protein interactions between IRFs and other proteins. PEST domain is thought to be important for protein stability in the IRF family of proteins. Thus, one would expect the mutation could change the protein stability impacting on its function. However, alanine and valine are amino acids which are very close according to their physical and chemical properties, and low GERP scores also suggest that the region is not evolutionary conserved (Figure 25).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
IRF5	g.128587524C>T (GRCh37)	NM_001098629.1	c.722C>T	p.Ala241Val	MS	6	0.0005	Tol (0.22)	Benign N/A	14.76	-3	likely benign

Figure 25. Pedigree of family 4 (top). Dashed lines in the pedigree indicate that in this family member WES was not performed. Schematic representations of the *IRF5* gene and IRF5 protein domains show the position of c.722C>T, (p.Ala241Val) mutation (bottom). Modified according to the references number 251 and 252. AF: allele frequency; Ala: alanine; C: cytosine; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; MS: missense; N/A: not available; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; SIFT: Sorting Intolerant from Tolerant prediction tool; T: thymine; Tol: tolerant; Val: valine.

As a consequence of mutation in the *IRF3*, it is expected that amino acid substitution occurs at position 302. This is located in the IAD domain of IRF3 (253), important for the interactions of IRF3 (Figure 26).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
IRF3	g.50165280A>G (GRCh37)	NM_001197122.1	c.907A>G	p.Ser303Gly	MS	6	<0.0001	Tol (0.14)	Benign (0.007)	12.97	-2.97	likely benign

Figure 26. Pedigree of family 4 (top). Dashed lines in the pedigree indicate that in this family member, WES was not performed. Schematic representations of the *IRF3* gene and IRF3 protein domains show the position of c.907A>G, (p.Ser303Gly) mutation (bottom). Modified according to the reference number 254. A: adenine; AF: allele frequency; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; G: guanine; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; Gly: glycine; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; MS: missense; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; Ser: serine; SIFT: Sorting Intolerant from Tolerant prediction tool; Tol: tolerant.

Although both variants are rare and genes *IRF5* and *IRF3* are associated with the disease phenotype of the patient, due to the presence of the variants in other family members not having SLE or ulcerative colitis or are otherwise healthy, as well as the prediction of *in silico* tools consistent with the expectation that amino acid substition would not influence the protein function, according to the AMCG criteria, both variants were classified as likely benign (Tables 18 and 19).

Table 18. Classification of the *IRF5* variant according to the ACMG criteria.

Pathogenicity Criterion	***IFR5*, (p.Ala241Val)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	**Yes**	rare alle, MAF = 0.0005
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	**Yes**	SLE, IBD
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP + 1 SP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***IFR5*, (p.Ala241Val)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	**Yes**	Present in sister with autoimmune thyroiditis, not consistent with IRF5-related disease phenotypes
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	**Yes**	SIFT= 0.22, CADD=14.76
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S + 1 SP	
INTERPRETATION	LIKELY BENIGN	

Table 19. Classification of the *IRF3* variant according to the ACMG criteria.

Pathogenicity Criterion		***IFR3*, (p.Ser303Gly)**
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	rare alle, MAF < 0.0001
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	Yes	SLE
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP + 1 SP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact		***IFR3*, (p.Ser303Gly)**
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in healthy family members
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	Yes	SIFF = 0.14, Polyphen2=0.007, CADD = 12.97
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S + 1 SP	
INTERPRETATION	LIKELY BENIGN	

The third variant relates to the *LIFR* gene. The gene of patient 4 contains c.2302A>G missense mutation in exon 16 (NM_001127671.1), resulting in a p.Arg768Gly. *LIFR* may be involved in bowel inflammation but has not been associated with SLE (255). Although SIFT predicted the effect of substitution could be deleterious (SITF = 0.02) and the variant attained a high CADD score (CADD = 22.7), the Polyphen2 prediction was completely the opposite. The region of exon 16, where the mutation occured, is under the evolutionary constraint (GERP

score = 2.16). Given the conflicting results for benign and pathogenic criteria, the variant is classified as VUS (Table 20).

Table 20. Classification of the *LIFR* variant according to the ACMG criteria.

Pathogenicity Criterion	***LIFR*, (p.Arg768Gly)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Rare alle, MAF = 0.0009
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	No	
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***LIFR*, (p.Arg768Gly)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in family members with different autoimmune diseases, while such phenotypes of LIFR-related disease are not described in the literature
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	No	
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S	
INTERPRETATION	NOT BENIGN	
REMARK	NOT PATHOGENIC, NOT BENIGN - VUS	

VUS: variant of uncertain significance.

After analysing the HLA genes, it was shown that the patient carried *HLA-DQA1*01:02* which is a risk allele for SLE (57, 58). She doesn't have the HLA risk alleles for ulcerative colitis (256).

5.6. Family 5

Case summary

WES was performed on a "trio" containing a proband case (patient 5) with cSLE and two healthy parents. The patient was diagnosed with cSLE and lupus nephritis at the age of 9. As a complication of the underlying disease and therapy, she developed secondary immunodeficiency followed by septic endocarditis, arthritis, multiorgan failure and compressive vertebral fractures.

Clinical report

The mother and father were healthy individuals who were not consanguineous. They have one son who is unaffected. The patient is the second child with no significant family history. She was otherwise a healthy child except for a perimembranous ventricular septal defect, which was followed up from birth.

At the age of 9, the girl was hospitalised in another clinical hospital due to prolonged fatigue, drowsiness and malaise accompanied by a purpuric rash on the lower extremities. A diagnostic assessment revealed splenomegaly, an elevated erythrocite sedimentation rate, anemia, hypergammaglobulinemia with inversion of the albumin-globulin ratio, microhematuria with boderline protenuria, decreased complement levels, positive ANA and positive aPL. A high dosage of peroral glucocorticoids were introduced into the therapy. However, proteinuria increased despite the therapy while microhematuria and hypocomplementia still persisted. A renal biopsy was performed and was interpretated as focal, segmental necrotizing glomerulonephritis with crescents and diffuse uneven proliferation with full house granular mesangial sample. The patient was then reffered to the University Hospital Centre Zagreb, Department for Paediatrics, Division of Clinical Immunology, Respiratory and Allergic Diseases, and Rheumatology.

From the clinical examination, to be highlighted are a cushingoid appearance, facial erythema, purpuric efflorescenes on the lower legs, moderate pretibial edema, systolic murmur and splenomegaly.

After reviewing the biopsy findings, a patohistologic diagnosis of lupus nephritis class III was established. The patient satisfied 5 out of 11 ACR-97 classification criteria for SLE (malar rash, hematologic, renal and immunologic disorder and positive ANA) as well as 7 of 17 SLICC criteria (4 clinical criteria: acute cutaneous lupus, non-scarring alopecia, renal disorder, hemolytic anemia, and 3 immunologic criteria: positive ANA and aPL with low complement).

The treatment continued with glucocorticoids and pulse cyclophosphamide therapy. After the first pulse of cyclophosphamide, the patient developed staphylococcal sepsis which became complicated with septic shock, acute respiratory distress syndrome, acute oliguric kidney injury and endocarditis. She required combined antimicrobial therapy, mechanical ventilation, continuous venovenous hemodialysis and five cycles of plasmapheresis, after which respiratory and renal function recovered and diuresis was established. After stabilisation, she developed a new sepsis caused by *Pseudomonas aeruginosa* originating from a central venous catheter.

It was decided not to continue cyclophosphamide therapy, and for the treatment of the SLE, hydroxychloroquine with mycophenolate mofetil were introduced with glucocorticoids. She was also treated with intravenous gamma globulins.

Unfortunately, a new complication soon followed - septic arthritis of the right talocrural joint. She was treated again with a combination of antibiotics.

After multiple sepsis, thoracic spine fractures were noticed, in the later stages of the disease, bisphosphonate therapy commenced with the use of a spinal orthosis. During regular follow-up, hypogammaglobulinemia was observed requiring regular intravenous immunoglobulin replacement therapy. Despite the therapy, infectious complications continued, first paronychia and then septic knee arthritis for which a synoviectomy was performed.

The last hospitalisation in the Department was when she was 12, due to bacterial pneumonia. Otherwise, the underlying disease has been well controlled.

Patient 4 fulfilled one inclusion criterion: severe clinical presentation (organ and life threatening, lupus nephritis class III, cardio-pulmonary involvement, requiring pulse glucocorticoids and systemic immunomodulators including immunoglobulins and plasmapheresis).

Genetic analysis

The stepwise approach was used to reduce the inital number of 26,320 variants in exome of the patient 4, including 25,007 SNVs, 9,394 homozygous, 16,641 heterozygous, 542 insertion and 666 deletion mutations. Based on the result of the first round of analysis, the total number of 11,296 coding SNVs and indels was selected for the second round. On removing alleles with MAF higher than 0.005, there were still 1,523 variants that needed to be narrowed down using *in silico* gene panels associated with SLE and vasculitis, which resulted in 23 variants that were analysed in detail. Combining the use of prediction tools on the impact of the mutation on protein function and data from tools which provide information on the conservation of the affected regions, as well as data from avalilable publications, only one gene with possible association with the disease was discovered (Figure 27).

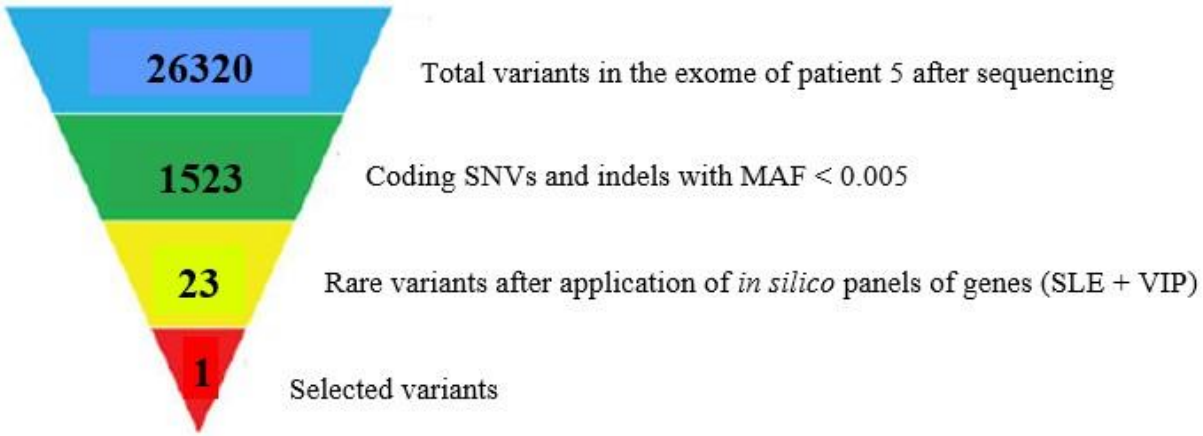
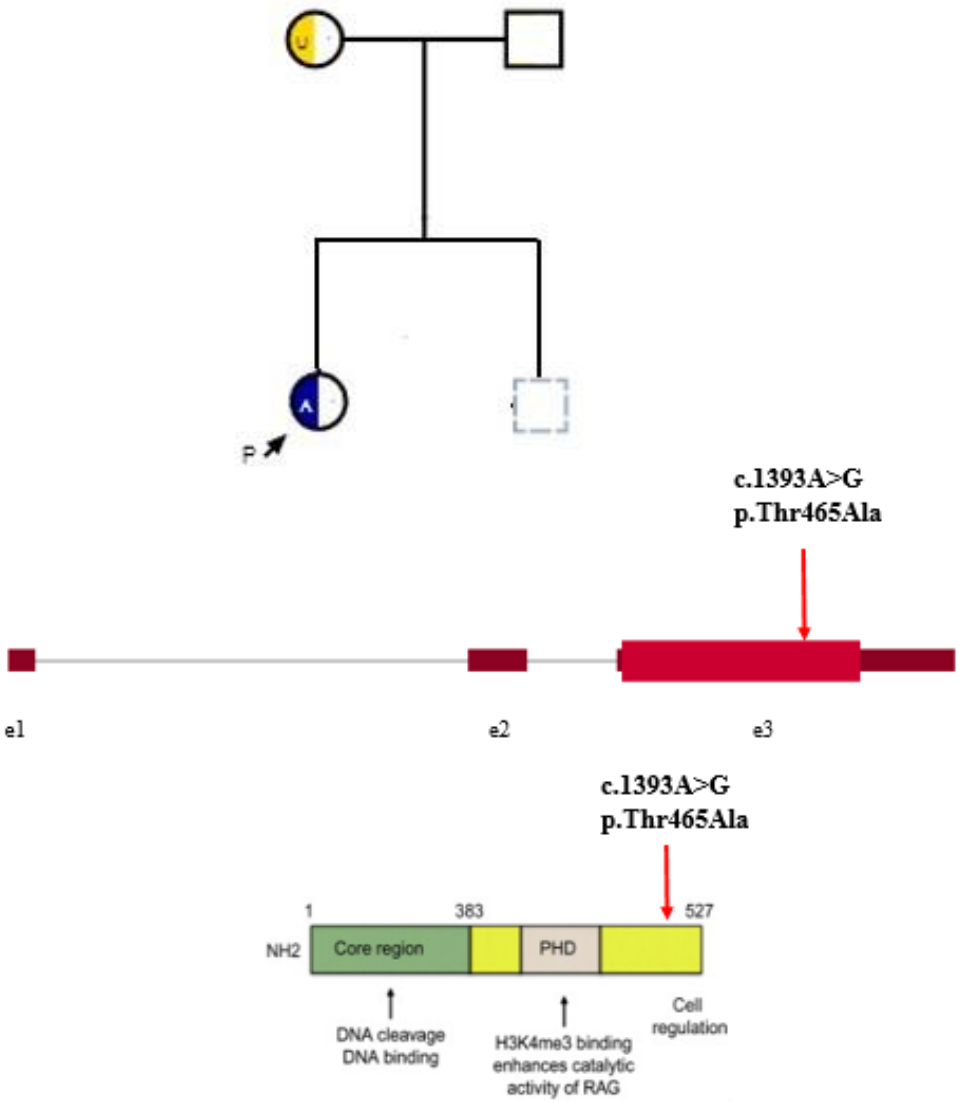


Figure 27. Schematic representation of the exome data-filtering approach used to identify variants in patient 5. MAF: minor allele frequency; SLE: systemic lupus erythematosus; SNVs: single nucleotide variants; VIP: Vasculitis and Inflammation Panel.

Patient 5 was found to have heterozyogous A>G mutation at chromosome position 36592776 (GRCh37) in the *RAG2* gene (NM_000536.3: c.1393A>G). This mutation resulted in a p.Thr465Ala amino acid change and obtained a high CADD score (CADD = 22.9). When the variant was analysed using SIFT, it was predicted to be tolerated, with a SIFT score of 0.07 and benign using the Polyphen2, with a score of 0.138. From the literature, it is known that the active core of RAG2 corresponds to the first two-thirds of the protein (257). Therefore, the N terminus of RAG2, which contains amino acids from position 1 to position 383, is important

for chromatin binding and multiple biochemical functions. The RAG2 carboxy-terminal region, which contains amino acids from position 384 to 527 is important for maintaining genomic stability. The mutation in patient 5 affects this part of the protein, since it is located at position 465. However, this is not the crucial part of this region, since residues 388-419 have been the most important for the interaction with the histones which enhance catalytic activity (Figure 28).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
RAG2	g. 36592776A>G (GRCh38)	NM_000536.3	c.1393A>G	p.Thr465Ala	MS	3	0	Tol (0.07)	Benign (0.138)	22.9	3.98	VUS

Figure 28. Pedigree of family 5 (top). Dashed lines in the pedigree indicate that in this family member WES was not performed. Schematic representations of the *RAG2* gene and RAG2 protein domains show the position of c1393A>G, (p.Thr465Ala) mutation (bottom). Modified according to the reference number 258. A: adenine; AF: allele frequency; Ala: alanine; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; G: guanine; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; MS: missense; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; SIFT: Sorting Intolerant from Tolerant prediction tool; Thr: threonine; Tol: tolerant; VUS: variant of uncertain significance.

The variant is not present in population databases and the heterozygous *RAG2* mutation is associated with SLE (259). However, the variant *RAG2*^{T465A} did not cosegregate in the family since the variant was inherited from an unaffected mother. Taking all the above into account, it was classified as VUS (Table 21).

Table 21. Classification of the *RAG2* variant according to the ACMG criteria.

Pathogenicity Criterion	***RAG2*, (p.Thr465Ala)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Absent in population databases
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	Yes	SLE
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP + 1 SP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***RAG2*, (p.Thr465Ala)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in unaffected mother
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	No	
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S	
INTERPRETATION	NOT BENIGN	
REMARK	NOT PATHOGENIC, NOT BENIGN - VUS	

VUS: variant of uncertain significance.

After examination of HLA alleles in patient 5, no risk allele was found (57, 58).

5.7. Family 6

Case summary

WES was performed on five members of family 6 counting a proband case with cSLE, renal tubular acidosis and autoimmune thyroiditis, the mother with autoimmune thyroiditis, a healthy father, an aunt on the father's side with rapidly progressive glomerulonephritis and a healthy grandfather on the father's side. Patient 6 is a 24-year-old female who was diagnosed with cSLE at the age of 14.

Clinical report

The patient is the first and only child of non-consanguineous parents. From the family history, it should be noted that the father's sister had rapidly progressive glomerulonephritis with renal failure, which was followed by a kidney transplantation but she eventually died. The grandfather on the mother's side suffered from type 1 diabetes.

At the age of 3 years, the patient had an epileptic seizure for which she was treated with carbamazepine for the following two years. Since the seizures did not repeat and the control EEG was normal, therapy was discontinued.

She was admitted to another hospital due to sudden muscle weakness in the whole body, prostration and an inability to move when she was 14 years old. Based on persitent hyperchloremic metabolic acidosis associated with hypokalemia, low serum bicarbonate, inappropriately high urine pH and positive urine anion gap, diagnosis of distal renal tubular acidosis was established and the patient was referred to the University Hospital Centre Zagreb, Department for Paediatrics, Division of Peadiatric Nephrology where a renal biopsy was undertaken. Multiplication of connective tissue and occasional mononuclear inflammatory cells were found in the medulla, with part of the detached epithelium of the tubule. No pathological changes were found in the glomeruli. Therapy with potassium citrate and sodium bicarbonate was prescribed.

After 7 months of controlled hospitalisation, the patient complained of pain in both knees, without swelling, and pain in the metacarpophalangeal joints of both hands and wrists which

passed spontaneously. Leukopenia with hypergammaglobulinemia and low C3, C4 and CH50 with positive ANA, SS-A and SS-B were present in the laboratory findings. She was referred to the University Hospital Centre Zagreb, Department for Paediatrics, Division of Clinical Immunology, Respiratory and Allergic Diseases, and Rheumatology. On admission, butterfly-shaped rashes covering the cheeks and nose, as well as mild non-scaring alopecia, were present while other clinical examinations proved to be unremarkable.

The patient fulfilled 4 out of 11 ACR-97 classification criteria for SLE (malar rash, photosensitivity, hematologic disorder and positive ANA) as well as 6 of 17 SLICC criteria (4 clinical criteria: acute cutaneous lupus, non-scaring alopecia, leukopenia, later she also developed thrombocytopenia, and 2 immunologic criteria: positive ANA, with low complement). Hydroxychloroquine and low-dose corticosteroid therapy was initiated with the continuation of the earlier treatment recommended by nephrologists.

Due to the established good disease control, glucocorticoids were discontinued from therapy after two years. Six months later she had a relapse of cSLE: fatigue, loss of appetite, nausea, vomiting, weight loss with leukopenia, thrombocytopenia and mild proteinuria in laboratory findings, therefore, glucocorticoids were reintroduced into her therapy with the addition of azathioprine. A further patient review found the occurence of autoimmune thyroiditis with the development of hypothyroidism which required thyroid hormone replacement therapy.

Patient 6 completed one inclusion criterion: a confirmed family history of autoimmune diseases (autoimmune thyroiditis in the mother; glomerulonephritis and type 1 diabetes in other members of the family).

Genetic analysis

In the exome of patient 6, as a result of WES, 31,705 variants were found: 28,689 SNVs, 11,858 homozygous, 18,585 heterozygous, 931 insertion and 932 deletion mutations. Using filters in the first step, all synonymous and splicing mutations were eliminated. The remaining 11,967 were then filtered to exclude those with MAF above 0.005, resulting in 1,998 variants. Gene panels for SLE and vasculitis helped to designate 39 variants for closer analysis and of these, 4 were finally selected for presentation (Figure 29).

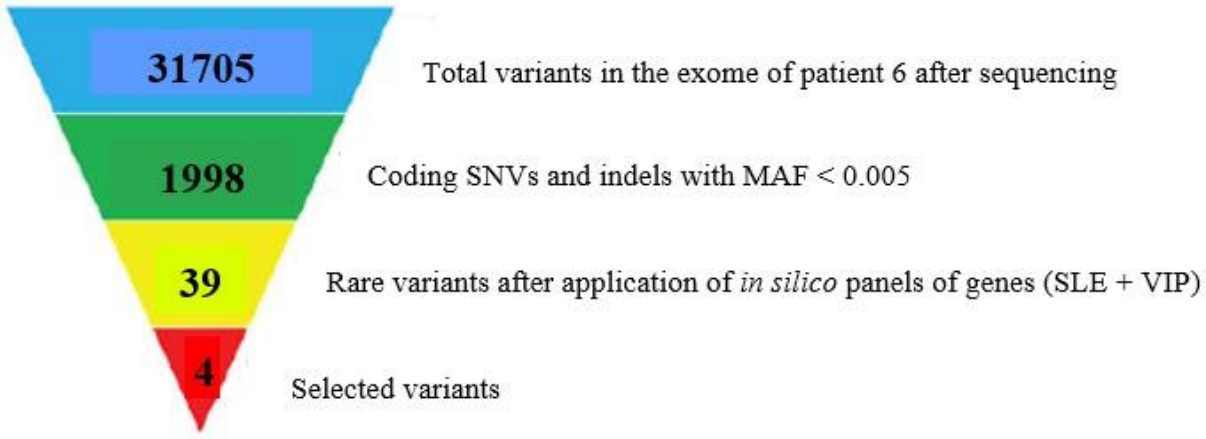
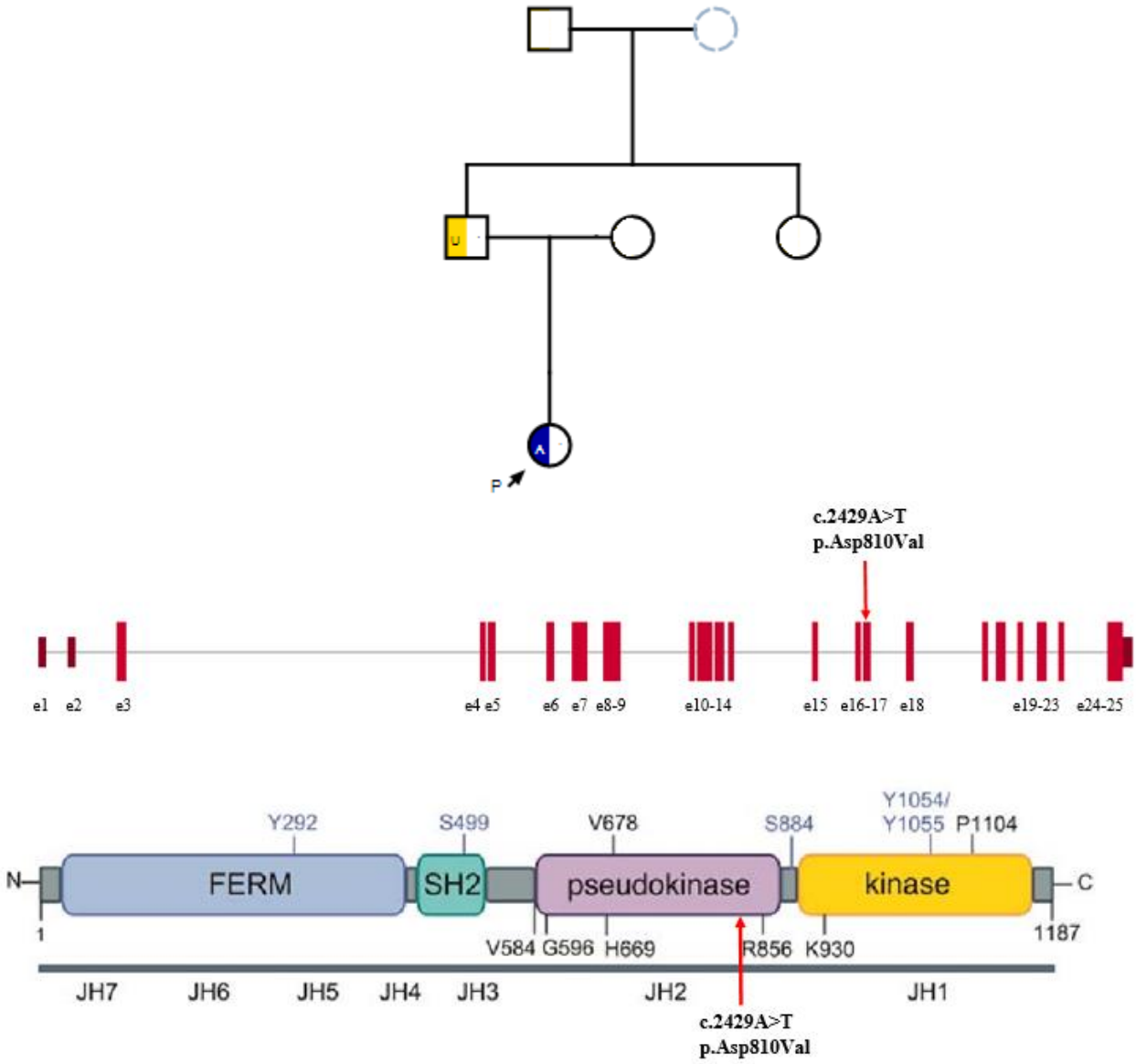


Figure 29. Schematic representation of the exome data-filtering approach used to identify variants in patient 6. MAF: minor allele frequency; SLE: systemic lupus erythematosus; SNVs: single nucleotide variants; VIP: Vasculitis and Inflammation Panel.

The highest score was obtained for the heteozygous *TYK2*^{D810V} variant with MAF<0.0001 which, according to the literature data, may be linked with SLE (260), which is consistent with the disease phenotype in the proband. From the genogram it shows there is no co-segregation of the variant in the family and the variant is inhereted from the healthy father. The A>T substitution in the part of exon 17 (NM_003331.4:c.2429A>T) results in an amino acid change at position 810 (aspartic acid changed with valine); the two amino acids have different physical and chemical properties, and this position is under evolutionary constraint (GERP score = 2.6). The SIFT predicts this substitution to be deleterious, consistent with the high scores obtained from Polyphen2 = 0.905 and CADD = 24.8. It is known that TYK2 protein contains seven JAK homology domains (JH1-JH7) and 4 structural domains (kinase, pseudokinase, SH2 and FERM domain). The amino acid position 810 is located with JH2 domain, which lacks catalytic activity, but has an essential regulatory function and mutations within this domain can impact on kinase activity (261). Therefore it is possible that this mutation would change the activity of the enzyme (Figure 30).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
TYK2	g.10468477A>T (GRCh37)	NM_003331.4	c.2429A>T	p.Asp810Val	MS	17	<0.0001	Del (N/A)	Pos Dam (0.905)	24.8	2.6	VUS

Figure 30. Pedigree of family 6 (top). Dashed lines in the pedigree indicate that in this family member WES was not performed. Schematic representations of the *TYK2* gene and TYK2 protein domains show the position of c2492A>T, (p.Asp810Val) mutation (bottom). Modified according to the reference number 262. A: adenine; AF: allele frequency; Asp: aspartate; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; Del: deleterious; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; MS: missense; N/A: not available; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; Pos Dam: possible damaging; SIFT: Sorting Intolerant from Tolerant prediction tool; T: thymine; Val: valine; VUS: variant of uncertain significance.

Given the conflicting results about pathogenicity, the variant is finally classified as VUS (Table 22).

Table 22. Classification of the *TYK2* variant according to the ACMG criteria.

Pathogenicity Criterion	***TYK2*, (p.Asp810Val)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Very rare, MAF <0.0001
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	Yes	SIFT = deleterious, Polyphen2 = 0.905, CADD = 24.8
PP4. Phenotype specific for disease	No	
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP + 1 SP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***TYK2*, (p.Asp810Val)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in unaffected father
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	No	
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S	
INTERPRETATION	NOT BENIGN	
REMARK	NOT PATHOGENIC, NOT BENIGN - VUS	

VUS: variant of uncertain significance.

The following three variants share the similar predicting scores of SIFT, Polyphen2 and CADD: *SAMHD1*^{V112I}, *DNASE1*^{V185I}, *BCL10*^{D127V}. Namely, all three variants are missense mutations and are rarely represented in the general population databases. On the other hand, as a result of these amino acid substitutions, their function was predicted not to have been significantly disrupted by the bioinformatic tools: SIFT indicated the mutations are tolerated, Polyphen2 showed that they are benign, while the CADD scores were not very high. All three variants were found in healthy family members, thus lacking the cosegregation in the family. Consequently all three variants were classified as probably benign. The difference between these three variants is in their association with the proband's phenotype: while there are published data about the influence of *SAMHD1* and *DNASE1* variants in SLE pathogenesis (263, 264), there are no data about *BCL10* variants in SLE (265) (Tables 23, 24, 25).

Table 23. Classification of the *SAMHD1* variant according to the ACMG criteria.

Pathogenicity Criterion	***SAMHD1*, (p.Val112Ile)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Very rare, MAF = 0.0003
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	No	
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***SAMHD1*, (p.Val112Ile)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in unaffected father
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	Yes	SIFT = 0.45, Polyhen2 = benign, CADD = 0.002
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S + 1 SP	
INTERPRETATION	LIKELY BENIGN	

Table 24. Classification of the *DNASE1* variant according to the ACMG criteria.

Pathogenicity Criterion	***DNASE1*, (p.Val185Ille)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Very rare, MAF = 0.0003
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	Yes	SLE
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP + 1 SP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***DNASE1*, (p.Val185Ille)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in unaffected mother
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	Yes	SIFT = 1, Polyphen2 = benign, CADD = 12.75
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S + 1 SP	
INTERPRETATION	LIKELY BENIGN	

Table 25. Classification of the *BCL10* variant according to the ACMG criteria.

Pathogenicity Criterion	***BCL10*, (p.Asp127Val)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Absent
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	No	
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***BCL10*, (p.Asp127Val)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in unaffected mother
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	Yes	SIFD = 0.86, Polyphen 2 = 0.001
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S + 1 SP	
INTERPRETATION	LIKELY BENIGN	

Regarding HLA analysis, patient 6 has as many as four SLE susceptibility genes: *HLA-B*08:01*, *HLA-DQA1*01:02*, *HLA-DQB1*02:01* and *HLA-DRB1*03:01* (57, 58).

5.8. Family 7

Case summary

The seventh proband (patient 7) is a 20-year-old female who suffers from cSLE (disease was diagnosed at the age of 16) with a confirmed family history of the autoimmune condition (mother has autoimmune thyroiditis). WES was performed on the three members of family 7 (proband case and two parents).

Clinical report

The patient is the child of non-consanguineous parents. The mother suffers from autoimmune thyroiditis and underwent surgery for uterine cancer. Other family history was unremarkable.

The first symptoms of SLE started at the age of 15 with polyarthralgias, morning stiffness of finger joints and acrocyanosis. She was hospitalised due to fever, chest pain, coughing and swelling of the right hand and wrist when she was 16 years old.

In the clinical status, areas of non-scarring alopecia have been noticed in the scalp with mild diffuse erythema of the face, slightly more pronounced on the cheeks and the root of the nose. Proximal interphalangeal joints of the fingers on the right hand were thickened, palpatory painful with terminally limited extension and limited flexion of the proximal and distal interphalangeal joints on both hands. The left talocrural joint was painful and thickened.

Radiological examinations verified thickened soft tissues in the metacarpophalangeal joints of both hands with paraarticular osteopenia of the proximal and distal interphalangeal joints, reduced intraarticular space of the proximal interphalangeal joints, but without any visible erosive process, and effusion around the flexor and extensor tendons of the right hand.

ANA was positive in high titer (1:25600) with positive anti-dsDNA, anti-Smith (anti-Sm) and anti-histone antibodies and low C3, C4 and CH50. She also had an elevated erythrocyte sedimentation rate, leukopenia and laboratoy signs of liver injury (alanine transaminase and aspartate transaminase levels elevated four times above the upper limit of normal). The autoantibodies for autoimmune liver diseases were negative. Other diagnostic tests for liver disease were negative.

The patient fulfilled 5 out of 11 ACR-97 classification criteria for SLE (malar rash, arthritis, hematologic disorder, immunologic disorder and positive ANA) as well as 8 of 17 SLICC criteria (4 clinical criteria: acute cutaneous lupus, nonscaring alopecia, arthritis and leukopenia, and 4 immunologic criteria: positive ANA, anti-dsDNA and anti-Sm with low complement).

She was treated with pulse doses of glucocorticoids, followed by glucocorticoids in gradually decreasing doses with hydroxychloroquine and azathioprine. During follow-up, satisfactory disease control without any complications was achieved.

Patient 7 satisfied one inclusion criterion: a confirmed family history of autoimmune conditions (autoimmune thyroiditis).

Genetic analysis

The WES discovered as many as 61,293 variants in the exome of patient 7: 55,629 SNVs, 23,032 homozygous, 36,473 heterozygous, 2,013 insertion and 2,177 deletion mutations. Using the step-wise approach, a total of 24,006 coding SNPs and indels were selected for further analysis. There were only 2,322 variants with MAF below 0.005. Using the filter with integrated gene panels for SLE and vasculitis, only 20 variants remained to be inspected using the SIFT, Polyhen2 and CADD tools accompanied by a GERP score and SiPhy. In the final round of analysis, after the reading available scientific publications, two variants were selected (Figure 31).

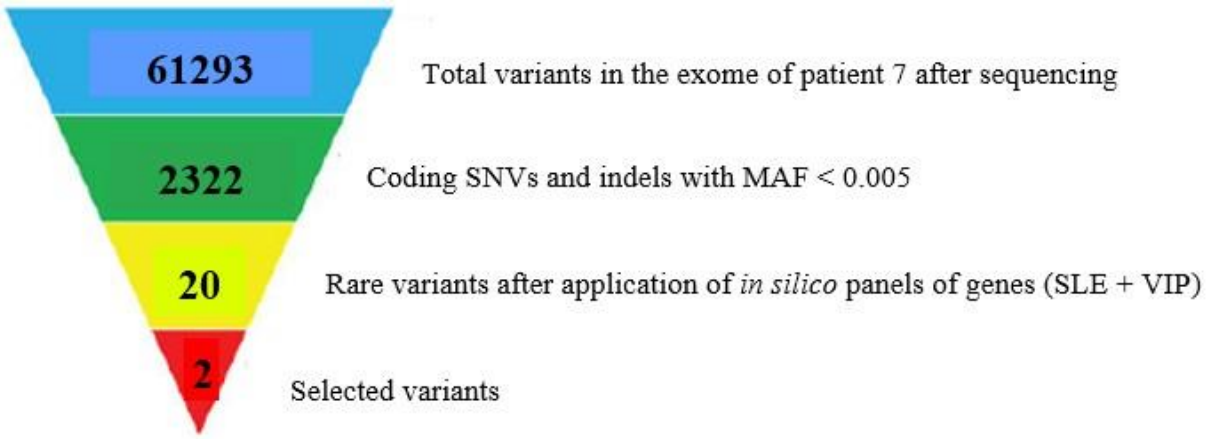
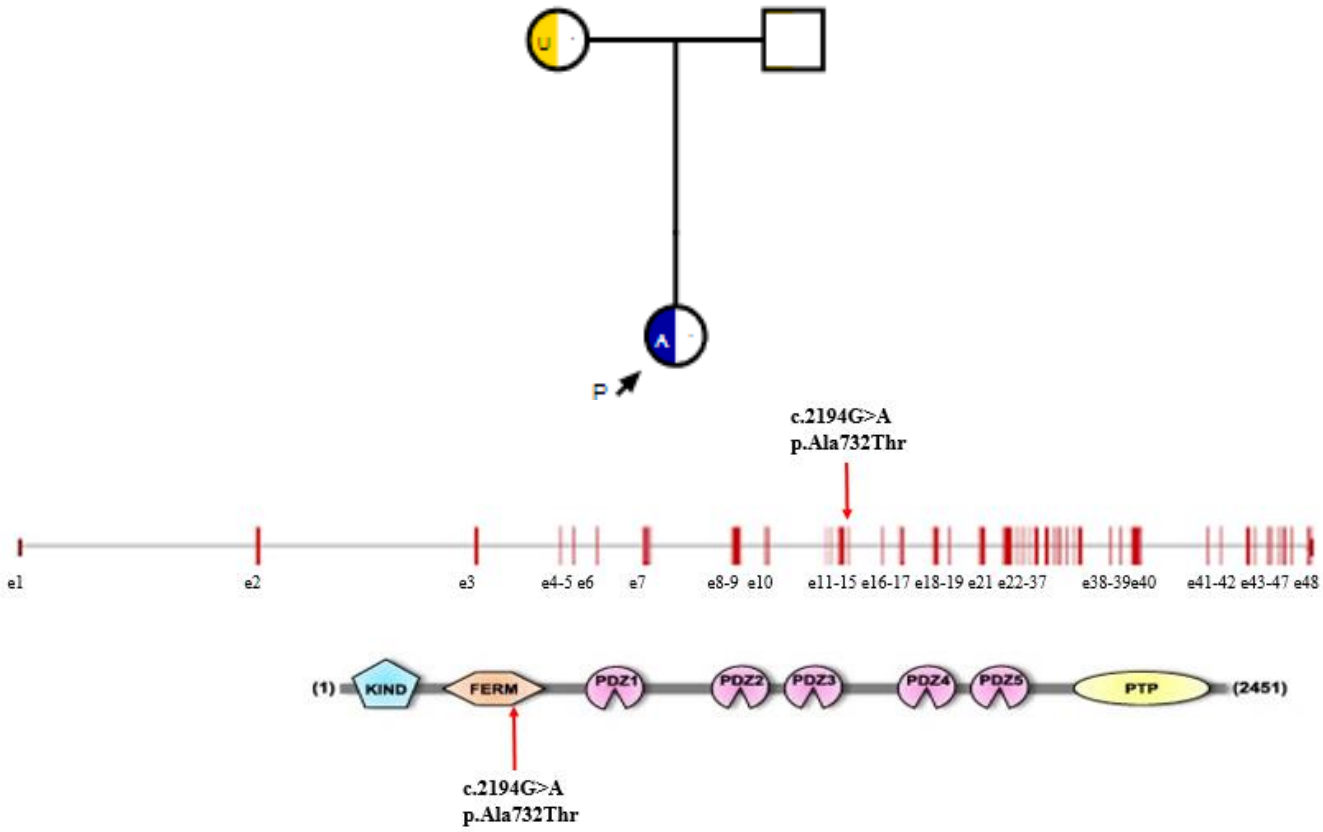


Figure 31. Schematic representation of the exome data-filtering approach used to identify variants in patient 7. MAF: minor allele frequency; SLE: systemic lupus erythematosus; SNVs: single nucleotide variants; VIP: Vasculitis and Inflammation Panel.

The highest scores obtained were the *PTPN13*^{A732T} and *CFD*^{A188T} variants.

The *PTPN13*^{A732T} (MAF = 0.0004) heterozygous missense variant is very rare in exon 15 (NM_006264.2:c.2194G>A), leading to an alanine to threonine substitution at codon 732, predicted to be disruptive for protein function, as indicated with SIFT = 0.04, Polyphen2 = 0.777, CADD = 24.8. PTPN13 is composed of an N-terminal KIND (Kinase non-catalytic C-lobe) domain, followed by a FERM (Four-point-one/Ezrin/Radixin/Moesin) domain, five PDZ domains and a tyrosine phosphatase domain, which is a carboxy-terminal catalytic domain, the most important for protein function (266). The mutation in patient 7 affected the amino acid position 732, which is within the FERM domain, and is involved in the interaction with other proteins and also thought to regulate the membrane localisation of PTPN13 (Figure 32).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
PTPN13	g.87656789G>A (GRCh37)	NM_006264.2	c.2194G>A	p.Ala732Thr	MS	15	0.0004	Del (0.04)	Pos Dam (0.777)	24.8	1.75	VUS

Figure 32. Pedigree of family 7 (top). Schematic representations of the *PTPN13* gene and PTPN13 protein domains show the position of c2194G>A, (p.Ala732Thr) mutation (bottom). Modified according to the reference number 267. A: adenine; AF: allele frequency; Ala: alanine; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; Del: deleterious; G: guanine; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; MS: missense; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; Pos Dam: possible damaging; SIFT: Sorting Intolerant from Tolerant prediction tool; Thr: threonine; VUS: variant of uncertain significance.

The FERM domain is not crucial for enzyme function, but it still may be very important. The variant was inherited from the mother with a different autoimmune disease compared to the proband, suggesting a lack of co-segregation, as a result of which the *PTPN13*^{A732T} was classified as VUS (Table 26), consistent with the data from the available publications about the association between *PTPN13* gene variants and SLE (268).

Table 26. Classification of the *PTPN13* variant according to the ACMG criteria.

Pathogenicity Criterion	***PTPN13*, (p.Ala732Thr)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Very rare, MAF = 0.0004
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	Yes	SIFD = 0.04, Polyphen2 = 0.777, CADD = 24.8
PP4. Phenotype specific for disease	No	
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP + 1 SP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***PTPN13*, (p.Ala732Thr)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in the mother who suffers from another autoimmune disease
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	No	
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S	
INTERPRETATION	NOT BENIGN	
REMARK	NOT PATHOGENIC, NOT BENIGN - VUS	

VUS: variant of uncertain significance.

The *CFD*^{A188T} variant is even more rarely presented in population databases with MAF < 0.0001. Using the prediction tools, it was shown that substitution of alanine with threonine should not cause loss of function of the CFD protein, since the score of SIFT and Polyphen2 were low, SIFT = 0.4, Polyphen2 = 0.003. Given the same variant was observed in unaffected family members and the lack of published cases of CFD-associated variants with phenotype of SLE (269), the variant was classified as likely benign (Table 27).

Table 27. Classification of the *CFD* variant according to the ACMG criteria.

Pathogenicity Criterion	*CFD*, (p.Ala188Thr)	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Very rare, MAF < 0.0001
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	No	
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	*CFD*, (p.Ala188Thr)	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in family members with different autoimmune diseases, not consistent with CFD-related diseases
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	Yes	SIFT = 0.4, Polyphen2 = 0.003
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S + 1 SP	
INTERPRETATION	LIKELY BENIGN	

No HLA alleles strongly associated with SLE were present in patient 7 (57, 58).

5.9. Family 18

Case summary

Patient 18 is a 14-year-old boy who was diagnosed with IgAV, complicated with pulmonal, gastrointestinal and renal involvement. WES was performed on the patient and two unaffected parents.

Clinical report

He was otherwise a healthy child of non-consanguineous parents with no significant family history. At the age of 12,, he sought medical attention when he began to complain of spontaneous pain in his wrists, knees, and ankles with the development of edema of the dorsum of the hands and a bright, maculopapular rash on the skin of the lower legs. The next day, with the progression of all the above symptoms, he also became febrile with a sore throat. On the third day of the illness, he was examined and admitted to the day hospital in another institution where he was rehydrated parenterally and treated with ceftriaxone. From the 5th day of the disease, with the development of diarrhea and vomiting accompanied by abdominal pain, the patient was admitted for inpatient treatment. A therapy with ceftriaxone was started together with the administration of ibuprofen, loratadine and ranitidine. Due to the progression of the rash, which evolved into purpura and severe pain in the abdomen and testicles and fluid retention, and despite human albumin replacement therapy, glucocorticoids were introduced into the therapy. In laboratory findings, an increase of C-reactive protein and proteinuria (1.34 g in 24 hour urine sample) were noticed. With the above therapy, joint pain and swelling regressed.

On the seventh day of the illness, he began to complain of a chest pain and dyspnea with a dry cough and drop in oxygen saturation. Decreased diuresis with fluid retention and hypertension also developed. The patient was transferred to the intensive care unit and furosemide and amlodipine therapy was introduced, to which regular diuresis and partial regulation of hypertension were established. As the course of treatment progressed, he remained dyspnoeic with a progression of radiological findings on the lungs in terms of acute respiratory distress syndrome (Figure 33).



Figure 33. Chest radiograph of patient 18 shows diffuse bilateral coalescent opacities (left). Repeated chest radiograph after treatment show normal intrathoracic status (right).

Due to the need for intensive and multidisciplinary treatment, the patient was transfered to the intensive care unit of the University Hospital Centre Zagreb, Department for Paediatrics.

He was diagnosed with IgAV according to EULAR/PRINTO/PRES-endorsed Ankara 2008 classification criteria: palpable purpura without thrombocytopenia accompanied with diffuse abdominal pain, arthralgia, renal involvement and IgA deposition were found in the renal biopsy specimen.

Pulse therapy with methylprednisolone was started immediately, followed by standard then gradually decreasing doses and other supportive measures. The respiratory symptoms gradually regressed and he was referred to the Division of Clinical Immunology, Respiratory and Allergic nDiseases, and Rheumatology. After stabilising the respiratory status, a kidney biopsy was perfomed and the findings were consistent with IgAVN (class II according to International Study of Kidney Disease in Children classification and $M_1E_1S_0T_0$-C_0 according to the updated Oxford classification). In accordance with SHARE recommendations, mycophenolate mofetil was additionally introduced into the therapy.

In further regular follow-ups, glucocorticoid therapy was discontinued after 6 months due to satisfactory disease control, while he is still taking mycophenolate mofetil and antihypertensive therapy.

Patient 18 fulfilled one inclusion criterion: severe clinical presentation (severe IgAVN, pulmonary involvement, required intravenous pulse glucocorticoids).

Genetic analysis

Proband 18 has 26,098 variants detected by WES, including 24,789 SNVs, 9,355 homozygous, 16,471 heterozygous, 556 insertion and 660 deletion mutations. Among them, there were 11,478 coding SNVs and indels. The number of variants below MAF < 0.005 was 1,625. *In silico* gene panels (SLE and vasculitis panel) helped to reduce the number of potentially interesting variants to 28. Among them, two had the highest score regarding the potential influence on protein function and phenotype of the patient and a third variant was selected after additional research of all *de novo* variants of which this was one, since using the described strategy didn't yield a likely variant (Figure 34).

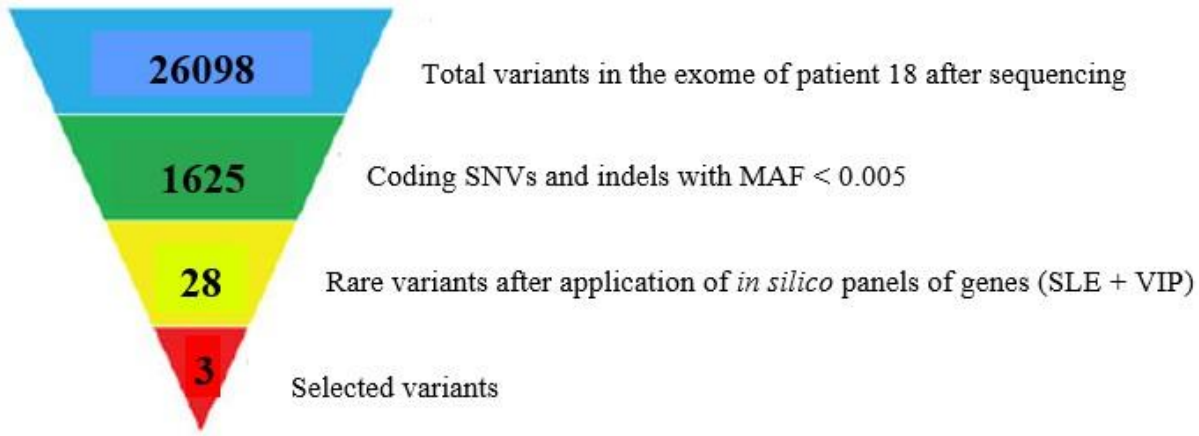
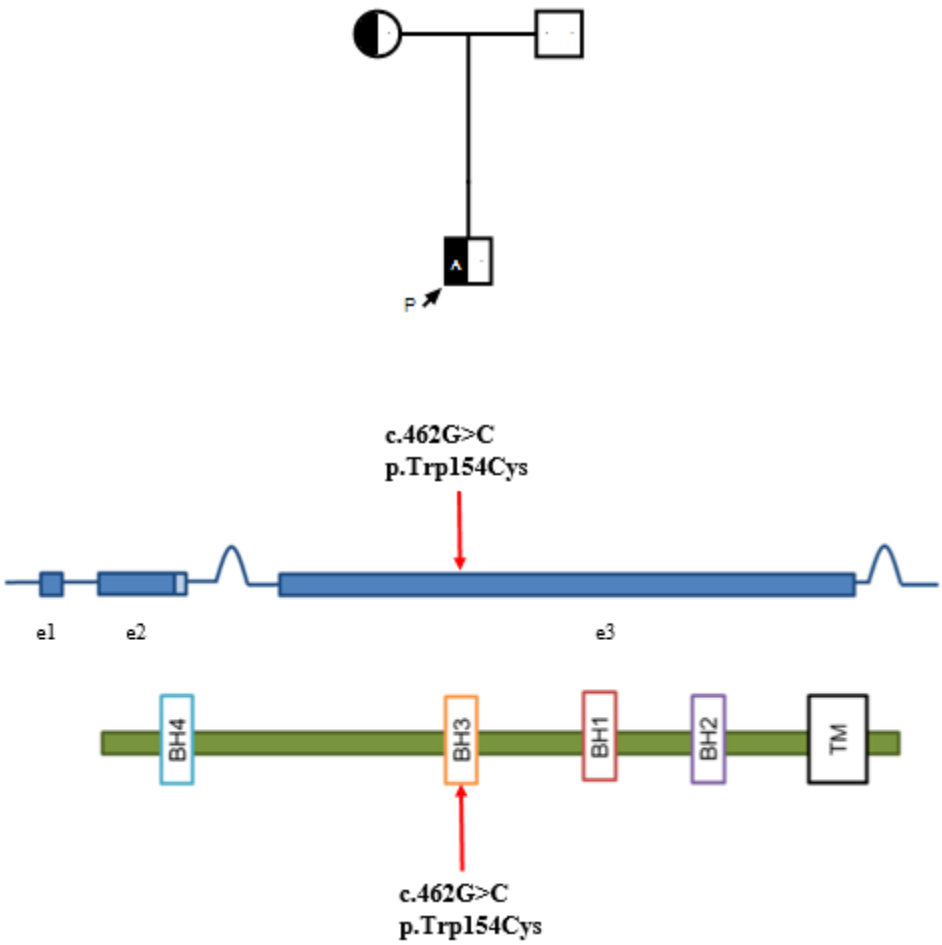


Figure 34. Schematic representation of the exome data-filtering approach used to identify variants in patient 18. MAF: minor allele frequency; SLE: systemic lupus erythematosus; SNVs: single nucleotide variants; VIP: Vasculitis and Inflammation Panel.

The proband is heterozygous for a highly damaging missense variant in *BAD* (NM_004322.3: c.462G>C) on chromosome 11, at chromosome position 64270254 (GRCh37), encoding a tryptophan to cysteine substitution at amino acid position 154 (p.Trp154Cys) of the

Bcl2 protein, predicted to be probably damaging (Polyphen2 = 0.999). When the variant was analysed using CADD, it also obtained a very high CADD score (CADD = 34). Measuring the variant conservation with the GERP score = 2.22 and Siphy Logodds = 15.323, it was found to be in line with the observation that nucleotide substitution occurred at the site which is under evolutionary constraint, predicting it may cause protein disruption.

Protein Bcl2 contains four conserved domains - BH1-4. BH1, BH2, and BH3, which participate in the formation of various dimer pairs as well as in the regulation of cell death. The BH3 represents the critical death domain, while the BH4 is vital as a death-repressor function (270). Position 154, affected in patient 18, is located within BH3, which explains the high scores obtained by the prediction tools. Furthermore, the two amino acids are in different physicochemical groups, aditionally supporting the possible influence on protein function (Figure 35).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
BAD	g.64270254G>C (GRCh38)	NM_004322.3	c.462G>C	p.Trp154Cys	MS	3	0.0005	N/A	Prob Dam (0.999)	34	2.22	VUS

Figure 35. Pedigree of family 18 (top). Schematic representations of the *BAD* gene and Bcl2 protein domains show the position of c.462G>C, (p.Trp154Cys) mutation (bottom). Modified according to the reference number 271. AF: allele frequency; C: cytosine; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; Cys: cysteine; G: guanine; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; MS: missense; N/A: not available; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; Prob Dam: probably damaging; SIFT: Sorting Intolerant from Tolerant prediction tool; Trp: tryptophan; VUS: variant of uncertain significance.

There is evidence in the literature that the Bcl2 expression was down-regulated in human umbilical vein endothelial cells, co-cultured with IgA1 and isolated from IgAV patients (272). However, since there are no published cases of patients with *BAD* variants and IgAV (273), and given the lack of cosegregation in the family, the variant was classified as a VUS (Table 28).

Table 28. Classification of the *BAD* variant according to the ACMG criteria.

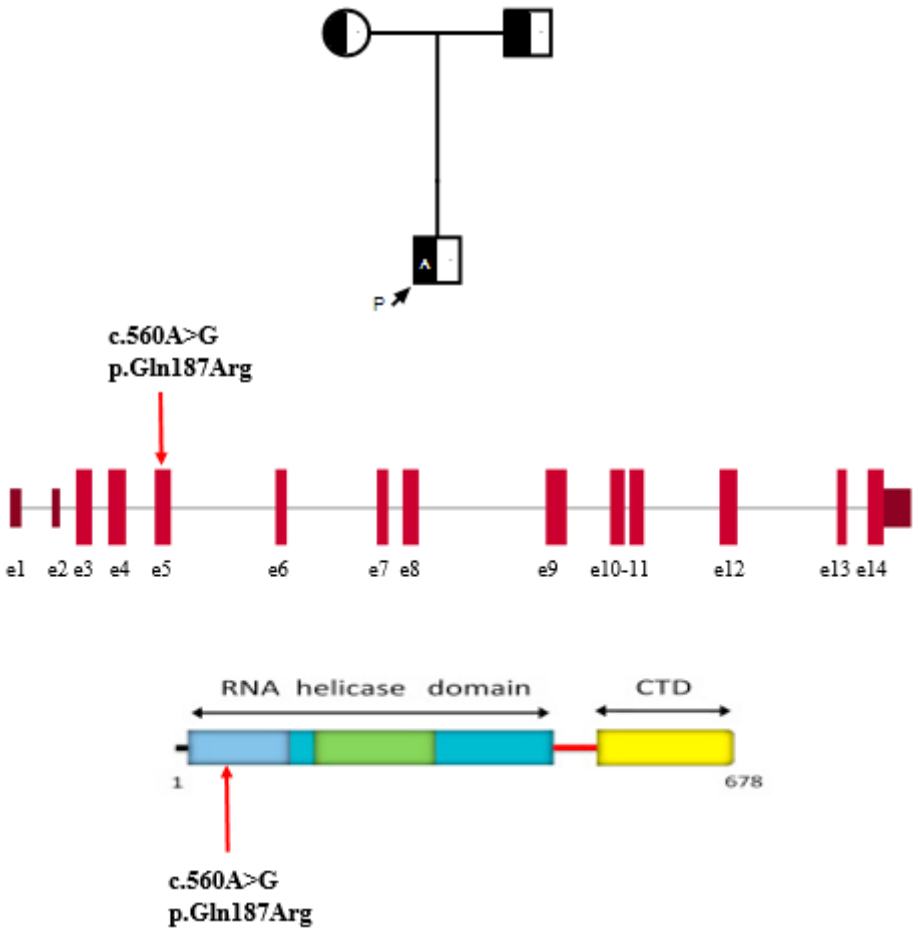
Pathogenicity Criterion	***BAD*, (p.Trp154Cys)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Very rare, MAF = 0.0005
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	Yes	Polyphen2 = 0.999, CADD = 34
PP4. Phenotype specific for disease	No	
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP + 1 SP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***BAD*, (p.Trp154Cys)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in healthy mother
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	No	
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S	
INTERPRETATION	NOT BENIGN	
REMARK	NOT PATHOGENIC, NOT BENIGN - VUS	

VUS: variant of uncertain significance.

The second highest score was obtained for the the *DHX58*^{G187R} variant on chromosome 17 (NM_024119.2: c.560A>G), at chromosome position 42110724 (GRCh37), leading to a glycine to arginine substitution at amino acid position 187 (p.Gln187Arg) of LGP2 protein, predicted

to be possibly damaging (Polyphen 2 = 0.904) with high CADD scores (CADD = 27.8), and conflicting results obtained by SIFT - tolerated substitution (SIFT = 0.23). The affected region is evolutionary conserved (GERP score = 2.54, Siphy Logodds = 13.7655). LGP2 consists of two core domains: the central ATP dependent DExD/H domain containing a conserved motif Asp-Glu-X-Asp/His (DExD/H), which is the most important for enzyme function and acts like ATPase, and the C-terminal domain which is important for RNA binding (274). The position 187 corresponds to the RNA helicase domain, therefore making it the most important part of the protein, which is in concordance with the high pathogenicity scores obtained with Polyphen2 and CADD (Figure 36).



Gene	Genomic Annotation	Reference sequence	cDNA Annotation	Protein Annotation	Coding Effect	Exon	gnomAD AF	SIFT (Score)	Polypen-2 (Score)	CADD Score	GERP (Score)	Classification
DHX58	g. 42110724A>G (GRCh38)	NM_024119.2	c.560A>G	p.Gln187Arg	MS	5	<0.0001	Tol (0.23)	Pos Dam (0.904)	27.8	2.54	VUS

Figure 36. Pedigree of family 18 (top). Schematic representations of the *DHX58* gene and LGP2 protein domains show the position of c.560A>G, (p.Gln187Arg) mutation (bottom). Modified according to the reference number 275. A: adenine; AF: allele frequency; Arg: arginine; c.: coding DNA sequence; CADD: Combined Annotation Dependent Depletion prediction tool; cDNA: complementary DNA; G: guanine; g.: genomic sequence; GERP: Genomic Evolutionary Rate Profiling score; Gln: glutamine; gnomAD: Genome Aggregation Database; GRCh37: Genome Reference Consortium Human genome build 37; MS: missense; p.: protein sequence; Polyphen-2: Polymorphism Phenotyping 2 prediction tool; Pos Dam: possible damaging; SIFT: Sorting Intolerant from Tolerant prediction tool; Tol: tolerant; VUS: variant of uncertain significance.

However, after segregation analysis, it was found that both unaffected parents also carried the same variant, and after literature research the direct association of *DHX58* mutations and IgAV could not be found (276), so the variant was classified as VUS according to ACMG criteria (Table 29).

Table 29. Classification of the *DHX58* variant according to the ACMG criteria.

Pathogenicity Criterion	***DHX58*, (p.Gln187Arg)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Very rare, MAF < 0.0001
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	No	
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***DHX58*, (p.Gln187Arg)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in healthy family members
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	No	
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S	
INTERPRETATION	NOT BENIGN	
REMARK	NOT PATHOGENIC, NOT BENIGN - VUS	

VUS: variant of uncertain significance.

The last variant, *MSH2*^{Q561L}, is a homozygous missense mutation on chromosome 2 (NM_000251.2:c.1681G>A), resulting in a change of glutamate in position 561 with lysine and predicted to be tolerated with SIFT = 0.07 and benign with Polyphen2 = 0.367, with conflicting results obtained by CADD (CADD = 24.5). The exon 11, where the mutation occurred, exhibits a pattern of conservation typical of a protein-coding exon (GERP score = 2.68, Siphy Logodds

= 20.2019). The MAF of <0.0001 indicates this is a very rare allele, however, it lacks segregation in the family given that it is present in both parents who are healthy. This, together with the prediction of low impact on protein function, obtained with SIFT and Polyphen2, and lack of association with IgAV in the literature (277), has led to the variant being classified as benign (Table 30).

Table 30. Classification of the *MSH2* variant according to the ACMG criteria.

Pathogenicity Criterion	***MSH2*, (p.Glu561Lys)**	
A. VERY STRONG (VSP)		
PVS1. Null variant	No	
B. STRONG (SP)		
PS1. Same aa change previously pathogenic	No	
PS2. De novo (confirmed parentity)	No	
PS3. Established function test - damaging	No	
PS4. Prevalence in patients > controls	No	
C. MODERATE (MP)		
PM1. Variant hotspot, functional domain	No	
PM2. Absent, rare in healthy databases	Yes	Very rare, MAF < 0.0001
PM3. For recessive, phase trans	No	
PM4. Change in protein length	No	
PM5. Same residue, different aa change pathogenic	No	
PM6. Assumed de novo (w/o parentity)	No	
D. SUPPORTING (SP)		
PP1. Co-segregation in the family	No	
PP2. Low benign variant frequency	No	
PP3. In silico damaging, multiple lines	No	
PP4. Phenotype specific for disease	No	
PP5. Reported pathogenic, w/o evidence provided	No	
TOTAL SCORE	1 MP	
INTERPRETATION	NOT PATHOGENIC	

Evidence of benign impact	***MSH2*, (p.Glu561Lys)**	
A. STAND-ALONE (SA)		
BA1. Frequency is >5% healthy databases	No	
B. STRONG (S)		
BS1. Frequency greater than expected for disorder	No	
BS2. Observed in a healthy adult individual when disease is fully penetrant at an early age	No	
BS3. Established function test - no damaging effect	No	
BS4. Lack of segregation	Yes	Present in healthy family member
C. SUPPORTING (SP)		
BP1. Missense variant where truncating variant cause a disease	No	
BP2. in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or in *cis* in any inheritance pattern	No	
BP2. In-frame deletions/insertions in a repetitive region without a known function	No	
BP3. In silico no impact, multiple lines	Yes	SIFD = 0.07, Polyphen 2 = 0.367
BP4. Found in a case with an alternate molecular basis for disease	No	
BP5. Reputable source recently reports variant as benign	No	
BP6. Synonymous (silent) variant AND the nucleotide is not highly conserved	No	
TOTAL SCORE	1 S + 1 SP	
INTERPRETATION	LIKELY BENIGN	

After evaluation of HLA genes in detail, it was found that one of them may be the risk allele associated with susceptibility to IgAV: *HLA-DQB1*05:01* (146).

6. DISCUSSION

Overall, this thesis analyses a cohort of patients with cSLE and IgAV characterised by a burdened family history in terms of autoimmune diseases, unusual clinical courses, syndromic features and/or resistance to common treatments, using WES to find new and rare genetic variants which could contribute to the pathogenesis or susceptibility to the disease, or explain the unusual phenotypic characteristics of patients with syndromic features.

Compared to similar studies, this research differs in that it included the Central European population with cSLE and IgAV, which aims to expand existing genetic databases since the current lack of data in this area makes variant interpretation more complex and compromises the implementation of new testing modalities such as polygenic risk scores. Patients from different ancestral backgrounds present different clinical manifestations and noticeably divergent gene expression which is illustrated by the example of cSLE and IgAV (13, 155, 278).

Another novelty is the innovative approach to patient selection. Most studies are based on patients with a familial presentation of disease and/or patients with early onset disease, while the patient selection system in this research is much more complex, allowing, for example, comparison of a group of patients with a familial presentation of a disease with those who have other autoimmune disease in the family and includes patients with syndromic forms of the two diseases.

An important factor in the success of determining relevant candidate genes is the accessibility of family members. With an increased number of patients available to do sequencing on, the chance of finding causative mutation increases. However, this number, as well as the number of unaffected family members necessary to discover the variant, varies depending on the nature of the phenotype being examined, and there is no recommended number. According to data from the literature, this number is in the range from a single patient to ten family members or unrelated patients to pinpoint candidates (279). The hope was that the 19 "trio" samples would help to pinpoint new and rare genetic variants which can be prioritised for future examination using functional studies.

Since each "trio" is unique in terms of its characteristics and genetic background, the results obtained in the course of the discussion will be commented on separately in respect of each "trio" in question.

6.1. Patient 1

The two most prominent aspects of patient 1 are the dysmorphic features, dominant on the face, although somewhat obscured by the edematous features secondary to glucocorticoid use and the atypical clinical course of the disease with general physical and neurologic deterioration and progressive deterioration of lung function due to interstitial lung disease. In addition to the diagnosis of cSLE, this patient was additionally diagnosed with Kabuki syndrome after WES. This is the first case of an SLE and Kabuki syndrome association, which was published in 2019 (232).

Kabuki syndrome is a rare multiple malformation disorder, today recognised as combined immunodeficiency associated with syndromic features and classified in the group of inborn errors of immunity according to the updated classification from the International Union of Immunological Societies Expert Committee (280). Although the syndrome is rare, the number of patients is probably higher than the estimated prevalence ranging from 1: 32,000 in Japan to 1: 86,000 in Australia and New Zealand, due to non-recognition of phenotypic traits, as was the case with this patient (281, 282).

There are five typical dysmorphic features that have been scored and included in the new diagnostic criteria: long palpebral fissures with everted lower eyelids in the lateral third (the most typical and needs to be present), elevated eyebrows with lateral third interruption or lacking denseness, short columella with lowered nasal tip, large, irregularly shaped ears and the presence of fetal fingertip pads. Other features may be present with different representation (283). Patient 1 has all five prominent and the most important dysmorphic features, although using the older scoring system from 2013 proposed by Makrythanasis et al. achieved only 5 out of a total of 10 points, while the patients with confirmed mutations had a mean score of 6.1 (226).

The mechanism of the syndrome has not been elucidated, but it is known to be associated with pathogenic variants in either histone-lysine N-methyltransferase 2D (*KMT2D*) or lysine-specific demethylase 6A (*KDM6A*), which are enzymes involved in the epigenetic regulation

of the number of other genes during embryogenesis, acting on histone, which results in an activated chromatin state of these genes. Both genes affect the opening of chromatin and foster gene expression. Neverheless, target genes and their functions are not known. It is also believed that *KMT2D* and *KDM6A* act as tumor suppressors (226).

A heterozygous pathogenic variant in *KMT2D* is present in about 75% patients, it is inherited in an autosomal dominant manner, has complete penetrance and it is estimated that in most cases it occurs *de novo* (284, 285, 286). A loss-of-function mechanism is responsible for Kabuki syndrome. Pathogenic variants seen tend to reduce catalytic activity of KMT2D, which consequently leads to an increase in methylated histone levels and decreases homeobox gene (developmental control genes) expression, accordingly affecting global cell differentiation. In the literature, atypical cases of the syndrome are described in terms of distinctive dysmorphic features, possibly associated with gain-of-abnormal-function, caused with a heterozygous pathogenic missense variant that involves exons 38 or 39 (287, 288). The minority of patients, aproximatelly 3-5%, have the pathogenic *KDM6A* variant inherited in an X-linked manner, and the most frequent type are splice site variants (284). For about 30% of patients clinically recognised as Kabuki syndrome, the genetic cause remains unknown. Possible candidate genes include *RAP1A*, *RAP1B* and *KDM6C* (284).

The variant in exon 34 of *KMT2D* (NM_003482.3:c.8626delC; 55 reads C, 56 reads delC) in patient 1 is a frameshift variant predicted to encode the protein (p.Gln2876Serfs*34) with the predicted consequence of changing glycine at position 2876 to serine, and terminating the protein at position Ter34. The mutation is estimated to have occurred in the evolutionary well-conserved region. The result of mutation is a truncated protein with predictive loss of gene function where loss of function is a known mechanism of disease. The variant has not been previously described and is not present in population databases. The unaffected parents don't carry the variant and the phenotype of the patient is unquestionably consistent with Kabuki syndrome. This variant meets the criteria to be classified as pathogenic according to the five-tier system for describing the clinical significance of genetic variants of ACMG and is associated with an abnormal phenotype in patient 1.

The truncated KMT2D protein would lead to major modifications of the methylation marks on targeted histones. It can be speculated that absence, or a short form of the protein encoded by *KMT2D,* leads to a more severe loss of function (228). This is consistent with the observation that patients with pathogenic truncating variants that occur in the first half of the gene, which

has 54 exons, may have more severe intellectual disability (228). In patient 1 the mutation occured at 34 exon, in the second half of the gene, which corresponds with the clinical picture of a mild to moderate range of intellectual disability. Furthurmore, it was observed that mutations in the terminal region of the KMT2D gene, i.e. in exon 52, may increase the risk for autoimmune diseases (229). Patient 1 has the most severe and serious type of autoimmune disorder previously not reported in Kabuki syndrome although the mutation was distant from the 3'-end of the *KMT2D* gene near the SET domain region, where the mutations in patients with Kabuki syndrome, associated with autoimmune diseases, usually occur.

In patients with Kabuki syndrome, numerous immunopathological manifestations are described, including immunodeficiences and autoimmune disorders, both being present in patient 1 (289, 290). Regarding autoimmune diseases associated with Kabuki syndrome, the most frequently described is immune thrombocytopenic purpura, with or without concurrent hemolytic anemia, followed by vitiligo and autoimmune thyroiditis (289). Other autoimmune diseases were reported less frequently, such as diabetes type 1, Crohn disease, membrane glomerulonephritis type 3 and lymphoid interstitial pneumonia (291). The possible mechanism of development of autoimmunity could be explained by the fact that both *KMT2D* and *KDM6A* are part of a protein complex, COMPASS (which is also involved in the epigenetic regulation of the *FOXP3* gene), which plays a crucial role during the differentiation of naive CD4+ T cells into T-regulatory cells and is important for maintenance of peripheral tolerance (289). Therefore, dysregulation of T lymphocyte differentiation results in the production of inadequate regulatory T cells and tolerance breakdown, which prompt to autoimmune disease.

As some clinical features may occur in both cSLE and Kabuki syndrome, such as positive antiphospholipid antibodies, the episode of convulsions, intestitial lung disease and persistent hypogammaglobulinemia in patient 1, the question remains whether these two conditions are associated suggesting potential overlap of Kabuki syndrome and SLE, or do they just represent accidental coexistence in the same patient? Given positive antiphospholipid antibodies have already been described in a patient with Kabuki syndrome but without SLE, these antibodies may be part of a clinical picture of SLE, but also an autoimmune feature of Kabuki syndrome (292). A similar problem can be found with the episode of convulsions in patient 1, which may be part of the spectrum of neurological manifestations of SLE but is often found in patients with Kabuki syndrome (293). Furthermore, patient 1 developed interstitial lung disease of open etiology. It is known that in SLE patients different pulmonary diseases may occur, especially interstitial pneumonia, which is reportedly found in 3-13% of patients with SLE (294), with the

most frequent intestitial pneumonia pattern referred to as being "unclassifiable" on high-resolution computed tomography (295), and radiological findings from patient 1 may also correspond to this pattern. On the other hand, there are reported cases of granulomatous and lymphocytic interstitial lung disease (GLILD) (296) in patients with Kabuki syndrome, in which radiological findings of patient 1 may also fit the picture although the results of the lung biopsy are inconclusive and a surgical lung biopsy has not been performed. Finally, a minority of patients with SLE have low immunoglobulin levels, but immune dysfunction is a common feature of Kabuki syndrome, especially hypogammaglobulinemia and IgA deficiency (297, 298).

Loss of function of *KMT2D* and *KDM6A* epigenetically leads to defective B-lymphocyte, since COMPASS complex helps in the transcription of the Activation Induced Deaminase complex gene involed in the somatic hypermutation and class switch recombination, which is essential to B-cell differentiation, and thus hypogammaglobulinemia and reduced memory B-cell numbers are often present in patients with Kabuki syndrome (229). A disrupted process of antibody affinity maturation and immunoglobulin class switching, may lead to low IgG and IgA levels and propensity for infections. The low immunoglobulin levels may aggravate immunosuppressive therapy in patients who have accompanying autoimmune disease, as was the case in patient 1, where the modification of immunosuppressive SLE therapy was necessary due to several cyclophosphamide-induced sepses (232).

Besides the pathogenic *KMT2D* variant, patient 1 has two additional variants in *C1R* and *C1S* genes, encoding for complement C1r and C1s subcomponent respectively. Both C1r and C1s are part of C1 complex, which comprises a single C1q molecule bound to two molecules of C1r and two molecules of C1s. Complement component C1q binds directly to pathogen surfaces or indirectly to antibodies bound to pathogens. Binding of C1q to a pathogen surface causes a conformational change in complex, which first leads to activation of C1r, which then causes activation of C1s that acts on the next two components of the classical pathway, C4 and C2. These are the first steps in the classical complement pathway (299). Homozygous C1r and C1s deficiencies are known monogenic forms of SLE (86). The mechanisms by which C1r and C1s deficiencies contributes to the development of SLE are not yet known in detail. It is a paradox that the disruption of complement activity is associated with autoimmune diseases since it is known that complement-activation products lead to increased inflammatory response, so one would expect that in persons with complement component deficiencies, the inflamation is decreased (300). It is even more difficult to understand why people with a deficiency of a late

complement factor do not usually suffer from inflammatory and autoimmune diseases but only from susceptibility to infections.

The patient is heterozygous for *C1R*^{M112I} and *C1S*^{D315N}, both result from missense mutations in evolutionary conserved regions and predicted to be pathogenic with high scores obtained using *in silico* prediction tools. However, since both variants are present in unaffected members of the family and under the established autosomal recessive mode of inheritance of monogenic forms of SLE caused by C1r or C1s deficiency, they are unlikely direct causes of SLE in patient 1.

To summarise, using WES in our patient allowed for SLE to be added to the list of documented autoimmune disorders in Kabuki syndrome and, more broadly, this case demonstrates the value of WES in elucidating the diagnosis of rare, complex and sometimes clinically unrecognised genetic conditions (232). Nevertheless, the strongest genetic influence regarding the SLE susceptibility in this patient is represented by the HLA genes, since she has as many as three HLA alleles associated with SLE.

6.2. Patient 2

Patient 2 is characterised by cSLE affecting a number of organ systems, including the central nervous system, kidneys, gastrointestinal system, musculoskeletal system, skin, associated with malformation syndrome, immunodeficiency and coagulopathy with a positive family history of malformation syndromes and unexplained deaths in young age. Due to the unusual clinical features and course of the disease, as well as the burdened family history of this patient, there was a suspicion of a genetic etiology of the disease. However, WES analysis did not yield a positive result, as expected: two candidate gene variants have been identified, whose significance and potential association with a phenotype and risk for disease development are commented on below.

The variant that seemed most associated with the phenotype is the missense heterozygous *ADAR*^{I939V} variant. The product of transcription and translation of this gene is the enzyme adenosine deaminase acting on the RNA (ADAR), also called double-stranded RNA-specific adenosine deaminase (DSRAD) or simply RNA-specific adenosine deaminase. The enzyme binds to the RNA and converts adenosine to inosine by deamination, which results in destabilisation of the double-stranded RNA helix. It is involved in the inhibition of certain

viruses replicating, such as human immunodeficiency (HIV) and hepatitis C viruses, by modifying their RNA. Furthermore, it is important for the modification of receptor proteins that interact with the neurotransmitters and is involved in the control of the innate immune response (301). The two most prominent diseases associated with different ADAR variants are dyschromatosis symmetrica hereditaria, inherited mainly in autosomal dominant mode, and Aicardi-Goutières syndrome, inherited mainly in autosomal recessive mode (238, 302).

Dyschromatosis symmetrica hereditaria is a rare pigmentary dermatosis, primarily affecting skin pigmentation, but sometimes different extracutaneous manifestations may occur. The most prominent clinical feature of the disease are numerous hyper- and hypopigmented macules in a reticular pattern, distributed symmetrically over the face, trunk, and distal part of extremities, occasionally accompanied with neurological abnormalities, mental deterioration, dystonia, developmental regression, autistic disorder, depression, seizure disorder, psoriasis, intracranial hemangiomas, Parry-Romberg syndrome and thalassemia, but even autoimmune abnormalities such as cutaneous lupus erythematosus and hyperthyroidism have been described in the literature (303). Although disease occurs predominantly in people of East Asian origin, sporadic cases have been found in other parts of the world as well. Patient 2 has many clinical features of this disorder, including pigmentary skin changes and some extracutaneous manifestations described in the literature. Additionally, the mother who also carried the $ADAR^{I939V}$ variant has a pigmentation disorder which is less pronounced than in the patient, with no other associated disorders. Skin changes in this disease appear in childhood, usually before the age of six, and remain through life without any changes after stabilisation in adolescence (303, 304). Patient 2 has compatibile skin changes from early childhood. On the other hand, the skin changes in the mother appeared in adulthood. In the literature, cases have been reported of late onset disease (304). Because the exact influence of gene defect on disease pathogenesis is unknown, the diagnosis generally relies on clinical examination (305). Histological examination has limited capabilities to differentiate between different reticulated pigmentary disorders (303). There are several factors that support this diagnosis of dyschromatosis symmetrica hereditaria in both the patient and the mother. First of all, the features of other pigmentation disorders, that may be considered as a differential diagnosis, such as the presence of atrophy and an absence of hypopigmented lesions, hyperpigmentation in the body's folds, accompanied by comedogenic lesions on the back and neck, symptoms of xerosis, atrophy, telangiectasia, and tumors in photo-exposed areas are absent both in the patient and her mother. Secondly, the presence of similar skin changes in two family members (the proband and the mother, although somewhat less

convincing in the mother) both carrying the *ADAR* - consistent with the high penetrance of the pathogenic *ADAR* variants. It remains unclear, however, whether the patient has the disease, and particularly how to explain the numerous extracutaneous manifestations which, although described in the literature, are not usually in such large numbers and predominance in the same patient. On the other hand, patient 2 does not meet the number of diagnostic criteria for Aicardi-Goutières syndrome such as basal ganglia calcifications, cerebral white matter abnormalities and cerebral atrophy etc. (306). Aicardi-Goutières syndrome is an inherited encephalopathy that affects newborn infants and usually results in severe intellectual and physical disability (306). In the early-onset form of the disease, infants are born with neurological and liver abnormalities (hepatosplenomegaly and elevated liver enzymes), while in later-onset form after the first weeks or months of normal development, a progressive decline in head growth, weak or stiffened muscles and cognitive and developmental delays occur. Typical clinical features include encephalopathy and/or significant intellectual disability, acquired microcephaly during the first year of life, dystonia and spasticity, sterile pyrexias, hepatosplenomegaly and chilblain lesions on the feet, hands and ears (307). Diagnosis is established based on the clinical findings, as well as characteristic brain abnormalities that can be seen in an MRI brain scan. Patient 2 did not have microcephaly, nor dystonia, spasticity, sterile pyrexias or chilblain lesions. However, she has splenomegaly, significant intellectual disability and thrombocytopenia that may be seen in patients with Aicardi-Goutières syndrome.

The variant $ADAR^{I939V}$ in exon 10 in patient 2 results in amino acid substitution of isoleucine for valine at the codon 939 in the evolutionary semi-conserved region, with positive predicting a moderate impact on its function. Furthermore, this region is within the tRNA-specific and double-stranded RNA adenosine deaminase (ADEAMc) domain, corresponding to exons 9 to 15 of *ADAR*, encompassing amino acids 839-1222 (235). The most frequent *ADAR* mutations are missense mutations located within this region. On the other hand, isoleucine and valine are amino acids within the same group of aliphatic amino acids, which implies that their physicochemical properties are similar. However, the *in silico* prediction tools SIFD, Polyphen2 and CADD are concordant in the prediction that this substution may disrupt the function of the protein. It can be hypothesised that this amino acid substitution may alter the activity of *ADAR* directly, or disturb the formation of the wild-type ADAR homodimers (235). An increased expression of IFN-I regulated genes (an IFN signature) was not demonstrated in the PBMCs from patient 2 (although analysis was undertaken when the patient was under treatment with immunosuppresive drugs which may influence the results). This is consistent

with the fact that the phenotypic characteristics of patient 2 do not completely conform to Aicardi-Goutières syndrome, in which a loss of ADAR1 activity leads to a dramatic upregulation of IFN-stimulated gene expression (308). On the contrary, the *ADAR* mutations in dyschromatosis symmetrica hereditaria have different characteristics and are not associated with the IFN signature (308). The variant $ADAR^{I939V}$ is present in the patient and mother with skin changes with which the diagnosis cannot be unequivocally confirmed, and is extremly rare in public databases. The functional analysis, in terms of quantifying the relative mRNA expression of *ADAR*, for this variant has not been reported in the literature. Thus, the definitive association of this variant with phenotypic traits remains unknown, although the variant is likely to be pathogenic but only partially explains the phenotype in the patient (since it is only compatible with altered pigmentation and doesn't have many other features). It can be speculated that this extremly rare variant somehow produces an intermediate phenotype between dyschromatosis symmetrica hereditaria and Aicardi-Goutières syndrome. Perhaps there is a presence of unidentified/unknown modifier genes which allow for a more pronounced and complex phenotype in the proband as opposed to the mother.

Another variant present in this patient is $SH2B3^{C133T}$. The gene *SH2B3* encodes for Src homology 2-B adaptor protein 3 (SH2B3). The protein is expressed in different tissues, with the highest expression in hematopoietic cells. It functions as a negative regulator in lymphopoiesis, megakaryopoiesis, erythropoiesis and helps regulate inflammation by suppressing cytokines such as TNF-alpha and IL-6 (309). Due to certain genetic variants, SH2B3 may fail to control an overactive immune response, leading to autoimmunity. *SH2B3* mutations have been reported in patients with various myeloproliferative neoplasms (310). *SH2B3* polymorphisms have also been reported to be linked with SLE (240, 311). The possible mechanism is not elucidated and it is supposed that the overexpression as well as the deficiency of this protein may be important in the development of autoimmune diseases. SH2B3 overproduction in lymphoid precursors resulted in a reduction in the number of both B and T cells, whereas the deficiency of the SH2B3 resulted in enhanced B-cell production.

Patient 2 has a missense variant in the *SH2B3* gene with the substitution of cysteine with threonine, two amino acids with the same group and with many similar properties, in the evolutionary semi-conservated region, and predicted not to effect the protein function significantly using SIFD, Polyphen2 and CADD. The variant is inhereted from an unaffected mother. It is a rare allele. Studies on the function, as well as patients, with SH2B3-related diseases and this particular variant have not been reported in the literature, so the potencial

influence of this variant in patient 2 remains unknown but since the variant is likely to be benign, it is not convincing.

In conclusion, it can be said that despite numerous syndromic features and an atypical picture of cSLE in this patient, the application of WES failed to identify a convincing pathogenic variant that would explain the most prominent phenotypic features and establish a genetic diagnosis of a monogenic form of SLE. Instead, a pathogenic variant in *ADAR* was found, which is not associated with cSLE but with a concomitant dermatologic disorder. Similar to the previous patient, the most strongly genetic association with SLE was demonstrated with HLA alleles, among which the patient has three previously confirmed to be linked with SLE susceptibility.

6.3. Patient 3

The specificity of patient 3 provides a more severe clinical picture of cSLE, with special emphasis on involvement of the gastrointestinal system, although with additional vascular anatomical variation of the celiac trunk with a questionable impact on the clinical picture. It should also be kept in mind that in this patient, a number of organs, including the heart, kidneys, gastrointestinal system, pancreas, musculoskeletal system and serous membranes, were affected. Taking into account the presence of the same disease in the mother, also with a more severe clinical picture, as well as the presence of systemic autoimmune diseases in the family, i.e. scleroderma, genetic influence or susceptibility on the phenotype in this patient was assumed.

The most important gene variant highlighted by WES in this patient was a rare variant resulting in insertion in the *CR1* gene. The protein product of this gene is complement receptor type 1, also known as the C3b/C4b receptor or CD35. Its main role is to bind C3b/C4b-opsonised foreign antigens, it therefore participates in processing and clearing complement opsonised immune complexes and has an important function in destroying microbes and initiating an immunologic response (312). The possible association with *CR1* polymorphisms and SLE was first observed in 1981, when a group of authors described CR1 deficiency in SLE patients, measuring the concentration of CR1 on erythrocytes from normal individuals and patients with SLE (313). They found defects in the CR1 reactivity in SLE patients, which could not be restored even after SLE patients had gone into remission and are thought to be inherited since the same defect was found frequently in the relatives of patients. The association with

SLE was explained by the fact that in SLE, there is an increased amount of antigen-antibody complexes, which could be the consequence of either overproduction of autoantibodies, as a result of polyclonal B-cell activation or altered suppressor T-cell function, or impaired catabolism of immune complexes. CR1 are involved in the clearance of immune complexes that have activated the immune system, so CR1 deficiency would result in decreased immune complex clearence. Later, the association of CR1 deficiency and SLE was called into question when Moldenhauer et al. found the same frequency of CR1 deficiency in normal cases and in patients with SLE (314). Nevertheless, meta-analysis from 2005 clarified the association of *CR1* polymorphisms with SLE (242). It seems that *CR1* polymorphisms causing functional changes, i.e. decreased expression of CR1 on erythrocytes, are not associated with SLE, suggesting no association of lower CR1 levels in SLE. Lower CR1 levels are indeed found in SLE patients, but they are acquired and not inherited, and are a consequence of the disease processes (315). On the other hand, *CR1* polymorphisms resulting in the different molecular weight of CR1, such as the S or B variant of CR1, characterised by an additional C3b binding site, are linked with increased SLE susceptibility although it is not known how the structural polymorphisms of the *CR1*, which did not differ in function, may play a role in SLE (314). However, a recently published study showed that even functional *CR1* polymorphisms, resulting in decreased CR1 expression on monocytes increased susceptibility to development of SLE and lupus nephritis in malaria endemic areas (243).

The variant in exon 25 of *CR1* (NM_000651.4:c.4052dup) in patient 3 is a frameshift variant predicted to encode the protein p.(Asp1351Glufs*23) with the predicted consequence of changing asparagine at position 1351 to glutamic acid and the new reading frame encountered a new translation termination (stop) codon. The duplication is estimated to be in an evolutionary weakly conserved region, however it was not possible to predict the function of the altered coding sequence of a protein with the available *in silico* tools. The variant is very rare in population databases and, until now, has not been reported in the literature, i.e. there is no description of a disease related to this variant, and functional studies have not been performed. This variant is present only in affected family members with the same disease and is inherited from an affected mother, which is why it seems that this variant could be involved in the pathogenesis of SLE in this patient. Further functional analyses are needed to verify this variant, especially considering the data from the literature suggesting that in pathogenic *CR1* variants in patients with SLE result in a change of CR1 protein molecular weight; in our case, the protein's length is altered, with additional changes in other amino acids. However, since only

homozygous CR1 mutations were described to be associated with the susceptibility to SLE, this variant is unlikely to cause SLE in the patient (243).

Patient 3 also has another interesting variant, BLK^{A71T}. The *BLK* gene encodes a non-receptor tyrosine kinase of the Src (Src is short for sarcoma) family of tyrosine kinases. BLK is known to be functionally involved in B-cell receptor signaling and B-cell development. It is normally expressed in B-cells. BLK may have oncogenic properties and expressional associations with malignancy (316). *BLK* polymorphisms are described to be associated with maturity-onset diabetes of the young, SLE and possiblly with rheumatoid arthritis (66, 317). More recently, the polymorphisms of the gene have been linked with several autoimmune diseases such as systemic sclerosis, primary Sjogren's syndrome, dermatomyositis and Kawasaki disease (243). It was shown that BLK may repress IRF5-mediated IFN-beta expression. Some loss-of-function *BLK* variants with impaired kinase activity may be associated with enhanced IFN-beta production. SLE patients with rare *BLK* variants also had increased expression of IFN signature genes compared to healthy controls (66).

BLK^{A71T} in patient 3 is a rare missense variant, resulting in substitution of alanine for thyrosine at codon 71. This region is predicted to be evolutionary conserved and it corresponds to the SH3 domain of the BLK protein, important for protein-protein interactions. Functional analysis of this variant was previosuly described (244, 318). It was demonstrated that the half-life of the protein is reduced. This effect was explained with the possibility of threonine residue of the mutated variant to be phosphorylated and phosphorilation may represent the signal for degradation by ubiquitin ligases. Functional studies have also shown that the variant severely reduces the binding of the BLK protein to an adaptor protein BANK1 (244). As a result of increased ubiquitination and proteasomal degradation, there is significant reduction of the level of endogenous BLK protein. However, the effect of reduced BLK expression on the function of human B lymphocytes is not fully understood. The reduction of BLK may result in lowering the thresholds for B-cell receptor signaling by insufficient propagation of inhibitory signals or their suppression, which may cause the loss of self-tolerance. High levels of BANK1, compaired to reduced levels of BLK in patients with the missense variant, may result in BLK being left without substrate, with the consequent reduction of the inhibitory activity of BLK on B-cell activation at the plasma membrane. In the case of the BLK^{A71T} variant, this effect may be compensated by decreased binding to BANK1, but the excessive proteasomal degradation of the kinase may still reduce the inhibitory signals of BLK. Ultimately, the lack of inhibition of activation and lack of tolerance control may be a major mechanism in the development of

autoimmunity in the case of the *BLK*^{A71T} variant (244). The variant increased the risk of SLE in the heterozygous state, since in the group of 178 selected samples of SLE patients and controls, the presence of the variant was observed in 2.17% of SLE patients and 0.92% of controls given the odds ratio of 2.31 (the risk for SLE is increased 2.31 compaired to healthy controls), although after adding an additional 1,103 new controls the risk decreased but was still present (odds ratio 1.79). The variant is rare with a frequency of 1-2% in the European population. In family 3 the variant is present only in the affected mother and daughter with the same disease, raising the possibility it may be the risk allele in this patient.

To sumarise: although WES did not obtain a result in terms of isolating a definitively pathogenic variant according to the definition of ACMG, which would be the cause of the disease, one promising variant was found, on which functional analyses were performed and a GWAS confirmed that it is associated with a higher risk of SLE and that it may be linked with the pathogenetic mechanism of the disease in terms of loss of immune tolerance. Patient 3 is one of the two patients in the study for whom no HLA risk alleles were found (57, 58). Her affected mother was also found to have no HLA risk alleles.

6.4. Patient 4

Patient 4 is very interesting from a clinical point of view due to the overlap of SLE and ulcerative colitis features. Most of the clinical manifestations and problems associated with the establishment of remission in this patient were related to ulcerative colitis and not SLE. Several family members suffer from various autoimmune diseases, but ulcerative colitis predominates.

It is well known that SLE tends to either coexist or overlap with various other autoimmune diseases including rheumatoid arthritis, systemic sclerosis, polymyositis, varieties of vasculitis, Sjogren's syndrome, mixed connective tissue disease and anti-phospholipid syndrome (319). The coexistence of the inflammatory bowel disease (IBD) and SLE is rare and represents a diagnostic challenge (320). It is not clear whether IBD and SLE co-exist by chance or if there is a real association and no exact data on incidence and prevalence of IBD and SLE co-occurrence is available. So far, the data regarding the association between IBD and SLE is mostly based on case reports and case series which indicate an infrequent association (320, 321). There was a previously reported prevalence of 0.4%, mainly seen in case reports (322). In a systematic review of the literature, the co-occurrence of SLE and Crohn's disease was reported to be even less frequent (320). In cases of concomitant diagnosis of IBD and SLE, IBD

may occur either before or after SLE diagnosis, but in most of the described examples from the literature, ulcerative colitis developed a mean of 10 years after the onset of SLE symptoms, which is exactly the opposite in the case of patient 4, in whom SLE was diagnosed 9 years after ulcerative colitis (323).

The distinction between IBD and SLE may be difficult to see, as patients with IBD may show similar clinical signs and symptoms to SLE, and IBD patients might fulfill some of the classification criteria for SLE and sometimes the laboratory test and radiographic findings may appear similar in the two diseases (324, 325). Between 15% and 75% of SLE patients may have gastrointestinal symptoms (326, 327). There are some differences: ulcerative colitis compaired to SLE presents more frequently as bloody diarrhea, abdominal pain and tenesmus. To be more confident in providing a definite diagnosis, a combination of imaging, endoscopic and histological information may be needed, as was the case in patient 4 (320). In SLE patients, colon involvement is remarkably segmental with focal distributed ulcerations, and on biopsies the architectural structure of the mucosa is preserved with an absence of cells typical in chronic inflammatory conditions, while ulcerative colitis has greater colon involvement and colonoscopy shows diffuse erythema, and friability of colonic mucosa and superficial ulcerations (328, 329). The most frequent lesions in the gastrointestinal tract of patients with SLE are chronic, non-specific mucosal inflammation and ischemic changes due to vascular lesions (330). Intestinal perforation rarely occurs in IBD, but is often observed in lupus enteritis, and may be helpful in differential diagnosis (330). In patient 4, features of ulcerative colitis were clearly expressed and present before the onset of SLE, with unequivocal endoscopic and histological findings.

Another source of confusion is the observation that certain medications used for treating IBD, including aminosalicyling acid, sulfasalazine and anti-TNF drugs, may cause drug-induced lupus (331). Sulphasalazine-induced lupus syndrome has been reported at various times in ulcerative colitis, including children (332). The clinical picture may resemble SLE with positive ANA, anti-dsDNA and anti-histones. Even severe and fatal clinical manifestations in terms of vasculitis affecting the central and peripheral nervous system and lungs are described (320). Discriminative features between drug-induced lupus and idiopathic SLE include complement levels, since drug-induced lupus is only rarely associated with low complement levels, presence of aCL, anti-Ro and anti-La antibodies, which are absent in drug-induced lupus, and different HLA haplotypes, where *HLA-B8* and *-DR3* occurs in idiopathic SLE and *HLA-DR4* is associated with drug-induced lupus (333, 334). In drug-induced lupus leukopenia,

anemia, and high titres of ANA antibodies return to normal after withdrawal of the drug and symptoms usually resolve over a period of four to six months (320). Patient 4 was treated with sulphasalazine almost 9 years before she was diagnosed with cSLE. She had positive ANA, anti-dsDNA, aCL antibodies, low complement levels and these laboratory features persisted even after sulphasalazine was withdrawn from therapy after achieving stable remission of ulcerative colitis. WES revealed she has *HLA-B*15:10* and **52:01*, as well as *HLA-DRB1*13:02* and **15:02*. The patient did not have any characteristics of drug-induced lupus other than the use of sulfasalazine prior to the onset of lupus symptoms. Therefore, it can be said with great certainty that the patient does not have a drug-induced lupus.

Another feature that can be found in both diseases, SLE and ulcerative colitis, are the positive anti-PR3 which are also present in this patient. According to the literature, positive anti-PR3 is significantly more prevalent in ulcerative colitis than in Crohn's disease patients (335, 336). Anti-PR3 may be helpful in the differentiation of Crohn's disease from ulcerative colitis and in the identification of ulcerative colitis patients with more extensive disease (336). ANCA of various specificities also occur in patients with SLE with a predominance of p-ANCA of MPO specificity (337). ANCA has been linked with higher disease activity in SLE patients, with a higher frequency of diffuse glomerulonephritis, worse kidney function, higher anti-dsDNA levels and lower complement levels (338). However, their potential role in organ injury still have to be investigated (339). Nevertheless, patient 4 did not develop renal involvement, nor had any severe manifestations of SLE. Most of the clinical manifestations in this patient were related to ulcerative colitis and not SLE.

There are conflicting data in the literaure concerning the common genetic susceptibility between SLE and IBD. The first evidence for a shared linkage region between IBD and SLE outside of the HLA locus was described in the gene region 16q12–13 region, which has been linked to SLE and contain caspase recruitment domain family member 15 (*CARD15*) gene variants associated with Crohn's disease (340). Using the data collected from 1305 SLE patients by de Jager et al. demonstrated that IBD-associated rs2066845 *CARD15* polymorphism may have a strong effect on the risk of developing SLE (340). The *CARD15* gene, also known as *NOD2,* encodes for the nucleotide-binding oligomerization domain-containing protein 2, also called caspase recruitment domain family member 15. It is an intracellular pattern recognition receptor and recognises muramyl dipeptide which is found in certain bacteria and stimulates an immune reaction (341). *CARD15* mutations are associated with IBD, Blau syndrome and there

are possible associations with psoriatic arthritis (342). However, another study on the IBD-associated mutations on *CARD15* reported no influence on SLE susceptibility (343).

Considering all the above, it is obvious that patient 4 does not have a monogenic form of SLE. Given the overlap syndrome involving cSLE and ulcerative colitis and the presence of various autoimmune diseases in several family members, with a predominance of ulcerative colitis, it was to be expected that there would be certain risk genes. It was presumed that the occurrence of autoimmune diseases in this family was not coincidental, but that it has a genetic background. The patient also had IgAV, which presented with a mild, common clinical picture and self-limiting course, but could also be triggered by susceptible genes inside and outside the HLA system. However, contrary to expectations, WES showed a small number of significant gene variants with a possible impact on the phenotype.

The most promising variant was $IRF5^{A241V}$. The gene *IRF5* encodes interferon regulatory factor 5, which is a member of the IRF family, a group of transcription factors with diverse roles, including virus-mediated activation of IFN, modulation of cell growth, differentiation, apoptosis, and immune system activity (344, 345). IRF5 is a direct transducer to IFN signaling, it is activated via phosphorylation and can induce transcription of IFN-alpha. IRF5 controls whether macrophages will promote or inhibit inflammation (346). High expression of IRF5 promotes M1 macrophages with inflammatory phenotype. *IRF5* mutations are associated with different diseases, but interestingly, with both SLE and IBD, it corresponds well with the patient's phenotype, as well as with rheumatoid arthritis and primary biliary cirrhosis (248, 347, 348, 349).

Dideberg et al. studied 12 polymorphisms in the *IRF5* gene in a cohort of 1,007 IBD patients and 241 controls (347). They found that six of the polymorphisms appear to be associated with IBD, but the strongest association was found with rs77571059 *IRF5* polymorphism which is commonly referred to as a CGGGG indel, since each allele has either three or four copies of the CGGGG repeat sequence. The four copy-number variant allows binding of additional SP1 transcription factors and is associated with increased expression of IRF5, but also TNF-alpha, IL-12p40, IL-8, IL-1β, and IL-10 (350). All these polymorphisms increase levels of IRF5. The association was particularly strong among the ulcerous colitis patients (347).

Sigurdsson et al. analysed 47 polymorphisms in the *IRF5* gene in 485 SLE patients and 563 controls and found that there were 18 associated with SLE, but the strongest and most independent association was found for two polymorphisms: *rs10488631* and *rs77571059* (248).

The *Rs7571059 IRF5* polymorphism was the same SNP studied by Dideberg et al. The authors showed increased binding of protein to the risk allele, *rs7571059*, and they showed increased expression of *IRF5* mRNA from a promoter containing that allele. In PBMCs from SLE patients carryng this risk allele it was demonstrated that there was increased expression of the IRF5 protein.

In patient 4, variant $IRF5^{A241V}$ is a missense variant resulting in amino acid substitution of alanine at the codon 241 with valine in the evolutionary weakly conserved region, resulting in predictions of only moderate substitution impact on the protein function using the GERP score and SiPhy, consistent with the predictions of SIFT, Polyphen2 and CADD that this change would not disrupt protein function. Alanine and valine are two amino acids from the same group of aliphatic amino acids with similar chemical characteristics, which further supports the fact that this substitution should not have a significant effect on protein function. However, the variant is rare, with a frequency of 0.01% in the ExAC and 0.05% in GnomAD and it is present only in three members of the family, and all affected by autoimmune diseases. The variant was not detected in any of the healthy family members. It was inhereted from the affected mother. In the literature there is no report of this variant in patients with IRF5-associated disease. There is no information about the functional analyses of the variant. Taking all this into consideration, the association of the variant with the phenotype of the patient and two affected members of the family is not clear and further studies are underway to clarify its pathogenicity.

Another variant in the gene of the *IRF* family, $IRF3^{S303G}$, is also present in this patient. IRF3 is a transcription factor that activates transcription of IFN-related genes and its most important function is antiviral activity (351). A critical role for IRF3 is in the control of central nervous system infection following HSV-1 exposition. Allelic variants in *IRF3*, resulting from heterozygous missense mutations are associated with the susceptibility to herpesviruses (351). From the point of view of patient 4, it is important to emphasise that recently it was published that a missense variant of *IRF3*, which changes serine to threonine on the last amino acid of IRF3, SNP rs7251, was associated with SLE, and significant correlation was observed with lupus nephritis (249). The risk allele is associated with increased expression of *IRF3*, thus resulting in the increased production of IFN I and proinflammatory cytokines, but it may also be involved in SLE pathogenesis through repression of regulatory T cells.

The variant $IRF3^{S303G}$ in patient 4 is a heterozygous missense variant, resulting in amino acid substitution and predicting that it does not significantly impair protein function according to the

in silico tools SIFT, Polyphen2 and CADD, which is concordant with the prediction using the GERP score and SiPhy, given the weak evolutionary conservation of the region in question. The variant is rare but not segragated from the disease, since it is also present in healthy family members. Therefore, it is unlikely to influence the phenotype or susceptibility to SLE in patient 4.

Filtering and prioritising genetic variants in patient 4 also highlighted the variant in the *LIFR* gene. The product of the *LIFR* gene is a subunit of a receptor for the leukemia inhibitory factor (LIF). LIF is a cytokine that affects the differentiation, survival, and proliferation of a number of cells. It was found to be important in inhibiting the growth of leukemia cells, but its signaling is also involved in the formation of bone and the development of nerve cells. It may also have an important function in normal development of the autonomic nervous system (352). Mutations in the *LIFR* gene are associated with a rare genetic syndrome characterised by bone anomalies, respiratory distress, feeding difficulties and hyperthermic episodes (255). However, recently, it was found that LIF and LIFR expression was increased in the inflamed colon in a mouse colitis model (353). Th17-cell differentiation triggered by interleukin-6 fosters inflammation in IBD patients. LIF, which belongs to IL-6 family cytokine, blocks Th17-cell differentiation and is responsible for repairing damaged intestinal epithelium in an inflamed colon (255).

Patient 4 has $LIFR^{R768G}$, resulting in amino acid substitution in a semi-conserved region. Both the GERP score and SiPhy predict this mutation may affect the protein function with moderate probability, while the *in silico* prediction tools, SIFD, Polyphen2 and CADD give conflicting results indicating that amino acid change may disrupt the protein function, which is further supported by the fact that arginine and glycine are amino acids in different groups, thus having different properties. The variant is not frequent in population databases. No data has been published regarding the association of the variant and LIFR-associated disease and the results of functional analyses are not known. The variant is present in three family members, who all have autoimmune disease. It can be concluded, that similar to $IRF5^{A241V}$, influence on the phenotype of patient 4 is not known and further functional studies are necessary to address this issue.

In conclusion, despite the clinically convincing segregation of phenotypes and the very rare overlap of SLE and ulcerative colitis, WES analysis did not identify a convincing monogenic cause of the conditions seen in this patient and family members. Some of the variants identified

warrant further functional characterisation and consideration of digenic or oligogenic genetic etiology. The only significant genetic background for SLE is represented by four susceptibility alleles (57, 58).

6.5. Patient 5

In the clinical picture of patient 5, the only feature that needs special attention are the severe and frequent infections that followed the administration of a single pulse of cyclophosphamide, with documented hypogammaglobulinaemia. Infections were so serious that they led to multiorgan failure, and continued even after regular substitution of immunoglobulins, by which IgG levels were maintained above 5 and 8 g/L respectively, and even three years after the first and last administration of cyclophosphamide. Therefore, the question arises as to whether this patient has cSLE and drug-induced hypogammaglobulinaemia or common variable immunodeficiency (CVID).

A number of immunosuppressive drugs used in the treatment of SLE have been implicated in causing hypogammaglobulinaemia, including high dose cyclophosphamide, azathioprine, mycophenolate mofetil, rituximab and long-term therapy with glucocorticoids (354, 355, 356, 357). However, the effect of low dose cyclophosphamide therapy on immunoglobulin levels is not well known (358). Drug-induced hypogammaglobulinaemia is potentially reversible with cessation of therapy, unlike CVID, although the duration of hypogammaglobulinaemia after drug discontinuation can be very prolonged (358).

Since decreasing IgG in our patient continued even three years after the cyclophosphamide cessation, accompanied by concentrations of IgA and IgM more than two standard deviations below the age mean in serum and recurrent infections, SLE-associated CVID, although uncommon, cannot be excluded (359). CVID has been described in patients after the diagnosis of SLE, but the majority of the patients reported developed hypogammaglobulinaemia after therapy with immunosuppressive drugs (359, 360). This makes it difficult to make a definitive diagnosis of CVID or drug-related hypogammaglobulinaemia, since a diagnosis of CVID depends on exclusion of all other known causes of hypogammaglobulinaemia, but the role of immunosupresive agents in the etiopathogenesis of CVID also cannot be excluded (360, 361). The findings in the literature suggest that CVID in SLE patients may be caused by an intrinsic B-cell defect or by an extrinsic factor with an effect on B-cell maturation, although there is no

common immune defect among all the patients, so a shared unique mechanism is not likely (359).

Considering that the other clinical features of SLE in our patient were classical, it was not to be expected that she has a monogenic form of the disease, but it could be expected that, due to a pronounced susceptibility to severe infections, WES results could indicate numerous genetic variants associated with immunodeficiencies. However, as with the previous patient, WES identified a few genetic variants that could have an impact on the phenotype, but could not establish a genetic diagnosis on their own.

The only variant to be highlighted is *RAG2*^{T465A} in recombination-activating gene 2, *RAG2*. It is a gene located on chromosome 11p12 which encodes protein RAG2 (362). Together with RAG1, RAG2 catalyses V(D)J recombination in developing lymphocytes during the early stages of T and B cell maturation. V(D)J recombination is the mechanism of somatic recombination, which results in rearranging variable (V), joining (J), and in some cases, diversity (D) gene segments for the variable regions of the antibody proteins and T-cell receptors. Thus, it is responsible for generating the highly diverse repertoire of immunoglobulins and T cell receptors.

Pathogenic variants in *RAG2* are associated with a broad spectrum of phenotypes ranging from severe combined immunodeficiency, leading to severe infections and early mortality, to delayed combined cellular and humoral immune defects associated with granulomas (363, 364). Mutations, both in *RAG1* and *RAG2*, can be severe, leading to null alleles, or mild, leading to hypomorphic alleles that can still preserve residual enzymatic activity. Null alleles predominate in T^-B^- severe combined immunodeficiency, since there is no productive rearrangement of the T- or B-cell receptor. Missense mutations predominate in Omenn syndrome and leaky severe combined immunodeficiency (365). *RAG2* mutations may be associated with autoimmune phenotype as well, which can vary in the spectrum from the presence of autoantibodies and immune-mediated cytopenias to destructive vasculitis (259). Autoimmune diseases resulting from pathogenic *RAG2* variants may be associated with disruption of T and B cell tolerance checkpoints, probably secondary to impaired receptor editing (366, 367).

While homozygosity and compound heterozygosity for mutations in the *RAG2* have been described in patients with severe combined immunodeficiency and Omenn syndrome, a heterozygous missense mutation has been found in patients with SLE and erosive arthritis (259). The patient described in the literature was diagnosed with SLE at the age of 23 and presented

with polyarthritis, Raynaud phenomenon and sicca symptoms and, as the disease progressed, developed pleuritis and pericarditis, urticaria-like rashes, and class V lupus nephritis. That patient also had a history of recurrent infections and significant T and B cell lymphopenia. Impaired receptor editing in that patient has been demonstrated by measuring rearrangements that involve the cryptic heptamer in the J-C intron (iRS-RS rearrangement) which was linked to heterozygous *RAG2* mutation (259).

In our patient, WES discovered novel missense heterozygous mutation in the *RAG2* gene which was not observed in large population cohorts. The $RAG2^{T465A}$ variant is a semi-conserved amino acid substitution. Using the GERP score, as a measure of sequence conservation across multiple species, the substitution is estimated to have moderate impact, therefore it may influence secondary protein structure. However, given the conflicting results of the *in silico* prediction tools, where SIFT and Polyphen2 indicate that the variant has no deleterious effects, and CADD, that the variant may have deleterious effects, but that these predictions have not been confirmed by published functional studies, and that the threonine residue is not highly conserved, as well as the fact that the same variant exists in unaffected mother, the actual association of this variant with the clinical picture of cSLE in the patient and with possible immunodeficiency remains unknown, but not convincing. Nevertheless, since this is a new, hitherto undescribed variant and, according to the literature, similar variants can be associated with autoimmunity and immunodeficiency, which are overlapping phenotypes in this patient, it would be worth measuring the iRS-RS rearrangement in order to determine the effect of this variant *in vivo*.

Taking into account all the above, while WES identified a possible genetic contribution to the phenotype in this patient, it did not establish a genetic diagnosis. This patient had one HLA risk allel (57, 58).

6.6. Patient 6

One particular feature of the clinical picture of this patient is the dominance of symptoms caused by distal renal tubular acidosis, with no glomerular involvement that is characteristic of lupus nephritis.

Renal tubular acidosis results from an ion transport disorder in the renal tubules which, in cases of distal renal tubular acidosis, is a defect in hydrogen ion excretion from the kidneys

(368). It can develop due to diabetes mellitus, rheumatoid arthritis, Sjögren syndrome, lysosomal storage diseases or drug use (368). Renal tubular acidosis has been reported in patients with SLE, but on rare occasions and mainly in the form of case reports or in small cohorts. The underlying mechanism of renal tubular acidosis development in SLE is not known, but it was suggested that hypergammaglobulinemia, immunological factors such as antibodies against the carbonic anhydrase enzyme, or tubulointerstitial inflammation may play a role (369). It has also been proposed that a high disease activity score may be a risk factor for renal tubular acidosis in SLE patients, but this was not confirmed in the largest cohort of patients with SLE and renal tubular acidosis (370). Patient 6 also didn't have a high disease activity score (the highest SLEDA-2K in the course of the disease was only 4).

In the literature, there are well documented cases of hereditary distal renal tubular acidosis associated with various mutations in genes encoding for anion exchanger 1 (AE1), a membrane protein responsible for the rapid exchange of chloride for bicarbonate, such as *SLC4A1*, or for subunits of V-ATPase, which transfers protons and plays a role in producing an electrochemical gradient across the membrane and regulation of pH inside and outside the cell, such as *ATP6V1B1*, *ATP6V0A4* (371). In these cases the disease is serious, with early onset in infancy or childhood (371). Renal tubular acidosis does not appear to be primary in patient 6, but secondary to SLE.

Other manifestations of SLE in patient 6 were typical, with a significant family history of sudden renal failure and death. However, based only on these features, it was not realistic to expect that it is a monogenic form of the disease. The WES results in this patient revealed four gene variants for which a possible influence on the phenotype and predisposition to disease onset (SLE) can be discussed. Mutations in genes associated with distal renal tubular acidosis have not been detected.

TYK2^{D810V} seemed to be the most promising genetic variant associated with the phenotype in this patient. The *TYK2* gene, located on chromosome 19p13.2, encodes non-receptor tyrosine-protein kinase 2 (372). TYK2 is a member of the Janus kinase (JAK) family and is involved in IFN-I, IL-6, IL-10, IL-12 and IL-23 signaling, most prominently in the signal transduction of IFN-alpha and beta (373). IFN-I has many functions in multiple adaptive and innate immune responses, but it can also activate autoreactive T/B cells and stimulate autoantibody production (29). IFN transduces signals through the JAK-STAT pathway. TYK2 is a protein that binds to the IFN-I receptor (IFNAR1) on the cell surface in its inactive form.

IFN-alfa binding to IFNAR1, TYK2 and JAK1 proteins leads to phosphorylation and recruitment of signal transducers and activators of transcription, STAT 1 and 2, which then translocate to the nucleus and regulate the expression of a number of IFN-stimulated genes (260).

In 2006, the first patient with Tyk2 insufficiency, as a result of a homozygous deletion in the *TYK2* gene, was described (373). This patient showed susceptibility to infections, including mycobacteria and suffered from atopic dermatitis with elevated serum IgE. Other patients with Tyk2 insufficiency experienced immunodeficiency characterised by recurrent intracellular bacteria, viral infections and, most typically, susceptibility to mycobacterial disease (374).

However, from the aspect of patient 6, the most interesting points are the observations regarding the association of *TYK2* polymorphisms with autoimmune diseases, especially with SLE. Earlier, it has been shown that locus 19p13.2, where *TYK2* is located, has been linked to SLE in white pedigrees stratified by the presence of anti-dsDNA antibodies (375). After that, Sigurdsson et al. identified polymorphisms in the *TYK2* gene that displayed strong signals of joint linkage and association with SLE (376). Two *TYK2* polymorphisms were analysed in 679 Swedish, Finnish, and Icelandic patients with SLE, 798 unaffected family members, and 438 unrelated control individuals. The SNP *TYK2* rs2304256 causes a substitution of valine to phenilalanine in the JH4 region of Tyk2, which is important for the interaction of Tyk2 with the IFN-alpha receptor 1 (IFNAR1). Interestingly, *in silico* prediction tools showed conflicting results regarding the disruptive effect of this amino acid substitution. The other SNP is *TYK2* rs12720356 which results in a substitution of isoleucine to serine in region JH2 of TYK2, crucial for binding IFN-I to IFNAR1, and proved to be damaging using the prediction tools. The authors speculate that the amino acid substitutions caused by the *TYK2* polymorphisms may reduce the function of TYK2, thus having an effect on the susceptibility to SLE. These results supported a disease mechanism that involves components of the IFN-I system. Furthermore, the results indicated that the reduced function of TYK2 plays a protective role for SLE, therefore the SNPs *TYK2 rs2304256* and *rs12720356* decrease the susceptibility to SLE.

On the other hand, recent studies suggest that *TYK2* variants are linked with different autoimmune diseases, including type 1 diabetes, psoriasis and multiple sclerosis, as well as with an increased risk for SLE (377, 378). Among the investigated polymorphisms, some were protective for some autoimmune diseases but posed a risk factor for others, which supports a different underlying pathogenic mechanism. The main significance of these associations is in

view of the development of small molecule selective TYK2 inhibitors to be used in clinical practice (379). A meta-analysis from 2018 suggested the association of the *TYK2 rs2304256 C* allele as a risk factor for SLE in Europeans but not in Asians (380). Recent meta-analysis comprising 9 different *TYK2* SNPs showed the A allele of the *rs280519* SNP was associated as a risk factor for SLE (included 14 studies, 13,969 cases and 29,167 controls), while the minor alleles of the remaining 8 SNPs proved protective against autoimmune diseases (260). The A allele of the *rs280519* SNP does not cause an amino acid substitution, but it is located in a splice site of the *TYK2* gene (381). Currently, no studies have evaluated regarding the impact of this SNP on *TYK2* function. It seems that at least some *TYK2* SNPs cause amino acid substitution decrease TYK2 activity and, consequently, the inflammatory response and apoptosis, which can explain the protective role they play against autoimmune diseases (260).

Therefore, different SNPs in the *TYK2* gene may be linked with autoimmune diseases, including SLE, but the results on individual SNPs are still inconclusive, especially due to the increased number of studies undertaken in the last few years on different ethnicities.

The $TYK2^{D810V}$ variant present in patient 6 is the result of amino acid substitution at codon 810 of the TYK2 protein, where the aspartic acid residue is located, and this region in exon, according to the GERP score, is moderatly conserved, suggesting that this missense variant may have a moderate impact on protein function. Another software, that implements rigorous statistical tests to detect bases under selection from multiple alignment data, indicating conservation and comparing the genomes from closely related species to identify functional elements in a reference genome and identify evolutionarily constrained sequences, SiPhy, also predicts moderate impact of the substitution on the protein function. Furthermore, the computational predictive tools SIFT, Polyphen2, CADD all suggest that this variant is likely to be disruptive, therefore it may impact on the protein function. There is a large physicochemical difference between aspartic acid and valine. Aspartate is a small, negatively charged residue, while valine is an aliphatic, branched hydrophobe. The variant is present in population databases but is rare, with a frequency of 0.01% in European alleles in the gnomAD. The variant has not yet been described in the literature in individuals with TYK2-related diseases, although it is present in ClinVar, and there are no published functional studies regarding the $TYK2^{D810V}$ variant. However, given the fact that the most described missense *TYK2* variants, resulting in amino acid substition, as in this case, so far decrease TYK2 activity and are protective against autoimmune diseases (260) and that the same variant is found in the unaffected father, the available evidence is currently insufficient to determine the role of this variant in the disease of

patient 6. Additional studies with larger sample sizes are necessary to clarify the impact of this variant on susceptibility for different autoimmune diseases and functional studies are also needed to elucidate what the impact is on TYK2 function.

Another variant detected in patient 6 is *SAMHD1*^{V112I}. *SAMHD1* gene encodes the Sterile Alpha Motif (SAM) domain and the Histidine-Aspartic (HD) domain-containing protein 1 (86). The primary function of the SAMHD1 protein is to block replication of retroviruses, especially HIV, in dendritic cells, macrophages, monocytes and resting CD4+ T lymphocytes. It was also observed on animal models that *SAMHD1* expression can be induced by IFN-I and it is suggested that SAMHD1 may also act as a negative regulator of the IFN response (382). In SAMHD1-depleted cells, single-stranded DNA fragments accumulate in the cytosol and induce expression of proinflammatory IFN-I, so SAMHD1 is included in the replication stress response, where prevents chronic inflammation (383). SAMHD1 mutations are responsible for increasing DNA damage and upregulation in IFN-stimulated genes.

Disease-causing, pathogenic variants in *SAMHD1* are described in patients with SLE, Aicardi-Goutières syndrome and chilblain lupus (263, 384). Patients with *SAMHD1* mutations presented with chilblain lupus, with or without central nervous system disease, arthritis, mental retardation and microcephaly (263). The patients with Aicardi-Goutières syndrome had homozygous mutations in *SAMHD1* resulting with biallelic null alleles, but some of them also had compound heterozygous mutations. Mutations result in a less functional SAMHD1 protein, and in loss-of-function of the SAMHD1 protein, which has been shown to be reduced or absent in patient cells (385). Several of the families were consanguineous. All of the mutations involved highly conserved residues, segregated with the disease, and all unaffected parents tested were heterozygous for the mutations (382). How exactly this protein dysfunction leads to immune system abnormalities, inflammatory damage to the brain and skin, and other characteristics of this syndrome is still not elucidated (386). It has been proven that circulating IFN-alpha is increased in these patients, but the molecular mechanism by which SAMHD1 deficiency causes IFN overproduction is not known. In patients with chilblain lupus, heterozygous mutation in the *SAMHD1* gene were identified, suggesting autosomal dominant inheritance (263). Very recently, a case-report of a child with heterozygous mutation in *SAMHD1*, chilblain lupus and normal interferon signature was published (387). This is an unusual finding, since it is thought that IFN overproduction is responsible for a lupus-like phenotype in patients with *SAMHD1* mutations.

Patient 6 has a variant *SAMHD1*^{V112I}, inherited from an unaffected father. The variant is the result of amino acid substitution at codon 112 of the SAMHD1 protein. According to the GERP score and SiPhy this region in exon is not evolutionary conserved suggesting that this variant may not impact on protein function. Additionally, both valine and isoleucine are aliphatic amino acids in the same group, so they have quite similar physicochemical properties. In line with this prediction are also algorithms developed to predict the effect of missense changes on protein structure and function, SIFD, Polyphen2 and CADD, according to which this variant is not likely to be disruptive. This variant was observed as part of a predisposition screen in an ostensibly healthy population with a frequency of 0.1% of European alleles in the Genome Aggregation Database. Given all of the above, it is unlikely that the variant *SAMHD1*^{V112I} is associated with the phenotype in patient 6.

Another variant found is *DNASE1*^{V185I}. *DNASE1* is a gene encoding Deoxyribonuclease 1 (Dnase1), an enzyme that cleaves DNA, facilitating chromatin breakdown and is responsible for DNA fragmentation during apoptosis (388). DNASE1 is a major nuclease present in the serum, urine, and secreta, and may have control over the removal of DNA from nuclear antigens at sites of high cell turnover, supporting its role in preventing SLE. In agreement with this, it was demonstrated that Dnase1-deficient mice present with the classic symptoms of SLE inclunding the presence of ANA, the deposition of immune complexes in glomeruli, and glomerulonephritis (389). It was found that Dnase1 activity in the serum of SLE patients is lower than in normal subjects, implicating that deficiency or reduction of Dnase1 is an important factor in the initiation of human SLE (389). A heterozygous pathogenic variant, inherited in an autosomal dominant manner in *DNASE1* has been identified in individuals diagnosed with a monogenic form of SLE (264). Affected patients presented with SLE phenotype with higher titers of anti-nucleosome antibodies (389, 390). In familial SLE with *DNASE1* mutation affected patients presented with positive ANA, high frequency of ANCA, and lupus nephritis (391). Common polymorphisms in *DNASE1* affecting the SLE phenotype were first described in Korean patients and were significantly associated with an increased risk of the production of anti-RNP and anti-dsDNA antibodies among SLE patients (392). Later, it has also been reported that polymorphisms in *DNASE1* are associated with polygenic, non-Mendelian forms of SLE (393). It was found that *rs1053874 DNASE1* SNP, characterised by a substitution of glycine to arginine, is linked with an increased susceptibility to SLE but was not related to Dnase1 activity nor with autoantibody production, calling into question the direct involvement of this specific SNP.

DNASE1^{V185I} in patient 6, similar to the *SAMHD1*^{V112I} variant, is related to amino acid substitution with two amino acids which share similarities in their physicochemical properties, with a prediction not to cause dysfuntion in Dnase1, and evolutionarily this region in exon is non conserved. The variant is described in population databases with a frequency of 0.3% and is inherited from an unaffected mother. As it was argued in the example of *SAMHD1*^{V121I}, it is not likely that this variant contributed to disease phenotype nor susceptibility to cSLE in patient 6.

Patient 6 also has a novel variant in the *BCL10* gene. It encodes the B-cell lymphoma/leukemia 10 protein involved in NFκB activation after stimulation of various receptors on lymphoid, myeloid, and epithelial cells, thus playing a role in the immune system (265). NFκB is a protein complex that controls transcription of DNA, cytokine production and cell survival. Diseases associated with *BCL10* include lymphoma, mucosa-associated lymphoid tissue (MALT) lymphoma and immunodeficiency (344). The patient with this immunodeficiency was a a boy born of consanguineous parents with homozygous mutations in the *BCL10* gene, resulting in complete absence of the protein in his cells, who suffered from numerous severe respiratory and gastrointestinal infections, and later developed secondary leucoencephalopathy and encephalitis and died at the age of 3 years.

The *BCL10*^{D127V} in patient 6 was inherited from a healthy mother. The variant results in amino acid substitution at codon 127, in the evolutionary non conserved region of the exon. The GERP score and SiPhy both predict substitution which does not affect protein function, while SIFT, Polyphen2 and CADD anticipate it is not likely to be disruptive. However, there is a large physicochemical difference between aspartic acid and valine. This variant has not been reported in the literature and is not observed in large population cohorts. It is not known what the significance of this variant is on the patient's 6 phenotype, but according to the data found so far from the literature related to the *BCL10* gene, none of the clinical characteristics in patient 6 match those associated with mutations in *BCL10*, so a causal relationship is unlikely.

Despite as many as four gene variants being analysed, none of them is convincing and cannot explain the unusual renal presentation in this patient on their own, or the possible association with kidney disease in a family member; hence no monogenic genetic diagnosis was established. A synergistic effect of a few rare alleles can't be excluded, but is difficult to study at present. Also, there were no HLA alleles associated with susceptibility to SLE (57, 58).

6.7. Patient 7

From the description of the clinical picture of patient 7, it is clear that she has a classic form of cSLE without any peculiarities in the clinical picture, in terms of an unusual course of the disease or resistance to conventional treatment, which would indicate a monogenic form of the disease. Therefore, it was to be expected that this is a polygenic form of SLE in which there is no causal gene whose mutation would be associated with the onset of the disease. Due to the presence of autoimmune diseases in the family and the fact that SLE and other autoimmune diseases share many common susceptible genes, one would expect new or previously described genetic variants which could contribute to the disease pathogenesis in this patient. In line with expectations, two rare gene variants were detected in this patient for which there is evidence in the literature in terms of association with immune dysregulation diseases. The following is a description of the functions of these gene variants and an explanation of their possible association with the onset of the disease and elements of the clinical picture of the patient.

The first variant, CFD^{A188T}, is located at chromosome 19p13.3. The *CFD* gene encodes complement factor D (CFD), a serine protease of about 24 kDa that circulates in the blood as a constitutively active enzyme (394). Adipocytes and macrophages predominantly synthesise this enzyme which has an essential role in the initiation and propagation of the alternative pathway of complement activation, triggering a natural defense against infections, and in the amplification loop of C3 activation, which is the central component of the complement system (395). It was shown that CFD has a very close similarity to amino acid and is homologous to mouse adipsin. This finding suggested that adipose tissue may play a role in immune system biology (396).

Missense and nonsense mutations in the *CFD* gene have been described in the literature resulting in reduced protein function (loss-of-fuction mutations) and were inherited under autosomal recessive mode (394, 397). The best-known examples are two families in which a homozygous mutation in the *CFD* gene resulted in CFD deficiency. The first documented mutation in the *CFD* gene, resulting in a complete CFD deficiency, was in a Dutch family with a high degree of consanguinity. The proband was a 23-year-old woman who suffered from a serious *Neisseria meningitidis* infection presented as septic shock and Waterhouse-Friderichsen syndrome from which she completely recovered (394). Interestingly, another family member had bacterial meningitis, but the other members with complete or partial CFD deficiency had no history of recurrent infections. It was concluded that CFD deficiency is a significant risk

factor for serious invasive bacterial diseases including meningitis although severe and life-threatening infections will not occur in every person with complete factor D deficiency. Another capitvating finding from the same case report is that some heterozygotes had low-normal CFD levels.

The second example is regarding a Turkish family of consanguineous parents in which two children had two missense mutations in the *CFD* gene, which resulted in undetectable CFD levels (397). Both children experienced severe *Neisseria meningitidis* infections and one child died. The parents and unaffected siblings were heterozygous.

It is known that complement activation has an important role in the pathogenesis of glomerulonephritis in SLE (269, 398). Namely, the deposition of immune complexes in the glomeruli of patients with SLE leads to complement activation. The alternative complement pathway is a factor of tissue damage via the C3 amplification loop. It increases the concentration of inflammatory mediators in tissue. CFD is indispensable for the formation of the althernative pathway C3 convertase, which is important for the amplification of complement activation, not only in alternative but also in the classical and lectin pathways. However, in animal models it was demonstrated that only a deficiency of complement components involved in the early activation phase of the classic pathway leads to the development of a lupus-like syndrome, which is complementary for C1q, C1r, C1s, C2, C3 C4A, C4B deficiency in humans and is linked with SLE susceptibility (83, 269). Thus, the deficiency of components involved in early althernative pathway activation, such as CFD, is not associated with the development of autoimmune disease but only to the susceptibility to *Neisseria meningitidis* infections (269). It is even shown that in the *MRL/lpr* (Murphy Roths Large Lymphoproliferative) mouse (murine model of SLE), CFD deficiency decreased the incidence of renal disease, probably due to the lack of alternative pathway activation.

Given that patient 7 has a heterozygous mutation in the *CFD* gene that should not significantly reduce CFD levels (394), an increased susceptibility to infections caused by *Neisseria menigitidis* would not be expected. Also, taking into account that homozygous mutations should not affect the predisposition to SLE (269), and could, on the contrary, act protectively for the development of lupus nephritis, it can be concluded that this gene variant is not associated with the disease or pathogenesis of SLE in this patient. The variant was considered, taking into account that it is a gene associated with immune functions, that the

variant is present only in family members with autoimmune diseases and that it is a rare variant in existing databases.

Another variant found in the patient was a heterozygous mutation in the *PTPN13* gene. It encodes for protein tyrosine phosphatase non-receptor 13, a protein which belongs to the Class I superfamily of tyrosine-specific phosphatases (399). Little is known about the physiological role of PTPN13. In mice models, it was shown that PTPN13 ablation was not associated with major changes (399, 400). These mice exhibited abnormal T-helper cell differentiation. Namely, in CD4+ PTPN13, deficient murine cells there was increased STAT4 and STAT6 activation. STAT (Signal Transducer and Activator of Transcription) proteins are latent cytoplasmic transcription factors that are activated by tyrosine phosphorylation. As a result of STAT4 and STAT6 activation, there is enhanced T helper 1 (Th1) and Th2 cell differentiation. This may be associated with improved immune defenses against *Klebsiella pneumoniae*, but also may affect motor neuron repair in mice. Thus, it has been proposed that PTPN13 plays a regulatory role in Th1 and Th2 polarisation. It is well known that Th cells contribute in immune system-related diseases: Th1 is involved in the development of autoimmune diseases and Th2 responses mediate allergy (401).

The *MRLlpr* mice are models for spontaneous lupus since this mouse strain develops autoimmunity with many of the features of lupus observed in humans. It was found that in *MRLlpr* spleens, *PTPN13* was upregulated (269). However, further studies are necessary to determine the role of this protein in human autoimmunity (402).

Furthermore, PTPN13 may function to regulate apoptosis of developing or activated T cells due to its interaction with FAS (403). PTPN13 reduces the FAS cell surface level and this prompted investigations into a possible involvement of PTPN13 in tumorigenesis. Several *PTPN13* variants were found to increase the risk of developing familial hepatocellular carcinoma, head and neck squamous cell carcinoma, lung and colorectal cancer (404, 405, 406). All the analysed mutations have an inhibitory effect on PTPN13 activity, thus reducing phosphatase activity (399). Some of them were missense and others were nonsense mutations, inherited under autosomal dominant mode.

The heterozygous $PTPN13^{A732T}$ variant present in the cSLE patient 7 and the mother, affected by another autoimmune disease, but not in the healthy father, was not observed in large population cohorts, and is inherited under autosomal dominant mode. This variant is a semi-conserved amino acid substitution, and may impact secondary protein structure as these

residues differ in some properties, reflected by the prediction tools (SIFD, Polyphen2 and CADD) as being possibly pathogenic. Namely, there is a physicochemical difference between alanine and threonine. Alanine is hydrophobic aliphatic amino acid with tendencies to form helices, while threonine is polar since it carries a hydroxyl group and tends to form beta-sheet structures (407). According to the literature, this substitution may result in reduced phosphatase activity. Given that this variant has not been reported in individuals with PTPN13-related diseases so far, the alanine residue is not highly conserved, and bioinformatic predictions of SIFD, Polyphen2 and CADD have not been confirmed by published functional studies, it is not possible to conclude how this variant may contribute to the development of autoimmune diseases in this case.

It can be pointed out that WES did not obtain a result on the basis of which risk alleles associated with the clinical picture or that its predisposition at the onset of the disease could be clearly identified, other than two HLA alleles for which such association was previously demonstrated in larger studies (57, 58).

6.8. Patient 18

Patient 18 presented with a severe clinical picture of an otherwise commonly self-limiting disease, IgAV, involving a number of organ systems and the need for treatment in the intensive care unit, however, after extensive diagnostic assesment, no elements were found to support another form of vasculitis, such as ANCA associated vasculitis or polyarteritis nodosa, it is therefore indisputably a severe form of IgAV.

Experience with WES analyses that covers family "trios" containing proband cases with IgAV is scarce according to the available literature. In a recently published study in which WES was performed on family members of patients with IgAV and other unrelated patients, it was found that the two genes, *MIF* and *MGAT5*, important for immune functions, could represent new susceptibility loci for IgAV (278). This paper also highlights the importance of using WES in detecting gene mutations important for understanding the pathogenesis of this disease. Not long ago it was discovered that single mutations in genes involved in the inflammatory pathway may cause monogenic vasculitides with a phenotype resembling a well-known vasculitis, including IgAV (408). On the other hand, IgAV can present showing atypical manifestations, more common in children younger than 2 years of age (409). In patients with atypical presentations of vasculitis, genetic defects should be considered.

In accordance with the above, WES was performed on this patient and on the two healthy parents and it detected several genetic variants, which are difficult to explain in terms of potential influence on the phenotype and onset of the disease.

The first variant that will be disscused is *BAD*^{W154C}, whose protein product is B-cell lymphoma 2 with associated antagonist of cell death (Bcl2). It is involved in initiating apoptosis, but can also interact with anti-apoptotic proteins and prevent them from stopping apoptosis. Bcl2 is additionally involved in metabolic functions, particulary in glucose homeostasis (410). Research indicated the link between autoimmunity and several genes involved in apoptosis. The *BAD* (also called *BCL2*) gene increases lymphocyte survival by inhibiting or delaying apoptosis (411). B lymphocytes of transgenic mice with overexpression of Bcl-2 showed polyclonal expansion and extended survival in vitro and these mice developed lupus-like syndrome (412). Mehrian et al. reported *BCL2*, *FUS-L*, and *IL-101* genes are associated with SLE in Mexican patients and that *BCL2* and *IL-101* have a synergistic effect (413). They concluded that overexpression of Bcl2 is associated with the inappropriate elimination of autoreactive lymphocytes. Some studies in SLE patients suggested that Bcl2 expression is elevated in both B and T lymphocytes (273). Cytokines involved in the pathogenesis of autoimmune diseases, such as IL-17 or BAFF, may modulate the expression levels of pro-survival Bcl-2 in SLE patients, consequently supporting the possibility that impairment of the Bcl2-regulated apoptosis can contribute to the rise of autoimmunity in men (414). Altered regulation of apoptosis and impaired clearance of inflammatory cells could lead to the persistence of inflammation and excessive tissue injury. In patients with IgAV, it was shown that in the acute phase of disease there is an increased percentage of apoptotic peripheral blood neutrophils and lymphocytes suggesting that increased apoptosis in the immune cells may play an important role in the early control of inflammatory response contributing to the self-limited nature of the disease (415). It was proposed that the interrupted apoptosis of neutrophils leads to vascular damage in leukocytoclastic vasculitis (416). IgA_1 from IgAV patients may induce apoptosis of endothelial cells through inhibiting the cytoskeletal proteins expression (417). Although there is currently no data in the literature on the association of Bcl2 and vasculitis, given that apoptosis disorders may play a role in the development of autoimmunity, including vasculitis, and Bcl2 has an important function in regulating apoptosis and is associated with autoimmunity, the link between Bcl2 cannot be excluded.

The *BAD*^{W154C} variant in patient 18 is a missense mutation, a substitution of aromatic amino acid tryphtophan to sulfur-containing cysteine, occuring in the moderatly conserved region of

Bcl2 protein. This is further supported by Polyphen2 and CADD predictions, so an adverse impact of this substitution on the protein structure may be expected. In addition, tryptophan and cysteine are amino acids with substantially different chemical properties. The variant is rare, its functional consequence is not known and the data about this variant and BCL2-associated diseases do not exist, it is present in both the patient and unaffected mother, therefore, at this point in time, it is not possible to conclusively interpret its significance i.e. pathogenicity.

In all three members of family 18, the variant in the *MSH2* gene, associated with cancer, is present, but in homozygous form only in the patient, while both parents are heterozygous. The *MSH2* gene is a tumor suppressor gene encoding for the DNA mismatch repair protein Msh2. The protein is involved in a system for recognising and repairing erroneous insertion, deletion, and mis-incorporation of bases that can occur during DNA replication and recombination, and in repairing some forms of DNA damage (418). It is known that heterozygous mutations in the *MSH2* gene may result in hereditary colorectal cancer, but may also be linked to other types of cancer (277). On the other hand, IgAV could be related to cancer in adults (419). Most of the cancers in patients with IgAV are mucosal (lung, colorectal and urinary bladder etc.) with a short median time between the diagnosis of cancer and IgAV (419). Although the pathogenetic association between cancer and IgAV is not elucidated, it is hypothesised that the occurrence of a cancer in a predisposed host with an abnormal IgA glycosylation process could participate in the pathogenesis of IgAV.

The variant $MSH2^{E561K}$ was found in patient 18 in homozygous form. The mutaton is located in coding exon 11, resulting in a change to the amino acid, from a glutamic acid to lysine at codon 561. *In silico* analyses indicate that this is an evolutionarily conserved residue, however computational prediction is inconclusive regarding the impact of this variant on protein structure and function. A variant has been reported in the literature in an individual from a family with Lynch syndrome, but has been shown not to affect the MSH2 protein function significantly (420). It can be found in the ClinVar database. On the other hand, there is no history of cancer in this family. Considering all the above, and in particular how it is not possible to clearly link the patient's phenotype and the functions of the *MSH2* gene, the clinical significance of this alteration remains unclear.

The third variant to be disscused is $DXH58^{Q187R}$. The *DXH58* (DExH-box helicase 58) gene encodes the protein product Laboratory of Genetics and Physiology 2 (LGP2). It was found that LGP2 is important for producing effective antiviral responses against many viruses, especially

the hepatitis C virus (421). In innate immunity, LGP2 plays an important role in the production of IFN-I through recognition of cytosolic viral RNA. Overexpression of LGP2 has been linked to decreased IFN production in cells after viral infection, while in the fibroblast of LGP2-deficient cells, increased IFN expression was observed (422). It has been speculated that the inhibiton of LGP2 due to the TNF receptor-associated factor (TRAF) family, proteins may have a beneficial effect on different conditions associated with interferonopathic systemic autoimmunity, arthritis, inflammatory bowel diseases, asthma, atherosclerosis, cancer, and other conditions (423). It is well known that dysregulation of IFN-I responses can result in the development of autoimmune diseases such as SLE, Aicardi-Goutières syndrome, dermatomyositis and primary Sjogren's syndrome (424). It was also hypothesised that IFN-I are dysregulated in vasculitis (424). However, this was not confirmed for ANCA associated vasculitis, where IFN-II, in particular IFN-gamma, is upregulated. Regarding IgAV and IFN-I, some studies have indicated that it may be important since infections are often the triggers for IgAV and pathogens activate Toll-like receptor-mediated signaling pathways, which induce gene expression of inflammatory cytokines and IFN-I (425, 426, 427). In this way, it might be possible to link the functions of the *DXH58* gene and the pathogenetic processes in IgAV although, for now, it has not been not observed that *DX58* mutations cause systematic inflammation or autoimmune disease (276).

Patient 18 carries the missense variant $DXH58^{Q187R}$. The amino acid change is predicted to have a moderate impact on LGP2 protein function with conflicting pathogenicity predictions indicated by the *in silico* tools. The variant is rarely described in public databases and is present in all three family members in heterozygous form. The possible influence on the phenotype is questionable, both from the aspect of the mechanism by which this gene variant may be important, and also from the aspect of the influence of amino acid substitution on the function of the protein it encodes.

In summary, in the patient with severe IgAV using WES, no variants were found that could reliably explain the severity of the clinical picture or the possible predisposition to the disease, except one HLA allele which, in a previous GWAS, was reported to be associated with susceptibility to IgAV (146).

6.9. Overview and future directions

Taken together, the results from the WES analysis conducted in the cohort of 19 "trios" yielded a new pathogenic variant in the *KMT2D* gene, explaining previously unrecognised syndrome features in a patient with Kabuki syndrome and cSLE. Kabuki syndrome was diagnosed after discovery of the *de novo KMT2D* variant, which is an interesting example of WES helping to refine diagnosis. In addition, likely pathogenic variants worth further functional analyses were identified in *ADAR1,* in a patient with dysmorphic features, skin pigmentation changes and cSLE, only partially explaining the very complex phenotype, as well as one likely pathogenic variant in *BLK*, in a patient with severe cSLE and multisystemic involvement with the same disease found in her mother, which also warrants further pathogenicity characterisation.

A number of likely benign variants and VUS were detected, among which, the most promising, in terms of contribution to the development of the disease and the impact on the clinical picture, are the variants in the *RAG2* and *TYK2* gene. By far the largest number of variants are likely to be benign or VUS. While such a result may seem disappointing, the high proportion of VUS is not an unusual WES finding. According to the results of a study performed not so long ago, although related to cancer risk, but which can be applied in general to the results of next-generation sequencing, the share of variants of unknown significance is high and in this study proved to be 40% (428). With the introduction of multigene panels, WES and WGS, the numbers of variants discovered per person has increased progressively and the problem of VUS interpretation has magnified (429). In fact, pathogenic variants often represent only a small percentage of all the variants established (430).

VUS are difficult to classify due to a lack of statistical evidence from large population databases, insufficient functional evidence and different evaluations by clinicians and researchers (430). This is particulary problematic when working in populations that are under-represented or not represented in the publicly available databases of human genetic variation, such as the Croatian population and the Slavic populations in general. Most VUS are missense or synonymous substitutions, substitutions of amino acid residues with similar biochemical properties or in-frame indels (430). If the mutations occur in non-coding regions, at less conserved residues, at splicing boundaries, or in less functionally relevant domains, their impact on the proteins and their functions are more difficult to uncover, compared to nonsense mutations. This underlines the need for more applications of complex and time consuming

functional analyses when dealing with VUS. Finally, clinicans and researchers may have different views and expectations from WES. From a research perspective, VUS and novel uncharacterised variants may be worth looking at because they can expose unexpected genetic and protein alterations involved in biochemical processes, although they are not always informative for clinical purposes (430). The functional test in interpretating genetic variants, especially VUS, are efficacious in VUS classification but are still burdened with some difficulties, such as poor feasibility in everyday practice, they are dependent on experimental models, are often performed using cell lines that do not necessary repeat and reflect patient biological processes, and often require large teams of research groups and are not applicable to all the various types of variants (430).

On the other hand, WES is not the perfect tool for discovering novel variants and the respective false negative rate is approximately 2% (431). Different algorithms for filtering WES data which are impossible to interpret indiscriminately due to the large amount of information, in addition to helping to identify new variants by narrowing the number of candidates, can be a problem because they lay too much importance on genes already known to have a function in the immune system, which is why they can identify obvious genes already established and yet, on the other hand, can miss genes whose function is unknown, therefore they are not detected.

Our cohort included patients whose history and clinical course of the disease strongly favoured monogenic forms of SLE (male gender, more severe progressions of the disease and presence of SLE in the family), but WES didn't only not detect genes that would cause the disease, but they didn't even find novel or rare gene variants in the group of genes linked to SLE, which is unexpected. However, in all of these cases, we haven't exhausted the possibilities of monogenic disease because we have only undertaken a panel-based approach (i.e. candidate genes): we have taken a first pass analysis, reducing approximately 20,000 genes in the genome to a discrete list of candidate genes. Therefore, the relatively restricted analysis of WES is the major limitation of this research.

Taken all together, it is possible that even a powerful test, such as WES, may not provide the answers for clinicans and researchers. Diagnostic rates of WES are in the range of 25-58% in a clinical setting (432), with an estimated mean diagnostic rate of 36%, while in a research setting, the rates have often been even lower, only 8-24% (433). Therefore, it still remains a fact that, statistically, it is more likely that WES would not be able to find the answer, which

can be frustrating for families, as well as for medical professionals, and also involves a financial burden.

Using WGS has the advantage of capturing more regions that might be missing when performing WES, but the high costs involved and the large amount of data that consumes a lot of time still limit the applicability of WGS (74, 207). Nevertheless, it can be an option for patients where there is a strong suspicion of monogenic forms of SLE or vasculitis (primarily patients with a very early onset of the disease, before the fifth year of life and with a positive family history), and where no candidate gene was found using WES.

WES is entering the phase where its capabilities have a broader use and there exist many opportunities for further research and to expand knowledge. For punctilious interpretation of WES data, it is important to continue to study individual genes and conditions to illuminate the genotype-phenotype correlations. Diseases such as cSLE and IgAV are complex and, although rare variants may have a strong influence, they cannot explain all the pathogenesis of complex autoimmunity since other environmental, epigenetic and infectious influences all play a role in the development of cSLE and IgAV.

In the new epoch of personalised medicine, implementation of next generation sequencing has improved the diagnostics and treatment of patients with autoimmune diseases. Nevertheless, such quantities of data have raised the issue regarding interpretation of genetic variants and their use for therapeutic purposes. Undeniably, pathogenic variants often represent only a small percentage of all the variants reported, while there is growing number of variants which we are still not able to clearly define and use in a clinical context, such as VUS, which limits the clinical utility of genetic information. This has to prompt the scientific community to develop methods to properly categorise VUS and escalate the amount of practicable information which can be derived from next generation sequencing.

7. CONCLUSIONS

1. Using WES *de novo* frameshift 1bp deletion in the *KMT2D* gene was detected, establishing the diagnosis of Kabuki syndrome in a patient with cSLE and syndromic features of a clinically unrecognised syndrome. The pathogenic variant in exon 34 (c.8626delC), has not been described previously and is predicted to truncate the protein (p.Gln2876Serfs*34) resulting in *KMT2D* loss of function.

2. The exact relationship between the cSLE and Kabuki syndrome is difficult to determine with certainty, as a number of clinical features may occur in both conditions, suggesting potential overlap. With this research, the SLE was added to the list of documented (auto)immune disorders in Kabuki syndrome, pointing to the value of WES as a diagnostic tool for rare, complex and sometimes clinically unrecognised genetic conditions.

3. The likely pathogenic variant in the exon 10 of *ADAR* gene (c.2815A>G) predicted to encode the protein (p.Ile939Val) was detected in patients with cSLE, dysmorphic features and skin pigmentation changes, only partially explaining the complex pheotype, suggesting dyschromatosis symmetrica hereditaria as additional diagnosis and raising the question of potential overlap with cSLE, not previously described in the literature.

4. One likely pathogenic and rare variant in exon 4 of the *BLK* gene (c.211G>A), predicted to encode protein (p.Ala71Thr), was discovered in a patient with severe cSLE and multisystemic involvement with the same disease in the first degree relative, which may have contributed to the complex pathogenesis of the disease, but further functional research is needed to confirm and determine their role in more detail.

5. Among the number of VUS in patients with cSLE in this cohort, the most promising, in terms of contribution to the development of the disease and the impact on the clinical picture, making them candidates for further function analyses, are *RAG2* (c.1393A>G) which is predicted to encode (p.The465Ala), and *TYK2* (c.2492A>T) which is predicted to encode (p.Asp810Val).

6. In patients with IgAV in this cohort, using WES, it was not possible to find genetic variants which could be associated with the pathogenesis of the disease. Two rare VUS, one in exon 3 of the *BAD* gene (c.462G>C) predicted to encode (p.Trp154Cys), and the second, in exon 5 of the *DHX58* gene (c.560A>G) predicted to encode (p.Gln187Arg), require further investigation, since these genes are involved in immune processes, such as apoptosis regulation and IFN-I production, with potential contribution to the rise of autoimmunity, including vasculitis.

7. In the patients with cSLE and IgAV, the most common and most consistent finding associated with genetic background of the disease remainded HLA alleles, for which the susceptibility to disease was previously demonstrated in large studies including GWAS.

8. Studies such as this one illustrate the degree of complexity which it should have the capability to handle in respect of the analysis of sequencing data and gene-discovery studies, as well as showing the need for continued research to extend the understanding of genes and their physiological repercussions on the autoimmunity so that new interrelations with human disease can be made.

9. Even though WES has proved to be an influential diagnostic instrument, there are still limitations to its capabilities, as shown by the results of this study and the difficulty in finding causative variants for most of the patients included.

8. SUMMARY

Contribution of the whole exome sequencing in the identification of genetic variants associated with childhood-onset systemic lupus and IgA vasculitis

Mario Šestan

PhD Thesis 2022

Introduction and aim. Childhood-onset systemic lupus erythematosus (cSLE) and IgA vasculitis (IgAV) are two complex autoimmune diseases with etiopathogenesis which are not fully understood. This research aimed to identify novel and rare gene variants using whole-exome sequencing (WES) in patients with cSLE and IgAV which may contribute to the etiopathogenesis of these diseases, and to expand existing genetic databases.

Subjects and methods. WES was performed on 17 "trio" groups containing a proband case with cSLE and parents (including other informative family members) and on 3 "trios" containing a proband case with IgAV, and parents with severe, atypical clinical features, syndromic characteristics, early onset of the disease, resistance to conventional therapy and/or a family pattern of occurrence. After completion of WES, data analysis and the identification of all the genetic variants of interest, the presence of an interferon gene expression signature in peripheral blood mononuclear cell samples was investigated on one patient.

Results. After performing WES and analysis of gene variants, novel and/or rare variants were detected in 8 patients. Among them, there were 7 patients diagnosed with cSLE and 1 patient with IgAV, while 1 patient was diagnosed with cSLE and IgAV in a different period. A total number of 20 variants were prioritised for more detailed analysis. WES analysis yielded a new pathogenic variant in the histone-lysine N-methyltransferase 2D gene (*KMT2D*), NM_003482.3:c.8626delC, predicted to truncate the protein (p.Gln2876Serfs*34) resulting in *KMT2D* loss of function, explaining a previously unrecognised syndrome which features in a patient with Kabuki syndrome and cSLE. In addition, likely pathogenic variants were identified in the adenosine deaminase acting on the RNA gene (*ADAR1*), NM_001111.3:c.2815A>G, predicted to encode the protein (p.Ile939Val) in a patient with dysmorphic features, skin pigmentation changes and cSLE, only partially explaining the very complex phenotype, as well as in the B lymphocyte kinase gene (*BLK*), NM_001715.2;c.211G>A, predicted to encode (p.Ala71Thr) in a patient with severe cSLE and multisystemic involvement with the same disease in her mother which is worth further functional analysis. Among the number of variants of uncertain significance (VUS) in patients with cSLE and IgAV, the most promising, in terms of contribution to the development of the disease and the impact on the clinical picture, are recombination activating 2, *RAG2* (NM_000536.3:c.1393A>G) predicted to encode (p.The465Ala), tyrosine-protein kinase 2, *TYK2* (NM_003331.4:c.2492A>T) predicted to encode (p.Asp810Val), B-cell lymphoma 2 associated antagonist of cell death, *BAD* (NM_004322.3:c.462G>C) predicted to encode (p.Trp154Cys), and DExH-box helicase 58, *DHX58* (NM_024119.2:c.560A>G) predicted to encode (p.Gln187Arg), making them candidates for further function analyses.

Conclusion. In the new epoch of personalised medicine, implementation of next generation sequencing has improved the diagnostics and treatment of patients with autoimmune diseases Nevertheless, such quantities of data raised the problem of interpretation of genetic variants and their use for therapeutic purposes. Undeniably, pathogenic variants often represent only a small percentage of all the variants reported, while there is growing number of variants which we are still not able to clearly define and use in a clinical context, such as VUS, which limit the clinical utility of genetic information. This has to prompt the scientific community to develop methods to properly categorise VUS and escalate the amount of practicable information from next generation sequencing.

9. SAŽETAK

Doprinos sekvenciranja cijeloga egzoma u otkrivanju varijanti gena povezanih sa sustavnim eritemskim lupusom s početkom u dječjoj dobi i IgA vaskulitisom

Mario Šestan

Doktorska disertacija, 2022.

Uvod i cilj. Sistemski eritemski lupus koji započinje u dječjoj dobi (cSLE) i IgA vaskulitis (IgAV) dvije su kompleksne autoimunosne bolesti još uvijek nepotpuno razjašnjene etiopatogeneze. Cilj ovog istraživanja bio je primjenom sekvenciranja cijelog egzoma (WES) u bolesnika cSLE-om i IgAV-om identificirati nove i rijetke genske varijante koje mogu doprinijeti etiopatogenezi ovih bolesti i proširiti postojeće genske baze podataka.

Ispitanici i metode. Skupina od 17 bolesnika s cSLE-om kojima su bila pridružena i oba roditelja (uključujući i ostale informativne članove obitelji) te 3 bolesnika s IgAV-om, također s pridružena oba roditelja, s teškom ili atipičnom kliničkom slikom, sindromskim obilježjima, ranim početkom bolesti, rezistencijom na konvencionalnu terapiju i/ili obiteljskom pojavnošću bolesti, podvrgnuta je WES-u. Nakon kompletiranja WES-a, analize podataka i identifikacije svih genskih varijanti od interesa, u jednog je bolesnika učinjeno određivanje interferonskog genskog potpisa iz mononuklearnih stanica periferne krvi.

Rezultati. Nakon učinjenog WES-a i analize genskih varijanti, nove i/ili rijetke varijante detektirane su u 8 bolesnika. Među njima bilo je 7 bolesnika s cSLE-om i 1 bolesnik s IgAV-om, a 1 bolesnik imao je cSLE i IgAV, ali u različitom periodu. Odabrano je 20 varijanti koje su podvrgnute detaljnoj analizi. Primjenom WES-a otkrivena je nova patogena varijanta u genu za histon-lizin N-metiltransferazu 2D (*KMT2D*), NM_003482.3:c.8626delC, za koju se predviđa da kodira trunkirani protein (p.Gln2876Serfs*34), čime dovodi do gubitka funkcije *KMT2D*, što je pomoglo objasniti prethodno neprepoznata sindromska obilježja u bolesnice s Kabukijevim sindromom i cSLE-om. Također su pronađene vjerojatno patogene varijante u genu za adenozin deaminazu koji djeluje na RNA (*ADAR1*), NM_001111.3:c.2815A>G, za koju se predviđa da kodira protein (p.Ile939Val), u bolesnice sa sindromskim obilježjima, promjenama pigmentacije i cSLE-om, kojom je samo djelomično moguće objasniti njezin kompleksni fenotip, te u genu za B limfocitnu kinazu (*BLK*), NM_001715.2;c.211G>A, za koju se previđa da kodira (p.Ala71Thr), u bolesnice s teškim cSLE-om i multiorganskim manifestacijama te istom bolešću u majke, a koje bi bilo korisno podvrgnuti daljnjim funkcijskim analizama. Među brojnim varijantama nesigurna značenja (VUS) u bolesnika s cSLE-om i IgAV-om, najperspektivnijima, u smislu mogućeg doprinosa u nastanku bolesti i utjecaja na kliničku sliku, pokazale su se varijante u genu za aktivaciju rekombinacije 2, *RAG2* (NM_000536.3:c.1393A>G) za koju se predviđa da kodira (p.The465Ala), u genu za tirozinsku proteinsku kinazu 2, *TYK2* (NM_003331.4:c.2492A>T), za koju se predviđa da kodira (p.Asp810Val), u genu za B-stanični limfom 2 agonist stanične smrti, *BAD* (NM_004322.3:c.462G>C), za koju se predviđa da kodira (p.Trp154Cys), te u genu za DExH-box helikazu 58, *DHX58* (NM_024119.2:c.560A>G), za koju se predviđa da kodira (p.Gln187Arg) pa su ove varijante kandidati za daljnje funkcijske analize.

Zaključak. U novoj epohi personalizirane medicine implementacijom sekvenciranja sljedeće generacije došlo je do poboljšanja dijagnostike i liječenja bolesnika s autoimunosnim bolestima. Međutim, tolika količina podataka otvorila je problem interpretacije genskih varijanti i njihove primjene u terapijske svrhe. Nedvojbeno, patogene varijante često predstavljaju samo mali postotak svih pronađenih varijanti dok raste broj varijanti koje još uvijek ne možemo jasno definirati i primijeniti u kliničkom kontekstu, kao što su VUS, što ograničava kliničku primjenjivost genskih informacija. Ovo bi trebalo potaknuti znanstvenu zajednicu na razvoj metoda kojima bi se na odgovarajući način kategorizirale VUS varijante i povećala količina praktičnih informacija koje možemo dobiti primjenom sekvenciranja sljedeće generacije.

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11. BIOGRAPHY

Mario Šestan, MD, was born in Karlovac on 4 June 1984. He graduated from the University of Zagreb School of Medicine in 2009 as the best student in the faculty with first-class honours and received Dean Award for the academic year 2003/2004 and Perović-Krmpotić Award as the best student in 2009. In 2010 he finished the internship at Clinical Hospital "Sveti Duh" in Zagreb after which he has worked at Department of Epidemiology in Institute of Public Health of the County of Karlovac in 2011, and as general practitioner in Community Health Centre Karlovac from 2013 to 2014. In 2014 Mario Šestan has begun with residency in paediatric infectious diseases at University Hospital for Infectious Disease "Dr. Fran Mihaljević", and from 2018 he is paediatric resident at Department of Paediatrics, University Hospital Centre Zagreb. He finished the PhD Program of Study "Biomedicine and Health Sciences" at University of Zagreb School of Medicine in 2021 and received scholarship from Profs Drs Ljiljana Zergollern and Kresimir Čupak foundation in 2019. So far, he is the author on 5 papers and the co-author on 14 papers published in journals indexed in Current Contents, Science Citation Index Expanded and Scopus as well as the author and co-author on more then 40 conference abstracts and co-author on module "Vasculitis" in Eular Textbook on Paediatric Rheumatology 2021. Mario Šestan is a Co-Principal Investigator from 2021 on a scientific-research project within Vasculitis Working Party of the Paediatric Rheumatology European Society: "Treatment of patients with severe cutaneous manifestations in IgA vasculitis - multicenter study" with Professor Marija Jelušić. He is Co-Investigator on many scientific-research projects, of which they are to be singled out Croatian Science Foundation Research Project "Histological, Clinical, Laboratory and Genetic Predictors of Outcome in Patients with Henoch-Schönlein Purpura and Nephritis", IP-2019-04-8822 led by Professor Marija Jelušić, "Genetic and cellular analysis of autoimmune and inflammatory disease" in colaboration with Centre for Personalised Immunology, Australian National University, Canberra, Australia, Australian NHMRC Centre of Research Excellence grant, "JIA classification study: The PRINTO Evidence-based Revision of the International League Against Rheumatism (ILAR) Classification criteria for juvenile idiopathic Arthritis", "Long-term PHARMAacovigilance for adverse effects in childhood arthritis focussing on immune modulatory drugs - PHARMACHILD", "The PRES European Network of Registres for Autoinflammatory Diseases in Childhood (Eurofever)" and a scientific-research project supported by the University of Zagreb "The possibilities of personalised medicine in treatment of children with autoimmune diseases" led by Professor Marija Jelušić.

SUPPLEMENT

List of *in silico* panel of genes associated with SLE used in primary filtering of exome data

A20	*BNIP3L*	*CRY1*	*FBXO22*	*IFIT3*	*INPP5D*	*MAPK13*
ABHD6	*C1QA*	*CRY2*	*FBXO9*	*IFITM2*	*INPPL1*	*MAP3K14*
ACE	*C1QB*	*CSF2RA*	*FBXW7*	*IFITM3*	*IRAK1*	*MASP1*
ACP5	*C1QC*	*CSK*	*FCER1A*	*IFNB1*	*IRAK1BP1*	*MASP2*
ACP6	*C1R*	*CTLA4*	*FCER2*	*IFNA1*	*IRAK2*	*MECP2*
ACTBL2	*C1S*	*CTSB*	*FCGR2A*	*IFNA21*	*IRAK3*	*MERTK*
ACTN1	*C2*	*CTSK*	*FCGR2B*	*IFNAR1*	*IRAK4*	*MIF*
ACTR2	*C4A*	*CTSS*	*FCGR3A*	*IFNAR2*	*IRF3*	*MIR146A*
ADA	*C4B*	*CUTC*	*FCGR3B*	*IKBKE*	*IRF9*	*MLLT4*
ADA2	*C4BPA*	*CXORF21*	*FCHSD1*	*IKZF1*	*IRF5*	*MMP1*
ADAR	*C6*	*CYBA*	*FCHSD2*	*IKZF2*	*TNPO3*	*MMP2*
ADAR1	*C8ORF12*	*CYLD*	*FGA*	*IKZF3*	*IRF7*	*MMP9*
ADARB1	*CASP10*	*DAK*	*FGG*	*IL10*	*IRF8*	*MPO*
ADRBK1	*CD19*	*DCK*	*FHOD1*	*IL10RA*	*ISG15*	*MS4A2*
AFF1	*CD2*	*DDX58*	*FKBP2*	*IL12A*	*ITGA1*	*MST1*
AGER	*CD226*	*DDX60*	*FLI1*	*IL12B*	*ITGA2B*	*MT1X*
AGMO	*CD28*	*DGUOK*	*GH1*	*IL12RB1*	*ITGA3*	*MX1*
ALOX12B	*CD44*	*DHCR7*	*GMPS*	*IL12RB2*	*ITGAM*	*MX2*
ANGPT1	*CD46*	*DHX58*	*GP1BA*	*IL17A*	*ITGB2*	*MYD88*
ANGPTL3	*CD79B*	*DNASE2*	*GRN*	*IL17B*	*JAK1*	*MYO1C*
ANKRD17	*CD80*	*DNASE2B*	*GTF2I*	*IL17RA*	*JAK2*	*N4BP1*
ANXA6	*CD81*	*DNASE1*	*GTF2IRD1*	*IL17RB*	*JAM2*	*NADSYN1*
APCS	*CDC16*	*DNASE1L3*	*GZMA*	*IL18R1*	*JAZF1*	*NAMPT*
APOB	*CDC45*	*DOK1*	*GZMB*	*IL18RAP*	*KCNN4*	*NBR1*
ARFIP2	*CDKN1B*	*DTX4*	*HCP5*	*IL1R1*	*KDM2A*	*NCDN*
ARID5B	*CECR1*	*EGR3*	*HERC5*	*IL1RAP*	*KDM2B*	*NCF2*
ARIH2	*CFI*	*EIF2AK2*	*HIP1*	*IL2*	*KLK*	*NCF4*
ARPC1B	*CFLAR*	*ELAVL1*	*HLX*	*IL21R*	*LAMC2*	*NDRG2*
AS1	*CHEK2*	*ELF1*	*HMGB1*	*IL21*	*LAP3*	*NEIL3*
ATG16L2	*CHML*	*ELMO2*	*HOXA7*	*IL22*	*LBH*	*NFKB1*
ATG5	*CIITA*	*EOMES*	*HSP90B1*	*IL23*	*LGMN*	*NKTR*
ATG7	*CISH*	*ETS1*	*HSPB1*	*IL23R*	*LIF*	*NLGN1*
AXL	*CKS1B*	*ETS2*	*ICOS*	*IL27*	*LIFR*	*NLRC3*
BACH2	*CLEC16A*	*FADS6*	*IFI16*	*IL27RA*	*LILRA6*	*NLRP4*
BAD	*CLTC*	*FAIM2*	*IFI35*	*IL32*	*LPP*	*NMI*
BANK1	*CNTROB*	*FASL*	*IFI44*	*IL4R*	*LRRC47*	*NR*
BCR	*COL25A1*	*FASLG*	*IFI44L*	*IL6*	*LYN*	*NR1D2*
BIRC2	*CR1*	*FBXL19*	*IFI6*	*IL6R*	*LYST*	*NR3C1*
BIRC3	*CR2*	*FBXL2*	*IFIH1*	*IL6ST*	*MAP3K5*	*NR5A2*
BLK	*CRP*	*FBXO18*	*IFIT1*	*ILT3*	*MAPK12*	*OAS1*

OAS2
OAS3
OASL
ODC1
PARK2
PARP2
PCBP4
PDHX-CD44
PFAS
PIK3CB
PLAUR
PLK3
PLSCR1
POLA2
POP5
PP2A
PPIAL4G
PPIL2
PPIL4
PPIL6
PPP6R3
PRDM14
PRIM1
PRKAR2B
PRKCG
PRKCH
PRKCQ
PSMA2
PSMA4
PSMA5
PSMA6
PSMB1
PSMB2
PSMB3
PSMB5
PSMB6
PSMC1
PSMC4
PSMD11
PSMD14

OAS2
OAS3
OASL
P2RY2
PARK2
PARP2
PCBP4
PDHX
PDHX-CD44
PFAS
PIK3CB
PLAUR
PLD2
PLK3
PLSCR1
POLA1
POLA2
POP5
PP2A
PPIAL4G
PPIL2
PPIL4
PPIL6
PPP6R3
PRDM1
PRDM14
PRIM1
PRKAR2B
PRKCB
PRKCD
PRKCG
PRKCH
PRKCQ
PSMA2
PSMA3
PSMA4
PSMA5
PSMA6
PSMB1

PSMB2
PSMB3
PSMB4
PSMB5
PSMB6
PSMB8
PSMB9
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PSMC4
PSMD11
PSMD14
PSMD6
PSMD7
PSME4
PTPN11
PTPN13
PTPN2
PTPN22
PTPN6
PTPRC
PTPRK
PTTG1
PXK
RAD51B
RAD51D
RFFL
RAD51L3
RAD54B
RAD54L
RAF1
RAP1B
RARS
RASA1
RASGRP3
RC3H1
RC3H2
RELB
REV1
RGS1
RIPK1

RIPK2
RNASE2B
RNASEH1
RNASEH2A
RNASEH2B
RNASEH2C
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RNF135
NH1
RPP21
RSAD2
RUNX3
SAMHD1
SFR1
SH2B3
SHFM1
SHH
SHISA6
SETD5
SIKE1
SKP1
SLA
SLC15A3
SLC15A4
SMC6
SMG7
SMPD1
SOCS1
SPG21
SPHK2
SPICE1
SPP1
SPRED2
STAT1
STAT2
STAT4
STAT6
SUGT1
SWI5
TAGAP

TAP1
TAX1BP1
TBK1
TBX21
TCF7
TDGF1
TESK1
TGFBI
THEMIS
TK2
TLR2
TLR4
TLR3
TLR6
TLR7
TLR8
TLR9
TMEM173
TMEM39A
TNF
TNFAIP3
TNFRSF10D
TNFRSF13B,
TNFRSF13C
TNFRSF1A
TNFRSF1B
TNFRSF4
TNFSF10
TNFSF13B
TNFSF4
TNIP1
TNIP3
TNXB
TOLLIP
TOP3B
TPRG1
TRADD
TRAF3IP2
TRAF5
TRAF6

TRAF2
TREX1
TRIM32
TRIM56
TSPAN6
TYK2
TYMS
TYRO3
UBA7
UBASH3A
UBE2L3
UBE2L6
UHRF1BP1
UNC93B1
USP18
VAV2
VAV3
VCL
VDR
VPRBP
WDFY4
XKR6
XRCC2
XRCC3
ZAP70
ZFP36L1
ZFP90
ZMIZ1
ZNF521

List of *in silico* panel of genes associated with vasculitis and inflammation used in primary filtering of exome data

ACTA2	*LRBA*	*MEFV*	*CFHR5*	*DNASE1L3*
BMPR2	*NCF1*	*MVK*	*CFI*	*PRKCD*
FBN1	*NCF2*	*NLRP12*	*CFP*	*RNASEH2A*
SLC2A10	*NCF4*	*NLRP3*	*MASP2*	*RNASEH2B*
TGFBR1	*PIK3R1*	*NOD2*	*MBL2*	*RNASEH2C*
TGFBR2	*PTEN*	*PLCG2*	*SERPING1*	*SAMHD1*
MYH11	*RET*	*PSMB8*	*COL3A1*	*TREX1*
RNF213	*SKIV2L*	*PSTPIP1*	*COL5A1*	*CECR1*
GUCY1A3	*SLC37A4*	*RBCK1*	*COL5A2*	*TRAP1*
MYLK	*TTC37*	*TMEM173*	*PLOD1*	*WDR1*
PRKG1	*WAS*	*TNFRSF1A*	*PRF1*	*UNC13D intron 1*
SMAD3	*AP3B1*	*TRNT1*	*SLC29A3*	*RAG2*
SMAD4	*CBL*	*AP1S3*	*STX11*	*BCL10*
TGFB2	*CORO1A*	*CARD14*	*STXBP2*	*CFD*
HFE	*CTPS1*	*NLRC4*	*UNC13D*	
RHOD	*IFNGR1*	*NLRP7*	*DNASE2*	
ELN	*IFNGR2*	*POMP*	*LYST*	
FBN2	*MAGT1*	*PSMA3*	*RAB27A*	
NOTCH1	*PIK3CD*	*PSMB4*	*APOA1*	
ADAM17	*SKI*	*PSMB9*	*APOA2*	
AICDA	*VPS13B*	*SH3BP2*	*FGA*	
BTK	*NF1*	*TNFAIP3*	*GSN*	
CD40LG	*B2M*	*TNFRSF11A*	*LYZ*	
COL7A1	*CTC1*	*PYCARD*	*TTR*	
CYBA	*STK4*	*NLRP6*	*APOE*	
CYBB	*CASP10*	*C1QA*	*SAA1*	
DCLRE1C	*CASP8*	*C1QB*	*SAA2*	
DOCK8	*FAS*	*C1QC*	*SAA4*	
FERMT1	*FASLG*	*C1R*	*APOC3*	
FOXP3	*NRAS*	*C2*	*APOA4*	
G6PC3	*SH2D1A*	*C3*	*CBS*	
GUCY2C	*XIAP*	*C4A*	*COL4A1*	
HPS1	*IL10*	*C5*	*CST3*	
HPS4	*IL10RA*	*C6*	*GLA*	
HPS6	*IL10RB*	*C7*	*HTRA1*	
ICOS	*IL1RN*	*C8A*	*NOTCH3*	
IKBKG	*IL36RN*	*C8B*	*ACP5*	
IL2RA	*LPIN2*	*C9*	*ADAR*	
ITGB2	*LYN*	*CFH*	*DNASE1*	