

IDENTIFICATION AND CLINICAL RELEVANCE OF  
PHENOTYPE MODIFYING GENETIC FACTORS  
IN MONOGENIC DISEASES

Ph.D. Thesis

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## LIST OF PUBLICATIONS

### Publications providing the basis of the dissertation

**I. Pap ÉM**, Farkas K, Tóth L, Fábos B, Széll M, Németh G, Nagy N.: Identification of putative genetic modifying factors that influence the development of Papillon-Lefèvre or Haim-Munk syndrome phenotypes. **Clin Exp Dermatol.** 2020 Jul;45(5):555-559. **IF: 1.977**

**II. Pap ÉM**, Farkas K, Széll M, Németh G, Rajan N, Nagy N.: Identification of putative phenotype-modifying genetic factors associated with phenotypic diversity in Brooke-Spiegler syndrome. **Exp Dermatol.** 2020.07.26. Accepted for publication. **IF: 3.368**

**III. Pap ÉM**, Széll M, Nagy N, Németh G: Genomikai és fenotípus vizsgálatok Brooke-Spiegler, Papillon-Lefèvre, és Haim-Munk szindrómában. **Magyar Nőorvosok Lapja.** Under review.

## 1. INTRODUCTION

### 1.1. General features of monogenic diseases

Monogenic diseases result from modifications in a single gene occurring in all cells of the body. Though they are relatively rare, they can affect millions of patients worldwide. Scientists currently estimate that over 10,000 of human diseases are known to be monogenic. Pure genetic diseases are caused by a single error in a single gene in the human DNA. The nature of the disease depends on the functions of the modified gene. The single-gene or monogenic diseases can basically be inherited in three different manners: dominant, recessive and X-linked.

Dominant diseases are monogenic disorders that involve damage to only one gene copy, recessive diseases are monogenic disorders that occur due to damages in both copies, while X-linked diseases are monogenic disorders that are linked to defective genes on the X chromosome. The X-linked alleles can also be dominant or recessive. These alleles are expressed equally in men and women, more so in men as they carry only one copy of X chromosome (XY) whereas women carry two (XX).

The global prevalence of all single gene diseases at birth is approximately 10/1000 (Orphanet Database, [www.orpha.net](http://www.orpha.net)). Monogenic diseases affect nationwide only a few patients, but altogether they affect a significant portion of the populations since their thousands of different types are known. In general, these disorders are less known than the common, multifactorial ones. They occur rarely in the everyday practice of the medical practitioners and less attention is paid for the research of these diseases (Kelsall *et al.*, 2013).

Monogenic diseases can vary greatly in their severity. They can cause mild, severe or very severe symptoms and can be associated with phenotypic diversity like common diseases. Their symptoms can significantly impair the life quality of the patient and they can also result in stigmatization and difficulties in socialization (Kelsall *et al.*, 2013).

## **1.2. Clinical management of monogenic diseases – the importance of genetic counseling**

Genetic counseling gives the patient information about how genetic conditions might affect the patient or his/her family. The genetic counselor or other healthcare professional will collect the patient's personal and family health history. They can use this information to determine how likely it is that the patient or his/her family member has a genetic condition. Based on this information, the genetic counselor can help the patient decide whether a genetic test might be right for the patient or his/her relative.

Based on your personal and family health history, your doctor can refer you for genetic counseling. There are different stages in your life when you might be referred for genetic counseling.

Before the patient becomes pregnant genetic counseling can address concerns about monogenic diseases that might affect the baby of the patient during infancy or childhood or the ability of the patient to become pregnant.

Genetic counseling is important while the patient is pregnant, it can address certain tests that may be done during the pregnancy to detect monogenic diseases that might affect the baby of the patient during infancy or childhood.

Genetic counseling can address concerns if a child is showing signs and symptoms of a monogenic disease.

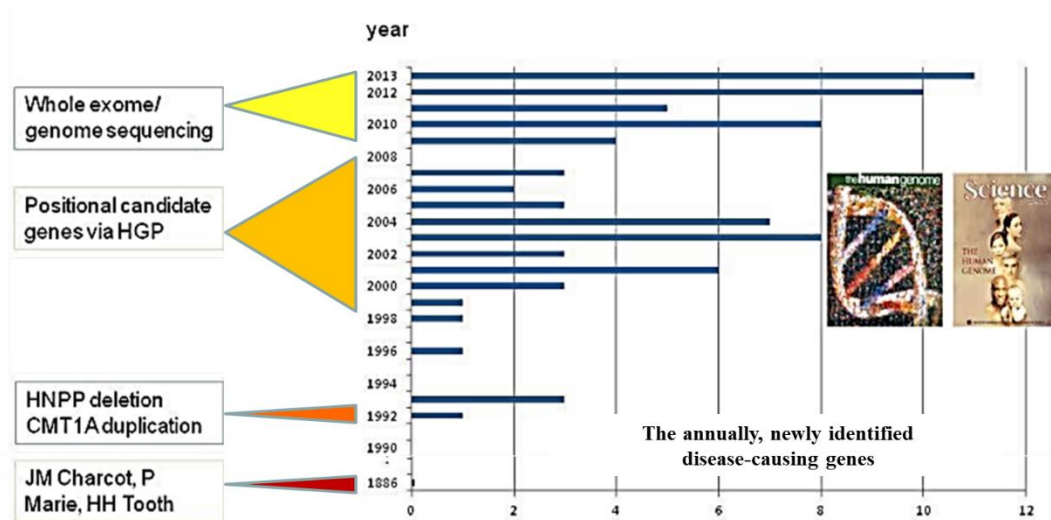
Genetic counseling can be helpful if the patient has symptoms of a monogenic disease or has a family history of a monogenic condition that makes him or her more likely to be affected with that condition.

Following the genetic counseling session, the patient might decide to have genetic testing. Genetic counseling after testing can help the patient better understand the test results and treatment options, help the patient deal with emotional concerns, and refer the patient to other healthcare providers and advocacy and support groups.

## **1.3. Recent milestones in the background of knowledge explosion in the understanding of monogenic diseases**

As a result of the Human Genome Project (HGP) and the development of next-generation sequencing technologies, nowadays we know more and more about

monogenic diseases. In the last 30 years, many disease causing genes and mutations have been identified and the genetic background of many monogenic diseases have been elucidated (Figure 1.). As an example we demonstrate the timeline of the discoveries of the genetic background of Charcot-Marie-Tooth (CMT) disease. However, it can occur that sequencing is unable to answer clinically relevant questions regarding phenotypic diversity and/or disease prognosis (Jarinova *et al.*, 2012; Timmerman *et al.*, 2014; Kiritsi *et al.*, 2015; Smith *et al.*, 2019).



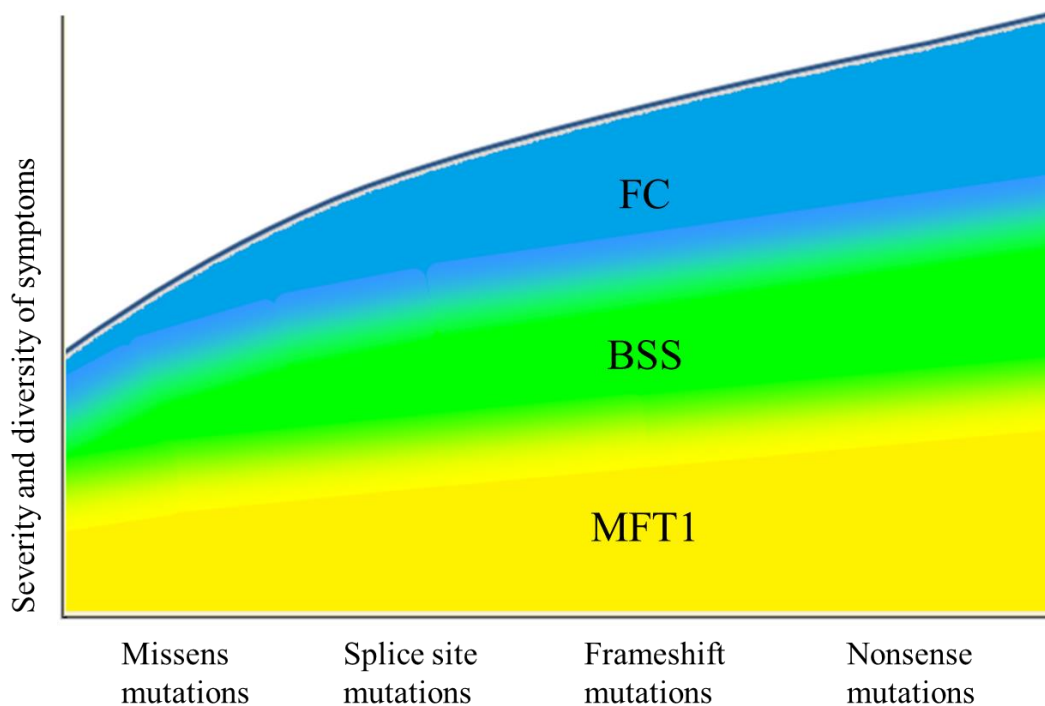
**Figure 1. Genetics of Charcot-Marie-Tooth (CMT) Disease within the Frame of the HGP Success.** The number of the annually, newly identified causative genes show two peaks: one is explained by the HGP, the other is the consequence of the fast development of sequencing, next-generation sequencing technologies (Timmerman *et al.*, 2014).

#### 1.4. The investigated monogenic diseases

In my thesis, I have summarized the results of my genetic investigations in monogenic diseases: the clinical variants of the cylindromatosis gene (*CYLD*) mutation-caused disease spectrum such as multiple familial trichoepithelioma type 1 (MFT1), familial cylindromatosis (FC) and Brooke-Spiegler syndrome (BSS) and the clinical variants of the cathepsin C (*CTSC*) mutation-caused disease spectrum such as the Papillon-Lefèvre syndrome (PLS) and the Haim-Munk syndrome (HMS). My investigations focused on the identification of putative phenotype modifying genetic factors in these rare monogenic diseases, which are responsible for the observed phenotypic differences among the affected patients carrying the same disease-causing *CYLD* or *CTSC* mutations.

#### 1.4.1. Clinical variants of the *CYLD* mutation-caused spectrum

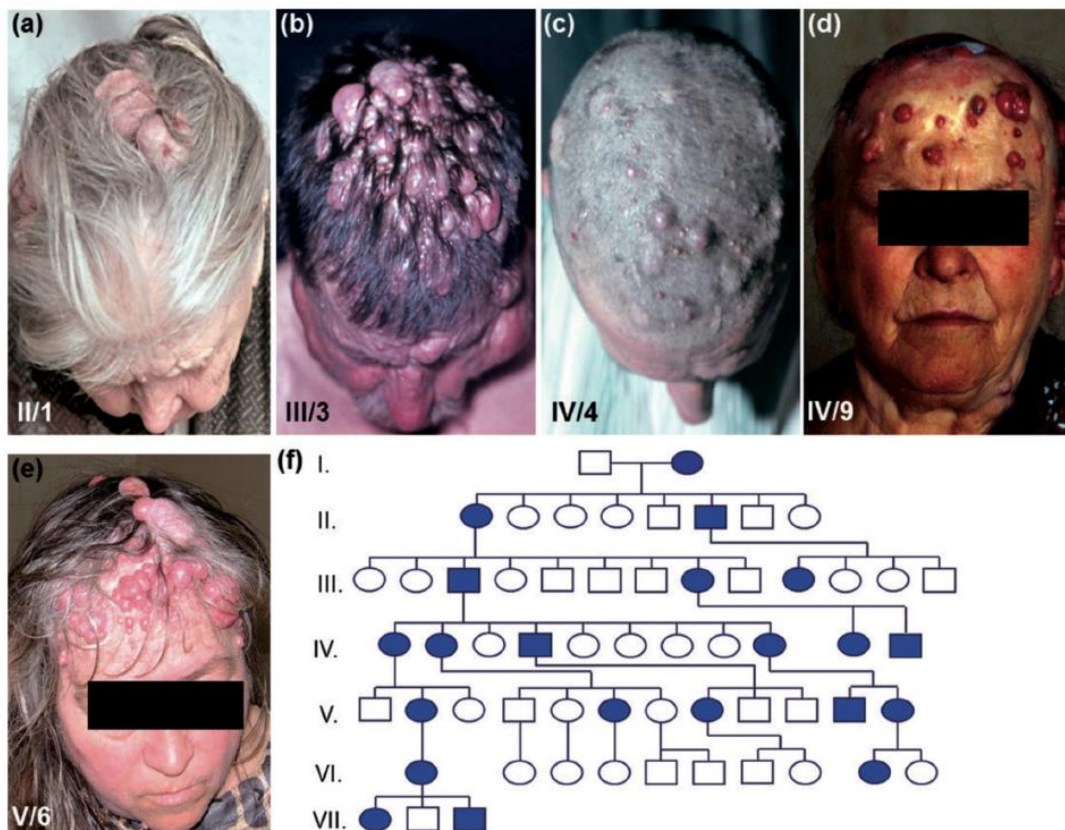
Brooke-Spiegler syndrome (BSS) is a rare monogenic skin disease characterized by the development of skin appendage tumors such as cylindromas, trichoepitheliomas and spiradenomas (Evans 1954; Bignell *et al.*, 2000). The first symptoms of BSS are small skin-colored papules, which occur in childhood and adolescence (Evans, 1954). These tumors grow slowly in size and continue to appear throughout the lifetime of the patient (Nagy *et al.*, 2015). Expression of the papules exhibits wide variation among and within affected families (Bignell *et al.*, 2000).



**Figure 2. The severity and diversity of the symptoms of the clinical variants of the *CYLD* mutation-caused spectrum.** In general, missense mutations of the *CYLD* gene is associated with the mildest symptoms and in approximately 50% of the cases they results in the development of MFT1. Nonsense mutations of the *CYLD* gene are associated with the most severe symptoms and with the highest diversity of the symptoms of the affected patients, they can lead to the development of FC, MFT1 and BSS with approximately equal frequency. (Nagy *et al.*, 2015).

BSS is transmitted as an autosomal dominant condition affecting males and females equally (Guggenheim and Schnyder, 1961). BSS and its phenotypic variants were independently mapped to chromosome 16q12-q13 by several groups (Fenske *et al.*,

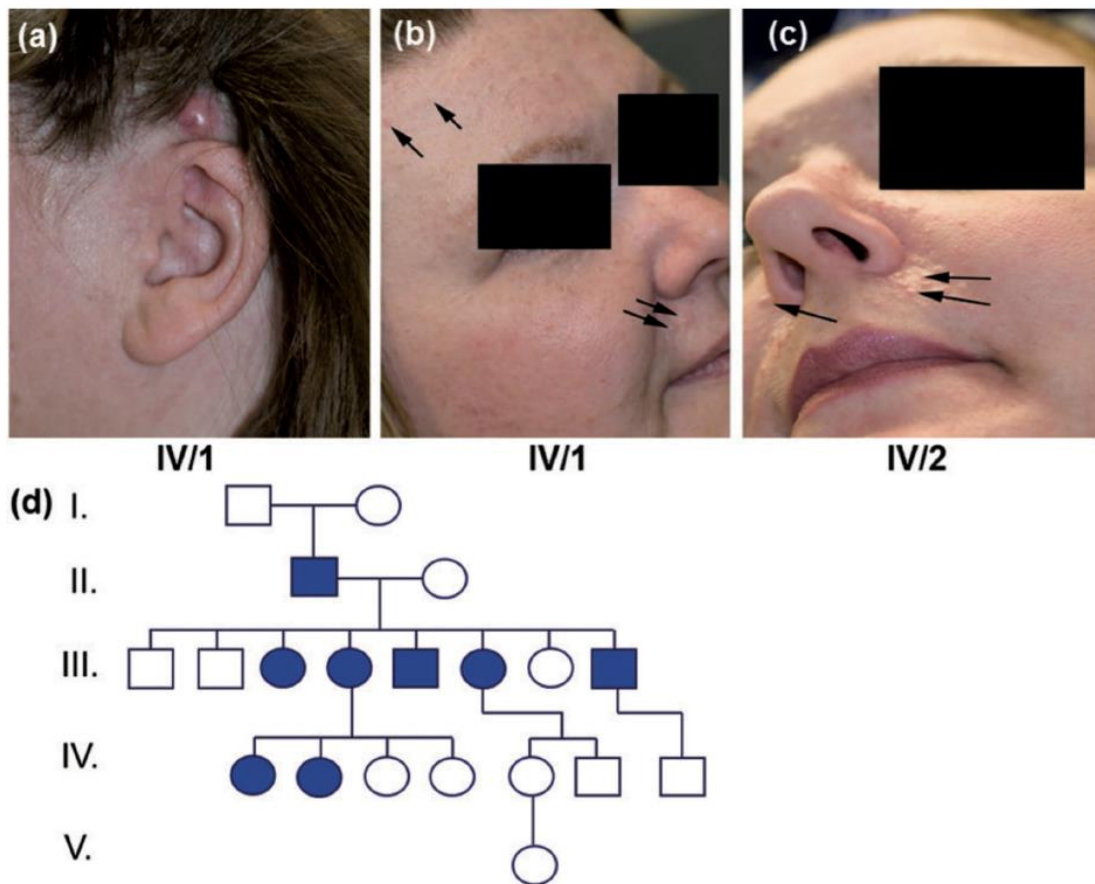
2000; Biggs *et al.*, 1995; Takahashi *et al.*, 2000). Within the mapped region, the cylindromatosis gene (*CYLD*) was identified as the causative gene responsible for the development of the disease (Bignell *et al.*, 2000). The *CYLD* gene (GenBank accession number NM\_015247) spans 56 kb and contains 20 exons, the first 3 of which are untranslated, and 19 introns. Of the 17 known splice variants, 13 affect protein coding regions, and the remaining produce non-coding transcripts (<http://ensemble.org>). The tumor suppressor *CYLD* gene encodes an enzyme with deubiquitinase activity. The CYLD enzyme posttranslationally modifies its target proteins by removing Lys63-linked ubiquitin chains (Kovalenko *et al.*, 2003). The protein interacts with several members of the NF- $\kappa$ B signaling pathway, including the TRAF2, TRAF6, NEMO and BCL3 proteins, acting as a negative regulator (Hutti *et al.*, 2009).



**Figure 3. The Hungarian BSS pedigree of Bukovinian (Romania) origin.** The clinical pictures of the severe hairy scalp symptoms of the affected individuals are represented from the (a) 2<sup>nd</sup>, (b) 3<sup>rd</sup>, (c and d) 4<sup>th</sup> and (e) 5<sup>th</sup> generations. (f) The pedigree contains 21 affected family members spanning 7 generations (Nagy *et al.*, 2013).



Concerning the so far reported phenotypes and the associated *CYLD* mutations, it is difficult to establish genotype-phenotype correlations in BSS. However, the elucidation of the genotype-phenotype correlations has significant clinical relevance in promoting the understanding of disease mechanism and contributing to the development of future therapeutic modalities. The picture is even more complex, since mutations of the *CYLD* gene have been identified in patients with phenotypic features of either BSS, FC or MTF1 suggesting that these syndromes are clinical variants of the *CYLD* mutation-caused spectrum (Figure 2., Rajan *et al.*, 2011; Nagy *et al.*, 2015).



**Figure 4. The investigated Anglo-Saxon pedigree from the North of England.** Clinical pictures of the affected individuals from the 4th generation show tumors (a) above the ear, (b) on the forehead, and (c) around the nose. (d) The pedigree contains 8 affected family members spanning 5 generations (Nagy *et al.*, 2013).

A Hungarian pedigree from Bukovina (Romania) affected by BSS (Figure 3.) and an English BSS pedigree (Figure 3.) from Northern England were included in this

study. The clinical phenotypes of the affected family members and the pedigrees are reported in detail in a previous paper from our research group (Nagy *et al.*, 2013). The patients of the Hungarian BSS pedigree were presented with severe symptoms: numerous large tumors arising from different skin appendages and developing on the scalp, face and trunk mainly (Nagy *et al.*, 2013). The patients of the Anglo-Saxon pedigree showed moderate severity: few tumors, small size arising from different skin appendages developing primarily on the face (Figure 4., Nagy *et al.*, 2013).

Out of the previously reported patients, two Hungarian and two Anglo-Saxon ones, affected by the different phenotypes of BSS, but carrying the same disease-causing mutation (c.2806C>T, p.Arg936X) in the *CYLD* gene (Nagy *et al.*, 2013) were enrolled to the study.

#### **1.4.2. Clinical variants of the *CTSC* mutation-caused spectrum**

Papillon-Lefèvre syndrome (PLS) and Haim-Munk syndrome (HMS) are characterized by overlapping dermatological and dental symptoms, including hyperkeratosis of the palms and soles as well as severe periodontitis (Selvaraju *et al.*, 2003; Nagy *et al.*, 2014). Patients with PLS can also develop mild mental retardation, calcification of the dura mater, hyperhidrosis and increased susceptibility to infections (Gorlin *et al.*, 1964; Haneke *et al.*, 1979; Dalgic *et al.*, 2011). Specific features of HMS include pes planus, arachnodactyly, acroosteolysis and onychogryphosis (Papillon *et al.*, 1924; Haim *et al.*, 1965; Hart *et al.*, 1999).

The prevalence of PLS is approximately four cases per million, and, to date, approximately 300 cases have been reported worldwide. Parental consanguinity has been noted in more than 50% of these cases (Hewitt *et al.*, 2004). The prevalence of HMS is approximately one case per million, and the majority of reported cases are descendants of a few consanguineous families from a religious isolate in Cochin, India. One unrelated Brazilian patient has also been reported. Fewer than 100 HMS cases have been reported in the literature to date (Papillon *et al.*, 1924; Haim *et al.*, 1965; Hart *et al.*, 1999).

The ratio of affected males to females is 1:1 for both syndromes. PLS and HMS are both inherited in an autosomal recessive manner and develop as a consequence of mutations of the *CTSC* gene (Toomes *et al.*, 1999; Adkison *et al.*, 2002).

So far 89 *CTSC* gene mutations have been identified (Sulák *et al.*, 2016). The majority of these mutations have been detected in PLS patients, whereas only 4% have been associated with HMS (Selvaraju *et al.*, 2003; Nagy *et al.*, 2014).

In light of the reported PLS and HMS phenotypes and the associated *CTSC* mutations, it was hypothesized that PLS and HMS are the same entity with different phenotypic appearances (Table I., Sulák *et al.*, 2016). Although it is difficult to establish genotype–phenotype correlations, the elucidation of these correlations is likely to have significant clinical relevance for the development of the different clinical variants (PLS and HMS), the disease mechanism and the development of future therapeutic modalities.

|                       | PLS   | HMS   |
|-----------------------|---|---|
| Clinical symptoms     | Periodontitis,<br>Palmoplantar hyperkeratosis | Periodontitis,<br>Palmoplantar hyperkeratosis<br>Arachnodactyly,<br>Acroosteolysis,<br>Pes planus, Onychogryposis |
| <i>CTSC</i> mutations | Any types of mutations                        | Any types of mutations  |

**Table I. Comparison of the symptoms of the clinical variants of the *CTSC* mutation-caused spectrum.**

The clinical phenotypes of the affected patients were reported in detail in a previous paper from our research group (Sulák *et al.*, 2016). A Hungarian woman presented with a typical HMS phenotype (Figure 5.): mild hyperkeratotic plaques were observed symmetrically on her palms and soles, onychogryphosis and arachnodactyly were noted on her fingers and pes planus on her soles.

A Hungarian male patient presented with the classical PLS phenotype (Figure 5.): moderate hyperkeratosis on his palms and soles. Both patients were missing all permanent teeth and using a permanent dental prosthesis. In our previous paper, we also reported haplotype analysis that raise the possibility that these patients are

siblings (Sulák *et al.*, 2016). It was not possible to genotype unaffected relatives (Sulák *et al.*, 2016).



**Figure 5. The clinical symptoms of the HMS and PLS Patients.** HMS Patient exhibited (a) mild hyperkeratosis on her palms, (b) onychogryphosis and arachnodactyly of her fingers and mild hyperkeratosis and (c) pes planus on her soles. PLS Patient was affected by moderate hyperkeratosis of his (d) palms and (f) soles, and exhibited no specific symptoms of HMS. (e) No other affected individuals are known in the family of the PLS Patient, and HMS Patient was brought up in state care without knowing her relatives (Sulák *et al.*, 2016).

The two previously reported Hungarian patients affected by PLS and HMS carrying the same disease-causing mutation (c.748C/T; p.Arg250X) of the *CTSC* gene were investigated (Sulák *et al.*, 2016) in the current study.

## 2. AIMS

### 2.1. Aims related to the *CYLD*-mutation caused clinical variants

In order to comprehensively profile coding variants and identify putative phenotype-modifying genetic factors - which could potentially explain the observed clinical differences between the Hungarian and the Anglo-Saxon BSS pedigrees carrying the same causative *CYLD* mutation - we performed whole exome sequencing (WES).

Here we report an investigation of Hungarian and Anglo-Saxon BSS pedigrees, which have been chosen as they both carry the same recurrent germline mutation in the *CYLD* gene (c.2806C>T, p.Arg936X), yet they show striking difference in their phenotypes (Nagy *et al.*, 2013). These two pedigrees provide an excellent opportunity to identify phenotype-modifying genetic factors that are responsible for the phenotypic diversity in BSS.

### 2.2. Aims related to the *CTSC*-mutation caused clinical variants

In order to identify putative phenotype-modifying genetic factors - which could explain the observed clinical differences between the PLS and HMS patients carrying the same causative *CTSC* mutation – WES was performed.

We have recently investigated two Hungarian patients, one with the symptoms of PLS and one with the symptoms of HMS, who nonetheless carry the same homozygous nonsense mutation (c.748C/T; p.Arg250X) of the *CTSC* gene (Sulák *et al.*, 2016). As there is currently no explanation for why one mutation can lead to these two different clinical variants (PLS and HMS), we became interested in the identification of phenotype-modifying genetic factors that could facilitate the understanding of the phenotypic differences between these patients.

### **3. PATIENTS AND METHODS**

#### **3.1. Patients**

The clinical details of the investigated patients are demonstrated in the introduction part of the thesis. Their clinical features have been reported in the previous publications of our research group (Nagy *et al.*, 2013; Sulák *et al.*, 2016).

#### **3.2. Methods**

##### **3.2.1. DNA samples**

Out of the previously reported patients, two Hungarian and two Anglo-Saxon ones, affected by the different phenotypes of BSS, but carrying the same disease-causing mutation (c.2806C>T, p.Arg936X) in the *CYLD* gene, were investigated (Nagy *et al.*, 2013). DNA samples from the patients (n=4) were used for WES (performed by UD-GenoMed Medical Genomic Technologies Ltd., Debrecen, Hungary; <http://www.ud-genomed.hu/>).

Two previously reported Hungarian patients affected by PLS and HMS carrying the same disease-causing mutation (c.748C/T; p.Arg250X) of the *CTSC* gene were investigated (Sulák *et al.*, 2016). DNA samples from both patients were subjected to WES, which was performed by UD-GenoMed Medical Genomic Technologies Ltd.

The quality of the DNA samples was evaluated by agarose-gel electrophoresis.

##### **3.2.2. Whole exome sequencing (WES)**

Briefly, 4 µg of DNA with a concentration of 100 ng/µL were used for library construction. A liquid chip capture system (Agilent Research Laboratories, Santa Clara, CA, USA) was used to efficiently enrich all human exon regions. High-throughput deep sequencing was subsequently performed on the Illumina (San Diego, CA, USA) platform. An exon kit (SureSelect Human All Exon V6 Kit; Agilent) was used for library construction and capture experiments, and a bioanalyser (Model 2100; Agilent) was subsequently used to verify the library

insert size. The Illumina platform was used for sequencing according to the effective concentration of the library and the data output requirements. High-throughput paired-end sequencing (paired-end 150 bp; PE150) was performed.

### **3.2.3. Bioinformatics analysis**

After WES was completed, bioinformatics analysis was performed, including quality assessment of sequencing data, single-nucleotide polymorphism (SNP) detection and whole exome association analysis.

The sequencing data quality control requirements were as follows: sequencing error rate of each base position < 1%, mean Q20 ratio > 90%, mean Q30 ratio > 80%, mean error rate < 0.1%, alignment rate for sequencing reads  $\leq$  95% and read depth of the base at one position  $\geq$  10X.

### **3.2.4. Single nucleotide polymorphism (SNP) testing**

SNP testing was performed as follows: high-quality sequences were aligned with the human reference genome (GRCh37/hg19) to detect sequence variants, and the detected variations were analyzed and annotated. Variants were filtered according to read depth, allele frequency, and prevalence in genomic variant databases such as ExAc (v.0.3), ClinVar and Kaviar.

Variant prioritization tools (PolyPhen2, SIFT, LRT, Mutation Taster, Mutation Assessor) were used to predict the functional impact of the variants. All the identified candidate variants were confirmed by direct sequencing (Delta Bio 2000 Ltd., Szeged, Hungary; <http://www.deltabio.hu/>).

## 4. RESULTS

### 4.1. Identification of three putative genetic variants responsible for phenotypic variants in BSS

| Gene            | SNP   | Polyphen2         | SIFT        | MutationTaster  |
|-----------------|---|-------------------|-------------|-----------------|
| <i>ABCA13</i>   | rs74790141  | Damaging          | Deleterious | Disease causing |
| <i>ARID1B</i>   | rs113232635                                       | Unknown           | Tolerated   | Normal          |
| <i>DECRI</i>    | rs550991042                                       | Unknown           | Tolerated   | Disease causing |
| <i>EPB41L4A</i> | rs17266567  | Damaging          | Deleterious | Disease causing |
| <i>FGFR2</i>    | rs765066758                                       | Benign            | Tolerated   | Disease causing |
| <i>HLA-A</i>    | rs1136741   | Damaging          | Tolerated   | Normal          |
| <i>HLA-A</i>    | rs1059563   | Damaging          | Tolerated   | Polymorphism    |
| <i>HLA-A</i>    | rs9260179   | Probably damaging | Tolerated   | Polymorphism    |
| <i>HLA-A</i>    | rs9260180   | Probably damaging | Tolerated   | Polymorphism    |
| <i>KALRN</i>    | rs78202770  | Damaging          | Deleterious | Disease causing |
| <i>KMT2C</i>    | rs74483926  | Benign            | Tolerated   | Polymorphism    |
| <i>LAMB1</i>    | Chr10:107638858, ENST00000222399, c.A293G, p.N98S | Damaging          | Deleterious | Disease causing |
| <i>MKI67</i>    | rs45438392  | Damaging          | Tolerated   | Normal          |
| <i>NBR1</i>     | rs202122812                                       | Probably damaging | Deleterious | Disease causing |
| <i>POM121L2</i> | rs61736098  | Damaging          | Deleterious | Normal          |
| <i>SMO</i>      | rs111694017                                       | Benign            | Tolerated   | Damaging        |
| <i>STAT3</i>    | rs1053023   | Unknown           | Unknown     | Polymorphism    |
| <i>TNSI</i>     | rs140104262                                       | Probably damaging | Deleterious | Disease causing |
| <i>TRAF3</i>    | rs1131877   | Benign            | Tolerated   | Polymorphism    |
| <i>TYWIB</i>    | rs181240185                                       | Probably damaging | Deleterious | Unknown         |

**Table II. WES identified 20 variants, which were all present in the Hungarian BSS patients, but not in the Anglo-Saxon ones.**



A comparison of the WES data from the Hungarian and Anglo-Saxon BSS patients carrying the same disease-causing mutation (c.2806C>T, p.Arg936X) in the *CYLD* gene identified 20 genetic variants (Table II.), which were all present in the Hungarian patients, but not in the Anglo-Saxon patients. Based on the results of variant prioritization tools and the data of the literature, three of the 20 variants were suggested as putative phenotype-modifying polymorphisms. According to our current knowledge the other 17 variants are not associated with the functions of the *CYLD* enzyme.

The three putative phenotype-modifying polymorphisms are the followings: the rs1053023 SNP of the *signal transducer and activator of transcription 3 (STAT3)* gene, the rs1131877 SNP of the *tumor necrosis factor receptor-associated factor 3 (TRAF3)* gene and the rs202122812 SNP of the *neighbor of BRCA1 gene 1 (NBR1)* gene. The rs1053023 polymorphism is located in the 3'UTR region of the *STAT3* gene, while the other two polymorphisms (rs1131877 and rs202122812) are common missense variants of the *TRAF3* and *NBR1* genes, respectively. Pathogenicity predictions of the identified phenotype-modifying factors are summarized in Table III.

| SNP<br>(gene)                  | Location<br>(variant<br>type) | SIFT<br>Polyphen2<br>MutationTaster                    | Clinical<br>associations              | References   |
|--------------------------------|-------------------------------|--|---------------------------------------|--|
| rs1053023<br>( <i>STAT3</i> )  | 3' UTR<br>variant             | —<br>—<br>Polymorphism                                 | Multiple<br>sclerosis<br>B-NHL<br>BSS | Lu <i>et al.</i> , 2005<br>Butterbach <i>et al.</i> , 2018<br>Pap <i>et al.</i> , 2020 |
| rs1131877<br>( <i>TRAF3</i> )  | Exonic<br>(Missense)          | Tolerated<br>Benign<br>Polymorphism                    | Postradiotherapy<br>toxicity<br>BSS   | De Ruyck <i>et al.</i> , 2011<br>Pap <i>et al.</i> , 2020                              |
| rs202122812<br>( <i>NBR1</i> ) | Exonic<br>(Missense)          | Deleterious<br>Probably<br>damaging<br>Disease causing | BSS                                   | Pap <i>et al.</i> , 2020   |

**Table III. Pathogenicity predictions and clinical associations of the identified phenotype-modifying factors.**

#### 4.2. Identification of two putative genetic variants responsible for phenotypic variants in HMS and PLS

A comparison of the WES data from the PLS and HMS patients carrying the same disease-causing mutation (c.748C/T; p.Arg250X) of the *CTSC* gene identified 35 putative genetic variants (Table IV.), which were present in the HMS patient, but not in the PLS one. The PLS patient did not carry any extra polymorphisms compared to the HMS patient.

| Gene             | SNP         | Polyphen2         | SIFT        | MutationTaster  |
|------------------|-------------|-------------------|-------------|-----------------|
| <i>HLA- A</i>    | rs145046067 | Probably damaging | Deleterious | Normal          |
| <i>HLA- A</i>    | rs150028516 | Probably damaging | Deleterious | Normal          |
| <i>HLA- A</i>    | rs9260156   | Unknown           | Unknown     | Disease causing |
| <i>HLA- B</i>    | rs1050683   | Benign            | Tolerated   | Normal          |
| <i>HLA- DQB1</i> | rs41552812  | Benign            | Tolerated   | Normal          |
| <i>HLA- DQB1</i> | rs1071637   | Damaging          | Deleterious | Normal          |
| <i>HLA- DRB1</i> | rs9269744   | Probably damaging | Deleterious | Disease causing |
| <i>HLA- DRB1</i> | rs3830125   | Benign            | Deleterious | Unknown         |
| <i>HLA- DRB1</i> | rs1136881   | Probably damaging | Deleterious | Disease causing |
| <i>HLA- DRB1</i> | rs1071752   | Benign            | Deleterious | Disease causing |
| <i>HLA- DRB1</i> | rs201614260 | Probably damaging | Tolerated   | Normal          |
| <i>HLA- DRB1</i> | rs148093782 | Benign            | Deleterious | Normal          |
| <i>HLA- DRB5</i> | rs41553512  | Probably damaging | Tolerated   | Disease causing |
| <i>HLA- DRB5</i> | rs41559420  | Benign            | Tolerated   | Disease causing |
| <i>HLA- DRB5</i> | rs1064587   | Benign            | Deleterious | Normal          |
| <i>HLA- DRB5</i> | rs701884    | Benign            | Deleterious | Disease causing |
| <i>HLA-DOA</i>   | rs2582      | Benign            | Unknown     | Disease causing |
| <i>HLA-DPB1</i>  | rs1042121   | Benign            | Tolerated   | Disease causing |
| <i>HLA-DQA1</i>  | rs12722039  | Benign            | Tolerated   | Disease causing |
| <i>HLA-DQA1</i>  | rs12722042  | Damaging          | Tolerated   | Disease causing |

|                 |             |                      |             |                 |
|-----------------|-------------|----------------------|-------------|-----------------|
| <i>HLA-DQA1</i> | rs35087390  | Benign               | Deleterious | Disease causing |
| <i>HLA-DQB2</i> | rs9276570   | Benign               | Tolerated   | Disease causing |
| <i>HLA-DQB2</i> | rs9276572   | Damaging             | Deleterious | Disease causing |
| <i>HLA-DRA1</i> | rs7192      | Benign               | Tolerated   | Unknown         |
| <i>KIR2DL1</i>  | rs199644153 | Benign               | Tolerated   | Normal          |
| <i>KIR2DL1</i>  | rs200746024 | Benign               | Tolerated   | Normal          |
| <i>KIR2DL1</i>  | rs38559888  | Benign               | Tolerated   | Normal          |
| <i>KIR2DL2</i>  | rs3810343   | Benign               | Tolerated   | Normal          |
| <i>KIR2DL2</i>  | rs35719984  | Benign               | Tolerated   | Normal          |
| <i>KIR2DL2</i>  | rs78713511  | Benign               | Tolerated   | Normal          |
| <i>KIR2DL2</i>  | rs200686594 | Benign               | Tolerated   | Normal          |
| <i>KIR2DL3</i>  | rs662386    | Benign               | Deleterious | Normal          |
| <i>MICA</i>     | rs41554412  | Damaging             | Tolerated   | Disease causing |
| <i>OBP2A</i>    | rs55695858  | Possibly<br>damaging | Tolerated   | Polymorphism    |
| <i>SH2D4A</i>   | rs34608771  | Benign               | Tolerated   | Polymorphism    |

**Table IV. WES identified 36 variants, which were all present in the HMS patient, but not in the PLS patient.**

Based on the results of variant prioritization tools and the data of the literature, two out of the 35 variants are suggested as putative phenotype-modifying polymorphisms. According to our current knowledge the other 33 variants are not associated with the functions of the CTSC protein.

| <b>SNP<br/>(gene)</b>           | <b>Location<br/>(variant<br/>type)</b> | <b>SIFT<br/>Polyphen2<br/>MutationTaster</b>      | <b>Clinical<br/>associations</b>       | <b>References</b>           |
|---------------------------------|--|---|--|-----------------------------|
| rs34608771<br>( <i>SH2D4A</i> ) | Exonic<br>(missense)                   | Tolerated<br>Benign<br>Polymorphism               | Development<br>of the HMS<br>phenotype | Pap <i>et al.</i> ,<br>2020 |
| rs55695858<br>( <i>OBP2A</i> )  | Exonic<br>(missense)                   | Tolerated<br>Possibly<br>damaging<br>Polymorphism | Development<br>of the HMS<br>phenotype | Pap <i>et al.</i> ,<br>2020 |

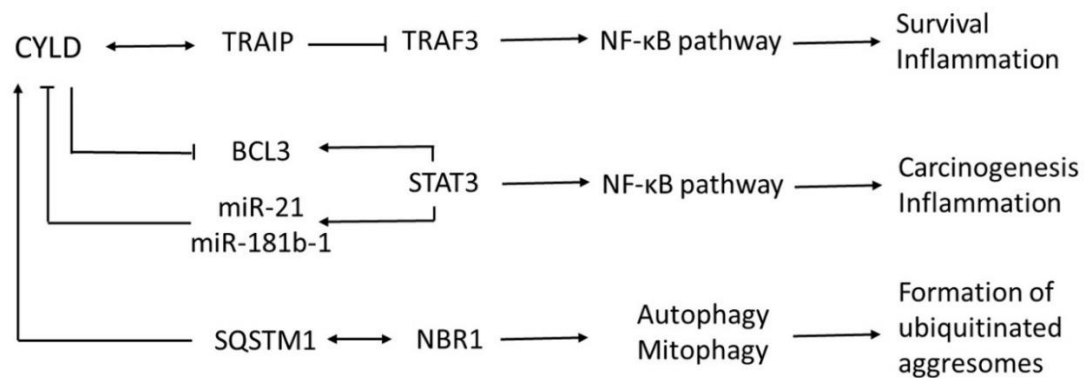
**Table V. Pathogenicity predictions and clinical associations of the identified phenotype-modifying genetic factors.**

The two putative phenotype-modifying polymorphisms are the followings: the rs34608771 SNP of the *SH2 domain containing 4A (SH2D4A)* gene and the rs55695858 SNP of the *odorant binding protein 2A (OBP2A)* gene. Both variants are common missense polymorphisms. Pathogenicity predictions for the identified phenotype-modifying factors are summarized in Table V.

## 5. DISCUSSION

Although the identification of disease-causing mutations is still extremely important for family planning and therapy, direct sequencing is unable to answer clinically relevant questions regarding phenotypic diversity and disease prognosis (Jarinova *et al.*, 2012; Timmerman *et al.*, 2014; Kiritsi *et al.*, 2015; Smith *et al.*, 2019). This limitation of direct sequencing was encountered with the Hungarian and Anglo-Saxon BSS pedigrees previously reported by our workgroup (Nagy *et al.*, 2013). Using haplotype analysis we have demonstrated that the presence of the same *CYLD* mutation in these geographically distant BSS families (the Hungarian one and the Anglo-Saxon one) is the consequence of two independent mutational events (Nagy *et al.*, 2013). These results suggest the position of this recurrent mutation is a mutational hot spot on the *CYLD* gene (Nagy *et al.*, 2013).

Comparing the WES data of the Hungarian and Anglo-Saxon BSS patients, we identified three putative phenotype-modifying genetic variants that potentially explain the striking phenotypic differences among patients carrying the same disease-causing *CYLD* mutation. The genes harbouring these genetic modifying variants code for proteins that are either directly or indirectly functionally linked to *CYLD* (Figure 6.)



**Figure 6. Schematic of the proposed mechanisms of the identified phenotype-modifying factors.**

STAT3 is a transcription factor, which is constitutively activated in a variety of human cancers and plays critical roles in cancer cell survival, metastasis and angiogenesis (Yu *et al.*, 20009). STAT3 is activated by interleukin-6 (IL-6) and

directly activates certain microRNAs (miRs), such as miR-21 and miR-181b-1 (Aggarwal *et al.*, 2009). MiR-21 and miR-181b-1 inhibit the enzymatic activity of CYLD, leading to increased nuclear factor- $\kappa$ B (NF- $\kappa$ B) activity. Thus, STAT3 is not only a downstream target of IL-6 but, with miR-21, miR-181b-1 and CYLD, is part of the positive feedback loop that underlies the epigenetic switch that links inflammation to cancer (Iliopoulos *et al.*, 2010). The B-cell CLL/lymphoma 3 (BCL3) protein, which directly interacts with the STAT3 protein, is deubiquitinated by the CYLD enzyme, and abnormal BCL3 ubiquitination has been associated with the development of basal cell carcinomas (Chaudhary *et al.*, 2015). It has been recently reported that *BCL3* serves as an oncogene in cervical cancer and its oncogenic effect is mediated by *STAT3* (Zhao *et al.*, 2016). Interestingly, the rs1053023 SNP of the *STAT3* gene has already been associated with multiple sclerosis (Lu *et al.*, 2005) and reported in B-cell non-Hodgkin lymphoma (Butterbach *et al.*, 2011). However, this is the first study that suggests a potential phenotype-modifying role for *STAT3* in BSS.

TRAF3 is a member of the TRAF family of proteins, which serve as both crucial intracellular adaptors and E3 ubiquitin ligases that mediate signaling after the activation of various receptors. Receptors that signal through TRAF proteins include those involved in inflammation, innate immune responses, cell death and, most notably, interact with the followings: tumor necrosis factor receptors, Toll-like receptors, RIG-1-like receptors and interleukin-1 receptors (Wang *et al.*, 2010; Hacker *et al.*, 2011). The TRAF-interacting protein (TRAIP) interacts with TRAF3, while TRAIP is reported to interact with CYLD (Chapard *et al.*, 2012). TRAIP expression is increased in basal cell carcinomas and in multiple breast epithelial cell lines with oncogenic potentials ranging from non-malignant to highly invasive (Almeida *et al.*, 2011). Mutations in *TRAF3* and *CYLD* leading to constitutive activation of NF- $\kappa$ B have been identified in cancers, including multiple myeloma and solid tumors (Harhaj *et al.*, 2012). The rs1131877 SNP of the *TRAF3* gene is highly predictive for the development grade  $\geq 2$  acute esophageal postradiotherapy toxicity (De Ruyck *et al.*, 2011), and here we have demonstrated its association with the phenotypic diversity in BSS.

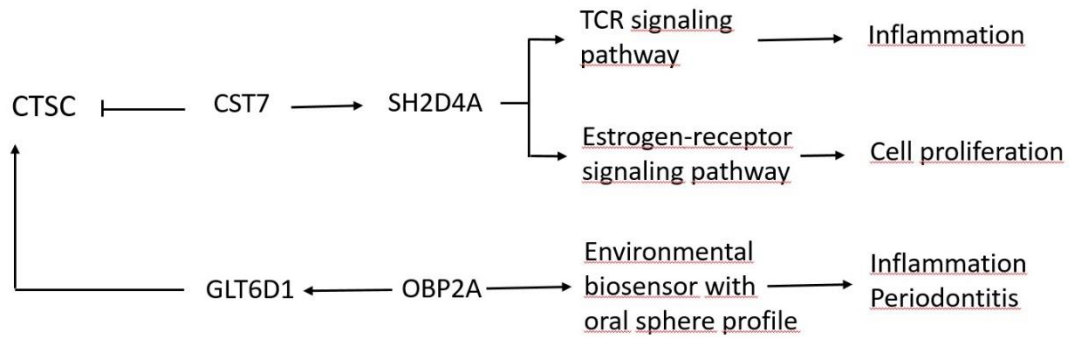
NBR1 is an autophagic adaptor protein involved in the efficient clearance of damaged mitochondria (Shi *et al.*, 2015). Briefly, upon mitochondrial damage, E3 ubiquitin ligases are recruited from the cytosol to depolarized mitochondria, where

they target damaged mitochondrial proteins for ubiquitination and bulk degradation by autophagy (Shi *et al.*, 2014). NBR1 is a functional homolog of sequestosome 1 (SQSTM1), another autophagic adaptor protein, which is a selective autophagy substrate that also acts as a cargo receptor for the degradation of other substrates (Svenning *et al.*, 2011). The interaction of CYLD with TRAFs is dependent upon SQSTM1, and the absence of SQSTM1 results in the reduction of the activity of the CYLD enzyme (Wooten *et al.*, 2008; Into *et al.*, 2010). The rs202122812 SNP of the *NBR1* gene has not been previously associated with any human diseases: this is the first study to indicate its clinical relevance in the development of the BSS-related phenotypic diversity.

The comparison of the WES data of the HMS and PLS patients identified a putative phenotype-modifying genetic variant (rs34608771 SNP) in the *SH2D4A* gene, which encodes a T-cell-expressed adapter protein that is expressed in T-cells, B-cells, macrophages and dendritic cells (Hashimoto *et al.*, 2000). SH2D4A regulates T-cell receptor (TCR) signal transduction in T-cells, and, in human T-cells, its expression increased in response to T-cell activation (Gonçalves *et al.*, 2018). SH2D4A is linked to cathepsin C via cystatin F. This latter protein is a cysteine-protease inhibitor expressed selectively in immune cells, such as T-cells, NK cells and dendritic cells (Obata-Onai *et al.*, 2002). The rs34608771 polymorphism of the *SH2D4A* gene has not been associated previously with any human diseases: this is the first study which links it to the development of the HMS clinical variant and raises its putative association with the phenotypic differences between PLS and HMS patients.

The other putative phenotype-modifying genetic variant (rs55695858 SNP) is located within the *OBP2A* gene, which encodes an odorant-binding carrier protein that has a known environmental biosensor function (Lacazette *et al.*, 2000). The OBP2A protein is expressed in the nasal structures, salivary and lachrymal glands and lungs, and, thus, has an oral sphere profile (Lapinski *et al.*, 2009 and 2019). OBP2A interacts with the glycosyltransferase 6 domain containing 1 (GLT6D1) protein, encoded by the *GLT6D1* gene, which has been identified as a susceptibility locus for periodontitis by genome-wide association studies and this association has been confirmed by several previous studies (Li *et al.*, 2009; Hasim *et al.*, 2015). Although genetic variants of the *OBP2A* gene have been implicated in influencing

the substrate-binding specificity of the encoded protein, none have previously been associated with the development of a human disease (Halfon *et al.*, 1998; Hamilton *et al.*, 2008). Since periodontitis is a major feature of the PLS and HMS phenotypes, we suggest that the rs55695858 SNP of the *OBP2A* gene might contribute to the phenotypic differences observed between PLS and HMS patients (Figure 7.).



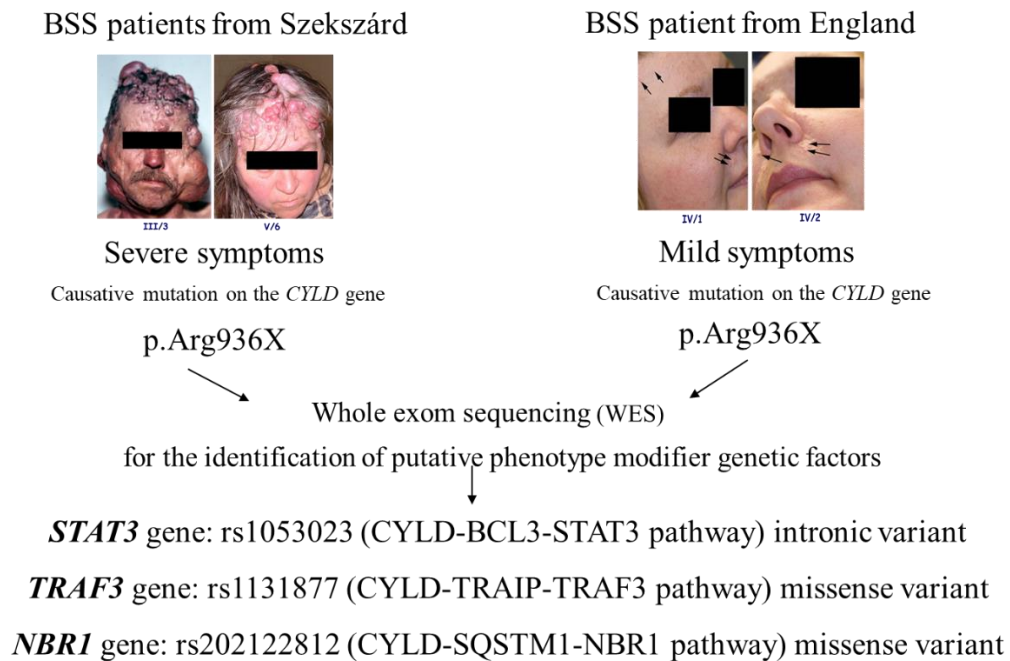
**Figure 7. Schematic of the proposed mechanisms of the identified phenotype-modifying factors.**

Further functional experiments are currently performed by our research group to unveil how the modifying effects of these polymorphisms are realized.



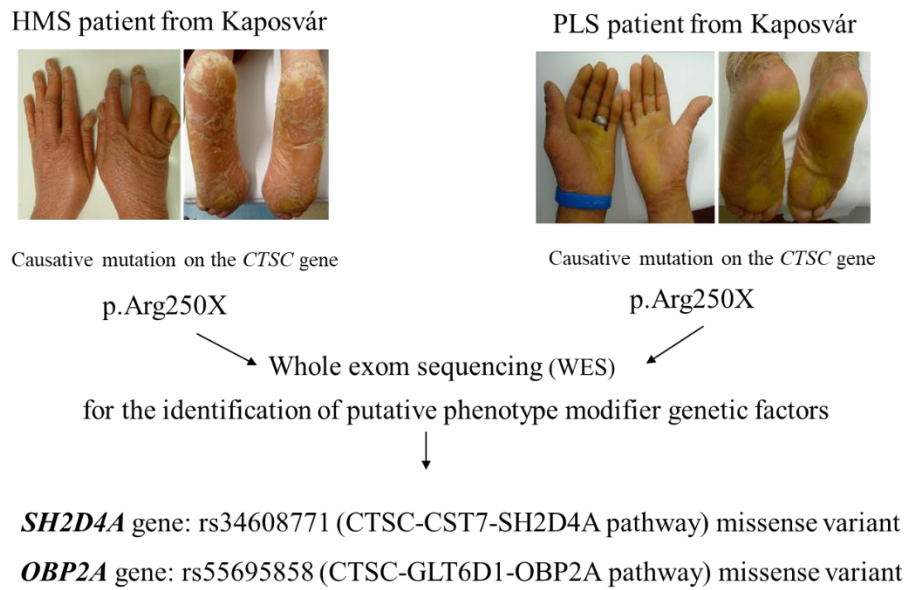
## 6. CONCLUSIONS

Our study aimed to explain the phenotypic differences in BSS patients carrying the same disease-causing *CYLD* mutation by identifying phenotype-modifying genetic polymorphisms (Figure 8.) and to elucidate the phenotypic differences in PLS and HMS patients carrying the same disease-causing *CTSC* mutation by identifying phenotype-modifying genetic polymorphisms (Figure 9.).



**Figure 8. The identified putative phenotype-modifying genetic variants can explain the differences in the severity of the clinical symptoms between BSS patients from Szekszárd and England carrying the same disease-causing *CYLD* mutation (p.Arg936X).**

It should be noted that, in addition to genetic factors, environmental or lifestyle factors might also contribute to the observed phenotypic differences between the investigated patients. Further functional studies are needed to prove the clinical relevance of the identified phenotype-modifying genetic factors and to describe the underlying mechanism that explain their phenotype-modifying roles. Our study contributes to the accumulating evidence supporting the clinical importance of phenotype-modifying genetic factors and their potential to facilitate the elucidation of genotype–phenotype correlations, phenotypic diversity and disease prognosis (Lee *et al.*, 2008).



**Figure 9. The identified putative phenotype-modifying genetic variants can explain the phenotypic differences between PLS and HMS patients carrying the same disease-causing *CTSC* mutation (p.Arg250X).**

Our finding suggests relevant clinical implications. Although the identification of disease-causing mutations remains extremely important for family planning and for the development of novel causative therapeutic modalities, it is not always able to answer clinically relevant questions about phenotype diversity and disease prognosis. According to our view, the next big challenge in human genetics and clinical genetics is to identify these variants. We have joined to this new direction with these investigations demonstrated in my thesis. We are convinced that in the future the results of such investigations will highly contribute to the explanation of the phenotypic differences observed in monogenic diseases and contribute to the deep exploration of the genotype-phenotype relationships. In the clinical practice - primarily in genetic counseling - this knowledge will help us to make a more careful and accurate assessment of the prognosis of monogenic diseases.

## 7. SUMMARY

My investigations focused on the identification of putative phenotype modifying genetic factors in two groups of rare monogenic diseases, which are responsible for the observed phenotypic differences among the affected patients carrying the same disease-causing *CYLD* or *CTSC* mutations.

We recently investigated a Hungarian and an Anglo-Saxon pedigree affected by Brooke-Spiegler syndrome (BSS). Despite carrying the same disease-causing mutation (c.2806C>T, p.Arg936X) of the *CYLD* gene, the affected family members of the two pedigrees exhibit striking differences in their phenotypes. To identify phenotype-modifying genetic factors, WES was performed and the data from the Hungarian and Anglo-Saxon BSS patients were compared.

Three putative phenotype-modifying genetic variants were identified: the rs1053023 SNP of the signal transducer and activator of transcription 3 gene, the rs1131877 SNP of the tumor necrosis factor receptor-associated factor 3 gene and the rs202122812 SNP of the neighbor of BRCA1 gene 1 gene and the functional relevance of the genetic variants were predicted by *in silico* methods.

We investigated two Hungarian patients suffering from different phenotypic variants (PLS and HMS) but carrying the same homozygous nonsense *CTSC* mutation (c.748C/T; p.Arg250X). To gain insights into phenotype modifying associations, WES was performed for both patients and the results were compared to identify potentially relevant genetic modifying factors.

WES revealed two putative phenotype-modifying variants: a missense mutation (rs34608771) of the *SH2 domain containing 4A (SH2D4A)* gene encoding an adaptor protein involved in the intracellular signaling of the cystatin F, a known inhibitor of the cathepsin protein, and a missense variant (rs55695858) of the *odorant binding protein 2A (OBP2A)* gene influencing the function of the cathepsin protein through the glycosyltransferase 6 domain containing 1 (GLT6D1) protein. In silico analysis and literature search results explained how the effected proteins are linked to *CTSC* and possibly modify its functions.

Our investigations contribute to the accumulating evidence on the clinical importance of the identification of phenotype-modifying genetic factors. In the future it will have high potential in the elucidation of genotype–phenotype correlations and disease prognosis.

## **8. LIST OF ABBREVIATIONS**

BCL3 = B-cell CLL/lymphoma 3

BSS = Brooke-Spiegler syndrome

CTSC = cathepsin C gene

CMT = Charcot-Marie-Tooth disease

CYLD = cylindromatosis gene

HMS = Haim-Munk syndrome

HGP = Human Genome Project

NBR1 = neighbor of BRCA1 gene 1 gene

NF- $\kappa$ B = nuclear factor- $\kappa$ B

PLS = Papillon-Lefèvre syndrome

SNP = single-nucleotide polymorphism

SQSTM1 = sequestosome 1

STAT3 = signal transducer and activator of transcription 3 gene

TRAF3 = tumor necrosis factor receptor-associated factor 3 gene

TRAIP = TRAF-interacting protein

WES = whole exome sequencing

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