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Cloud-based data collection and multidisciplinary approach in diagnosis of naevoid basal cell carcinoma syndrome

PhD Thesis

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Szeged,
2023

LIST OF PAPERS RELATED TO THE SUBJECT OF THE THESIS

I. Pál M, **Vetró É**, Nagy N, Nagy D, Horváth E, Bokor AB, Varga A, Seres L, Oláh J, Piffkó J, Széll M.: Whole-Exome Sequencing Identified Two Novel Pathogenic Mutations in the *PTCH1* Gene in BCNS.
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IF: 2.976 Q3

II. **Vetró É**, Oláh J, Kalmár T, Maróti Z, Horváth E, Széll M, Piffkó J, Seres L.: Multidisciplinary approach for mapping genetic variants in naevoid basal cell carcinoma syndrome. Newly identified patched 1 mutation in half-sisters.
Asian J. Dent. Sci. 2022;5,7–14.

III. **Vetró É**, Oláh J, Nagy D, Széll M, Piffkó J, Seres L.:
A Gorlin–Goltz-szindróma genetikai aspektusai [Genetic aspects of Gorlin-Goltz syndrome]
Orv Hetil. 2020;161:2072-2077. [Hungarian]
IF:0.54 Q4

LIST OF PAPERS NOT RELATED TO THE SUBJECT OF THE THESIS

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IF: 2.046 Q2

Contents

List of abbreviations

ACMG	-	American College of Medical Genetics and Genomics
BCC	-	Basal cell carcinoma
BWA	-	Burrows–Wheeler Aligner
CBCT	-	Cone-beam computed tomography
CGH – array	-	Comparative genomic hybridisation - array
CT	-	Computed tomography
dbSNP	-	Database of Single Nucleotide Polymorphisms
DNA	-	Deoxyribonucleic acid
ECT	-	Electrochemotherapy
EDTA	-	Ethylenediaminetetraacetic acid
EVS	-	Exome Variant Server
ExAC	-	Exome Aggregation Consortium
GATK	-	Genome Analysis Toolkit
GLI	-	Glioma associated oncogene
GLI 1	-	Glioma-associated oncogene family zinc finger 1
GLI 2	-	Glioma-associated oncogene family zinc finger 2
GLI 3	-	Glioma-associated oncogene family zinc finger 3
Hh	-	Hedgehog protein
KC	-	Keratocyst
MLPA	-	Multiplex ligation-dependent probe amplification
MRI	-	Magnetic resonance imaging
mRNA	-	Messenger ribonucleic acid
NBCCS	-	Nevoid basal cell carcinoma syndrome
NGS	-	Next-generation sequencing
OPG	-	Orthopantomogram
PCR	-	Polymerase chain reaction
PTCH1	-	Patched 1 gene
Ptch1	-	Patched 1 protein
PTCH2	-	Patched 2 gene
RNA	-	Ribonucleic acid
SALSA	-	Selective Adaptor Ligation, Selective Amplification

SHH	-	Sonic Hedgehog signaling pathway
SMO	-	Smoothened gene
Smo	-	Smoothened protein
SUFU	-	Suppresses of Fused gene
UV	-	Ultraviolet
WES	-	Whole exome sequencing
WGS	-	Whole genome sequencing

1. Introduction

Naevoid basal cell carcinoma syndrome (NBCCS) also known as Gorlin - Goltz syndrome or basal cell nevus syndrome is a rare, hereditary condition that is characterized by a wide range of developmental disorders and a predisposition to different malignancies.

Reports of the disease that fit the description of Gorlin syndrome go back as far as 1894 when Jarisch and White published an article about a patient whose symptoms clearly resembled what we now call NBCCS **[White 1894**]. Nearly seven decades later, in 1960, Gorlin and Goltz recognized the symptoms as a syndrome, based on their findings **[Gorlin 1960]**.

1.1. Epidemiology

To obtain precise information regarding its incidence and prevalence is difficult, due to its rarity and the fact that individuals with milder features may not be recognized. The estimated prevalence varies over a wide range, the most quoted one is 1:57.000 but in Italy, it was reported as 1:256.000 **[Evans 1991, Kimonis 1997]**. Its incidence at birth was assessed to be 1:19.000 [**Farndon 1992**]. NBCCS affects both women and men in a rather equal distribution. As for races, all are affected and have been reported worldwide, although African Americans and Asians represent only 5% of the cases.

1.2. Etiopathogenesis – Hedgehog signaling pathway

NBCCS is an autosomal dominant disorder which can be caused by mutations in the protein patched homolog 1 (PTCH1) gene on chromosome 9q22.32., and protein patched homolog 2 (PTCH2) gene on chromosome 1p34.1. or suppressor of fused homolog (SUFU) gene on chromosome 10q24.32. **[Johnson 1996, Spadari 2022]**.

In most cases, mutations of the PTCH1 gene are responsible for the development of NBCCS **[Farndon 1992, Chenevix-Trench 1993, Compton 1994**]. PTCH1 is a tumour suppressor gene which encodes the patched-1 transmembrane receptor (Ptch1) that recognizes the hedgehog (Hh) protein **[Lam 2013, Bresler 2016]**. As shown in **Figure 1**, in the lack of ligand Hh, PTCH1 exerts an inhibitory effect on Smoothened (SMO), a downstream protein in the Sonic Hedgehog signaling pathway (SHH). This inhibition is ceased upon Hh binding to Ptch1, during a catalytic process that does not demand a direct interaction between Ptch1 and Smo **[Aza-Blanc 1997, Méthot 1999**]. Despite investigations, the molecular mechanism of the interplay

among Ptch1 and Smo remains undefined, but a new theory assumed that Ptch1 acts as a sterol pump, eliminating the oxysterols made by 7-dehydrocholesterol reductase **[Gong 2018]**. Functional disturbance of protein ptch1 results in the accumulation of oxysterols around SMO. Therefore, this accumulation leads to the activation of the nuclear transcription factors glioma-associated oncogene homologs GLI1, GLI2 and GLI3. Due to this cascade, the activated GLI turns on the expression of Hh-targeted genes **[Rimkus 2016, Bresler 2016]**.

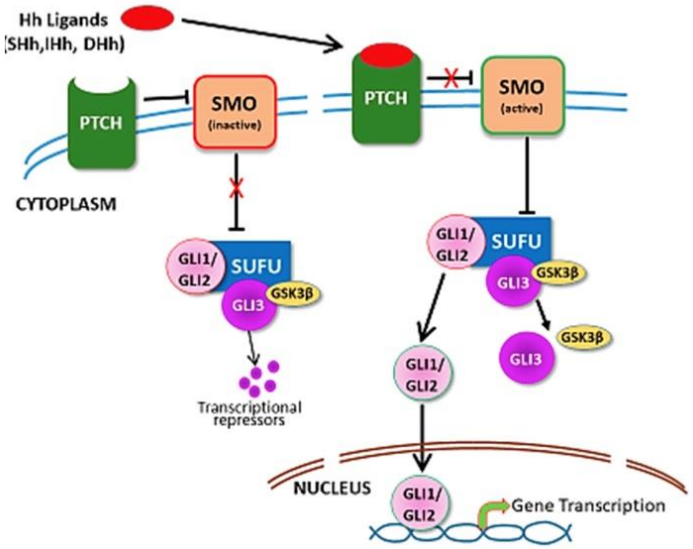

Figure 1. The Sonic Hedgehog signaling pathway [**Bhateja 2019**]

In a few cases, variations in the PTCH2 gene, which also encodes a transmembrane receptor protein, have been found in patients meeting the criteria for NBCCS **[Smyth 1999]**. However, it has been suggested that occasional variants in PTCH2 may not be conclusive.

SUFU gene is a negative regulator of the SHH pathway and it encodes a cytoplasmic protein, which inhibits the GLI1 protein. Thus, mutations in the SUFU gene result in activating the SHH pathway.

All the above-mentioned genes and proteins play an essential role in the SHH signaling pathway. This signaling cascade bears a mandatory part in cell growth and differentiation, in the regulation of embryogenesis and in the development of the brain, spinal cord and teeth **[Hui 2011, Taipale 2011, Choudhry 2014]**. SHH also plays an important role in nervous system cell-type specification and in limb patterning. Malfunctions of the signaling pathway were proven to be involved in over 50% of cancer types and may lead to developmental disorders, affecting the differentiation processes of embryogenesis, tissue regeneration, and cell division processes **[Pál 2023]**.

1.3. Clinical features

Signs and symptoms of NBCCS are extremely variable; the integumentary, central nervous, genitourinary, cardiovascular, ocular, auditory and even skeletal systems may be affected. Most patients suffering from NBCCS experience the development of multiple jaw keratocyst (KCs) and/or basal cell carcinomas (BCCs) (**Figure 2**).
The most common and relevant signs of NBCCS are the following:

1.3.1. Integumentary system

Basal cell carcinomas are present in 50-97% of all affected patients, mostly between puberty and 35 years of age, although they can appear even in early childhood (2 years of age) **[Ortega 2008, Kiran 2012]**. On the other hand, studies have claimed that only 40% of black patients affected by NBCCS develop BCC **[Kimonis 1997]**. These lesions show high clinical and histopathological heterogeneity. Syndromic BCCs and non-syndromic basal cell carcinomas cannot be distinguished by histopathological characteristics, but the risk of recurrent BCC with NBCCS is higher than that of non-syndromic BCC. Usually, they occur on the face, chest and back, in the form of skin-coloured, pink or pigmented lesions. The numbers of these basal cell carcinomas may vary from a few to hundreds and show differences in size from a few millimetres to several centimetres in diameter (**Figure 2**). BCCs usually grow in an indolent manner, but in the absence of treatment, they become locally invasive.

Other common skin signs are *palmar and plantar pits* (>2), which affect approximately 30-65% of patients **[Gutierrez 1986, Lo Muzio 2008]**. They usually appear in the second decade of life as a result of the partial or entire lack of the cutaneous keratinized layer in the palm and in the sole of the foot **[Spadari 2022]**. The number of pits permanently increases with age **[Gutierrez 1986]**. They are manifested as depressed, pink or flesh-coloured dimples having a diameter of 2-3 mm and a depth of 1-3 mm. The lesions become more visible after soaking the hand and feet in warm water for a short time **[Evans 2018]**. Overall, palmar and plantar pits are harmless lesions so they do not require any intervention.

Many other skin-related symptoms have been reported in NBCCS patients, including but not limited to multiple nevi, benign dermal cysts, epidermoid cysts, and hairy skin patches, but these symptoms are less common than BCCc and plantar and palmar pits **[Lo Muzio 2008]**.

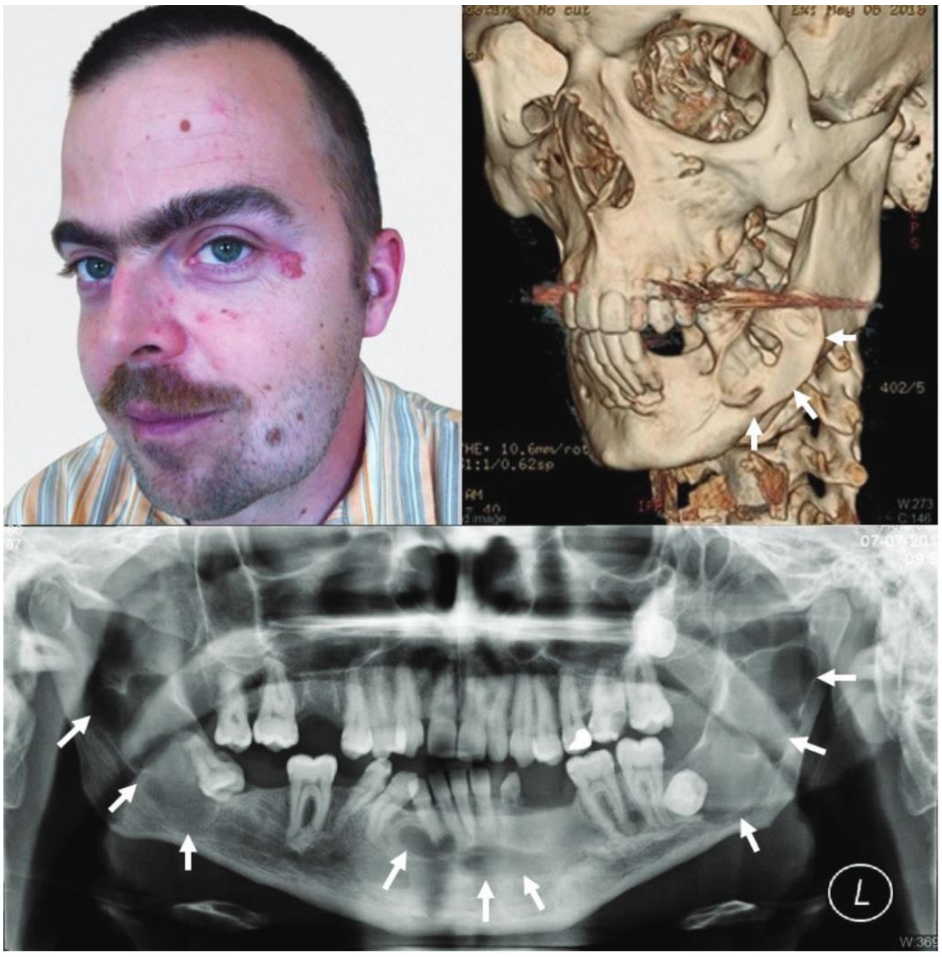

Figure 2. Clinical features of an NBCCS patient. Multiple BCCs on the face and multiple jaw keratocysts (white arrows) in the mandible.

1.3.2. Multiple jaw keratocysts

80% of NBCCS patients are affected by *multilocular jaw keratocysts (KCs)*, which are asymptomatic, but locally aggressive lesions in most cases **[Lo Muzio 2008]**. KC's usually first appear during adolescence and rarely develop after the age of 30. Clinical signs may include facial asymmetry, swelling, displaced, impacted teeth and pain. Definitive diagnosis of KC is largely based on histological examination of samples obtained during surgery. Histologically the epithelium of KC can show budding of the basal lamina into the underlying connective tissue with the development of separated microcysts, which have been labelled as satellite or daughter cysts **[Gorlin 2004]**. Since multiple jaw keratocysts may be the first and sole symptom of NBCCS, oral and maxillofacial surgeons and dentists can be the first to detect the syndrome.

Other orofacial defects are cleft lip and palate, malocclusion, dental agenesis, dental ectopy and dental heterotopy, which may arise in NBCCS patients **[Ruprecht 1987, Soekarman 1991]**.

1.3.3. Central nervous system

Lamellar calcification of the falx cerebri affects 70-85% of the patients diagnosed with NBCCS **[Kimonis 1997]**. The calcification of the falx cerebri can be seen early in childhood with salient radiological findings, which can highlight the symptom in the background. Mineralization of other regions of the nervous system may involve the tentorium cerebelli (20-40%), the diaphragma sellae (60-80%) and the sella turcica (25%) **[Ratcliffe 1995]**.

1.3.3.1. Medulloblastoma

While in the sporadic form, the peak incidence of medulloblastoma is between 7 and 8 years of age, NBCCS-associated medulloblastoma appears during the first two years of life **[Lo Muzio 2008]**. Approximately 5% of all individuals with NBCCS are affected by this insidious brain malignancy **[Cowan 1997]**. The male-to-female ratio is 3:1. Alarming symptoms of this primitive neuroectodermal tumour can include slow suction reflexes, the presence of hydrocephalus and hyporeactivity **[García-Espinosa 2021]**. In the case of early-onset medulloblastoma, NBCCS should be suspected, nevertheless, NBCCS-associated medulloblastoma shows a more favourable prognosis than the sporadic form of that **[Amlashi 2003]**.

1.3.4. Skeletal system

Musculoskeletal abnormalities occur frequently in approximately 60% -75% of the patients. These abnormalities might be present at birth, but will not be apparent clinically. *Sprengel deformity, bifid-, fused-, splayed-, underdeveloped ribs, frontal bossing, pectus deformity, vertebral fusion, syndactyly, and polydactyly* can be seen in NBCCS **[Kimonis, Evans 2018**]. Severe skeletal defects ruining the quality of life have been reported, but are uncommon, usually, these anomalies do not cause any problems.

Relative *macrocephaly* is likely to be observed as the first awareness-raising symptom after birth, which has been reported in 50% of cases. The term „relative macrocephaly" is used to describe the phenomenon that a head circumference is not above two standard deviations from the mean but appears disproportionately large compared to the weight and height parameters of the patient **[Williams 2008]**. Macrocephaly (frontal bossing, coarse facial features, and facial milia) is one of those externally recognizable clinical features which affect approximately 60% of NBCCS individuals **[Evans 2018]**.

1.3.5. Cardiovascular, genito-urinary and ocular systems

NBCCS can be diagnosed in 3-5% of all patients with a *cardiac fibroma*. Fibroma of the heart may be asymptomatic, but its initial signs in children may include unexplained heart failure or arrhythmias **[Bossert 2006]**.

25-50% of women affected with NBCCS are diagnosed with *ovarian cysts and fibromas*. These lesions are often bilateral and they do not seem to cause fertility problems, however, torsions of the ovary may occur **[Lo Muzio 2008]**. 5-10 % of males suffer from *hypogonadotropic hypogonadism, gynecomastia or cryptorchidism*.

Myriad ophthalmologic features have been reported in association with NBCCS and the most common ones are *congenital cataracts, hypertelorism, retinal anomalies, eyelid cysts, exophthalmos, internal strabismus, rotatory nystagmus* **[Chen 2015]**.

The high phenotypic variability of NBCCS can lead to delayed diagnoses and, subsequently, delayed treatments that greatly increase associated morbidity.

1.4.Genetic background

NBCCS exhibits an autosomal dominant mode of inheritance with variable expression and nearly complete penetrance **[Boutet 2003]**. 70 to 80% of patients with NBCCS have a familial aggregation of symptoms and de novo pathogenic variants are responsible for 20-30% of cases **[Okamoto 2014]**. In rare cases, gonadal or somatic mosaicism may occur **[Kijma 2012)]**. In approximately 50-85% of NBCCS cases sequence variations of the *PTCH1* gene, in 6–21% duplication or deletion of exon(s) of the *PTCH1* gene, in approximately 5% sequence variations of the *SUFU* gene, and in 1% deletion or duplication of exon(s) of the *SUFU* gene play role in the development of the disease **[Evans 2002]**. In 15-27% of patients, the genetic mechanism causing NBCCS remains unknown. In general, NBCCS patients with *SUFU* variants have an increased risk of developing medulloblastoma compared to those carrying *PTCH1* mutations **[Spiker 2018, Pál 2023]**.

Tumours in patients with NBCCS are believed to develop according to the two-hit hypothesis, also known as the Knudson hypothesis **[Levanat 1996]**. The patients usually carry one heterozygous germline variant of the PTCH1 or SUFU genes. Development of the tumours requires most tumour suppressor genes for both alleles to be inactivated. The inactivation of the remaining normal allele is usually the consequence of "second hit" somatic variants of the *PTCH1* or *SUFU* gene. Second-hit mutations can be induced by, for example, ultraviolet light or ionizing radiation. In addition to the standard two-hit model, haploinsufficiency, dominant-negative isoforms and epigenetic silencing may be involved in the development of the tumours [**Pan 2010]**.

Both PTCH1 and SUFU are also involved in other genetic diseases. Mutations in the *PTCH1* gene have been associated with holoprosencephaly type 7 [**Richieri-Costa 2017]**. *SUFU* mutations have been described in familial meningioma, desmoplastic medulloblastoma, and Joubert syndrome-32, the latter of which is a developmental disorder that is characterized by delayed psychomotor development, intellectual disability, dysmorphic facial features, and postaxial polydactyly **[Aavikko 2012, Brugiéres 2012, Serpieri 2022]**.

Variants of the PTCH1 gene were first reported in 1996 [**Hahn 1996]**. From the reported variants, only 69 are pathogenic or likely pathogenic, and the rest are classified as having unknown significance or as being benign.

The first SUFU variant associated with NBCCS was published in 2009. Currently, 28 pathogenic or likely pathogenic unique variants have been reported for SUFU **[Pastorino 2009]**.

The first suggestion of a pathogenic role for *PTCH2* in NBCCS was published in 1999 **[Smyth 1999, Pál 2023]**. Since the *PTCH2* gene encodes a protein that is highly homologous to PTCH1, the question emerged as to whether it is also a disease-causing gene in NBCCS. Using single-stranded conformational polymorphism analysis, a truncating mutation of the *PTCH2* gene was identified in a patient with medulloblastoma **[Smyth 1999]**. Subsequently, a few additional publications described cases in which variants of *PTCH2* were linked to NBCCS **[Fan 2008, Xu 2008]**. However, direct evidence to support this proposed association is lacking. To date, no pathogenic *PTCH2* variants associated with the NBCCS phenotype have been reported. Thus, it is not known whether *PTCH2*-caused NBCCS is, for example, a rarely reported subtype with a milder phenotype than NBCCS arising from other mutations or *PTCH2* does not predispose to NBCCS but, instead, is a phenotype modifier **[Fuji 2013**, **Smith 2022]**. Somatic mutations in *PTCH2* have been implicated in the development of BCCs and medulloblastoma **[Smyth 1999, Pál 2023]**.

1.5.Diagnosis

The diagnosis of NBCCS is based on the recognition of the aforementioned characteristic clinical findings and the identification of a pathogenic genetic variant.

Diagnostic criteria were first established by Evans et al. in 1993 **[Evans 1993]**. In 1997, it was modified slightly by Kimonis et al **[Kimonis 1997]**. The late updates were proposed in 2011 when Bree et al. made some suggestions regarding the major criteria **[Bree 2011]**.

Currently, a diagnosis of NBCCS requires (1) one major criterion and molecular confirmation or (2) the presence of two major diagnostic criteria or (3) one major and two minor diagnostic criteria. Major criteria include (a) multiple BCCs or one BCC by the age of 20 years, (b) odontogenic keratocysts of the jaw (as proven by histology), (c) palmar or plantar pitting, (d) bilamellar calcification of the falx cerebri, (e) medulloblastoma and (f) first-degree relatives with NBCCS. Minor criteria include macrocephaly, congenital malformations, skeletal abnormalities, radiologic abnormalities and ovarian or cardiac fibromas **[Spiker 2023]** (**Table 1**).

Major criteria

1. Odontogenic keratocysts of the jaws proven by histopathology
2. Bilamellar calcification of the falx cerebri
3. Palmar or plantar pits (3 or more)
4. Multiple (>2) BCCs or one under 20 years
5. First-degree relatives with NBCCS
6. Medulloblastoma

Minor criteria

1. Macrocephaly
2. Skeletal abnormalities
   - *Bifid, fused ribs*
   - *Polydactyly*
   - *Syndactyly*
   - *Sprengel deformity*
   - *Pectus deformity*
3. Ocular system
   - *Congenital cataracta*
   - *Glaucoma*
   - *Strabismus*
   - *Coloboma*
4. Facial features
   - *Frontal bossing*
   - *Hypertelorism*
   - *Wide nasal bridge*
   - *Coarse facial features*
   - *Cleft lip and/or palate*
   - *Facial milia*
5. *Lymphomesenteric cysts*
6. *Cardiac fibroma*
7. *Ovarian fibroma*
8. *Intellectual deficit*

Table 1. Diagnostic criteria for NBCCS according to Bree et al.

1.5.1. Radiological aspects of diagnosis

Radiological findings are of great importance in establishing a proper diagnosis. Not to mention the fact that in the absence of major features, which are often not recognizable until the teen years, it is radiological signs that can predict the presence of the syndrome. Besides, regular follow-ups of particular symptoms require the use of radiological methods **[Bree 2011]**. However, based on the abovementioned Knudson hypothesis, authors currently suggest that because of the attendant radiation exposure, radiological examinations without therapeutic consequences should be avoided as much as possible **[Verkouteren 2022]**.

Multiplex jaw keratocysts can be screened with an *orthopantomogram (OPG)*. Lesions are characterized by unilocular or multilocular radiolucencies. Most commonly the molar-ramus region is affected in the mandible, followed by the incisor-canine region **[Lo Muzio 2008]** (**Figure 2**). In the preoperative planning of these possibly extensive cystic lesions, *cone-beam computed tomography (CBCT)* or *CT* scans are very helpful tools because of the higher spatial resolution **[Borghesi 2018]**.

Magnetic resonance imaging (MRI) should be considered in children with a PTCH1 mutation, in order to rule out the presence of medulloblastoma when characteristic clinical features or atypical psychomotor development are present. Likewise, baseline MRI is recommended in children with a SUFU mutation **[Verkouteren 2022]**.

Additional examinations, including *posteroanterior (PA) and lateral skull X-rays* for ectopic calcification; *chest X-ray* for evaluation of bifid ribs; *posteroanterior chest* and *lateral spine* for scoliosis and vertebral deformities are reasonable methods for establishing the diagnosis of NBCCS **[Bree 2011]**.

Cardiological and genito-urinary symptoms can be evaluated by *ultrasound* **[Verkouteren 2022]**.

1.5.2. Genetic aspects of diagnosis

The attitude regarding the importance of genetic testing in the management of NBCCS has changed over the past decade. Previously, these methods played a less significant role in the therapeutic strategy, due to these investigative technique's limited availability and high costs **[Verkouteren 2022]**. Recently, laboratory techniques have improved rapidly, which has resulted in easier, faster and more affordable genetic testing panels **[Onodera 2020]**.

Consequently, authors suggest that genetic testing for NBCCS is necessary for the following situations: [1] establishing the diagnosis in the lack of sufficient phenotypic criteria; [2] for patients at risk who do not meet the diagnostic criteria but have an affected family member; [3] prenatal testing in the presence of a recognized mutation in the family **[Spiker2023, Spadari 2022]**.

In NBCCS the suggested stepwise sequence of performing a serial test of a single gene is the following: [1] PTCH1 sequence analysis; [2] PTCH1 deletion/ duplication analysis; [3] SUFU sequence analysis; [4] SUFU deletion/ duplication analysis; [5] PTCH1 ribonucleic acid (RNA) analysis **[Evans 2018, Spadari 2022]**. The importance of testing the PTCH2 gene remains controversial, due to the insufficient evidence of its causative role **[Lo Muzio 2013]**.

Besides serial testing of a single gene, the application of multigene panels and more comprehensive genomic testing may help to map mutations in probands **[Smith 2016, Bholah 2014, Klein 2005, Marsch 2005**].

Sequence variations of the *PTCH1* and SUFU genes are responsible for approximately 50-85% and 5% of NBCCS cases, respectively. However, formerly Sanger sequence analysis was considered the gold standard, aiming at identifying the exact order of nucleotide bases, but this method is time- and cost-consuming **[Stuppia 2012, Onodera 2020]**.

Next-generation sequencing (NGS) techniques, including whole exome sequencing (WES), are more eligible for high-throughput and large-scale sequencing and detecting point mutations, not to mention the fact that they are faster and more affordable than their predecessors **[Xia 2021].** By nature, WES is not able to identify large deletions or duplications which can be the causative types of mutations in 6-21% of patients affected by NBCCS. This situation can be solved by the usage of multiplex ligation-dependent probe amplification (MLPA) which is a specific PCR assay and an advantageous tool to scan large deletions and duplications, however, it cannot detect point mutations or mutations not reported before **[Lo Muzio 2013, Xia 2021]**.

If all the above-mentioned tests fail to detect alterations, but the symptoms are suspicious for NBCCS, then DNA samples from two different BCCs can be tested for PTCH1 and SMO to reveal the possibility of postzygotic mosaicism in the background **[Verkouteren 2021]**.

If a variation is detected, the relevance of the mutation and its effect on the protein function should be verified **[Richards 2015, Verkouteren 2022]**. Solely verified pathogenic, or likely pathogenic variants can validate the diagnosis and ascertain the clinical signs **[Richards 2015]**. Besides all efforts in 15-27% of patients, the genetic mechanism causing NBCCS remains unknown **[Pál 2023**].

1.6.Treatment and surveillance

The treatment and surveillance of NBCCS always need a multidisciplinary approach. The diversity and the course of symptoms highlight the need for the collaboration of different specialities and life-long follow-ups of the patients **[Škodrić-Trifunović 2015]**.

1.6.1. Basal cell carcinoma

Therapeutic recommendations for BCCs have been thoroughly discussed in the international literature **[Work 2018, Peris 2019]**. The patient's age and gender along with the size, depth and location of lesions, all influence the choice of the treatment. Multiple BCCs associated with NBCCS should be considered and managed as locally aggressive basal cell carcinomas **[Peris 2019]**.

In general, *surgical excision* is the gold standard to treat these lesions, its curative rate is 95% **[Drucker 2018]**. *Mohs micrographic surgery* is suggested for extensive and recurrent lesions where the clinical margin is not apparent **[Quazi 2020]**. Despite the effectiveness of surgical interventions, the accompanying pain and discomfort and the risk for cosmetic and functional defects have prompted researchers to develop other treatment methods **[Mitropoulos 2008]**.

Electrochemotherapy (ECT) is a durable and effective option for primary BCC **[Clover 2020]**. This minimally invasive technique is based on the combination of electroporation and chemotherapy. Electric pulses create temporary pores in the cell membrane, allowing molecules, such as drugs to enter the cells **[Pichi 2018, Clover 2020]**.

Another possibility for patients who are not candidates for surgery can be the systemic treatment with *Smoothened (Smo) inhibitors*. As it was discussed previously, in NBCCS the mutated patched-1 transmembrane receptor is not able to inhibit Smo, leading to the continuous induction of hedgehog genes, which results in the development of multiple BCCs **[Bánvölgyi 2020]**. Smo inhibitor vismodegib (Erivedge, Genentech/ Roche) is an amenable option for managing lesions which are either irresectable or of which surgical therapy would result in unfavourable aesthetic and functional outcomes. Consequently, Smo inhibitors can be the optimal choice of treatment in NBCCS when a substantial number of lesions would require expansive and multiple surgeries **[Sekulic 2017]**. However, the success of Smo inhibitor therapy can be limited by its drug-related adverse events. Muscle spasms, dysgeusia, loss of

appetite, fatigue and universal alopecia may occur during the treatment, which can severely impact the quality of life **[Bánvölgyi 2020]**.

From the development of the first BCC, dermatologic follow-ups should be scheduled every 4-6 months or on an individual basis. The avoidance of excessive exposure to UV radiation and adequate sun-protective measures are also very important **[Peris 2020]**.

1.6.2. Multiple jaw keratocysts

The aim of the management of jaw keratocysts (KCs) is always to remove the cyst and be attentive to preserving dental elements, neurovascular structures and functionality.

The most conservative methods are *marsupialisation* and *decompression*. The objective of these procedures is to reduce the intracystic pressure, considering this can promote bone remodelling inside the cystic cavity. Subsequently, the size of the cyst may decrease, allowing the removal of the residual lesion. Both are particularly effective in the treatment of paediatric patients and the management of extensive lesions **[Spadari 2022]**.

Enucleation is the primary method of the removal of KCs, which consists of the eradication of the lining epithelium in its entirety, in a single surgical procedure **[Morgan 2005]**. Enucleation can be supplemented by the usage of Carnoy's solution and peripheral ostectomy. Carnoy solution is a cauterizing mixture of agents (by volume: 75% ethanol, 25% concentrated acetic acid) that seems to be able to dissolve epithelial remnants **[Donnelly 2021]**. Peripheral ostectomy is an additional surgical manoeuvre, which consists of the removal of 1,5-2 mm of bone with a rotary instrument in cystic margins **[Khalil 2023]**. Both fore-mentioned procedures aim to decrease the presence of residual satellite cysts, as these epithelial islands could be the source of recurrence. According to previous studies, one of the most troublesome features of KCs in patients with NBCCS was that the probability of recurrence is 60%, compared to the sporadic form which was 17,6% **[Myoung 2001, Kim 2022]**.

The lowest recurrence rates have been observed related to *„en block" or segment resection*. This aggressive technique, which involves the removal of an entire part of the jaw bones without maintaining continuity, should be reserved only for KCs that have perforated the cortical plate of the bones **[Spadari 2022]**.

Long-term follow-ups must be done after the removal of KCs. Clinical and radiographic examinations are suggested every 6 months and the surveillance period of recurrence should be more than 10 years according to the literature **[Kim 2022, Spadari 2022]**.

1.6.3. Paediatric patients

Paediatric patients with NBCCS require special attention from early childhood. Given the risk of medulloblastoma, an MRI scan is suggested as soon as possible and should be repeated until 8 years of age **[Spiker 2023]**. Cardiac and pelvic ultrasound examinations are recommended to evaluate cardiac and ovarian fibromas. If a suspicion arises that the affected child may have a sense organ, mental, or developmental disorder, then neurological, psychological speech, vision and hearing screening should be performed **[Bree 2011]**.

2. Objectives

Our aim was to conduct a retrospective and prospective cohort study to evaluate patients affected by NBCCS. The main objectives of the study are the following:

1) set up a multidisciplinary team with experts in the diagnosis and management of NBCCS
2) retrospective and prospective, cloud-based data collection of NBCCS patients
3) unveil the major and minor characteristics of the probands
4) subject to probands genetic investigation
5) find a correlation between phenotypic and genotypic values
6) examine the utility of MLPA end WES in mapping the genetic background of NBCCS
7) organize the management and surveillance of affected probands.

3. Materials and methods

4.1. Ethical permission

Following the approval of the local Scientific and Research Ethics Committee of the Medical Research Council (license number: 58523-4/2017/EKU; 19.02.2018.) an online spreadsheet-based questionnaire has been created and made accessible for experts involved in the study. Written informed consent was obtained from all the enrolled patients according to a protocol approved by the Hungarian National Public Health Centre, in adherence to the Helsinki guidelines. All the enrolled individuals underwent pre- and post-test genetic counselling at the Department of Medical Genetics, University of Szeged (Szeged, Hungary).

4.2. Data collection

A multidisciplinary team, including oral and maxillofacial surgeons, dermatologists, radiologists, and clinical geneticists worked out a diagnostic protocol for all patients suspected of NBCCS.

4.3. Patients

Sixteen Hungarian patients from 11 families fulfilling the diagnostic criteria of NBCCS were enrolled in this study during an eight-year period.

The inclusion criteria of the study were those updated by Bree et al. in 2011 regarding the diagnosis of NBCCS:

(1) one major criterion and molecular confirmation
(2) the presence of two major diagnostic criteria
(3) one major and two minor diagnostic criteria.

All those patients who did not display any symptoms of NBCCS were excluded from the study.

No attempt was made to confirm the possible presence of postzygotic mosaicism.

4.4. Radiological examination

Panoramic radiography, chest x-ray, posterior-anterior skull radiography, and abdominal and pelvic ultrasound are performed in all cases.
If a genitourinary or a central nervous system disorder is suspected in radiology or upon clinical symptoms, magnetic resonance imaging of the region of interest is carried out, too
.

4.5. DNA Extraction

Genomic DNA was extracted from venous blood mixed with the anticoagulant EDTA using the DNeasy® Blood & Tissue Kit (QIAGEN, Germany), as described in the manufacturer's instructions. For quantification, Qubit Fluorometric Quantification instrument was used according to the manufacturer's instructions.

4.6. Whole-Exome Sequencing

Genotypes of patients were determined using next-generation sequencing. Library preparation was carried out using the SureSelectQXT Reagent Kit (Agilent Technologies, Santa Clara, CA, USA). Pooled libraries were sequenced on an Illumina NextSeq 550 NGS platform using the 300-cycle Mid Output Kit v2.5 (Illumina, Inc., San Diego, CA, USA). Adapter-trimmed and Q30-filtered paired-end reads were aligned to the hg19 Human Reference Genome using the Burrows–Wheeler Aligner (BWA). Duplicates were marked using the Picard software package. The Genome Analysis Toolkit (GATK) was used for variant calling (BaseSpace BWA Enrichment Workflow v2.1.1. with BWA 0.7.7-isis-1.0.0, Picard: 1.79 and GATK v1.6-23-gf0210b3).

The mean on-target coverage achieved from sequencing was 71× per base, with an average percentage of targets covered greater or equal to 30× of 96% and 90%, respectively. Variants passed by the GATK filter were used for downstream analysis and annotated using ANNOVAR software tool (version 2017 July 17) **[Wang 2010]**. Single-nucleotide polymorphism 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 analysed and annotated. Variants were filtered according to read depth, allele frequency and prevalence in genomic variant databases, such asExAc (v.0.3) and Kaviar. Variant prioritization tools (PolyPhen2, SIFT, LRT, Mutation Taster, and Mutation Assessor) were used to predict the

functional impact. For variant filtering and interpretation, VarSome and Franklin bioinformatic platforms [https://franklin.genoox.com, accessed on 18 May 2023] were used according to the guidelines of the ACMG **[Richards 2015, Kopanos 2019, Pál 2023]**.

All the identified candidate variants were confirmed via bidirectional capillary sequencing. PCR amplification was set up using DreamTaq™ Green PCR Master Mix ready-to-use solution (Thermo Scientific™), as described in the manufacturer's instructions. Reaction conditions were as follows: initial denaturation at 95 °C for 1 min, denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, extension at 72 °C for 30 seconds with 35 repeated cycles, and a final extension at 72 °C for 10 min. The following primers were used:

Exon6 Fw: 5′ ctacaaggtggatgcagtgg 3′

Exon6 Rev: 5′ aagtgaacgatgaatggacac 3′

Exon11 Fw: 5′ gctggtggcagagtcctaac 3′

Exon11 Rev: 5′ gcagccagtgacacatcatc 3′

Exon14 Fw: 5′ atgggtattctccgtacaca 3′

Exon14 Rev: 5′ gaagcaatctgatgaactccaaa 3′

Exon18 Fw: 5′ aaaggcctggaggctatga 3′

Exon18 Rev: 5′ gcccagacataaacaaaactt 3′

4.7. Multiplex-Ligation-Dependent Probe Amplification

To assess larger genetic aberrations, we used SALSA MLPA Probemix P067 PTCH1 (MRC-Holland, Netherlands) containing probes for 23 of the 25 exons in the *PTCH1* gene (LRG_515; no probes are included for exons 1 and 9), according to the manufacturer's instructions. Amplicon fragment length analysis was performed on an ABI 3500 Genetic Analyzer (ThermoFisher Scientific, Waltham, MA, USA) and analyzed using Coffalyser.net software (MRC-Holland, Amsterdam, The Netherlands).

4.8. Data processing

The process of gathering data and sharing between specialists was implemented by applying Google Sheets (Google LLC, CA, USA). This spreadsheet application is freely available on the web and also on mobile devices. From a scientific point of view, simultaneous and real-time collaboration between multiple users is a highly attractive feature of it. As a cloud-based application, Google Sheets provides an online data storage service, with access to documents from any device with an internet connection. Additionally, it saves the workflow automatically, eliminating the possibility of losing data and keeping a detailed version history, allowing tracking modifications. The system allows different levels of permission for each user, which makes it practical to collaborate on tasks with different stakeholders.
The main advantages of Google Sheets are listed below:

1) Real-time collaboration
2) Cloud-based data collection
3) Automatic saving and version history
4) Integration with other Google services
5) Wide range of functions and formulas
6) Sharing and different levels of permission
7) Free and cost-effective
8) Data visualization and charting possibilities

4.9. Statistical analysis

Statistical analyses were carried out using VassarStats (https://faculty.vassar.edu/lowry/VassarStats.html, accessed on 18 May 2023).

4. Results

In our Hungarian NBCCS cohort, the male/female ratio was 7:9. The clinical phenotypes of the affected patients are summarized in **Table 2**. The most frequent clinical manifestations were histologically proven odontogenic keratocyst of the jaw (13 patients, 81%), multiple BCCs (nine patients, 56%) and congenital malformations (seven patients, 43%) including hypertelorism (3 patients, 18%), cleft lip- and palate (3 patients, 18%), bifid, fused ribs (1 patient, 6%) and polydactyly (1 patient, 6%). Bilamellar calcification of the falx cerebri and macrocephaly were detected in four patients (25%). Palmar and plantar pits were present in three patients (18%). Fifty per cent of the investigated patients were aware of a first-degree relative affected by NBCCS.

	Major Criteria						Minor Criteria				
Patient number	Odontogenic Keratocysts of the Jaw	Multiple BCCs	Calcification of the Falx Cerebri	Pits	Medullo-blastoma	First-Degree Relative with NBCCS (patient number)	Macro-cephaly	Bifid, Fused Ribs	Congenital Malfor-mations	Poly-dactyly	Result of the Genetic Screen
1	+		+						+		No variant identified
2	+		+								No variant identified
3		+		+		4,5					No variant identified
4	+	+	+	+		3,5					No variant identified
5	+	+		+		3,4					No variant identified
6	+					7			+		No variant identified
7	+	+	+			6	+		+		No variant identified
8	+	+									p.Leu297Pro
9	+	+									p.Gln527Ter
10	+	+				11					p.Q714Ter
11						10			+		p.Q714Ter
12	+						+		+	+	p.Cys998Ter
13	+	+				14					p.Asn272SerfsTer11
14	+					13					p.Asn272SerfsTer11
15	+	+					+		+		DelEx2
16	+						+	+	+		p.Val580_Val582del

Table 2. Summary of the clinical phenotypes presented by the investigated NBCCS patients. + indicates the presence of the trait.

WES identified three novel likely pathogenic mutations of the *PTCH1* (NM_000264.5) gene: the c.2994C>A p.Cys998Ter heterozygous nonsense mutation in patient 12, the c.814_818del p.Asn272SerfsTer11 heterozygous frameshift mutation in patients numbers 13 and 14 and the c.1737_1745del p.Val580_Val582del heterozygous deletion in patient 16 **(Figure 3, Table 3**). The heterozygous frameshift variant (p.Asn272SerfsTer11) is located in the sixth exon of the NM_000264.5 variant, which has 24 exons. Based on the American College of Medical Genetics (ACMG) variant classification guideline (**Richards 2015**), this variant is classified as pathogenic, as null variants (frameshift) in the *PTCH1* gene are predicted to cause loss-of-function (a known mechanism of the disease). The affected exon contains 10 additional pathogenic frameshift variants (PVS1) in the database. The identified frameshift variant is not present in the gnomAD population database (accessed on 18 May 2023) (PM2). The identified frameshift variant results in a premature termination codon occurring in 11 amino acids downstream of the frameshift, which may either cause nonsense-mediated RNA decay or the production of a severely mutated protein that is missing in most of the 3′ ends. Therefore, we hypothesized that the identified novel variant has a severe loss-of-function impact on protein functions. The other novel variant (p.Cys998Ter) is a heterozygous nonsense variant in the eighteenth exon of the *PTCH1* gene. Based on the ACMG variant classification guidelines (**Richards 2015, Pál 2023)**, this variant is also classified as pathogenic, as null variants (nonsense) in the *PTCH1* gene are predicted to cause loss-of-function (a known mechanism of the disease). The affected exon contains eight other pathogenic nonsense variants (PVS1). The identified nonsense variant, which is not present in the gnomAD population database (PM2), results in the formation of a premature termination codon, likely causing nonsense-mediated RNA decay or a severely mutated protein that is also missing much of the 3′ ends of the protein. Therefore, we hypothesized that the identified novel variant has a severe loss-of-function impact on protein functions. The third novel variant (c.1737_1745del p.Val580_Val582del) is a heterozygous deletion variant in the thirteenth exon of the PTCH1 gene. This variant has not been found in either dbSNP (Database of Single Nucleotide Polymorphisms), ClinVar, ExAC (Exome Aggregation Consortium), HGMD or EVS (Exome Variant Server) public databases. Based on the ACMG guidelines and the results from the analysis with the Franklin Variant Effect Predictor (www.franklin.genoox.com, accessed on 18 May 2023), these novel variants are considered to be disease-causing in relation to NBCCS.

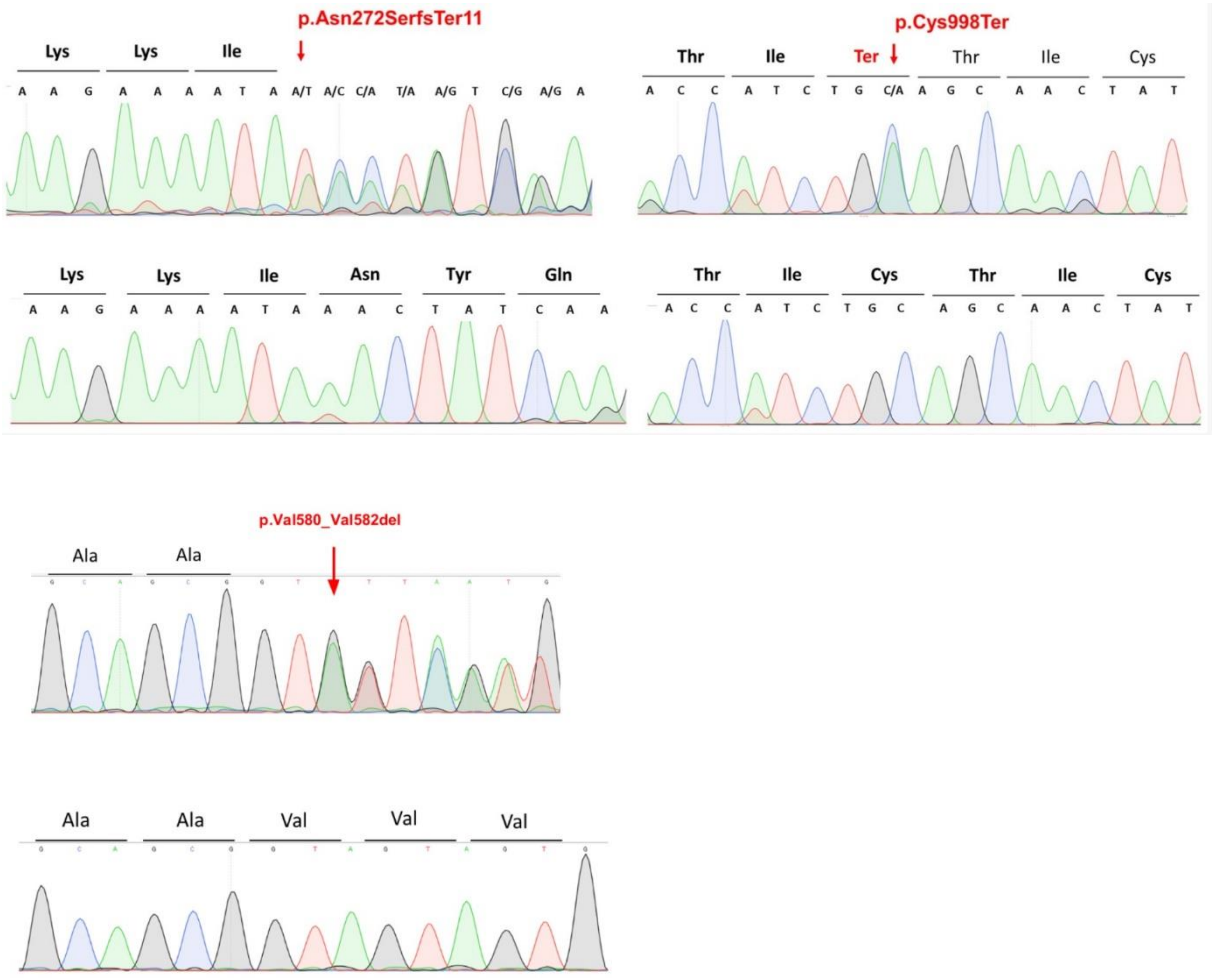

Figure 3. Sequencing results of the three novel mutations of the *PTCH1* gene.

WES also revealed three recurrent pathogenic variants of the *PTCH1* gene: the c.890T>C p.Leu297Pro heterozygous missense mutation in patient No.8, the c.1579C>T p.Gln527Ter heterozygous nonsense mutation in patient No.9, and the c.2140C>T p.Gln714Ter heterozygous nonsense variant in patients No.10 and No.11 (**Table 3**).

Gene	cDNA variant	Protein variant	Clinical Significance	Novelty
PTCH1	c.814_818del	p.Asn272SerfsTer11	Likely pathogenic	Newly identified by this study
PTCH1	c.1737_1745del	p.Val580_Val582del	Likely pathogenic	Newly identified by this study
PTCH1	c.2994C>A	p.Cys998Ter	Likely pathogenic	Newly identified by this study
PTCH1	c.890T>C	p.Leu297Pro	Likely pathogenic	Recurrent
PTCH1	c.1579C>T	p.Gln527Ter	Pathogenic	Recurrent
PTCH1	c.2140C>T	p.Q714Ter	Pathogenic	Recurrent

Table 3. Summary of results from the genetic screening of the investigated BCNS patients.

All mutations identified via WES were validated with Sanger sequencing.

From the MLPA results, a pathogenic deletion of the second exon of the *PTCH1* gene was identified in patient No.15 (**Figure 4**). WES did not identify pathogenic variants of the *PTCH1* gene for this patient.

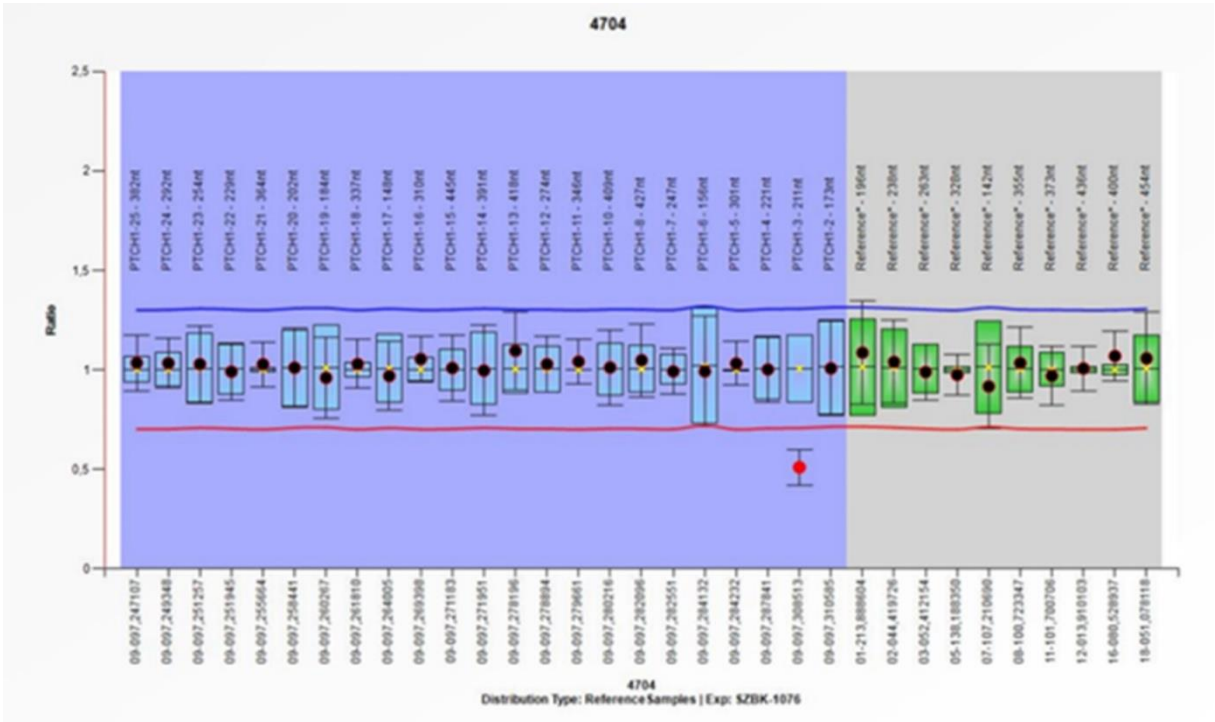

Figure 4. MLPA analysis of the *PTCH1* gene in patient No.15.

Causative mutations in the *SUFU* and *PTCH2* genes were not detected in our cohort.

The genetic backgrounds of seven of the eleven investigated NBCCS families were determined (63.6% diagnostic yield), and correspondingly, missing heritability accounted for 36.4% in this Hungarian cohort. We found a significant association between the presence of *PTCH1* pathogenic variants and the presence of the NBCCS clinical phenotype (Chi2 test: p = 0.0041).

5. Discussion

Because of the variable expressivity, a significant part of the NBCCS patients is not recognized as they are scattered amongst different medical specialities. Due to the low incidence and diagnostic difficulties, studies with a large number of patients are hardly available **[Jones 2011]**.

16 patients with NBCCS were enrolled in our Hungarian cohort study. Taking the prevalence of NBCCS into account in this study, we performed a closely thorough examination of the tertiary health care region of South-East Hungary, which covers 1.5 million inhabitants.

By comparing our results with the most quoted authors regarding the phenotypic manifestations of NBCCS, we found that in our study the prevalence of hypertelorism (18%), calcification of falx cerebri (25%), macrocephaly (25%) and skeletal abnormalities were lower than in the other studies **[Kimonis 1993, Shanley 1994, Lo Muzio 2008, Kimonis 2007, Fernández 2022]**. However, the prevalence of cleft lip and palate (18%) was higher than that found in any other surveys. Two previous studies found a higher prevalence of this characteristic than the average. In Japan, the ratio of cleft patients was 9%, while in Switzerland it was 11% **[Rehefeldt-Erne 2016]**. The frequency of the manifestation of BCCs and jaw keratocysts in the affected patients did not show differences compared to previous studies.

If we had applied the criteria proposed by Kimonis et al. for the diagnosis of NBCCS, one patient (patient No.11) would not have been recognized in our cohort. This proband fulfilled one major and one minor criterion. On the other hand, in seven patients, no pathogenic variations in the tested genes were found even after WES and MLPA. Interestingly four (patients No. 3, 4, 5, 7) out of these patients had three major signs of NBCCS based on either diagnostic criteria and they should have been evidently considered NBCCS. These findings unequivocally highlight the deficiencies of the up to now proposed diagnostic approaches.

In addition, we reviewed the cohort studies on the investigation of NBCCS. In 2007, Rupprecht et al. published their observations on 8 patients affected with NBCCS collected over a 12-year period. The study retrospectively analysed the epidemiological, clinical, radiological and histological data on the probands, but the research did not intend to investigate the genetic aspects of NBCCS **[Rupprecht 2007]**. In 2016, Rehefeldt and her colleagues systematically examined the clinical signs of the syndrome in the context of Zurich register, without aiming at clarifying the genetic background of the examined 30 patients **[Rehefeldt-Erne 2016]**. One

year later an Australian study was published which showed the most similarity with our cohort in terms of the method of genetic investigation. The recruited 19 patients underwent Sanger sequencing and MLPA or CGH-array-based tests. However, at the beginning of the study they applied Sanger sequencing and just the latter samples were examined by next-generation sequencing. In this study, from the tested 11 patients 6 (54.5%) were found to have a pathogenic PTCH1 mutation **[Huq 2018]**. In the same year, the Maastricht University Medical Center published the results of a multicentre retrospective cohort study, which examined the period between 1999 and 2015. In this study, patients with characteristic signs of NBCCS were genetically tested for PTCH1 mutations by Sanger sequencing. With the sole application of Sanger, the mutation detection rate was 21% **[Cosgun 2018]**. Also, in that year, Gianferante et al. shared their observations on 18 well-characterized NBCCS families. WES and aCGH techniques were used to detect mutations of all genes which can be affected in NBCCS and as well as 155 genes that play a role in the SHH pathway. 89% of the families were affected with PTCH1 mutations, but the other genes tested showed no alterations **[Gianferante 2018]**.

As a consequence of the above, cohort studies, which examine the manifestations of NBCCS frequently report a low number of cases, use different methods in terms of genetic testing and are not able to find genotype-phenotype correlations in NBCCS.

In our cohort study, we recognized three novel and three recurrent variants of the *PTCH1* gene using the WES and MLPA methods. The *PTCH1* gene encodes a 1447 amino acid transmembrane protein (Q13635), which has 12 known transmembrane regions and six extracellular, five intracellular, N-terminal and C-terminal domains (**Figure 5**) **[Lindström 2006]**. The identified c.814_818del p.Asn272SerfsTer11 novel variant is located in the first large extracellular loop, whereas the c.2994C>A p.Cys998Ter novel variant is located in the second large extracellular loop of the PTCH1 protein (**Figure 5**). Both of these large extracellular loops are required for the binding of N-SHH to the patched protein **[Marigo 1996]**. These mutations are located in evolutionarily conserved regions of the PTCH1 protein (AMINODE evolutionary analysis, www.aminode.org, accessed on 18 May 2023) (**Figure 5c**). The identified c.1737_1745del p.Val580_Val582del variant is located within the fifth transmembrane helix of the PTCH1 protein, in an evolutionarily conserved region (AMINODE evolutionary analysis). This region is part of the sterol-sensing domain of PTCH1. Both truncating variants are predicted in silico to cause early nonsense-mediated mRNA decay and, thus, most probably lead to haploinsufficiency or loss of function (NMDEscPredictor, shinyapps.io, accessed on 18 May 2023).

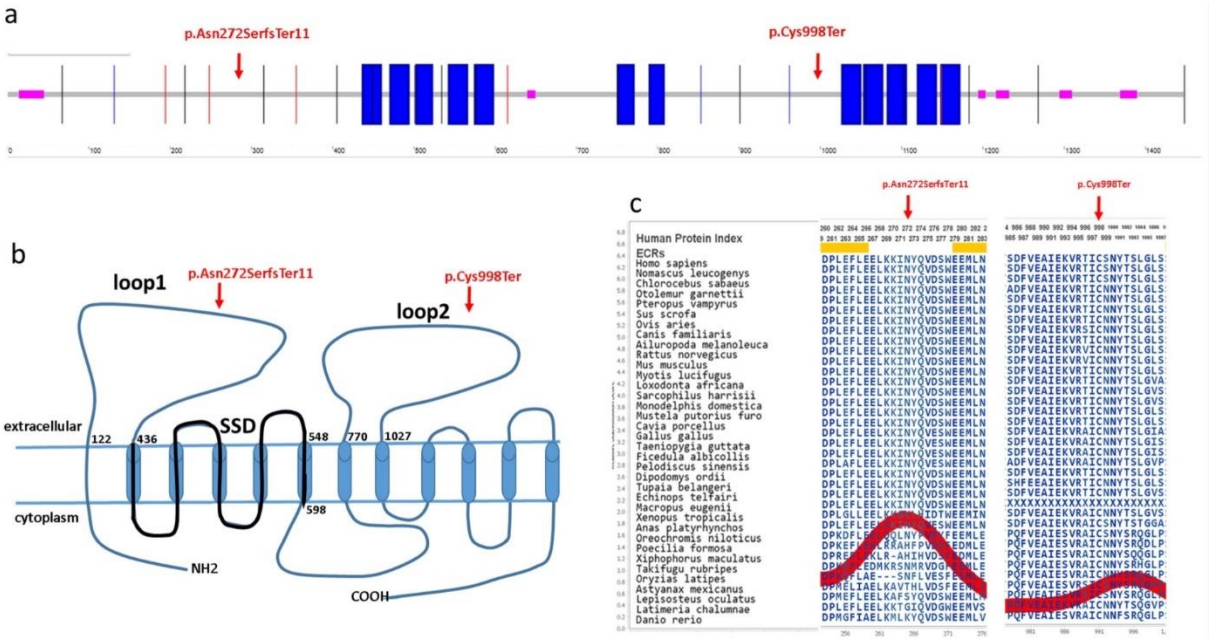

Figure 5. Location of the first and second novel variants in the PTCH1 protein. (**a**) Schematic representation of the PTCH1 protein (SMART protein, https://smart.embl-heidelberg.de/, accessed on 18 May 2023). (**b**) Predicted structure of the PTCH1 protein **[Guo 2013]**. Thick line denotes the sterol sensing domain (SDD). (**c**) Evolutionary conservation of the affected p.Asn272SerfsTer11 and p.Cys998Ter regions (Aminode, https://www.aminode.org/search, accessed on 18 May 2023). The red line represents the relative rate of amino acid substitution calculated at each protein position.

Pathogenic mutations in the *PTCH1* gene have been found in all domains, but mutations occur most frequently in the first and second large extracellular loops and in the third intracellular domain **[Lindström 2006]**. The identified recurrent p.Leu297Pro variant is a likely pathogenic missense variant, which was previously reported in the Netherlands **[Reinders 2018]**. The recurrent p.Gln714Ter pathogenic nonsense variant was identified in Australia **[Marsch 2005]**, and the p.Gln527Ter pathogenic nonsense variant occurs relatively frequently and is reported worldwide **[Chidambaram 1996, Pál 2023]**.

The MLPA technique was used to investigate the possibility of large deletions in the *PTCH1* gene. The method identified a deletion of exon 2. As WES did not identify any pathogenic variants of the *PTCH1* gene in the MLPA-positive patient, our result suggests the importance of this combination of screening methods for the genetic diagnosis of NBCCS.

PTCH1 mutations reported for NBCCS patients are predominantly nonsense or frameshift mutations (64%), followed in frequency by splice site mutations (13%), large insertions or deletions (12%), and missense mutations (8%) **[Kato 2017]**. In good agreement with these observations, our study identified four nonsense variants, one large deletion, one small deletion and one missense mutation.

Patients with *SUFU* pathogenic variants are reported mostly with medulloblastoma and without jaw keratocysts **[Evans 2002]**. Our results are in good agreement with this observation since none of the investigated patients presented with medulloblastoma and none had causative variants in *SUFU*.

The pathogenetic role of *PTCH2* variants in NBCCS has not yet been proven. Our study did not identify any pathogenic variants of the *PTCH2* gene, and neither does it support the direct involvement of *PTCH2* variation in the development of NBCCS **[Smith 2022]**.

WES analysis is usually not able to detect non-coding variants further than +/− 20 base pairs from exon-intron boundaries. However, intronic or even deep intronic mutations can create strong cryptic splice acceptor sites leading to frameshift mutations, which can lead to premature stop codons **[Anna 2018]**. The identification of deep intronic splicing mutations is challenging **[Dirix 2023]**. Bholah and collages demonstrated the importance of comprehensive transcript mutation analysis for individuals with clinically diagnosed Gorlin syndrome when *PTCH1* variants have not been detected by genomic sequencing or by copy number analysis **[Bholah 2014]**. Pathogenic mutations can occur deep within the introns of disease-causing genes. Deleterious DNA variants located more than 100 base pairs away from exon-intron junctions most commonly lead to pseudo-exon inclusion due to the activation of non-canonical splice sites or changes in splicing regulatory elements. Additionally, deep intronic mutations can disrupt transcription regulatory motifs and non-coding RNA genes **[Vaz-Drago 2017].**

Since 1996, when the first pathogenic mutations were identified in *PTCH1* **[Hahn 1996]**, a high number of disease-causing variants have been reported for this gene. These discoveries reflect the enormous development in the field of mutation screening technologies. In this study, WES and MLPA were successfully applied to identify the genetic background of approximately 56% of the investigated NBCCS patients. A recent study (2022) with a similar approach from a group in the UK determined the genetic background of 75% of the patients in their study **[Maroilley 2019]**. These results further emphasize that even high-throughput genetic screening methods,

such as WES, have limitations and that missing heritability is an issue for rare diseases such as NBCCS **[Maroilley 2019, Pál 2023]**.

Missing heritability affects both common and rare diseases and is mainly associated with common and complex diseases where promising modern technological advances, such as genome-wide association studies, were unable to uncover the complete genetic mechanism of the disease **[Maroilley 2019]**. Missing heritability is likely to be caused by several conditions. In rare diseases, it can be the consequence of high phenotypic diversity. Such diversity can be caused by strikingly different phenotypes associated with different variants of the same gene as well as high genetic heterogeneity caused by variations in distinct genes that produce similar phenotypes **[Maroilley 2019]**. Mosaicism can be another explanation **[Matthews 2017, Raggotte 2017, Peron 2018]**. Regarding NBCCS, mosaic patients may have PTCH1 mutation in the tumour-forming skin, while the peripheral blood leukocytes do not carry the mutation. A further cause of missing heritability can be that the probands have mutations elsewhere in the Hh pathway or other tumour suppressor genes **[Klein 2005]**.

To resolve this phenomenon in rare diseases, novel technological approaches, such as the promising techniques of whole-genome sequencing (WGS) or epigenetic analysis, should be implemented in clinical practice.

Many large-scale genome sequencing projects are ongoing globally, but the clinical implementation of the results of these projects is, for the most part, lagging **[Stranneheim 2021]**. Whole genome sequencing (WGS) analysis can be used to detect and interpret single-nucleotide variants, insertions and/or deletions, uniparental disomy, copy-number variations, balanced structural variants, and short tandem repeat expansions **[Stranneheim 2021]**. The availability of WES and WGS has drastically impacted genetic diagnostics, and the clinical genetics speciality is undergoing rapid development **[Stranneheim 2021]**. The clinical application of WGS can contribute to the genetic diagnosis of rare diseases and additionally help identify novel disease-causing genes **[Stranneheim 2021, Pál 2023]**. In particular, the integration of WGS into the healthcare setting could potentially reduce missing heritability in rare diseases and increase the genetic diagnostic rate of monogenic diseases, including NBCCS.

WES has recently been used more and more frequently in diagnosis; however, a significant proportion of patients remain undiagnosed after sequencing their genome. New approaches, based on functional aspects of the genome, including epigenomics, are beginning to emerge **[Stranneheim 2021]**. Increasing numbers of reports describe functionally relevant alterations

of the genome that do not involve mutation of the nucleotide sequence. Moreover, a considerable number of studies reveal the appearance of aberrant epigenetic modifications of nucleic acids in association with the occurrence of diseases, including cancer, diabetes, Alzheimer's disease, and many others. A better understanding of the exact roles of epigenetic modification in biological processes and human diseases might make it possible to identify biomarkers for diagnosis and treatment. Notably, remarkable efforts have been made to establish technologies to facilitate the accurate detection and mapping of epigenetic modification. Shortly, the integration of epigenetic analysis in healthcare settings might also help to reduce missing heritability for rare diseases and increase the genetic diagnostic rate in diseases such as NBCCS **[Pál 2023]**.

To understand this complicated disorder better, information on a large number of patients is necessary. Insomuch as NBCCS is a rare hereditary condition, researchers are under the necessity of assembling small pieces of information gained from long-run cohort studies and genetic databases. In this genetic puzzle, newly identified pathogenic mutations can help to elucidate the genetic background and the genotype-phenotype correlation of NBCCS.

Based on our results, we hypothesize that the application of WGS and/or an epigenetic approach should be applied to unsolved NBCCS cases to attempt to resolve missing heritability for NBCCS.

6. Summary of new findings

1.) We have successfully introduced a cloud-based, multidisciplinary, real-time data collection method to obtain clinical and genetic data on a rare hereditary disease.
2.) According to our best knowledge, in Europe, our survey was the first genetic cohort study in which all the probands underwent genetic testing with the application of multiplex ligation-dependent probe amplification and whole exome sequencing.
3.) With the combined application of whole exome sequencing and multiplex ligation-dependent probe amplification we have successfully recognized three novel pathogenic variants of the PTCH1 gene.
4.) The prevalence of cleft lip and palate patients in our cohort is the highest ever has been reported.
5.) Our findings justify the assumption that all of the currently applied diagnostic systems for NBCCS have their shortcomings and they are incapable of setting up the diagnosis conclusively.

7. Acknowledgements

We are grateful to the families and patients whose generosity has made this study possible.

I would like to express my sincere gratitude to my supervisor Dr. László Seres for the continuous support of my PhD study and research.

Besides my advisor I would like to thank Dr. Margit Pál and Professor Márta Széll for the irreplaceable and essential help making the study possible.

I am earnestly grateful to Professor József Piffkó for the help and support he has given me throughout the past 14 years.

I would also like to thank all my colleagues involved in the research for their help and dedicated work.

Finally, I am extremely grateful for my whole family and friends for the supportive background, the empathy and the encouragement they gave me.

I dedicate my thesis to my parents and Flóra Bandula for their endless love, support and encouragement throughout my pursuit for education.

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