APPLICATION OF NEXT GENERATION SEQUENCING IN NEUROLOGY – RETROSPECTIVE STUDY AT THE DEPARTMENT OF MEDICAL GENETICS AND BIOLOGY, FACULTY OF MEDICINE RIJEKA

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UNIVERSITY OF RIJEKA
FACULTY OF MEDICINE

INTEGRATED UNDERGRADUATE AND GRADUATE UNIVERSITY STUDY OF MEDICINE IN ENGLISH

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GRADUATION THESIS

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List of Abbreviations and Acronyms

ADHD	Attention-deficit/hyperactivity disorder
ALT	alternate allele
ASD	autism spectrum disorder
BBB	blood brain barrier
bp	base pairs
CAS	childhood apraxia of speech
CNA	copy number alteration
CNS	central nervous system
CNV	copy number variant
CSF	cerebrospinal fluid
DM	diabetes mellitus
DNA	Deoxyribonucleic acid
ECTS	European Credit Transfer and Accumulation System
EEG	Electroencephalography
EMG	Electromyography
EMNG	Electromyoneurography
FH	Family history
FISH	Fluorescence In Situ Hybridization
GABA	Gamma-Aminobutyric acid
GER	gastroesophageal reflux
GRCh38	Genome Reference Consortium Human build 38
GTAC	Genetic Testing Advisory Committee
INDEL	Insertion and/or Deletion
MRI	magnetic resonance imaging
NFT	Neurofibrillary tangle
NGS	Next Generation Sequencing
PCR	polymerase chain reaction
PDGF	Platelet derived growth factor
REF	reference allele
SBS	sequencing by synthesis
SIDS	sudden infant death syndrome
SNP	single nucleotide polymorphism
TCS	transcriptome sequencing
TP53	Tumor protein 53
TS	targeted sequencing
VC	variant calling
VSD	ventricular septal defect
WES	whole exome sequencing
WGS	whole genome sequencing

1. Introduction

Genetic science is advancing at a rapid speed and the possibilities for using new technologies and the increasing understanding of the genetic background of disease in clinical practice for the diagnosis and especially for personalized individually adapted treatments seem endless. However, in practice, the use of genetic technologies such as Next-Generation-Sequencing (NGS), are rather sparse. There are numerous studies dedicated to the investigation of reasoning for the delay in the use of new advancements that could greatly benefit patients and revolutionise treatment as we know it. Findings suggest, among other reasons, that low access to genetic experts and a lack ability to apply medical genetics among all clinical specialties is reducing the application of genetic testing in the clinical hospital setting. (1)

Therefore, the study performed in this paper aims to evaluate the current application of NGS in the field of neurology at the clinical hospital center Rijeka.

1.2 Importance of Genetics

The idea of genetics was first discovered by the scientist Gregor Mendel in 1865 when he performed an experiment with phenotypically different plants of the same species, where he published his theories about inheritance. Over a century ago, in 1906, the term "genetics" was coined as the science of heredity. In the following years, Mendelian genetics and the arising chromosomal theory of inheritance fused, which marks the beginning of "classical genetics" as is today. However, it was not until the 1950s that DNA was discovered, enabling scientist to take our understanding of genetics and inheritance to the next level. Until today, with new methods, scientists are still unravelling the mysteries of genetics and inheritance.(2)

Today we define genetics as "a branch of biology that deals with the heredity and variation of organisms. [It describes] the genetic makeup and phenomena of an organism, type, group, or condition."(3)

The prevalence of genetic diseases may be low among the general population, but our understanding and diagnosis of those is crucial to the 10% of Americans suffering from a rare disease, 80% of those being of genetic origin.(1) In addition, the application of genetics is expanding beyond genetic syndromes and our genetic makeup is becoming increasingly important in the research of what has been assumed to be acquired diseases, such as cancers.(4) Genetics impacts

all clinical specialties, and the small number of specialized board certified medical geneticists cannot cover all implications of genetics.(1) In Croatia, the clinical field of genetics currently does not exist.(5) Thus, is it crucial that clinical specialists are trained in the applications of medical genetics within their field of medicine. (1)

Unfortunately, the majority of physicians (63%) in the United States disagree with the statement: "I feel that my genetics training adequately trained me to order and use genetic tests", in a study performed at Kanas University. Other similar studies revealed similar outcomes.(1,5) Genetics training, for most physicians, is limited to courses of medical genetics during medical school, which have been held since 1980. Physicians who graduated before this, have practically no training in the subject.(1)

At the University of Rijeka, Faculty of Medicine, medical genetics is taught during a mandatory course during the fifth year of medical school, comprising 3 ECTS.(5)

Although most physicians perceive advancements in genetic knowledge as important, studies show that physicians feel inadequately trained to properly use the existing genetic tools in clinical practice.(1) A general lack of medical genetics experts(4,5) exacerbates the gap between the potential of genetic tools in clinical patient care and their application in hospital settings.

1.3 Next Generation Sequencing

Next generation sequencing, or NGS, is one of the most widely used methods of performing DNA sequencing of current times. However, it probable that NGS will be outdated in the near future, as research in genomics is evolving at a high speed and new 3rd and 4th generation sequencing methods are already appearing on the research horizon. The first human genome sequencing was performed in 2001, and since 2005 NGS methods have been developed and used in research and clinical practice around the world. (6)

The method of NGS has been available for clinical application at the clinical hospital center Rijeka since 2018.

NGS technologies have been developed to overcome the limitations of formerly advantageous sanger sequencing methods, which was used to perform the first human genome sequencing. The basic principle of action is the performance of parallel sequencing of millions of small DNA fragments to generate a large amount of data in a short amount of time. (7) The exact mechanism by which this is achieved differs between the numerous manufacturers and is

technologically too advanced to be thoroughly described in this research, however, is outlined briefly.

1.3.1 Procedure of Next Generation Sequencing

NGS technologies use advancements in polymerase chain reaction (PCR), primers and base specific fluorescent markers to prepare a relatively small DNA sample for sequencing by NGS. This enables NGS to sequence numerous genes and base pairs, using a small sample of DNA, in a short amount of time, at a low cost. Sequencing up to several billion nucleotides within a single day or even several hours is a great technological success, compared to preceding sequencing methods, such as sanger sequencing, enabling methods including whole genomes sequencing (WGS), whole exome sequencing (WES), variant calling (VC), targeted sequencing (TS) and transcriptome sequencing (TCS). The number of base pairs sequenced in a short time is made possible by reading billions of sequences ranging from 50-300 base pairs simultaneously, generating large scale results in a short period of time.
Multiple manufacturers have developed the technical hardware to perform NGS analysis, their instruments differentiating slightly in technique and outcome. The most common approach to NGS methodology is sequencing by synthesis (SBS), used in 90% of sequencing worldwide. (SBS technique can be used with different sequencing techniques, including fluorescence, pH measurement, and thermal detection, based on the manufacturer). Performance ranges from 150bp to 550bp per sequence and from 13 million reads per day to 130 million reads per run in a few hours. Even in this high output of sequencing error is minimal, at about 1-1,5%. These outcomes, at high speed and low cost while being exceptionally accurate enable the use of NGS not only for large scale studies and genomic research opportunities, but also for the use in clinical practice, enabling a personalized approach to medicine. (6)

Second generation sequencing differs from prior methods by using pyrophosphate synthesis with luminescence to determine the nucleotide sequence, rather than fluorescent labelled nucleotides. This change is what enables the technique to perform the sequencing of billions of sequencing reactions in parallel. Other techniques have also been developed, such as sequencing by oligonucleotide ligation and detection or using the measurement of pH values cause by the release of protons during the polymerization process. The main factor in differentiating Next Generation Sequencing techniques from other generations is simply that parallel sequencing of previously amplified DNA fragments using PCR takes place. (8)

While NGS technology is still the most widespread sequencing technique used, 3rd generation sequencing technologies have already been developed, and may obliterate the need for NGS altogether. Their methods aim at elimination of long DNA preparation times by amplification, which decreases the occurrence of error during sequencing. This also avoids NGS limitations such as long CG base pair repeats. (7)

1.3.2 Interpretation of NGS results

In order to identify genomic variants using NGS technologies, the sequencing data obtained must be analyzed. This is done using advanced biotechnology. The large files of so called "sequencing reads", containing the raw information of DNA sequence of the analyzed material, is aligned to the human reference genome: Genome reference Consortium Human Build 38 in a process referred to as "read mapping". During this process the sample DNA sequence is compared to the human reference genome, detecting variations. The GRCh38 is an artificially constructed genome which has the ability to accommodate for natural genetic variants of the population. The process of detecting theses genetic differences in regions of the genome that are likely to cause disease is variant calling.(9)

NGS can detect several different types of possible genetic variations, including long variants such as copy number variants (CNVs) and copy number alterations (CNAs) and is especially useful in the detection of short variants. The most common types of variants found in the genome are single-nucleotide polymorphisms (SNPs), which describes the substitution of one base for another in the sequence. (9)

This can change the translated protein sequence in one of 3 ways: the changed sequence can transcribe to the same amino acid, essentially not changing the structural or functional outcome of the protein. This is called a silent mutation. The altered base pair may also lead to the translation of a different amino acid in the formation of a protein, which may lead to a change in structure and function or lack of function of the specific protein. The most drastic change is the formation of a stop codon by the base substitution, leading to an immature stop of transcription and usually results in a non-functioning protein due to severe structural changes and loss of integrity of the protein. These are referred to as missense or nonsense mutations, respectively.(10)

Other single nucleotide variants (SNVs) as well as short insertions and deletions of less than 20 base pairs (INDELs) can be detected during variant calling.(9) Similarly the change of base sequence can result in an altered amino acid sequence or lead to the insertion of a premature stop codon transcription pattern, leading to loss of integrity of proteins and structural and functional alterations, phenotypically presenting as a disorder.(10)

Limitations of NGS technologies include the detection of long sequence variants. While some larger CNVs and CNAs can be detected, many long sequence variants such as larger inversions and deletions are not detected.(9) In addition to rearrangements, long repeat sequences are difficult to detect using current NGS technologies.(11) These variants can include the deletion or duplication of partial or whole chromosomes.(9) Alterations of this magnitude can better be detected using other methods, such as karyotyping or FISH (12), although newly emerging NGS technology, third generation techniques, are looking to overcome these limitations and provide effective detection of all genomic rearrangements.(11)

For SNPs there are two possible outcomes in the analysis of reads. The sequence can align with the corresponding allele of the reference genome, being defined as a reference allele (REF), or it may differ from the reference genome as an alternate or variant allele (ALT). In the analysis of alternate variants, it is important to consider the zygosity of the variant allele within the proband. Humans are diploid, meaning they have two copies of each chromosome, one of paternal and one of maternal origin. These chromosomes are considered homologous as they encrypt the same genetic information, however, the genetic material and the sequence of the DNA can be very different. Thus, it is of great possibly that a variant found in one chromosome is not present in the other. As a result, there are three possible genotypes that may occur in a person for every allele, homozygous for the reference (REF/REF), meaning no alternate sequence was detected of this allele in either chromosome, homozygous alternate allele (ALT/ALT), both chromosomes showing a variant for the specific allele or heterozygous (REF/ALT), in which one of the homologous chromosomes presents with a variant allele, the other, however, is compliant with the reference genome.(9)

Determining the zygosity of a person for a variant gene, provides with two major clinical considerations - clinical presentation of the disease and inheritance. A major benefit in determining genetic variations and diagnosing diseases of genetic mutations is determining the risk of the same genetic anomaly in future pregnancies of the parents. Naturally, in a homozygous variant where the ALT is present in both maternal and paternal chromosomes, the risk of a future offspring inheriting the alternate variant is higher than if the variant is present in merely one parent

chromosome, as in heterozygotes. Another factor to consider in estimating the risk of offspring inheritance is the nature of the inherited disease, which can be dominant or recessive. This refers to the expression of disease in heterozygotes. In dominantly inherited disease heterozygotes of an ALT will be affected by the disease, in recessive patterns of inheritance, only homozygotes are considered affected, while heterozygotes are carriers of the disease.(13,14) The risk of inheritance of genetic anomalies in hetero- and homozygotes in autosomal dominant and recessive diseases are presented in figures 1 and 2, respectively.

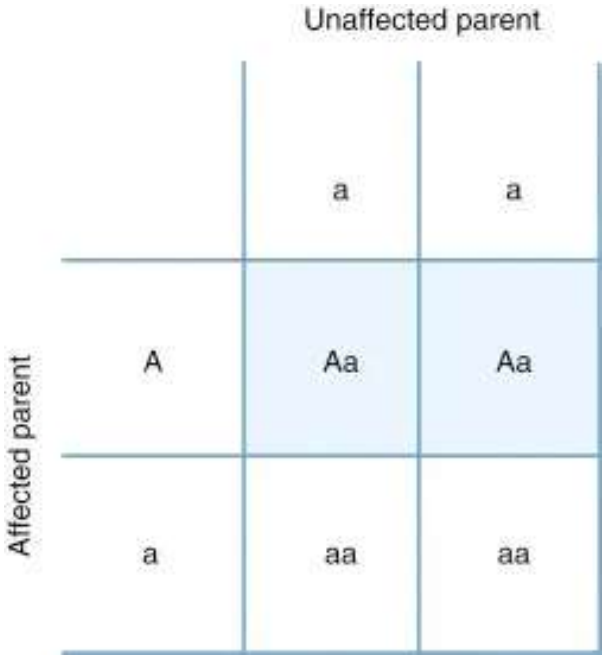

Figure 1. *Punnett square illustrating the risk of inheritance of an autosomal dominant disease with one parent affected in a heterozygous manner (Aa, ALT/REF) and an unaffected parent homozygous for REF (aa). The risk for their offspring to be affected is 50% with ALT/REF (Aa), and the chance of a genetically and phenotypically healthy offspring is also 50% (REF/REF, aa).This image was obtained from "basicmedical key"*(15).

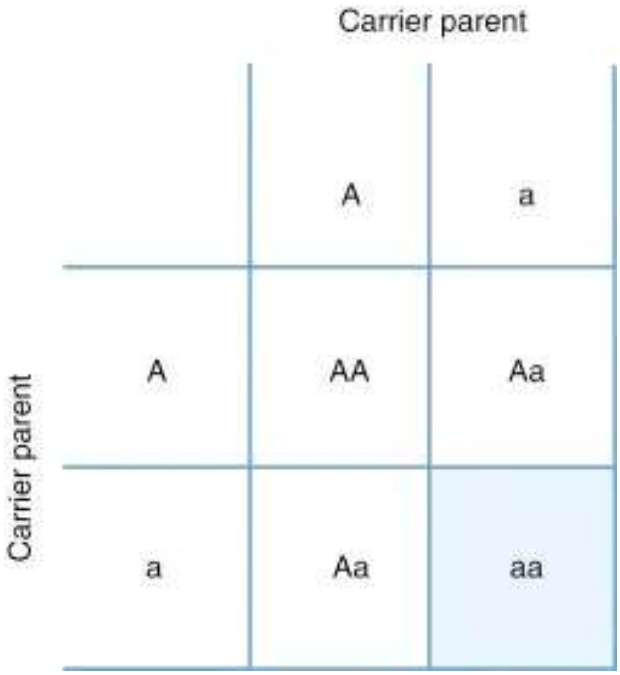

Figure 2. *Punnett square illustrating the risk of inheritance of an autosomal recessive disease with both parents presenting in heterozygous manner (ALT/REF) as carriers of the disease (Aa). The risk of an offspring inheriting the disease is 25% (homozygote ALT/ALT, AA) and there is a 50% chance of an offspring presenting a carrier (heterozygote ATL/REF, Aa). The chance of a phenotypically and genetically healthy offspring (REF/REF) is 25% (aa).This image was obtained from "basicmedical key"*(16)

.

Once variants are detected in the DNA of a proband, they must be interpreted to validify their relevance in a certain clinical presentation. This is done using bioinformatic tools such as REVEL* or CADD* for variant annotation. Moreover, external resources such as large sequencing databases or disease knowledge databases are used by annotation software such as ANNOVAR* to interpret the findings of numerous variants for their significance regarding the clinical presentation of the patient.(9)

The outcome of variant interpretation determines the likelihood of a present variant being the cause for the disease or disorder a person is experiencing clinically. This is an important consideration for the accuracy of diagnosis using NGS. The presence of a variant in the genome does not automatically conclude that any symptoms that appear phenotypically in the patient are caused by the mutation and inaccurate interpretation of variants could potentially lead to missed diagnoses and inaccurate treatment of acquired disorders. The relevance of a variant for the clinical presentation is determined during the process of variant annotation by comparison of the presence of the specific variant and the presence of a group of symptoms in patients with the specific mutation that is documented in large databases containing all known information

about not only genetic information and known variants, but also the clinical consequences of all mutations. Based on the interpretation of variants found, positively interpreted results are classified into three possible outcomes: pathogenic variants (class 5), likely pathogenic variants (class 4) and variants of unknown significance (class 3). Class 1 and 2 variants detected are likely benign, they are not linked to the clinical picture of the patient and are therefore not considered as results of NGS, and they most likely appear as individual genetic heterogenicity. Their relevance is limited to research possibilities and expansion of knowledge and information in databases, as a high prevalence of these classes of variants in patients with no clinical presentation of disease increases the certainty that these variants are insignificant regarding genetic diseases. Similarly, the larger the research database is for a specific variant found linked to a specific clinical picture, the higher the certainty that this variant is in fact the causative agent for a genetic disorder causing this clinical picture. Class 5 variants have the highest certainty, meaning the variant and the clinical picture in the proband match numerous accounts of the same mutations linked with the symptomatology. In class 4 mutations, either the prevalence is lower, the variant slightly differs in its alteration from similar variants in the same allele or a close allele in the same gene. Another possibility is the presence of a known variant for a clinical picture that differs from the presentation of a patient, the patient may lack characteristic symptoms of the known disease caused by the variant, or present with other symptoms not typically associated with the disease. All these factors decrease the certainly, based on research databases, that the variant present is responsible for the phenotype. Nevertheless, the outcome is similar enough that is it reasonable to assume the known disorder associated with the mutation or similar variants is present in each patient, hence the term likely pathogenic variants. In variants of unknown significance, the alterations from the database information are more significant, decreasing the certainty of a variant being seen as a causative agent. Knowing the complexity of the human genome, it would be arrogant to assume that the current knowledge found by databases is sufficient in its extent to exclude the possibility of new found causative aberrations for a disease. Thus, a variant is determined of unknown significance, usually indicating that databases do not show sufficient accounts of this variant in disorders, however compared to similar variants in the same gene and the clinical picture of both, it is interpreted that there is a reasonable possibility that this variant could by a causative alteration.(17–20)

1.3.3 Indications for Next Generation Sequencing in Neurology

The major possibilities of the use of NGS technologies in routine clinical diagnostics are precise diagnoses and the ability to implement personalized treatments. It is currently being applied routinely for the diagnosis of: inherited diseases, solid tumors, hematologic malignancies, infectious diseases, human leukocyte antigen analysis and non-invasive prenatal screening for the detection of fetal chromosomal defects. (21)

The use of NGS in a clinical setting for diagnostics of genetic disorders and cancers has become increasingly available and can be seen as the gold standard diagnostic method. (8)

The early anticipation, that neurologic disorders would have a high demand for genetic testing and would be one of the early specialities to benefit from the use of NGS in clinical diagnostics has by now been realized and is increasingly beneficial. The use of NGS adds value, especially in the field of Neurology, where the involvement of genetic mutations and gene variations in the etiopathogenesis of disease is a factor of high relevance and frequency, as over 80% of all known coding genes are expressed in the brain. Due to the complex nature of neurologic pathways, involving numerous genes, many of which present in a similar manner clinically. On the contrary, it is also true that different variations of the same gene may present itself in an entirely different neurological pathologic presentation. Therefore, neurologic disorders can be described as genetically and phenotypically heterogenous.

Considering the fact, many neurologic diseases, and especially those attributed to genetic changes, cannot be cured, and are treated symptomatically, regardless of etiology, it is important to consider the value neurologic testing brings to the treatment of a patient, and justify a procedure, such as NGS, financially and ethically. The benefit of performing tests and diagnosing an underlying genetic cause for patients' presentation is fourfold: first, the obvious advantage of establishing genetic diagnosis of any kind is the possibility of family genetic counselling, discussing risks and diagnosing or initiating treatments in earlier stages for other affected members and considerations for family planning. Secondly, it may benefit the patient care by specific prognostic value and differential disease management information regarding the syndrome. Third, improper therapies and additional testing, especially costly or straining invasive testing methods may be spared. And finally the patients can be given research opportunities to participate in clinical trials for new therapies or guidelines. (22)

1.4 Genetics in Neurology

1.4.1 Movement disorders

Movement disorders can be classified as hypokinetic movement disorders and hyperkinetic movement disorders and are mainly characterized by involuntary postures or movements.

Hyperkinetic movement disorders include dystonia, chorea, athetosis, stereotypies, myoclonus, tics, and tremors. Hypokinetic movement disorders include a wide group of neurological diseases including: muscular dystrophies, paraplegias, paresis, myotonia, and rare diseases such as Parkinson's disease. The main distinguishing feature of this group of disorders is muscle weakness. The pathophysiology of movement disorders is poorly understood, but likely involves brain anomalies of the basal ganglia or cerebellar circuits.(23)

"Dystonia is a movement disorder characterized by sustained or intermittent muscle contractions causing abnormal, often repetitive, movements, postures, or both. Dystonic movements are typically patterned, twisting, and may be tremulous. Dystonia is often initiated or worsened by voluntary action and associated with overflow muscle activation." (24) Due to the complex nature and wide variability of disease, dystonia patients are classified along two axes, clinical features, and etiological descriptors. Importance considerations are the age of onset, body distribution, temporal pattern, inheritance, and the presence of associated features. Treatment options include dopa agonists, GABA receptor agonists, botulinum toxin and neurosurgical procedures such as deep brain stimulation and intra-thecal baclofen. Unfortunately, in most children with dystonia, only mild to moderate benefit can be achieved with therapeutic options. (25)

Tremors are rhythmic, regular oscillatory movements of a part of the body around a joint axis. They commonly occur as a symptom of other movement disorders, such as Parkinson's or Huntington's disease. Tremors are classified as resting tremors, action tremors or postural tremors.(26)

Chorea refers to involuntary, irregular, non-repetitive, dance-like movements. Affected children appear hyperactive or fidgety. Primary chorea occurs in genetic diseases such as Huntington's disease, Ataxia-telangiectasia, benign hereditary chorea or spinocerebellar ataxia or it can result from acquired causes including infections, injuries, immune-mediated disorders or drug-induced. (23)

Different movement disorders can co-exist in a wide range of diseases, as well as primary disorders, making the diagnosis of specific movement disorders challenging.(23)

1.4.2 Developmental disorders and morphological brain anomalies

A developmental disorder is "a condition (such as autism or dyslexia) that is typically marked by delayed development or impaired function especially in learning, language, communication, cognition, behavior, socialization, or mobility"(27).

Morphological brain anomalies and developmental disorders are usually detected early in childhood, during obstetric examinations and ultrasound during pregnancy or failure of a child to reach certain milestones in early childhood, such as verbal or other communication skills or motor advancements.

A delay in language skills is the most common sign of a developmental disorder and surveillance of children reaching language milestones at a certain age is a key tool for the investigation of development. Causes of delayed language development can be primary, in a familial presentation that is likely caused by genetic variants, or secondary from a wide variety of etiologies. Children with developmental disorders of language impairment have long-term disadvantages, affecting their language and social skills and often impairing academic performance throughout life. They are at higher susceptibility for behavioral and mental health disorders. The incidence of all forms of speech and language delay (including mild forms) is around 15%, measured in toddlers at the age of 2, however 50% of children with language delay will reach normal developmental milestones by the age of 3 to 5 years. (28)

Common disorders leading to impairment of language and speech development include, but are not limited to ADHD, autism spectrum disorder (ASD), childhood apraxia of speech (CAS), dysarthria in syndromic conditions causing structural brain malformations. Risk factors for acquired language delay include male gender, low birth weight and low socioeconomic status.(28)

Treatment of language and speech disorders is performed by specialist speech therapist, in concordance with a multidisciplinary team, as for many developmental disorders, especially genetic syndromes, will have concomitant symptoms.(28)

Structural malformations of brain development represent a major cause for severe developmental disorders, usually presenting with combined symptoms of language and speech delay or

absence, motor disabilities and intellectual disorders. Another common symptom with major structural brain anomalies is epilepsy, which is described below. Several genetic aberrations have been linked to neuronal migration disorders with cause lissencephaly-pachygyria-severe band heterotopia, including the LIS1, DCX, ARX, RELN, VLDLR, ACTB, ACTG1, TUBG1, KIF5C, KIF2A and CDK5 genes. Other major malformations causing neurological developmental defects include microcephaly, corpus callosum dysgenesis, cerebellar hypoplasia and tubulinopathies.(29)

1.4.3 Epilepsy and other seizure disorders

Epilepsy is a common, well known neurological disorder characterized by the occurrence of seizures.(30) It is etiologically heterogenous, with underlying genetic factors being described in studies determining familial presentations for decades. With advanced technologies, more and more genetic variants causing primary epilepsy syndromes are being detected. While the genetic basis for epilepsy can be monogenic in some cases, most causes are more complex involving polygenetic inheritance and epigenetic factors.(29)

Epilepsy can be divided into Generalized Epilepsy, defined by generalized spike wave activity measured in an EEG, and Focal Epilepsy, which can be unifocal or multifocal and is diagnosed clinically, although focal epileptic EEG patterns are often detected. Seizure types presented in generalized epilepsy include absence seizures, myoclonic, atonic, tonic, and tonic-clonic seizures. Focal epilepsy seizures can be focal motor seizures, focal non-motor seizures, focal tonic-clonic seizures, as well as focal aware or impaired awareness seizures. The main differentiating factor is that the patient has never experience a generalized seizure. Patients who experience both generalized seizures and focal seizures are categorized as combined generalized and focal epilepsies. The etiology of epilepsy can be structural, related to brain malformation, which have been described previously (1.4.2. developmental disorders and morphological brain anomalies), genetic, infectious, metabolic, immunologic or unknown.(31)

Many childhood-onset epilepsies are well regulated with anti-epileptic therapy and seizures regress into remission with increasing age. The prognosis for remission is worse in epilepsy due to a presumed or confirmed genetic background. The highest rate of reoccurrence of seizures is in children with additional neurological dysfunction at birth. (30)

1.4.4 Sensory neurologic disorders

The body's special sensory functions include taste, smell, sight, and hearing. These functions are controlled by specific cranial nerves and executed by sensory organs. Damage or malformations of any of these structures lead to an impairment of sensory functions.

The sense of smell is perceived by diffusion of small particles in specialized cells in the nose, the information being transported to the olfactory center of the brain via the olfactory nerve, or the first cranial nerve. Lesions of the olfactory nerve are most commonly of traumatic etiology, and lead to a perceived lack of taste in food and inability to smell. Smell has a large involvement in experiencing taste, thus with lesions of the olfactory nerve, both senses are impaired. Other etiologies of olfactory nerve lesions are tumor masses compressing the nerve, usually meningioma, suprasellar masses or nasopharyngeal tumors. Transient loss of smell can occur following viral infections, especially in the older population. It may also appear as an early sign of neurodegenerative diseases such as Parkinson. In the pediatric population the differential may include congenital aplasia of the bulbus olfactorius in hypogonadotropic hypogonadism.(32)

The sense of taste is perceived by specialized cells on the tongue. Neurologically, the hypoglossal nerve and the glossopharyngeal nerve interpret taste on the anterior and posterior tongue, respectively. Lesions of these cranial nerves will result in loss of taste in the part of the tongue they innervate. Due to the double innervation of taste, lesions in these nerves are typically diagnosed due to loss of other non-functions including movement of the tongue and muscles involved in swallowing.(32)

Sight is perceived by the complex properties of the structures of the eye enabling light refraction onto a delicate membrane of sensory cells connected to the optic nerve. Dysfunctions in any part of this process leads to impairment of sight, including mild defects such as miosis or mydriasis to complete blindness. By the neurological pathway via which the optic nerve passe through the brain, vision loss appears in a specific part of the field of vision according to the location of the lesion. The most common etiology is the compression of the optic chiasm by a pituitary gland tumor causing bitemporal hemianopsia.(33)

Hearing is processed by the eighth cranial nerve, the vestibulocochlear nerve. Sounds are transmitted through the middle and inner ear. Lesions of the vestibulocochlear nerve can lead to abnormal hearing such as tinnitus or deafness. Some etiologies of hearing defects include

Meniere's syndrome, acoustic neuroma, autoimmune or ischemic defects of the inner ear. The vestibulocochlear nerve is also associated with balance, therefore neurologic causes of hearing defects are usually also associated with vertigo. Structural defects of the ear can also lead to deafness.(32)

1.4.5 Dementia

Alzheimer's disease is characterized by dementia, typically appearing in elderly patients. The course of the disease is slowly progressive. In about 5% of Alzheimer's patients, symptoms appear at an early-onset and follow a more aggressive course of disease. This early-onset form of Alzheimer's has been recognized to be a familial form of the disease, inherited in an autosomal dominant fashion, that can be linked to mutations in three causative genes, PS1, PS2 and the APP gene. At least 230 different mutations within one of the genes have been identified in patients with familial Alzheimer's disease, thus showing the disease's heterogenicity. Similarly, to sporadic Alzheimer's, amyloid plaques and NFTs are detected in the brain, causing brain atrophy and neuronal loss. In addition, certain genetic mutations have distinctive features including cotton wool plaques, severe cerebral amyloid angiopathy or Lewy bodies. Specific criteria that indicate the need for genetic testing due to high suspicion of autosomal dominant early-onset familial Alzheimer's disease are age of onset of cognitive impairment and dementia under 65 years, family history, typically in at least 3 generations and an aggressive course of disease. Most patients with early-onset Alzheimer's will experience symptoms in their early 40's, although some may show signs of disease as young as their twenties. The mean course of disease is 8-10 years. There is currently no successful course of treatment for Alzheimer's patients.(34)

1.4.6 Brain Tumors

Brain tumors have the highest mortality among all cancers. In the pediatric population, the most common brain cancers are Medulloblastomas and low-grade gliomas. In adults, high-grade gliomas and meningiomas are more common. Regardless of malignancies, or grade of the tumors, any mass in the brain can cause elevation of intracranial pressure leading to herniation and death. The brains protection by the blood brain barrier (BBB) leads to impermeability of some therapeutic agents, further exacerbating poor outcomes. The main pathophysiological features

of any tumor include uncontrolled cell proliferation, evasion of apoptosis, angiogenesis, avoidance of immune surveillance and invasion. All these processes are regulated by genetic or epigenetic factors involved in signaling of cell cycle pathways. Brain tumors have been linked to several well-established genetic tumor markers including TP53 inactivation, PDGF overexpression and the involvement of CDK4/RB1 pathways. Some specific types of brain tumors present specific genetic markers such as a homozygous deletion of CDKN2A in oligodendrogliomas, deletions at chromosome 17p or isochromosome 17p in medulloblastomas. Identifying general and specific genetic markers of brain tumors is important to develop targeted therapeutic methods and improve the clinical treatment and outcome in brain tumors. (35)

2. Aims and objectives

The purpose of the study conducted is to research the applications of NGS in Neurology at the Faculty of Medicine, University of Rijeka in the time period from 2018 to 2022. The goal of the study performed is to evaluate the usefulness of the diagnostic method in a spectrum of neurologic diseases with high frequency of causative genetic variants in the etiology of the disease, specifically highlighting patient outcomes. The main questions to be answered in the study conducted are:

1) What are indications and applications for the use of NGS in the department of neurology?
2) What are the outcomes of NGS when performed in a spectrum of neurologic diseases at the Faculty of Medicine?

3. Participants and study design

3.1 Retrospective study of NGS performance in Neurology

A retrospective study of the application of NGS in Neurology was performed using the filed data stored in the department of Genetics, Faculty of Medicine, University of Rijeka. From all existing files, which date back to 2018, when NGS testing was first introduced at the University of Rijeka, all cases referred to genetic testing by the department of Neurology and pediatric

Neurology were considered, precisely from 2018 to 2022. All relevant data was obtained from the files. Names and other means of identification of patients were not obtained, nor were patients actively involved in the study, due to which no ethical approval or patients consent forms were needed for the performance of this study. Information extracted from each neurological patient is: age on day of NGS testing, sex, basic overview of presenting clinical features, indication for NGS testing, NGS result and final diagnosis. For Patients with positive NGS results the documentation includes the gene affected, the variant present, zygosity, the class of variant and interpretation of NGS provided by the official report.

4. Results

4.1. General Information

With this data, the findings were divided into seven classes of neurologic diseases: hypokinetic movement disorders, hyperkinetic movement disorders (excluding dystonia), dystonia, epilepsy and other seizures disorders, morphological brain anomalies and developmental disorders, sensory neurological disorders, dementias (excluding Parkinson) and neoplasms. For patients with multiple neurological manifestation of different classes of disease, they are classed according to the indication found on the official NGS report that was provided by the clinician referral. These groups of diseases are described and analyzed independently.

An overview of the number of NGS performed per year in neurology is visualized in table 1.

Table 1. Number and percentage of NGS performed at University of Rijeka in Neurology and pediatric Neurology per year (2018-2022)

	2018		**2019**		**2020**		**2021**		**2022**		**TOTAL**	
NEUROLOGY	2	1,7%	17	14,7%	15	12,9%	21	18,1%	27	23,3%	82	70,7%
PEDIATRIC NEUROLOGY	1	0.9%	3	2,6%	3	2,6%	4	3,4%	23	19,8%	34	29,3%
TOTAL	3	2,6%	20	17,2%	18	15,6%	25	21,6%	50	43,1%	**116**	

From a total of 116 neurologically indicated NGS performed at the University of Rijeka, 82 (71%) patients tested were referred by the department of Neurology, while 34 (29%) patients were referred by the department of pediatric neurology. It can also be perceived, that the option of performing NGS has been used increasingly with time from initially 3 (2,6%) patients in 2018 up to 50 (43%) patients in 2022. The data may suggest that the department of pediatric neurology has just begun to value NGS testing as their numbers in test have risen dramatically from 3-4 (2,6-3,4%) tests performed per year from 2019-2021 and being responsible for almost 50% (23 of 50) of NGS referrals in the year 2022. The number of initiated tests by the department of Neurology shows a slightly rising trend from 17 (15%) patients in 2019 to 27 (23%) patients in 2022 (excluding the first year of performance of NGS). Therefore, we may assume that this trend and the number of NGS performed on neurologic patients in the clinical hospital center Rijeka will continue to increase.

Of all NGS performed (116), at total of 31 different genetic variants were detected across 27 different genes in 27 patients, providing a positive diagnostic outcome in 23% of cases (23,3%). The most common types of variants found were substitutions, often causing a missense mutation, as well as frameshift mutations and premature stop codon insertions (nonsense mutations). An overview of all variants detected is illustrated in table 2. A detailed description of results is documented below, in before mentioned classes of neurologic diseases.

Table 2. Genetic variants discovered by NGS, (Group refers to the class of neurologic disease as divided in the following: 4.2. hyperkinetic movement disorders (excluding dystonia), 4.3. Dystonia, 4.4. hypokinetic movement disorders, 4.5. developmental disorders and morphological brain anomalies, 4.6. epilepsy and other seizure disorders, 4.7. sensory neurologic disorders)

GROUP	GENE	ALLELE	VARIANT	CLASS	ZYGOSITY	DIAGNOSIS
4.2.	ATM	c.1564_1565delGA, c.8147T>C	Frameshift variant, missense variant	Pathogenic variant (5), pathogenic variant (5)	Heterozygous, heterozygous	recessive form of ataxia-telangiectasia (OMIM: 208900)
4.2.	HTT	CAG trinucleotide repeat 20+/-1 and 42+/-1	Inframe trinucleotide repeat expansion	Pathogenic variant (5)	heterozygous	autosomal dominant Huntingtons disease (OMIM:143100)
4.2.	PANK2	c.894G>A, c.1043A>G	Substitution	Likely pathogenic (4), variant of unknown significance (3)	Heterozygous, heterozygous	neurodegeneration with iron accumulation in the brain 1 (OMIM:607236

4.2.	PRKCG	c.167T>A	substitution	Variant of unknown significance (3)	heterozygous	spinocerebellar ataxia, type 14 (OMIM:605361)
4.2.	MPV17	c.414G>A, c.122G>A	Substitution substitution	Likely pathogenic (4), pathogenic (5)	Heterozygous, heterozygous	autosomal recessive axonal Charcot-Marie-Tooth neuropathy type 2EE (CMT2EE) (OMIM:618400)
4.3.	GNAL	c.394A>G	Missense variant	Variant of unknown significance (3)	heterozygous	autosomal dominant dystonia, type 25 (OMIM: 315073)
4.3.	GNB1	c.352G>C	Missense variant	Likely pathogenic variant (4)	heterozygous	autosomal dominant intellectual retardation type 42 (OMIM:616973)
4.3.	YY1	c.1123C>T	Premature stop codon	Likely pathogenic (4)	heterozygous	dominantly inherited Gabriele-de Vries syndrome (OMIM:617557)
4.3.	GNAO1, TAFT1	c.529C>T	Premature stop codon	Variant of unknown significance /3), likely pathogenic (4)	Heterozygous, heterozygous	developmental and epileptic encephalopathy 17 (OMIM:615473), X-linked recessive syndromic neurodevelopmental disorder (OMIM: 617493)
4.3.	CHD8	c.5017C>T	Premature stop codon	Likely pathogenic (4)	heterozygous	autism spectrum disorders (OMIM:615032), phenotype childhood-onset progressive dystonia (PMID:34415117)
4.3.	SLC20A2	c.99delT	Frameshift variant	Pathogenic (5)	heterozygous	autosomal dominant basal ganglia calcification, idiopathic, type 1 (OMIM:213600)
4.4.	SGCA	c.409G>A	Missense variant	Likely pathogenic (4)	homozygous	limb girdle muscular dystrophy, type 2D (OMIM: 608099)
4.4.	ZFYVE26	c.6278dupC, c.4153C>T	Frameshift variant, premature stop codon	Likely pathogenic (4), likely pathogenic (4)	Heterozygous, heterozygous	spastic paraplegia 15 (OMIM:270700)
4.4.	SPAST	c.1315T>G	Missense variant	Variant of unknown significance (3)	heterozygous	spastic paraplegia 4 (OMIM: 182601)
4.4.	C19orf12	c.204_214delCGGGGGGCTGT	Frameshift variant	Pathogenic (5)	homozygous	neurodegeneration with iron accumulation in the brain 4 (OMIM: 614298)
4.4.	CLCN1	c.2680C>T	Premature stop codon	Pathogenic (5)	homozygous	autosomal recessive congenital myotonia (OMIM: 255700)
4.4.	MME	c.467delC	Frameshift variant	Pathogenic (5)	homozygous	autosomal recessive axonal Charcot-Marie-Tooth disease type 2T (OMIM:617017)
4.4.	KMT2B	c.4789C>T	substitution	Likely pathogenic (4)	heterozygous	childhood-onset dystonia 28 (OMIM:617284) and/or intellectual developmental disorder,

						autosomal dominant 68 (OMIM:619934)
4.5.	DNM1L, FAR1	c.1713+2T, c.304A>G	Consensus splice, missense variant	Variant of unknown significance (3), variant of unknown significance (3)	Heterozygous, homozygous	encephalopathy due to defective mitochondrial and peroxisomal fission 1(OMIM:614388), peroxisomal fatty acyl-CoA reductase-1 disorder (OMIM:616154)
4.5.	ARX	c.1497delG	Frameshift variant	Likely pathogenic (4)	hemizygous	X-linked lissencephaly type 2 (OMIM:300215)
4.5.	DYRK1A	c.691C>T	substitution	Pathogenic (5)	heterozygous	autosomal dominant mental retardation type 7 (OMIM:614104)
4.5.	DDX3X	c.1432_1433delAG	Frameshift variant	Likely pathogenic (4)	heterozygous	Snijders Blok type of X-linked dominant intellectual developemtal disorder (OMIM:300958)
4.5.	HECW2	c.3909C>G	Missense variant	Variant of unknown significance (3)	heterozygous	neurodevelopmental disorder with hypotonia, seizures and absent language (OMIM:617268)
4.6.	KAT6A	c.4645G>A	Missense variant	Likely pathogenic (4)	heterozygous	autosomal dominant Arboleda-Tham syndrome (OMIM:616268)
4.7.	NR2F1	c.169C>T	Premature stop codon	Pathogenic (5)	heterozygous	Bosch-Boonstra-Schaaf optic atrophy syndrome (OMIM:615722)

4.2. Hyperkinetic Movement Disorders (excluding Dystonia)

Dystonia is excluded here due to its high prevalence in Rijeka and is considered as an own entity (4.3.).

In this class of disorders 20 Patients were investigated, 5 resulting in positive genetic aberrations for their neurologic symptomatology (25%), as well as 2 non-symptomatic patients positive for a familial variant (10%). Additionally, 3 Patients had pending results at the time of conducting the study and one report file was missing (20%). 9 Patients NGS result was negative (45%). All results can be seen in table 3.

Table 3. Hyperkinetic movement disorders (excluding dystonia)

SEX	AGE	CLINICAL FEATURES	NGS RESULT	FINAL DIAGNOSIS
F	52	tremors in right hand during movement for 10 years, tremor of head and voice for 4 years, difficulty walking for 2 years, falls, knee pain, difficulty writing, lips feel heavy, difficulty swallowing, progressive hearing and vision loss, restlessness and fatigue	Negative	-
F	23	difficulty walking, speaking, and tremors of hand, head and voice, atactic peroneal gait with circumduction to left, inability to stand on heels and toes, foot muscle hypotrophy, instability in Romberg test, dysmetria of upper and lower extremities	Positive (class 5)	recessive form of ataxia-telangiectasia (OMIM: 208900) (ATM gene)
F	38	No documentation of clinical presentation	negative	-
M	59	throbbing in his head for last 20 years, last 2 years instability, balance disorders, speech disorders, MRI shows brain atrophy, wide base gait, dysmetria to left, dysarthria, uncertain tandem gait, no weakness of extremities, uvula to left, corner of mouth lower on left, asymmetry on left side, normal swallowing	negative	-
F	54	right sided tremors since 2015, progression of symptoms and paraesthesia's of right hand with dystonic attitude and stiffness, MRI shows signs of leukoencephalopathy, EMNG severe chronic radicular lesion of L5 and S1 on left side, BEAR neuronal lesion above pons, pain on right side of body and in neck, DATSCAN 2020 medium-severe form of Parkinson's disease	Negative	-
F	24	No clinical features of disease, mother affected by parkinsonism	negative	-
F	38	No documentation of clinical presentation	negative	-
F	50	dementia, repeats actions, rapid weight loss, difficulty moving, ataxia when walking, hand tremors with change of handwriting, whole body muscle spasms for 30min while conscious, temporally disoriented, dysmetria in coordination tests, pos. Babinski on right	negative	-
M	69	dementia, involuntary movements, , epileptic seizures, falls, personality change, avoidance of society, chorea of limbs and trunk, impaired executive functioning, delayed recall, impaired attention and abstract thinking, similar symptoms in mother and siblings	Positive (class 5)	autosomal dominant Huntingtons disease (OMIM:143100)

F	64	No clinical features of disease	Positive (class 5)	presence of family variant in ATM gene
M	69	No clinical features of disease	Positive (class 5)	presence of family variant in ATM gene
F	55	walking and speech disorder for 10 years, progressively worsening, dysarthric speech, dysmetria in heel-knee and finger-nose test, atactic gait, no headaches, history of stroke, MRI shows mild cortical atrophy, FH: mother Alzheimer's disease	Negative	-
M	59	Difficulty with walking and writing, progressive nature, MRI shows punctiform hyperintense areas in frontoparietal lobe bilaterally	negative	-
F	75	history of bilateral subdural hematoma, mild right sided weakness, involuntary head movements, choreatic movements of hands, FH: father had similar complaints	Report missing	
F	24	treated as dystonia, MRI under anaesthesia changes "tigers eye"	Positive (class 4,3)	neurodegeneration with iron accumulation in the brain 1 (OMIM:607236)
M	45	Difficulty walking and writing since childhood, now progressing, no family history, MRI shows cerebellar atrophy	Positive (class 3)	spinocerebellar ataxia, type 14 (OMIM:605361)
F	15	walking difficulties, neuropathic gait, no difficulties or weakness of upper extremities, abnormal neuroimaging, EMNG lower extremities indicate lesion of motor and sensory nerves bilaterally	Positive (class 4,5)	autosomal recessive axonal Charcot-Marie-Tooth neuropathy type 2EE (CMT2EE) (OMIM:618400)
M	64	onset at age 46 with tremor of right hand and micrographia, visual hallucinations, vivid dreams, anxious, low mood, pain in spine and shoulders, discrete rigor of neck, right sided rigor, bradykinesia, postural tremor bilaterally, no intention tremor, hypoesthesia of lower legs, negative FH	Results pending	
F	33	tremor of right hand for 8 months, onset of tremor of left hand 3 months ago, carpal tunnel on right, FH: grandfather unknown disease at 36yo, grandfather's sister's daughter Parkinson's disease	Results pending	
M	11mo	No documentation of clinical features	Results pending	

4.2.1. Patients with positive findings

A 23-year-old female patient presented with difficulty walking and speaking, and tremors of the hands, head and voice. Examination showed atactic peroneal gait with circumduction to the

left, inability to stand on heels or toes, her foot musculature was hypotrophic, Romberg test was instable and she had dysmetria of both extremities. She was referred to NGS due to suspicion of cerebellar degeneration. The working diagnosis was not confirmed; however, two variants of the ATM gene were detected, which represent an established cause of a recessive form of ataxia-telangiectasia. The variants detected were a frameshift variant on c.1564_1563 due to a deletion and a missense variant caused by substitution on c.8147. Both variants are classified as pathogenic variants, class 5. The patient is a heterozygote. Both her parents, age 64 and 69, though displaying no clinical features of the disease, were tested for the same genetic aberration, revealing that her mother was affected by the deletion frameshift variant and her father was affected by the missense variant caused by substitution. This suggests that in the patient, the interplay of both variants seemingly was the causative variant for her clinical presentation, as each variant individually does not evoke symptoms.

A male patient, 69, suffered from dementia, involuntary movements, epileptic seizures, and frequent falls. Family members described a recent change in personality of the patient and he was progressively avoiding society. On examination chorea of limbs and trunk were discovered, as well as impaired executive functioning, delayed recall, attention deficit and impaired abstract thinking. Similar symptoms were described in the patient's mother, sister and 3 brothers, strong suggesting an underlying genetic cause. He was referred to NGS due to presentation of extrapyramidal syndrome and dementia. The test results indicated a pathogenic variant in the HTT gene, an in-frame nucleotide expansion of CAG in two alleles (20+/1 and 42+/1), which is a confirmed cause of autosomal dominant Huntington's disease with the presence of an allele with CAG expansion in the range associated with fully penetrant Huntington's disease.

A 24-year-old female who had been treated for dystonia showed characteristic changes in an MRI under anesthesia, which were consistent with findings in Parkinson's disease. Due to these findings, she was referred to NGS due to suspicion of Parkinson's disease. NGS revealed that the patient was heterozygous for two substitutions of the PANK2 gene, a class 4, likely pathogenic variant on c.894 and a class 3 variant of unknown significance on c.1043. Pathogenic biallelic variants in the PANK2 gene, as represented in the patient, represent a confirmed cause of neurodegeneration with iron accumulation in the brain type 1, which presents with Parkinson and dystonia and shows the typical "eye of the tiger" sign on an MRI scan as had been detected in the patient.

The next patient was a male, 45, with difficulties walking and writing since childhood, which had been progressing recently. His family history was negative. An MRI performed showed

cerebellar atrophy. Due to the findings on the MRI scan, he was referred to NGS with suspicion of ataxia. The NGS report confirmed the suspicion, showing a substitution on c.167 of the PRKCG gene, a variant of unknown significance for which he was a heterozygote. As pathogenic heterozygous variants of the PRKCG gene are associated with spinocerebellar ataxia, type 14, the NGS report concluded the present variant to be a highly likely cause of the clinical presentation of the patient.

Finally, a 15-year-old patient with walking difficulties and neuropathic gait showed "abnormal neuroimaging". Unfortunately, the report was not specific. Additionally, an EMNG performed indicated a lesion of motor and sensory nerves bilateral of the lower extremities. The patients' upper extremities showed no signs of disease. The working diagnosis of the patient was a non-specific polyneuropathy, whether the lesion was peripheral or axonal was unclear. The NGS performed due to diagnosis of polyneuropathy showed two substitutions in the MPV17 gene, on c.414 and c.122, which were classified as likely pathogenic and pathogenic, respectively. Pathogenic biallelic variant of the MPV17 gene represent a confirmed cause of autosomal recessive axonal Charcot-Marie-Tooth neuropathy type 2EE.

4.3. Dystonia

Dystonia was the most common indication for NGS at the clinical hospital center Rijeka, and for that reason will be considered as a separate entity.

28 Patients were sent to be tested for genetic causes of Dystonia in Rijeka between 2018 and 2022. Of these, 19 test resulted in negative outcome (68%). 3 Results are pending at the time of this research (11%). Thus, the number of positive genetic diagnosis is 6 (21%) of 28 ordered NGS. The analysis of all patients is found in table 4. Patients with positive genetic test result will be highlighted individually.

Table 4. Dystonia

SEX	AGE	CLINICAL FEATURES	NGS RESULT	FINAL DIAGNOSIS
F	33	No documented findings	negative	-
F	50	Difficulty speaking, walking, dystonic movements, spasm of leg, areflexia of right arm	negative	-
F	36	tremors, dystonic posture of the body, involuntary movements of small muscles, blepharospasm, torticollis	Positive (class 3)	autosomal dominant dystonia, type 25 (OMIM: 315073)

F	27	juvenile rheumatic arthritis, abdominal muscles spasms from age 15 provoked by food intake, inability to straighten the trunk	Negative	-
M	54	tremors of the head 10 years ago, normal neurological exam, normal MRI	Negative	-
M	35	worsening spasms, involuntary movements of the trunk and neck into extension, worsening with walking and sitting	Negative	-
F	26	generalized dystonia, speech difficulty, gait, pronounced neck dystonia with torticollis to the left	Positive (class 4)	autosomal dominant intellectual retardation type 42 (OMIM:616973)
M	31	developmental delay due to pierre robbin syndrome, dysarthric speech, dyskinesias of the fingers and facial muscles ineffective therapy with akineton and rivotril, slight dysmetria on both sides, oromandibular dystonia and dystonic movements of the hands	Positive (class 4)	dominantly inherited Gabriele-de Vries syndrome (OMIM:617557)
M	71	head and hand tremors, dystonic head position, balance and gait disturbances, more pronounced on left, sister is affected by the same symptoms	Negative	-
M	58	Cervical dystonia, blepharospasm, jaw opening dystonia, spasmodic dysphonia, symptoms worsen as day progresses, botulinum toxin ineffective	Negative	-
F	40	cervical dystonia for 16 years, responds well to botulinum therapy, appetite affected, torticollis	Negative	-
F	47	difficulty walking, generalized dystonia, physical therapy, gait and tremor of hands	Positive (class 3)	neurodevelopmental disorders and unwanted movements including dystonia (OMIM:615473, 617493,,PMID:28747448, 30642806, 29758257), X-linked recessive syndromic neurodevelopmental disorder
F	78	head tremors, speech disorder, onset 2-3years ago with dizziness, no headaches, feels cramping sensation in feet, mild dementia, voice tremor	Negative	-
F	41	dystonia since puberty, numbness, slow right hand, neck affected, voice tremor, good response to botulinum toxin, negative family history, involuntary movements, dystonic position of hands	Positive (class 4)	childhood-onset progressive dystonia (PMID:34415117)
F	11	wheelchair, appearance of transient torticollis (left) and laterocollis in 2018, currently rapid onset painful laterocollis with worsening after traction procedure, FH: mother 2x transient torticollis	negative	-
F	56	cervical dystonia for 15 years, MRI shows non-specific demyelinating changes, no FH	Negative	-
F	55	Torticollis since birth, jerks in head for 10 years increase with stress, dystonic movements of the head to the right, tense paravertebral muscles	Negative	-
F	61	Difficulty speaking, speech dysphonic, no variations of voice during day, no FH	Negative	-
F	69	symptoms of cervical dystonia and dystonic tremor of the head	Negative	-
M	55	involuntary movement of head, no automatism or aura, worsening with stress, bradykinesia and tremor of right hand, head tremor, sensory tick to left, cervical dystonia, EEG shows epileptic changes, MRI shows cyst of pineal gland, history head trauma in 1992 with concussion	Negative	-

F	63	No symptoms of disease, familial variant detection due to positive family anamnesis (daughter)	negative	-
M	68	No symptoms of disease, familial variant detection due to positive family anamnesis (daughter)	negative	-
F	16	Anxiety attacks followed by tingling in extremities and right side of face, last few min to 30min, after attack feels very tired, first attack stress induced, occurrence regardless of surrounding, neuroimaging shows calcifications in basal ganglia	Positive (class 5)	autosomal dominant basal ganglia calcification, idiopathic, type 1 (OMIM:213600)
M	56	Symptoms of dystonia	Negative	-
F	44	spasm of individual muslces, pronounced cervical dystonia, right laterocollis, trunk shows lateropulsion	Negative	-
M	48	generalized form of dystonia, good response to therapy	Results pending	
M	12	tics since 3yo, first was squinting of eyes, progression to vocal and motor tics, dystonic spasms, severe cramping lasting 3 weeks, pain like cramp in neck, difficulty walking, pronounced fatigue	Results pending	
M	51	tremor of head since 1993, FH: mother and sister have same symptoms	Results pending	

4.3.1. Patients with positive findings

The first patient to be considered is a 36-year-old female who presented with tremors, dystonic posture, involuntary movements of small muscles, blepharospasm and torticollis. She was referred to NGS testing with a working diagnosis of blepharospasm-type dystonia and cervical dystonia. NGS revealed a missense variant caused by substitution of adenosine to guanine at c.394 on the GNAL gene. The patient presented as heterozygous for the variant classified as class 3, variant of unknown significance. The report concluded that the variant was likely responsible for the clinical presentation of the patient, since similar heterozygous variants in the GNAL gene represent an established cause of autosomal dominant dystonia, type 25.

A female, 26, presented with symptoms of generalized dystonia, speech difficulty, gait and pronounced cervical dystonia with torticollis to the left side. NGS was indicated due to the diagnosis of dystonia and revealed a variant in the GNB1 gene. The missense variant caused by substitution of guanine to cytosine in c.352 is classified as class 4, likely pathogenic, variant and was present as heterozygous in the patient. The NGS report concluded that the patient suffers from autosomal dominant intellectual retardation type 42.

Furthermore, a 31-year-old male with a history of developmental delay due to diagnosed Pierre Robin syndrome at birth, presented with dysarthric speech, both-sided dysmetria and dyskinesias of fingers and face, which were irresponsive to therapy with akineton and rivotril. On

examination dystonic movements of the hands and signs of oromandibular dystonia were recognized. NGS performed due to suspicion of dystonia revealed the diagnosis of Gabriele-de Vries syndrome. The syndrome is caused by heterozygous loss of function mutations of the YY1 gene, in this case specifically a premature stop codon was initiated due to a substitution variation in c.1123. The variant was described as likely pathologic for Gabriele-de Vries syndrome (class 4). Even though the syndrome is typically associated with mild cognitive impairment and various congenital anomalies, the NGS report stated that movement disorders in the form of progressive dystonia have been described in at least 2 patients with loss of function mutations in the YY1 gene, thereby declaring the present variant a likely cause of symptoms.

A 47-year-old female patient with general dystonia had difficulty walking. Examination showed gait and tremor of hands. She was undergoing physical therapy. NGS was performed due to the diagnosis of dystonia. Genetic testing revealed two separate variants in the GNAO1 and TAF1 genes. The first was a premature stop codon insertion at c.529 on the GNAO1 gene which has unknown significance (class 3 variant). The report also stated the presence of a likely pathogenic variant in the TAF1 gene, the specifics of which unfortunately are not documented. The patient was heterozygous for both variants. Loss of function mutations in the GNAO1 gene have been linked to several disorders including developmental and epileptic encephalopathy type 17, which presents with convulsions, developmental delay and dystonia, or neurodevelopmental disorders and unwanted movements including dystonia. Variants of the TAF1 gene are associated with an X-linked recessive syndromic neurodevelopmental disorder, which has been reported in females due to non-random inactivation of the X-chromosome. It could not be concluded which variant or disorder resulting from a variant in the genes was responsible for the clinical picture of the patient. An interplay of variants in both genes may be plausible.

The next patient, a female, 41, had been diagnosed with dystonia since puberty. She presented with numbness, a slow right hand and voice tremor that responded well to treatment with botulinum toxin. On examination cervical involvement was noticed, she performed involuntary movements and showed a dystonic positioning of the hands. Her family history was negative for dystonia. NGS showed a likely pathogenic (class 4) premature stop codon insertion at c.5017 on the CHD8 gene, which, as a heterozygote, is a highly penetrant risk factor for autism spectrum disorders. Recently the phenotype associated with nonsense variants, as described in the patient, have been extended to a broad spectrum of neurodevelopmental disorders including childhood-onset progressive dystonia. Due to the symptomatic presentation of the patient the latter can be described as the most likely diagnosis made based on her genetic aberration.

The final patient in the group of dystonia was a 16-year-old female presenting with the occurrence of anxiety attacks followed by a tingling sensation in her extremities and the right side of her face. The attacks lasted a few minutes up to 30min and during a recovery phase the patients felt exhaustion. The first attack seemed stress induced, occurring after starting a new career and ending a relationship, however, the patient described the following attacks to have occurred regardless of her surroundings or emotional state. Neuroimaging was performed and showed calcifications in the basal ganglia. She was referred to NGS for genetic testing with a differential diagnosis of Fahr's disease and dystonia. NGS revealed a heterozygous pathogenic (class 5) frameshift variant caused by deletion of tyrosine at c.99 of the SLC20A2 gene, which is a known cause of autosomal dominant basal ganglia calcification, idiopathic, type 1. In this case the diagnosis of dystonia was excluded due to genetic testing results.

4.4. Hypokinetic Movement Disorders

Of the overall 27 patients included in this study who have undergone NGS due to the occurrence of characteristic signs of a hypokinetic movement disorder with suspected underlying genetic variants, 11 resulted in negative findings (41%). This is interpreted in a way that with current knowledge of variants in genetic material that may or certainly cause specific diseases, no relevant variants are found that can sufficiently explain the clinical features they present with. For 8 of the patients tested within this group of disorders, NGS successfully provided a diagnosis of genetic nature (30%), and additional 2 familial variants were found in tested family members of a diagnosed patient (7,4%). The remaining 6 patients cannot be commented on in this study, due to missing/pending results (22%). The results of the study analysis of patients can be found in table 5. All patients showing positive NGS results are considered individually.

Table 5. Hypokinetic movement disorders

SEX	AGE	CLINICAL FEATURES	NGS RESULT	FINAL DIAGNOSIS
F	7	Muscles weakness of neck, hip and trunk; lab: hyperchloremia, hyperpotassaemia	Positive (class 4)	limb girdle muscular dystrophy, type 2D (OMIM: 608099)
F	14	Chronic respiratory insufficiency, heart defects, MRI changes of brain, psychomotor retardation, osteoporosis, FH: SIDS	negative	-

F	56	Muscle spasms of whole body, joint stiffness, muscle weakness, neck pain, meningioma	negative	-
F	40	Spastic paraplegia; atrophy and hypoesthesia of hands, lower legs and feet; progressive nature (onset age 15 legs, age 20 hands), wheelchair, inability to feed self	Positive (class 4)	spastic paraplegia 15 (OMIM:270700), neurodegenerative disorder
M	54	Involuntary leg movements	Negative	-
M	40	Weak gait since age 6, wheelchair since age 12, DM1	negative	-
M	35	walk with limp and waddling, crutches until age 14, DM1	negative	-
F	57	No clinical features of disease, FH: 2 children with hypokinetic movement disorders	negative	-
M	68	No clinical features of disease, FH: 2 children with hypokinetic movement disorders	Negative	-
F	10	slowed motor and speech development, motor restlessness, attention and concentration difficulties, elevated creatinine kinases, myopathic altered EMNG, normal MRI, karyotype normal	negative	-
F	53	Weakness in both extremities, progressive nature, difficulty walking since 2013, hyperreflexia, normal MRI	Positive (class 3)	spastic paraplegia 4 (SPG4 ili SPAST-HSP, OMIM: 182601)
F	62	No clinical features of disease, positive familial diagnosis (daughter)	Positive (class 4)	Presence of familial variant in heterozygous state
M	64	No clinical features of disease, positive familial diagnosis (daughter)	Positive (class 4)	Presence of familial variant in heterozygous state
F	16	No documented findings	Positive (class 5)	neurodegeneration with iron accumulation in the brain 4 (NBIA4, OMIM: 614298)
M	21	Clumsiness in childhood, weakness of muscles of extremities, positive myotonic phenomenon of hands, no FH	Positive (class 5)	autosomal recessive congenital myotonia (OMIM: 255700)
F	51	Progressive weakness of extremities from age 40, hypotrophy of handy, lower legs and feet, peroneal gait, hypoalgesia, thermaesthesia, hyporeflexia, EMG shows motor neuron loss, neurography shows slowing of conduction and amplitude reduction, FH: sister affected by same symptoms	Positive (class 5)	autosomal dominant and autosomal recessive axonal Charcot-Marie-Tooth disease type 2T (OMIM:617017)
F	46	Weakness of thigh muscles, feet, no visible atrophy, areflexia of lower extremity, upper extremity normal, EMG shows motor neuron loss, neurography shows slowing of conduction and amplitude reduction, FH: sister affected by same symptoms	Positive (class 5)	autosomal dominant and autosomal recessive axonal Charcot-Marie-Tooth disease type 2T (OMIM:617017)
F	54	development of hoarseness, disturbances in speech, stretching and swallowing with sudden onset, weight loss, gets tired during the day, MRI shows hypoplasia, EMNG generalized damage mostly in small muscles of hands and feet, hyperreflexia, tremors, dysarthria	negative	-

F	71	bilateral almost complete ptosis, fixed bulbs, hearing problems, diagnosed oculopharyngeal form of muscular dystrophy	negative	-
F	47	left sided weakness, MRI shows no signs of CNS demyelinating disease, tremor of left hand, dysmetria on left, paretic gait on left, hemihypoesthesia on left side	Negative	-
F	17	dystono-dyskinetic form of cerebral palsy, feeding difficulties, hypotrophy protein-energy deficit, epilepsy, progressive neuromuscular disease	Positive (class 4)	childhood-onset dystonia 28 (OMIM:617284) and/or intellectual developmental disorder, autosomal dominant 68 (OMIM:619934)
F	63	recurrent falling, gradual progressive difficulty walking, leg spasms, hypotonia of legs	Results pending	
F	25	occasional involuntary jerks of hands and legs, impaired visual field (binasal hemianopsia), muscle weakness, severe headaches, worsened after infection with COVID19, psychiatric problems, normal female karyotype, negative for fabrys disease	Results pending	
M	10	sudden weakness in legs, could not walk properly with right sided upper and lower limb weakness and left sided facial weakness, mild headache, 4days prior febrile 39°C for 2 days and then subfebrile for next 2 up to attack	Results pending	
M	10	preterm birth at 34weeks, dystonia in childhood, since 9 months pain in legs, waddling gait, clumsy, falls often, difficulty standing up, headaches	Results pending	
F	5	Arnold-Chiari malformation detected during suboccipital osteoclastic craniotomy due to loss of consciousness, epilepsy	Results pending	
M	15	difficulty in relaxation of muscles, EMNG shows myotonic bursts of paravertebral muscles	Results pending	

4.4.1. Patients with positive findings

The first patient was a young female, 7 years old, who suffered from muscle weakness of the neck flexor muscle group and the group of muscles responsible for adduction of the trunk and hip. Additionally, lab results showed hyperCKemia, due to which muscular dystrophy was suspected. She was referred for NGS with the indication of suspected limb girdle type muscular dystrophy. NGS revealed a missense variation in the SGCA gene caused by substitution of Guanine to Adenosine in c.409, for which the patient was homozygous. This gene variant is categorized in class 4, a likely pathogenic variant, which confirms the working diagnosis of muscular dystrophy as pathogenic variants of this gene represent an established cause of limb girdle type muscular dystrophy, type 2D.

Second, a 40-year-old female patient with a long history of difficulty walking since the age of 15, presented in a wheelchair with atrophy of the lower legs and feet and contractures at the ankle joints, as well as progressive loss of muscle function in the hands from the age of 20 with severe spastic paraplegia, inabling the patient to feed herself. Moreover, the patient presented with polyneuropathic hypoesthesia affecting the hands and legs below the knee. Her working diagnoses included spinocerebellar degeneration, specifically SMA type 6, spastic paresis, possible hereditary mixed polyneuropathy or other chronic motor neuropathy and leukoencephalopathy. NGS was performed due to chronic motor neuropathy revealing spastic paraplegia type 15, a neurodegenerative disorder with is characterized by progressive spasticity affecting lower limbs and is also associated with other neurologic dysfunction including intellectual disability, hearing and visual defects and a thin corpus callosum. It may be added that unrelated to the muscular symptoms, documentation also showed suspicion of possible retinitis pigmentosa, indication symptomatology consistent with visual disturbances. Responsible for her spastic paraplegia type 15 was a biallelic variant in the ZFYVE26 gene, the first variation being a frameshift variant caused by duplication of Cytosine on c.6278, and the second a premature stop codon due to Tyrosine on c.4153. Both transcript changes are classified as class 4, likely pathogenic variants, and present as heterozygous variants in the patient. Both her parents were tested for the variant, revealing that while none had symptoms, the mother was positive for the duplication variant on c.6278 and the father of the patient had the premature stop codon. This suggests that an interplay of both variants resulted in the clinical picture of the patient.

A female patient age 53 presented with difficulty walking since 2013 due to muscle weakness in her lower extremities, upper extremities were also affected by weakness. The features were described to be of progressive nature. Examination of the patient showed hyperreflexia and a previous MRI performed showed normal brain morphology. NGS was performed due to suspicion of hereditary spastic tetraparesis. The working diagnosis could be confirmed due to the finding of a missense variant caused by a substitution of cytosine for tyrosine on c.1315 in the SPAST gene. The variant, for which the patient was heterogenous, is categorized as class 3, a variant of unknown significance, however, due to the alignment of the patient's clinical presentation and the symptoms of spastic paraplegia type 4 caused by pathogenic heterozygous variants in the SPAST gene, the NGS report considered it very likely to be the cause of disease.

Unfortunately, no findings of clinical presentation or history of disease were found for the 16-year-old female patient referred to NGS based on neurodegeneration with iron accumulation with suspicion for C19orf12 related disorder. NGS confirmed a class 5 variant (pathogenic

variant) of the C19orf12 gene. The variant was caused by a deletion in the region of c.204_214 leading to a frameshift mutation. Variants of the C19orf12 gene represent an established cause of neurodegeneration with iron accumulation in the brain type 4, regardless of zygosity. The patient presented as a homozygote for the variant.

A 21-year-old male patient presented with a history of appearing clumsy in childhood and now complained of weakness of the muscles in the extremities. Examination showed a positive myotonic phenomenon in the hands and no muscle atrophy. His family history was inconspicuous. NGS was indicated by suspicion of congenital myotonia, possibly Thomsen or Becker type. A premature stop codon insertion by substitution for tyrosine on c.2680 of the CLCN1 gene revealed as a pathogenic variant that represents an established cause of autosomal recessive congenital myotonia. The patient was a homozygote for the variant, confirming the working diagnosis.

The following two patients are sisters affected by the same neurological disease, due to which they are considered simultaneously. The first was a 51-year-old female presenting with progressive weakness beginning at the age of 40. Both upper and lower extremities were affected, she was not able to stand on heels or toes. Examination showed hypotrophy of hands, lower legs and feet, peroneal gait and sensory disturbances (hypoalgesia, thermoanesthesia). She showed hyporeflexia in both extremities and areflexia of the feet. Her younger sister, 46, presented with similar weakness limited to her lower extremities. She also could not stand on heels or toes, showed inability to stand up using the muscles of the thigh. On examination she had no visible muscle atrophy, but showed areflexia of the entire lower extremity. Previous studies conducted were EMG and neurography, which show motor neuron loss and slowing of conduction and amplitude reduction, respectively, in both the sisters. NGS was indicated due to working diagnosis of polyneuropathy, specifically suspected Charcot-Marie-Tooth Neuropathy. Sequencing revealed the same frameshift variant caused by a cytosine deletion on c.467 of the MME gene. This class 5 variant presented as homozygous in both sisters, representing a pathological variant causing autosomal dominant axonal Charcot-Marie-Tooth disease type 2T.

The last patient to be described in this group of neurological disorders is a 17-year-old female presenting with progressive neuromuscular disease, spastic tetraparesis, normal development until 4.5years when difficulty in walking appeared progressing to the need for the use of a wheelchair by the age of 7. She also had numerous other notable neurologic conditions including epilepsy, a dystono-dyskinetic form of cerebral palsy and feeding difficulties resulting in the need for PEG recently due to hypotrophy protein-energy deficit. Previously an MRI study

revealed deep white matter atrophy in parietal and occipital lobes and enlarged ventricles. NGS was performed primarily due to cerebral paralysis and revealed a class 4 (likely pathogenic) variant in c.4789 KMT2B gene due to substitution of cytosine for tyrosine. The female was heterozygous for the variant. The NGS report confirmed that variants in the KMT2B gene can be the cause of two distinct neurologic disorders, childhood-onset dystonia type 28 and/or autosomal dominant intellectual developmental disorder type 68. Considering the clinical picture presented, findings indicate a likelihood that the patient was affected by both disorders arising from this variant.

4.5. Developmental disorders and morphological brain anomalies

In this category, 14 patients underwent testing by NGS, 5 of which resulted in positive results for underlying genetic disorders (36%). At the time of analysis 4 results were pending and unfortunately one report was missing entirely (36%). With only 4 negative results for genetic disorders (29%), this group of disorders had the highest outcome of genetic diagnosis.

Table 6. Developmental disorders and morphological brain anomalies

SEX	AGE	CLINICAL FEATURES	NGS RESULT	FINAL DIAGNOSIS
F	1 mo.	cyst discovered prenatally, status without asymmetry and pathological motor pattern, good muscle tone	negative	-
F	1	Inability to sit independently or turn in lying position, reaction to sound and light, no eye contact, partial head control, infantile spasms, hand jerks several times per day, hypotonia of extremities, abnormal EEG, normal reflexes, history of septic shock and cardiac arrest, brain MRI shows brain atrophy and hypomyelination	Positive (class 3), (class 3)	encephalopathy due to defective mitochondrial and peroxisomal fission 1 (EMPF1, OMIM:614388), peroxisomal fatty acyl-CoA reductase-1 disorder (OMIM:616154)
M	5	pregnancy complications, normal psychomotor development, prominent forehead, dolichocephaly, back skin change similar to cutis marmorata, speech therapist, MRI shows pronounced cisterna magna, small volume of right cerebellum, rotated vermis, mother has history of medically indicated abortion due to down syndrome	negative	-

M	14 d	cystic formations in fetal head, mild venticuomegaly, oligohydramnios, brachycephlic fetal head seen prenatally, convulsions starting 10min after delivery (blinking, smacking, rowing hand then generalized tonic clonic seizures), hyptonia, dolichocepahalic head, low set ears, hig forehead, pronounced neurocranium, micrognathia, high palate, micropenis, MRI shows pachygyria, agenesis of corpus callosum and colpocephaly	Positive (class 4)	X-linked lissencephaly type 2 (OMIM:300215)
M	2	global developmental dely, difficult in communication and speech-language, craniofacial dysmorphia, microcephaly, atpical febrile convulsions, abnormal brain MRI (unmyelinated zones and microbleeding)	Positive (class 5)	autosomal dominant mental retardation type 7 (OMIM:614104)
F	10	global developmental delay in motor skills, speech development, cognitive deviation, low vision, dysmorphia, malformation of brain (ventriculomegaly, partial agenesis of corpus callosum, epilepsy), epilepsy and tremors with EEG changes	Positive (class 4)	Snijders Blok type of X-linked dominant intellectual developmental disorder (OMIM:300958)
F	9	slow development, speech difficulties, short attention and concentration, motor restlessness, low results of wechsler intelligence test (slow processing of information, cognitive defect in working memory, mothers help in personal hygiene, difficult social interaction), cardiac abnormalities (transposition of great arteries, VSD, ASD, coarctation of aorta)	negative	-
F	16	well controlled epilepsy, fetal hypertrophy, syndactyly of 6 fingers on hand and congenital heart defects, global developmental delay of motor, speech, and social interactions difficult, macrocephaly	No report	
F	4	difficulty in speech development, short term eye contact, difficulty to follow instructions, deaf, had hearing aid, history of VSD	negative	-
F	6	absence of expected physiological development, difficulties in all developmental areas, averbal, seizures with nonspecific EEG changes, respond well to therapy, eye-contact made, strabismus, nystagmus, microcephaly, malformation of CNS	Positive (class 3)	neurodevelopmental disorder with hypotonia, seizures and absent language (OMIM:617268)
F	16	doesn't make contact, doesn't speak, repeats vocalizations, grabs other people tightly with her hands, grabs toys and throws them, sits on floor in w position, walks on wider base, walks unsteadily with arms in the air, kyphotic posture with straightened lumbar lordosis, both feet in incorrectable planovalgus, in wheelchair pushed by mother	Results pending	
F	6	good psychomotor development, at age of 2 noticed improper social interaction, no playing with other children, motorically very active, auto-aggressive with light and sound stimuli, likes sensory stimuli, occasional flickering eyelids maybe related to light stimuli, non-verbal communication with mother, understands simple commands, no sense of danger or fear	Results pending	

M	5	eye contact present, social skills below average for age, tearful and frightened during exam but cooperation possible, head bitemporally narrowed, ears otostatic, myopathic muscular stature, mouth half open, articulations less pronounced, left eye ball more medial, hypermobile joints, feet in planovalgus, skin show 2 cafe au lait lesion 2x1cm, hypotonia at 3 months	Results pending
M	1	MRI shows asymmetric ventricles, ventriculomegaly esp. in area of frontal horns, arachnoid cysts on right apicotemporal and prepontine bilaterally, macrosomic microcephalic child, congenital stridor and laryngomalacia, GER, development normal	Results pending

4.5.1. Patient with positive findings

A one-year-old female child was examined at a regular check up with her pediatrician, who noticed she could not sit independently or turn to lying position and she did not form eye contact. She reacted to sound and light. The patient displayed infantile spasms and hand jerks several times per day, the performed EEG was abnormal. On motor examination hypotonia of the extremities was detected. Reflexes were normal. The patient had suffered from septic shock and cardiac arrest a few months prior. Further MRI studies showed brain atrophy and hypomyelination. Her extensive working diagnoses included a wide range of possible encephalopathies, such as epileptic encephalopathy, leukoencephalopathy, or non-specific encephalopathy. Epilepsy may be part of her differential. It is clear, however, that the girl likely suffered from psychomotor retardation due to an unknown cause. A metabolic disorder was suspected and NGS performed to detect possible genetic causes of encephalopathy and/or metabolic disorders. Two gene variants were detected by NGS on different genes. The first was a consensus splice variant of unknown significance (class 3) on c.1713 of the DNML1 gene, for which the girl was a heterozygote. Pathogenic variants in the DNML1 gene are an established cause of encephalopathy due to defective mitochondrial and peroxisomal fission type 1. The second variant was a missense variant of unknown significance (class 3) on c.304 of the FAR1 gene, for which she was affected by in a homozygous form. Pathogenic homozygous variants in the FAR1 gene are a known cause of peroxisomal fatty-acyl-CoA reductase-1 disorder, which is characterized by severely delayed psychomotor development, growth retardation with microcephaly and seizures. While both variants were classified as of unknown significance, the clinical picture of the patients suggests it is very likely that these variants of the genes did in fact cause the disorder in the patient. It is very possible that the girl suffered from both.

A 14-day-old male was referred to NGS after cystic formations in the head, ventriculomegaly, a brachycephalic head and oligohydramnios were detected on prenatal examinations. Ten minutes after delivery of the baby, he had started convulsing. First the convulsions started as blinking, smacking, and rowing his hand, then they progressed to generalized tonic-clonic seizures. Upon further examination after birth, distinct facial features including a dolichocephalic head, low set ears, high forehead with pronounced neurocranium, micrognathia, high palate and micropenis were noted. The baby appeared overall hypotonic. An MRI study was conducted which further showed pachygyria, agenesis of the corpus callosum and colpocephaly. NGS was performed due to high suspicion of newborn lissencephaly. The suspicion was confirmed. NGS detected a likely pathogenic (class 4) frameshift variant of the ARX gene due to a deletion of guanosine in the c.1497 allele. This represents a confirmed cause of X-linked lissencephaly type 2.

A two-and-a-half-year-old male presented with global developmental delay, having difficulties in communication and speech-language formation. The patient showed craniofacial dysmorphia, that was not further described, microcephaly and an abnormal brain MRI with unmyelinated zones and microbleeding. He had a history of atypical febrile convulsions. NGS revealed a variant in the DYRK1A gene, that was pathogenic (class 5) and represents an established cause of autosomal dominant mental retardation type 7, characterized by intellectual disability, impaired speech development, autism spectrum disorder and microcephaly. The boy was heterozygous for the substitution variant on c.691 of the DYRK1A gene.

A 10-year-old female patient was referred to NGS due to a global developmental delay in motor skills, speech development, cognitive deviation, low vision, and dysmorphic features, which were not described further, the patient had previously known malformations of the brain, imaging studies showed ventriculomegaly and partial agenesis of the corpus callosum. Furthermore, epilepsy with tremors and EEG changes, including a previous episode of status epilepticus, accompanied her clinical picture, either as its own diagnosis or part of her syndromic picture. The NGS report concluded the patient's diagnosis is Sneijder's Block type of X-linked dominant intellectual developmental disorder, due to a heterozygous, likely pathogenic frameshift variant caused by a deletion on c.1432_1433 of the DDX3X gene.

A female patient, 6, suffered from an absence of expected physiological development and seizures with EEG changes that respond well to therapy. She had difficulties in all developmental areas, especially pronounced was her lack of speech development, the girl was completely averbal. She could establish eye-contact on examination. Her vision, however, was likely

impaired due to her strabismus and nystagmus. Imaging showed microcephaly and a malformation of the CNS. NGS revealed a missense variant of unknown significance caused by substitution on c.3909 of the HECW2 gene. The girl was a heterozygote for the variant that is an established cause of neurodevelopmental disorder with hypotonia, seizures and absent language.

4.6. Epilepsy and other Seizure Disorders

In the group of epileptic disorders, there is only one positive result to further investigate (11%). However, it is necessary to point out that epileptic seizures are a common symptom in other neurologic disorders or may appear as secondary diagnosis in cases discussed previously. The results of the analysis performed on patients with clinical presentation of solely or primarily epileptic or other seizures are highlighted here.

Table 7. Epilepsy and other seizures disorders

SEX	AGE	CLINICAL FEATURES	NGS RESULT	FINAL DIAGNOSIS
F	33	Non-specific epilepsy, MRI shows no signs of cerebral vasculitis, focal atrophies on left hemisphere, takes antiepileptics	Negative	-
F	1	epileptic seizures, nystagmus, short eye twitching, opisthotonos, tone variable to elevated, difficult dorsiflexion of foot, multiple malformations, tested for di George syndrome and metabolic screening performed (neg)	Positive (class 4)	autosomal dominant Arboleda-Tham syndrome (OMIM:616268)
F	29	No clinical features of disease, positive family anamnesis of genetic aberration causing epilepsy (daughter)	Negative	No family variant
M	33	No clinical features of disease, positive family anamnesis of genetic aberration causing epilepsy (daughter)	Negative	No family variant
F	8	seizures, involuntary hand movements all day, EEG normal	negative	-
M	9	febrile convulsion with foam in mouth, eyes bulged out, mouth changed color, non-responsive to call, hand shaking bilaterally, lasted up to 5 minutes, pathological EEG, twin brother has similar symptoms of delayed speech and febrile convulsions, history mother: 2 spontaneous abortions, gestational diabetes, smoked during pregnancy	negative	-

M	43	epilepsy since 15yo, seizure with minimal increase in body temp, talking incoherent, stare (pseudotemporal absence), then hypermotor seizures in clusters, cyanosis of face, transition to bilateral tonic-clonic seizures, video EEG shows seizures with oral automatisms, left hand automatisms, takes antiepileptic medication	Results pending
F	42	episodes of speech production difficulties, onset of tics in late adolescence, EEG and MRI normal, psychomotor development normal, no CNS inflammatory disease, FH: father has similar epilepsy	Results pending
M	11mo	No clinical features documented	Results pending

4.6.1. Patients with positive findings

The patient, an almost 2-year-old female, presented with epileptic type seizures. Moreover, her eyes showed signs of a disorder, including nystagmus, short eye twitching, opisthotonos and elevated tone. Due to multiple malformations, which are not further specified in documentation, she was tested for di George syndrome and a metabolic screening was performed. Due to both tests yielding no explanation for her symptomatology, the girl was referred to NGS with the indication of epilepsy. NGS revealed a likely pathogenic (class 4) missense variant caused by a substitution of guanine for adenosine on the c.4645 allele of the KAT6A gene, which, when present in a heterozygous form, is a confirmed cause of autosomal dominant Arboleda-Tham syndrome. The NGS report states that although epilepsy is not a frequent feature of the syndrome, it has been described in patients with pathogenic variants of the KAT6A gene, therefore, the class 4 variant was likely the causative agent for the clinical picture of the patient.

4.7. Sensory Neurologic Disorders

Sensory neurological disorders include all disorders related to the special sensory organs of the body, the eyes, ears nose and tongue. Sensory disorders related to touch are not included in this category. Moreover, involvement of sensory systems in other syndromes or as a secondary disorder are not listed here.

In the group of sensory disorders, primary deficits of sight, hearing, taste, and smell are considered. In the clinical hospital center Rijeka, from 2018 to 2022, 8 patients were referred to NGS for establishment of possible genetic disorders related to a disturbance in sensory systems. A diagnosis of genetic disease could be made in two cases (25%). 4 cases were negative in regards to genetic causes (50%) and 2 results are still pending currently (25%).

Table 8. Sensory neurologic disorders

SEX	AGE	CLINICAL FEATURES	NGS RESULT	FINAL DIAGNOSIS
F	31	noticed poor vision at start of school, better in dark, depression of retinal sensitivity, concentric narrowing, thin optic nerves on MRI, visual acuity 20%, not improved with glasses	Positive (class 5)	Bosch-Boonstra-Schaaf optic atrophy syndrome (OMIM:615722)
F	33	noticed poor vision at start of school, better in dark, depression of retinal sensitivity, concentric narrowing, thin optic nerves on MRI, visual acuity 20%, not improved with glasses	Positive (class 5)	Bosch-Boonstra-Schaaf optic atrophy syndrome (OMIM:615722)
F	66	No clinical features of disease, family anamnesis of optic disorder (two daughters)	negative	Causative genetic aberration not determined
M	65	No clinical features of disease, family anamnesis of optic disorder (two daughters)	Negative	Causative genetic aberration not determined
F	43	progressive external ophthalmoplegia, ptosis for over 10 years, sus. neuroborreliosis, MRI normal	Negative	
F	71	bilateral almost complete ptosis, fixed bulbs, hearing problems, diagnosis of oculopharyngeal form of muscular dystrophy in 2000, symptoms appeared in form of double vision at 22yrs, history of cardiorespiratory arrest with loss of consciousness fall and skull fracture in 2008, no other signs of muscle weakness	Negative	
F	73	blind since 1989 due to paresis, started as double vision, after 90s progression of ptosis until 2016 complete ptosis, MRI hypotrophy of eye muscles, no difficulty swallowing or walking	Results pending	
M	8mo	no family history, ABR on both sides without response, microcephaly, development normal, personal history: bronchiolitis, bronchopneumonia and rotavirus gastroenteritis, mother had gestational diabetes in pregnancy	Results pending	

4.7.1. Patients with positive findings

The two patients with positive results for their visual disturbance are sisters, who were both diagnosed with the same syndrome following NGS. Therefore, they are considered together. The two females, 31 and 33 years of age, both noticed poor vision at the start of school and that their vision improved in the dark. Examination showed a depression of retinal sensitivity,

concentric narrowing, and thin optic nerves on an MRI in both the women. They also shared the same visual acuity of 20%, and for neither it improved with glasses. NGS was recommended due to 2 members of the same family sharing the same symptoms and suspicion of an optical neuropathy with nystagmus and exophoria. NGS revealed a class 5 pathogenic premature stop codon caused by a substitution variation on c.169 of the NR2F1 gene. Both girls were heterozygotes for the variant. The pathogenic variant of the NR2F1 gene represents an established cause of Bosch-Boonstra-Schaaf optic atrophy syndrome. Due to both sisters being affected by the disorder, their parents were also recommended genetic testing for the variant, however, their NGS resulted in negative findings.

4.8. Other indications (Dementia and Neoplasms)

Other neurologic diseases that indicate NGS for diagnosis at the clinical hospital center include dementia without extrapyramidal signs indicating Parkinson's disease as cause for the dementia. A common example of genetic dementia is early-onset Alzheimer's disease.

Table 9. Dementia (excluding Parkinsonism)

SEX	AGE	CLINICAL FEATURES	NGS RESULT	FINAL DIAGNOSIS
M	40	progressive memory impairment, confusion, forgetting to close the door of the house, forgets conversations, places he went to, PET scan shows decreased glucose metabolism bilaterally temporoparietally, MRI shows cortical atrophy, CSF shows decreased ratio of beta amyloid	Negative	-
M	68	diagnosis of Alzheimer's disease 2yrs ago, progressive, nervous, disoriented, incontinent, sluggish	negative	-

Furthermore, neoplasms may occur due to genetic anomalies or increase the risk for the development of certain cancers or benign tumor formations, indicating a possible need for genetic testing in certain types of brain tumors.

Table 10. Neoplasms

SEX	AGE	CLINICAL FEATURES	NGS RESULT	FINAL DIAGNOSIS
M	22	headaches bilateral, location temporal, pressure like, of medium intensity, difficulty with balance and speech, nausea and vomiting, dysdiadochokinesis on right with rebound phenomenon, mild vertical nystagmus, MRI showing multiple tumor of cerebellum surgery showed hemangioblastoma, FH: father symptoms of dysdiadochokinesis, suspicion of von-Hippel-Lindau syndrome	Results pending	

F	24	no specific symptoms of disease, frequent headaches up to 2x month lasting 2-3days generalized location, FH: father and brother have cerebellar hemangioblastomas, suspicion of von-Hippel-Lindau syndrome	Results pending
M	55	personal history of several surgeries due to brain tumors of cerebellum - recurrent hemangioblastomas, progressive cerebellar symptoms of gait instability (atactic gait), impaired coordination and ataxic dysarthria, dysmetria of all extremities, bilateral dysdiadochokinesis, FH: son has similar symptoms, suspicion of von-Hippel-Lindau syndrome	Results pending

For the purpose of this research both indications are listed as "other neurologic disorders" due to lack of representation. There have only been sporadic cases in which NGS has been performed on patients with a clinical presentation of dementia from causes other than Parkinsonism and CNS neoplasms at the clinical hospital center Rijeka, and none of the results yielded any genetic aberrations. Thus, the lack of number of cases and negative outcome of these sporadically performed NGS cannot be interpreted and cannot be included in the further discussion of the applications of NGS in Neurology.

5. Discussion

5.1 Hyperkinetic movement disorders

In a study conducted in Italy, patients with genetic variants causing hyperkinetic movement disorders including dystonia, chorea, athetosis, myoclonus, tremors, tics, ataxia, and stereotypies were analyzed to evaluate the frequency of genetic variants in these disorders as well as the possibility of specific hallmarks for genetic causes of hyperkinetic movement disorders. Possible hallmarks examined included clinical features, age of symptom onset and age at diagnosis. The results showed that in a significant number of patients features of multiple hyperkinetic movement disorders were present, and in some patients a mixture of hyper- and hypokinetic features was observed. More significantly the study pointed out that the age of onset of hyperkinetic features range from birth to 14 years of age, with 58% of cases being diagnosed between ages 1-6 and 25% before the age of one.(36)

In this study conducted at the university of Rijeka, the age of patients positive for genetic aberrations causing hyperkinetic movement disorder other than dystonia, which is reviewed separately, are 23, 69, 64, 69, 24, 45 and 15 years of age. It is important to highlight that this refers to the age of the patient at the time of genetic testing, not the age at the onset of symptoms.

Patient history does not clearly indicate the age at onset of symptoms for all patients and can therefore not be evaluated accurately. It can be said, however, that the mean diagnostic delay in the research performed in Italy was 6 years, with the maximum delay being 14 years.(36) It can be considered, that the patients older than 60 with positive diagnosis were two parents with no clinical features who were tested as carriers for a variant causing a hypertonic movement disorder in their 23-year-old daughter. A 69-year-old patient tested positive for Huntington's disease which can present at an older age. Additionally, the middle-aged man was described to suffer from hyperkinetic movements since childhood. However, the results of the research performed cannot underline the results of the study conducted in Italy. While the mean age of patients testing negative for genetic variants in hyperkinetic movement disorders is slightly elevated, 50 compared to 44, it is not significant in terms of being indicative for the probably of genetic findings.

Furthermore, the analysis of clinical features does not show significant differences in severity or quality of any specific symptoms that may show an inclined indication for genetic testing in hyperkinetic movement disorders.

Following, Dystonia is considered individually as it is the most common indication for NGS at the clinical hospital center Rijeka.

5.2. Dystonia

Dystonia is a term used to describe a complex, heterogenous group of disorders characterized by muscle contractions causing involuntary repetitive movements or abnormal postures in patients. Dystonia can be focal or generalized and is definition has been revised numerous times in the past decades due to the complex nature of the disease presentations and etiologies. Even in its earliest descriptions from 1911, its presentation was indicative of an inherited disorder, due to its occurrence primarily in one geographic and ethnic group. Now, over 100 years later, it is known that there are, in fact, numerous monogenic and polygenic disorders presenting as forms of dystonia. Other forms, however, have etiologies not related to any genetic variations.(25) This imposes challenges in the diagnostics of this wide spectrum group of movement disorders, in that it needs to be evaluated and decided whom to undergo genetic testing options. As Dystonia is the third most common hyperkinetic movement disorder(25), it is obvious that

testing all for underlying genetic causes would be a potential waste of resources, considering the amount of non-genetic causes.

This problematic can be observed in the research performed at the clinical hospital center Rijeka, where, as documented previously, only 6 of 28 performed NGS yielded positive results for genetic causes of dystonia.

Of these 6 patients 5 were female and one male. In the negative group the proportion of females and males is 12:7(10), indicating a slightly higher prevalence of diagnosis of dystonia in Rijeka. Their ages range from 16-47 with the median age of patients with genetic causes for dystonia being 33. In contrast, within the negative group of patients, the age range is 11-78 with a median age of 51 years. While the remark must be made, that the age represents the age at the time of testing, and due to the fact that NGS is a more recent development that has only been available since 2018, therefore many patients have suffered from dystonia for many years prior, it is still quite apparent that the age of patient with an underlying genetic cause of disease is significantly lower than in the group of patients for which genetic testing did not aid their further diagnosis. This outcome is not surprising considering research shows that in detectable causes of dystonia the age of onset is typically in childhood. The median age of onset of symptoms ranges from 9 years in the TORIA gene to a median age of onset at 38years in the GNAL gene at the upper end of the age spectrum. Dystonia appearing in later adulthood is typically not related to genetic causes. For this reason, dystonia has recently extended its classification to include the age of onset as it gives significant diagnostic and prognostic value. The age of onset is divided into five categories: infancy (birth – 2 years), childhood (3 – 12 years), adolescence (13 – 20 years), early adulthood (21 – 40 years) and late adulthood (> 40 years). In addition to the age of onset being indicative to the possible cause of the disease, research also shows it may be a valuable prognostic factor for the progression of the disease. Dystonia appearing in adulthood is less likely to present in a progressive nature, whereas dystonic features starting in childhood have a tendency to progress to a generalized form of the disease.(25)

This is somewhat difficult to accurately evaluate for patients with Dystonia in Rijeka, due to an incongruency of documented patient history. For all patients with positive genetic results, the history of clinical presentation shows either an onset during childhood or puberty with worsening of symptoms over time or findings consistent with a generalized form of dystonia. In the larger group of negative findings only some individuals present with signs of early onset and/or progressive generalized dystonia, such as the 11-year-old girl with postural anomalies, a 35-year-old male with what is described as worsening spasms, or a 27-year-old with muscle spasms

from age 15. For the majority, documentation is described as long lasting static focal presentation of dystonia, as seen in a 54-year-old male with tremors of the head 10 years ago, a 40-year-old female with cervical dystonia for 16 years, a 78-year-old female with tremors and a speech disorder for 2-3years, a 56-year-old female with cervical dystonia for 15 years, a 69-year-old patient with focal cervical dystonia, and several more, as seen in table 3.

Overall, the results of the conducted research quite clearly underline current research of dystonia, indicating that disease presentation with onset in adulthood and especially static, non-progressive focal presentation of dystonic movements such as cervical dystonia present for years with no progression to more generalized forms is highly unlikely to result from genetic variations.

Another thought to consider with the diagnostic testing of elderly patients for dystonia is the potential value of the outcome of the procedure. For patients past their reproductive age, testing for underlying genetic causes for present disorders loses its benefit of calculating the risk of inheritance and its possible use for family planning. In terms of treatment options, it seems as though the temporal pattern of the appearance of symptoms is closely linked to the treatment response for certain treatment. Therefore, it may be of more value for effective patient treatment to determine patterns of dystonic symptoms, which can be persistent, paroxysmal, diurnal, or action-specific.(25) For some patients, temporal patterns such as diurnal fluctuations were specified, and for all genetically positive patients the pattern is persistent. Treatment options for dystonia include oral medication, botulinum toxin and surgical interventions.(25) Hence, genetic diagnosis can be very important to plan patient care and can potentially spare patients from unnecessary surgery. On the other hand, therapy used in patients with dystonia also seems to be indicative of the Etiology of the disease. As the results show, some patients that were tested for genetic causes of dystonia had received botulinum toxin as a treatment and responded well to the therapy. All patients stated to be responding to given therapy have a negative genetic testing result. Limitations of this observation are lack of mentioning of treatment protocols if most of the patients.

In conclusion, signs that indicate a likely underlying genetic cause in dystonia patients are a young age of onset of symptoms, involvement of the extremities and/or trunk, persistent symptoms, progressive nature of the disease and poor response to therapy. In patients that do not exhibit indicative signs, genetic disease is very unlikely and the performance of NGS in these cases has poor prognostic value and little to no benefit for patient treatment. Therefore, the

application of diagnostic NGS for every patient with dystonia can be seen as a waste of resources.

5.3. Hypokinetic movement disorders

Similarly, as in hyperkinetic movement disorder, the same study conducted in Italy concluded that for hypokinetic disorders, specifically hypokinetic-rigid syndrome, and parkinsonism. Even though the median age of onset of symptoms in this group of disorders is twice as high as for hyperkinetic disorders, the median age is 16 years, which is still very young. The mean age of diagnosis is 23 for patients exhibiting symptoms of hypokinetic movement disorders.(36)

In the study conducted at the clinical hospital center Rijeka, patients with hypokinetic movement disorders were diagnosed with muscular dystrophy, spastic paraplegia, myotonia, polyneuropathy (specifically Marie-Charcot-Tooth disease), and neurodegeneration with iron accumulation. The disorders are more specific and rarer compared to the disorders evaluated most in Italy. The age of diagnosis for this group of patients is very high at a mean age of 38 years. Many affected patients are diagnosed in their late 40s to 50s. Although, it should be mentioned that for most cases in this group it is stated that symptoms of disease have been persistent or progressive since childhood. The recent NGS tests ordered late 2022, which cannot be analyzed as their results are not yet present, however, it can be observed that all pending tests are in patients aged 5-25 (with one exception) as seen in table 1. This may indicate that the high age of patients with hypokinetic movement disorders in Croatia may be due to NGS being a new possibility for diagnosis. The average age of onset of symptoms cannot be calculated accurately. Furthermore, a striking finding in this group of patients is the overall high rate of genetic findings, especially when compared to hyperkinetic movement disorders. While 11 NGS tests performed came back negative, 10 patients were positive for genetic aberrations linked to their hypokinetic movements. Hence, in this group of disorders, diagnostic success rate with NGS is almost 50%. This raises the question, if hypokinetic movement disorders have more genetic causes overall, leading to their higher success in diagnostics with NGS.

5.4. Developmental disorders and morphological brain anomalies

Developmental disorders and morphological brain anomalies seemingly yield the highest positive outcome for NGS diagnosis, with over 50% of positive genetic results. All patients referred to NGS with the indication of developmental delay and/or morphological brain anomalies are pediatric patients, with an age range of birth – 16 years, and a median age of 5. The diagnoses made include, encephalopathies, lissencephaly, neurodevelopmental, and intellectual developmental disorders, that even in the patients diagnosed at an older age have presented with dramatic signs of developmental delay from a very young age (<1 year). Common secondary presentations include symptoms of other disorder groups considered here, including hypokinetic movement disorders, hyperkinetic movement disorders and epilepsy. This is in concordance with other studies conducted, also showing a strong prevalence of movement disorders and epilepsy coinciding with developmental disorders.(36) In the study conducted in the clinical hospital center Rijeka, 100% of patients with genetic causes of developmental delay present with epileptic or other seizures and 60% have concomitant movement disorders. 100% have documented morphological changes of the brain detected by MRI, and in 60% seizures were confirmed by abnormal EEG findings.

For all patients with negative findings after NGS performance, it is striking that none have documented seizures or movement disorders as secondary symptoms to developmental delay. 50% have no documented MRI findings with symptoms of developmental delay and the other 2 patients have signs of brain malformations with good developmental development. Therefore, the results of the study can conclude that the presence of both developmental delay and detectable structural brain malformations are strong indicators for an underlying genetic aberration. The presence of epileptic seizures or convulsions and movement disorders, that can be either hyperkinetic or hypokinetic in patients with developmental delay are another strong indicator for positive outcomes when performing NGS diagnostics.

While the high prevalence of positive genetic outcomes in this group of pediatric patients, which is underlines by the general finding that the prevalence of positive NGS results is higher in the group of patients referred by pediatric neurology compared to adult neurology, could suggest better clinical judgement and patient selection for NGS by pediatric neurologist, it must also be considered that the incidence of genetic disorders and their diagnosis is generally higher within the pediatric population.

5.5. Epilepsy and other Seizures

NGS has become the gold standard and primary method in diagnosis of primary epilepsy.(37) In 2019, a working group in Ontario put together recommendations for NGS in epilepsy to reduce the chance of uncertain and secondary results, as epilepsy is a very common neurological condition with many genetic and acquired causes.(38) Additionally, epilepsy like seizures may occur as a symptom of other disorders, in many diseases including other neurologic disorders, infectious, or metabolic diseases. Due to the wide variety of causes of epilepsy, it would not be substantial as an indication for NGS in all cases. However, in 15-30% of cases will benefit from molecular diagnosis. Therefore, guidelines should be made to identify distinct presentation of likely hereditable forms of seizure disorders. This consists of a pretest evaluation involving counseling with the patient to discuss the possible benefits and limitations of genetic testing for the patient and determining the likelihood of results of unknown significance and determining a specific gene panel consisting of known genes for a certain presentation of epilepsy to avoid secondary incidental findings that are not significant for the clinical presentation of the disease. These specific epilepsy panels include: focal epilepsy, progressive myoclonic epilepsy, early infantile epilepsy, childhood-onset epilepsy, epilepsy association with brain malformations, STAT epilepsy panel and comprehensive epilepsy panel. In addition, before consideration of genetic testing, individuals should undergo a detailed assessment by a neurologist including performance of an EEG, MRI, a metabolic work-up, an assessment of syndromic features indicative of genetic syndromes. Patients who meet the GTAC criteria for consideration of genetic testing are sent to NGS.(38) Poor response to antiepileptic therapy is common in patients with familial epilepsy.(36)

In alignment with these considerations, it should be noted that most patients presenting with seizures or epilepsy are considered in other groups of disorders due to seizures appearing concomitant to another disorder. Only patients whom present with primary epilepsy are considered in the evaluation of the application of NGS in epilepsy and other seizure disorders.

In the research performed at the clinical hospital center in Rijeka only one female was found to have a relevant genetic variant when NGS had been performed primarily due to epileptic seizures. Even so, the patient had secondary malformations and the genetic results revealed a genetic syndrome in the one-year-old girl. The other two young children and three young adults tested for genetic causes of epilepsy yielded negative results. The overall number of patients tested in year, from 2018-2022, was only nine patients, which, considering the prevalence of

epilepsy in the population is very high, could indicate that many epileptic patients are not diagnosed with genetic disorders as patients with primary epilepsy are not tested by NGS methodology.

Overall, due to the lack of patients in this category, representing either positive or negative results, comparison of the data is insufficient to observe links between the clinical features of epilepsy and an increased risk for genetic causes that could possibly suggest guidelines for testing of patients with primary epilepsy.

5.6. Sensory disorders

Evaluation of results obtained within the group of sensory neurological disorders is difficult to generalize since out of 6 patients with NGS results for a sensory neurological disorder, 4 are within one family, including both positive results. 100% of positive genetic sensory disorders at the clinical hospital center Rijeka are for Bosch-Boonstra-Schaaf optic nerve atrophy syndrome. However, considering the patients affected are related, this is not representative for the population of Rijeka. Both patients have a typical presentation of symptoms that are likely genetic, progressive loss of vision with an onset of symptoms in early childhood that shows poor response to conventional therapy. MRI changes as a sign of structural disease may be an indicator for a genetic background. Finally, the fact that directly related family members (two sisters) present with the same symptoms is an obvious sign that genetic diagnostic is likely necessary for accurate diagnosis.

Considering most cases of blindness can be attributed to acquired conditions, occurring in the population over the age of 50, such as cataracts, age related macular degeneration, glaucoma, and diabetic retinopathy (39), it is difficult to evaluate whether genetic aberrations for blindness are being missed by not using NGS routinely in blindness or if the prevalence is very low.

Other sensory disorders are not represented at all in patients undergoing NGS at the clinical hospital center Rijeka, which is suggestive that they are not considered for diagnostic NGS currently.

6. Conclusions

Concluding the findings discussed above, indications for genetic diagnostic testing using NGS in the clinical hospital center Rijeka include hyperkinetic and hypokinetic movement disorders, developmental delay, brain malformations, seizure disorders which are mainly limited to epilepsy, sensory disorders limited to blindness, and sporadic cases of dementia with suspicion for Alzheimer's disease and brain tumors. The success rate for accurate diagnosis of genetic disorders is significantly higher within the pediatric population compared to the adult neurology. Possible causes are the increased incidence of genetic disorders presenting from a young age, which is a main indicator for a high risk of genetic variants throughout the literature. For all groups of disorders, the mean age at onset of symptoms is during childhood. Delay of diagnosis is very high in adults that have yielded positive diagnosis. This may be explained by the fact that NGS testing has only been available in Rijeka since 2018, and diagnosis of genetic diseases could not be established for many patients with the onset of clinical signs of disease. Another possibility could be that pediatric neurologists have more experience with genetic disorders and can execute better clinical judgement and patient selection for NGS referral. However, other studies would be necessary to confirm this hypothesis.

Considering the high incidence of genetic disorders in the pediatric population, it is striking that referrals for NGS testing are almost three times higher in adult neurology. In addition, a high negative outcome rate in this population subgroup suggests the need for better patient selection, especially for the adult population. Particularly, dystonia patients can be mentioned here, as they are the most common indication for NGS in adult neurology and yield the highest rate of negative results. The findings of this study, as well as similar studies conducted, show that hereditable dystonia presents mostly in childhood, and the disorder is progressive in nature, usually involving the trunk and extremities. Therefore, guidelines for indication of NGS testing in dystonia can be adapted to exclude patients with an onset of symptoms of dystonia over the age of 50 and patients with static symptoms, especially when limited to a cervical distribution of disease. Temporal patterns of presentation and response to treatment may also be considered when selecting dystonia patients for NGS, although these factors variable. Developmental delay and concomitant presentations of seizures and/or movement disorders with or without obvious structural brain anomalies are very likely to show genetic aberrations, thus this should be a clear indication for NGS, while structural anomalies or developmental delay without MRI changes have shown no detectable genetic disorders in the study performed. A general lack of patients with hereditary epileptic syndromes may indicate insufficient testing of patients with this

presentation, due to the overall high incidence of hereditary genome variants leading to primary epilepsy. Other groups of disorders are difficult to interpret due to their overall rarity.

Furthermore, a general consideration when performing NGS is the value of the outcome, positive or negative, for the further treatment of the patient. The value of NGS decreases with increasing age of the patient.

In conclusion, the guidelines for NGS indications in Neurology could be optimized for better use of resources in the future. Due to the rapid technological advancements and research load in the field of genetics, it has become increasingly important of specialists of other fields to invest time understanding the genetic basis of disorders within their field. A lack of clinical geneticists in Croatia, and around the world, imposes a challenge for physicians to adequately keep up with rapidly changing guidelines for diagnostic genetic testing.

7. Abstract

Genetics is rapidly advancing, with new technologies revolutionizing research. However, opportunities provided by NGS seemingly cannot be replicated in clinics, due to lack of genetic expertise among specialists. NGS is a DNA sequencing method, allowing rapid detection of genetic variants in probands by comparison to a reference genome. NGS is an important diagnostic tool in the field of Neurology, as the prevalence of genetic factors in etiology is high, such as in a multitude of causes for movement disorders, developmental delay and morphological brain anomalies, epilepsy, and other neurological diseases.

The aim of this study was to assess the usefulness of NGS. A retrospective study was performed, evaluating the indications for NGS in Neurology and pediatric Neurology from 2018-2022, at the University of Rijeka.

Of 116 NGS tests performed across 5 years, 31 pathological variants could be detected across 27 genes, providing a diagnosis for 27 patients (23%). The highest outcome of positive genetic results occurred in developmental anomalies, while the lowest, compared to negative findings, was in dystonia.

Genetic movement disorders were found to be more frequent in the younger population, which is in concordance with previous research. Diagnosed developmental delay is a strong indicator for NGS, especially with concomitant structural brain malformations, seizures, or movement disorders. NGS is the gold standard for the diagnosis of primary epilepsy, indicating that in Rijeka epilepsy is an underrepresented indication for NGS.

In conclusion, the guidelines for applications of NGS in Neurology could be optimized, with emphasis on genetic education of neurologists.

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9. CV

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