Molecular diagnostics

Iveta Valaskova

Aplications of Molecular Genetic Testing

Genetic testing, through interrogation of DNA, RNA, can provide critical information for the detection of heritable disease genotypes for a number of different applications

- **Diagnostic testing**
  Testing for a gene pathological variant in symptomatic individuals as a diagnostic aid
  Testing requires genetic counseling.
- **Newborn screening**
  Testing is used to screen populations to identify prevalent genetic pathological variant in asymptomatic infants.
  The purpose of the screening is to identify affected babies early in life to allow for appropriate intervention before irreversible damage occurs.
- **Presymptomatic testing**
  Testing for a pathogical variant in asymptomatic individuals in order to predict or asses the risk of disease in the future.
  These applications include testing for diseases in which lifestyle changes, increased medical surveillance, or medical intervention might be beneficial if the pathological variant is known.
  Testing requires extensive pretest and posttest counseling.
- **Carrier screening**
  Testing for a pathological variant in an autosomal recesive disorder in asymptomatic individuals for the purpose of family planning and genetic counseling to determine probability of disease in childern.
  Test requires extensive pretest and posttest counseling.
- **Prenatal diagnosis**
  Testing fetal cells/tissues for mutations to determine if a fetus is affected with a disease early in the pregnancy.

- **Carrier screening**

Might be recommended in various situations including

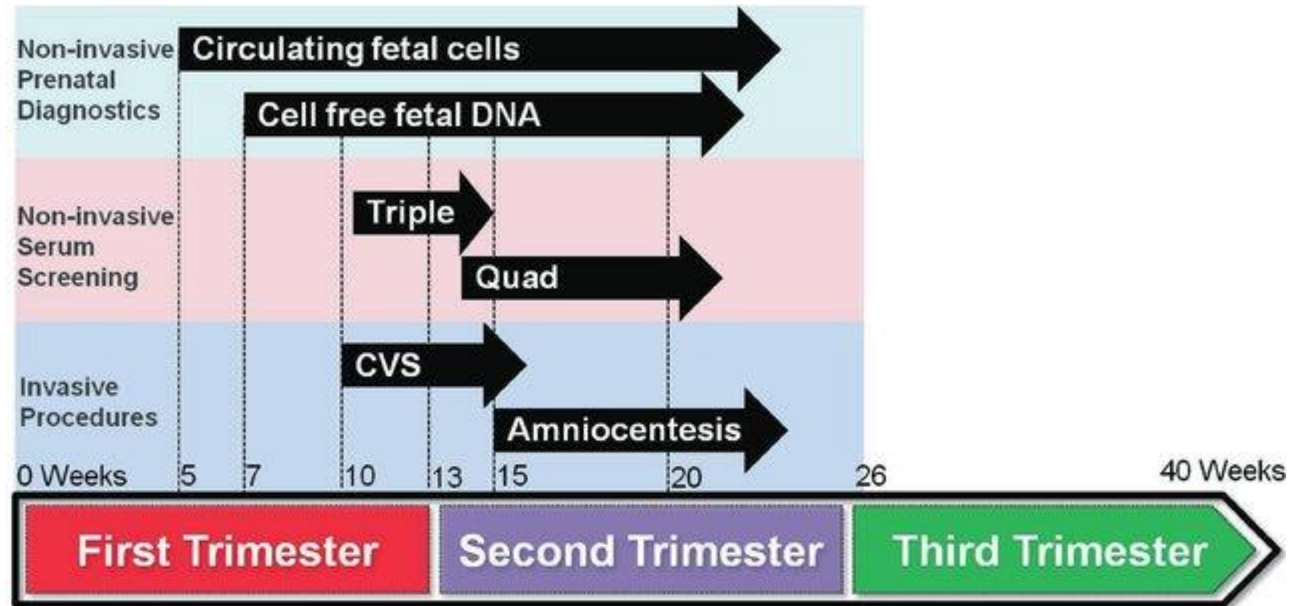
1. If one or both partners have a family history of disease
2. If one or both partners are members of a population or ethnic group with a higher incidence of the disease
3. If partners are seeking preconception or prenatal testing
4. General population screenig

Newborn Screening

Newborn Screening (NBS) is a public health program where infants are screened shortly after birth for a list of conditions not clinically evident early in life. NBS looks for serious developmental, genetic, and metabolic disorders that would not otherwise be detected in time for life-altering treatment.
For these disorders, early detection and treatment is essential to preventing irreversible mental or physical disabilities, even death.
NBS does not diagnose diseases, but identifies which babies need *additional* testing to confirm or rule out these diseases.
Although these diseases are very rare, they are treatable if caught early.

Molecular Newborn screening: Cystic fibrosis, Spinal muscular atrofy, Severe Combined Immunodeficiency Disease

- **Prenatal diagnosis**



A comparative schematic of prenatal diagnostic techniques and their applicability with respect to the progress of pregnancy:

- conventional invasive procedures (amniocentesis, chorionic villi sampling); Testing can be done at 8 to 12 wk gestation by chorionic villus sampling (CVS) or at 14-16 wk by amniocentesis.
- serum screening techniques (triple and quad screens - ultrasonography);
- non-invasive prenatal diagnostics (cell-free fetal DNA sampling methods).

- **Prenatal diagnosis**

Prenatal diagnosis means diagnosis before birth. It’s a way to see if developing baby has a problem.
These tests help find genetic disorders before birth.
Some parents have increased risk of having a baby with a genetic disorder or other problem.
Knowing about problems before the baby is born may help parents. They may be able to make better decisions about health care for their infant. Certain problems can be treated before the baby is born.
Other problems may need special treatment right after delivery.
In some cases, parents may even decide not to continue the pregnancy.

Molecular genetic testing

Can be used a direct or indirect analysis

Direct testing

detects the specific disease-causing pathogenic variants or foreign DNA sequence

Indirect testing (gene tracking)

This type of testing is commonly referred to as linkage analysis.
Polymorphic markers closely associated with the disease-causing gene are used
to assess whether an individual has inherited the pathogenic allele of the disease-causing gene
responsible for the disease phenotype.

Linkage analysis is based on tracking the inheritance of polymorphic markers in a family with a genetic disease.
If the markers and the disease-associated gene are in proximity, then the likelihood of a recombination event occurring
between them is minimal. Thus, coinheritance of the markers and the disease-associated gene is likely.
The advantage of linkage analysis is that the gene of interest need only be mapped to a chromosomal location.
Limitations to this technology include significant labor and turnaround-times, the need to analyze samples
from many family members, and the possibility of having to use numerous markers to obtain informative data.

Direct testing

provides evidence of a pathological variant responsible for producing the illness.
It is determined whether the sequence of the DNA (nucleotide sequence) has changed.

These assays require that pathological variant and/or the gene sequence of interest is known.

Sequence changes testing methods can be divided into two groups:

1. Scoring – methods for detecting specific sequence changes

2. Scanning – methods for scannig a gene for any sequence change

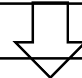
Direct testing

Checking for patogenic variant of gene/genes causing disease

analysed entirely using test
for specific sequence changes
SCORING

An example is Achondroplasia

analysed entirely using test
for specific sequence changes
SCORING



Sequencing of gene/genes
SCANNING

An example is Cystic fibrosis

Sequencing of gene/genes
SCANNING

An example is LQT syndrome

Scoring - methods for detecting specific sequence changes

Searching for known sequence change is possible for:

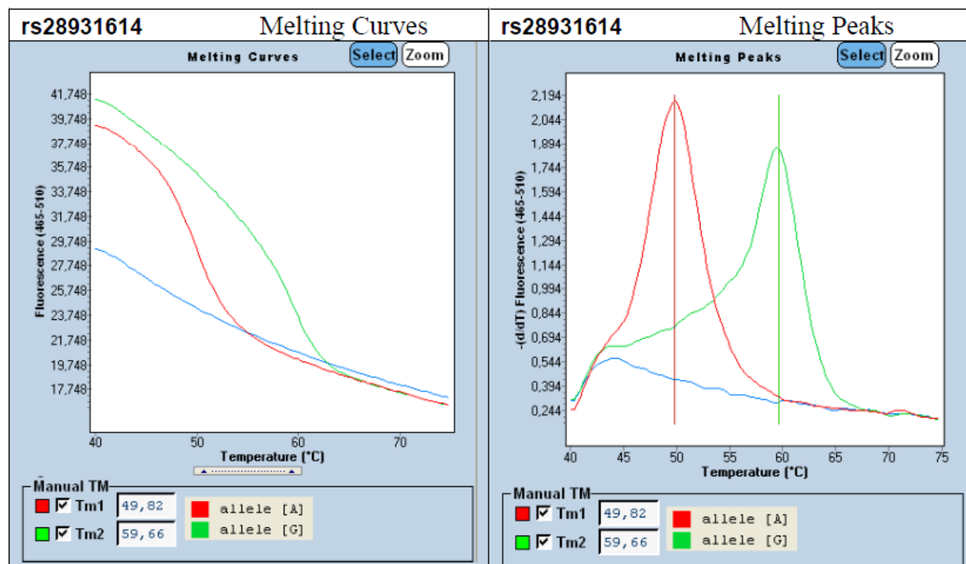
- The disease in question may always be caused by exactly the same sequence change
  An example is **Achondroplasia**

Achondroplasia

is the most common of the skeletal dysplasias that result in marked short stature (dwarfism)

arise from a change in the same base pair of *FGFR3*, that are autosomal dominant
around 98% of persons with achondroplasia have a c.1138G>A gene change,
and 1% have a c.1138G>C mutation

targeted mutation analysis is the routinely employed molecular test



method of genotyping by melting point analysis
on High Resolution Melting (HRM)
on the LightCycler® 480 System platform
using the LightSNiP assay.

Scoring - methods for detecting specific sequence changes

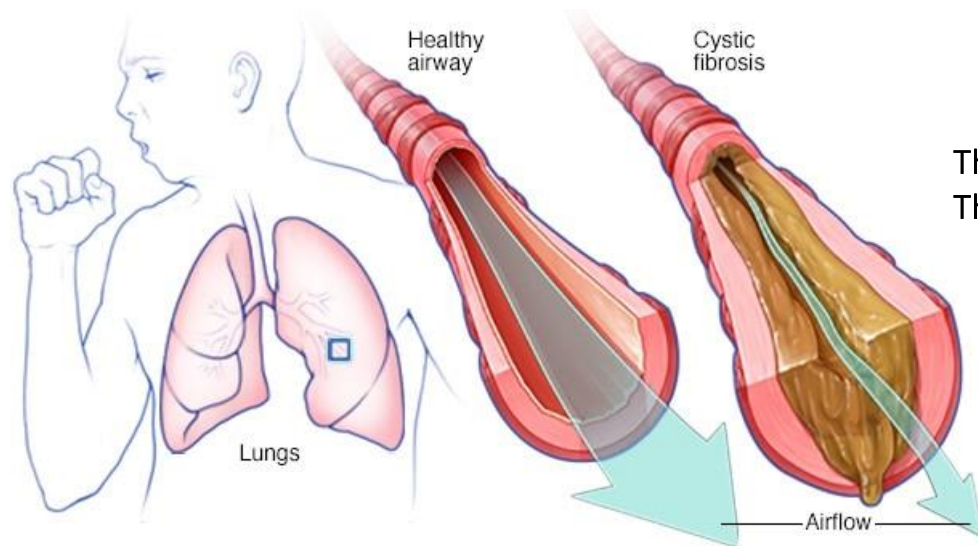
Searching for known sequence change is possible for:

- The disease in question may always be caused by exactly the same sequence change
  An example is **Achondroplasia**

- A disease may be caused by various different sequence changes, but one or a few variants may be so frequent in a particular population that it is worth first checking for these befor going on to a more general search.
  An example is Cystic Fibrosis*; over 2000 different variants have been reported, but 65% of all pathogenic variants of CFTR gene in Europeans are one particular deletion of 3 nucleotides p. F508del*

Cystic fibrosis

In people with CF, patogenic variants in the cystic fibrosis transmembrane conductance regulator (CFTR) gene cause the CFTR protein to become dysfunctional.
When the protein is not working correctly, it's unable to help move chloride -- a component of salt -- to the cell surface.
Without the chloride to attract water to the cell surface, the mucus in various organs becomes thick and sticky.



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The airways fill with thick, sticky mucus, making it difficult to breathe.
The thick mucus is also an ideal breeding ground for bacteria and fungi.

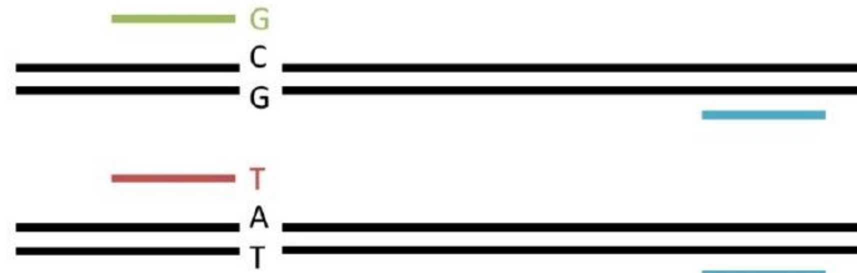
Although over 2000 different variants in the CFTR gene have been described in cystic fibrosis patients,
most of these have been seen in only one or a very few cases.
A small number of patologiocal variants are relatively common.
Mutation testing in cystic fibrosis therefore starts by checking for these common mutation.

Cystic fibrosis

Most genetic tests only screen for the most common CF mutations: **Allele-specific polymerase chain reaction**

→ rozděleno do 2 PCR

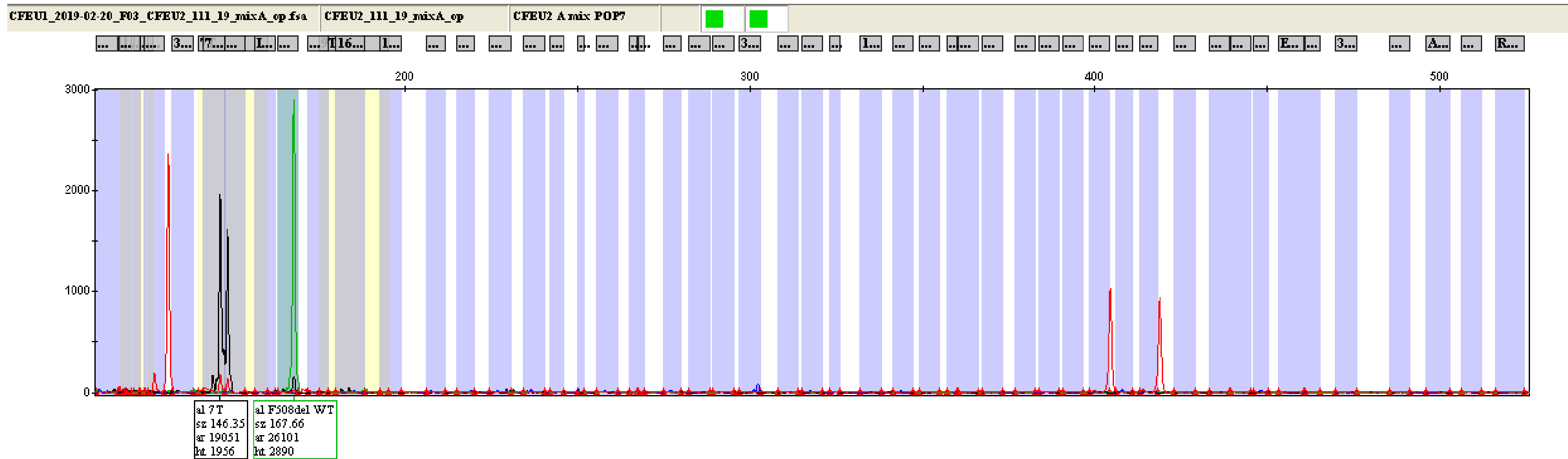
1. F-WT+R
2. F-MT+R

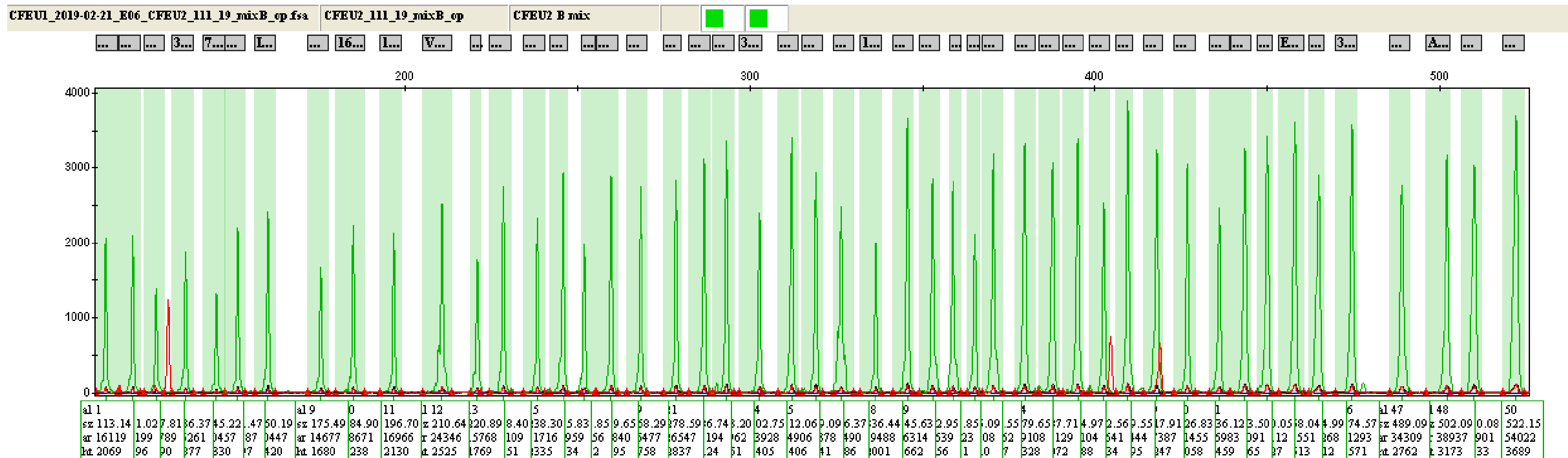


Allele-specific polymerase chain reaction (AS-PCR) is a technique based on allele-specific primers, which can be used to analyze single nucleotide polymorphism.
The *allele-specific PCR* is also called the (amplification refractory mutation system) ARMS-PCR corresponding to the use of two different primers for two different alleles.
One is the mutant set of primers which are refractory (resistant) to the normal PCR, and the other is the normal set of primers, which are refractory to the mutant PCR reaction.
The 3’ ends of these primers are modified such that one set of the primer can amplify the normal allele while others amplify the mutant allele.
This mismatch allows the primer to amplify a single allele.

Elucigene® CF-EU 2v1

MT + WT pro F508del (+STR)





WT - F508del (+STR)

typical electropherogram obtained through ARMS analysis

Scoring - methods for detecting specific sequence changes

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- The test may be to check somebody for a family pathogenic variant that has already been identified and characterized in other family members

- The test may be to check samples from haelthy controls to make sure a variant found in a patients is not a non-pathogenic variant present in normal population

Scanning- methods for scanning a gene for any sequence changes

A dignostic laboratory often needs to check every exon of candidate gene in a patient to look for patogenic variants.

Given the average sizes of exons and introns (145 bp and 3365 bp, respectively)
this usually means PCR amplifying and **sequencing** each exon individually.

Various methods were developed to save sequencing costs by scanning each exon quickly and cheaply to eliminate those that apparently contained no variants (SSCP, DGGE, dHPLC).

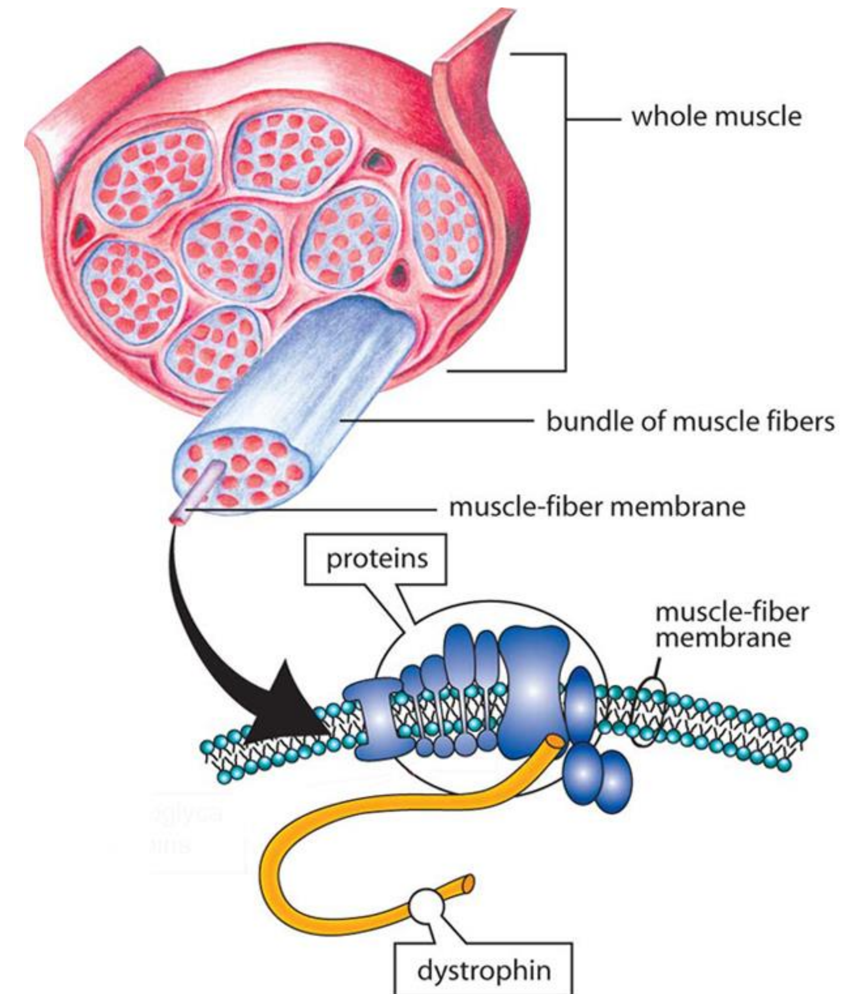
The cost of sequencing has now fallen to the point that this approach is seldom used,

except for scanning a gene to check for deletions or duplications of whole exon
An example Duchenne muscular dystrophy (DMD)

Duchenne muscular dystrophy (DMD)

a genetic disorder characterized by progressive muscle degeneration and weakness due to the alterations
DMD occurs because the mutated *DMD* gene fails to produce any functional protein called *dystrophin*
that helps keep muscle cells intact.

The other dystrophinopathy is Becker muscular dystrophy (BMD, a mild form of DMD)
Individuals with BMD genetic mutations make dystrophin that is partially functional, which protects their muscles from degenerating as badly or as quickly as in DMD.



Muscles are made up of bundles of fibers (cells).
A group of interdependent proteins along the membrane surrounding each fiber helps to keep muscle cells working properly.
When one of these proteins, dystrophin, is absent, the result is Duchenne muscular dystrophy (DMD);
poor or inadequate dystrophin results in Becker muscular dystrophy (BMD).

Duchenne muscular dystrophy (DMD)

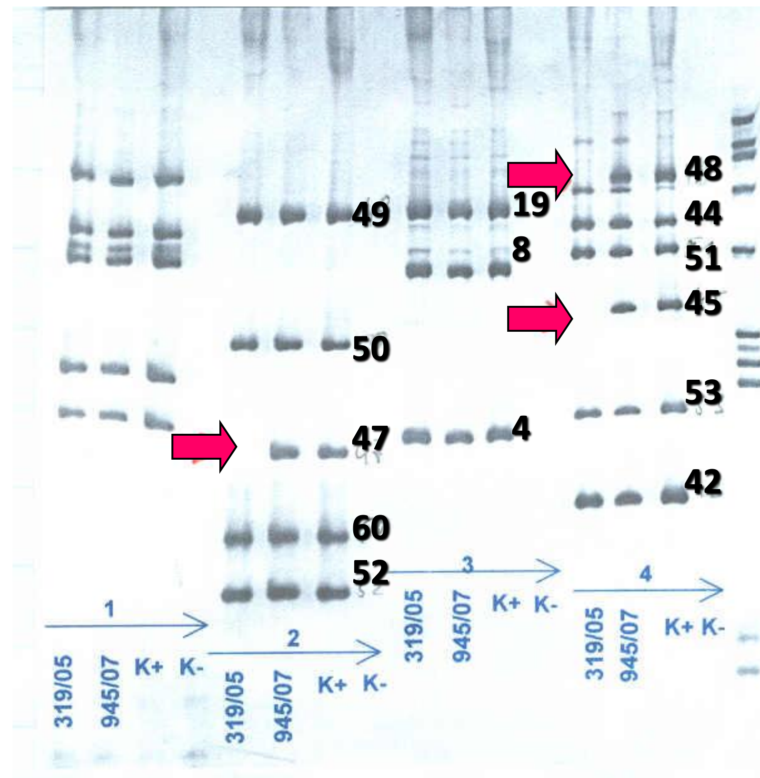
has an X-linked recessive inheritance pattern
and is passed on by the mother, who is referred to as a *carrier*.

The dystrophin gene is the largest gene yet identified in humans and is located in the short arm of the X chromosome, in the Xp21.2 locus (a locus is the position of a gene on a chromosome).

The majority (60 - 70 %) of mutations of the dystrophin gene are deletions of one or more complete exons.

These deletions are relatively easy to detect in an affected male.

Duchenne muscular dystrophy (DMD)



confirmed DMD:
deletion DMD exons 45, 47, 48

4 sets multiplex PCR

Tube 1
Pm 535 pb
exon 3 410 pb
exon 43 357 pb
exon 13 238 pb
exon 6 207 pb

Tube 2
exon 49 439 pb
exon 50 271 pb
exon 47 181 pb
exon 60 139 pb
exon 52 113 pb

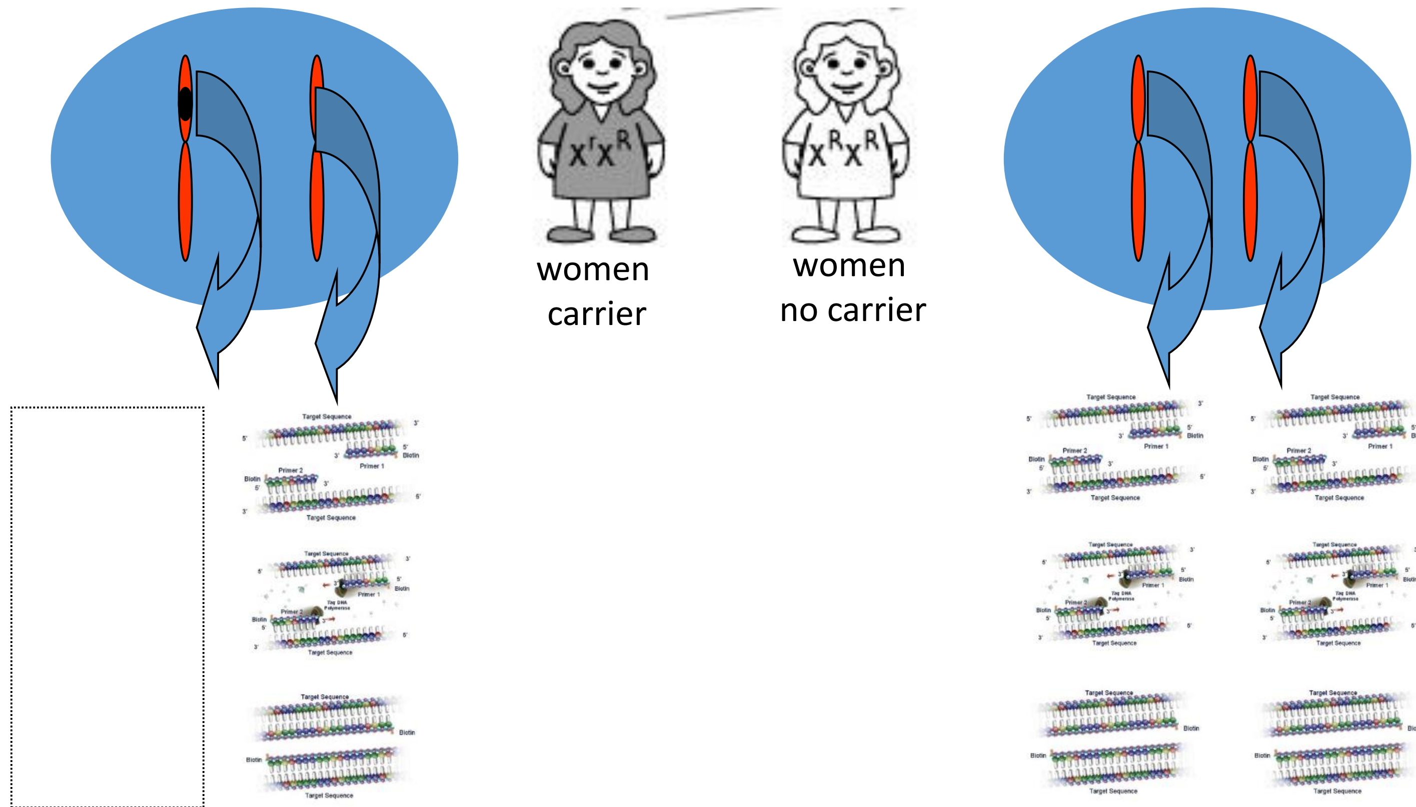
Tube 3
exon 19 459 pb
exon 8 360 pb
exon 4 196 pb

Tube 4
exon 48 506 pb
exon 44 426 pb
exon 51 388 pb
exon 45 307 pb
exon 53 212 pb
exon 42 155 pb

To check for partial deletions, individual exons of the gene are amplified by PCR.
Primers are designed to match sequences in the introns flanking an exon,
so that the product contains the complete exon and some intronic sequence.
Each exon gives a different sized product by varying the amount of intron included.
A series of PCR reactions can be performed in one operation (multiplexed).
The mix of products from all PCR amplifications is run on an electroforetic gel.

Duchenne muscular dystrophy (DMD)

Carrier testing in women is more difficult than testing a boy because a carrier would be hetorozygous and every exons of the dystrophin gene would amplify from her normal chromosome.

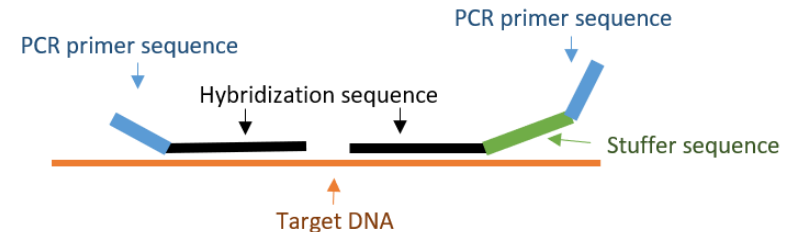


A quantitative test is required - **MLPA**

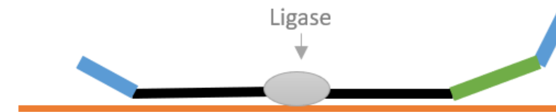
Duchenne muscular dystrophy (DMD)

Multiplex ligation-dependent probe amplification (**MLPA**) is a variation of the multiplex PCR that permits amplification of multiple targets with only a single primer pair.
It detects copy number changes at the molecular level, and software programs are used for analysis.

1 – Denaturation; 2 – Hybridization



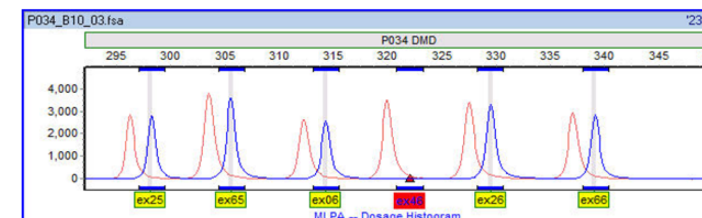
3 – Ligation



MLPA consists of the following steps:
1. Denaturation
2. Hybridization
3. Ligation
4. Amplification (by PCR)
5. Fragment Separation and Data Analysis

4 – Amplification

5 – Fragment separation and Data analysis



1 - Denaturation and 2 – Hybridization

Denaturation involves separation of the annealed DNA strands, so that double-stranded DNA becomes single-stranded.
Hybridization involves hybridizing the DNA sample to specific probes. Because it is a multiplex technique, you can analyze each sample by up to 60 probes simultaneously, thus targeting different sites!
These probes have a primer sequence that binds to the PCR-primer in the amplification process.
All different probes will have the same primer binding sequence.
Additionally, the probes also have a hybridization sequence complementary to the target site that will allow the probe to bind to the DNA. Both probes will hybridize on adjacent sites on the DNA strand.
One of the probes from the pair contains a stuffer sequence, which is different in length for each target site.
The length of the stuffer sequence changes between different probes, allowing multiplexing.
So, you can expect each amplification product to have a unique length!

3-Ligation

The ligation step will bind the two probes together. In this step, a specific enzyme called DNA ligase is used.
It binds the probes that are already hybridized on adjacent sites of the DNA strand at the target site.
The ligase used in MLPA protocols is ligase-65, an NAD-dependent ligase enzyme.
Both probes contain the binding sites for PCR-primers. This means, if we were to use the probes as a single molecule, we would obtain an amplification product, even without the DNA target site, thus giving us non-specific amplification.
The enzyme ligase is extremely specific: if there are any mismatches between the probe and the target site, the ligase will not be able to bind the probes and no amplification would occur.
Consequently, MLPA detects specific point mutations, and even distinguishes between pseudogenes and the real target gene.

4-Amplification

The next step is amplification, which is essentially a polymerase-chain reaction (PCR). For the PCR step, a polymerase, dNTPs, and a forward and reverse primer are added. Since all of the probes have the same PCR-primer sequence, it will only be necessary to add one pair of universal primers to study all of our targets. The forward primer is fluorescently labelled, allowing visualization and quantitation during analysis.

5-Fragment Separation and Data Analysis

After amplification, the fragments are separated by capillary electrophoresis. Capillary electrophoresis separates fragments based on their length, and shows different length fragments as peak patterns, called an electropherogram. Each amplicon has a different known size, due to the stuffer sequence on each specific probe, and therefore each amplicon can be quantified during data analysis.

The data obtained by capillary electrophoresis will be the input for the analysis. MRC- Holland provides a free software for data analysis – Coffalyser.

By comparing each sample to a set of reference samples, we can obtain a probe ratio. This probe ratio will inform us of how many copy numbers a gene has. Since most human genes are diploid, if the sample presents two copies, the ratio will be 1.0; i.e. the sample probes have obtained the same number of genes as the reference sample.

However, if the ratio is 0.5 there was only one copy of the gene in the individual, which probably means a heterozygous deletion of the target gene. If, on the other hand, the ratio is 1.5, there is, probably, a heterozygous duplication of a gene.

MRC-Holland offers many different kits that may have the solution for your problems. However, if you are trying to find something a bit more obscure, or study something that isn’t in any kit, you can design your own probes. I advise you to read carefully the protocol for synthetic probe design.

Scanning- methods for scanning a gene for any sequence changes

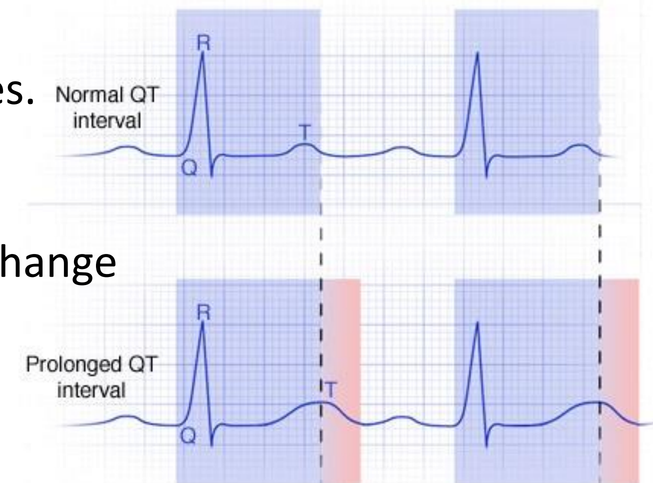
LQT syndrome

Long QT syndrome (LQTS) is an abnormal feature of the heart's electrical system
that can lead to a potentially life-threatening arrhythmia called torsades de pointes (pronounced torsad de pwant).
Torsades de pointes may result in syncope (fainting) or sudden cardiac death.

Currently, there are three major LQTS genes (*KCNQ1, KCNH2*, and *SCN5A*)
that account for approximately 75% of the disorder.

Approximately 75% of patients with a clinically certain LQTS diagnosis have patogenic variants
in one of three major LQTS-susceptibility genes *KCNQ1, KCNH2*, and *SCN5A.*
The 10 minor LQTS-susceptibility genes collectively account for less than 5% of LQTS cases.

Possible testing methods that could be used to check for the presence of the sequence change
are **Sanger sequencing and Massively parallel sequencing.**



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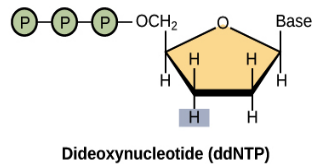
Scanning- methods for scanning a gene for any sequence changes

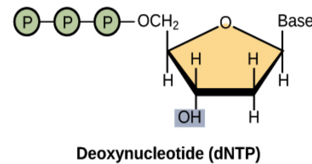
DNA sequencing

is the process of determining the sequence of nucleotide bases

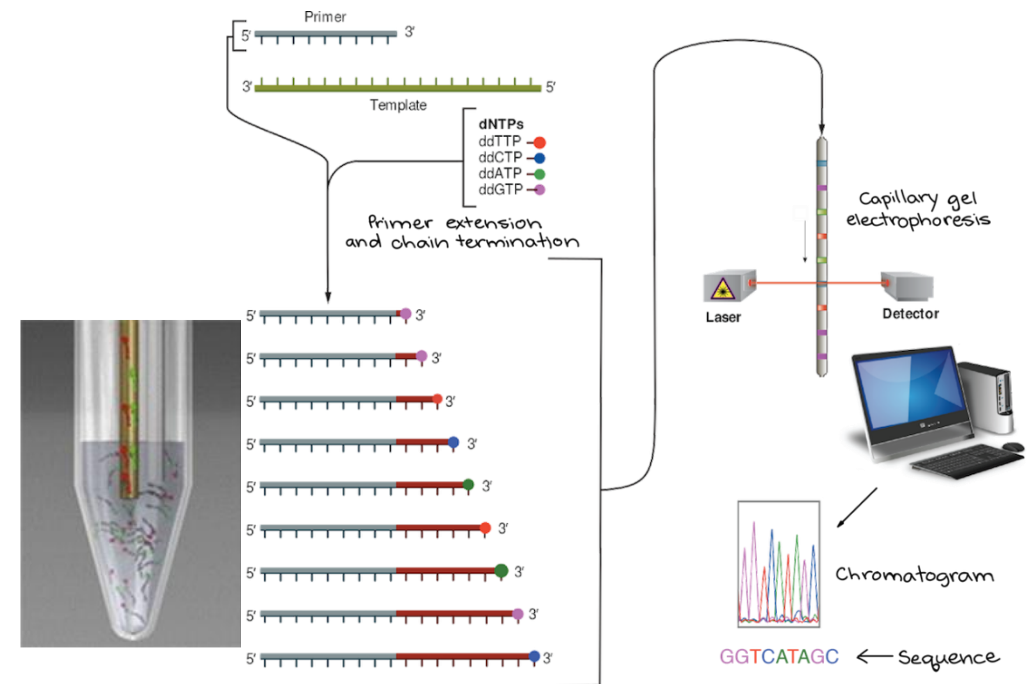
For almost all of past 30 years all DNA sequencing has been based on a single basic technic, dideoxy or **Sanger sequencing.**

DNA fragments are enriched by PCR and sequenced with a combination of regular deoxynucleotides and terminating labeled dideoxynucleotides (ddNTPs), each with a base-specific color.





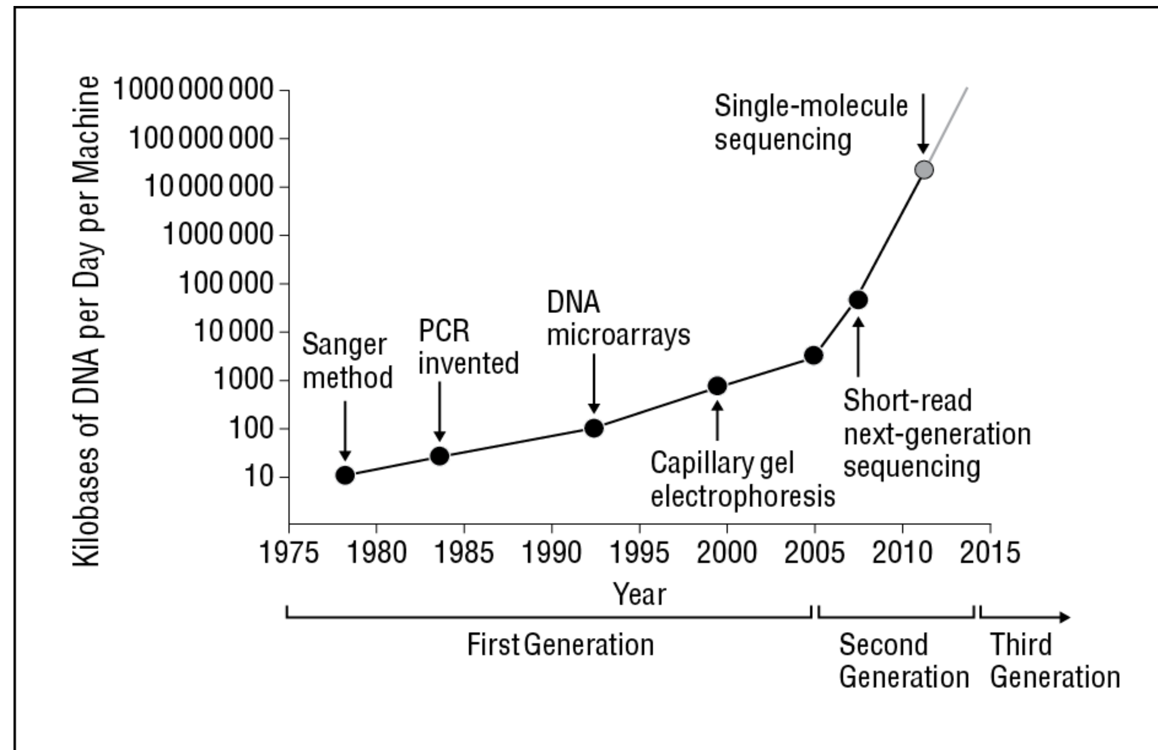
Different fragment lengths are generated and size separated by capillary electrophoresis, and the location of each of the ddNTPs is identified by excitation with a laser.



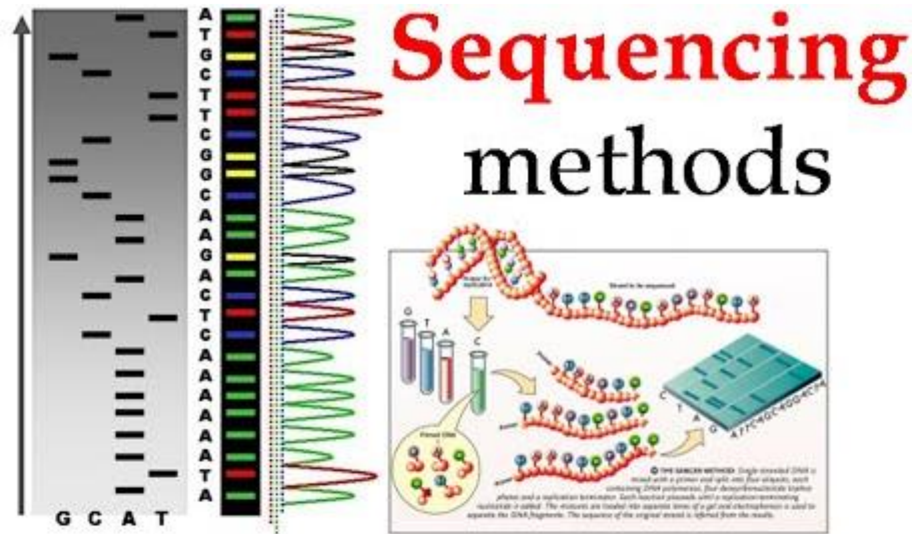
DNA sequencing

is the process of determining the sequence of nucleotide bases

Several revolutionary new sequencing technologies have burst onto the scene.
Collectively called „next generation sequencing“ or „massively parallel sequencing“



DNA sequencing



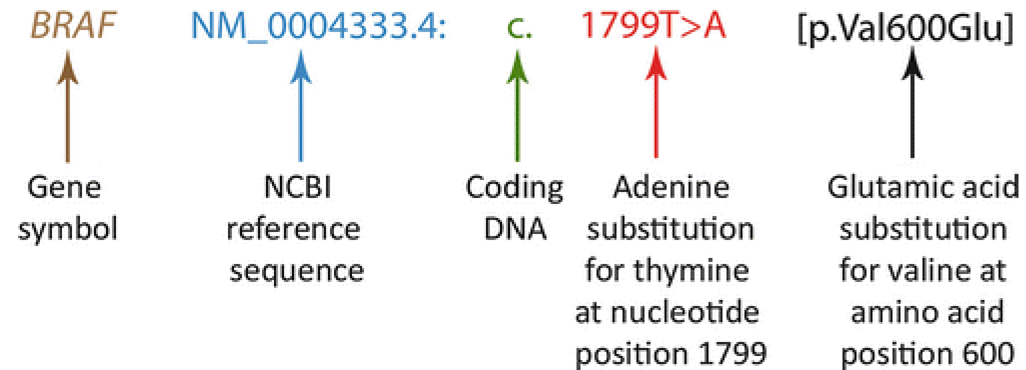
Basic methods

- Maxim-Gilbert sequencing
- Sanger sequencing

High-throughput methods

- Long- read sequencing methods
  - Single molecule real time sequencing (SMRT)
  - Nanonopore DNA sequencing
- Short- read sequencing methods
  - **Massively parallel sequencing**
  - 454 sequencing
  - Illumina sequencing
  - SOLiD sequencing
  - Ion Torrent sequencing

Sequence variant nomenclature



https://www.slideserve.com/tyra/mutation-nomenclature

Classification System for sequence variants

ACMG-AMP GUIDELINES

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Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology

Sue Richards, PhD[1], Nazneen Aziz, PhD[2,16], Sherri Bale, PhD[3], David Bick, MD[4], Soma Das, PhD[5], Julie Gastier-Foster, PhD[6,7,8], Wayne W. Grody, MD, PhD[9,10,11], Madhuri Hegde, PhD[12], Elaine Lyon, PhD[13], Elaine Spector, PhD[14], Karl Voelkerding, MD[13] and Heidi L. Rehm, PhD[15]; on behalf of the ACMG Laboratory Quality Assurance Committee

Proposed Classification System for Sequence Variants Identified by Genetic Testing

Class	Description	Probability of being Pathogenic
5	Definitely Pathogenic	>0.99
4	Likely Pathogenic	0.95–0.99
3	Uncertain	0.05–0.949
2	Likely Not Pathogenic or of Little Clinical Significance	0.001–0.049
1	Not Pathogenic or of No Clinical Significance	<0.001

5 classes linked to validated quantitative measures of causality/ pathogenicity

Class	Clinical Testing	Surveillance Recommendations if At-Risk Relative is Positive	Research Testing of Family Members
5	Test at-risk relatives for variant	Full high-risk surveillance guidelines	Not indicated
4	Test at-risk relatives for variant*	Full high-risk surveillance guidelines	May be helpful to further classify variant
3	Do not use for predictive testing in at-risk relatives*	Based on family history (and other risk factors)	May be helpful to further classify variant
2	Do not use for predictive testing in at-risk relatives*	Treat as "no mutation detected" for this disorder	May be helpful to further classify variant
1	Do not use for predictive testing in at-risk relatives*	Treat as "no mutation detected" for this disorder	Not indicated

All 5 classes are linked to clinical recommendations

Thank you for your attention!

Contact: valaskova.iveta@fnbrno.cz