# Molecular genetic diagnostics of monogenic diseases

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#### Molecular genetic diagnostics of rare diseases:

- Neuromuscular diseases
- Epilepsies
- Skin diseases
- Connective tissue diseases
- Metabolic diseases

## Molecular genetic diagnostics of monogenic diseases

## Why are we actually finding out?

## **1. Confirmation of clinical diagnosis**

- > psychological support
- > prediction of the course of the disease
- > specific treatment certain disease, certain mutation (example at the end of the lecture)

### **2. Segregation of variants/disease in family members**

- early treatment (in preclinical phase)
- > genetic counseling testing of partner, preimplantation diagnostics, prenatal diagnostics

## Molecular genetic diagnostics of monogenic diseases

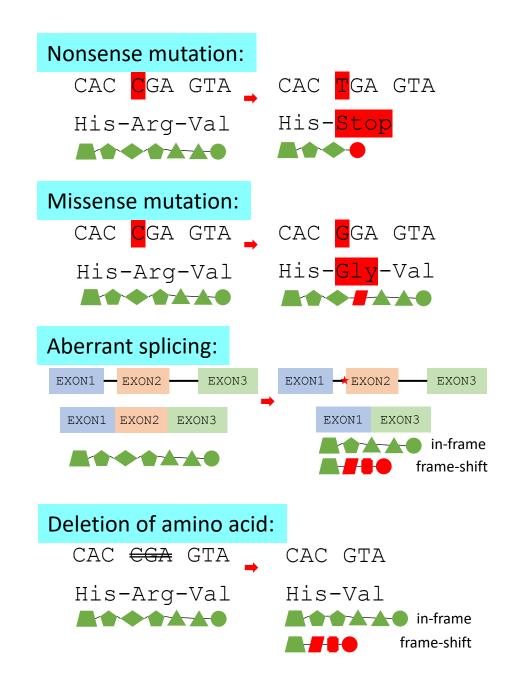
Germline mutation/mutations in **one gene**, not large deletions/insertions containing several genes:

identification of small scale variants:

- nucleotide substitutions --> nonsense mutation (creating premature stop codon)
  - $\rightarrow$  missense mutation (change of amino acid)
  - $\rightarrow$  affecting the splice site (aberrant splicing)

small deletions/insertions -> change in amino acid chain (without/with creating premature stop codon)

whole exon deletions / duplications (copy number variations, CNV)



## Molecular genetic diagnostics of monogenic diseases

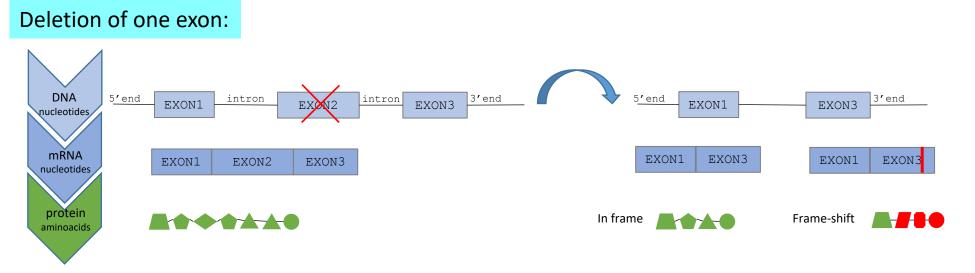
Germline mutation/mutations in one gene, not large deletions/insertions containing several genes:

identification of small scale variants: nucleotide substitutions, small deletions / insertions

> whole exon deletions/duplications (copy number variations, CNV) - in-frame - change in amino acid chain without

creating premature stop codon

frame-shift - change in amino acid chain with creating premature stop codon



## Molecular genetic diagnostics of monogenic diseases

*Material:* DNA isolated from the whole blood

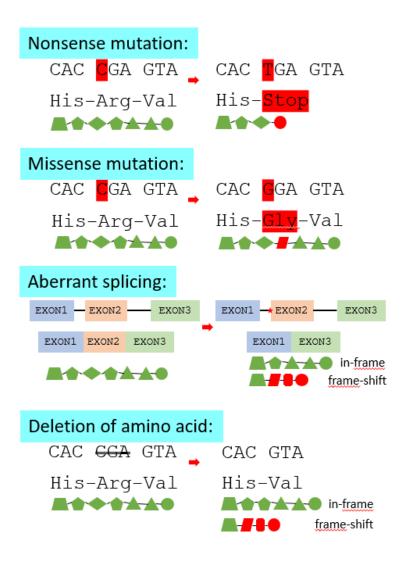
- 1. Classic Sanger sequencing
- 2. Next generation sequencing
- 3. MLPA CNV detection
- 4. RP-PCR detection of repeat expansions
- 5. Southern blot and hybridization detection of repeat expansions / deletions

identification of small scale variants: nucleotide substitutions, small deletions / insertions

#### > Method description:

1. PCR (polymerase chain reaction, amplification of known target sequence)

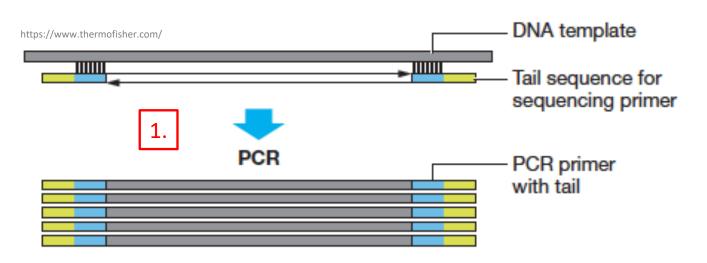
- 2. sequencing
- 3. result



identification of small scale variants: nucleotide substitutions, small deletions / insertions

#### > Method description:

1. PCR (polymerase chain reaction, amplification of known target sequence) > 2. sequencing > 3. result



#### **1. PCR**

- PCR amplifies a specific region of a DNA
- specific primers complementary to the target region
- reagents: DNA polymerase, primers, deoxynucleoside triphosphates (dNTPs), buffer solution, bivalent cations (typically magnesium)
- volume of 10–100 µL in small reaction tubes
- thermal cycler heats and cools the reaction tubes to achieve the temperatures required

#### PCR procedure

- 1. start denaturation 94°C/6min
- 2. denaturation
- 3. annealing
- 4. elongation
- 5. final elongation
- 94°C/1min 60°C/1min cycling steps2-4; 20-40x
- 72°C/1min
- 72°C/6min

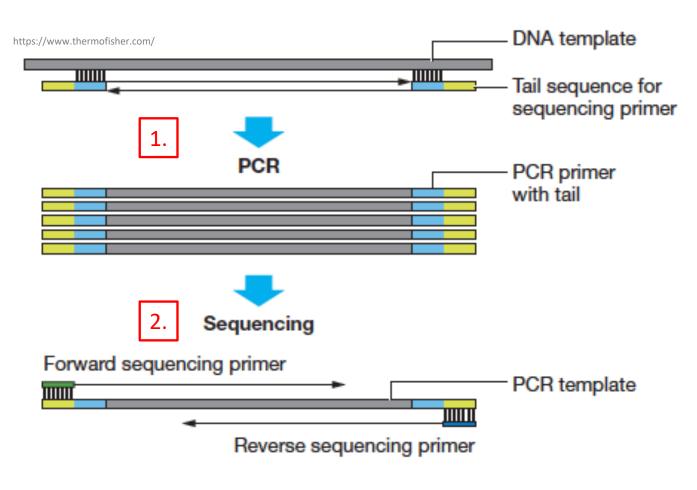




identification of small scale variants: nucleotide substitutions, small deletions / insertions

#### > Method description:

1. PCR (polymerase chain reaction, amplification of known target sequence) > 2. sequencing > 3. result



#### 2. sequencing

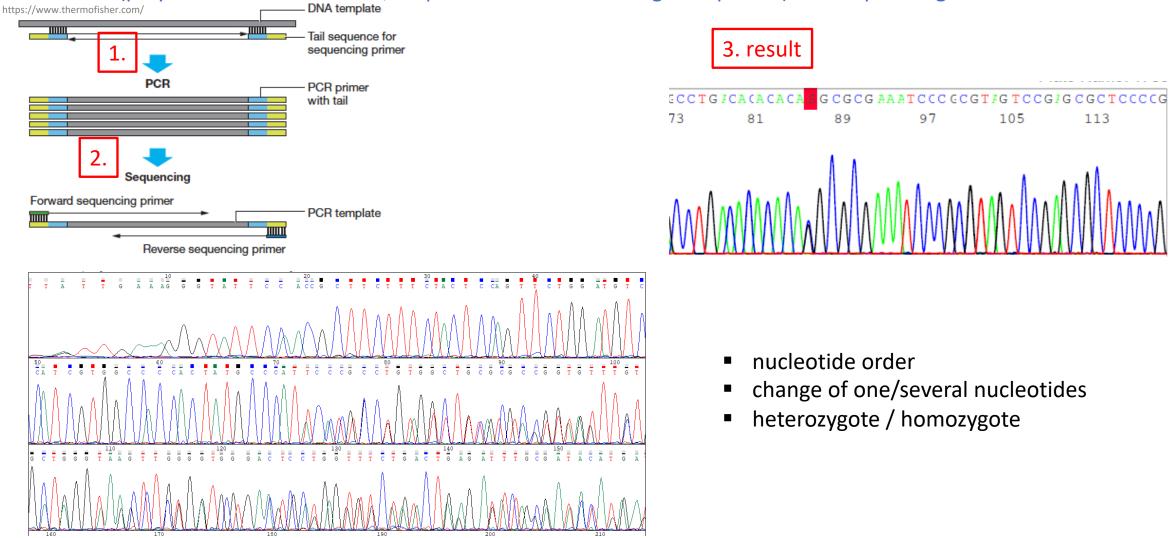
- determining of nucleotide order
- chain termination method
- one primer
- sequencing reaction in cycler > capillary electrophoresis



identification of small scale variants: nucleotide substitutions, small deletions / insertions

#### > Method description:

1. PCR (polymerase chain reaction, amplification of known target sequence) > 2. sequencing > 3. result



A. Sequencing of the certain part of the gene including the position of pathogenic variant

- segregation of variant in family members
- B. Sequencing of the whole gene by several PCR reactions:
  - in past: gene by gene approach (time-consuming and costly)
  - gene with clear clinical-genetic relationship, not a very long gene (example: phenylketonuria)

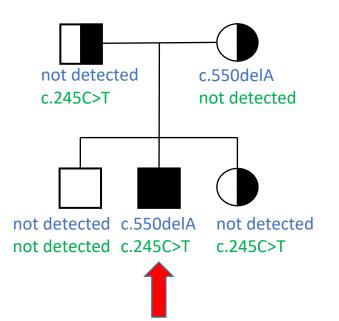
A. Sequencing of the certain part of the gene including the position of pathogenic variant

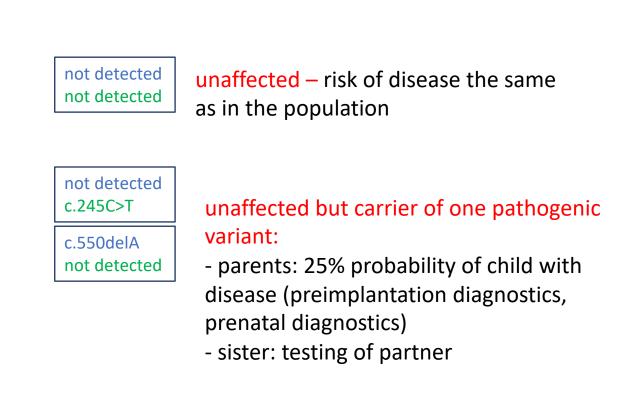
segregation of variant in family members

Patient with **autosomal recessive** limb girdle muscular dystrophy, 2 pathogenic variants in *CAPN3* gene *CAPN3*: c.245C>T and c.550delA

#### Presence of variants in:

- mother
- father
- brother
- sister





A. Sequencing of the certain part of the gene including the position of pathogenic variant

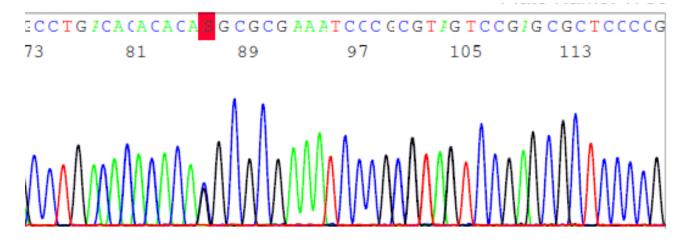
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B. Sequencing of the whole gene: gene with clear clinical-genetic relationship, not a very long gene example: phenylketonuria

## Phenylketonuria (PKU)

## autosomal recessive metabolic disease (deficiency of phenylalanine hydroxylase); gene PAH (12q23.2)

- diagnosed by newborn screening, on the basis of increased phenylalanine and the ratio phenylalanine/tyrosine
- increased Phe and Phe/Tyr is the indicator for DNA analysis of the PAH gene encoding the phenylalanine hydroxylase
- in 98% of cases, two pathogenic variants in the *PAH* gene are identified
   = clinical diagnosis of PKU is confirmed





## Molecular genetic diagnostics of monogenic diseases

- 1. Classic Sanger sequencing
- 2. Next generation sequencing
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- 5. Southern blot and hybridization detection of repeat expansions / deletions

*Material:* DNA isolated from the whole blood

- > 1. DNA samples are converted into sequencing libraries
  - DNA is randomly sheared into smaller fragments by mechanical or enzymatic methods
  - adapters for sequencing and multiplexing are added to DNA ends
  - regions of interest within the library are captured using oligonucleotide probes (hybridization)
  - probe-targeted fragment complex is separated from other fragments that are not bound to probes
  - amplification of targeted regions

#### oligonucleotide probes:

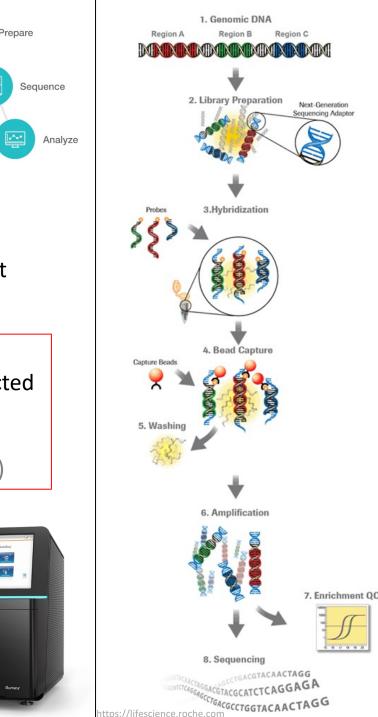
 targeted panel sequencing - selected sets of genes or gene regions

X Tor

- whole exome sequencing (WES)
- whole genome sequencing (WGS)

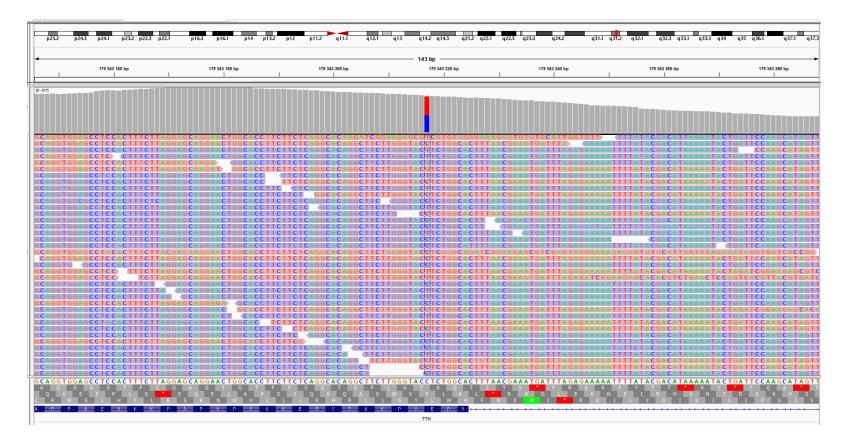
#### 2. sequencing

- NGS of targeted regions
- sequences millions of fragments in a massively parallel fashion
- improving speed and accuracy while reducing the cost of sequencing



#### > 3. data analysis

- the instrument software identifies nucleotides (a process called base calling) and the predicted accuracy of those base calls
- by commercial software or bioinformatics pipelines
- includes alignment of NGS reads
- identification and annotation of sequence variants





**Result:** > identification of small scale variants: nucleotide substitutions, small deletions / insertions

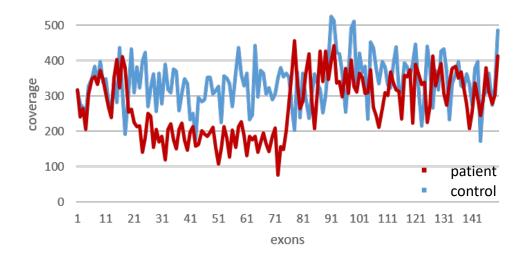
whole exon deletions / duplications (copy number variations, CNV)

Identification of a large number of sequence variants

A 4AJ91581	8	c 171	D 2	E F 1E+08 SNV	G		H A	No	J Homozyg	K 99.245	L 189	M 217	N 0.4655	406	P 408	Q A/A	R	EDAR .	T U ectodyspl EDAR	V W NM 022336.3 ± 1	X	Y NM 022		AA	AB	AC	AD AE not spec Conflict	AF
dAJ91584	1314	172	2		G		A	No	Homozyg	100	193			382		A/A	No	EDAR	ectodyspl EDAR	NM 022336.3 c.7		NM 022					not spec Conflict	
dAJ91581	1314	173	2	15+08 SNV	A		G	No	Homozys	100	272	270	0.4982	542	542	G/G	No	EDAR	ectodyspl EDAR	NM 022336.3 c.6							not prov Uncerta	
dAJ9158f	120	174	2	15+08 SNV	С		т	No	Heterozyg	48,092	107	90	0,4569	197	404	C/T	No	ERCC3	ERCC excl ERCC3	NM_000122.1:c.*2						0,0182 ×	Xeroderm Likely_b	oei 15/15, 15/1
dAJ9158f	82	175	2		С		т	No	Heterozyg	49,072	138		0,4908	271		C/T	No	ACVR1	activin A r ACVR1	NM_001105.4x.*1							Progressi Benign	
dAJ9158	178	176	2		с		T	No	Homozyg	99,52	301		0,4808	626	628		No	ACVR1	activin Ar ACVR1	NM_001105.4 c.6							Progressi Benign	
dAJ91581	166	177	2	2E+08 SNV	G		A	No	Heterozyg	48,958	145	141		286		A/G	No	ACVR1	activin A r ACVR1	NM_001105.4 c.2							Progressi Benign	5/12, 4/11,
dAJ91584 dAJ91584	723	178	2	2E+08 Delet 1609583 Delet			-	No	Heterozyg	47,222 47,239	120	105	0,469	227		complex		ITG86	integrin s ITG86	NM_000888.4:c.22 NM_000888.4:c.22							not_spec Benign	
dAJ91581	900	179	2		on AA		-	No	Heterozyg Heterozyg	49.66	114	114	0,5	328		complex C/T	No	ITG86	integrin s ITG86 integrin s ITG86	NM_000888.4/2.2							not_spec Benign	8/13.10/13
dAJ91581	636	181	2		a			No	Heterozys	45,307	101		0,4757	208		4/3	No	ITGA6	integrin s ITGA6	NM 000; NP 001				0.3126	0.281	0.2516 1	Epidermo Benien	
dAJ91581	725	182	;				â	No		51.724	70		0.4575	153		A/G	No	TGAS	integrin a ITGA6	NM 000210.3 c 1				0.4081			Epidermo Benign	1/20,1/23,
dAJ91581	511	183	2		c		Ť	No		51,263	150		0.4967	302	591	сл	No	ITGA6	integrin s ITGA6	NM 000210.3 c.2				0.2128	0.2974		Epidermo Benign	15/26 15/2
dAJ91581	400	184	2	1733690 Insert	ion -		T	No		37,104	120	137	0.4669	257	671	·/T	No	ITGA6	integrin s ITGA6	NM 000210.3 c.*?				0,1391	0.1696		Epidermo Likely b	
dAJ91581	801	185	2	25+08 SNV	A		G	No	Heterozya	46,296	143	168	0.4598	311	670	A/G	No	ITGA6	integrin s ITGA6	NM 000210.3 c.**	35AxG: N	N NM 000					Epidermo Benien	26/26.25/2
dAJ9158f	706	186	2	1784047 Insert	ion -		AAAG	No	Heterozys	59,77	26	48	0,3514	74	123	complex	No	AGPS	alkylglyce AGPS	NM_003659.3:c.*1	814_181	1 NM_003				P	Rhizomel Benign	20/20
dAJ9158f	692	187	2		A		G	No	Heterozyg	44,828	10	10	0,5	20		A/G	No	AGPS	alkylglyce AGPS	NM_003659.3:c.*1						8	Rhizomel Benign	
dAJ9158f	67	188		1784048 Deleti				No	Heterosyg	39,535	16		0,4074	27		complex		AGPS	alkylglyce AGPS	NM_003659.3:c.*1								20/20
dAJ9158	666	189		1784048 Deleti		A I	•	No	Heterozyg	46,512	17		0,4333	30		complex		AGPS	alkylglyce AGPS	NM_003659.3:c.*1							Rhizomel Conflict	
dAJ9158	704	190		2E+08 SNV	С		т	No	Heterozyg	46,784	114		0,4672	244	510		No	AGPS	alkylglyce AGPS	NM_003659.3:c.*5		NM_003					Rhizomel Benign	
dAJ91581	740	191		1784067 Insert 1784068 Delet			т	No	Heterozyg	45,614	77		0,4577	142	320		Yes	AGPS	alky/glyce AGPS	NM_003659.3:c.*5		NM_003					Rhizomel Benign	
dAJ91581 dAJ91581	75	192	2		on TIT		- A	No	Heterozyg	43,621				244		complex A/C		AGPS	alkyigiyce AGPS	NM_003659.3:c.*3 NM_003659.3:c.*4							Rhizomel Uncerta	
dAJ91581	738	195	2		-		A C	No	Heterozys Heterozys	50,145 43,258	141		0,4221 0,4872	244	539		No No	AGPS	alkylglyce AGPS alkylglyce AGPS	NM_003659.3:c.N		NM_003					Rhizomel Benign Rhizomel Benign	
6AJ91581 6AJ91581	341	194	2	26408 SNV 26408 SNV	G		c	No	Heterozyg	45,258	120		0,4872	254		C/G	No	COL3A1		NM_000659.3:c.N				0.1667	0 1264		Khizomeli Benign Ehlers-Da Benign	20/20
dAJ91581	340	195	2				4	No	Heterozy	44,828	108		0,4955	199		A/G	No	COLSAL		NM 000090.3 c 1		NM 000		0,1667			Chiers-Da benign Ehlers-Da Benign	26/51
dAJ91581	628	197	2		7		ĉ	No		47,826	95	95	0,4874	190		C/T	No	COL341		NM 000090.3 c 2		NM 000			0.5158		Ehlers-Da Benign	
dAJ9158	1525	198	2	25+08 SNV	÷		ä	No		99,178	290		0.4775	555		G/G	No	COLSAL	collagen t COL3A1					0,200	0,0100		not spec Benien	50/51
dAJ9158f	519	199	2	1898772 Deleti	on AG			No		52,717	149		0.4806	310		complex	No		collagen t COL3A1								Ehlers-Da Benign	
dAJ91581	1321	200	2	2E+08 SNV	A		G	No	Homozyg	99,342	237		0.4936	468		G/G	No	COL5A2	collagen t COLSA2			NM 000						48/54
dAJ91581	1320	201	2	2E+08 Delet	on T			No	Homozyg	83,125	88	117	0,4293	205	245	-/-	No	COL5A2	collagen t COL5A2	NM 000393.4 c.2	086-12del	NM 000					not spec Benign	
dAJ91581	1322	202	2	2E+08 SNV	т		с	No	Homozyg	99,468	290	296	0,4949	586	588	C/C	No	COL5A2	collagen t COL5A2	NM_000393.4 c.13	311A-G	NM_000	No					21/54
dAJ9158f	1522	203	2	25+08 SNV	G		т	No	Homozyg	100	257	289	0,4707	546	546	Т/Т	No	COL5A2	collagen t COLSA2	NM_000393.4 c.3	LSC»A	NM_000	No				not_spec Benign	2/54
dAJ9158f	992	204	2	25+08 SNV	т		с	No	Heterozyg		103		0,4747	217		C/T	No		uncharac LOC1019								Congeniti Benign	45/45, 53/3
dAJ9158f	1511	205	2	2E+08 SNV	A		G	No		97,786	215			388		G/G	No		ATP bindi ABCA12								not_spec Benign	
dAJ9158	599	206	2	2E+08 SNV	т		c	No	Heterosyg	49,588	192			383	776		No		ATP bindi ABCA12					0,1855	0,2331		Congenit: Benign	
dAJ9158	1310	207	2		A			No	Homozyg	99,237	224		0,4121	381	383		No		ATP bindi ABCA12							C	Congenit: Benign	
dAJ91581	178	208	2		c		T	No		99,628	199		0,4877	408		т/т	No	FN1 FN1	fibronect FN1 fibronect FN1	NM_0015 NP_997								39/44, 39/4
dAJ91581 dAJ91581	175	209	2		c		<u> </u>	No		50,327	185		0,4935	371	755		No	PN1	fibronect FN1	NM_0011NP_0012								28/44, 34/4
dAJ91581	1/5	210	2		C		c	No	Heterozys Heterozys	48,413	52		0,4/5/	185		C/G	No	FN1	fibronect FN1	NM_001306129.1 NM_001306129.1								28/44, 28/4
dAJ91581	113	212	;		č			No	Heterozyg	43,11	94	99	0,4522	193	446		No	FN1	fibronect FN1	NM 001306129.1								
da19158	113	213	;	2E+08 SNV	Ť		e.	No	Heterozya	47.386	120		0.4937	237		G/T	No	EN1	fibronect FN1	NM 001306129.1								20/44 20/4
dAJ91581	113	214	2		Ť		Ğ	No	Heterozya	52,098	120		0.4894	235		G/T	No	EN1	fibronect FN1	NM 001306129.1								20/44 20/4
da19158f	178	215	2		T		G	No	Homozyg	100	284		0.4731	559		6/6	No	FN1	fibronect FN1	NM 0011 NP 001								17/44 17/4
dAJ9158f	103	216	2	2162748 Insert	ion -		AC	No		84,821	136	164	0,4533	300	355	AC/AC	No	FN1	fibronect FN1	NM 001306129.1	c.1942-1	8 NM 212						
dAJ9158f	170	217	2	2E+08 SNV	т		A	No	Heterozys	44,295	97	97	0,5	194	442	A/T	No	FN1	fibronect FN1	NM 0013 NP 0013	2 NP 997	6 NM 212	Yes					1/44, 1/44,
dAJ91581 0	hyba	218	2	2E+08 SNV	С		G	No	Heterozyg	52,83	55	82	0,4015	137	266	C/G	No			NM_001306129.1	c270Gx	C; NM_001						
dAJ9158	424	219	2	2E+08 SNV	С		т	No	Heterozyg	47,619	97	109	0,4709	206	451	C/T	No		SWI/SNF SMARCAL							0,2079 \$	Schimke_ Benign	1/18
dAJ9158	50	220	2	25+08 SNV	т		A	No	Heterozyg	48,663	155	151		286	583		No			NM_025; NP_079				0,0185			Selective_ Conflict	
dAJ91581	305	221	2		С		G	No	Heterozyg	45,074	142		0,4897	290		C/G	No	PAX5	paired bo PAX3	NM_001127366.2				0,1231	0,1394		Craniofac Benign/	
dAJ9158f	1294	222	2	2E+08 SNV	A		G	No		99,257	195		0,4841	378		G/G	No	PAXS	paired bo PAX3	NM_000438.5:c.1						C	Craniofac Benign	
dAJ91581	105	223	2	2E+08 SNV	G		A	No	Heterozyg	51,667	99	84		183		A/G	No	MLPH	melanoph MLPH	NM_001042467.2								1/13, 1/15,
dAJ9158	211	224	2	2E+08 SNV	T		c	No	Heterozyg	48,276	136		0,4494	247	516		No	MLPH	melanoph MLPH	NM_001(NP_077								6/14, 5/13,
dAJ9158	211	225	2		G		A	No	Heterozyg	52,149	151		0,4757	288		A/G	No	MLPH	melanoph MLPH	NM_001(NP_001								6/14, 5/13,
dAJ91581 dAJ91581	104 210	226	2	2E+08 SNV 2E+08 SNV	C		<u>.</u>	No	Heterozyg	48,997 46.108	136		0,4925	268	550		No	MLPH MLPH	melanoph MLPH	NM_001042467.2 NM_001(NP_077)								6/13, 7/15, 6/13, 7/15,
dAJ9158( dAJ9158)	210	227	2	26+08 SNV 26+08 SNV	C		T G	No	Heterozyg Homozyg	46,108	107		0,4592	235	295		No	MLPH	melanoph MLPH melanoph MLPH	NM_001( NP_077)								6/15, 7/15, 9/16, 9/17
dAJ91581	184	228	2		÷		с С	No	Homozyg		281		0,4712	295	295		No	MIPH	melanopt MLPH melanopt MLPH	NM_001(NP_001)								9/16, 9/17 8/14, 8/13
34101501	112	229			-			No	nomozyg		127		0,4991	260	504		No			NM_001042457.3								0/14, 0/15

Identification of whole exon deletions / duplications: by comparing the number of reads for individual exons





#### Patient's case:

Patient has complained of difficulties in running a climbing stairs since his early teens. Patient has elevated creatine kinase levels. Proximal weakness in the upper and lower limbs has been progressive and he displays wasting of trunk muscles and slight hyperlordosis.

#### Neurologist requests analysis of genes associated with muscular dystrophy.

## **Muscular dystrophies and myopathies**

- to date, 162 genes associated with clinical manifestation of muscular dystrophy/myopathy
- clinical, biochemical, pathological,.... findings are mostly not specific enough for selection of a gene for molecular genetic analysis
- Which gene to analyse?

#### In past before NGS:

- genes analysed sequentially by classical DNA sequencing
- starting with a gene with the most likely mutation occurrence > negative result > another gene
- TIME AND FINANCIALLY CONSUMING
- only a certain number of genes analysed

#### NGS era:

- all genes associated with the disease analysed at the same time (in parallel) = **targeted panel**
- FAST AND RELATIVELY CHEAP

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#### NGS era:

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We use panel including all 162 genes



**Result:** > identification of small scale variants: nucleotide substitutions, small deletions / insertions

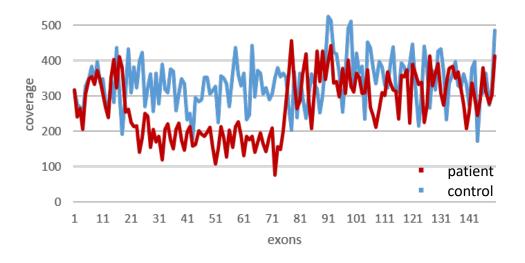
whole exon deletions / duplications (copy number variations, CNV)

Identification of a large number of sequence variants

6			~	$\checkmark$	JX																									
A dAJ91581	8	c 171	0 2	E 15408	F	G	н	1	1	к	L 189	M 217	N 0.4655	406	P 408	Q	R	S EDAR	T U	V NM 0223	w	x		222 No	AA	AB	AC		AE	AF
dAJ91581 dAJ91581	128/	1/1	2			G	A	No	Homozyg Homozyg	100	189	189		382	382		No No	EDAR	ectodysp EDAR ectodysp EDAR	NM 0223				222: No 222: No				not_spec Co not_spec Co		
dAJ91581	1314	172				6	G	No	Homozyg	100	272		0,4948	542	542		No	EDAR	ectodysp EDAR	NM 0223								not_spec Co not_prov/Un		
dAJ91581	120	174	,			ĉ	T	No	Heterozya	48.092	107		0.4569	197	404		No	FROCS	ERCC excl ERCC3	NM 0001								(eroderm Lik		
dAJ9158f	82	175	2			c	T	No	Heterozya	49.072	138		0.4908	271	563		No	ACVR1	activin Ar ACVR1	NM 0011								Progressi Ber		
dAJ9158f	178	176	2	25+08	SNV	с	т	No	Homozyg	99,52	301	325	0,4808	626	628	т/т	No	ACVR1	activin Ar ACVR1	NM 0011	105.4 c.6	BOGSA; N	MINM	001: No				Progressi Ber		8/12, 7/11,
dAJ9158	166	177	2	26+08	SNV	G	A	No	Heterozyg	48,958	145	141	0,493	286	582	A/G	No	ACVR1	activin A r ACVR1	NM_0011	105.4 c.2	70CsT; NI	M NM	001: No				Progressi Ber	nign S	5/12, 4/11,
dAJ9158	723	178	2				-	No	Heterozyg	47,222	120	105	0,469	227	480	complex	Yes	ITG86	integrin s ITG86	NM_0008								not_speci Ber	ign	
dAJ9158	306	179		1609583		AA	-	No	Heterozyg	47,239	114	114	0,5	229		complex		ITG86	Integrin s ITG86	NM_0008								not_spec Ber		
dAJ9158f	9	180	2			с	т	No	Heterozyg	49,66	171	157	0,4787	328	659		No	ITG55	Integrin s ITG86	NM_0008										3/13, 10/13
dAJ91581	636	181	2	25+08		G	A	No	Heterozyg	45,307	101	107	0,4856	208	460		No	ITGA6	integrin s ITGA6	NM_0003					0,312			Epidermo Ber		7/26, 7/25,
dAJ91584 dAJ91584	725	182	2			A .	G	No	Heterozys	51,724	70	83		153	288		No	ITGA6	integrin s ITGA6 integrin s ITGA6	NM_0002 NM_0002					0,408			Epidermo Ber Epidermo Ber		15/26 15/2
dAJ91581	400	184		1733690		C	T	No		37,104	120	137	0,4967	257	671		No	ITGA6	integrin s ITGA6	NM 0002					0.139			spidermo bei Spidermo Lik		
dAJ91581	801	185		25+08			G	No		46,296	143		0,4598	311	670		No	ITG46	Integrin s ITGA6	NM 0002					0,235	0,1090		pidermo Ber		
dAJ9158	706	186		1784047		2	AAAG	No	Heterozya	59.77	26		0.3514	74			No	AGPS	alkylelyce AGPS	NM 0036								Rhizomel Ber		20/20
dAJ9158f	692	187		25+08		A	G	No		44.828	10	10	0.5	20	46		No	AGPS	alkylglyce AGPS	NM 0036								Chizomeli Ber		0/20
dAJ9158f	67	188	2	1784048	Deletion	TA		No		39,535	16	11	0,4074	27	67	complex	No	AGPS	alkylglyce AGPS	NM_0036									Č (	20/20
dAJ9158	666	189		1784048		TATA	-	No	Heterozyg	46,512	17		0,4333	30		complex		AGPS	alkylglyce AGPS	NM_0036								Rhizomeli Co		
dAJ9158	704	190		26+08		С	т	No		46,784	114		0,4672	244	510		No	AGPS	alkylglyce AGPS	NM_0036			NM_					Rhizomel Ber		20/20
dAJ9158f	740	191		1784067			т	No		45,614	77		0,4577	142	320		Yes	AGPS	alkylglyce AGPS	NM_0036								Rhizomel Ber		20/20
dAJ9158f	75	192		1784068			•	No	Heterozyg	43,621	70		0,4895	143		complex		AGPS	alkylglyce AGPS	NM_0036								Rhizomeli Un		
dAJ9158(	737	193	2	25+08		c	A	No		50,145	141		0,4221	244	500		No	AGPS	alkylglyce AGPS	NM_0036			NM_					Rhizomeli Ber		20/20
HAJ9158	738	194				T			Heterozyg	43,258	120		0,4872	234	539 493			AGPS COL3A1	alkylglyce AGPS collaren t COL3A1	NM_0036			NM_		0.165	0.1264		Rhizomeli Ber Ehlers-Da Ber		20/20
AJ9158	541	195	2			G	C A	No	Heterozyg	44,444	108		0,4955	214	495		No	COLSA1 COLSA1		NM_0000 NM_0000				000 - 000 No	0,166			chiers-Da Ber Chiers-Da Ber		6/51
dAJ91581	628	190				-	C A	No		44,828	95	97	0,4874	199	400		No	COLSA1		NM 0000				000 No	0,164			chiers-Da bei Chiers-Da Bei		32/51
4419158	1525	198				-	G	No		99.178	290		0.4775	555	558		No		collagen t COLSA1						0,28	0,5136		not_spec Ber		50/51
dAJ9158	519	199	2	1898772		AG		No		52.717	149		0.4806	310		complex			collagen t COLSA1									Ehlers-Da Ber		51/51
dAJ91581	1321	200	2	26+08	SNV	A	G	No		99,342	237		0,4936	468	470		No	COL5A2	collagen t COLSA2					000: No						18/54
dAJ91581	1320	201	2	26+08	Deletion	т	-	No	Homozyg	83,125	88	117	0,4293	205	245	-/-	No	COL5A2	collagen t COL5A2	NM_0003	93.4 c.2	086-12de	NM.	- 1000				not_speci Ber	nign	
dAJ9158	1322	202	2			т	с	No	Homozyg	99,468	290	296	0,4949	586	588	C/C	No	COL5A2	collagen t COL5A2				NM_	000: No						21/54
dAJ9158f	1522	203	2			G	т	No	Homozyg	100	257	289	0,4707	546	546		No		collagen t COL5A2					000! No				not_spec Ber		2/54
dAJ9158f	992	204	2	25+08		т	с	No		53,053	103	114		217	410		No		uncharac LOC1019									Congeniti Ber		15/45, 53/5
dAJ9158	1311	205	2			A	G	No		97,786	215		0,4459	388	394		No		ATP bindi ABCA12									not_speci Ber		
dAJ9158	599	206	2			T	C	No	Heterozyg	49,588	192	191		383	776		No		ATP bindi ABCA12						0,185	0,2331		Congenit: Ber		14/45, 22/5
dAJ9158	1310	207	2			A	T	No	Homozyg	99,237	224	157		381	383		No No	ASCA12 EN1	ATP bindi ABCA12 fibronect FN1	NM_0156 NM_0015								Congenit: Ber		9/45, 17/53 99/44, 39/4
dAJ91581	1/8	208				0	-	No	Homozyg	50.327	185		0,4877	371	735		No	FN1	fibronect FN1	NM_0011										59/44, 59/4 54/44, 34/4
dAJ91581	175	210	;			c	-	No	Heterozys	48,413	97		0,4933	185	378		No	FN1	fibronect FN1	NM 0013										28/44, 28/4
dAJ9158	126	211	;			G	c	No		51.351	52	63		115	229		No	FN1	fibronect FN1	NM 0013										
dAJ91581	113	212	2			c	T	No	Heterozyg	43,11	94	99	0,487	193	446		No	FN1	fibronect FN1	NM 0013										
dAJ91581	113	213	2			т	G	No	Heterozyg	47,386	120	117	0,4937	237	492		No	FN1	fibronect FN1	NM_0013									;	20/44, 20/4
dAJ9158	113	214	2	26+08	SNV	т	G	No	Heterozyg	52,098	120	115	0,4894	235	445	G/T	No	FN1	fibronect FN1	NM_0013										20/44, 20/4
dAJ9158f	178	215	2			т	G	No	Homozyg	100	284		0,4731	539	539		No	FN1	fibronect FN1	NM_0011									1	17/44, 17/4
dAJ9158(	103	216		2162748		-	AC	No	Homozyg		136			300	355		No	FN1	fibronect FN1	NM_0013										
dAJ9158f	170	217	2			Ŧ	A	No		44,295	97	97	0,5	194	442		No	FN1	fibronect FN1	NM_0013										1/44, 1/44,
dAJ91581 0		218	2			c	G	No	Heterozyg	52,83	55	82		137	266		No			NM_0013										
dAJ9158 dAJ91581	424	219	2			C	T	No		47,619 48.663	97 135	109	0,4709	206	451		No		SWI/SNF - SMARCAL Wot famil WNT10A	NM_0011 NM 025;					0.018	0.0127		ichimke_Ber Selective Co		
dAJ91581 dAJ91581	305	220	2			C	G	No	Heterozyg	48,663	155		0,472	286	645		No	WNT10A PAX8	Writ famil WNT10A paired bo PAX3	NM_025; NM_0011								Selective_ Co Craniofac Ber		
6AJ91581 6AJ91581	1294	221				ă.	G	No		45,074	142		0,4897	378	380		No	PAXS	paired bo PAX3	NM 00011					0,125	0,1594		Craniofac Ber Craniofac Ber		
dAJ91581	105	222	;			â	A	No		51,667	99	84	0,4641	183	361		No	MLPH	melanopt MLPH	NM 0010							1	oraniorac del		L/0, 2/9, 2/1 L/13, 1/15,
dAJ9158	211	224	2			T	ĉ	No	Heterozya	48,276	136	111	0.4494	247	516		No	MLPH	melanoot MLPH	NM 0010										5/14. 5/13.
dAJ91581	211	225	2			G	A	No		52,149	151	137	0,4757	288	549		No	MUPH	melanopt MLPH	NM 0010										5/14, 5/13,
dAJ91581	104	226	2			с	т	No		48,997	136		0,4925	268	550		No	MLPH	melanoph MLPH	NM_0010										5/15, 7/15,
dAJ91581	210	227	2	25+08	SNV	С	т	No	Heterozys	46,108	107	126	0,4592	233	511	с/т	No	MLPH	melanopit MLPH	NM_0010	NP_077	0 NP_077	70 NM	24: Yes					i i	5/13, 7/15,
dAJ9158f	302	228	2	26+08	SNV	A	G	No	Homozyg	100	156	139	0,4712	295	295	G/G	No	MLPH	melanoph MLPH	NM_0010	NP_077	0 NP_077	70 NM	024: Yes					9	9/16, 9/17
dAJ91581	184	229	2	26+08			C	No	Homozyg	99,153	281	200	0.4991	561	564		No	MLPH	melanook MI PH					024; Yes						8/14.8/13.

Identification of whole exon deletions / duplications: by comparing the number of reads for individual exons





Interpretation of causality

Genet Med. 2015 May ; 17(5): 405-424. doi:10.1038/gim.2015.30.

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 [Chair, ACMG],

1.Benign sequence variants2.Likely benign sequence variants3.Sequence variants of uncertain significance4.Likely pathogenic sequence variants5.Pathogenic sequence variants

Patient's case:

Patient has complained of difficulties in running a climbing stairs since his early teens. Patient has elevated creatine kinase levels. Proximal weakness in the upper and lower limbs has been progressive and he displays wasting of trunk muscles and slight hyperlordosis.

#### **Result:**

#### A) Identification of pathogenic variant/variants

e.g. two pathogenic variants in *CAPN3* > confirmed diagnosis of limb girdle muscular dystrophy *CAPN3* (NM\_000070.3): c.245C>T p.(Pro82Leu) / c.550delA p.(Thr184Argfs\*36)

#### B) Identification of variants of uncertain significance: e.g. variant in DYSF

- genetic-clinical correlation
- segregation of variant in family
- type of inheritance

#### C) Only benign variants identified - diagnosis was not confirmed

- > pathogenic variant in unanalyzed gene, in noncoding region
- ≻ WES, WGS

#### **Limitations of NGS:**

- panel + WES: analysis of coding regions sequencing about 95-98% of selected regions
- occurrence of pseudogene, regions with high similarity: difficult non-specific mapping
- is not a suitable method for diseases associated with the expansion / deletion of repetitive sequences

## Molecular genetic diagnostics of monogenic diseases

- 1. Classic Sanger sequencing
- 2. Next generation sequencing
- 3. MLPA CNV detection
- 4. RP-PCR detection of repeat expansions
- 5. Southern blot and hybridization detection of repeat expansions / deletions

*Material:* DNA isolated from the whole blood

- gold standard for CNV detection (whole exons deletions/duplications)
- targeted analysis of a specific gene/genes
- available for certain genes



MRC

#### First example:

Boy has delayed motor function acquisition and proximal muscle weakness. Due to severely elevated creatine kinase levels (15,000 U/L), neurologist suspects a clinical diagnosis of Duchenne muscular dystrophy.

#### Duchenne muscular dystrophy,

gene *DMD* (chromosome X):

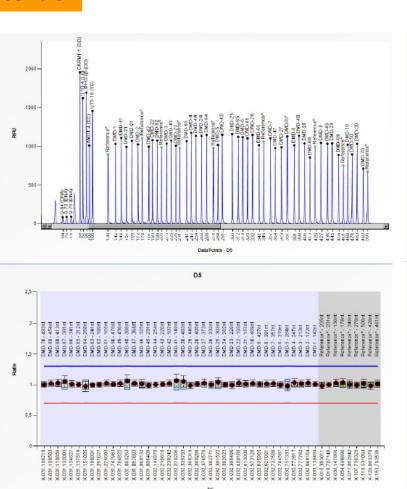
- whole exon deletions (68%) and duplications (10%)
- MLPA is the first choice method
- man = affected woman = carrier

#### **Result:**

due to the large DMD size, the MLPA has two parts, the second part is not shown

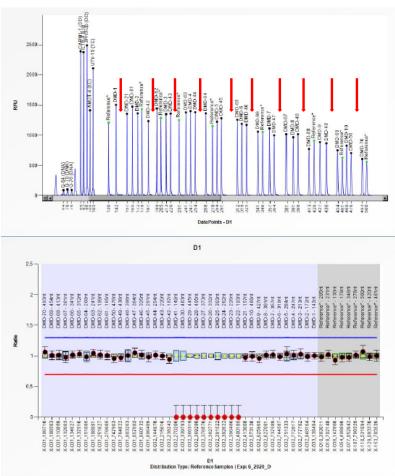
- deletion of exons 22-41 hemizygous
- out-of-frame deletion leading to creation premature stop codon
- confirmed diagnosis of Duchenne muscular dystrophy





Distribution Type: Reference Samples | Exp: 6 2020

#### 10 exons deletion, hemizygous, man



#### First example:

**Duchenne muscular dystrophy**, gene *DMD* (chromosome X):

whole exon deletions (68%) and duplications (10%)

2000

E 1500-

- MLPA is the first choice method
- man = affected  $\geq$

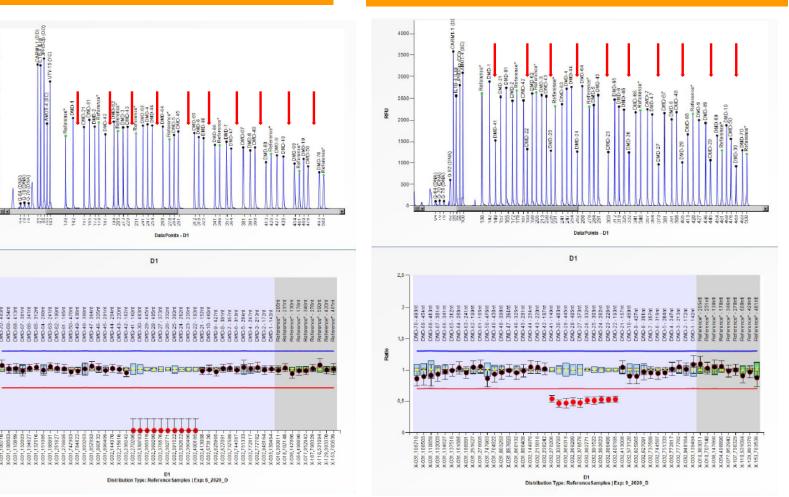
woman = carrier

#### 10 exons deletion, hemizygous, man

D1

D1 Distribution Type: Reference Samples | Exp: 6\_2020\_0

10 exons deletion, heterozygous, woman



#### **Boy's mother**

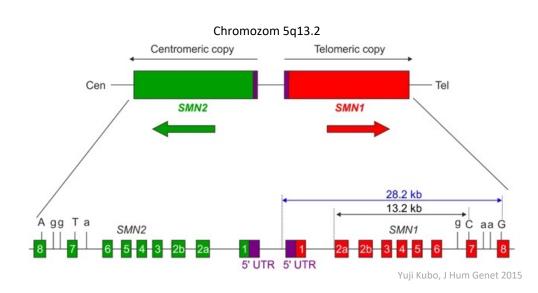
#### **Result:**

due to the large DMD size, the MLPA has two parts, the second part is not shown

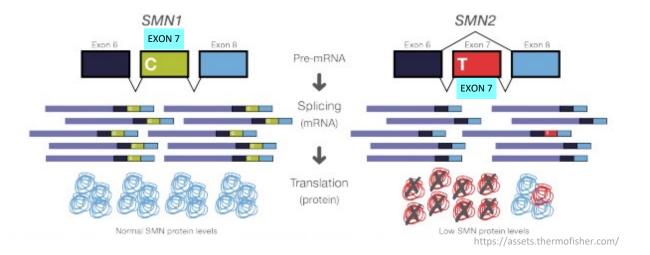
- deletion of exons 22-41 heterozygous •
- out-of-frame deletion leading to • creation premature stop codon
- mother is carrier of Duchenne muscular dystrophy

#### Second example: Spinal muscular atrophy (SMA), gene SMN1

- autosomal recessive disease
- incidence: 1 in 6,000 10,000 live births
- second most frequent fatal disease with autosomal recessive inheritance (after cystic fibrosis)
- characterized by degeneration of alpha motor neurons
- newborn screening



- > 95% caused by homozygous deletion of the SMN1 gene
- SMN1 has its almost identical copy SMN2 gene
   (SMN1 and SMN2 are homologous to except for few nucleotides)
- copy number variation of SMN1 and SMN2 in human genome
- clinical severity is modified by copy number the SMN2 gene



Second example:

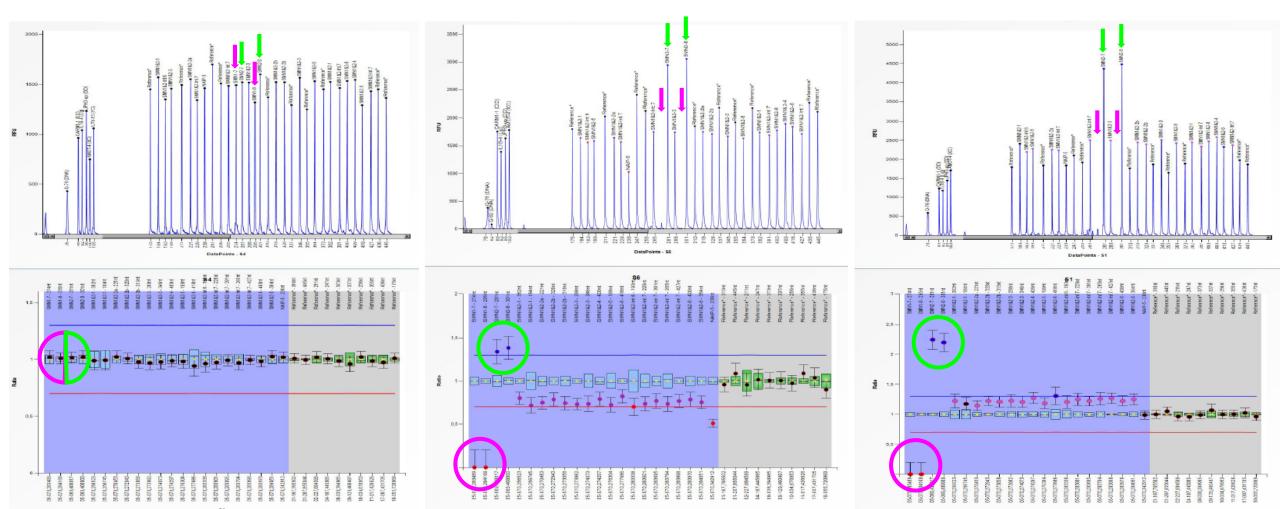
Spinal muscular atrophy (SMA), SMN1

- > patients with homozygous *SMN1* deletion
- heterozygous carriers of SMN1 deletion
- SMN2 copy number

Control: 2x SMN1, 2x SMN2

#### SMA: 0x SMN1, 3x SMN2

#### SMA: 0x SMN1, 5x SMN2



## Molecular genetic diagnostics of monogenic diseases

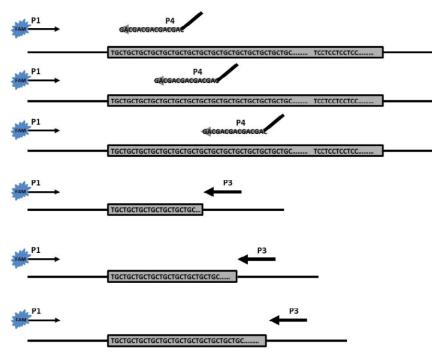
- 1. Classic Sanger sequencing
- 2. Next generation sequencing
- 3. MLPA CNV detection
- 4. RP-PCR detection of repeat expansions
- 5. Southern blot and hybridization detection of repeat expansions / deletions

*Material:* DNA isolated from the whole blood

## 4. RepeatPrimed-PCR (RP-PCR)

#### > Method description:

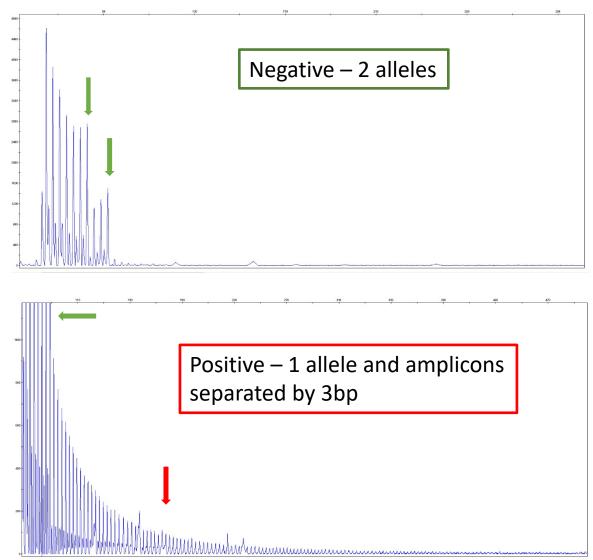
- PCR with three primers
- characteristic profile of amplicons of increasing length, which differ by the length of a repeat unit (3 bp)



V. Mootha, Inv. ophthal. & vis. science 2014

- detection of repeat expansions (usually three nucleotides)
- presence / absence of expansion
- does not determine length of expansion (number of repeats)

**RP-PCR** and fragment analysis:





Patient is displaying subtle signs of myotonia as well as ptosis and weak eye lid closure. Neurologist requested testing for myotonic dystrophy 1.

## Myotonic dystrophy type 1

- > expansion of CTG repeat in 3'UTR of DMPK (9q13.32)
- > autosomal dominant inheritance
- correlation between number of repeats and severity of the phenotype:
  - 5-37 repeats unaffected
  - 38–50 repeats premutation, asymptomatic
  - 51–149 repeats mild adult-onset form
  - 150–1000 repeats classic MD1
  - >1000 repeats congenital form MD1

#### 20x CTG

tccgcggccg gcgaacgggg ctcgaagggt ccttgtagcc gggaatg<mark>ctg ctgctgctgc tgctgctgct gctgctgctg ctgctgctgc tgctgctgct gctgctgggg ggatcacaga ccatttcttt ctttcggcca ggctgaggcc ctgacgtgga tgggcaaact gcaggcctgg</mark>

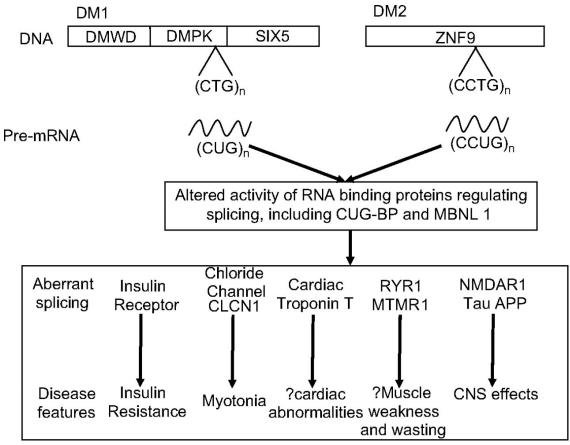
#### 140x CTG

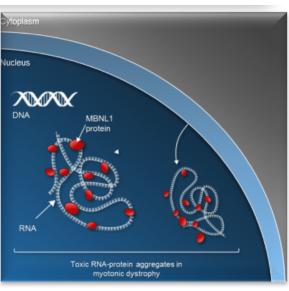
tccgcggccg gcgaacgggg ctcgaagggt ccttgtagcc gggaatgctg ctgctgctg tgctgctgct gctgctgct ctgctgctgc tgctgctgct gctgctgct ctgctgctgc tgctgctgct gctgctgctg ctgctgctgc tgctgctgct gctgctgcg ggatcacaga ccatttcttt ctttcggcca ggctgaggcc ctgacgtga tgggcaaact gcaggcctgg

anticipation - the number of repeats tends to increase in size over generations. Expansion of the CTG repeats commonly occurs during meiosis. As a result, children of affected individuals tend to have severe symptoms and earlier onset than their parents.

### Myotonic dystrophy type 1 – mechanism:

- toxic effect of expansion
- accumulation of RNA with expansions in the nucleus, sequestration of RNA-binding protein > formation of nuclear inclusions
- altering mRNA splicing of other genes





Mignon, IONIS-DMPK Clinical Program in Myotonic Dystrophy

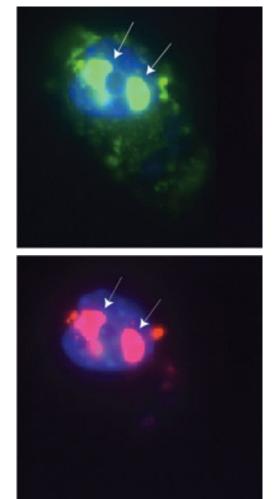


Image shows the location of the Mbnl1 splicing factor (green) and the second image shows the location of RNA repeats (red) inside the cell nucleus (blue). The white arrows point to two large foci in the cell nucleus where Mbnl1 is sequestered with RNA. Photos by Hongqing Du

Turner, Journal of Neurology, Neurosurgery & Psychiatry 2010

### 4. RepeatPrimed-PCR (RP-PCR)



Patient is displaying subtle signs of myotonia as well as ptosis and weak eye lid closure. Neurologist requested testing for myotonic dystrophy 1.

### **Myotonic dystrophy type 1 – result:**

presence of expansion

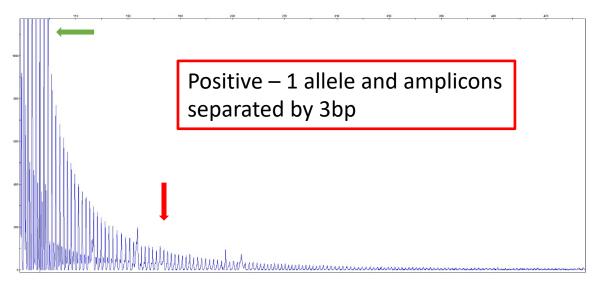
confirmed diagnosis of myotonic dystrophy type 1

#### not the length



Solution: Southern blot and hybridization

**RP-PCR** and fragment analysis:



# Molecular genetic diagnostics of monogenic diseases

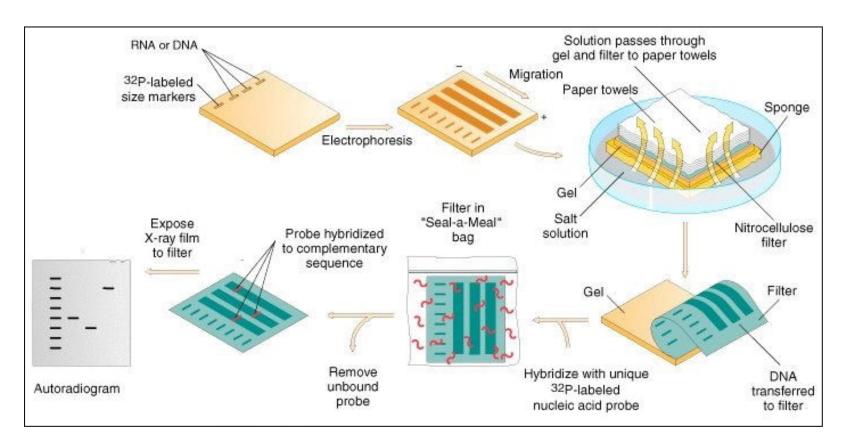
- 1. Classic Sanger sequencing
- 2. Next generation sequencing
- 3. MLPA CNV detection
- 4. RP-PCR detection of repeat expansions
- 5. Southern blot and hybridization detection of repeat expansions / deletions

*Material:* DNA isolated from the whole blood

detection of repeat expansions / deletions
determination of the size

### > Method description:

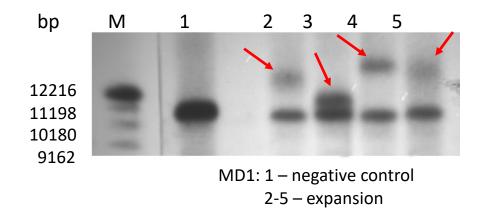
- DNA is cleaved by a restriction endonuclease
- electrophoresis
- transfer to membrane
- hybridization with radioactive labeled probe
- autoradiography



#### Example:

### **Myotonic dystrophy type 1 – result:**

- presence of expansion
- not the length

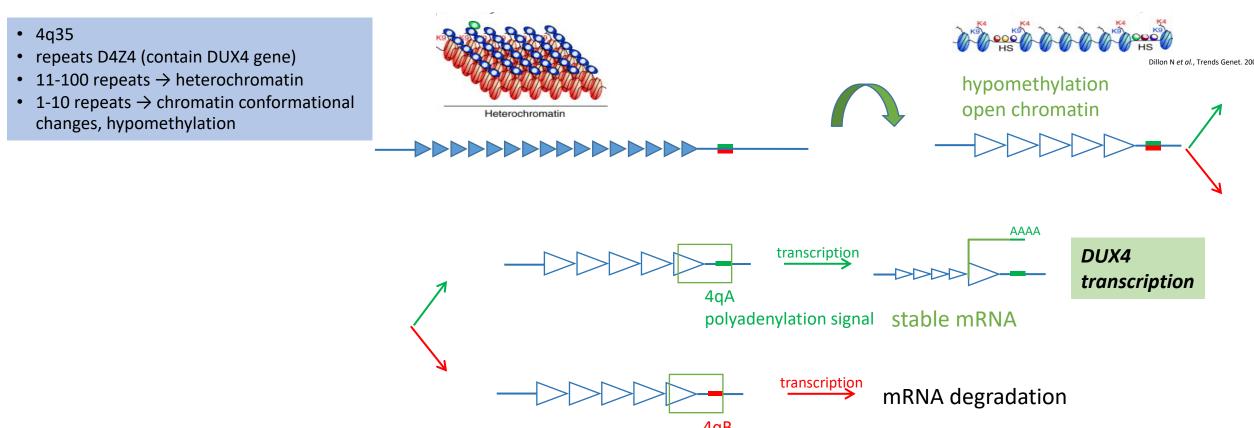


according to the size of the fragment, we determine the number of repeats

#### Example:

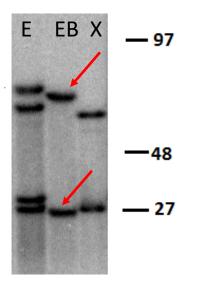
### Facioscapulohumeral dystrophy 1 (FSHD1)

- the third most prevalent muscular dystrophy, AD inheritance
- weakness and wasting of the face, shoulder and upper arm muscles, with later involvement of the trunk and lower extremities
- FSHD develops through complex genetic and epigenetic events that converge on a common mechanism of toxicity with mis-expression of the transcription factor DUX4



### Example: Facioscapulohumeral dystrophy 1 (FSHD1)

- 4q35, repeats D4Z4 (contain DUX4)
- 11-100 repeats  $\rightarrow$  unaffected
- 1-10 repeats  $\rightarrow$  affected



we determine the number of D4Z4 repeats according to the size of the product

# Molecular genetic diagnostics:

- the results must be interpreted with knowledge of the molecular nature of the disease and knowledge of the structure and function of encoded protein
- the results must be interpreted in relation to the patient 's phenotype and results of other patient examinations (biochemistry, pathology, NMR, EMG, etc.)
- it is necessary to return to the results of already examined patients with an unconfirmed genetic diagnosis and test them with new techniques and perform new interpretations of the identified sequence variants
- it is necessary to participate in international quality control of DNA diagnostics for individual diseases

### **Example of specific treatment - certain disease, certain mutation**

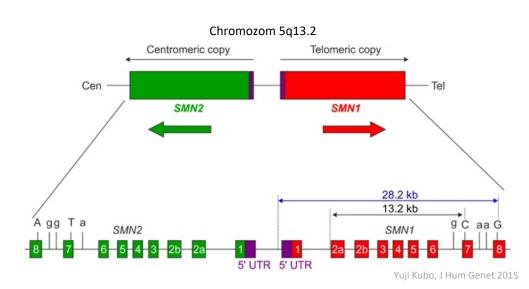
Spinal muscular atrophy (SMA)

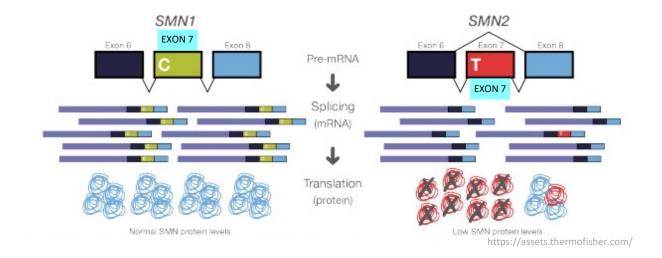
# Spinal muscular atrophy (SMA)

- gene SMN1, autosomal recessive disease
- incidence: 1 in 6,000 10,000 live births
- second most frequent fatal disease with autosomal recessive inheritance
- characterized by degeneration of alpha motor neurons
- newborn screening started last year

- > 95% caused by homozygous deletion of the SMN1 gene
- SMN1 has its almost identical copy SMN2 gene
   (SMN1 and SMN2 are homologous to except for few nucleotides)
- copy number variation of SMN1 and SMN2 in human genome

Not enough SMN protein, so the motor neurons shrink and die. As a result, the brain can't control voluntary movements, especially motion in the head, neck, arms and legs.

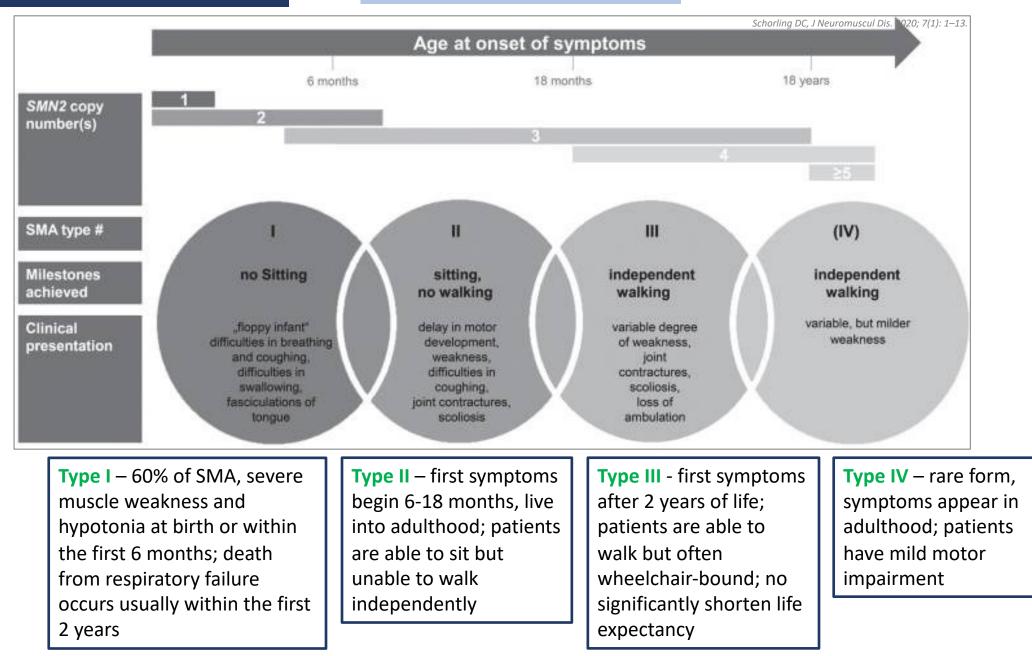




Clinical severity is modified by copy number the SMN2 gene

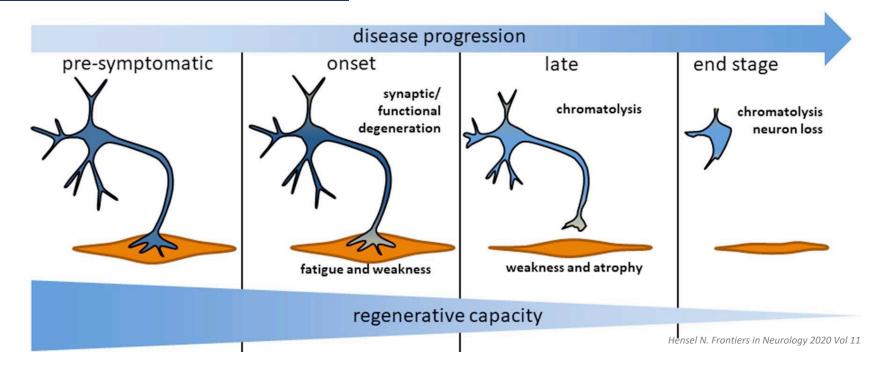
## Spinal muscular atrophy

### 4 clinical types of SMA



# Spinal muscular atrophy

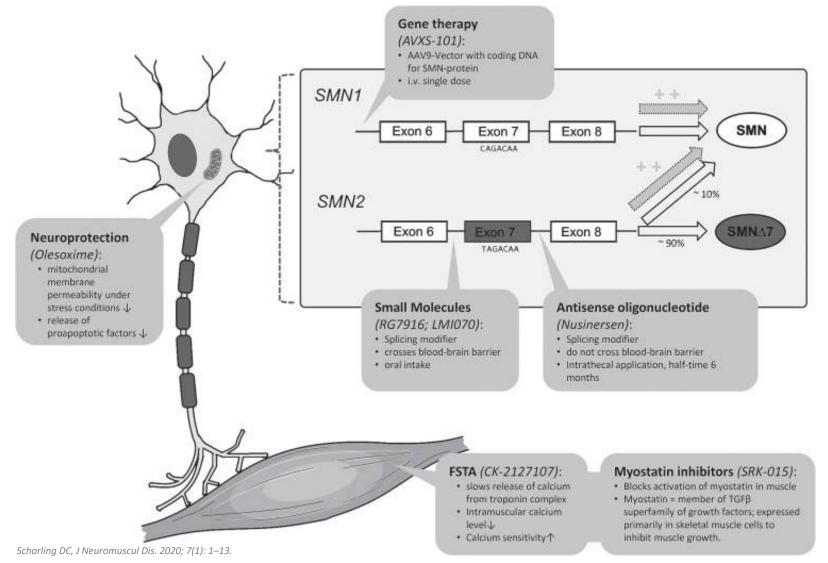
### Motoneuron degeneration in SMA



- functional degeneration of central synapses and neuromuscular junctions and subsequent axonal damage > motoneuron loss
- complete loss of motoneuron is a irreversible change
- The beneficial effects of SMA therapies are dependent on disease duration at the time of intervention. Disease duration before treatment is critical and a delayed intervention leads to a less efficient rescue. The effect of SMA therapies is strongest in pre-symptomatic patients.

### **SMA therapies**

- 1. modifying splicing of SMN2 (production of more amount of full length mRNA)
- 2. replacing the SMN1 gene



1. modifying splicing of *SMN2* (production of more amount of full length mRNA)

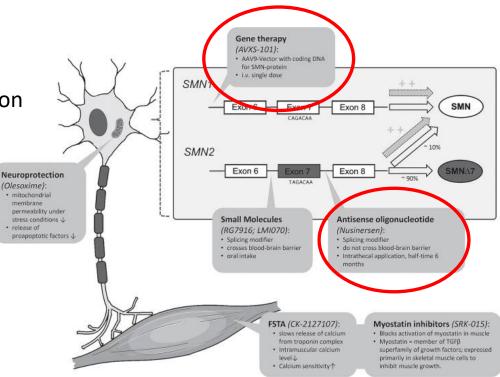
### A. Nusinersen (Spinraza®)

- an antisense-oligonucleotide (ASO) that enhances the inclusion of exon 7 in mRNA transcripts of SMN2
- administered intrathecally
- B. Risdaplam (Evrysdi®)
  - administered orally

### 2. replacing the SMN1 gene

### C. Onasemnogene Abeparvovec-xioi (Zolgensma®)

- children younger than two
- one-time intravenous infusion
- adeno-associated virus 9 (AAV9) delivering cDNA which codes the full length SMN protein
- = replacement of a missing or faulty *SMN1* gene with a functioning gene



# SMA neonatal screening pilot project in Czech republic

- > early detection of neonates in the preclinical asymptomatic stage
- > treatment before irreversible complete loss of motoneuron

# QUESTIONS?