LMU KLINIKUM DER UNIVERSITÄT MÜNCHEN

MGZ Medizinisch Genetisches Zentrum

cDNA analyses of the MMR genes *MLH1, MSH2, MSH6,* and *PMS2* investigate the effect of VUS upon splicing, detect unexpected splicing defects, and find allelic losses indicating a germline defect

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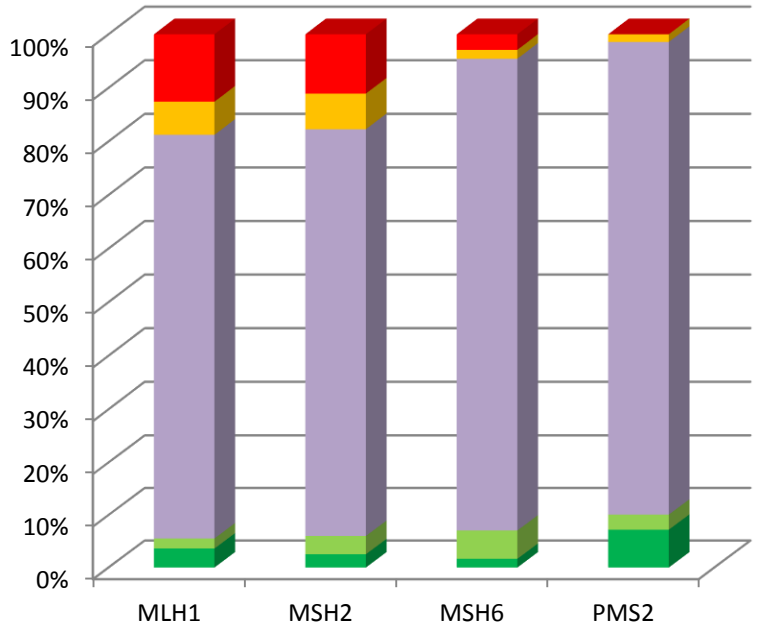
Pathogenicity assessment by RNA-analysis

- missense variants
- splice site variants
- intronic
- synonymous
- 5`/ 3`UTR
- exon duplication
- ins/del of one amino acid



Prerequists for RNA based pathogenicity assessment

- Allelic balance
- Normal splicing pattern
- Efficiency of NMD
- Abnormal splicing pattern

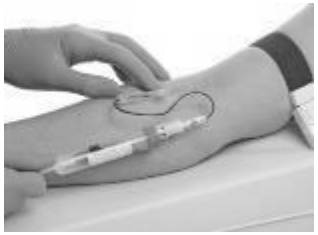
⇒ Cut-off definition by analysing more than 900 cDNAs

⇒ Full lenght cDNA analysis protocol - EMMR working group

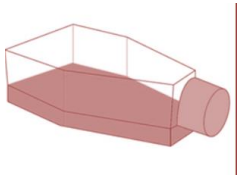
Isolation of patient's total RNA

RNA derived from patient blood samples

2x heparine



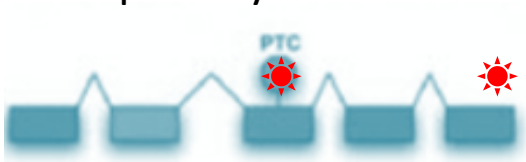
short-term lymphocyte cultures



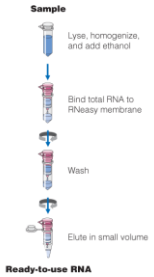
NMD-inhibition

+puromycin

-puromycin

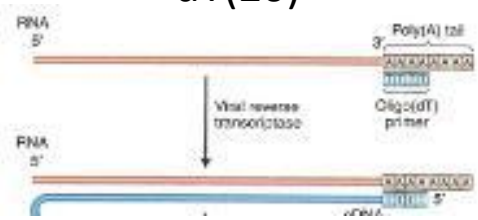


RNA isolation



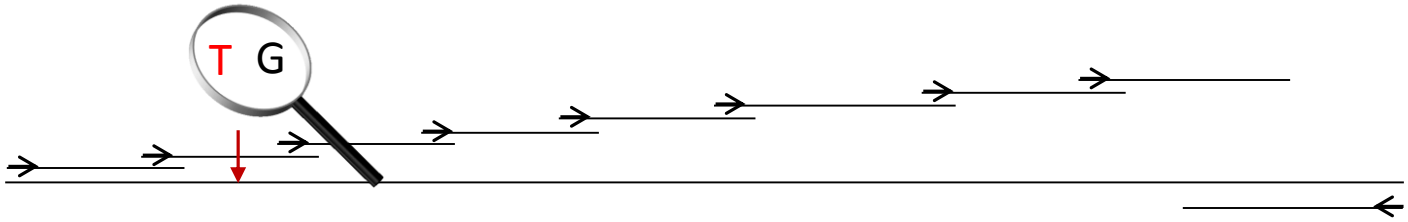
cDNA synthesis

dT(20)



72-96 h, 37°C **Nonsense-Mediated-mRNA-Decay (NMD)** 48°C, 75-90 min

early truncating > 55 nt prior to last exon-junction



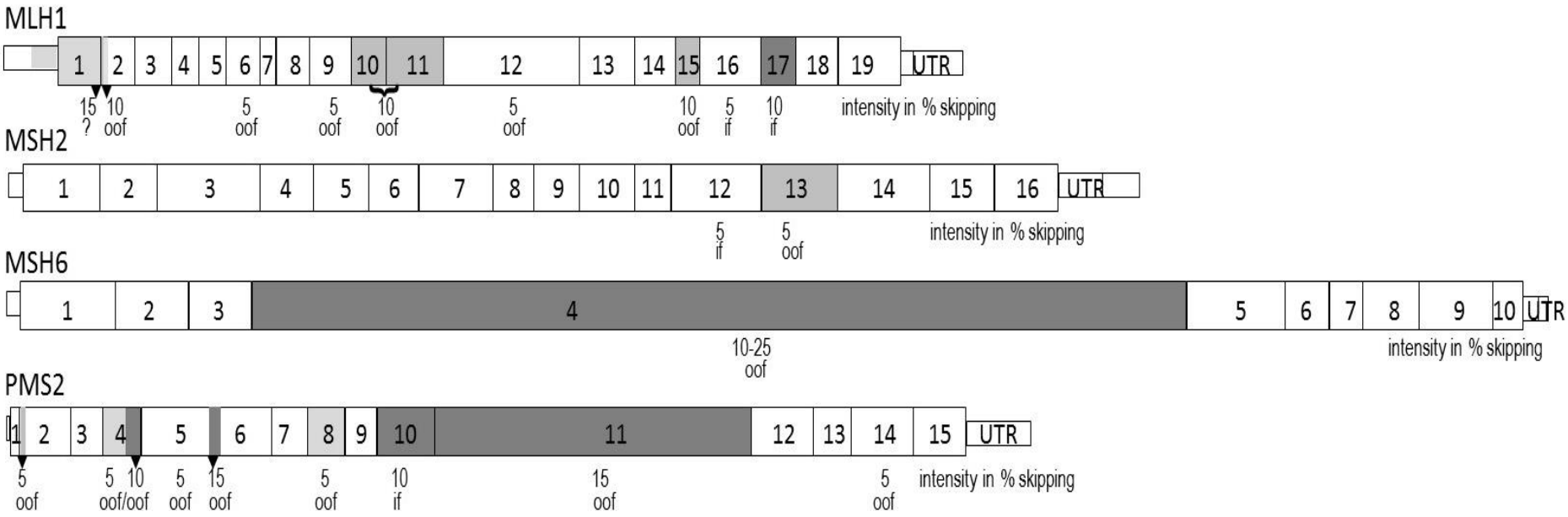
MLH1 ~ 2.5 kb

MSH2 ~ 3 kb

MSH6 ~ 4.2 kb

PMS2 ~ 2.8 kb

Alternative splicing in controls



-> alternative splicing in cDNA-P and cDNA+P usually 0-10%

-> gene- and exon-specific exceptions with higher values

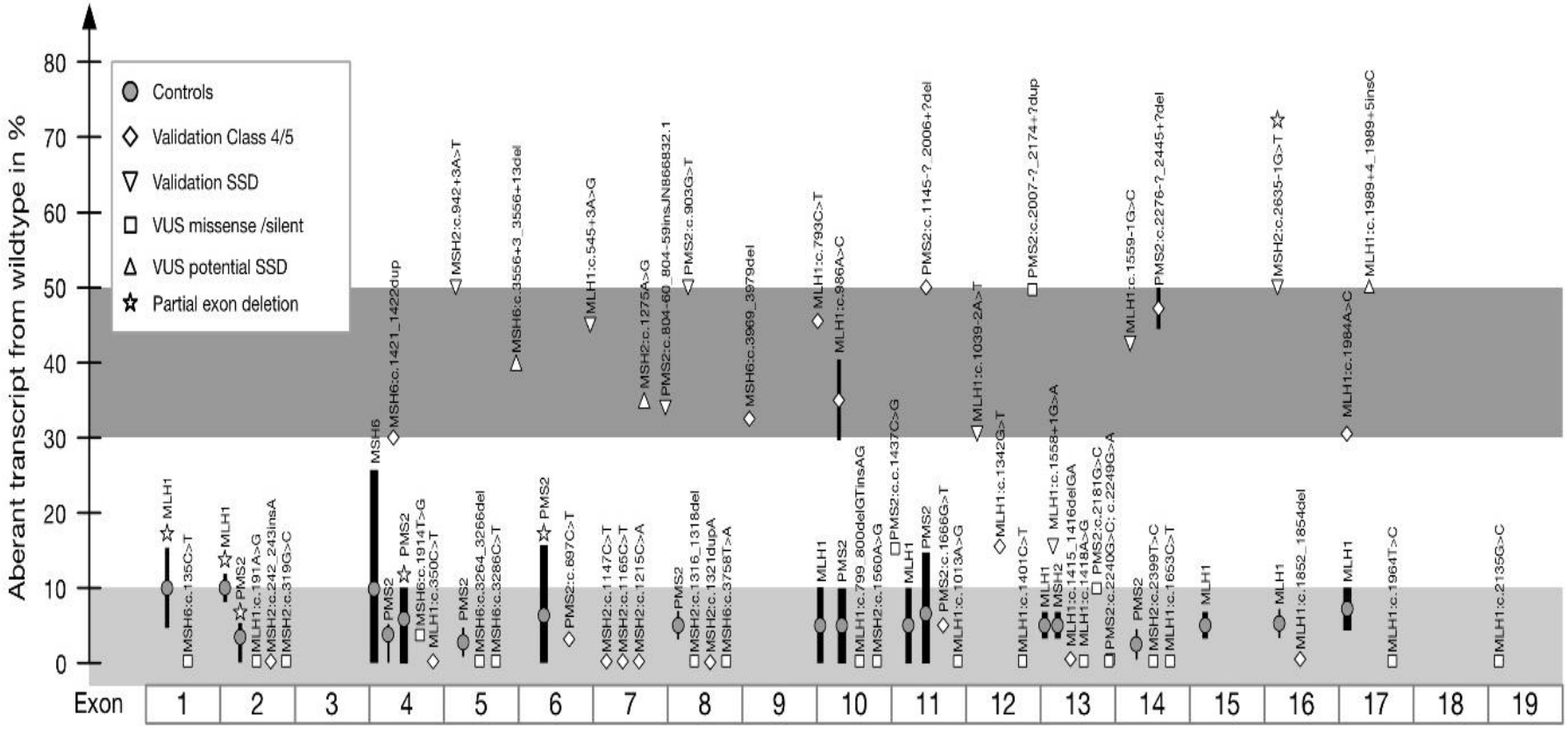
of up to 15% for *MLH1* exon 1q, *PMS2* exon 6p and exon 11,

of up to 25% for *MSH6* exon 4

Cut off definition

Allelic balalnce	**50% +/- 10%**
Allelic loss	**< 10%**
Normal splicing	**up to 10%, some exceptions**
Abberrant splicing	**above 30%**

Universal protocol used within the european mismathch repair consortium



Testing 5 Class 5 variants

5 variants predicted to affect one amino acid (putative missense/indel), class 4-5:

	***MLH1* class 4-5 variants**	**Effect on mRNA splicing**
	• c.793C>T p.(Arg265Cys)	• c.793C>T p.(Arg265Cys)
	• c.986A>C p.(His329Pro)	• c.986A>C p.(His329Pro)
	• c.1984A>C p.(Thr662Pro)	• c.1984A>C p.(Thr662Pro)
	• c.1852_1854del p.(Lys618del)	• c.1852_1854del p.(Lys618del)
	• c.350C>T p.(Thr117Met)	• c.350C>T p.(Thr117Met)
	bioinformatical prediction:	**experimental analysis:**
RNA analysis	**5 not predicted as splice defects**	**splice site defect: 2 complete, 1 partial**
protein function	**3 predicted deficiency**	**5 MMR deficiency**

Testing 25 class 3 variants

variant	cDNA result	re-classification
3 potential SSD	all splicing-defects	2x class 5 (complete SSD)
13 missense variants	all splice-neutral, biallelic	0, remain class 3 due to unclear
1 exon duplication	duplication verified	1x class 4 (funct. protein domain
4 synonymous variants	all splice-neutral, biallelic	4x class 2
4 VUS + pathogenic variant	all splice-neutral, biallelic	2x re-classification to class 2

-> re-classification of 36% of VUS

-> splicing defects 12%

Testing MMR deficient unsolved patients

26 „mutation-negative“ patients with unsolved IHC loss in CRC:

MLH1/PMS2 in 16 patients

PMS2 in 5 patients

MSH2/MSH6 in 2 patients

MSH6 in 3 patients

- **5/26 abnormal cDNA results (19%)**

cDNA result	**genomic cause**
MLH1 NMD-P, PTC+P	*MLH1* truncating variant (overseen)
MLH1 NMD-P, SSD oof+P	*MLH1* splice site variant (overseen)
PMS2 NMD-P, SSD ins oof+P	PMS2 partial intron inclusion
MLH1 allelic loss –P/+P	?
MLH1 allelic loss –P/+P	?

- **21/26 normal FLT = no germline deficiency in MMR gene analyzed**

Summary

- protocol for full-length cDNA analysis for the four MMR transcripts
- definition of cut-off values for allelic representation, normal and aberrant splicing
- testing the system on different variants

  missense variants (class 4-5) -> 60% splicing defects

  25 different types of VUS class 3 -> 12% splicing defects, 36% re-classified

- testing the system on 26 MMR deficient, unsolved patients

  19% with abnormal cDNA result = MMR-defect

  81% with normal cDNA results = no MMR-defect in germline

⇒ Transfer to NGS-bases RNA-analysis by using these data to calibrate the system and educate bio-IT systems

⇒ Creating a meaningfull algorithm for pathogenicity assessment

Thanks to all contributors

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cDNA analysis using NGS technologies

Next step:

Lab-project

Use the defined cut-offs to validate

automated, NGS-based cDNA analyses

IT-project

Improve skills of bio-IT

Classification of MMR gene variants

InSiGHT guidelines

Variant Interpretation Committe

class 1	2	3	4	5
non-pathogenic	probably non-pathogenic	VUS uncertain significance	probably pathogenic	pathogenic

- splice site variants
- intronic
- synonymous
- 5`/ 3`UTR
- exon duplication
- predicted missense variants
- ins/del of one amino acid

→

- mRNA splicing/expression
- ↓
- functional protein assays

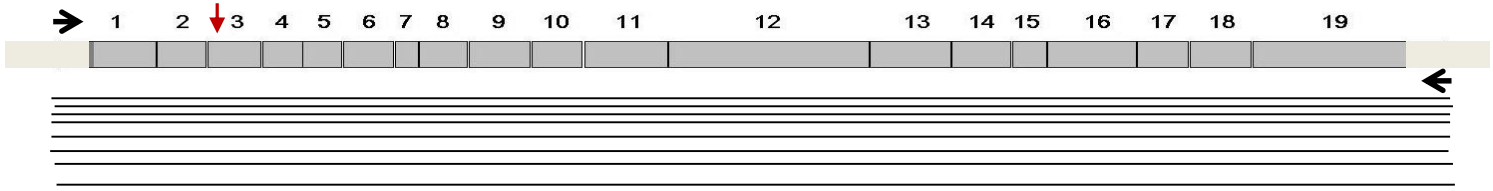
=> RNA based analysis for pathogenicity assessment of sequence variants

↓

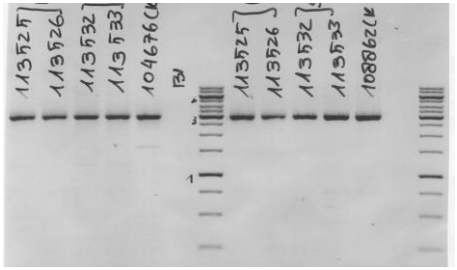
Thompson et al. Nat Genet. 2014

Full-length transcript amplification and sequencing

- LR-PCR from cDNA: primers in first and last exon



- agarose gel electrophoresis



MLH1 ~ 2.5 kb

MSH2 ~ 3 kb

MSH6 ~ 4.2 kb

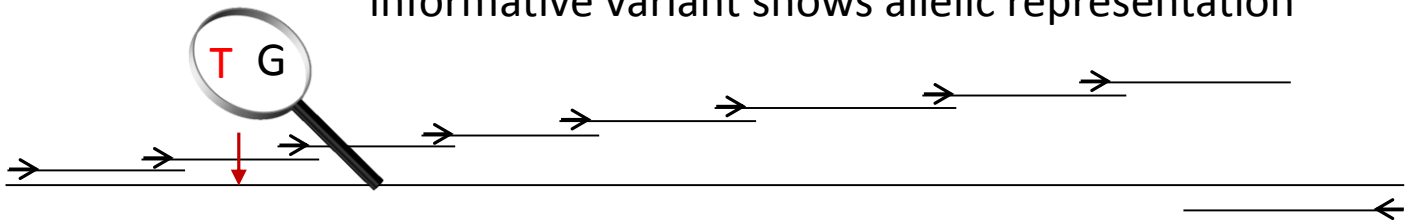
PMS2 ~ 2.8 kb

successful FLT amplification 98% PBL cDNA

- Sanger sequencing

complete sequence analysis in overlapping reads of one orientation

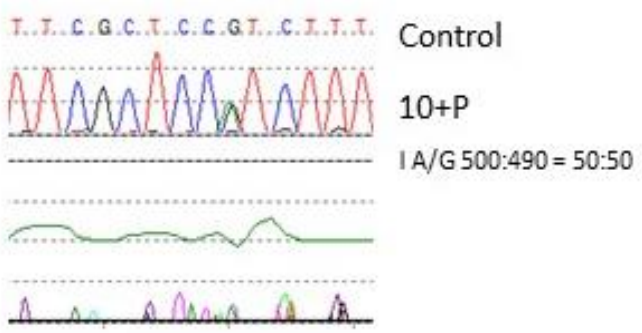
informative variant shows allelic representation

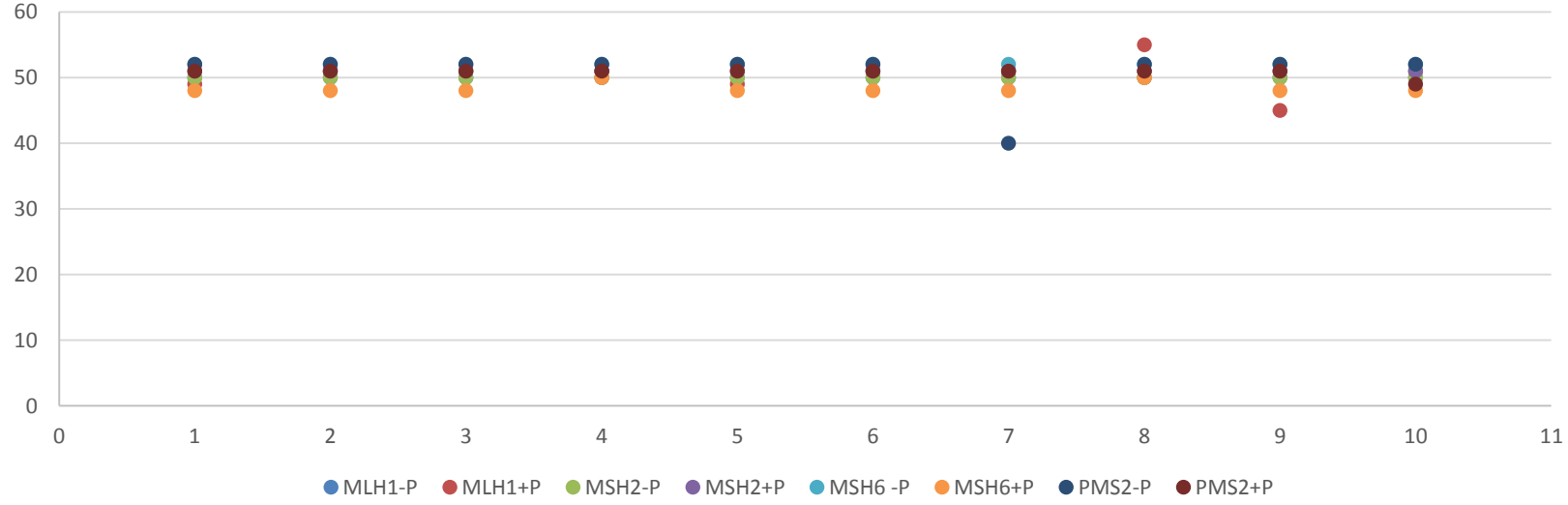


Allelic representation in controls

peak heights in sequence electropherogram -> relative intensities in %

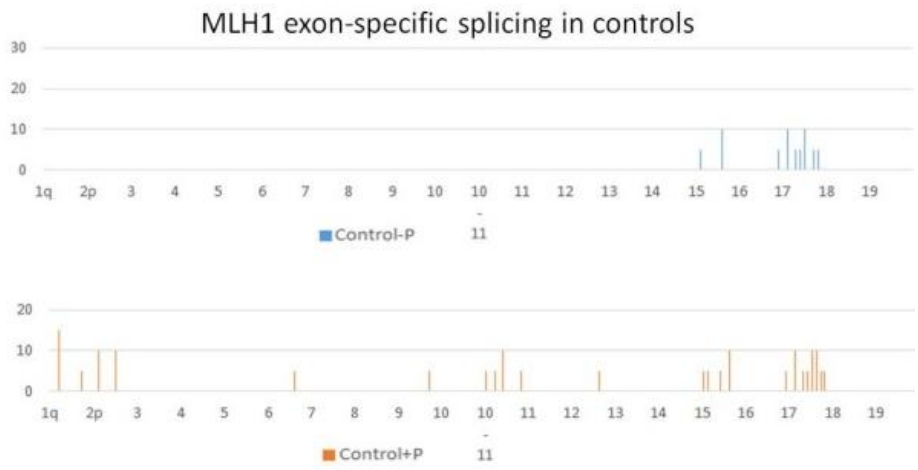
results compared between cDNA-P and cDNA+P

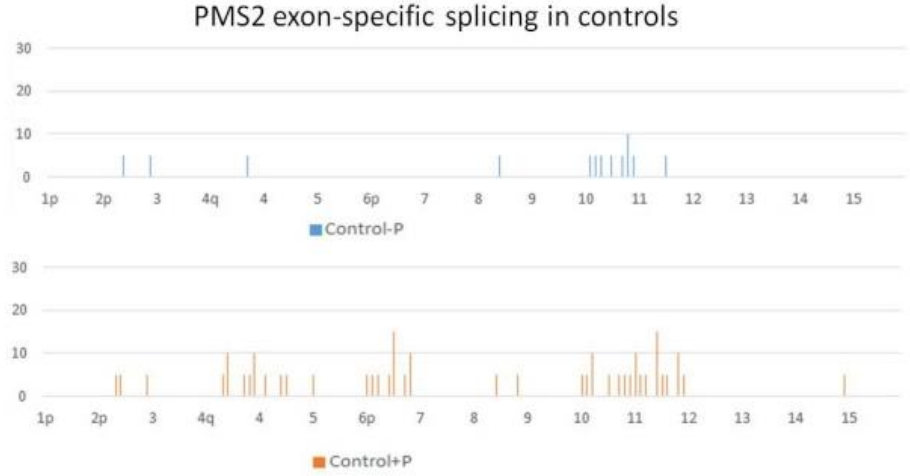




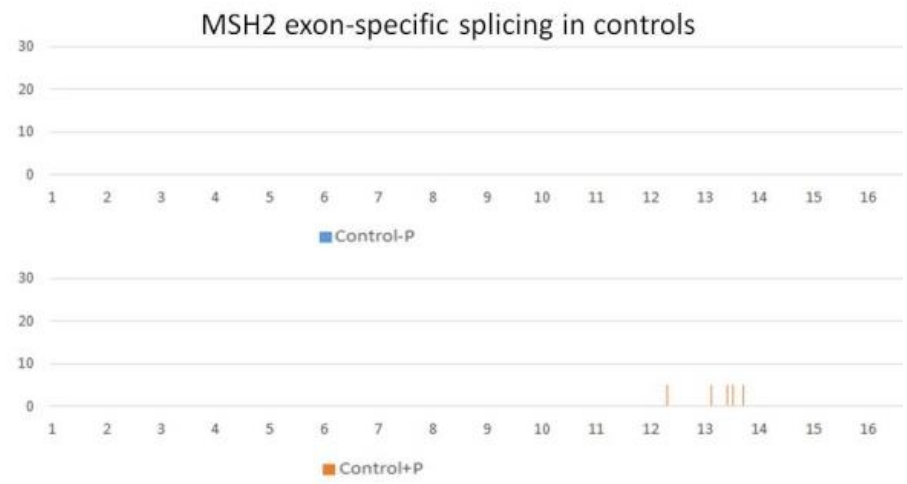
Alternative splicing in controls

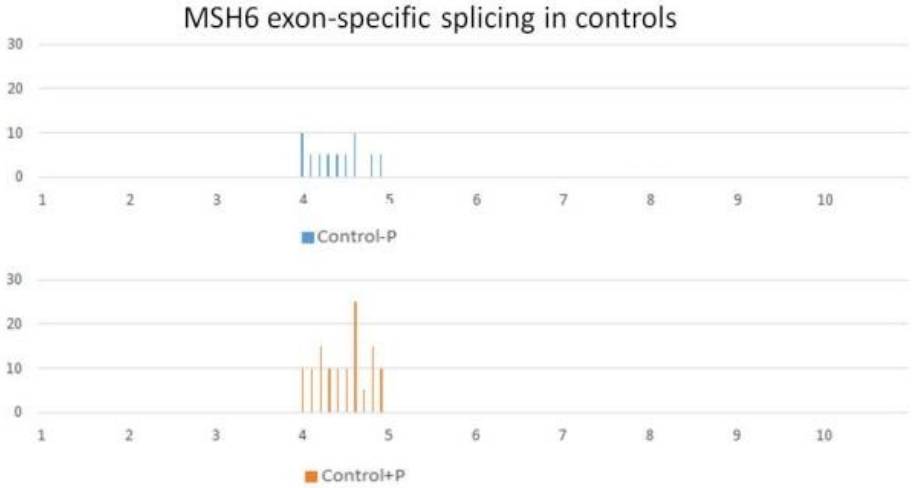
10 MMR-proficient controls for each MMR full-length transcript in cDNA –P and +P





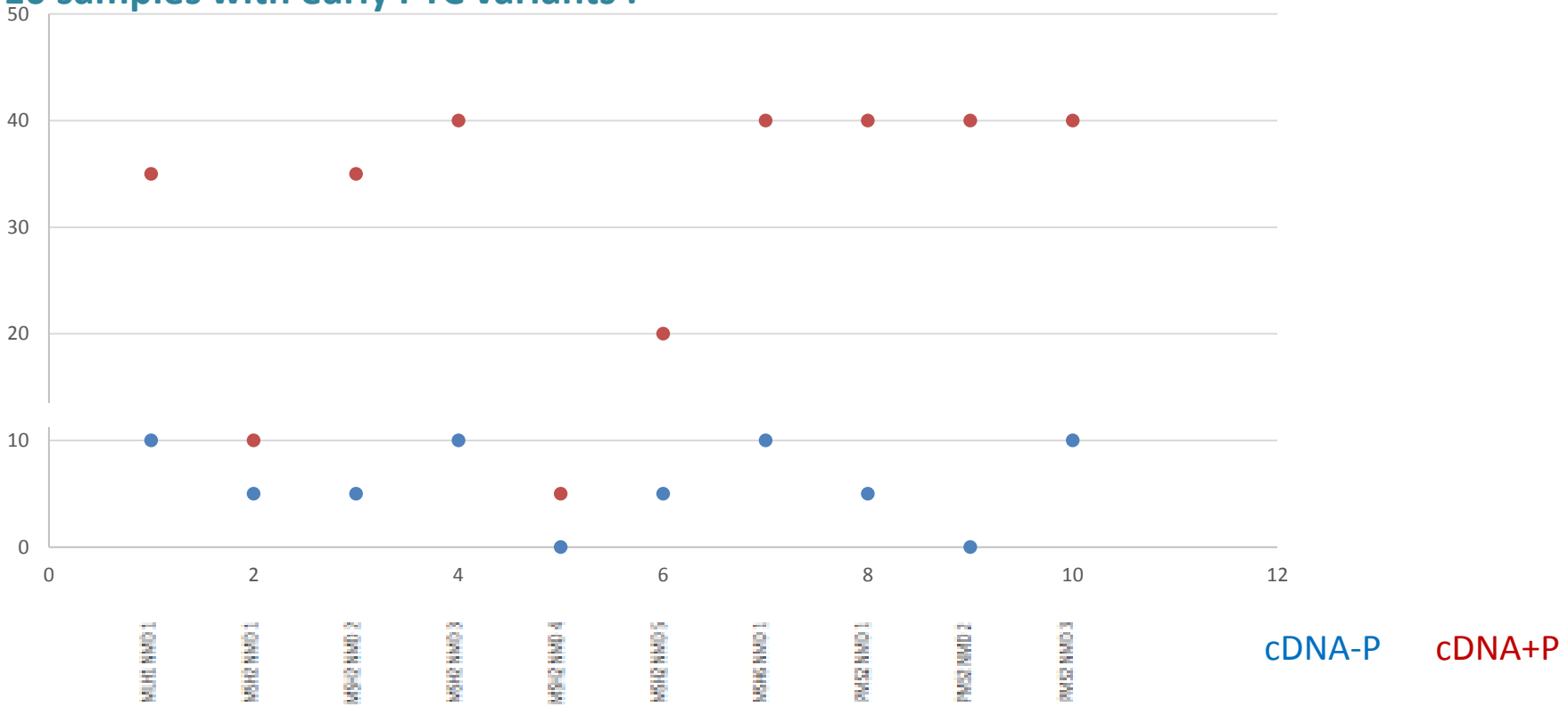
cDNA-P cDNA+P





NMD-inhibition in controls

10 samples with early PTC variants :



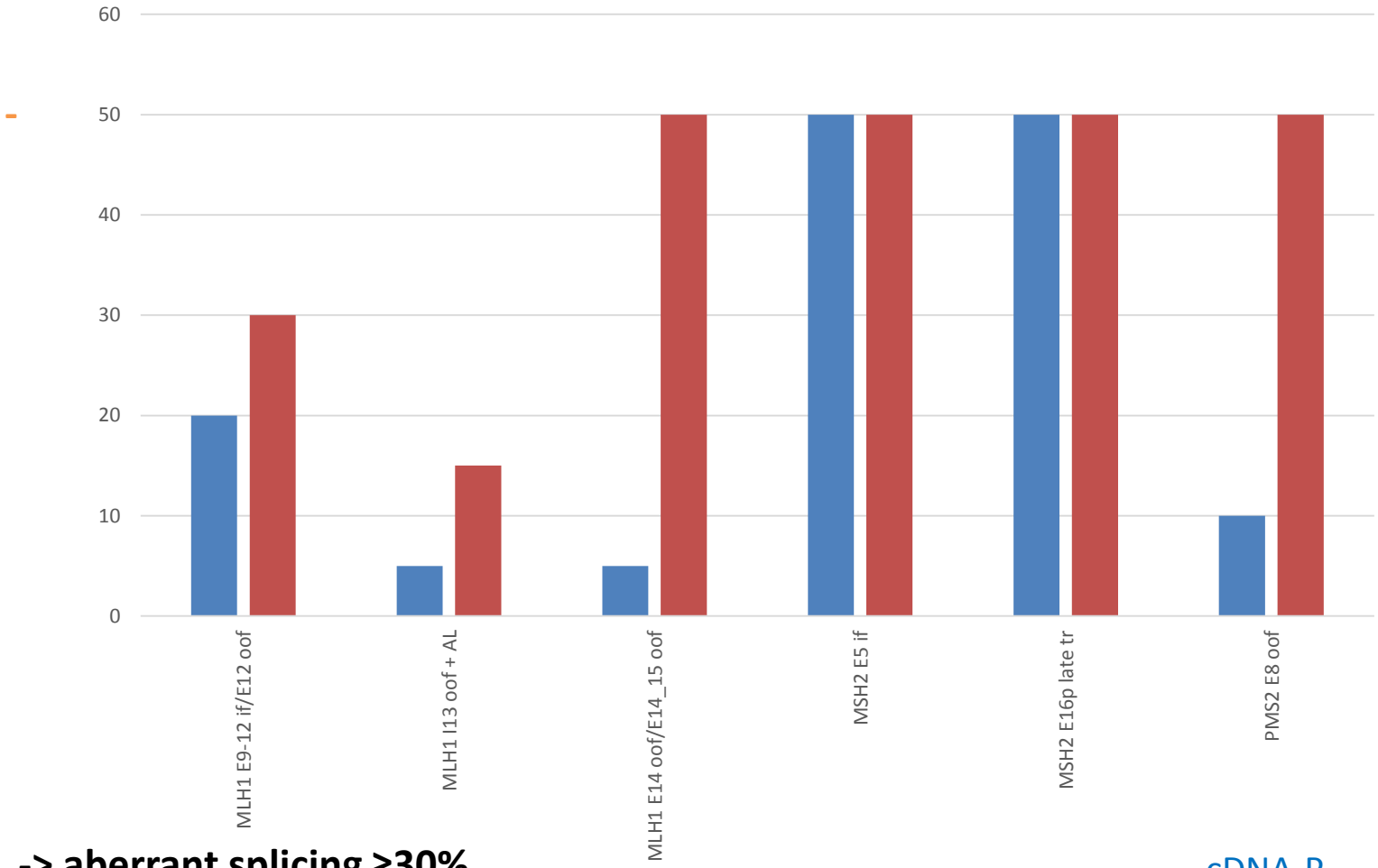
-> NMD = allelic loss = AR of 0-10%

-> NMD-block usually works, but informative variant is needed for calculation of AR

-> splicing in PTC transcripts may be enhanced (15% in 1/10 cDNA+P samples)

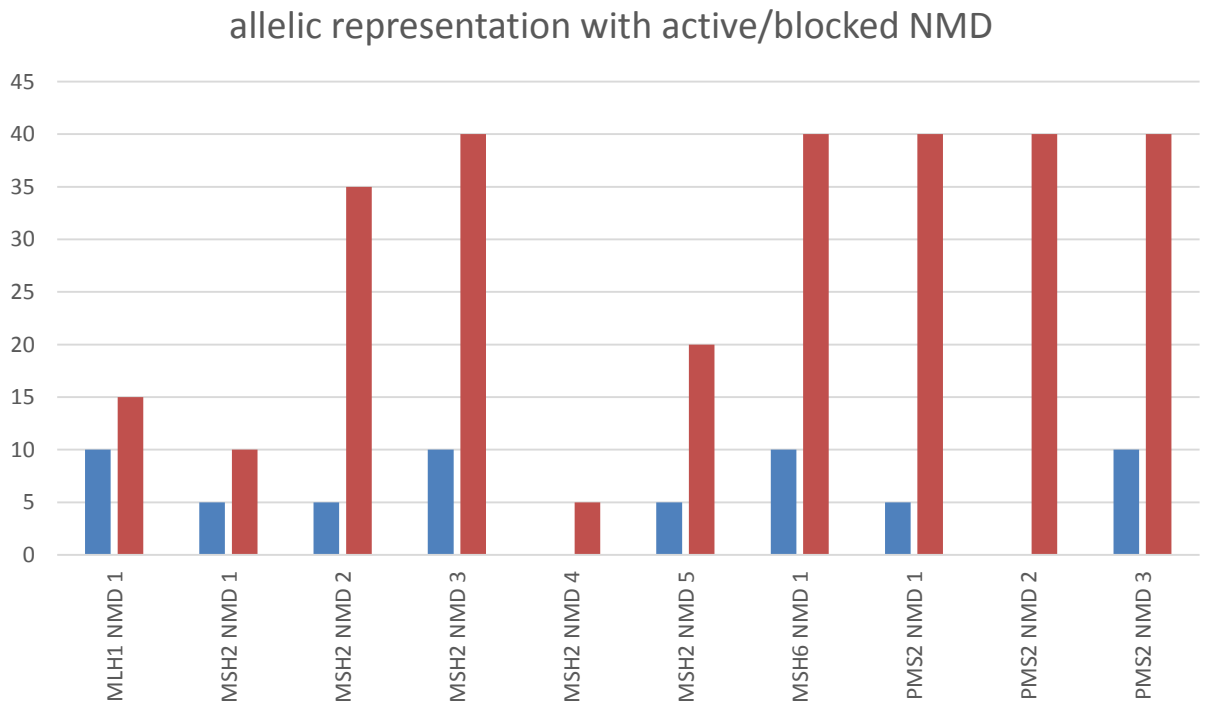
Definition of aberrant splicing

6 variants of class 4-5 predicted as splice site defect/exon skipping:



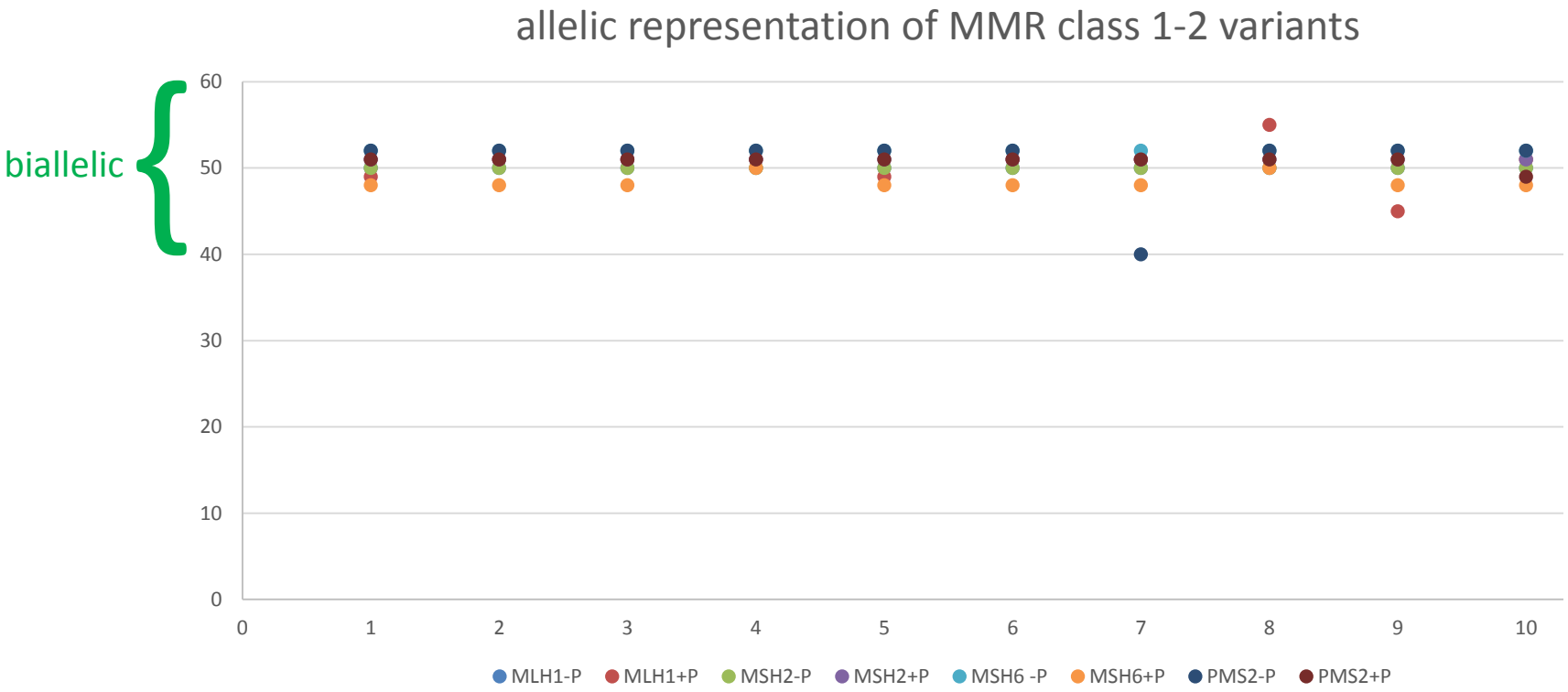
-> aberrant splicing ≥30%

-> Splicing above alternative pattern/10% <30% = unclear, needs further investigation or AR


allelic representation with active/blocked NMD
45
40
35
30
25
20
15
10
5
0
MLH1 NMD 1
MSH2 NMD 1
MSH2 NMD 2
MSH2 NMD 3
MSH2 NMD 4
MSH2 NMD 5
MSH6 NMD 1
PMS2 NMD 1
PMS2 NMD 2
PMS2 NMD 3

Allelic representation of benign variants in controls

10 MMR-proficient controls, informative class 1-2 variant for each MMR gene



-> biallelic= allelic representation of 50% +/-10%

Definition of aberrant splicing

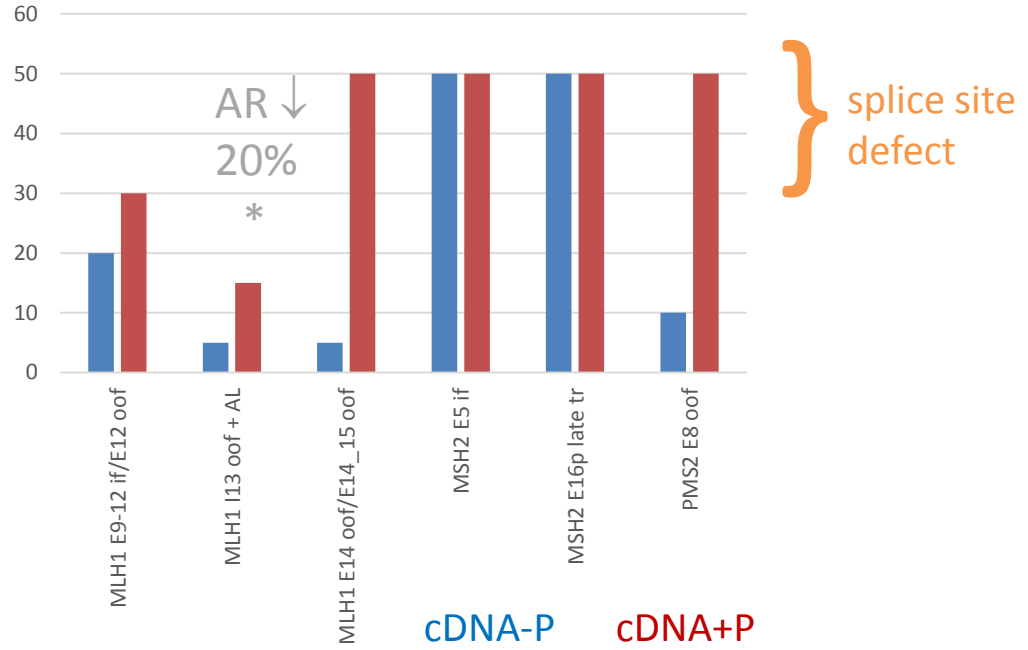
6 variants of class 4-5 predicted as splice site defect/exon skipping:

in-frame SSD

- 50±10% intensity in cDNA-P/+P

out-of-frame SSD -> NMD

- 0-20% intensity in cDNA-P (allelic loss)
- 30-40% intensity in cDNA+P
- exception: 15% SSD + allelic reduction 20%



-> aberrant splicing ≥30%

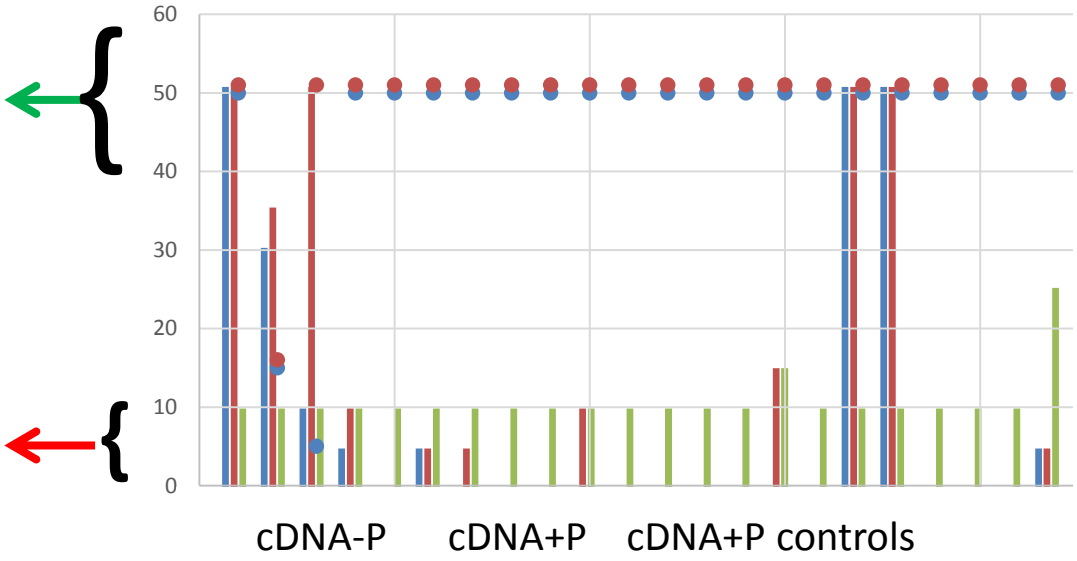
-> Splicing above alternative pattern/10% <30% = unclear, needs further investigation or AR

InSiGHT: demonstrate allele-specific splicing defect in absence of wildtype transcript

partial splicing defects = unclear clinical relevance, class 3

Testing VUs

Patient samples with 25 different types of class 3 MMR variants:



splicing in exon of VUS:

splice site defect

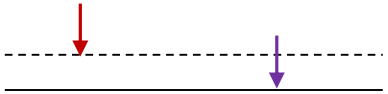
alternative splicing

Pros and cons of full-length transcript analysis

- universal protocol for coding MMR variants to investigate their effect on splicing

  allows re-classification of VUS

  determines the allelic status *in cis/trans* of VUS to a pathogenic variant

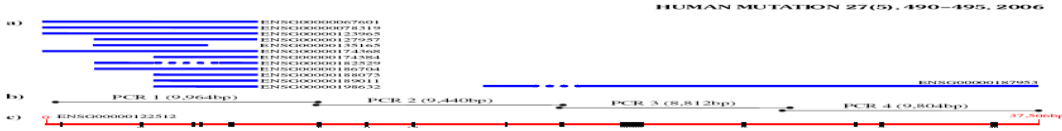


- selection for wildtype FLT

  allows detection of allelic loss by informative variant (transcript integrity test)

  excludes irrelevant transcripts using alternative first exons/poly-adenylation

- discrimination against *PMS2* pseudogenes



- needs informative variant for significant cDNA result (not in all samples, especially *MSH2*)
- semi-quantitative assesment of allelic representation and splicing
- requires good RNA quality for FLT amplification
- labour-intensive analysis with hands-on-time