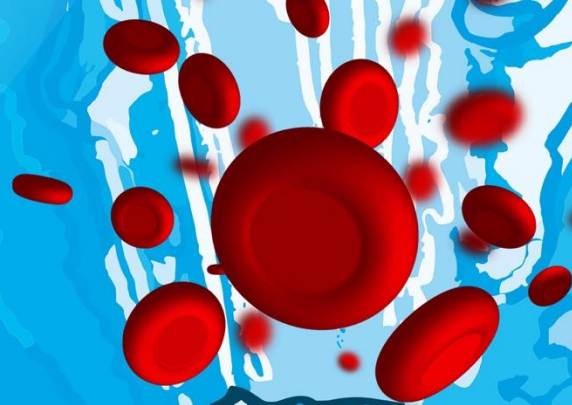




Meeting del CLUB DEL

## Oltre la genetica: integrazione con le scienze omiche

Roberta Russo, PhD

Associate Professor of Medical Genetics  
Dept Molecular Medicine and Medical Biotechnology  
University Federico II  
CEINGE Biotechnologie Avanzate Franco Salvatore  
Naples, Italy



# Globulo Rosso 2026

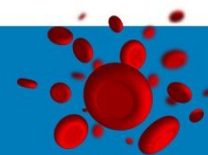
NAPOLI

28-29 SETTEMBRE 2026

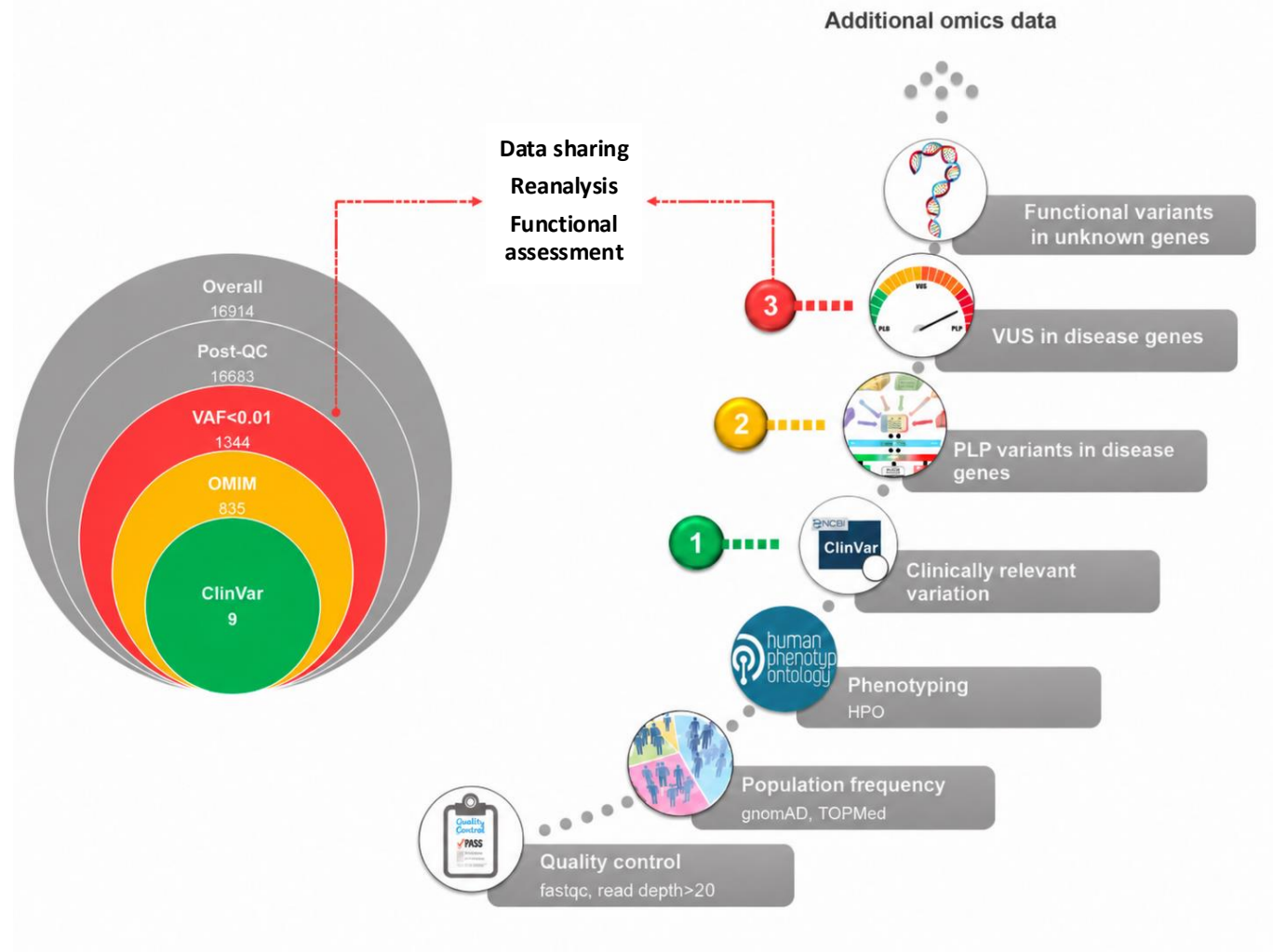
Complesso dei SS. Marcellino e Festo

## Disclosures

| Company name | Research support | Employee | Consultant | Stockholder | Speakers bureau | Advisory board | Other |
|--------------|------------------|----------|------------|-------------|-----------------|----------------|-------|
|              |                  |          |            |             |                 |                |       |
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|              |                  |          |            |             |                 |                |       |



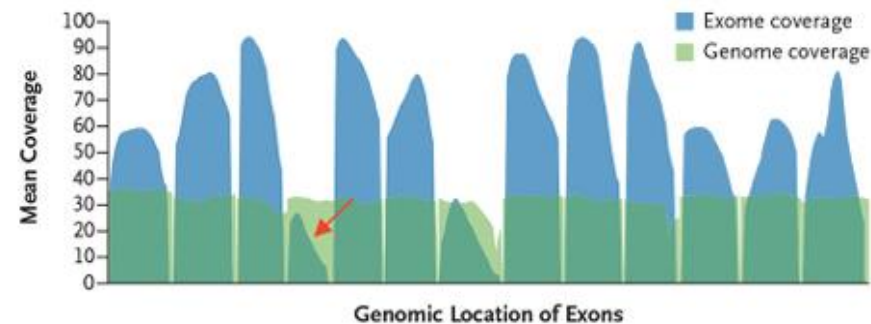
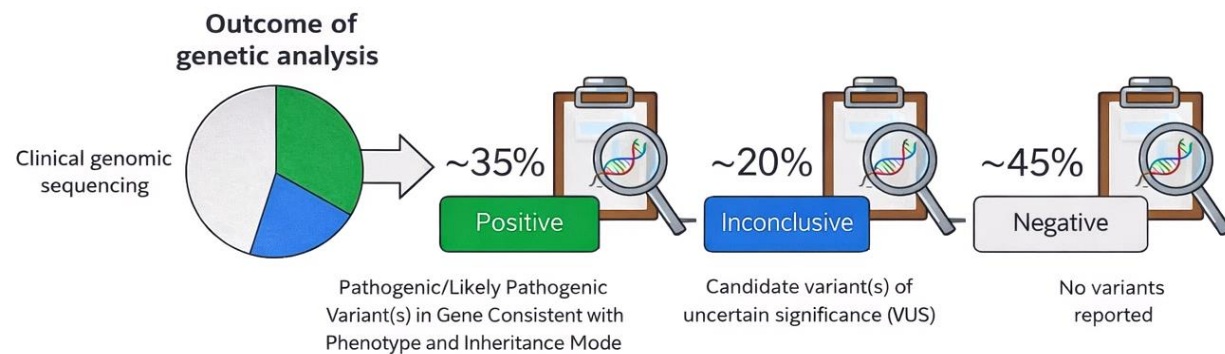
# Different diagnostic situations in current genetic testing



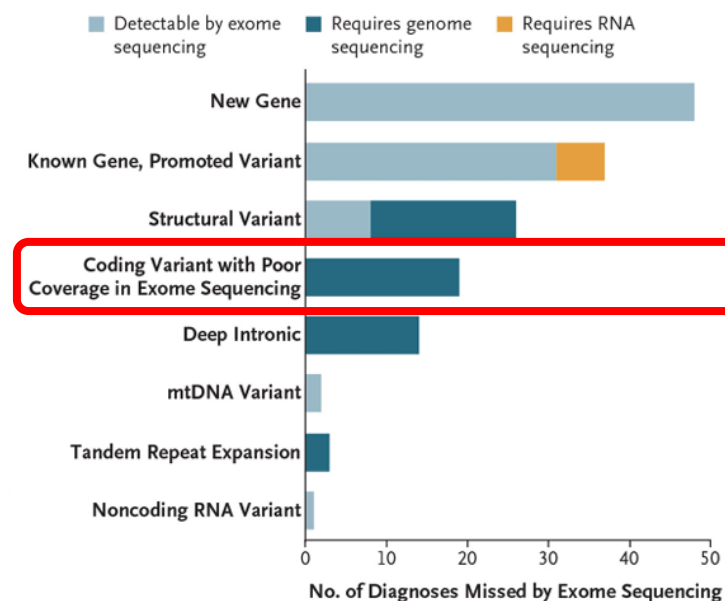
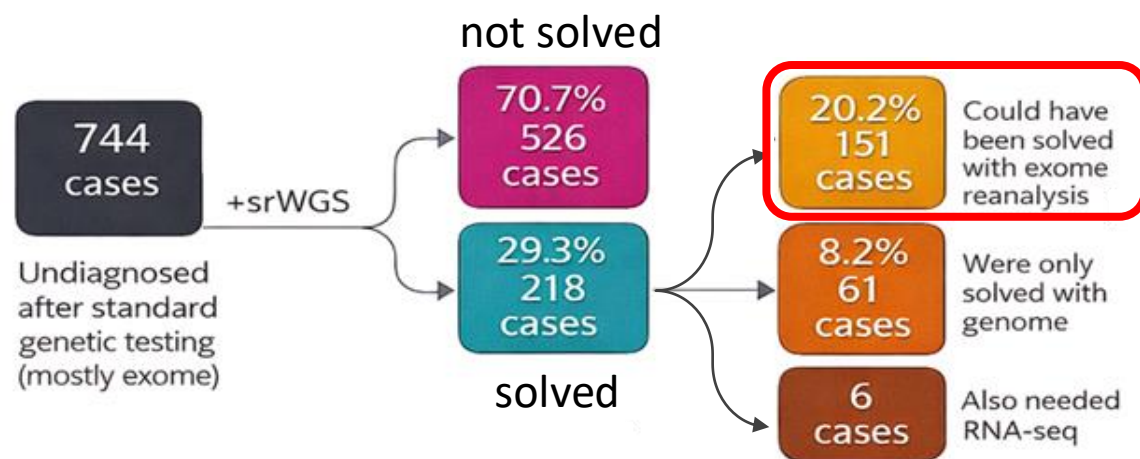
1. Identification of P/LP variants with well-established association with disease phenotype
  2. Identification of new P/LP variants in genes with known association with the phenotype
  3. Identification of **variants of unknown significance** in genes with known association with the phenotype
- ✓ A fourth case should be considered, i.e., the identification of functional variants in genes with unknown association with the phenotype

Adapted from Licata L, et al. *Frontiers in Molecular Biosciences* 2023

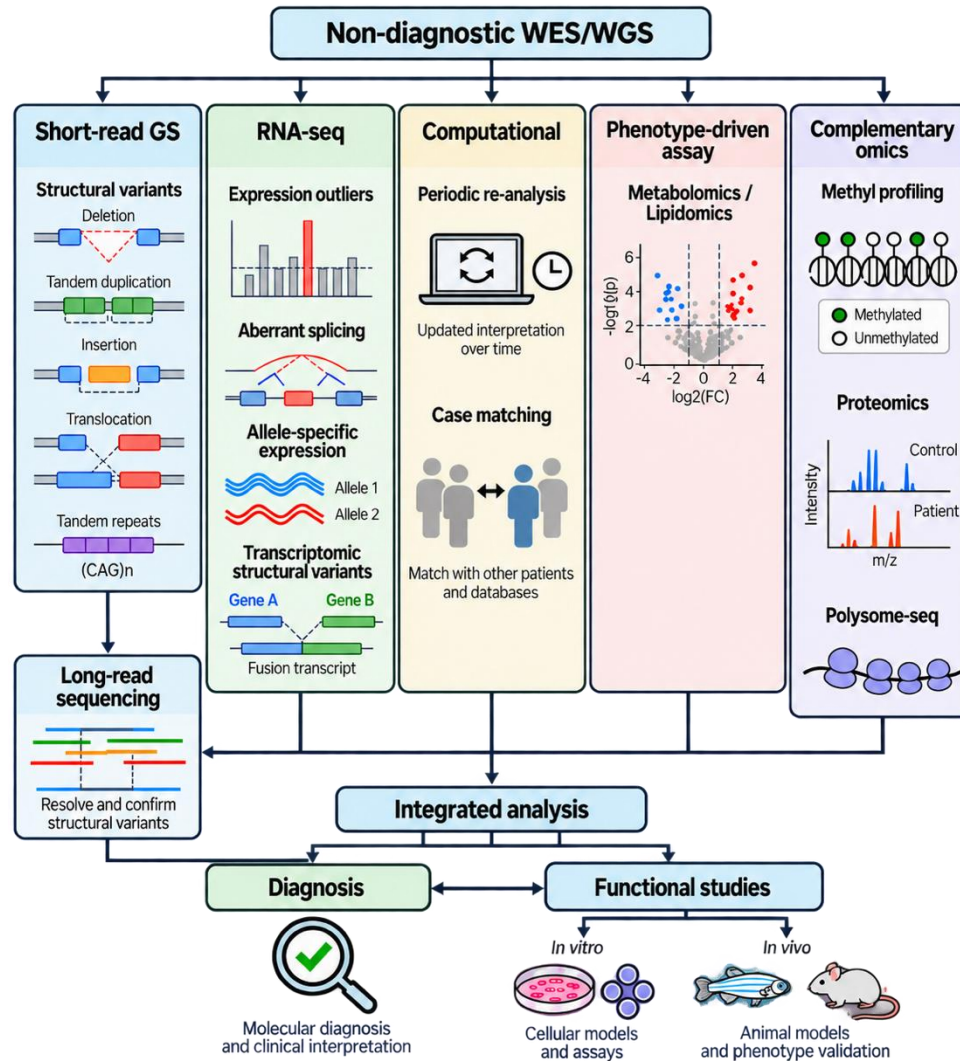
# NGS<sub>(short-read)</sub> diagnostic yield



## ✓ WGS after WES



Wojcik MH, et al. N Engl J Med. 2024



✓ There are several technologies and methods to improve the diagnosis of hereditary diseases when WES/WGS are unrevealing

1. Computational approaches → periodic re-analysis, CNV detection
2. Long-read sequencing → complex or structural variants
3. RNA-seq
4. Methyl profiling
5. Proteomics
6. Metabolomics

# Main unmet need: assigning clinical significance to VUS

❑ A VUS represents uncertainty, not a diagnosis

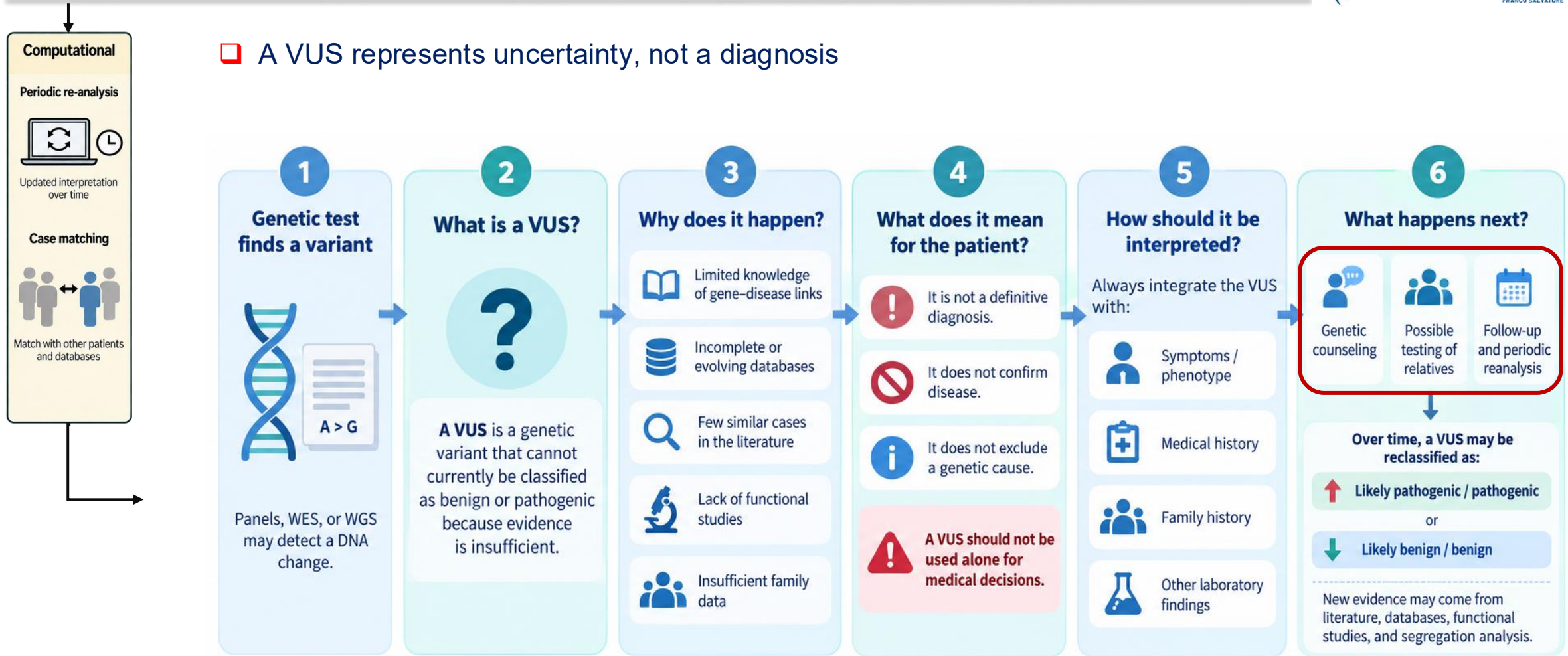
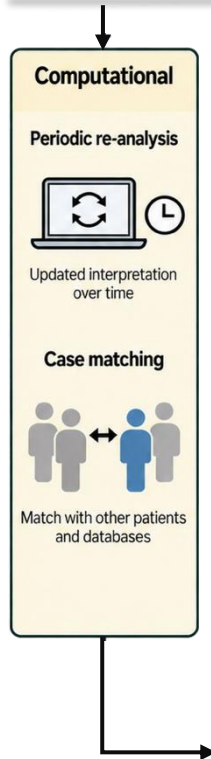


Figure created with ChatGPT (OpenAI) from author-provided scientific content; final content reviewed by the author

# Why reanalysis matters in variant interpretation



- ❑ Data reanalysis is important because
- Variant classification is dynamic
  - The phenotype may change over time
  - Hot-VUS deserve closer attention

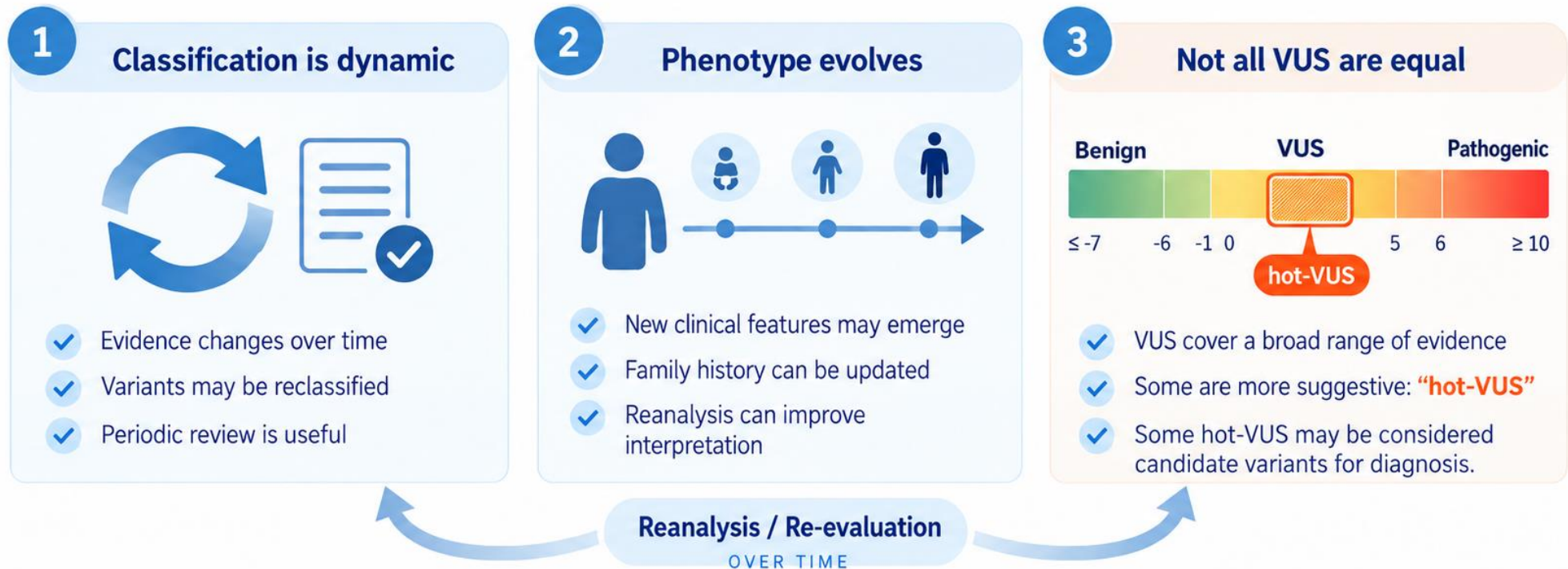
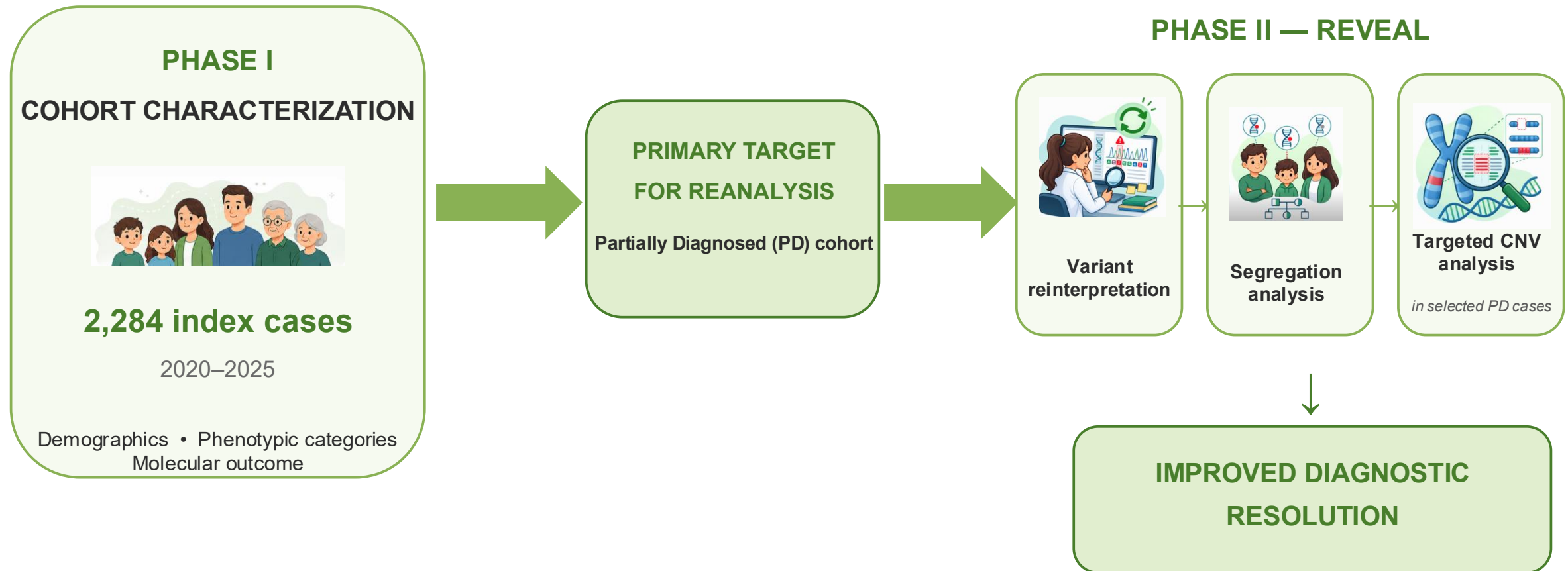


Figure created with ChatGPT (OpenAI) from author-provided scientific content; final content reviewed by the author

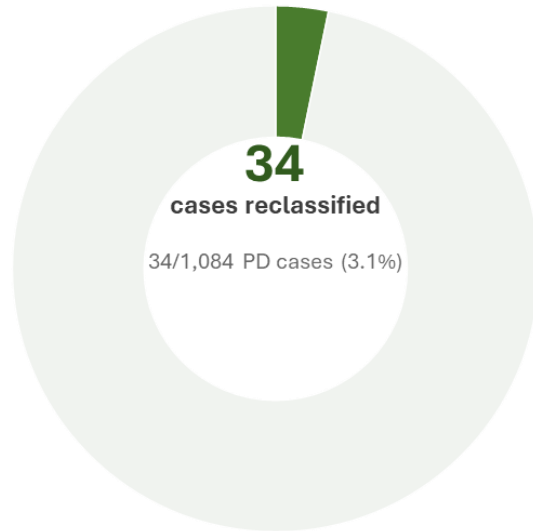
- Clinical exome sequencing (CES) cohort → to establish a standardized reanalysis workflow for partially diagnosed patients



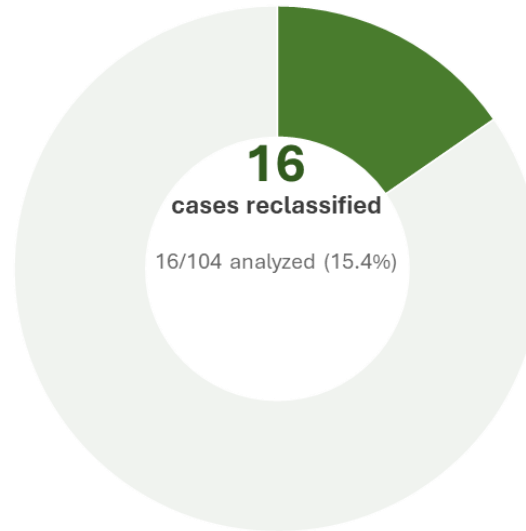
Manno M, et al. In preparation (2026)

# Systematic reanalysis of NGS data (clinical exome seq)

## Case-level analysis



Variant reassessment



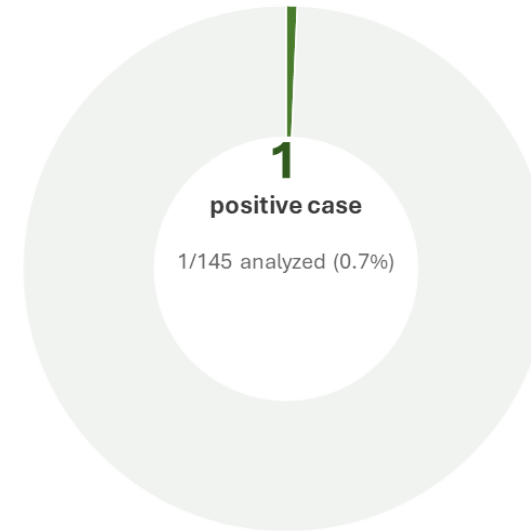
Segregation analysis

ACMG evidence from segregation

PS2: 9 – de novo occurrence

PM3: 7 – variants confirmed in trans

2/16 cases also required variant reassessment



Targeted CNV analysis

↑ 2,3%  
diagnostic rate



**51 additional molecular diagnoses**

34 variant reassessment • 16 segregation • 1 targeted CNV

Manno M, et al. In preparation (2026)

- ❑ Systematic review and meta-analysis
- ❑ NGS resolves nearly half of suspected CHA cases, with the highest yield in familial and red cell membrane disorder cohorts

10

Included studies

885

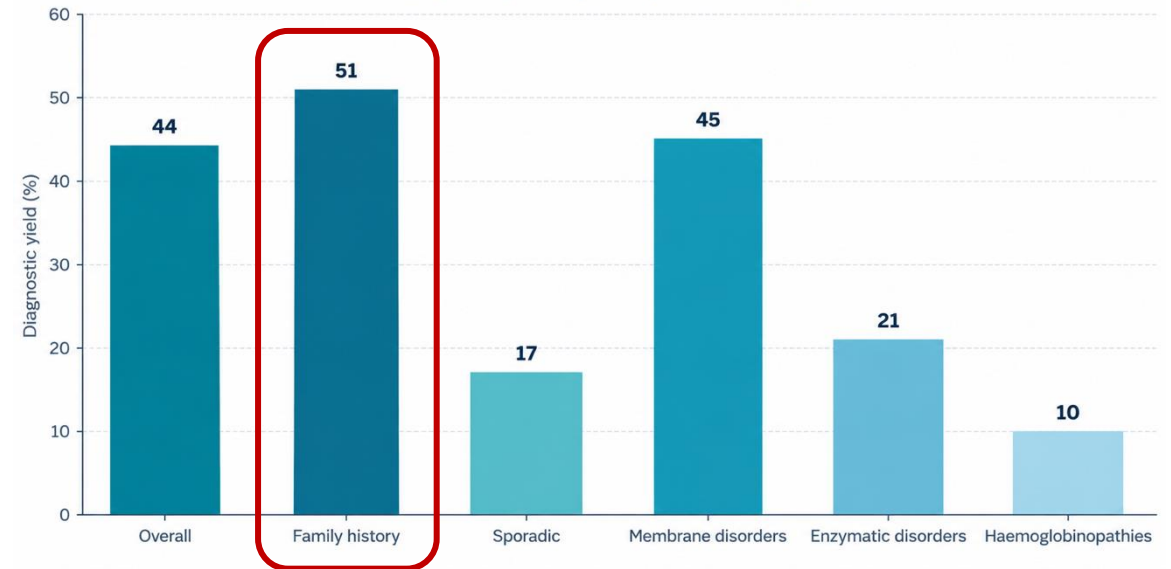
Patients

44.3%

Pooled positive detection rate  
95% CI 32.4–56.3

## NGS Diagnostic Yield in Congenital Haemolytic Anaemia

Overall, by family history, and by disease subtype



Xie & Zhong, *Clinical and Experimental Medicine* 2025

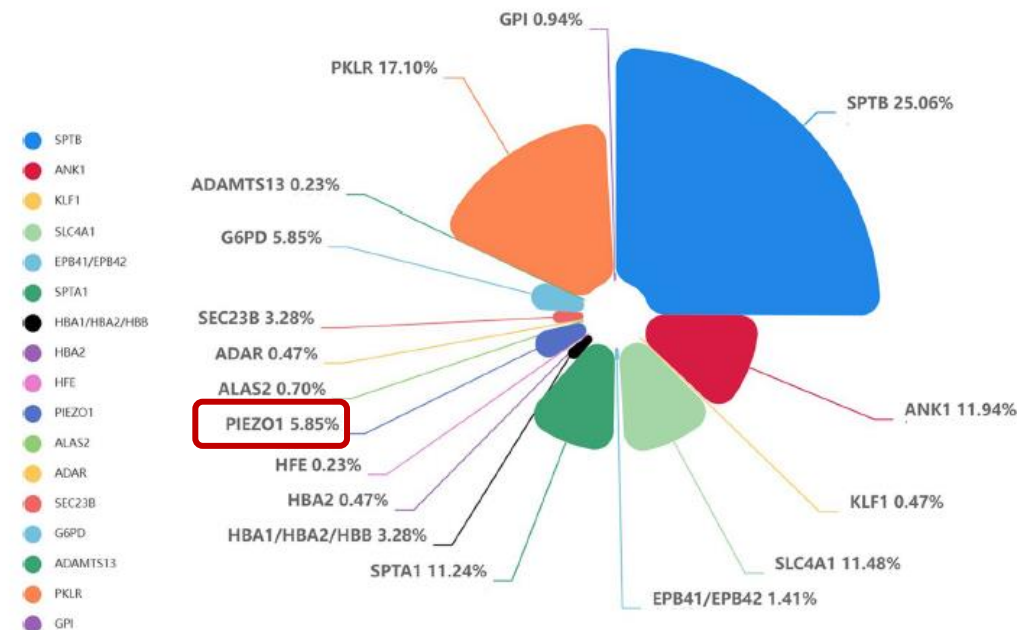
# Next-generation sequencing in congenital hemolytic anemia

❑ Clinical implications: prioritize NGS around core genes

**76.82%**

of positive variants mapped to five genes

|          |        |
|----------|--------|
| ● SPTB   | 25.06% |
| ● PKLR   | 17.10% |
| ● ANK1   | 11.94% |
| ● SLC4A1 | 11.48% |
| ● SPTA1  | 11.24% |



Suspected CHA

NGS panel / WES

Molecular diagnosis

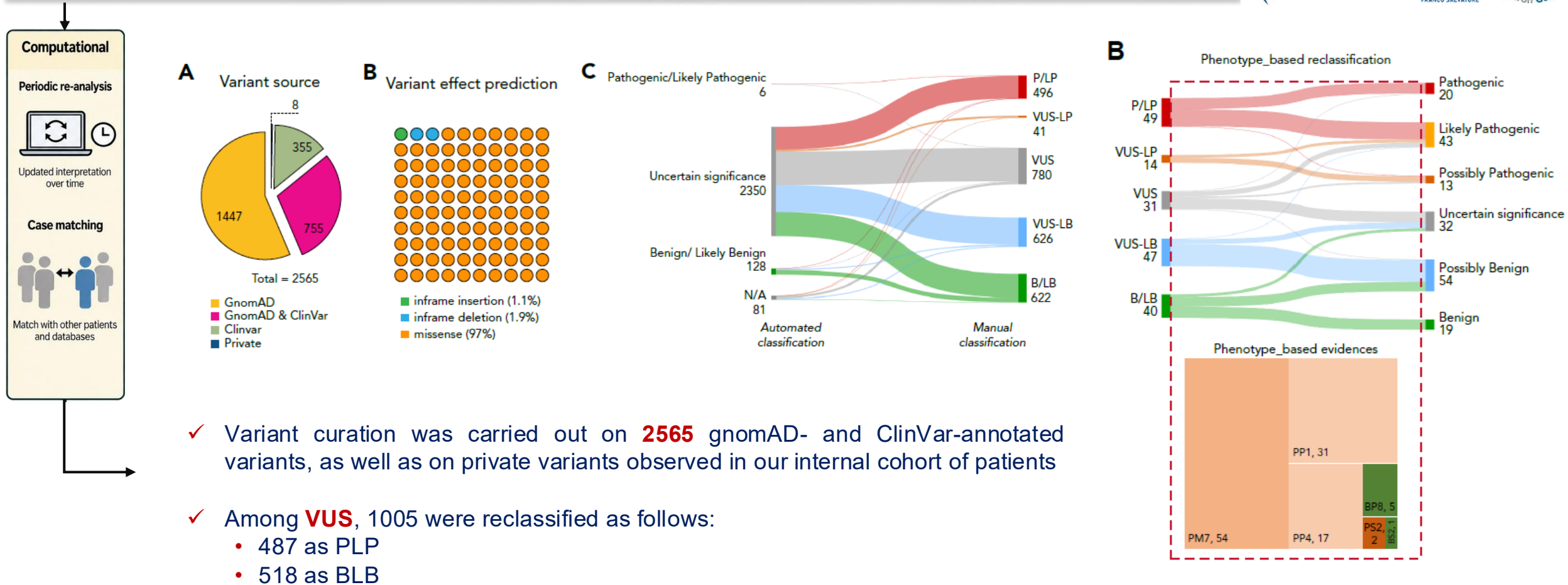
Care & counseling

## Caveat

Rare subtypes and WGS-vs-WES comparisons remain underpowered

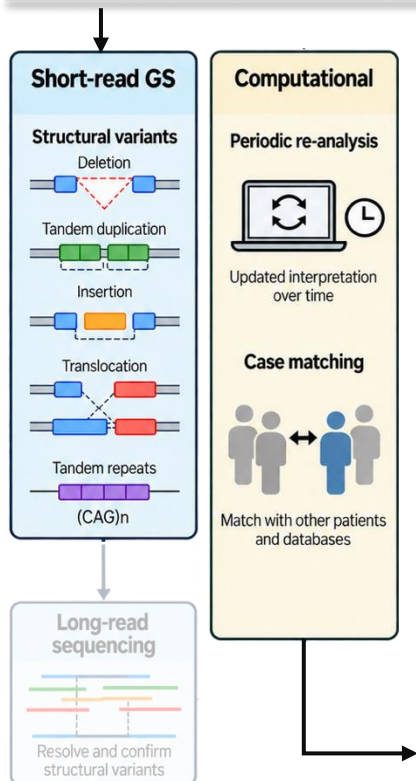
Xie & Zhong, Clinical and Experimental Medicine 2025

# Refined classification of *PIEZO1* variants



- ✓ Variant curation was carried out on **2565** gnomAD- and ClinVar-annotated variants, as well as on private variants observed in our internal cohort of patients
- ✓ Among **VUS**, 1005 were reclassified as follows:
  - 487 as PLP
  - 518 as BLB
- ✓ **Phenotype-based reclassification** of 39 variants from 56 patients. Among them, **13 VUS** were classified as diagnostic pathogenic variants (10 P/LP and 3 possibly pathogenic)

# CNV analysis from NGS<sub>(short-read)</sub> data



- ❑ Copy-number variations (CNVs) contribute to the pathogenesis of **up to 15% of rare monogenic cases**
- ❑ **Coverage uniformity** determines CNV confidence



## WGS

Whole-genome sequencing

- PCR-free, low bias, uniform genome-wide coverage
- Best for accurate CNV calling and breakpoint detection

✓ **Best overall choice**

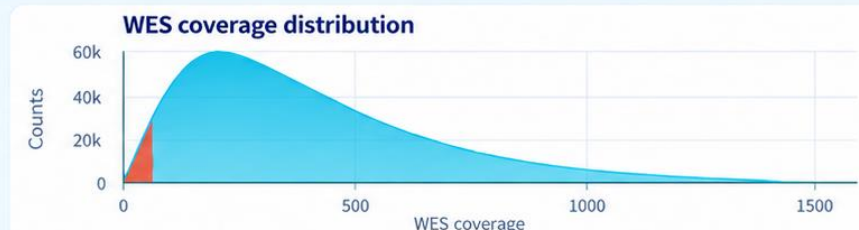


## WES

Whole-exome sequencing

- Exon-focused, uneven coverage in intronic/non-coding regions
- WES-CNV can add ~6–18% diagnostic yield

! **Useful, but limited for single-exon CNVs**

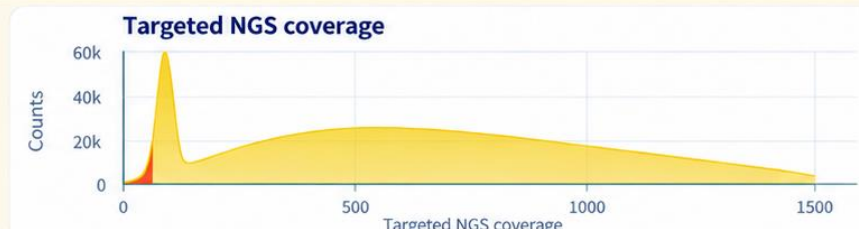


## Targeted NGS panel

Targeted sequencing (gene panels)

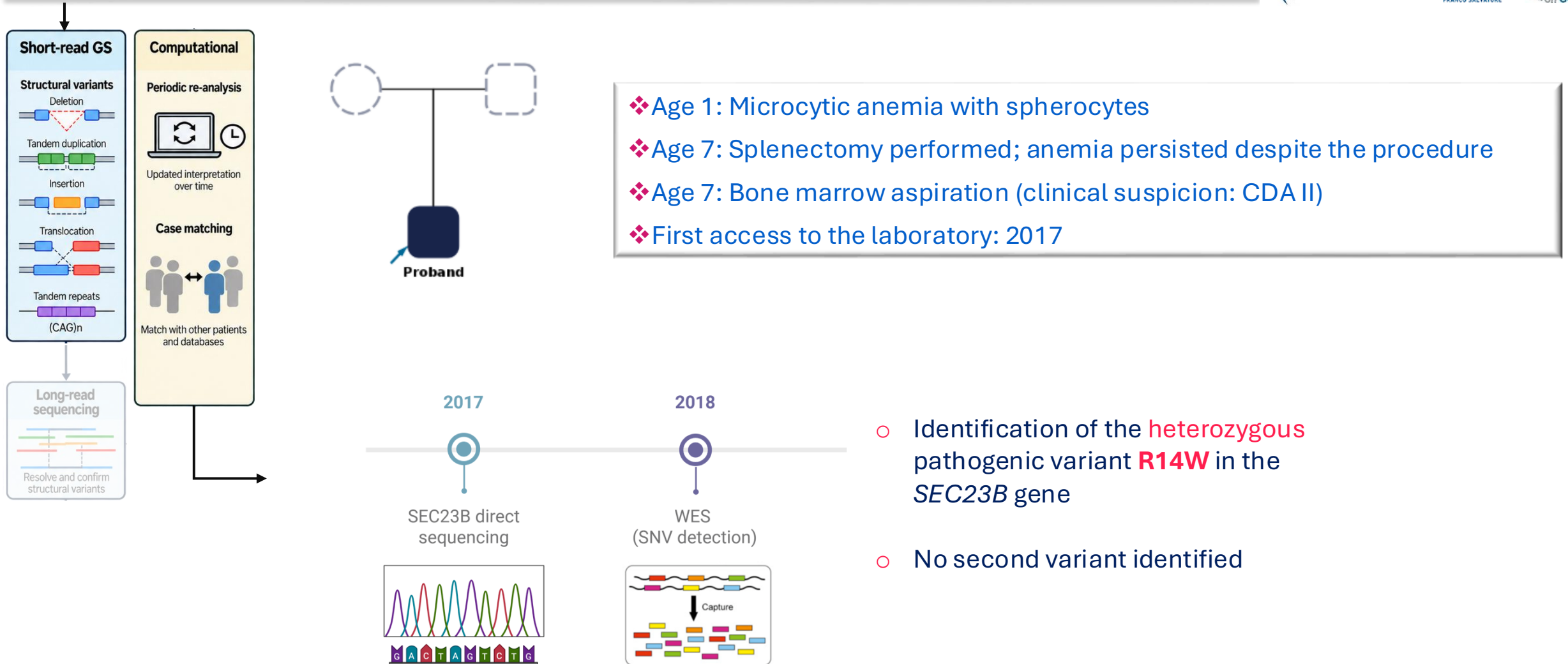
- Narrow capture and irregular coverage
- Higher false-positive / false-negative risk

! **Needs CNV-aware pipeline + orthogonal validation**

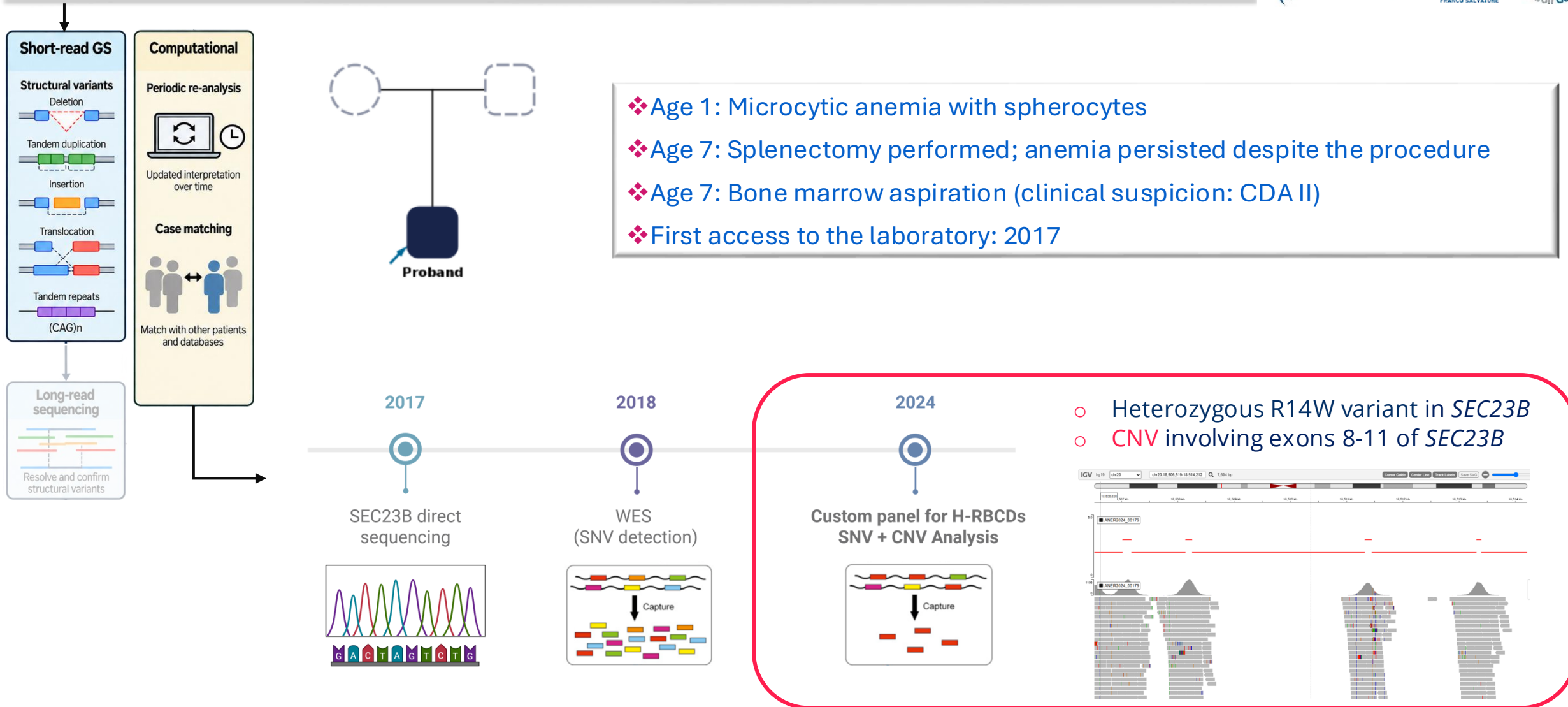


Trudsø LC, et al. PLoS One. 2020; Royer-Bertrand B, et al. Genes (Basel). 2021; Louw N. Front Genet. 2023

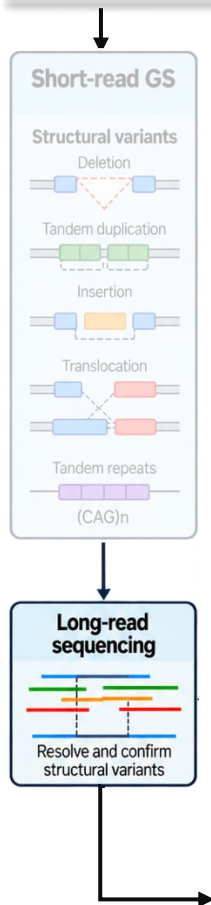
# CNV analysis from NGS<sub>(short-read)</sub> data: an example



# CNV analysis from NGS<sub>(short-read)</sub> data: an example



# Long-read sequencing (LRS): same genes, longer answers



## ☐ Detectable variant types

- ✓ Structural variants (SVs)
  - Large insertions/deletions
  - Inversions
  - Translocations
  - Complex rearrangements
- ✓ Repeat expansions
  - Tandem repeat disorders
- ✓ Copy number variants (CNVs)
- ✓ Variants in highly repetitive or GC-rich regions
- ✓ Phasing of variants
  - Haplotype reconstruction
  - Allele-specific effects
- ✓ Epigenetic modifications
  - DNA methylation profiling

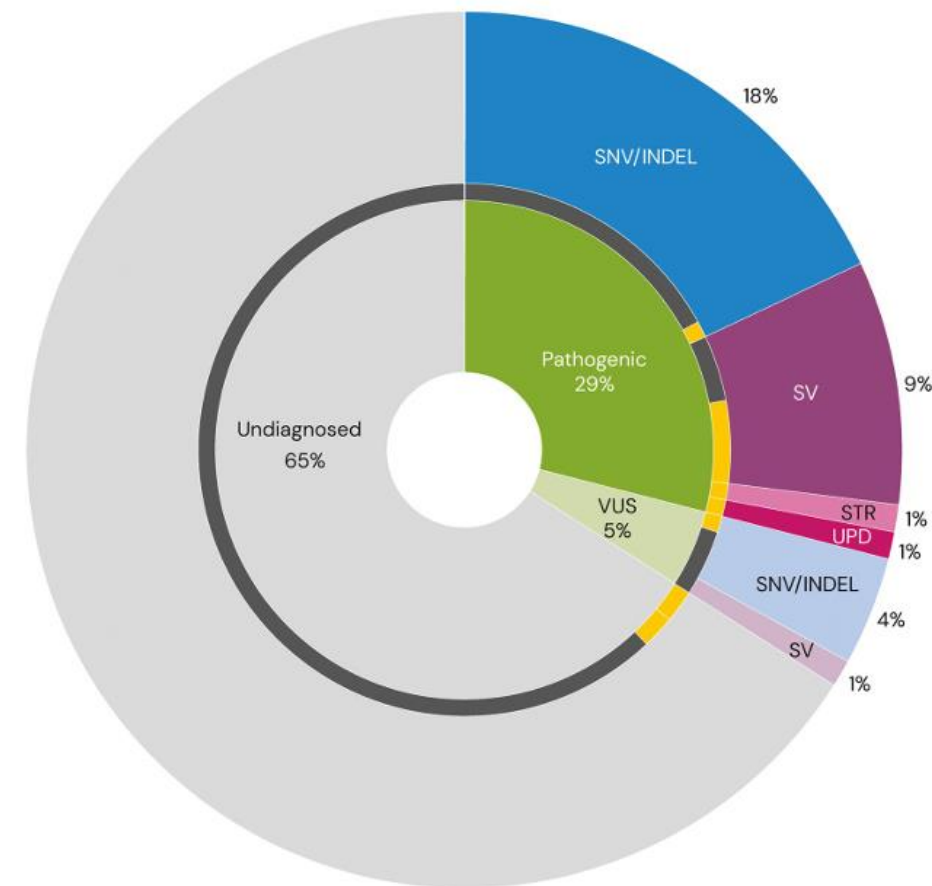
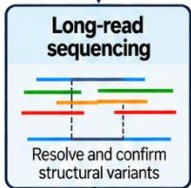
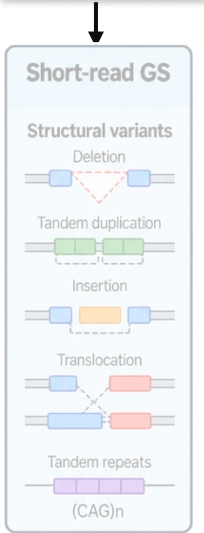


Figure created with ChatGPT (OpenAI) from author-provided scientific content; final content reviewed by the author

Ek et al. Genome Medicine 2026

# Long-read sequencing (LRS): same genes, longer answers



- ✓ Main indications for LRS in the diagnosis of hereditary red blood cell disorders
  - Genome-wide LRS enables the detection of sequence variants as well as
  - DNA methylation status
  - High sequence similarity

❑ Pilot study of 10 selected cases



| Phasing: <i>SPTA1</i> + alpha LELY<br>RESOLVE COMPLEX ALLELES                                                          | 6 cases |
|------------------------------------------------------------------------------------------------------------------------|---------|
| <b><i>SPTA1</i></b><br>SPECTRIN<br>ALPHA, ERYTHROCYTIC                                                                 |         |
| <b>Purpose</b><br>Resolve phase between pathogenic <i>SPTA1</i> variant and c.6531-12C>T (alpha LELY modifier allele). |         |
| <b>Example variants</b><br>p.Arg774* p.Leu155dup p.Trp597* VUS / LP / P mix                                            |         |
| <b>Patients</b><br>LRS_1, LRS_2, LRS_3, LRS_6, LRS_7, LRS_8                                                            |         |

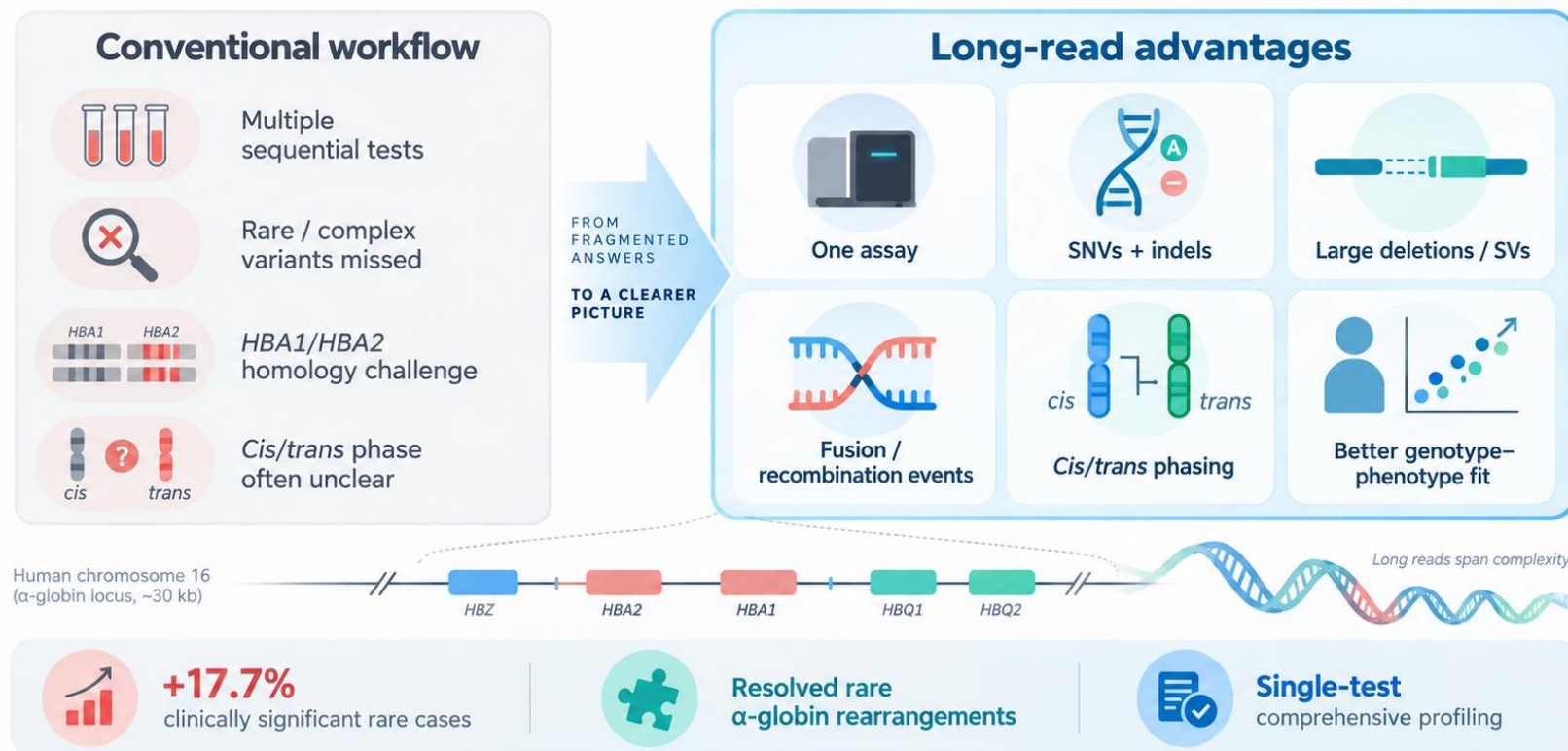
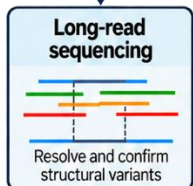
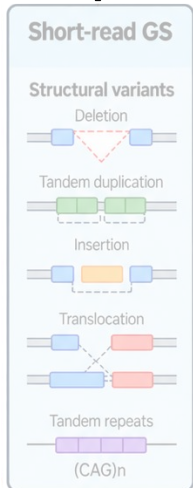
| Skewed X-inactivation<br>EXPLAIN VARIABLE PHENOTYPE                                             | 2 cases                     |
|-------------------------------------------------------------------------------------------------|-----------------------------|
| <b><i>G6PD</i></b><br>GLUCOSE-6-PHOSPHATE<br>DEHYDROGENASE                                      | <br>HETEROZYGOUS<br>FEMALES |
| <b>Purpose</b><br>Clarify phenotype in heterozygous females with multiple <i>G6PD</i> variants. |                             |
| <b>Example variants</b><br>p.Asn126Asp p.Asp181Val<br>p.Arg365Cys p.Ala335Thr                   |                             |
| <b>Patients</b><br>LRS_4, LRS_5                                                                 |                             |

| Phasing: SNV + CNV<br>LINK VARIANTS ACROSS<br>STRUCTURAL CHANGE                                                      | 2 cases                                      |
|----------------------------------------------------------------------------------------------------------------------|----------------------------------------------|
| <b><i>SEC23B</i></b><br>SEC23 HOMOLOG B<br>(COPII COMPONENT)                                                         | <br>DUPLICATION<br>(chr20:18507016-18513388) |
| <b>Purpose</b><br>Determine phase of <i>SEC23B</i> p.Arg14Trp with heterozygous duplication chr20:18507016-18513388. |                                              |
| <b>Example variants</b><br>CNV VUS                                                                                   |                                              |
| <b>Patients</b><br>LRS_9, LRS_10                                                                                     |                                              |

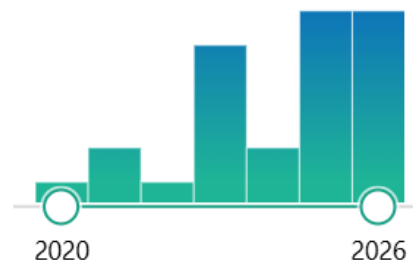
Figure created with ChatGPT (OpenAI) from author-provided scientific content; final content reviewed by the author

# Long-read sequencing (LRS): same genes, longer answers

- ✓ Main indications for LRS in the diagnosis of hereditary red blood cell disorders
- Genome-wide LRS enables the detection of sequence variants as well as
  - DNA methylation status
  - High sequence similarity

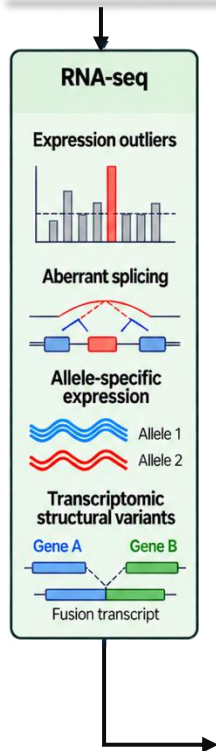


PubMed: "long-read sequencing" and "thalassemia"



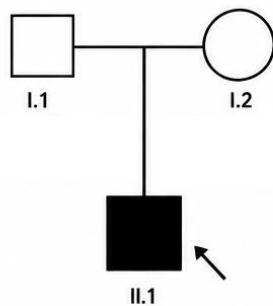
Jiang F et al. *Gene*, 2022  
Zhou X et al. *Human Mutation*, 2026  
Shi Q et al. *Reproductive Biology and Endocrinology*, 2026  
AGTC Genomics, 2026

# Integration of DNA-seq and RNA-seq



- ✓ Main indications for RNA-seq in the diagnosis of hereditary red blood cell disorders
  - Functional assessment of variants affecting RNA splicing
  - Detection and quantification of aberrant transcripts
  - Identification of alterations in transcript structure or expression associated with regulatory variants

## 1 Clinical case and pedigree



- 7-year-old child with suspected Fanconi anemia
- Family history consistent with autosomal recessive inheritance

## 2 DNA-seq findings

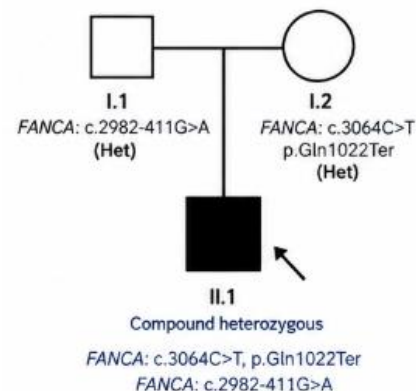
Targeted NGS  
(*FANCA* and related genes)

Pathogenic nonsense variant in *FANCA*  
c.3064C>T  
(p.Gln1022Ter)

WGS  
(genome-wide)

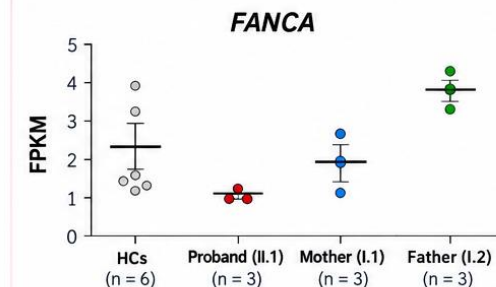
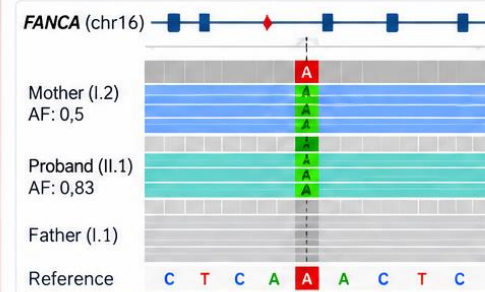
Deep intronic *FANCA* variant  
c.2982-411G>A

- WGS was then used to investigate the missing second allele and identified a deep intronic *FANCA* variant.

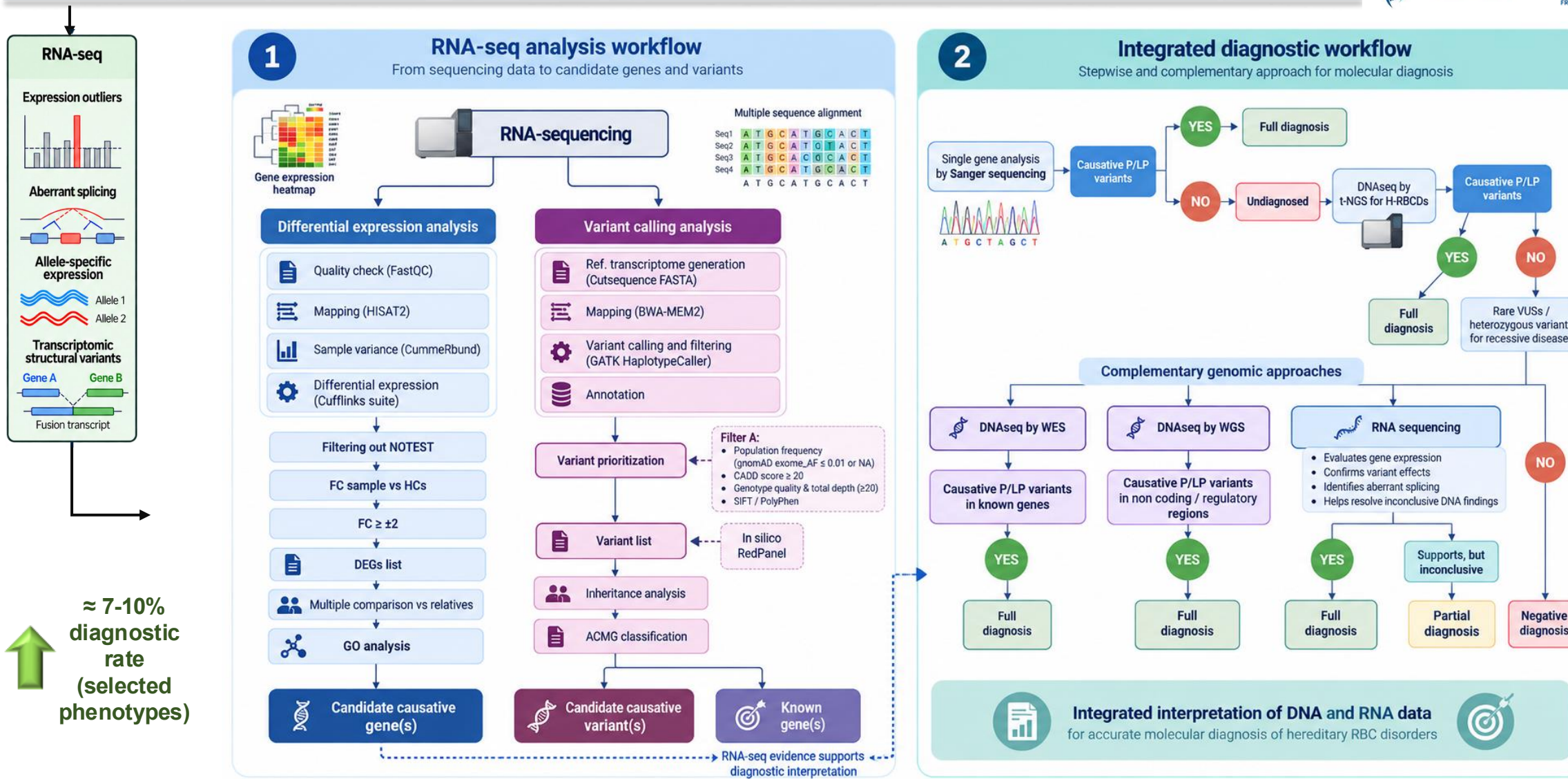


**Integrated  
DNAseq/RNAseq analysis**

## 3 RNA-seq support

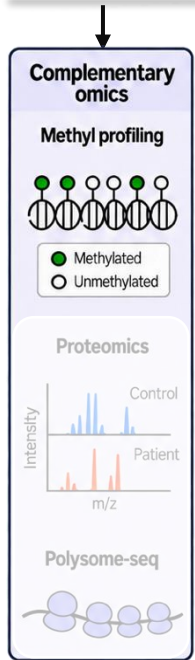


# Integration of DNA-seq and RNA-seq



# DNA methylation episignature for DBA diagnosis

## Cohort and workflow



86 patients

80 molecularly confirmed  
6 gene-orphan cases  
Controls matched by age, sex, and array type

EPIC v2 DNAm arrays

206 CpG probes

differentially methylated  
probes define the pan-DBA  
signature

SVM/MVP classifier

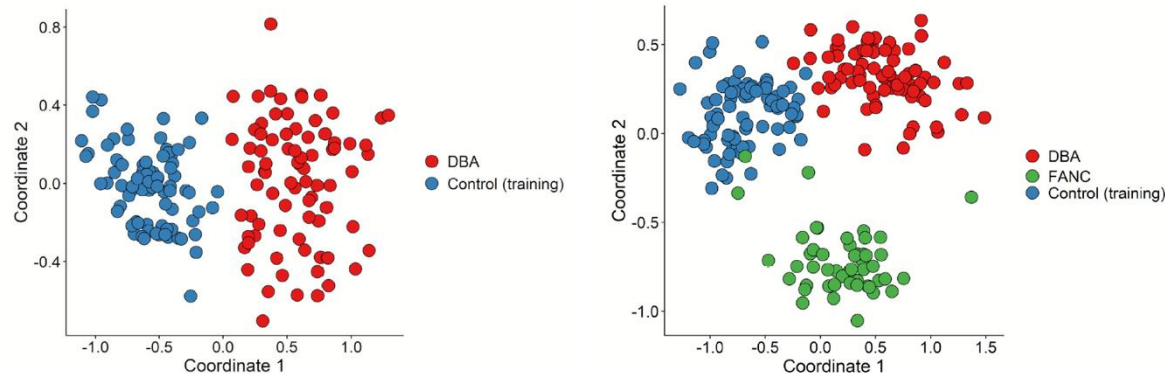
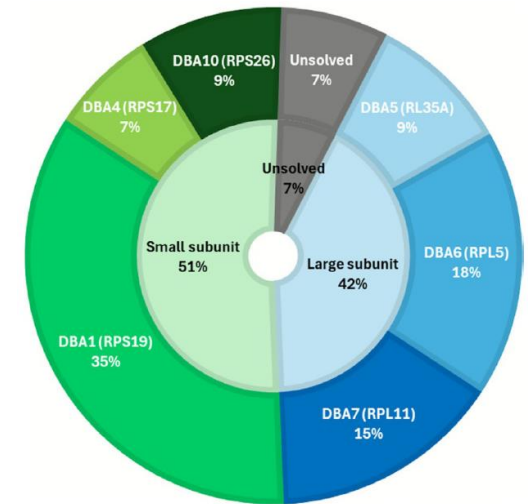
**1.00**

reported sensitivity in  
confirmed molecular DBA cases

**~1.00**

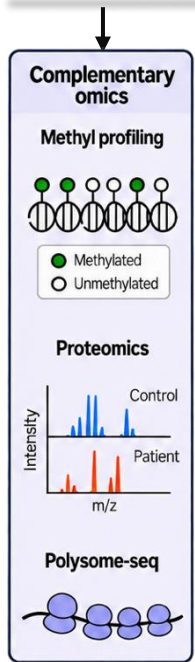
reported specificity against  
controls and other disorders

Peripheral blood DNAm profiling can act as a  
biomarker for DBA diagnosis and variant  
resolution



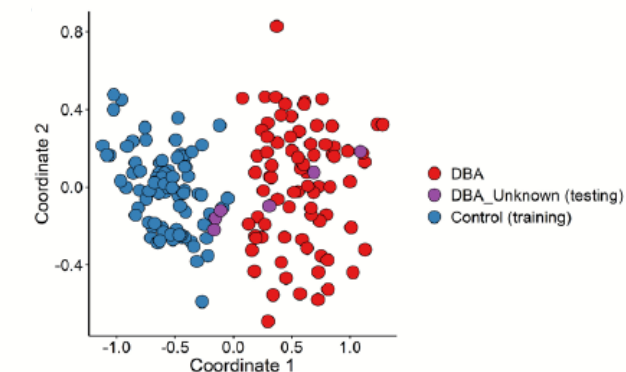
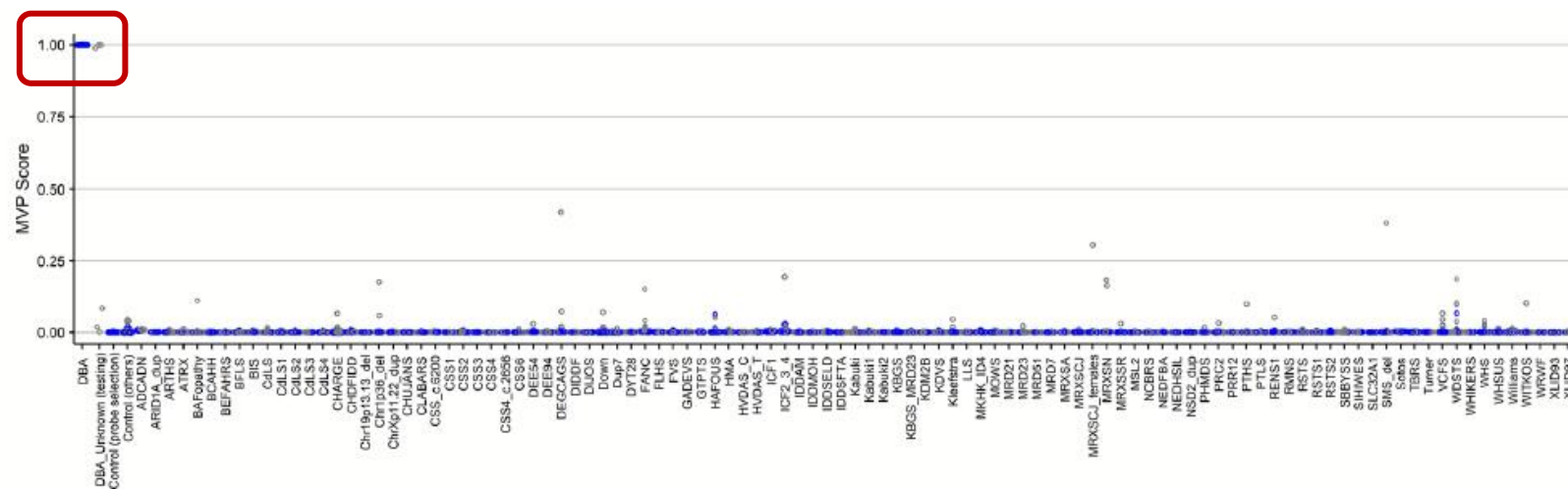
- A single pan-DBA DNAm signature with high specificity
- DNAm profiles clearly distinguish DBA cases from controls
- The pan-DBA DNAm signature remains distinct from the Fanconi anemia signature despite overlapping bone marrow failure phenotypes

# DNA methylation episignature for DBA diagnosis



## Diagnostic value

- In six clinically suspected but genetically unresolved cases, three showed the DBA methylation pattern
- In two of these three cases, WGS identified intronic/deep-intronic variants in *RPL5* and *RPL26*, which were subsequently validated by functional assays, leading to a conclusive molecular diagnosis



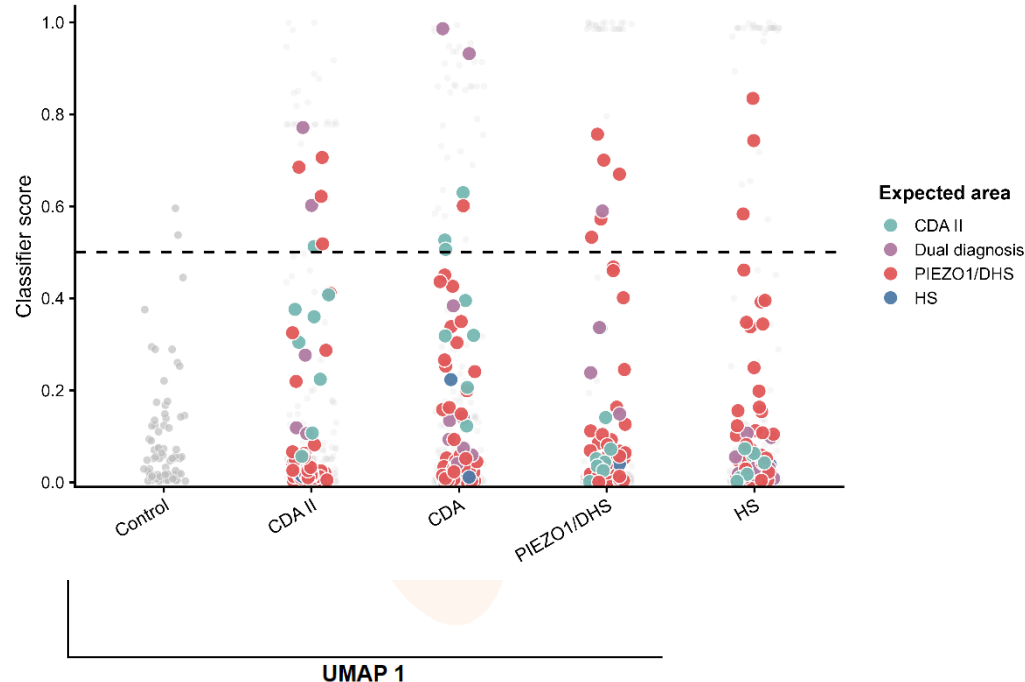
**DNAm profiling is proposed as a practical, stable, and specific diagnostic biomarker for suspected DBA**

## Diagnostic value

- Can support VUS interpretation and molecular diagnosis when standard testing is incomplete

### Episignature classifier framework

Projection cohorts evaluated using a uniform classification threshold of 0.50

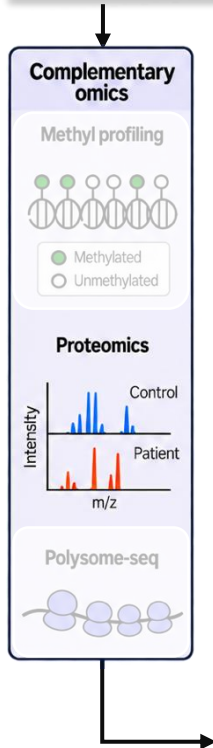


### ❖ Disease-specific methylation signatures in **100 molecularly confirmed patients**

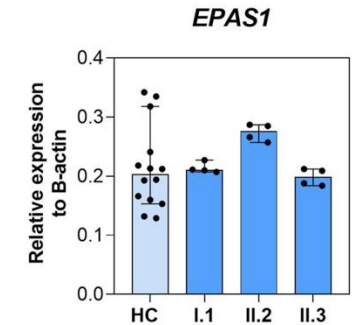
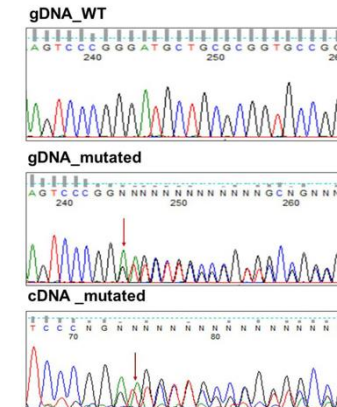
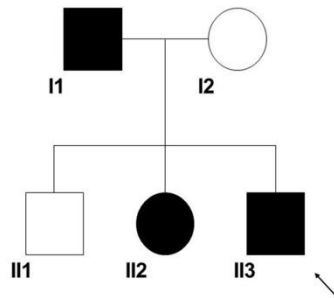
- CDA I, CDA II
- PIEZO1-DHS,
- HS
- G6PD deficiency

- **89 age- and sex-matched healthy controls**

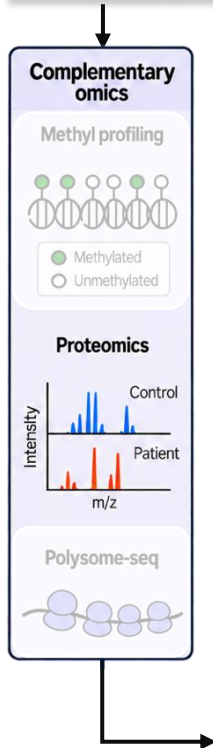
### ❖ Disease-specific classifiers can define undiagnosed patients



- ✓ Main indications for proteomics in the diagnosis of hereditary disorders
  - Functional assessment of variants predicted to alter protein synthesis or stability
  - Detection and quantification of reduced or absent protein expression
  - Confirmation of loss-of-function effects, even when transcript levels are preserved



- 8-year-old boy with chronic hereditary anemia
- Autosomal dominant family history
- Targeted NGS identified a novel heterozygous **EPAS1** frameshift variant
- Variant segregated with the anemic phenotype
- **EPAS1** mRNA expression was preserved ...



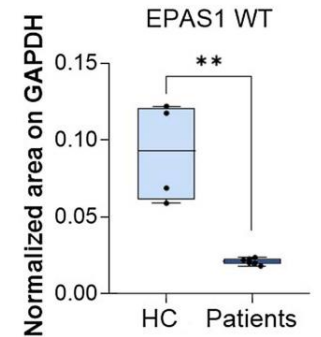
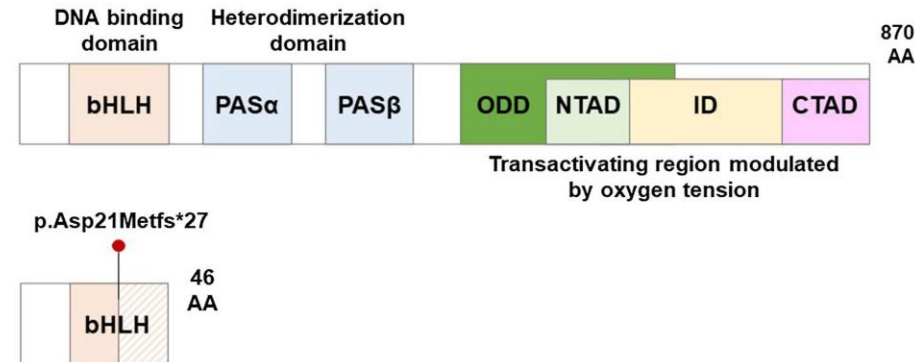
- ✓ Main indications for proteomics in the diagnosis of hereditary disorders
  - Functional assessment of variants predicted to alter protein synthesis or stability
  - Detection and quantification of reduced or absent protein expression
  - Confirmation of loss-of-function effects, even when transcript levels are preserved

## Protein reference sequence

MTADKEKKRSSERRKEKSRDAARCRRSKETEVFYELAHLEPLPHSVSHLDKASIMRLAISFLRTHKLLSSVCSSESEAEADQQMDNLY  
LKALEGFIAVVTQGDIMFLSENISKFMGLTQVELTGHISIFDFTPCDHEEIRENLSKNGSGFGKSKDMSTERDFMRMKCTVNRGRT  
VNLKSATWKLHCTGQVKVYNNCPHNSLCGYKEPLLSCLIMCEPIQHPSHMDIPLDSKTFLSRHSMDMKFTYCDRITELIGYHPEELL  
GRSAYEFYHALDSENMTKSHQNLCTKGQVVSQGYRLAKHGGYVWLETQGTVIYNPRNLQPQCIMCVNYVLSIEKNDVVFSMDQTESLFEK  
PHLMAMNSIFDSSGKGAVSEKSNFLFTLKEEPEELAQLAPTGPDAIISLDFGNQNFEESSAYGKAILPPSPQWATELRSHSTQSEAGSLP  
AFTVPQAAAGSTTPSATSSSSSCSTPNPEDYYSLDNDLKIEVIEKLFAMDTEAKDQCSTQTDNFELDLETLAPYIPMDGEDFQLSPIC  
PEERLLAENPQSTPQHCFSAMTNIFQPLAPVAPHSPFLLDKFOQQLESKKEPEHRPMSSIFFDAGSKASLPPCCGQASTPLSSMGGRSNT  
QWPPDPLHFGPTKNAVGDQRTFLGAAPLGPVSPPHVSTFKTRSAKGFARGPDVLSAMVALSNKLLKRLQLEYEEQAFQDLSGGDPP  
GGSTSHLMWKRMLNLRGGSCPLMPDKPLSANVPNDKFTQNPMLRGLGHLRHLPLQPSSAISPGENSKSRFPQCYATQYQDYSLSAAHKV  
SGMASRLLGPSFESYLLPELTRYDCEVNVPLVGSSTLLQGGDLLRALDQAT\*

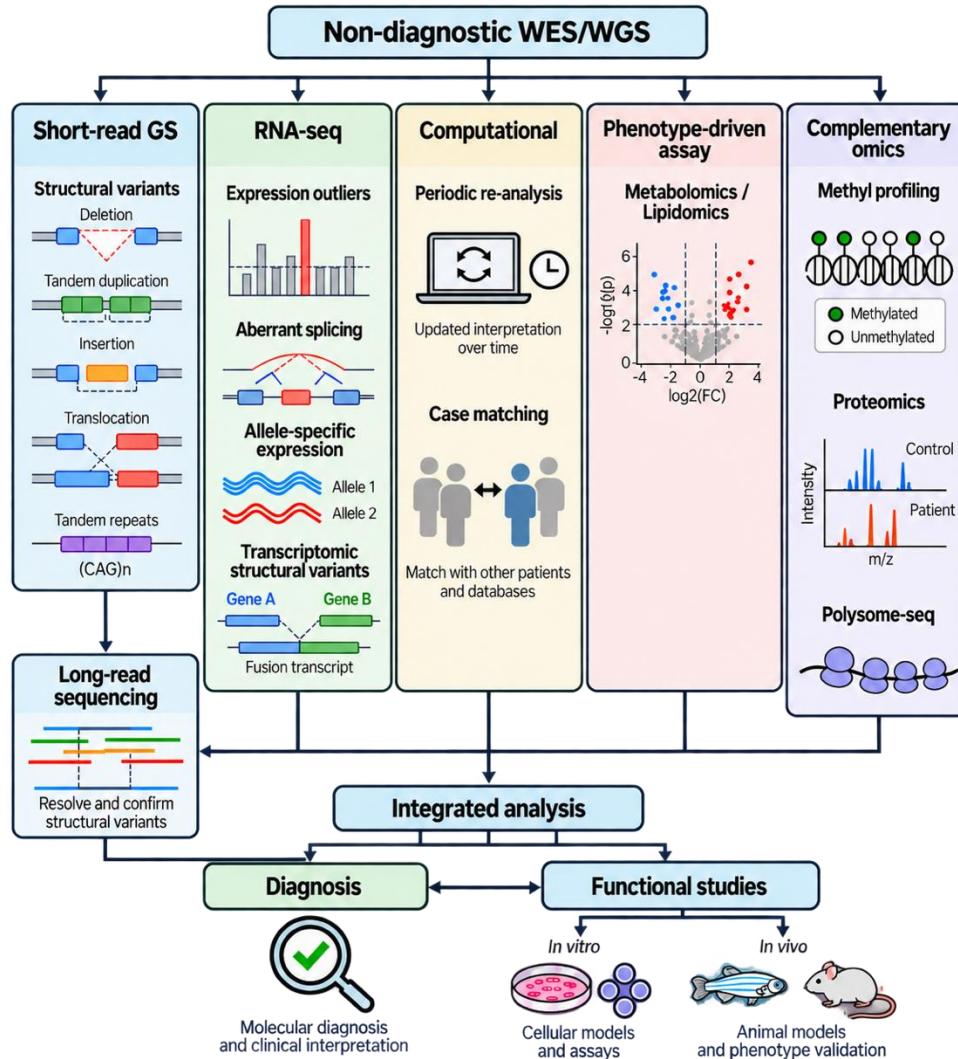
## Protein mutated sequence

MTADKEKKRSSERRKEKSRMLRGAGGARRRRCSMSWPMSCLCPTV\*



- ... but EPAS1 protein levels were markedly reduced
- Functional data support EPAS1 haploinsufficiency as the disease mechanism

➤ Key message: variant-specific effects can produce phenotypes opposite to the classical gene-associated disorder



- ✓ Several technologies and approaches can improve the diagnosis of hereditary red blood cell disorders when WES/WGS are non-diagnostic
- ✓ Different methods address different diagnostic gaps, including structural variants, aberrant splicing, expression changes, and functional abnormalities
- ✓ Many of these approaches are complementary and should be integrated to maximize diagnostic yield
- ✓ An integrated multi-omic approach can ultimately increase diagnostic yield

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Patients and their families



2023-2026



2023-2026



CN3 2022-2026

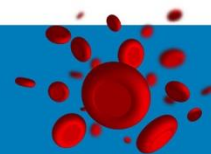


2025-2027

MEETING DEL CLUB DEL GLOBULO ROSSO 2026

NAPOLI, 28-29 SETTEMBRE 2026

**MEETING DEL CLUB DEL GLOBULO ROSSO 2026**



**NAPOLI, 28-29 SETTEMBRE 2026**

# Challenges in routine NGS genetic testing

- Phenotyping
- Variant interpretation
- Complex inheritance

- Sensitivity of mutation detection
- High sequence similarity
- Chromosome imbalance and rearrangements

