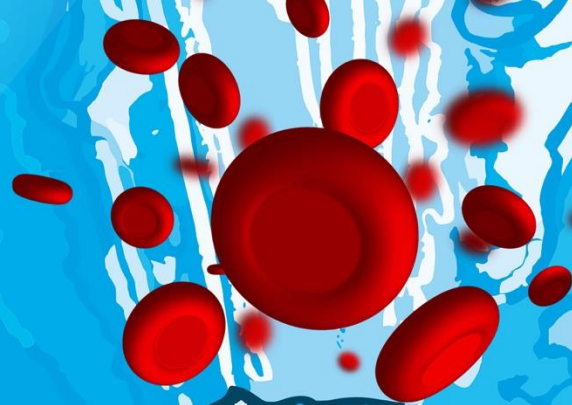




Meeting del CLUB DEL

***Firme epigenetiche nei disordini ereditari del globulo rosso:
un nuovo supporto alla diagnosi molecolare***

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Dip. Medicina Molecolare e Biotecnologie Mediche
Univestità degli studi di Napoli Federico II
CEINGE – Biotecnologie Avanzate Franco Salvatore

**Globulo
Rosso** 2026

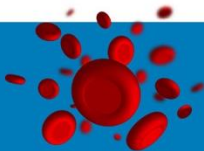
NAPOLI

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Complesso dei SS. Marcellino e Festo

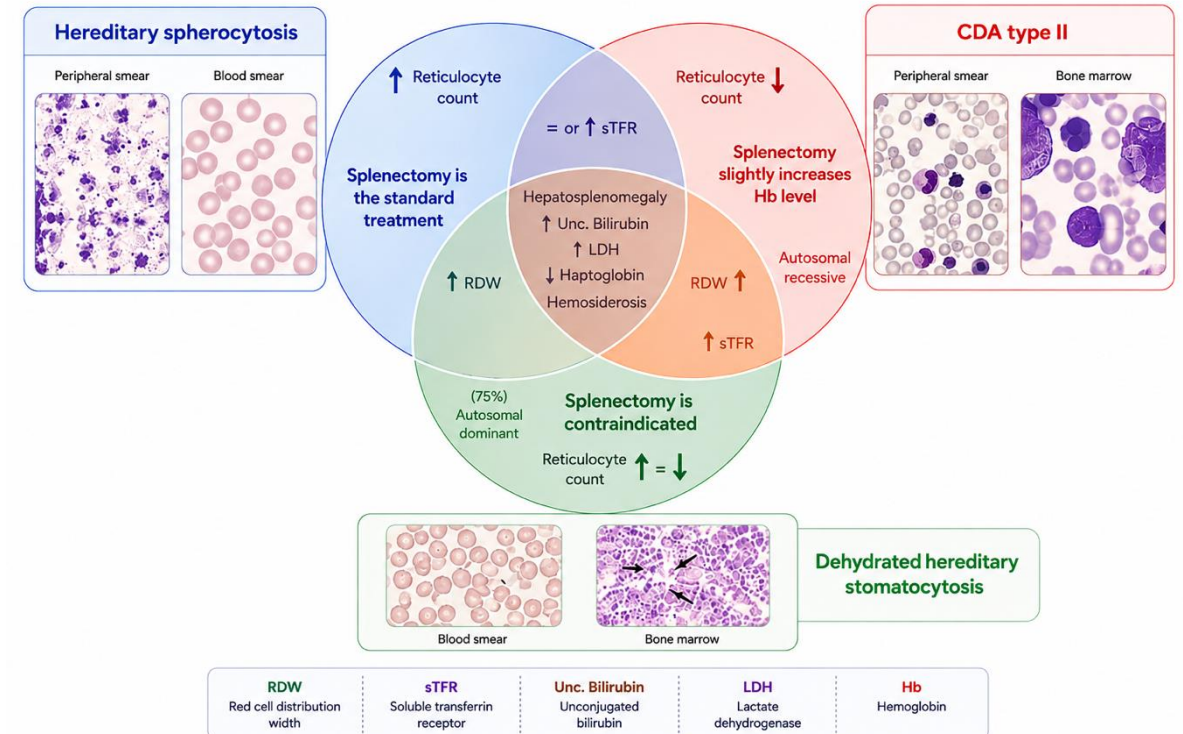
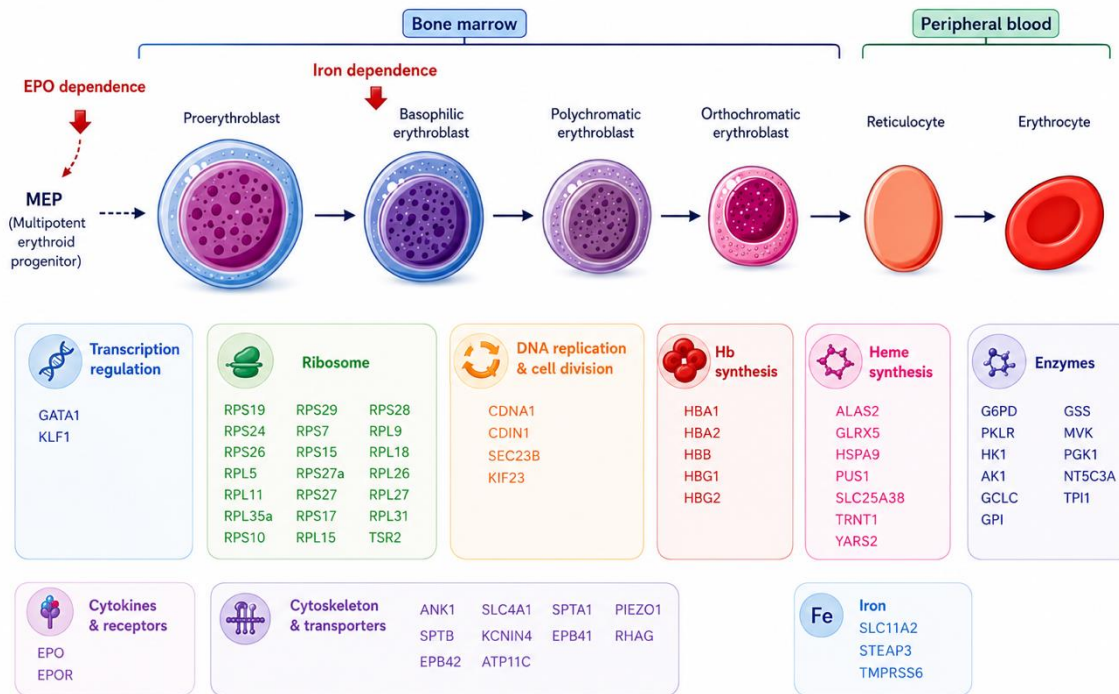
Disclosures

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



Clinical and genetic variability of inherited red blood cell disorders

*Inherited red blood cell disorders (H-RBCDs) are **heterogeneous** conditions characterized by **overlapping phenotypes** and challenging variant classification due to the variable penetrance of causative variants.*

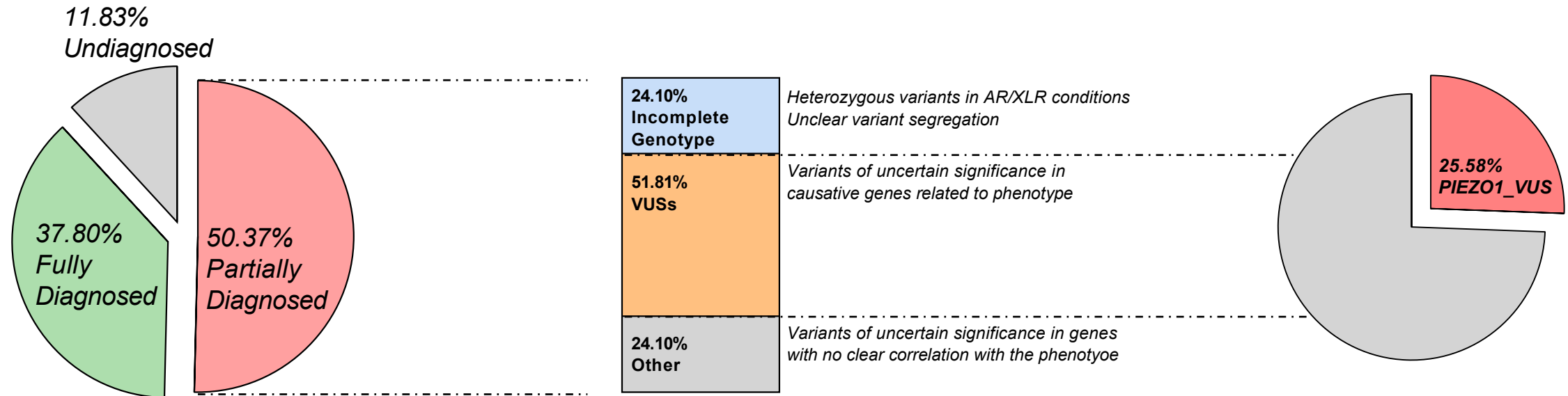


✓ > 100 genes associated with H_{RBC}-D

✓ Overlapping clinical features

Clinical and genetic variability of inherited red blood cell disorders

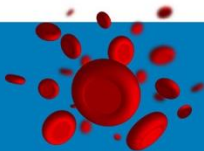
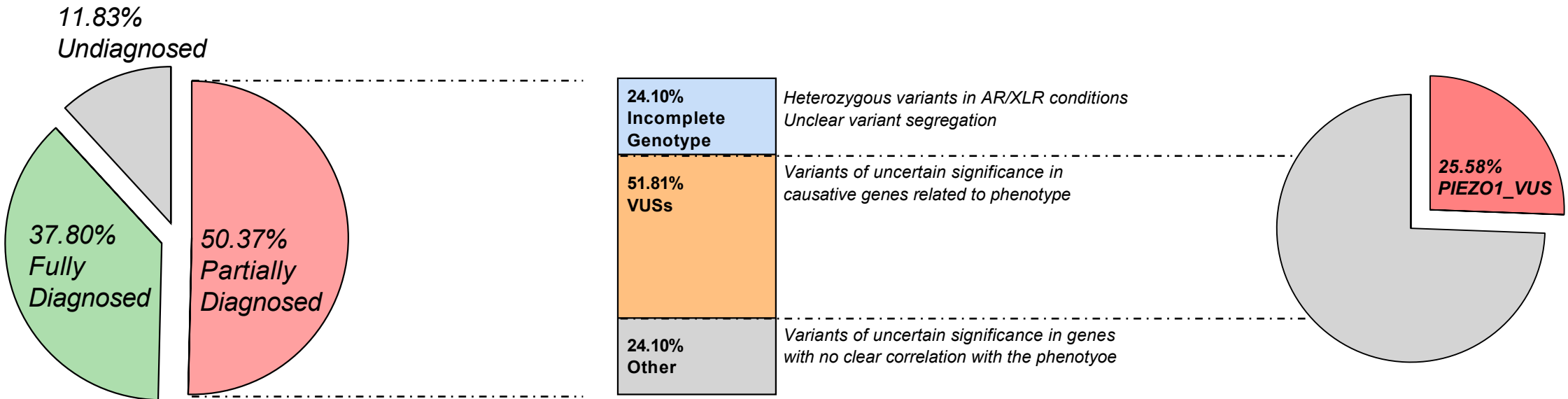
*Inherited red blood cell disorders (H-RBCDs) are heterogeneous conditions characterized by overlapping phenotypes and **challenging variant classification** due to the variable penetrance of causative variants.*



Probands enrolled between 2020-2025 (n=820) at Medical Genetics Lab – CEINGE-Biotecnologie Avanzate Franco Salvatore

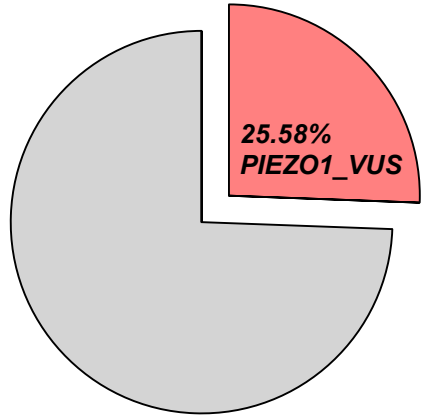
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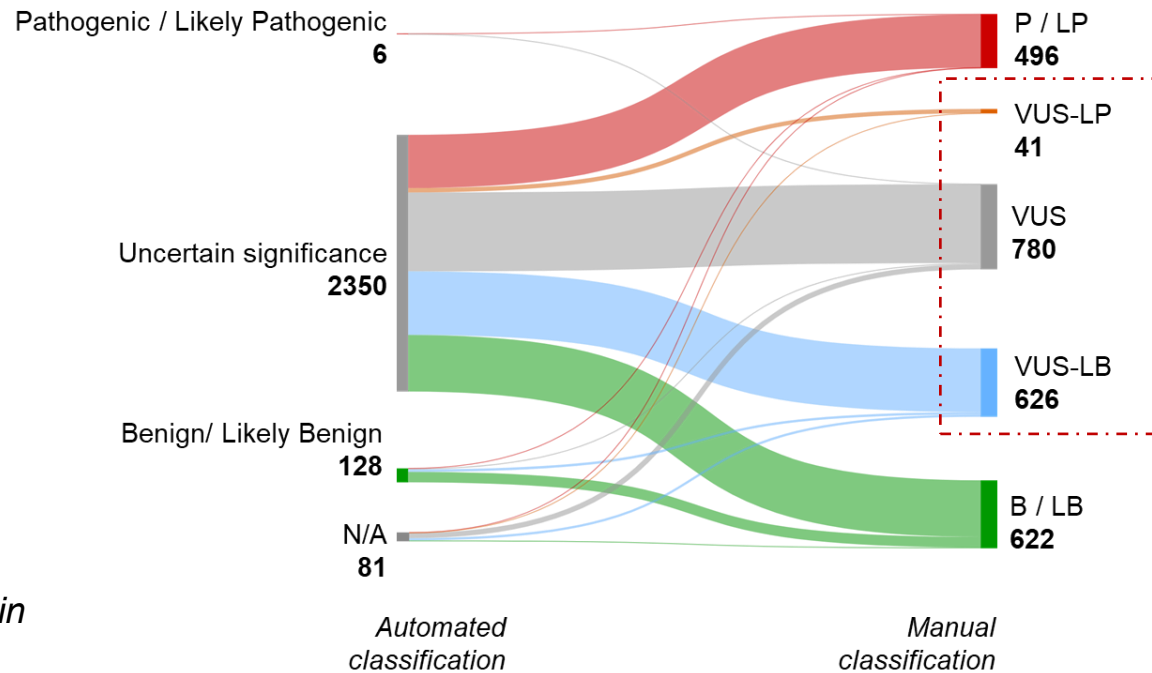


Clinical and genetic variability of inherited red blood cell disorders

*Inherited red blood cell disorders (H-RBCDs) are heterogeneous conditions characterized by overlapping phenotypes and **challenging variant classification** due to the variable penetrance of causative variants.*



More than 25% of VUS are in PIEZO1 gene.



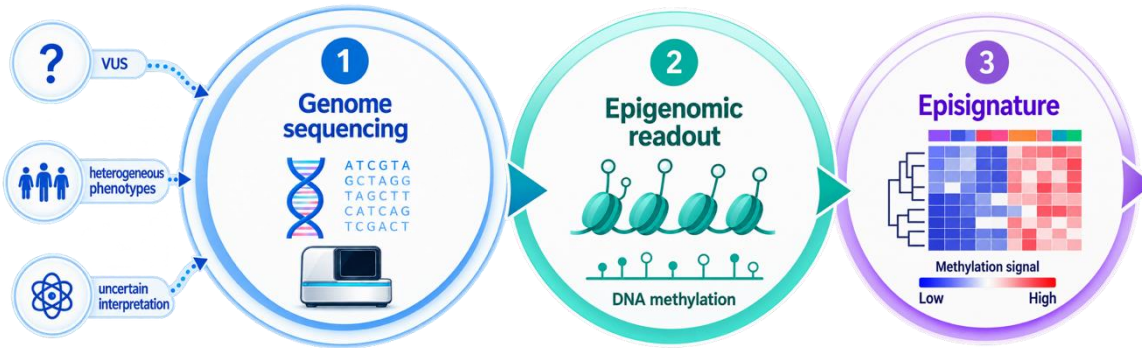
Although refined approaches for variant reclassification, more than 1000 variants in PIEZO1 remains of unknown significance

Rosato et al. Blood. 2026

Refining molecular diagnosis with epigenomic signatures

The **high burden of unresolved and partially diagnosed cases** reveals a clear **diagnostic gap** that genome sequencing alone cannot fully bridge. **Complementary approaches** able to translate **uncertain genetic findings** into more **definitive molecular diagnoses** are needed.

- ✓ Genome sequencing identifies variants.
- ✓ DNA methylation can reveal whether they generate a **reproducible disease-specific molecular state**.

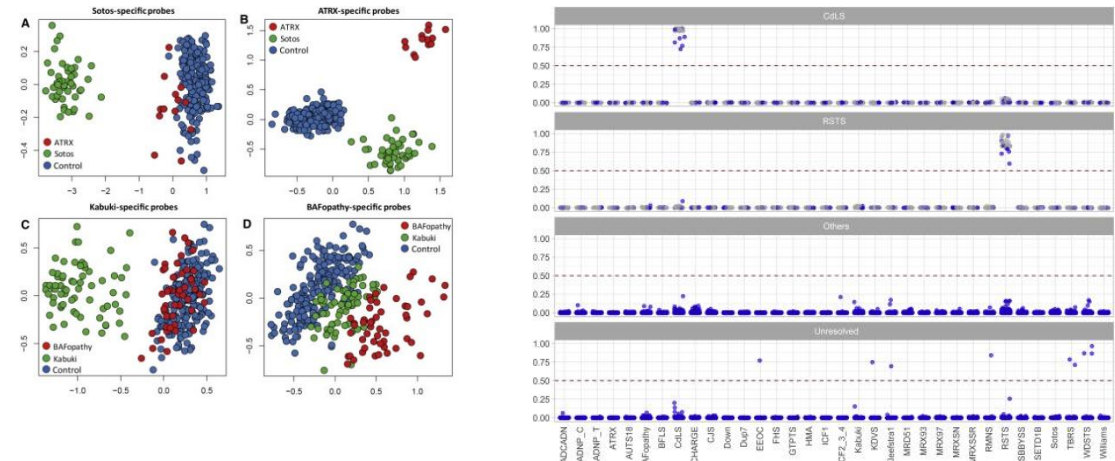


Episignature consists of a characteristic pattern of **hypermethylation and hypomethylation in hundreds or thousands of CpGs** scattered throughout the genome. It is a "functional signature" of the disease

Refining molecular diagnosis with epigenomic signatures: state of the art

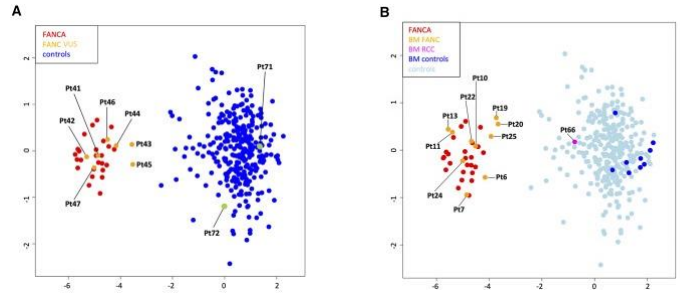
Am J Hum Genet. 2020 Mar 5;106(3):356-370. doi: 10.1016/j.ajhg.2020.01.019. Epub 2020 Feb 27.

Evaluation of DNA Methylation Episignatures for Diagnosis and Phenotype Correlations in 42 Mendelian Neurodevelopmental Disorders



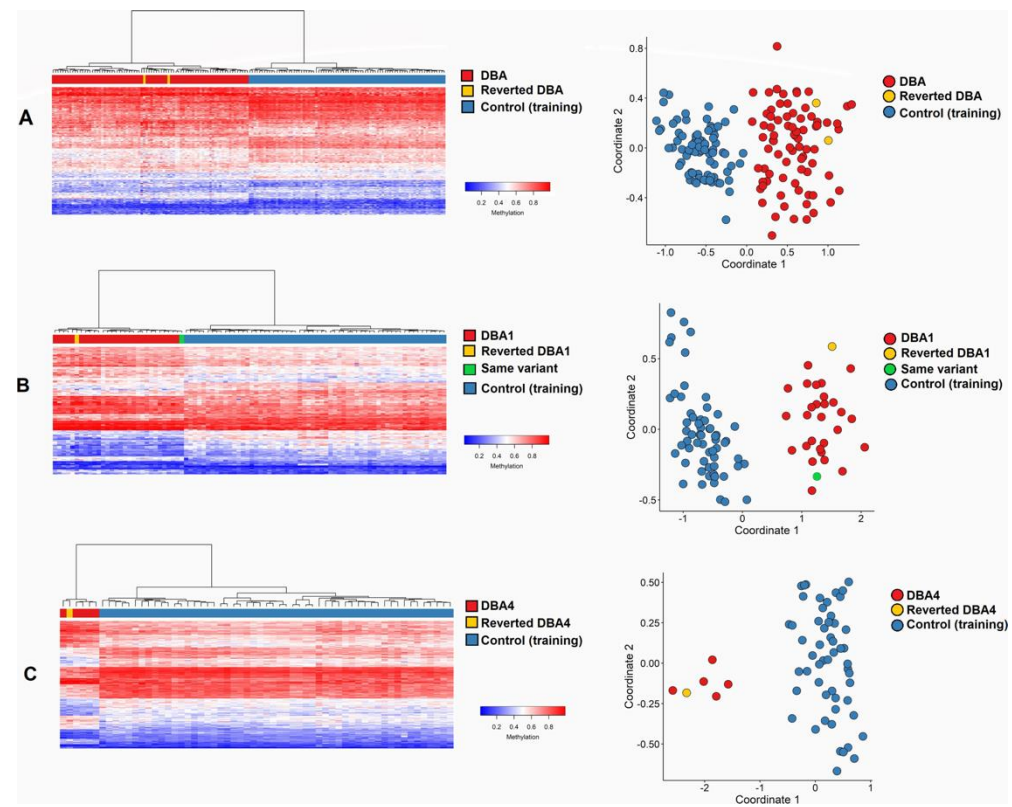
Am J Hum Genet. 2023 Nov 2;110(11):1938-1949. doi: 10.1016/j.ajhg.2023.09.014. Epub 2023 Oct 20.

Identification of a robust DNA methylation signature for Fanconi anemia



Am J Hematol. 2026 Feb;101(2):228-241. doi: 10.1002/ajh.70141. Epub 2025 Nov 17.

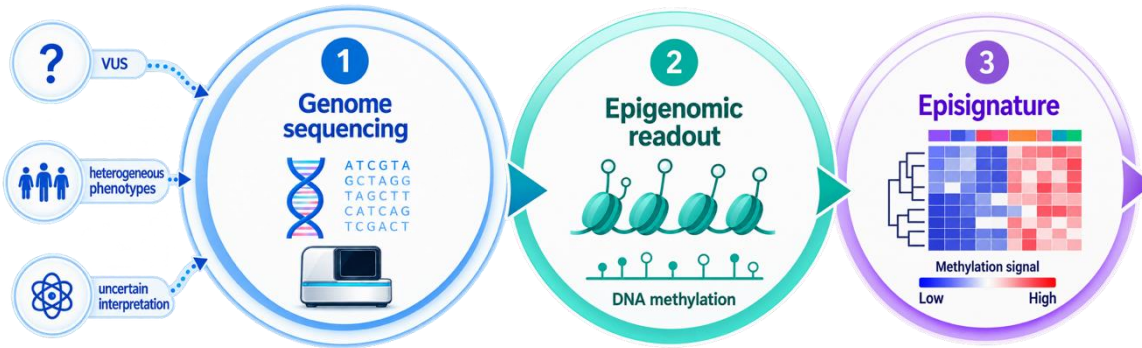
DNA Methylation Episignature as a Novel Diagnostic Tool for Diamond-Blackfan Anemia Syndrome



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Aim 1: To define a disease-specific methylation signatures for hereditary red blood cells defects

Study design



The **training set** (n=100) includes patients carrying PLP variants in causative genes of the following defects:

Hereditary spherocytosis (HS)

G6PD-deficiency

Congenital dyserythropoietic anemia type I (CDA I)

Congenital dyserythropoietic anemia type II (CDAII)

Dehydrated Hereditary stomatocytosis (DHS)

The **negative control group** (n = 89) consists of healthy individuals, matched for age and gender and with no PLP variants in the analyzed genes

An independent **test set** (25% of training set) includes patients carrying PLP variants but **not included to generate signatures**

Bisulfite

Unmethylated cytosines (C) are chemically converted into uracils (U)

PCR

Uracils (U) are amplified as thymines (T) during PCR

Identification of **Differentially Methylated Probe (DMP)**

$\Delta\beta = \text{mean } \beta_{\text{target}} - \text{mean } \beta_{\text{reference}}$

Candidate filters:

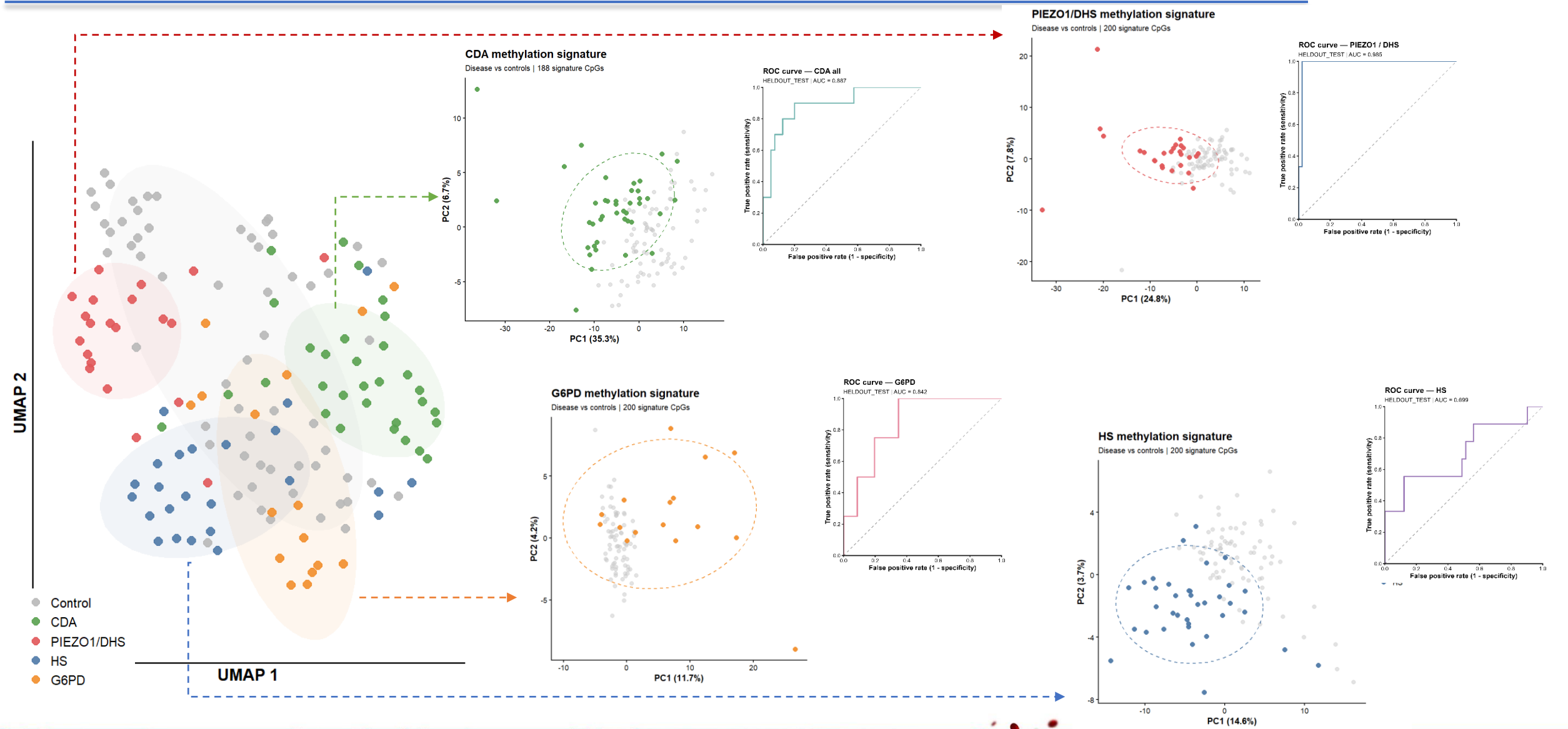
FDR	< 0.05	statistical confidence
$ \Delta\beta $	≥ 0.05	minimum effect size
Top rank	1000	initial candidate pool
AUC filter	top 50%	single CpG discrimination
Correlation	$r < 0.65$	non redundant CpGs

Quarello P, et al. Am J Hematol. 2026 Feb
Aref-Eshghi E, et al. Am J Hum Genet. 2020

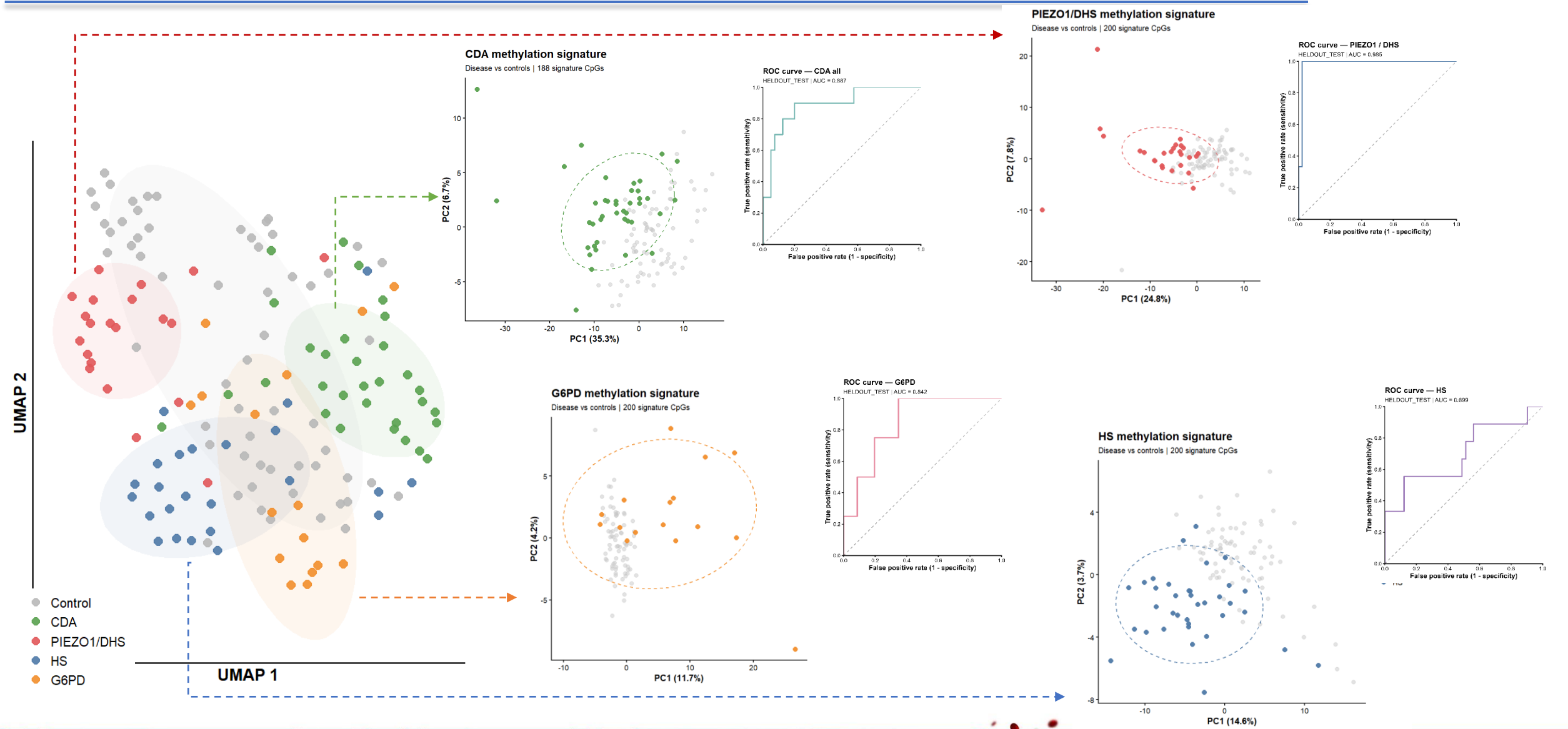
Final signature

A selected set of CpGs that together capture disease-discriminating methylation information

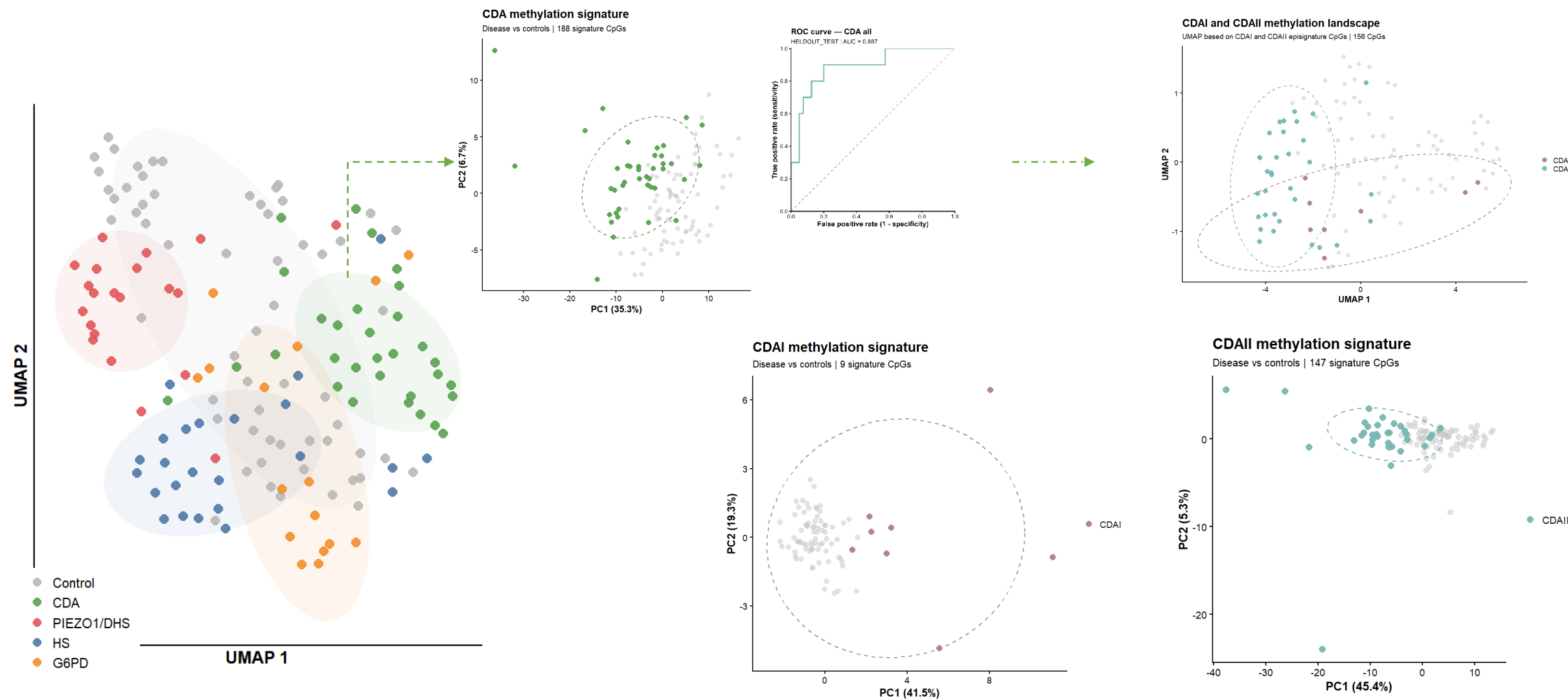
Disease-specific methylation profiles showed different degrees of separation



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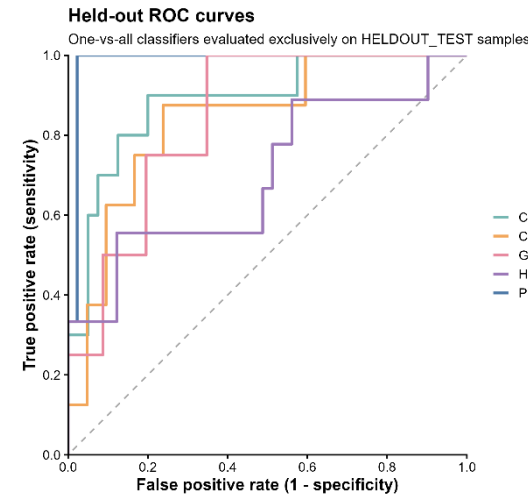
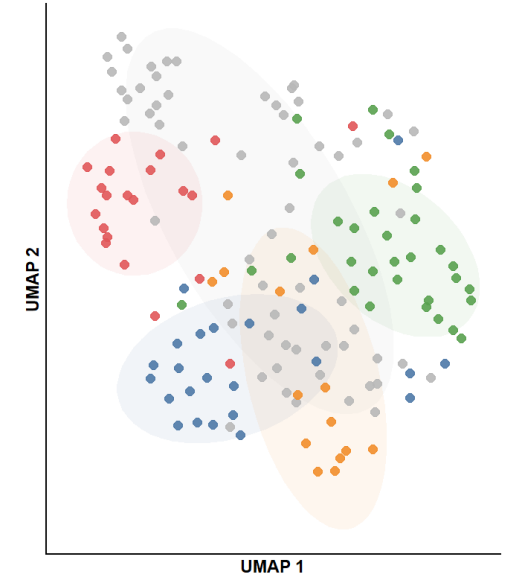
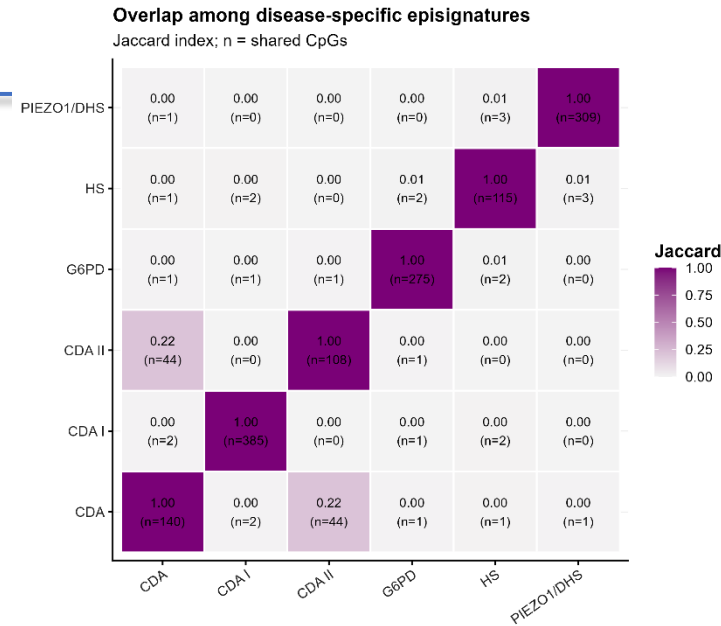


Disease-specific methylation profiles showed different degrees of separation

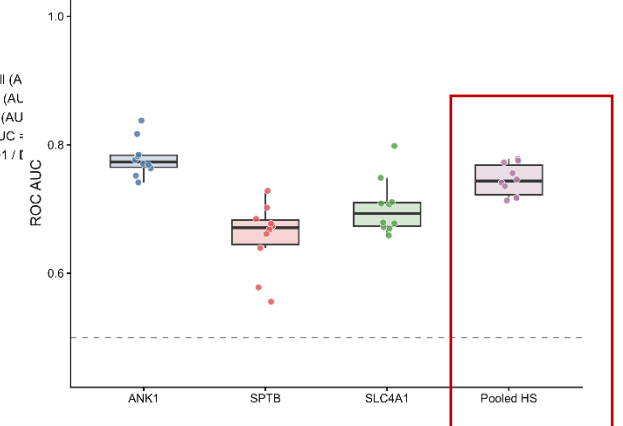


Conclusion (i)

- We generated a final **non-redundant** disease-associated episignatures;
- Disease-specific methylation profiles showed different degrees of separation;
- PIEZO1/DHS showed the clearest disease-associated pattern;
- HS showed the **weakest separation** between affected patients and healthy controls. This is likely related to **genetic heterogeneity** (training set includes **PLP variants** in *ANK1*, *SLC4A1* and *SPTB*)



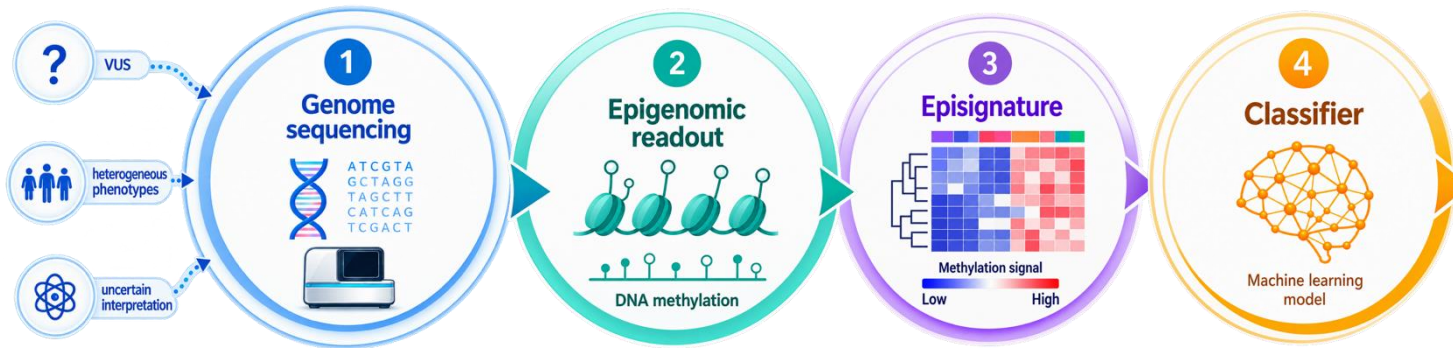
Does gene stratification improve the HS classifier?
Repeated family-aware nested cross-validation



Refining molecular diagnosis with epigenomic signatures

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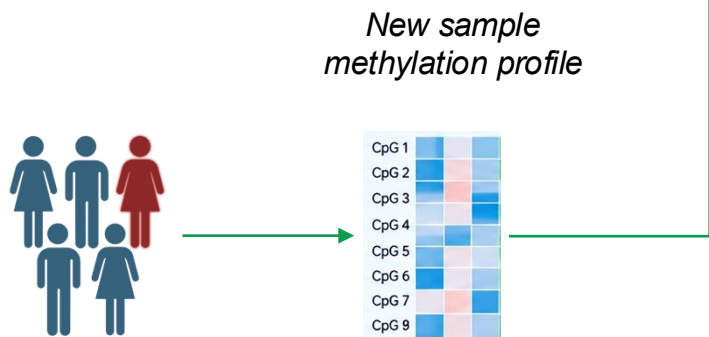
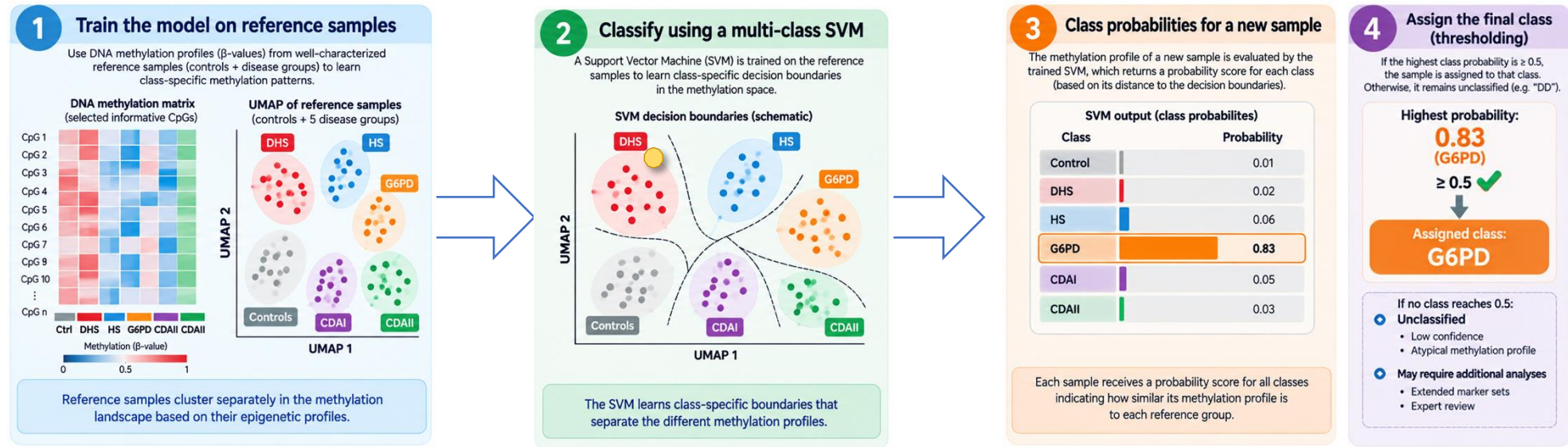


Aim 1: To define a disease-specific methylation signatures for hereditary red blood cells defects

Aim 2: To build a methylation-based classifier to reach a definitive molecular diagnosis

What does our multiclass methylation classifier do?

One methylation profile is compared simultaneously against several reference episignatures



A multiclass classifier compares one **sample against multiple reference** episignature
and **returns the most likely molecular class**

Study design

Define
positive group

Define
reference group

Rank CpGs

Final signature

Train classifiers

Score all samples

The **training set** (n=100) includes patients carrying PLP variants in causative genes of the following defects:



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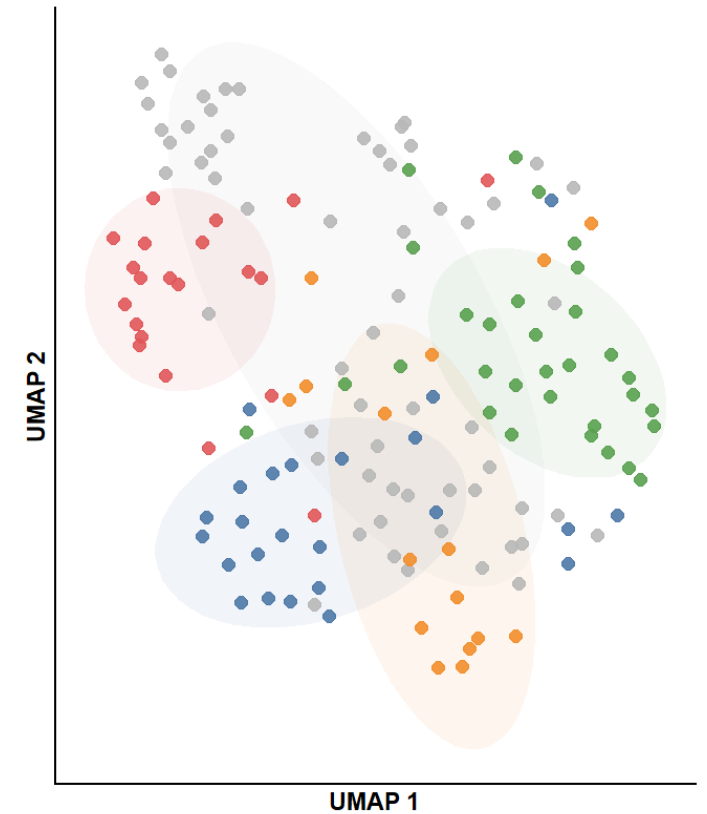
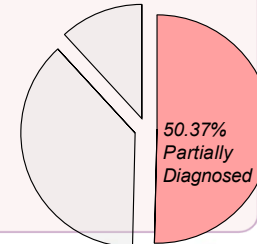
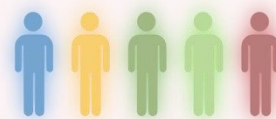
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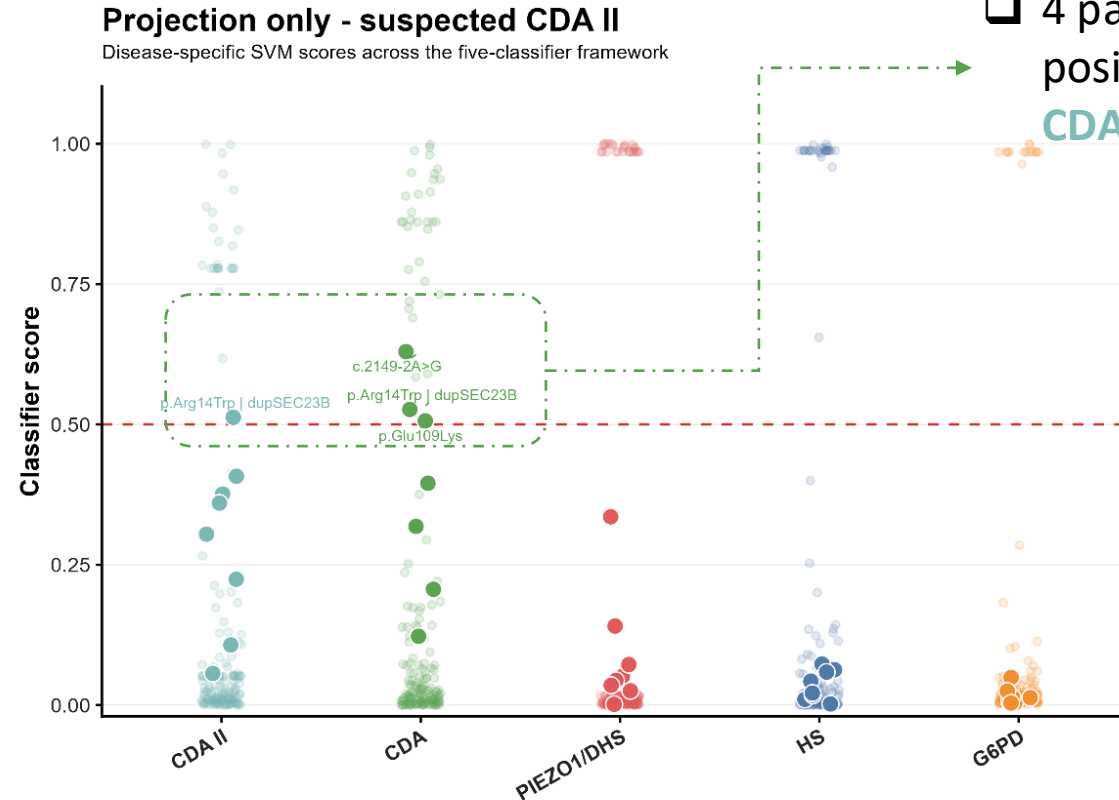
An independent **projection set** including:

- **VUS in *PIEZO1*** (n=49);
- **CNVs of unknown significance** (n=4)
- **Monoallelic variants in *SEC23B*** (n=6)
- **Dual *PIEZO1/SEC23B* inheritance** (n=5)



Application of methylation-based classifier to molecularly unresolved cases: **PtD CDAs**

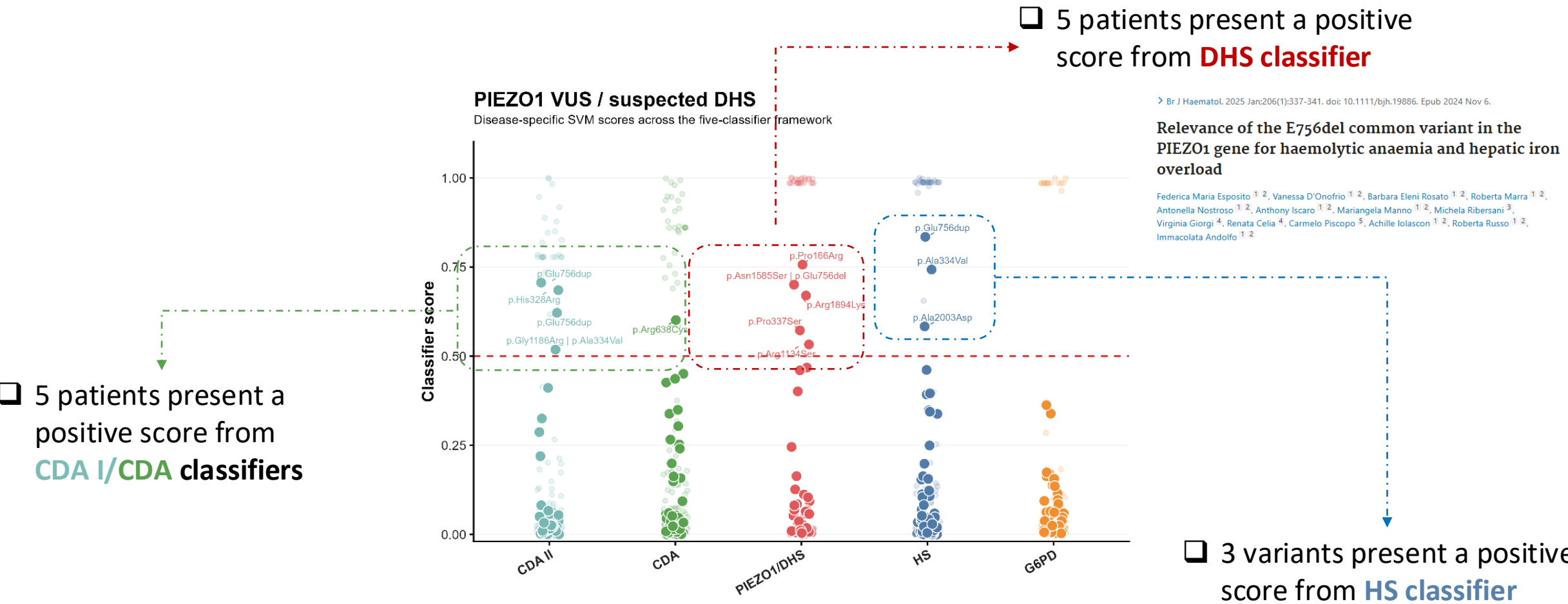
- 2 patients carrying a CNV and p.Arg14Trp → confirmed **compound heterozygosity** and **pathogenicity** of the CNV



- 4 patients present a positive score from the **CDA I/ CDA classifier**

- 2 patients carrying only **heterozygous pathogenic variants** → rational extension to **Whole Genome Sequencing**

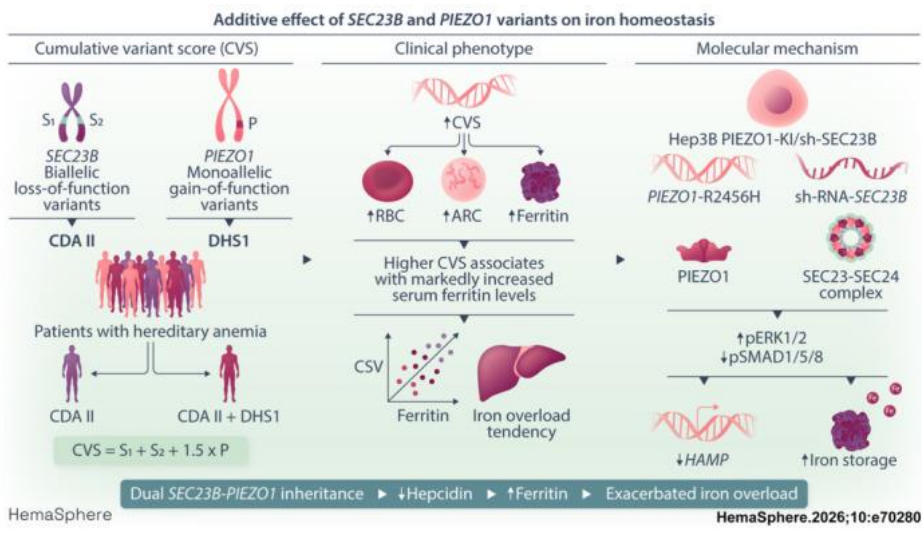
Application of methylation-based classifier to molecularly unresolved cases: **PIEZO1_VUSs**



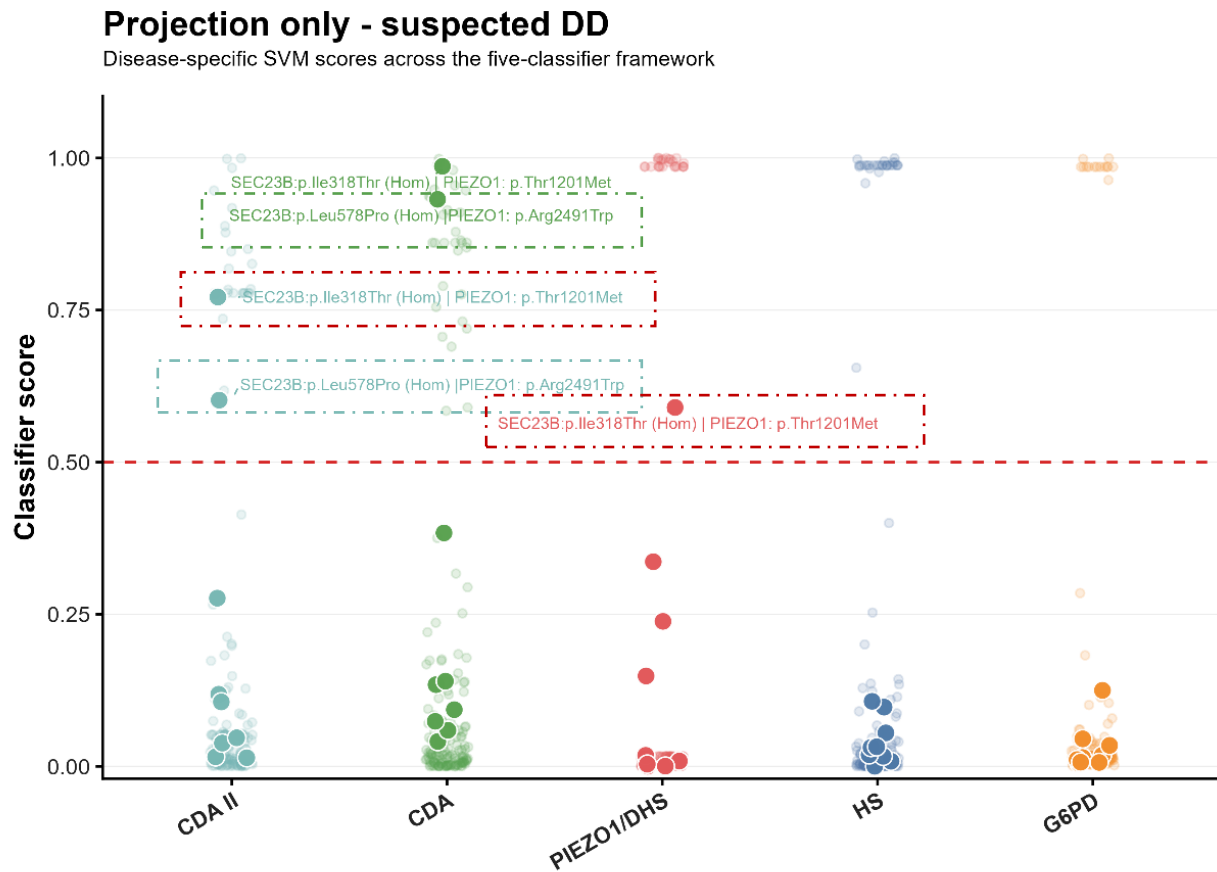
Application of methylation-based classifier to molecularly unresolved cases: **Dual *PIEZO1*/*SEC23B***

> HemaSphere. 2026 Jan 23;10(1):e70280. doi: 10.1002/hem3.70280. eCollection 2026 Jan.

Additive effect of multiple genetic variants in *SEC23B* and *PIEZO1* on iron metabolism dyshomeostasis in hereditary anemias



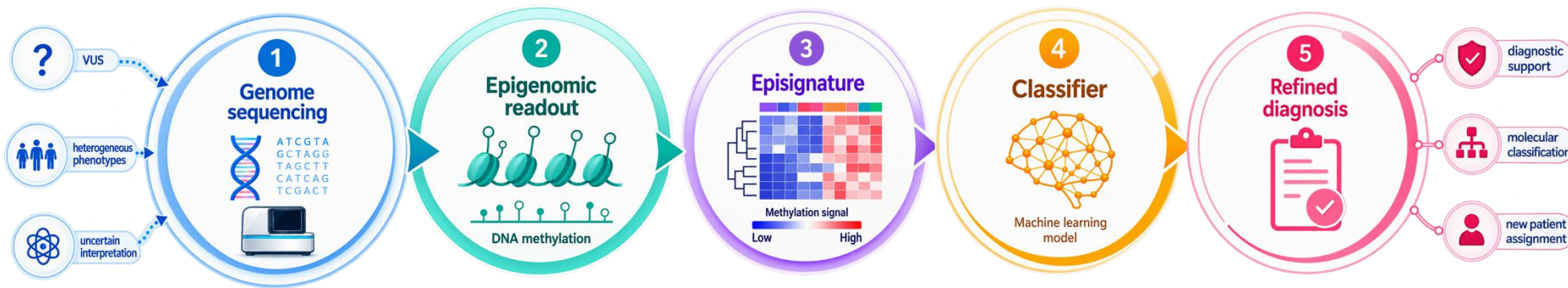
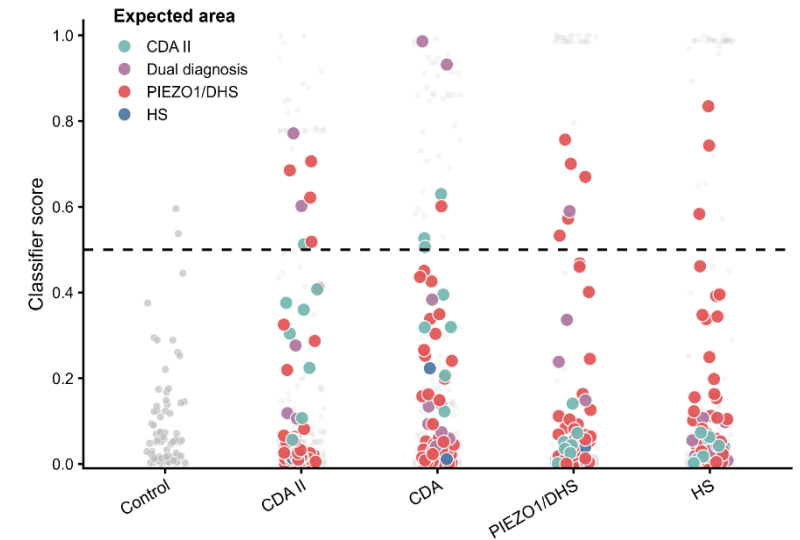
❑ 1 patient present a positive score only from the **CDA II /CDA all classifier**



❑ 1 patient present a positive score from both the **CDA II classifier** and the **DHS classifier**

Conclusion (ii)

- We generated a **multiclass methylation classifier**, showing **high preliminary performance** with limited off-target positivity
- Multiclass SVM enables **simultaneous** molecular-epigenetic **classification across disease groups**.
- **Projection** of independent/**unresolved samples** demonstrated the **potential of episignatures** to **resolve** genetically **inconclusive cases** and **prioritize** them for targeted **genomic re-analysis**.



DNA methylation adds an independent diagnostic layer beyond sequence variation alone.

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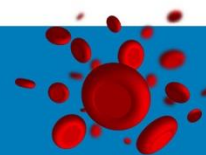
The "Epigenetic- Lab
Prof. Lorenzo Chiariotti
Mariella Cuomo
Rosa Della Monica



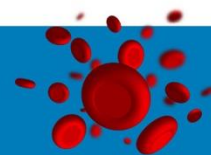
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FEDERICO II

Bioinformatic Service for NGS - CEINGE –
Biotechnologie Avanzate Franco Salvatore

Prof. Mario Capasso
Vito Alessandro Lasorsa

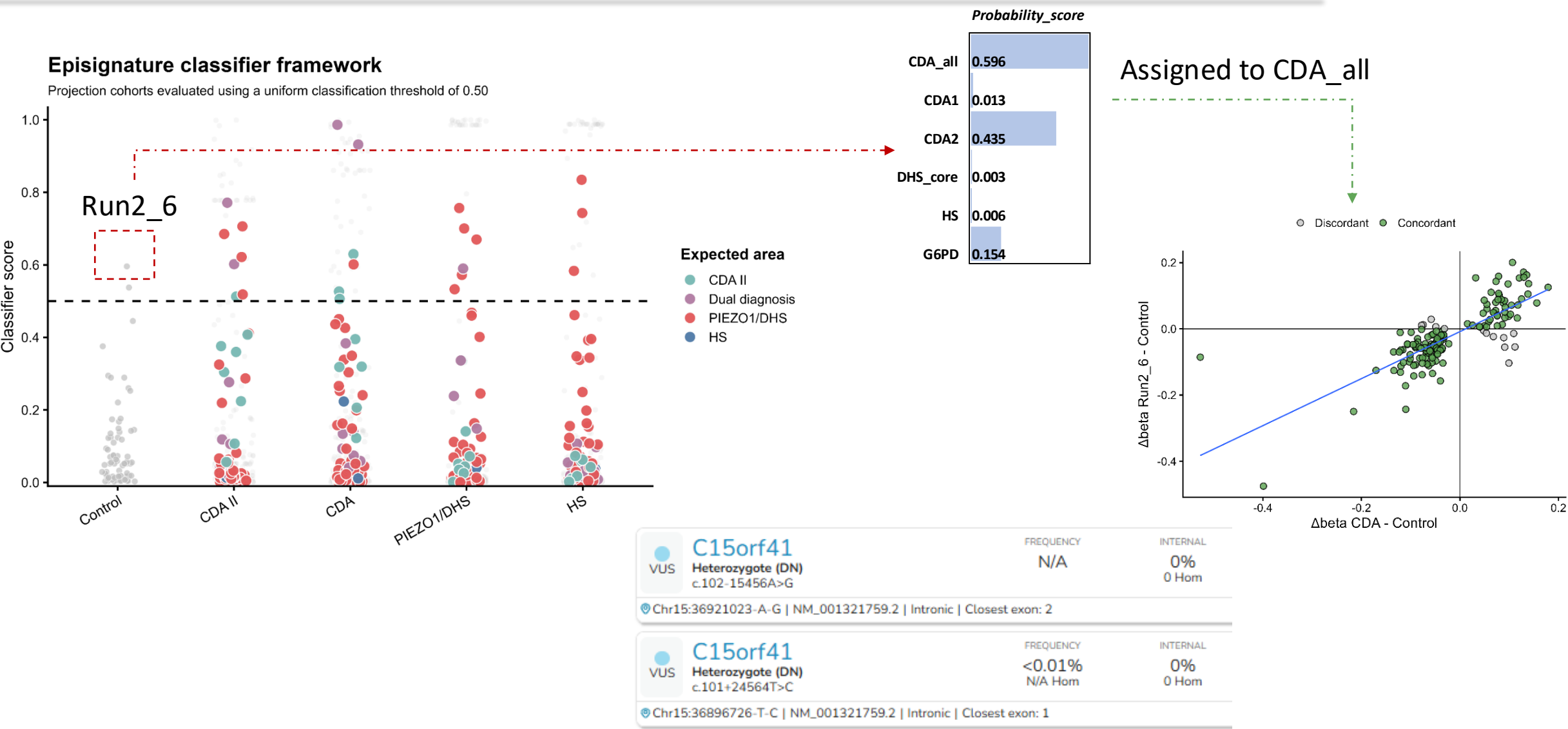


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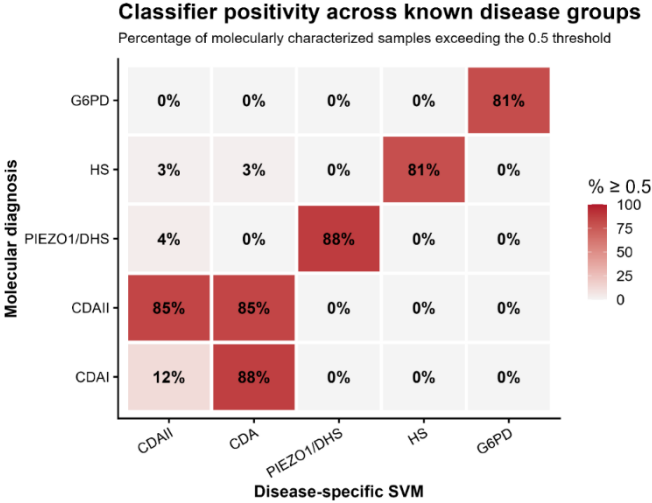
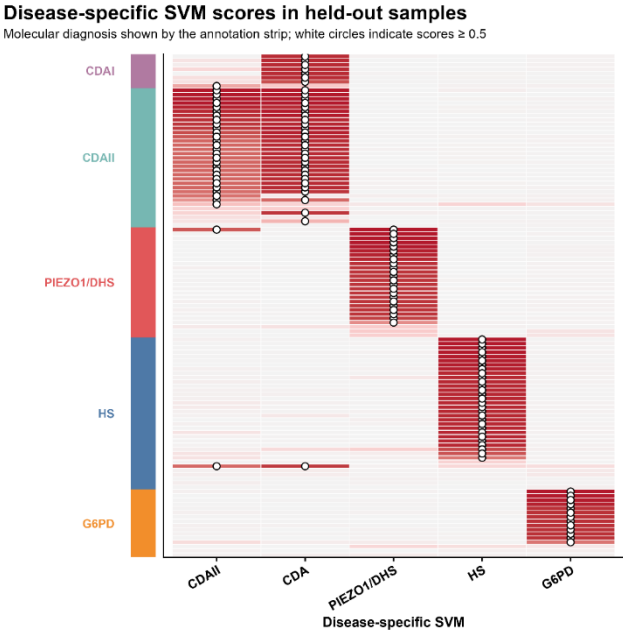
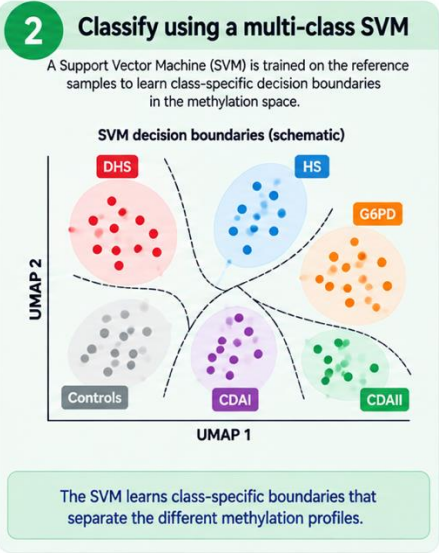
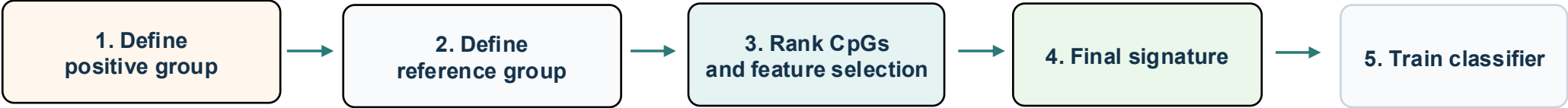


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Application of methylation-based classifier to molecularly unresolved cases



Train classifier



In held-out molecularly characterized samples, the disease-specific SVMs showed **high concordance with the expected molecular diagnosis**, with limited off-target positivity.