San Giuseppe Moscati - Avellino
Azienda Ospedaliera di Rilievo Nazionale e di Alta Specializzazione

Avellino, Hotel de la Ville
March 30-31, 2023

1ST SYMPOSIUM ON INNOVATIVE THERAPIES IN HEMATOLOGY

Molecular bases of congenital dyserythropoietic anemias

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NO CONFLIT OF INTEREST TO DECLARE

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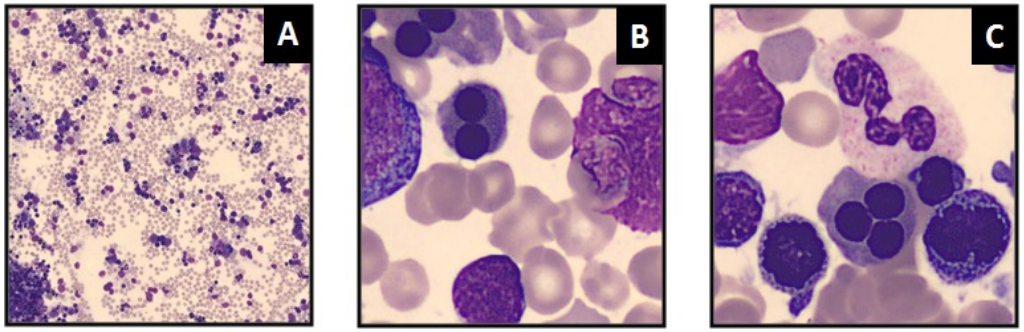
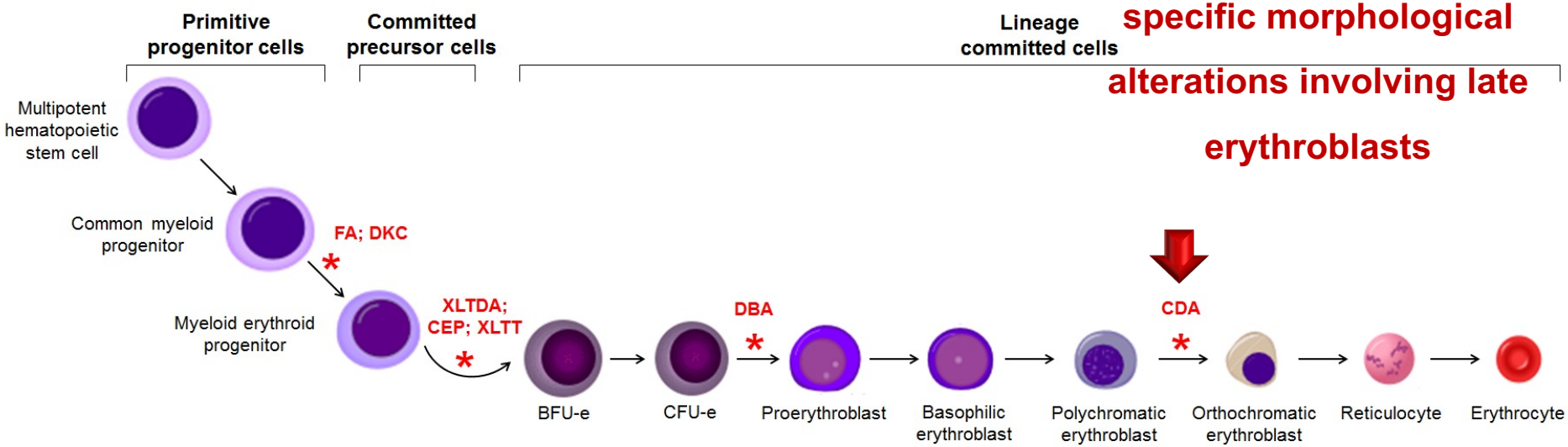
Congenital Dyserythropoietic Anemias



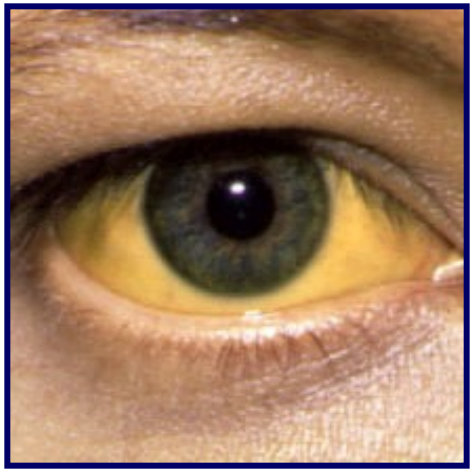
- ✓ **CDAs** are **Mendelian** diseases affecting the normal differentiation-proliferation pathway of the erythroid lineage
- ✓ They belong to a subtype of **bone marrow failure syndromes** characterized by **monolineage** involvement and morphological abnormalities in **erythroid** precursor cells

Erythroid hyperplasia with specific morphological alterations involving late erythroblasts

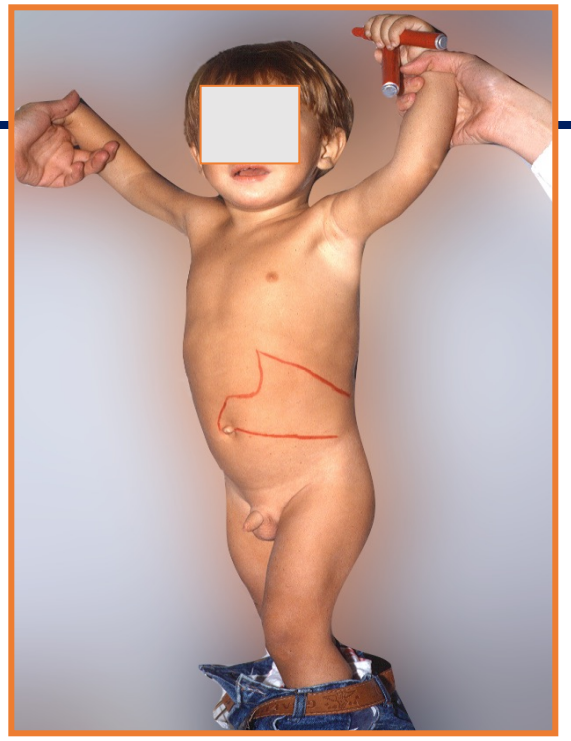


Clinical findings of CDA-s

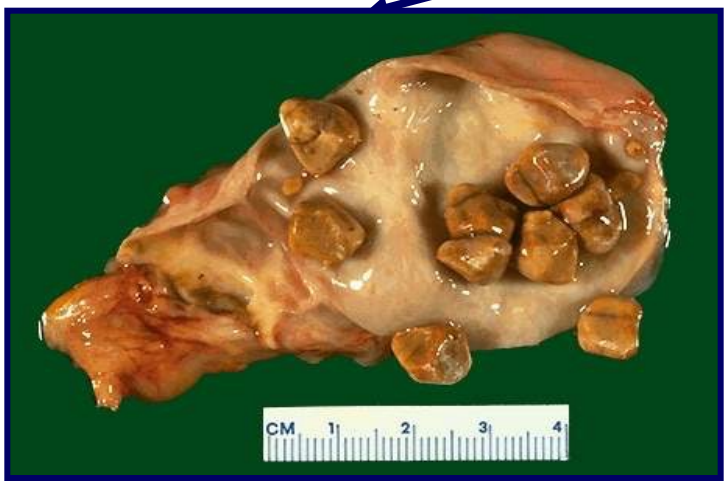
Anemia



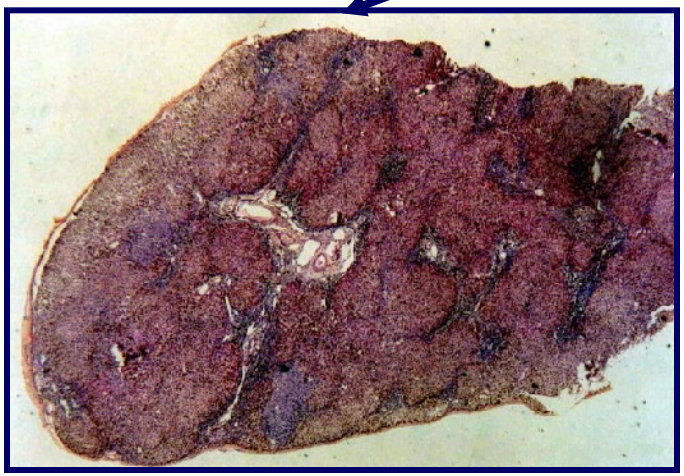
Jaundice



Splenomegaly



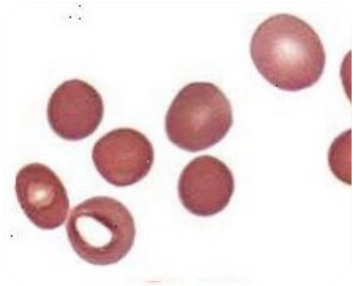
Gallstones



Iron overload

Differential diagnosis

HS



↑ Reticulocyte count

Splenectomy is the standard treatment

Autosomal dominant (75%)

↑ RDW

Normal or slightly increased sTFR

↑ Unc. Bilirubin

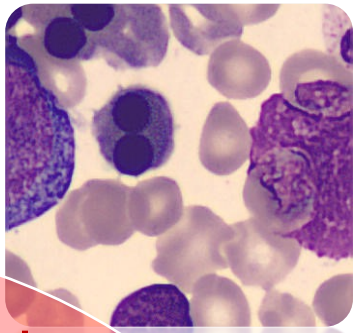
↑ LDH

↓ Haptoglobin

Hepatosplenomegaly

Hemosiderosis

CDA II



Reticulocyte count ↓

Splenectomy slightly increases Hb level

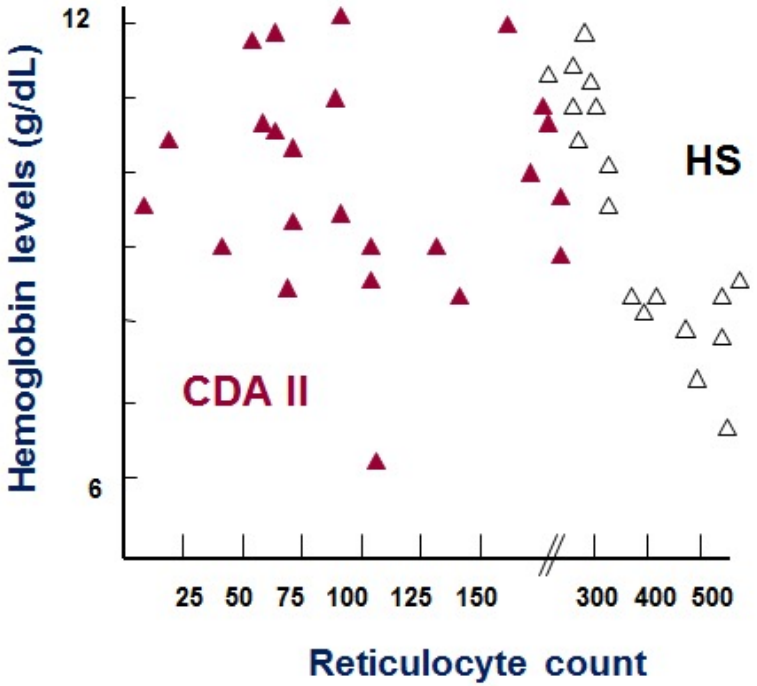
Autosomal recessive

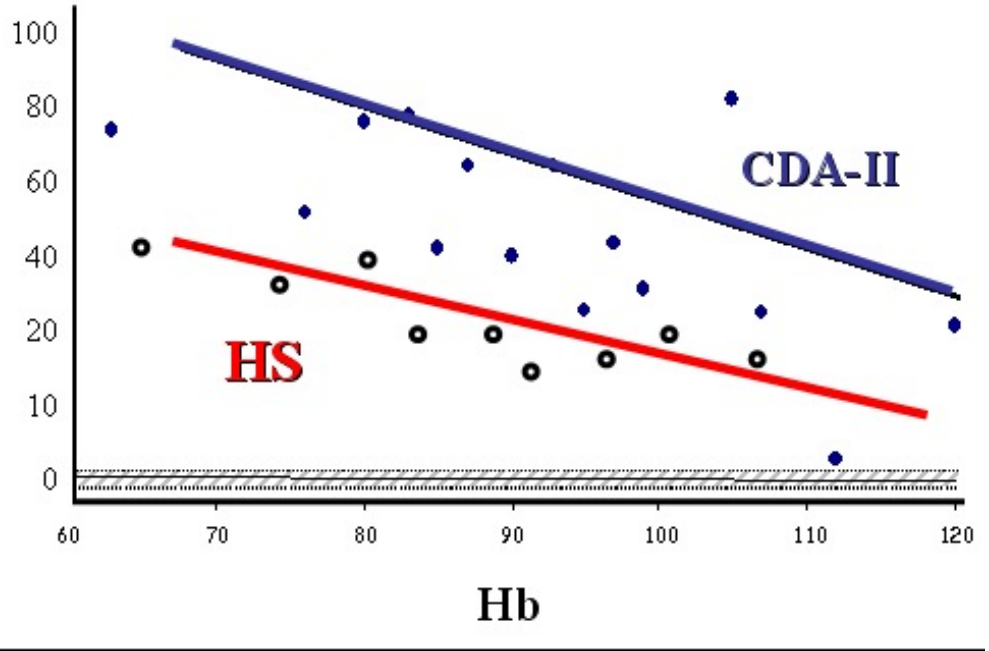
RDW ↑

↑ sTFR

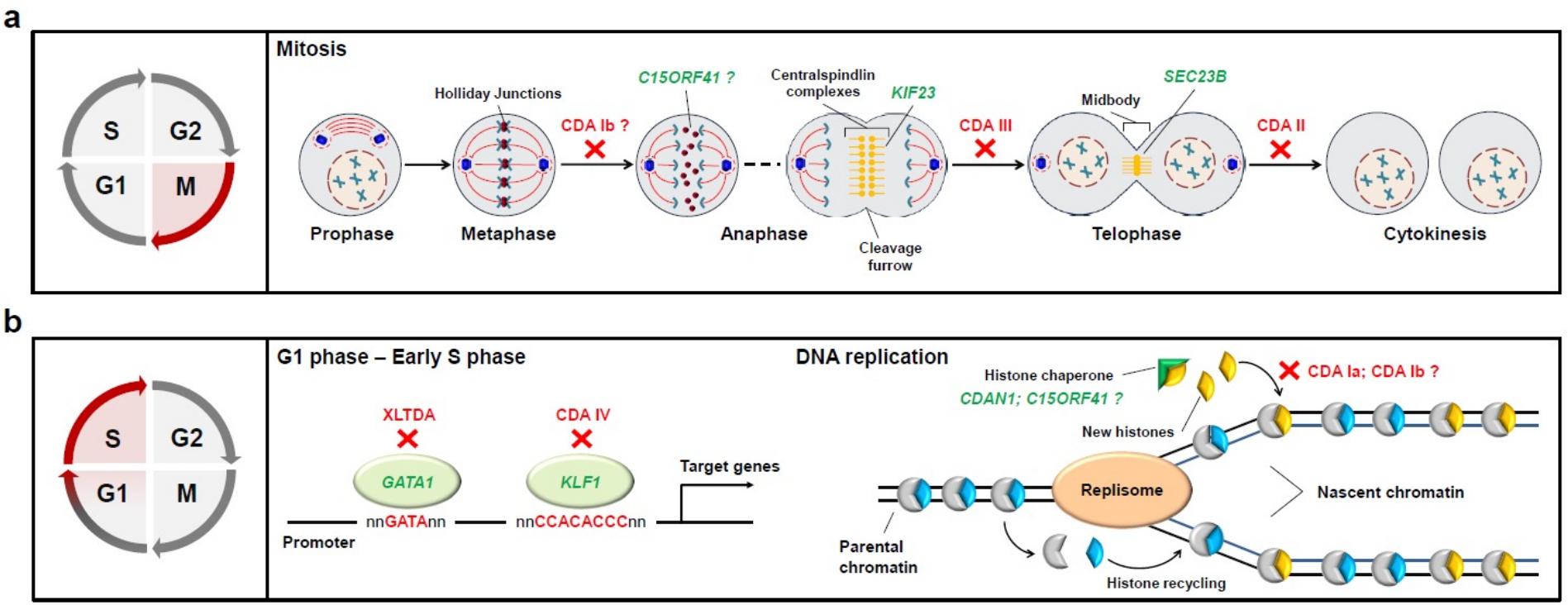
Differential diagnosis

Regression of Hb vs sTfR in 15 subjects affected with CDA II





Pathogenic mechanisms of CDA

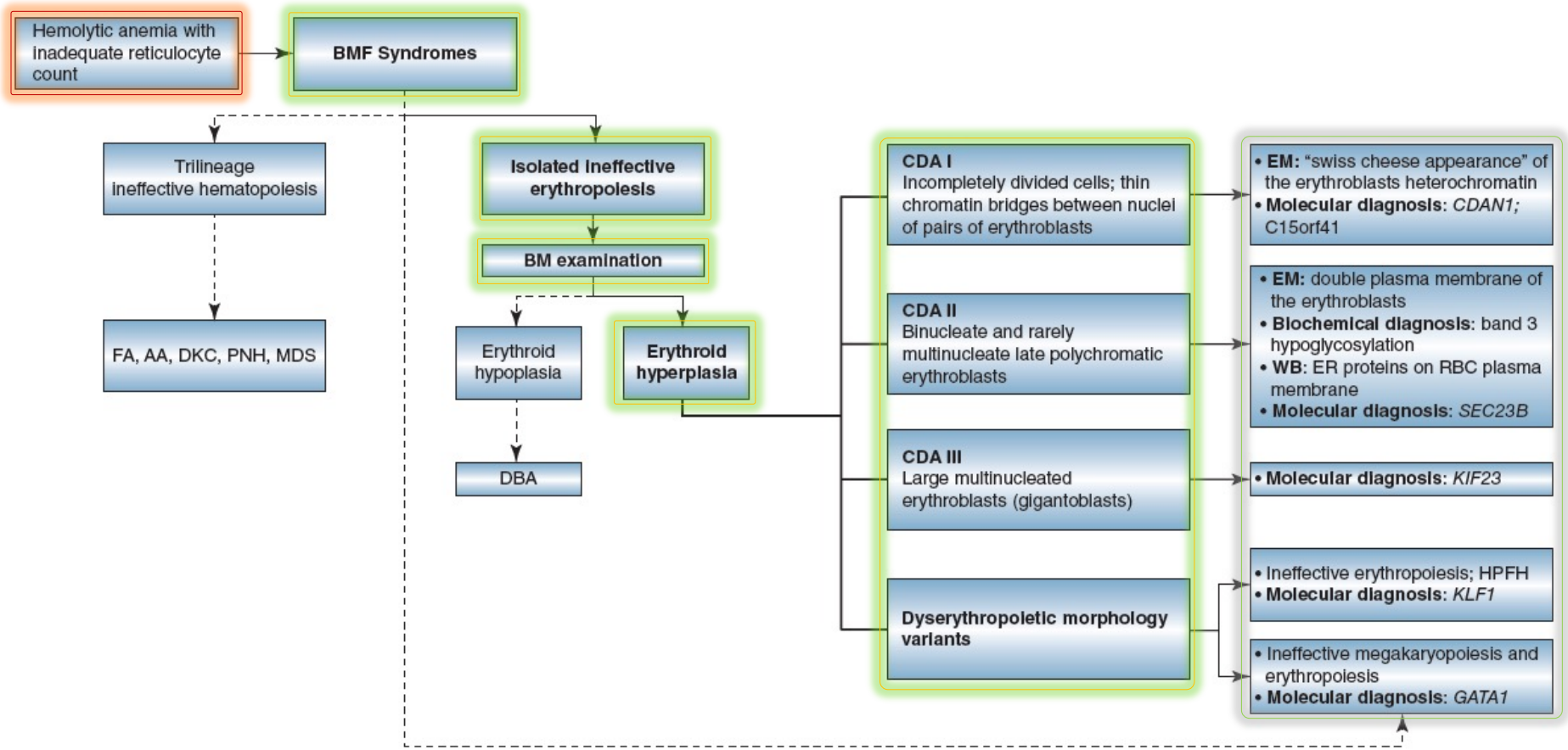


a. The pathogenic mechanisms of CDA II, CDA III, and probably CDA Ib could be due to **deregulation** of mechanisms involved in **cell division**

b. The pathogenic mechanisms of transcription factor-related CDA, as well as of CDA Ia-Ib could be due to **impairment** of mechanisms involved in **DNA synthesis and chromatin assembly**

Gambale A, Iolascon A, Andolfo I, Russo R. Expert Rev Hematol. 2016

Traditional diagnostic workflow for CDAs



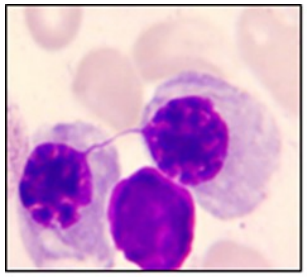
ESH EUROPEAN SCHOOL OF HAEMATOLOGY
An institution for continuing education

Williams Hematology, 9th Edition, Chapter 39 by A. Iolascon - McGraw-Hill

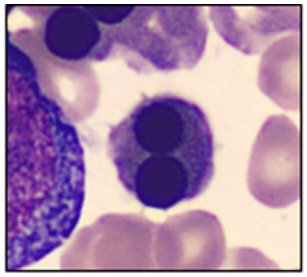
Different subtypes of CDAs

Table 1. Characteristic features of different types of congenital dyserythropoietic anemia

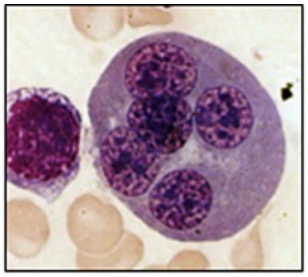
CDA type	I	II	III familial	III sporadic	Variants
Inheritance	Autosomal-recessive	Autosomal-recessive	Dominant	Variable	Autosomal-dominant or X-linked or recessive
Cases reported	> 300	> 450	2 families	< 20	~ 70
Bone marrow morphology (light microscopy)	Abnormal chromatin structure, chromatin bridges	Bi-nuclearity Multinuclearity of mature erythroblasts	Giant multinucleated erythroblasts	Giant multinucleated erythroblasts	CDA I-like CDA II-like others
BM EM findings	"Spongy" hetero-chromatin, invagination of cytoplasm into the nucleus	Peripheral cysternae beneath the plasma membrane	Clefts in hetero chromatin, auto-phagic vacuoles, intranuclear cisternae	various	various
Mutated Gene	CDAN1 C15ORF41	SEC23B	KIF23	Unknown	KLF1 GATA-1 unknown
Associated dysmorphology/organ involvement	Skeleton	Variable, rare	Monoclonal gammopathy, myeloma, angioid streaks	Variable	CNS others



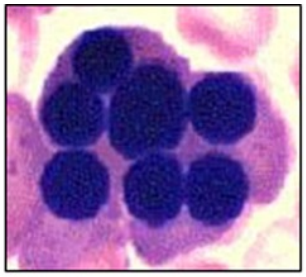
CDA type I



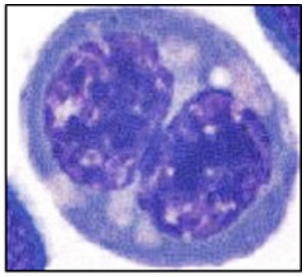
CDA type II



CDA type III familial



CDA type III sporadic

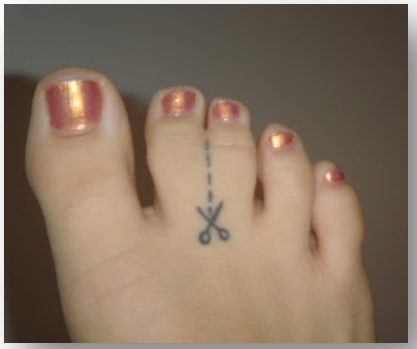


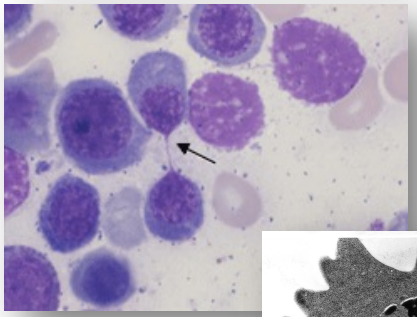
CDA variants

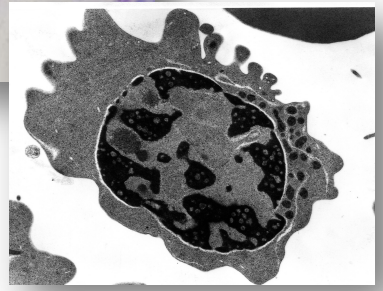
Iolascon A et al, Blood 2013

Main features of CDA I patients

- ✓ Clinical features: Anemia (often ***macrocytic***) with neonatal appearance; jaundice; splenomegaly; common complication: hemosiderosis
  → **Morphologic body abnormalities** (**20%**): skeletal malformations, syndactyly in hands or feet, absence of nails, or supernumerary toes
- ✓ Morphology: **Megaloblastoid erythroid hyperplasia;** nuclear bridges (BM)
  → EM: spongy-appearing nuclei and invagination of the cytoplasm in the nucleus
- ✓ Inheritance: Autosomal recessive
  Locus: 15q15.2 → ***CDAN1*** **(CDA Ia)**
  Locus: 15q14 → ***C15ORF41*** **(CDA Ib)**







Therapy: - Transfusion
- IFN
- Iron chelation
- BMT
- Gene therapy ?

Roy NB, Babbs C. BJH 2019

Main features of CDA II patients

✓ Clinical features:

- Average age of onset symptoms: 3.7 ± 0.6 y
- Mean age of diagnosis: 22.2 ± 1.7 y
- Normocytic mild anemia: **Hb 9.6** ± 0.2 g/dL with **MCV 87.3** ± 1.0
- Reticulocyte index: 1.7 ± 0.1
- Mean serum ferritin: 464.8 ± 55.9 ng/mL
- Splenomegaly: 102/122, **83.6% of patients**
- Transfusion dependency: 25/126, **19.8% of patients**

✓ Biochemical features:

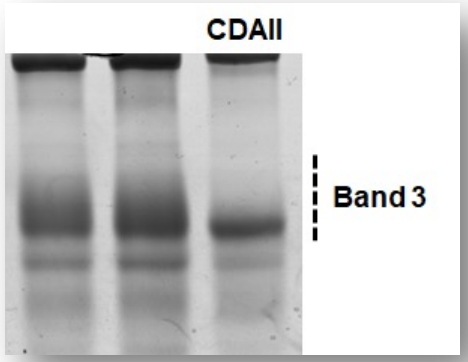
- Hypoglycosylation of band 3 at SDS-PAGE: **95.1% of patients**

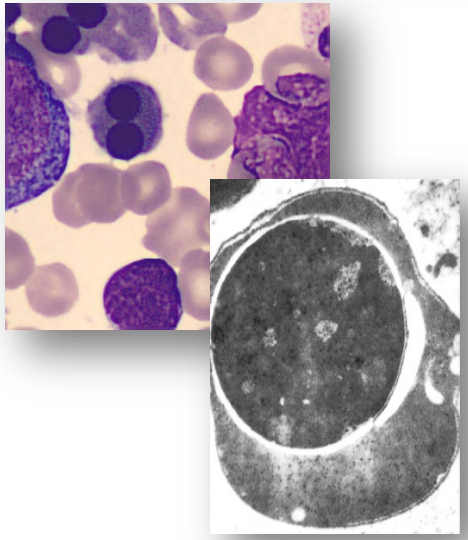
✓ Morphology: erythroid hyperplasia;
bi-nucleated erythroblasts **> 10%**

→ EM: double-membrane appearance

✓ Inheritance: Autosomal recessive

Locus: 20p11.23 → ***SEC23B***





Therapy: - Transfusion
- Iron chelation
- Splenectomy
- Luspatercept
- BMT
- Gene therapy ?

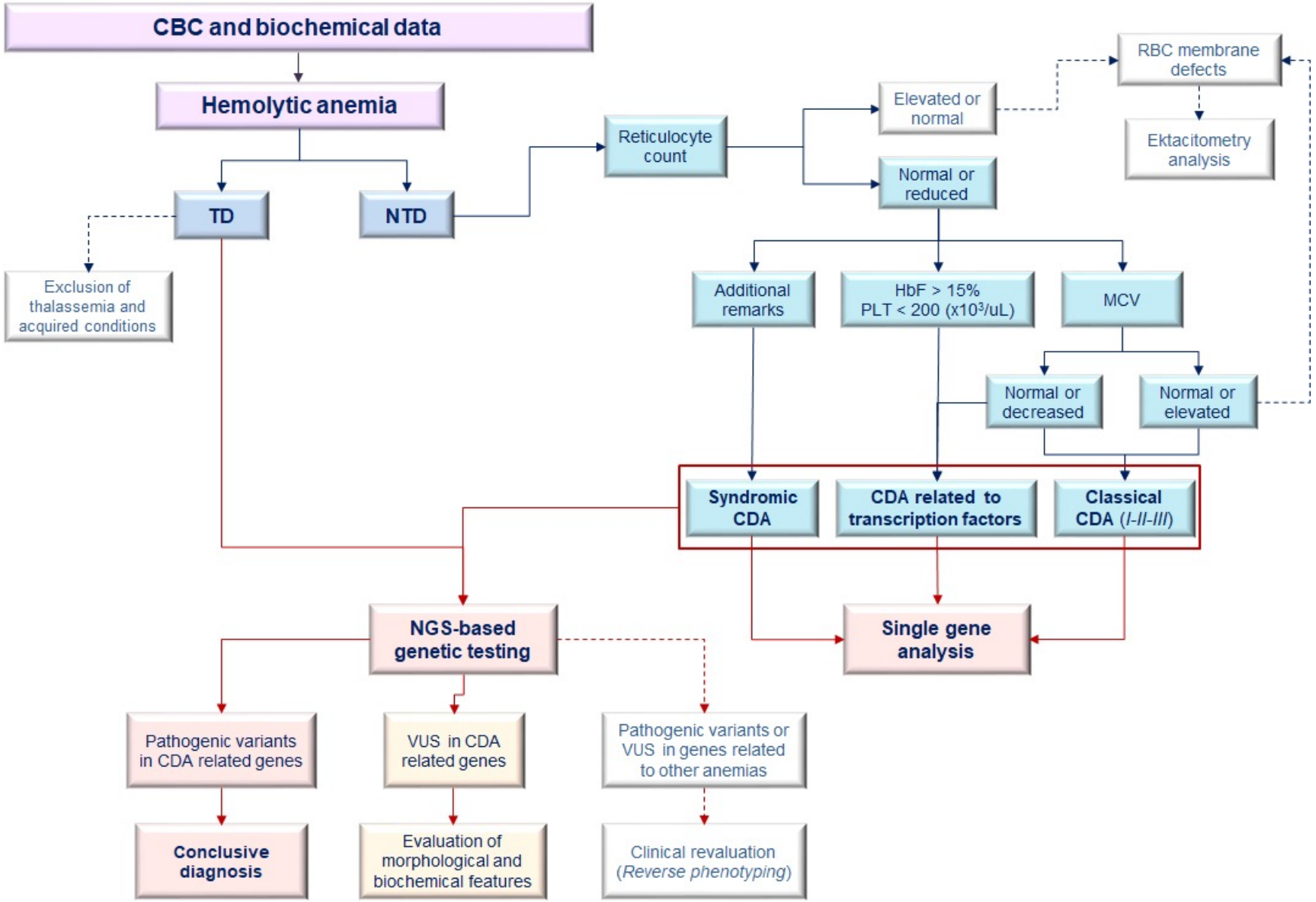
Russo R. et al. Am J Hematol. 2014

CDA variants

Disease symbol	Gene Inheritance	Main clinical features	Bone marrow morphological features
CDA IV	*KLF1* Autosomal dominant	Hemolytic anemia, generally severe, with normal or slightly increased reticulocyte count, and markedly elevated fetal hemoglobin levels	Erythroid hyperplasia with bi- or multi-nucleated erythroblasts; immature erythroid progenitors with atypical cytoplasmic inclusions, invagination of the nuclear membrane, and marked heterochromatin
XLTDA	*GATA1* X-linked recessive	Macro-thrombocytopenia, bleeding tendency, and mild-to-severe anemia	Erythroblasts with megaloblastic features, bi- and multi-nucleation, and nuclear irregularities; small dysplastic megakaryocytes with signs of incomplete maturation and reduced number of alpha granules
MJDS	*LPIN2* Autosomal recessive	Hypochromic microcytic anemia; chronic recurrent multifocal osteomyelitis and inflammatory dermatosis	Microcytosis and dyserythropoiesis
EIEE50	*CAD* Autosomal recessive	Autism, developmental delay, and generalized epilepsy; mild CDA II-like anemia with marked anisopoikilocytosis and abnormal glycosylation of the erythrocyte proteins band-3 and RhAG	Erythroid hyperplasia with dyserythropoiesis, bi- and tri-nucleated erythroblasts, prominent cytoplasmic bridging
-	*VPS4A* De novo autosomal dominant	Microcephaly, hypotonia, global developmental delay, structural brain abnormalities, cataracts; hemolytic anemia	Dyserythropoiesis with bi-nucleated erythroblasts and cytoplasmic bridges
-	*ALAS2* X-linked dominant	Macrocytic anemia with iron overload in female individuals	Erythroid hyperplasia with dyserythropoiesis; rare erythroblasts with siderotic granules (no excess iron or sideroblasts)
-	*COX4I2* Autosomal recessive	Exocrine pancreatic insufficiency, dyserythropoietic anemia, and calvarial hyperostosis	Erythroid hyperplasia with dyserythropoiesis
MEVA	*MVK* Autosomal recessive	Mevalonate kinase deficiency associated to CDA II-like anemia	CDA II-like morphological abnormalities of erythroblasts

CDA IV, CDA type IV; XLTDA, X-linked thrombocytopenia with or without dyserythropoietic anemia; MJDS, Majeed syndrome; EIEE50, early infantile epileptic encephalopathy-50; MEVA, mevalonic aciduria.

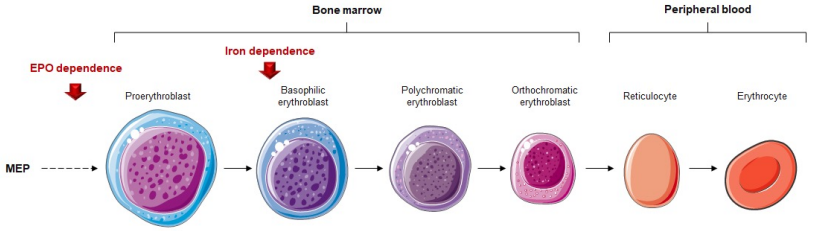
New diagnostic workflow for CDA



Iolascon A et al. Blood 2020 ,

Genetic and phenotypic heterogeneity of H-RBCDs

✓ > 100 genes associated to RBCDs



Transcription regulation
GATA1
KLF1

Ribosome
RPS19 RPS29 RPS28
RPS24 RPS7 RPL9
RPS26 RPS15 RPL18
RPL5 RPS27a RPL26
RPL11 RPS27 RPL27
RPL35a RPS17 RPL31
RPS10 RPL15 TSR2

Cytokines & receptors
EPO
EPOR

DNA replication & cell division
CDNA1
CDIN1
SEC23B
KIF23

Cytoskeletion & transporters
ANK1 SLC4A1
SPTA1 PIEZO1
SPTB KCNN4
EPB41 RHAG
EPB42 ATP11C

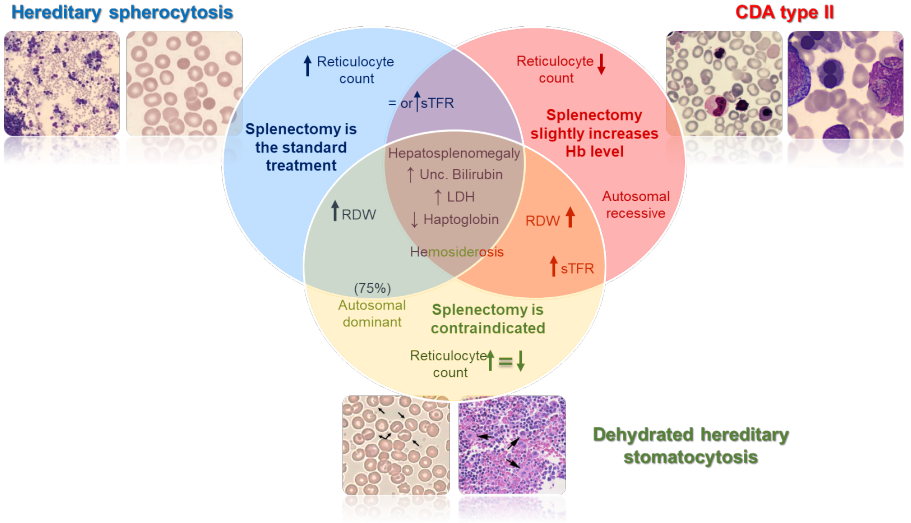
Hb synthesis
HBA1
HBA2
HBB
HBG1
HBG2

Heme synthesis
ALAS2
GLRX5
HSPA9
PUS1
SLC25A38
TRNT1
YARS2

Enzymes
G6PD GSS
PKLR MVK
HK1 PGK1
AK1 NT5C3A
GCLC TPI1
GPI

Iron
SLC11A2
STEAP3
TMPRSS6

✓ Overlapping clinical features



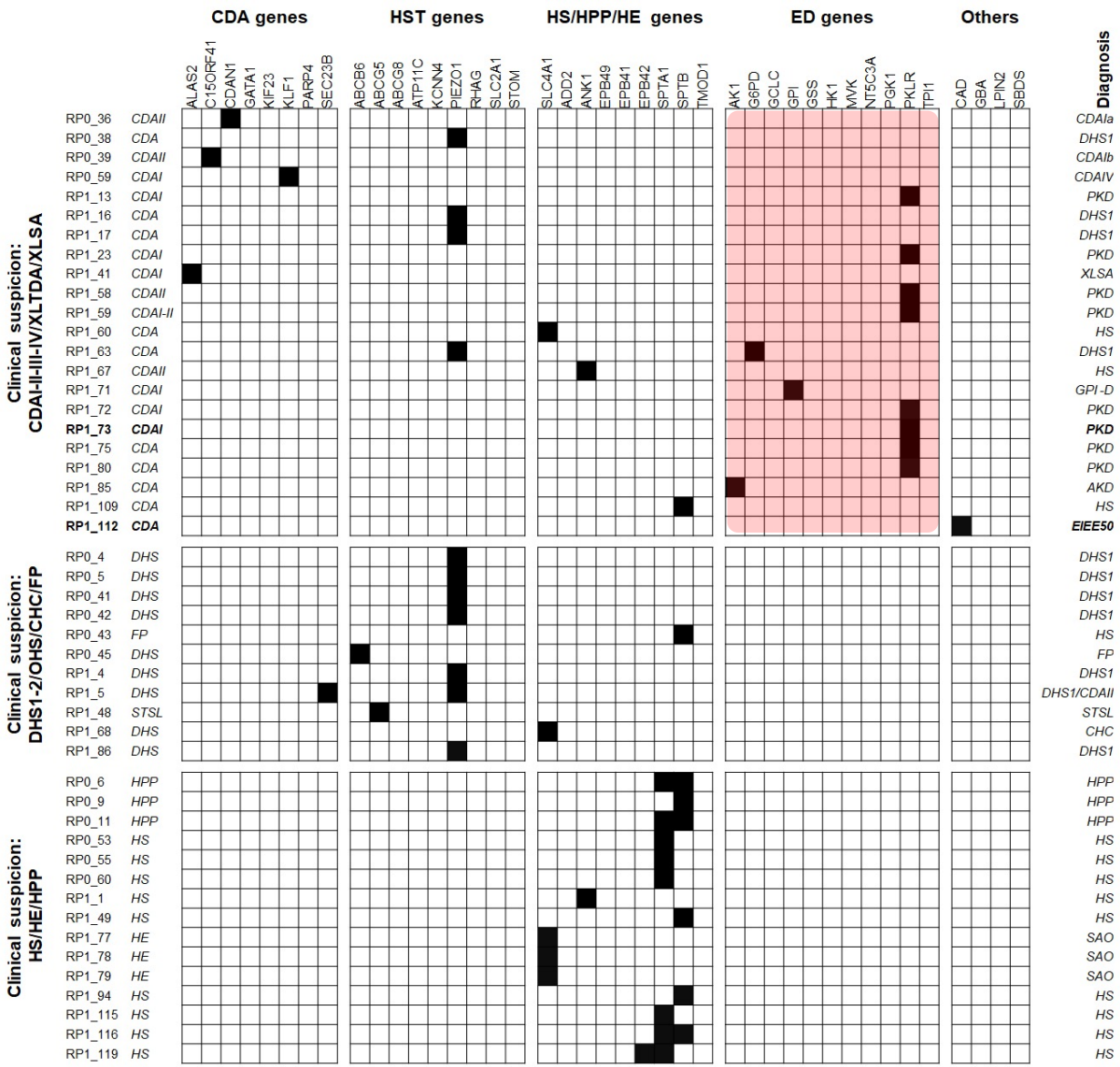
NGS-based genetic testing of rare hereditary anemias

- ✓ Several targeted-NGS panels for differential diagnosis of HA have been generated:

N° genes	N° patients	N° families	HA subtypes	Diagnostic yield (%)	Reference
33	57	57	CDA; DBA; Sideroblastic Anemia; RBC enzymatic defects	38.6	Roy NB, et al. BJH 2016
40	10	10	RBC membrane defects; CDA; RBC enzymatic defects	90.0	Del Orbe Barreto R, et al. Int J Lab Hematol 2016
28	15	15	RBC membrane defects; RBC enzymatic defects	86.7	Agarwal AM, et al. BJH 2016
600	10	3	RBC membrane defects	100.0	He Y, et al. Gene 2017
71	74	62	RBC membrane defects; CDA; DBA; RBC enzymatic defects	64.9	Russo R, et al. AJH 2018
43	59	59	RBC membrane defects; RBC enzymatic defects; HA modifiers	84.7	Choi HS, et al. Orphanet J Rare Diseases 2019

- ✓ The overall diagnostic yield obtained by these panels ranges between 35% and 65%

NGS-based genetic testing of HA



- ✓ The multi-gene approach modified the original diagnosis in **45.8%** of patients (**non-matched phenotype-genotype**)
- ✓ 81.8% of **non-matched patients** were clinically suspected to suffer from **CDA**

Russo R, et al. Am J Hematol. 2018

➢ **36.4%** of **CDA** patients within our cohort exhibited mutations in ***PKLR*** gene → overlapping phenotypes among these disorders

TABLE 3 Clinical features of CDA patients conclusively diagnosed as PK deficiency

	RP1_13	RP1_23	RP1_58	RP1_59	RP1_72	RP1_73	RP1_75	RP1_80
Age (years)	1.4	5.2	2.0	1.7	7	0.8	1.6	14
Onset symptoms (years)	At birth	Neonatal	Neonatal	At birth	4	At birth	At birth	At birth
Gender	Male	Female	Male	Male	Female	Female	Male	Male
Ethnicity	Turkish	Turkish	Colombian	Turkish	Italian	Turkish	Hungarian	Venezuelan
Complete blood count								
RBC (10^6/μL)	2.1	2.9	2.6	2.9	3.2	1.7	1.7	2.7
Hb (g/dL)	6.8	7.7	7.6	7.9	9.6	5.5	6.1	9.5
Ht (%)	18.0	23.4	21.6	23.3	29	15.8	17.5	32
MCV (fL)	104.9	80.6	82.0	81.2	89.6	90.1	103.6	117.8
MCH (pg)	32.5	26.1	29.0	28.1	33	31.4	35.3	35.2
MCHC (g/dL)	-	32.4	35.0	34.4	36.8	34.9	34.3	29.9
RDW (%)	-	13.7	14.0	13.2	-	14.9	16.7	18.2
PLT (10^3/μL)	387.0	287.0	361.0	276.0	295	284	362	1010
Retics %	0.6	0.1	3.2	1.8	7.2	2.0	8.56	18.2
Retics abs count (x10^3/μL)	12.8	3.8	83.5	51.5	23.3	35.2	144.7	215.0
Transfusion rate	8/year	7–8/year	25/year	12/year	-	6/year	10/year	12/year
Bone marrow examination	Erythroid hyperactivity, 10% double nucleated normoblasts (asymmetric nuclei)	Hypercellular with megaloblastic changes in erythroid cells	-	Erythroid hyperactivity, megaloblastic elements (bi- and multi-nucleated with internuclear bridges)	Erythroid hyperactivity with dyserythropoiesis, mostly bi- and multi-nucleated with internuclear bridges	Normoblasts with double nuclei and internuclear bridges	Hypercellular with megaloblastic changes and bi-nucleated normoblasts	Erythroid hyperactivity with dyserythropoiesis
Laboratory data								
Total bilirubin (mg/dL)	1.7	1.9	3.7	6.1	5.6	3.5	2.2	7
Unconjugated bilirubin (mg/dL)	0.5	1.5	3.1	5.4	5	3.1	2.1	6.3
Ferritin (ng/mL)	554	2554	1042	389	132	-	198	238
PKLR molecular analysis								
HGVS (coding[a]; protein; status)	c.1349A>G; p.Asp450Gly; Hom	c.1117-1G>C; Hom	c.1116 + 2T>G; Hom	c.67_68delTA; p.Leu23Cysfs* 55c.287C>A; p.Pro96Gln; Comp het	c.1492C>T; p.Arg498Cysc. 994G>A;p. Gly332Ser Comp het	c.353A>G; p.Asn118Ser; Hom	c.1594C>T; p.Arg532Trpc. 1529G>A; p.Arg510Gln; Comp het	c.1528C>T; p.Arg510Ter; Hom

Hom, homozygous; Comp het, compound heterozygous.
[a]Reference Transcript ID: NM_000298.

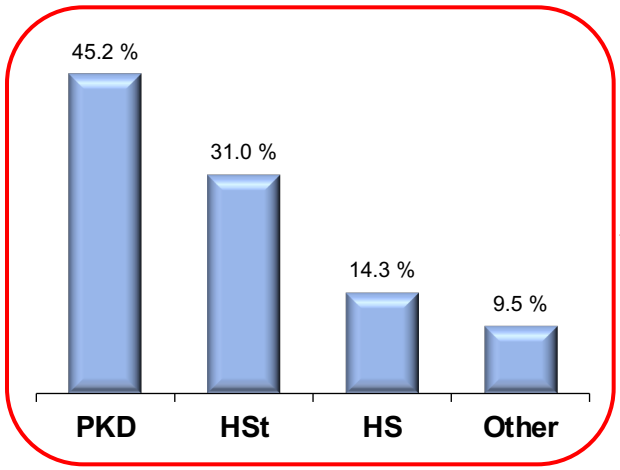
- Bone marrow features mostly resembling those of CDA I patients
- TD patients → enzymatic assay is not reliable

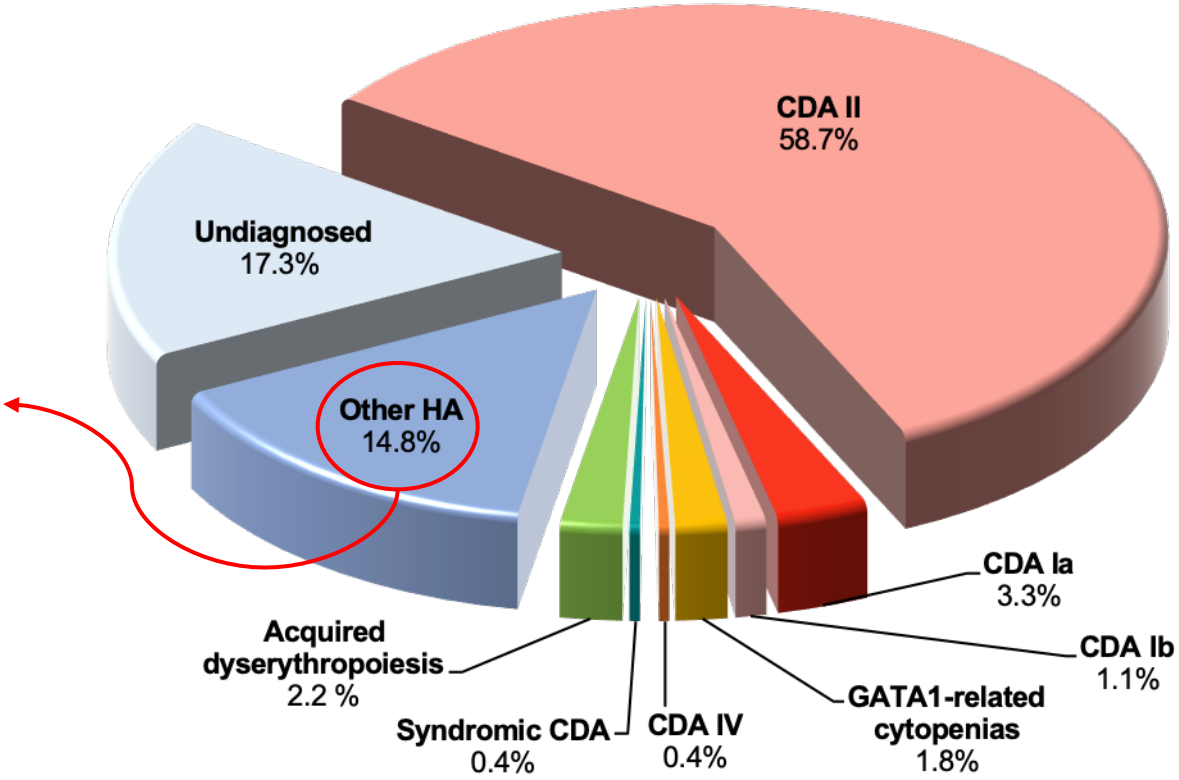
Russo R, et al. Am J Hematol. 2018

Different sub-types of congenital dyserythropoietic anemias

- ✓ CDA patients enrolled by the Medical Genetics Unit of Naples:

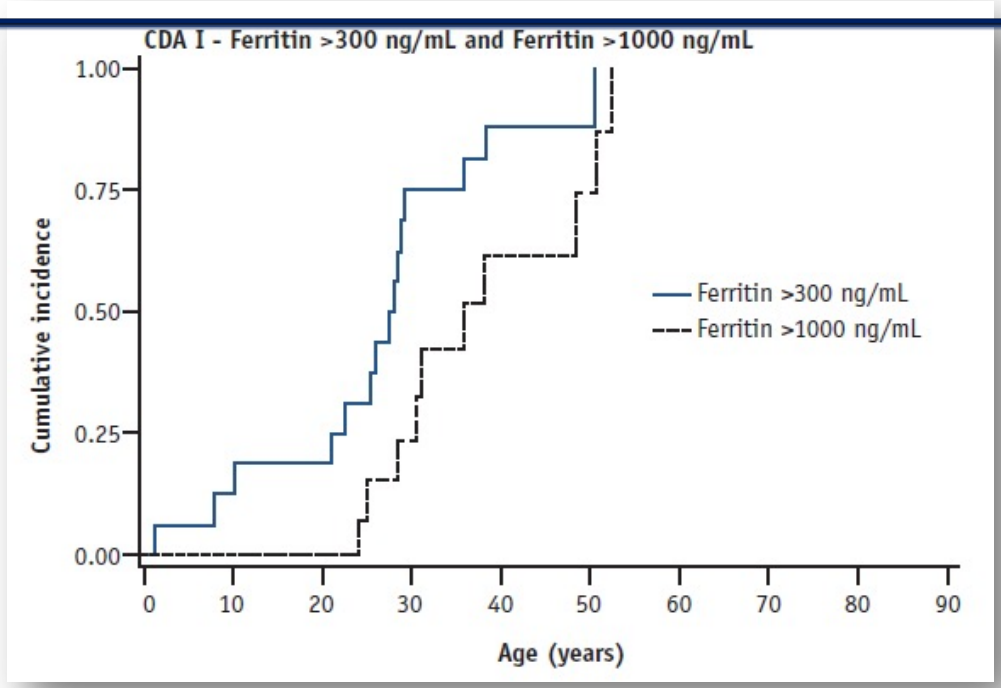
  271 affected subjects





Iolascon A, Russo R, Andolfo A.- Blood 2020

Iron overload in CDAs

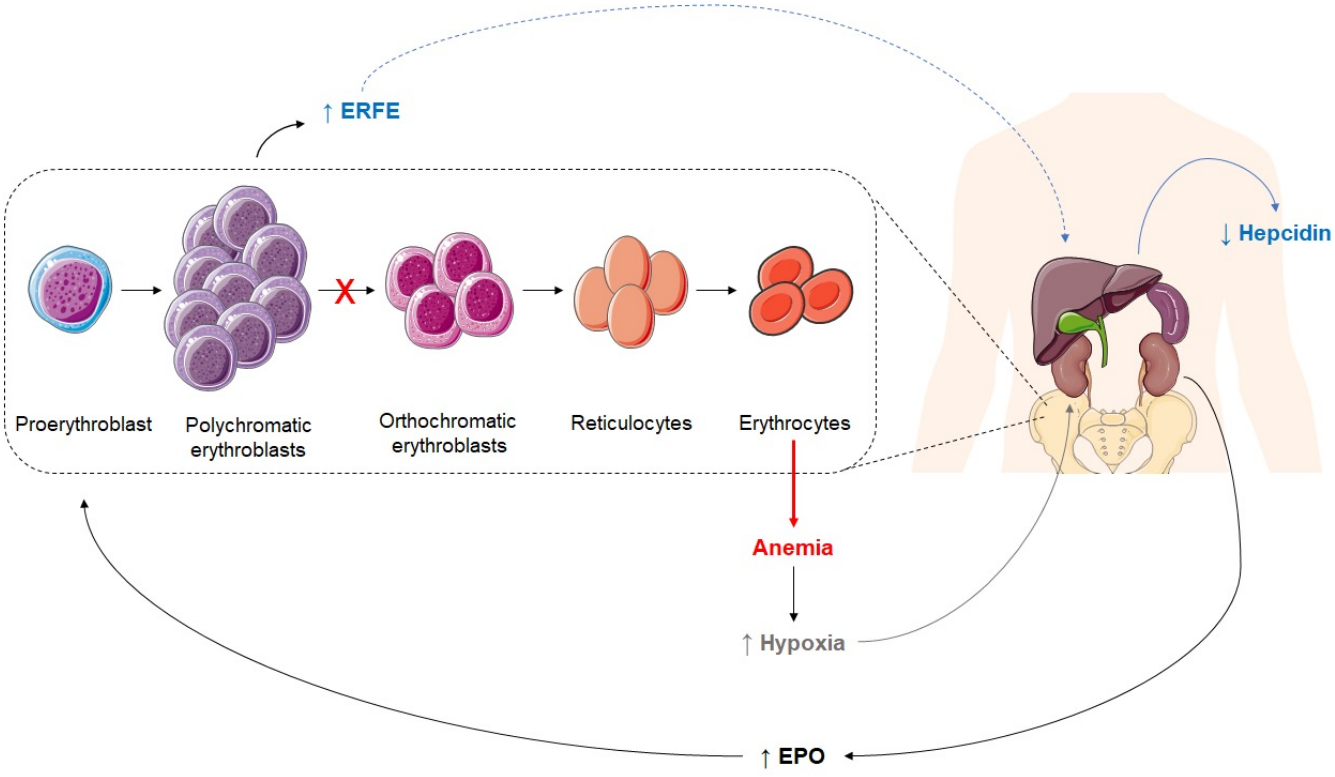


Almost all CDA I and II patients accumulate iron with a steady increase in ferritin values during their life

CDA I and II are hallmarked by **ineffective erythropoiesis**, **iron overload**, and **reduced expression of** hepatic hormone **hepcidin**

Physiopathology of CDAs (systemic level)

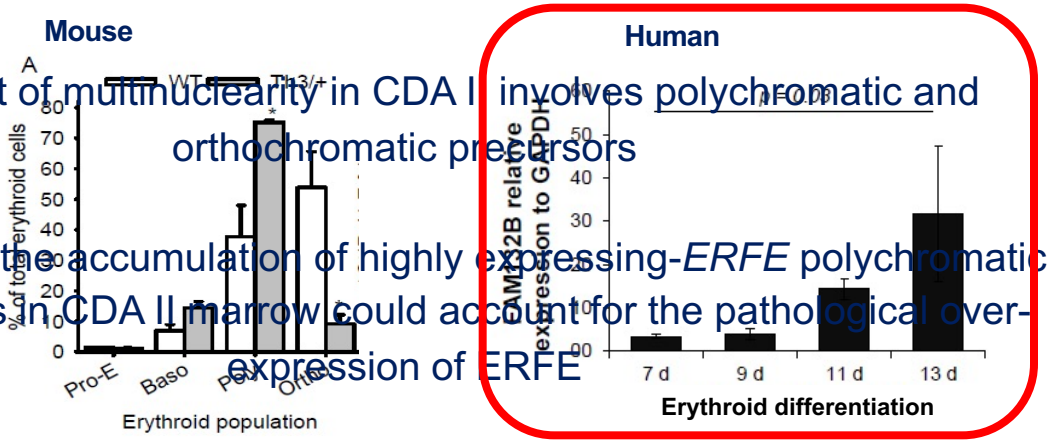
✓Anemia with reduced reticulocyte count



✓ **EPO** is not able to increase the production of RBCs

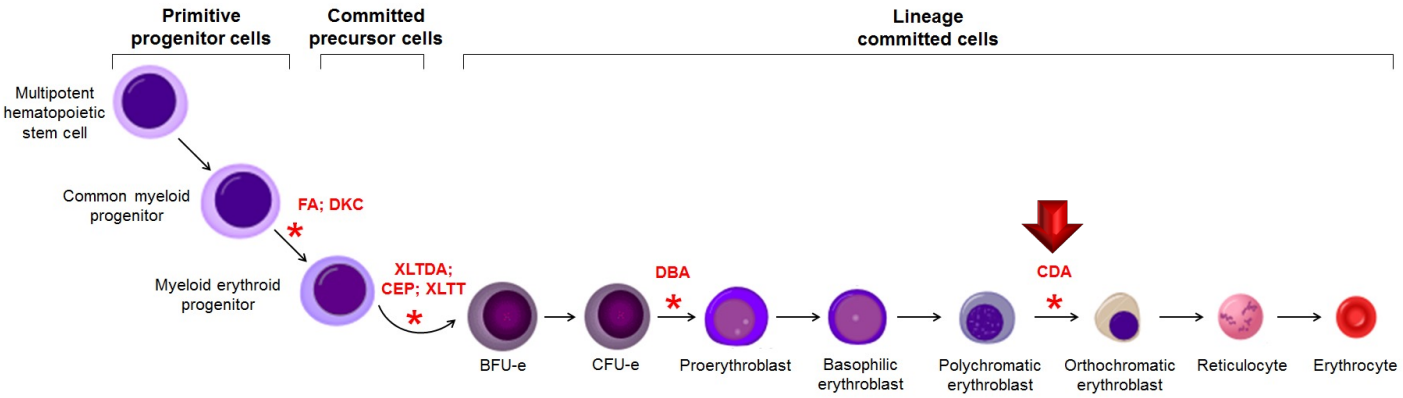
Iolascon A, Andolfo A, Russo R. Blood 2020

Polychromatic erythroblasts are the main source of ERFE in human and mice



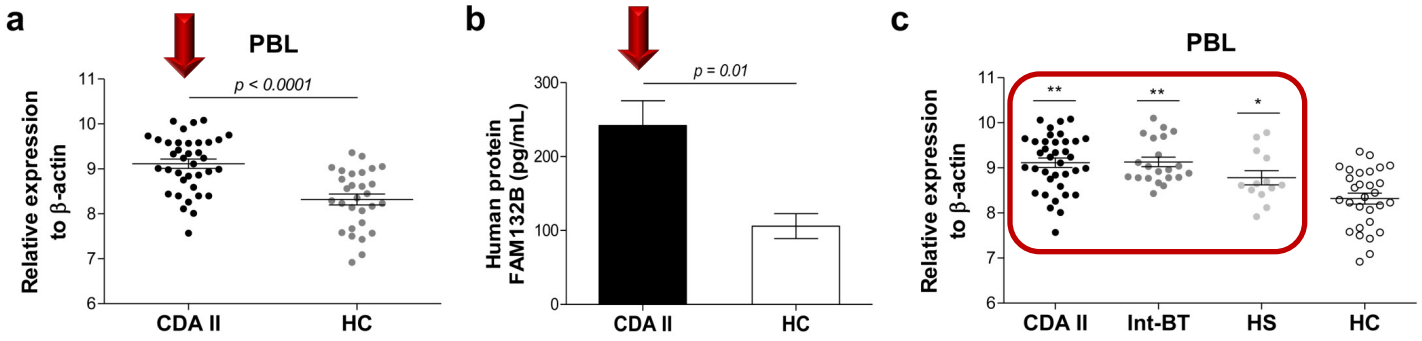
The onset of multinuclearity in CDA I involves polychromatic and orthochromatic precursors

Hypothesis: the accumulation of highly expressing-*ERFE* polychromatic erythroblasts in CDA II marrow could account for the pathological over-expression of ERFE



Williams Hematology, Nineth Edition, Chapter 39 by A. Iolascon - McGraw-Hill

Increased levels of ERFE-encoding FAM132B in patients with Congenital Dyserythropoietic Anemia type II



**p<0.0001; *p<0.05 vs HC group

- ✓ CDAII patients exhibit over-expression of ERFE at both gene and protein level
- ✓ β-thalassemia (BT)-intermedia patients, exhibiting iron overload likewise for CDAII patients, show over-expression of *ERFE*

These data suggested that the marked increased ERFE expression observed in both CDAII and BT-intermedia patients is mainly due to the ineffective erythropoiesis

Russo R et al. Blood 2016

SEC23B Loss-of-Function Suppresses Hepcidin Expression by Impairing Glycosylation Pathway in Human Hepatic Cells

Table 1. CDA II patients enrolled in the study.

Analysis	Units	Chronic Anemia		*p*-Value †	Reference Range
		Mild Hb ≥ 10.0 g/dL	Moderate/Severe Hb < 10.0 g/dL		
N		10	18	-	-
Gender	male/female	3 (0.3)/7 (0.7)	10 (0.6)/8 (0.4)	0.19	-
Age at sampling	years	28.6 ± 4.3	17.0 ± 5.7	0.19	-
Hemoglobin	g/dL	10.9 ± 0.2	8.6 ± 0.3	0.00001	11.5–15.5
ARC	$\times 10^3/\mu L$	51.5 ± 6.0	77.7 ± 14.7	0.21	20–90
TSAT	%	90.5 ± 6.5	76.7 ± 4.6	0.09	15–39
hERFE	ng/mL	40.5 ± 11.9	43.2 ± 9.3	0.86	0.1–3.8
EPO	mIU/mL	51.6 ± 11.1	151.1 ± 27.4	0.01	3.1–14.9
sTfR	mg/L	3.8 ± 0.5	4.2 ± 0.5	0.59	0.78–1.89
Hepcidin	nM	5.6 ± 2.3	6.2 ± 1.9	0.85	male: 40.10 female: 23.27
Hepcidin/ferritin	-	0.02 ± 0.01	0.03 ± 0.01	0.46	-
Ferritin	ng/mL	559.7 ± 234.4	369.2 ± 115.2	0.42	22.0–275.0
Ferritin/age §	-	20.5 ± 6.9	57.9 ± 19.9	0.21	-

ARC, absolute reticulocyte count; TSAT, transferrin saturation; sTfR, soluble transferrin saturation; EPO, erythropoietin; hERFE, human erythroferrone. Quantitative variables data are presented as mean ± SEM. Qualitative variables data are presented as n (%)/n (%); † Student's *t*-test for quantitative unpaired data; chi-square tests for categorical data. § Normalization of ferritin using "Ferritin level/dosage age ratio," as described by [27].

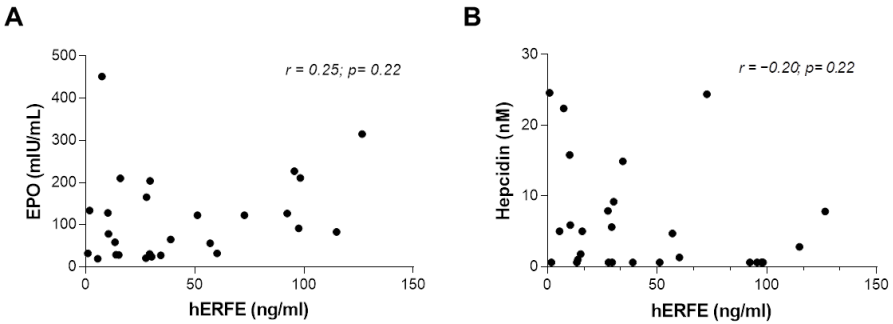
- ✓ 28 patients with **iron overload** (TSAT > 45%)
- ✓ Patients stratified according to the degree of anemia:
  - i. mild (Hb ≥ 10.0 g/dL)
  - ii. moderate/severe (Hb < 10.0 g/dL)

Overall patients exhibited

- reduced **hepcidin**
- high **ferritin** levels
- increased **EPO**

However, **no significant correlation between ERFE, hepcidin and EPO levels** was observed

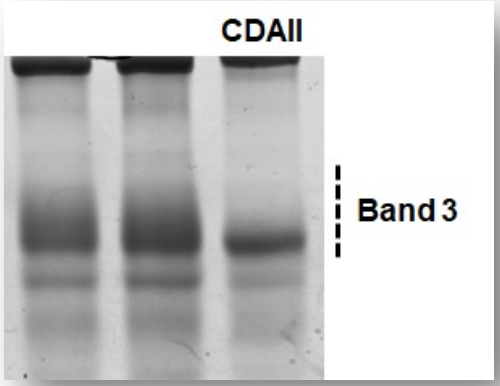
ERFE levels were inadequate to explain hepcidin suppression in CDA II patients



Rosato BE, Marra R, D'Onofrio V, Del Giudice F, Della Monica S, Iolascon A, Andolfo I, Russo R. IJMS 2022.

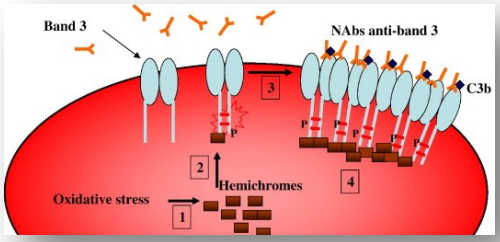
SEC23B Loss-of-Function Suppresses Hepcidin Expression by Impairing Glycosylation Pathway in Human Hepatic Cells

- ✓ Biochemical studies have shown that **CDAII** is **associated** with **reduced glycosylation** activity
- ✓ Erythrocytes presented a band 3 that was thinner than usual and also **migrated slightly faster** on SDS-PAGE
- ✓ Abnormalities in *SEC23B* **disturb** the **endoplasmic reticulum** to **Golgi trafficking** and **affect** different **glycosylation pathways**

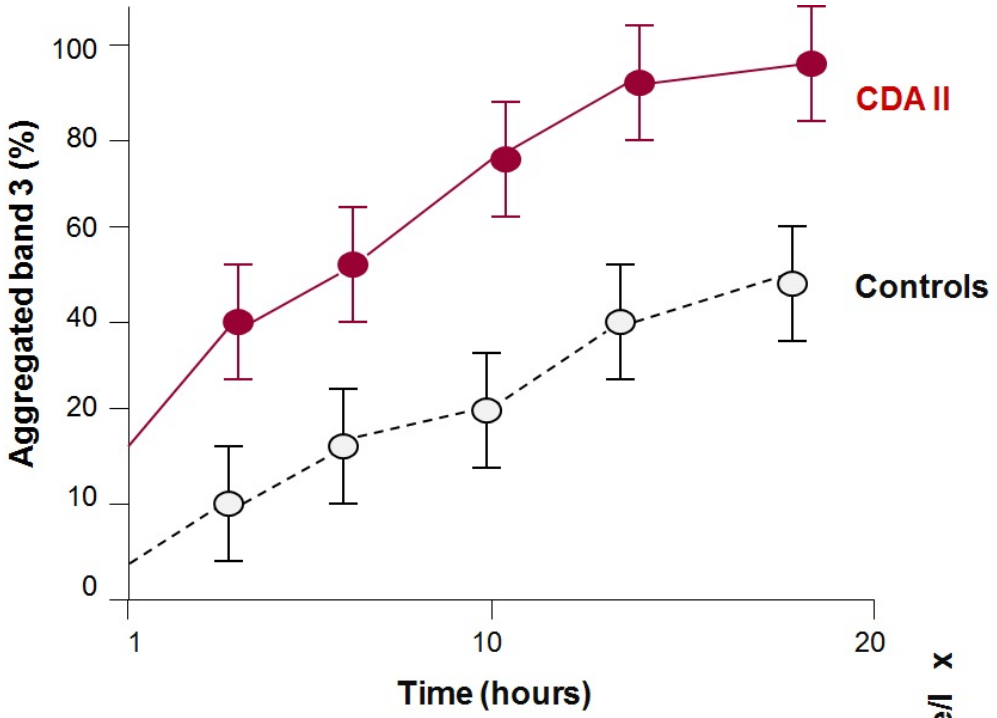


Defective glycosylation as pathogenetic mechanism of CDAII

This defective glycosylation is **not confined** to **erythrocyte** but is also to **hepatic** cells

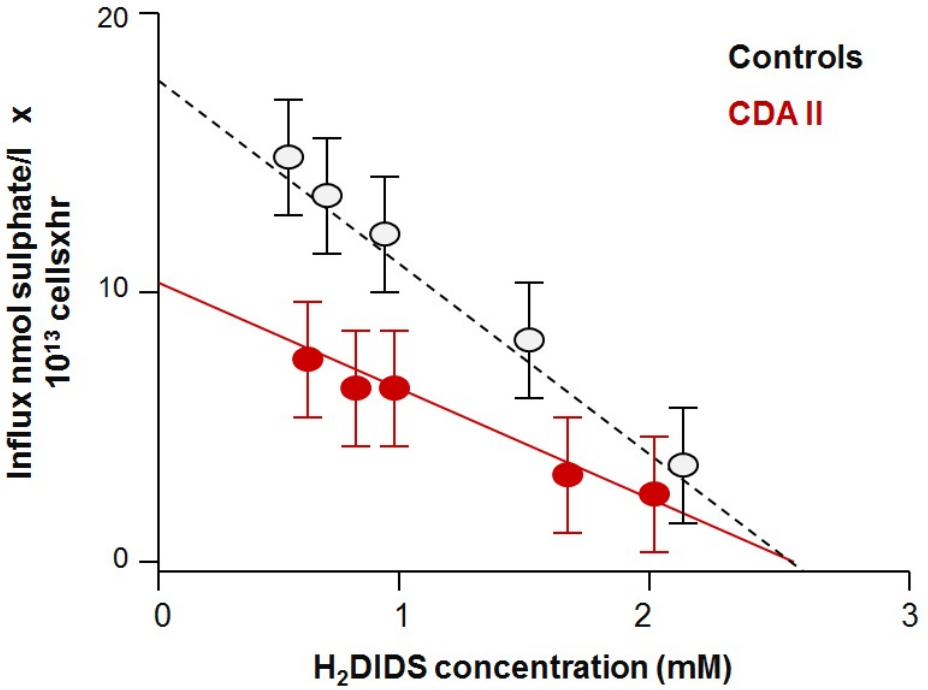


De Franceschi et al. Exp Hematol 1998
Iolascon et al. Haematologica 2009
Zhao, N et al. Biochemistry 2013



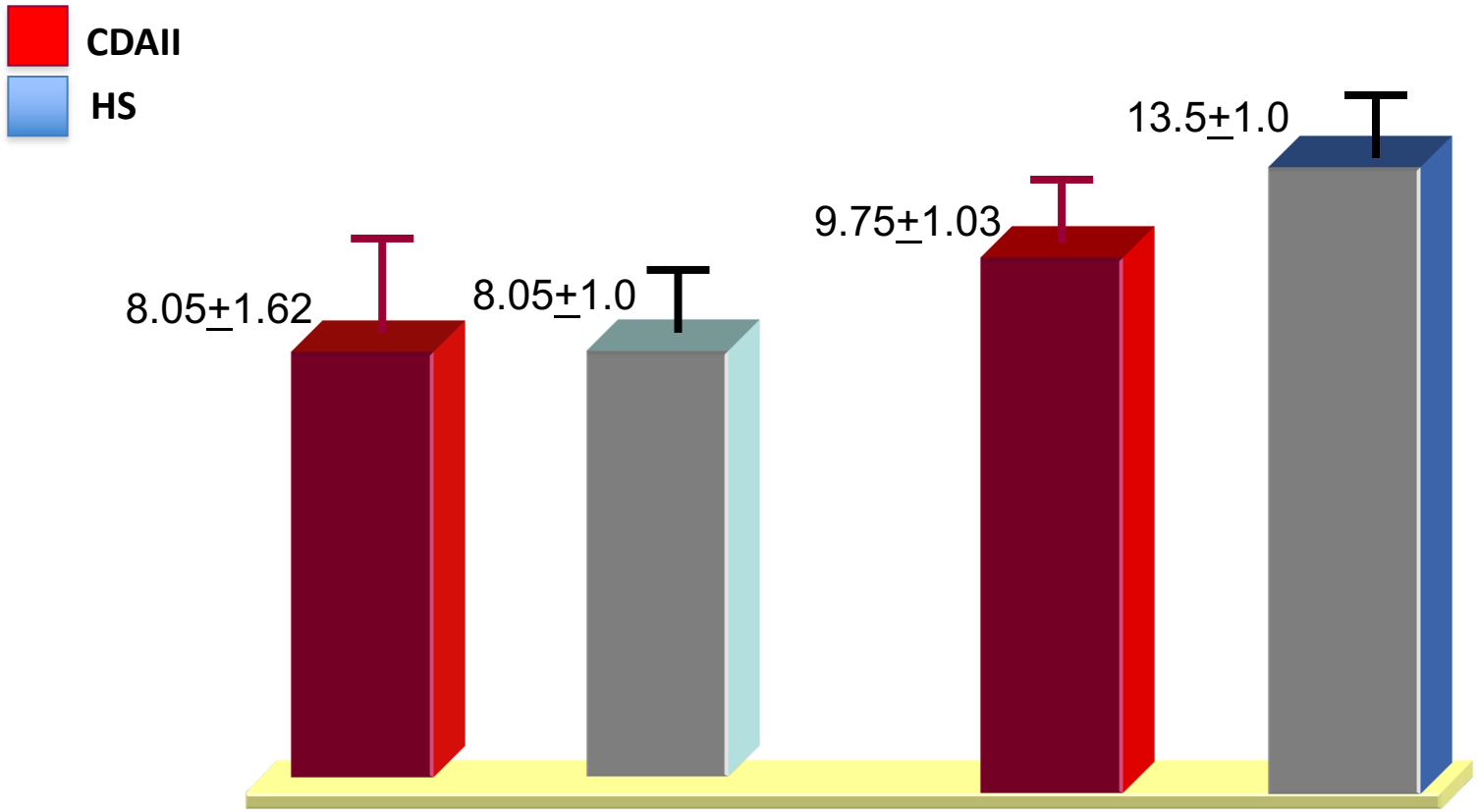
Time course of the aggregation of band 3

H_2DIDS inhibition of sulphate flux



Hemoglobin level and splenectomy

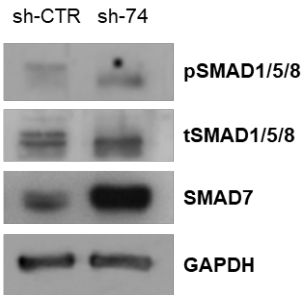
✓ Hb level in 19 and 35 patients affected with CDA-II and HS respectively

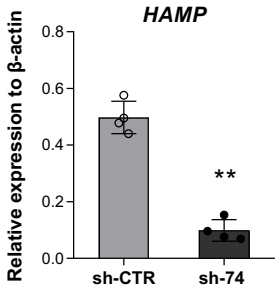


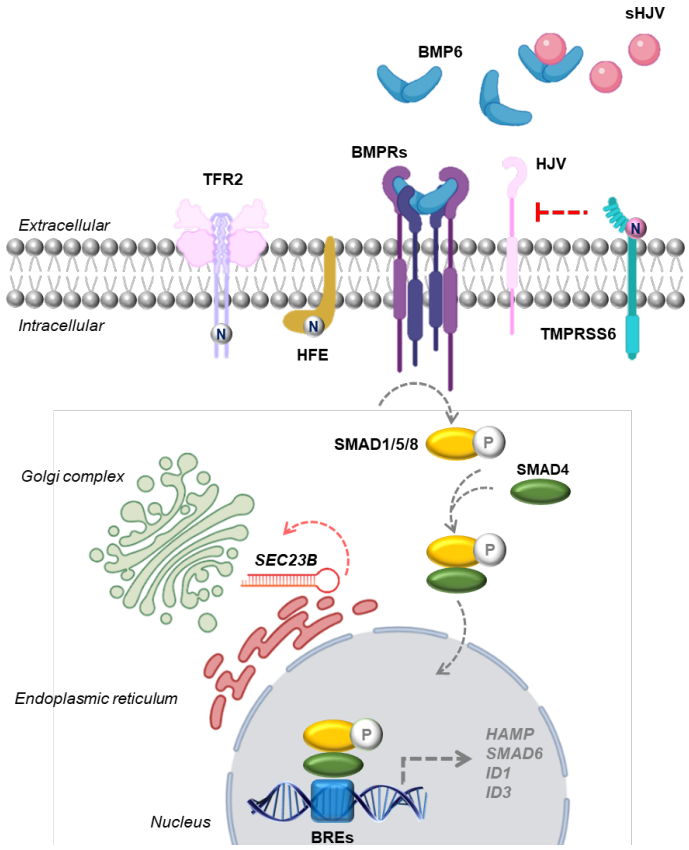
p<0.001

SEC23B Loss-of-Function Suppresses Hepcidin Expression by Impairing Glycosylation Pathway in Human Hepatic Cells

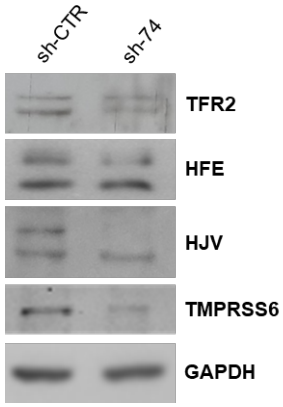
✓ **The silencing of SEC23B** impaired activation of **BMP/SMADs signaling pathway** leading to hepcidin suppression…..







✓ ... altering glycosylation of the **hemochromatosis associated membrane proteins (TFR2, HFE, HJV)**



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Acknowledgments

Medical Genetics Lab CEINGE
Roberta Russo
Immacolata Andolfo
Barbara Eleni Rosato
Roberta Marra
Federica Del Giudice
Gianna Gargiulo
Vanessa D'Onofrio
Federica Maria Esposito
Antonella Nostroso
Anthony Iscaro

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