Tips and tricks to diagnose congenital red blood cell disorders

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INTRODUCTION
(Anemia)

• **Anemia**: Condition in which the number of red blood cells (RBC) or the hemoglobin concentration within them is lower than normal
  - Insufficient to meet physiological needs

• **Global public health problem (WHO)**
  - Affects almost 27% of the world population (1.93 billion people) (2013)
    - 42% of children < 5 years of age
    - 40% of pregnant women
  - Increases morbidity and mortality

(Anemia)

- **Aetiologies:** Any condition that decreases the quantity of functional hemoglobin or decreases the quantity of RBC
Inherited Hemolytic (Anemias)

INTRODUCTION

HEMOGLOBINOPATHIES

MEMBRANE DISORDERS

ENZYME DEFICIENCIES

NGS

CONCLUSION

Inherited hemolytic anemias

Hemoglobinopathies

- Thalassemias
  - Alpha thalassemias
  - Beta thalassemias
  - Sickle cell disease

- Others (Hb C, Hb E, unstable Hb variants, modified oxygen affinity Hb variants)

Enzyme deficiencies

- G6PD deficiency
- PK deficiency
- Other rare enzyme deficiencies

Membrane disorders

- Membrane composition
- RBC hydration
  - Hereditary spherocytosis
  - Hereditary elliptocytosis
  - Hereditary stomatocytosis
  - Hereditary xerocytosis
    - SAO
    - Classical hereditary elliptocytosis
    - HPP
HEMOGLOBINOPATHIES
Laboratory techniques

1. Separation and quantification of Hb fractions

2. If presence of Hb variant
   - Use of a second method based on a different separation principle

3. Give a conclusion
   - Interpretation of the different techniques

Laboratory techniques

• Capillary electrophoresis
• HPLC
• Isoelectric focusing (IEF)

• Each technique is based on a different principle of separation of Hb fractions
  ➢ Variable sensitivity and specificity
Laboratory techniques

• Important to use a quantitative method to determine:
  ➢ Hb variant level
  ➢ Hb A2 level
  ➢ Hb F level

• No Hb variant ≠ No Hemoglobinopathies
Thalassemias

• Among the most common genetic disorders in the world:
  ➢ ±1.5% of the world population carry β-thalassemia
  ➢ ±20% of the world population carry α+-thalassemia

• Approx. 56,000 infants born annually with major thalassemia

• Epidemiology:
  ➢ Prevalent from sub-Saharan Africa, through the Mediterranean region and Middle East, to the Indian subcontinent and East / South-East Asia
  ➢ Increased incidence in our country due to migration of groups with a high frequency of thalassaemic mutations
Thalassemias

• Pathophysiology:
  - Due to genetic defects in the α- or β-globin genes
  - Impaired globin chain synthesis → Decreased Hb synthesis
Thalassemias

• **Thalassemia syndromes:** Highly complex

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Genotype</th>
<th>MCV</th>
<th>Anemia</th>
<th>Expected Disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alpha thalassemia</strong></td>
<td></td>
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<tr>
<td>silent carrier</td>
<td>$\alpha^a / \alpha^a$</td>
<td>nl</td>
<td>none</td>
<td>NTDT</td>
</tr>
<tr>
<td>minor</td>
<td>$\alpha^a / \alpha^a$ or $\alpha^a / \alpha^b$</td>
<td>low</td>
<td>mild</td>
<td>NTDT or TDT</td>
</tr>
<tr>
<td>HbH disease</td>
<td>$\alpha^a / \alpha^a$</td>
<td>low</td>
<td>moderate</td>
<td>NTDT or TDT</td>
</tr>
<tr>
<td>Bart’s syndrome</td>
<td>$\alpha^a / \alpha^a$</td>
<td>low</td>
<td>fatal</td>
<td></td>
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<tr>
<td><strong>Beta thalassemia</strong></td>
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<tr>
<td>minor</td>
<td>$\beta^a / \beta^a$ or $\beta^a / \beta^b$</td>
<td>low</td>
<td>mild</td>
<td>NTDT</td>
</tr>
<tr>
<td>intermedia</td>
<td>$\beta^a / \beta^a$ or $\beta^b / \beta^b$</td>
<td>low</td>
<td>moderate</td>
<td>NTDT or TDT</td>
</tr>
<tr>
<td>major</td>
<td>$\beta^a / \beta^a$ or $\beta^b / \beta^b$</td>
<td>low</td>
<td>severe</td>
<td>TDT</td>
</tr>
</tbody>
</table>

**Genotype interaction**

- **Homonymous**
  - $\beta^a$ or severe $\beta^0$-thalassaemia
  - Mild $\beta^a$ or severe $\beta^+$-thalassaemia
  - Mild $\beta^a+{\beta}^+$ or severe $\beta^+$-thalassaemia
  - $\delta^a$-thalassaemia
  - $\delta^a$ or severe $\delta^a$-thalassaemia
  - Hb Lepore
  - Hb C/J'' or severe $\beta^+$-thalassaemia
  - Hb C/mild $\beta^+$-thalassaemia
  - Hb D/Punjabi or severe $\beta^+$-thalassaemia
  - Hb O/Arab

- **Compound heterozygous**
  - $\beta^a$-thalassaemia trait to intermediate (variable)
  - $\beta$-thalassaemia trait to intermediate (variable)
  - Thalassaemia major
  - Thalassaemia intermediate to major (variable)
  - Milder thalassaemia intermediate (variable)
  - Severe thalassaemia intermediate
  - Mild thalassaemia intermediate
  - Mild to severe thalassaemia intermediate (variable)
Thalassemias

• Phenotypic diagnosis:
  - Quantitative separation of Hb fractions:
    - Beta-thalassemia trait: Hb A₂ > reference values
    - Alpha-thalassemia: Hb A₂ ± reference values

Reference intervals and diagnostic thresholds are method dependant!
**Thalassemias**

- **Molecular analysis:**
  - **ALWAYS** in prenatal diagnosis
  - To confirm the diagnosis of $\alpha^0$-thalassemia trait (genetic counselling)
  - Other situations: to be discussed

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**INTRODUCTION**

HEMOGLOBINOPATHIES

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ENZYME DEFICIENCIES

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CONCLUSION
Unstable Hb variants

- Approx. **150 Hb variants**: 
  - Chronic or episodic hemolysis
  - Mild, moderate or severe hemolytic anemia (depending on the severity of the molecular defect)

- Group of disorders characterized by **clinical, laboratorial and genetic heterogeneity**.

- They undergo rapid **denaturation, precipitation** and **degradation** within the RBC 
  - Misleading electrophoretic results

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A<sub>0</sub> = 97.2%
A<sub>2</sub> = 2.4%
F = 0%
CS = 0.4%

A<sub>0</sub> = 86.6%
A<sub>2</sub> = 2.6%
F = 0.4%
CS = 0%

Waneesorn J. et al., 2011
Unstable Hb variants

• **Diagnosis of unstable Hb variants:**
  - **Blood smear:** Heinz bodies
  - **Laboratory tests:**
    - Heat test
    - Isopropanol test
  - **Genetic analysis**

• **Automated detection of unstable hemoglobin variants by Sysmex XE- & XN-Series analyzers**
  - Low fluorescence signals and **major cluster analysis interferences**
  - Lysis of the RBC and release of unstable Hb may interfering with the leukocyte staining process?
Altered oxygen affinity Hb variants

**High oxygen affinity:**
- >120 Hb variants described
- **Autosomal dominant** transmission
- Deliver **less oxygen** than normal Hb A:
  - Mild tissue hypoxia
  - Increased EPO production
  - Polycythemia
- **Clinical features:**
  - No hepato- or splenomegaly (≠ PV)
  - Possible symptoms of hyperviscosity (headaches, vertigo, tinnitus, and paresthesia in the extremities)
  - Increased risk of thrombosis in later life

**Low oxygen affinity:**
- At least 70 Hb variants described
- **Increased** oxygen delivery to the tissues
  - Compensatory decrease in erythropoiesis
- **Clinical features:** depends on the decrease of oxygen affinity
  - Moderate decrease: Anemia
  - Marked decrease (p50> 50 mmHg): Cyanosis
Altered oxygen affinity Hb variants

• Diagnosis of altered oxygen affinity Hb variants:
  ➢ Phenotypic detection of Hb variants: hemoglobin electrophoresis, HPLC or capillary electrophoresis (+ unstable Hb test)
  ➢ p50 measurement: HEMOX Analyzer (TCS Scientific)


Within 7H after blood collection!
MEMBRANE DISORDERS
Red blood cell membrane disorders

Structural Disorders
- Hereditary Spherocytosis (HS)
- Hereditary Elliptocytosis (HE)

Ionic Transport Disorders
- Hereditary Stomatocytosis (HSt)
- Hereditary Xerocytosis (HX)

- Hereditary Ovalocytosis (HO)
  (Southeast Asian ovalocytosis SAO)
Hereditary spherocytosis

- First described in 1871 as microcythemia in a case history by 2 Belgian physicians

- **Most common inherited haemolytic anemia:**
  - **Prevalence:** 1/2000 – 1/5000 in Northern Europe
  - Probably **higher** (undiagnosed mild cases)

- **Highly heterogeneous group of disorders:**
  - **Clinical severity:** fully compensated haemolysis to transfusion-dependant anemia
  - **Protein defect:** ankyrin, band 3, spectrin and protein 4.2
  - **Mode of inheritance:** 75% dominant; 25% recessive/de novo
  - **Age of diagnosis**
Hereditary spherocytosis

- Pathophysiology:

⇒ Membrane loss (microvesicles)
Hereditary spherocytosis

• Pathophysiology:
Diagnostic approach

• Clinical features and Family history

• Laboratory findings

  1. First line tests
     • RBC morphology
     • Biological haemolysis parameters
     • Erythrocyte and Reticulocyte parameters

  2. Screening tests
     • Cryohaemolysis test
     • Eosine-5-maleimide binding test

  3. Diagnostic tests
     • Ektacytometry
     • SDS-PAGE
HS Clinical features & Family history

• Neonatal period/infancy:
  ➢ Neonatal jaundice
  ➢ Hb level:
    ▪ Normal at birth
    ▪ Rapid fall in Hb levels within the 1st month after birth
  ➢ Anemia: mostly improves during the 1st year of life

• Childhood/Adulthood:
  ➢ Persistent jaundice, anemia, splenomegaly, gallstones
  ➢ Hemolysis: can be compensated in adult
  ➢ Sudden onset of acute anemia

• Positive family history in 75% of cases

Send a pre-transfusion blood specimen or wait for complete clearance of residual transfused RBC
Laboratory findings

1. **First line tests**

   - **RBC morphology:**
     - Spherocytes, mushroom cells, acanthocytes, etc.
     - Good PPV in HS when associated with a family history and compatible RBCs indices

   - **Hemolysis parameters:**
     - Low total Hb level (but can be normal if compensated)
     - Low or absent haptoglobin level
     - Hyperbilirubinemia
     - High serum LDH level

   - **Erythrocyte and Reticulocyte parameters**

   + Direct antiglobulin test (DAT)
   + Enzymopathy tests (G6PD, PK)
   + Haemoglobinopathy tests
   + ...
Erythrocyte/Reticulocyte parameters & HS

• Abnormal RBC indices have been proposed but their sensitivities are low
  ➢ ↑ MCHC
  ➢ ↑ RDW
  ➢ ↑ Hyperdense cells (Advia analyzer)

• Development of additional parameters based on complete blood count (CBC) and reticulocyte parameters on last generation hematology analyzers:
  ➢ Many publications about their effectiveness as HS first tier screening tool
# Erythrocyte/Reticulocyte parameters & HS

<table>
<thead>
<tr>
<th>Analyzers</th>
<th>Parameters of interest</th>
<th>Definition</th>
<th>Usefulness in HS</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siemens (Advia 2120)</td>
<td>% Hyper</td>
<td>% Hyperdense RBCs (MCHC &gt; 41 g/dL)</td>
<td>% Hyper ↑</td>
<td>Membrane loss associated with increased permeability to monovalent cations</td>
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<tr>
<td></td>
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<td></td>
<td>➢ Sodium pump hyperactivity</td>
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<td></td>
<td></td>
<td>➢ Na⁺ loss &gt; K⁺ entry</td>
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<td></td>
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<td></td>
<td></td>
<td>➢ Dehydration</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>➢ ↑ MCHC</td>
</tr>
<tr>
<td>Abbot Diagnostics (CELL-DYN</td>
<td>% HPR</td>
<td>% Hyperchrome RBCs (MCHC &gt; 41 g/dL)</td>
<td>% HPR ↑</td>
<td>Reduced deformability of the spherocytes</td>
</tr>
<tr>
<td>Sapphire)</td>
<td></td>
<td></td>
<td></td>
<td>➢ Incapacity to increase their volume in hypo-osmotic solution</td>
</tr>
<tr>
<td>Beckman-Coulter (DxH800, LH755,</td>
<td>MSCV</td>
<td>Mean Sphered Cell Volume</td>
<td>MSCV &lt; MCV</td>
<td>Membrane loss and decreased surface area in HS</td>
</tr>
<tr>
<td>LH780)</td>
<td></td>
<td></td>
<td></td>
<td>➢ Already occur during erythropoiesis</td>
</tr>
<tr>
<td></td>
<td>MRV</td>
<td>Mean Reticulocyte Volume</td>
<td>MRV ↓</td>
<td></td>
</tr>
<tr>
<td>Sysmex (XN5, XT-4000i, XE-5000)</td>
<td>IRF</td>
<td>Immature Reticulocyte Fraction</td>
<td>Rét/IRF ↑</td>
<td>High reticulocyte count without an equally elevated IRF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>➢ Hypothesis: abnormal/decreased coloration of the reticulocytes</td>
</tr>
<tr>
<td></td>
<td>% Microcytic RBCs</td>
<td>% Microcytic RBCs (MCV &lt; 60 fl)</td>
<td>Micro R ↑</td>
<td>Spherocytes = small hyperdense cells</td>
</tr>
<tr>
<td></td>
<td>(MCV &lt; 60 fl)</td>
<td></td>
<td></td>
<td>➢ ↑ microcytic RBCs %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>➢ ↑ Hyper-haemoglobinized % = ↓ hypo-haemoglobinized %</td>
</tr>
</tbody>
</table>

> Parameters not standardized, method dependant.
Laboratory findings

2. Screening tests

- **Cryohemolysis test:**
  - Based on the increased susceptibility of spherocytic RBCs to cold (0°C) in hypertonic conditions
  - Measure the % of cryohemolysis compared to a normal control

- **Eosin-5’-maleimide (EMA) binding test:**
  - Flow cytometric test based on the decreased of Band 3 protein in HS RBC
  - EMA dye binds to Band 3 protein
  - Measure % of decreased mean fluorescence intensity (MFI) compared to controls
Laboratory findings

3. Diagnostic tests:

- **Osmotic gradient ektacytometry:**
  - Analysis of the RBC deformability in changing osmotic environment conditions with a constant shear stress applied

- **Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS Page):**
  - Determines the type and the extend of membrane protein deficiency

- **Molecular analysis (NGS)**
ENZYME DEFICIENCIES
G6PD deficiency

- **Most common** enzymatic disorder of RBCs
  - Affecting **400 to 500 million** people worldwide
  - **Global distribution** but more common in areas in which malaria is endemic

- **Mode of inheritance:** X-linked disorder
  - Men are more commonly affected than women

- **Clinical expression:** from **asymptomatic** to **episodic** to **chronic hemolysis**
  - Depends on the degree of the enzyme deficiency
  - Determined by the characteristics of the G6PD variant (>130)

<table>
<thead>
<tr>
<th>Class</th>
<th>Residual G6PD activity (% of normal)</th>
<th>Clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>&lt;10</td>
<td>CNSHA (NNJ, acute exacerbations)</td>
</tr>
<tr>
<td>II</td>
<td>&lt;10</td>
<td>None in the steady state</td>
</tr>
<tr>
<td>III</td>
<td>10-60</td>
<td>None in the steady state</td>
</tr>
<tr>
<td>IV</td>
<td>100</td>
<td>None</td>
</tr>
<tr>
<td>V</td>
<td>&gt;100</td>
<td>None</td>
</tr>
</tbody>
</table>
G6PD deficiency

• Physiopathology:

INTRODUCTION
HEMOGLOBINOPATHIES
MEMBRANE DISORDERS
ENZYME DEFICIENCIES
NGS
CONCLUSION

Potential triggers of oxidative stress:
1. Infection (most common)
2. Oxidant drugs
3. Fava beans

Glucose-6-Phosphate dehydrogenase deficiency

Glucose

Glucose

Glucose 6-phosphate

Glucose 6-phosphate

Glucose 6-phosphate

6-Phosphogluconate

Glycolysis

2 ATP

2 NADH

2 Pyruvate

2 Lactate

Pentose phosphate pathway

G6PD

Glucose 6-phosphate dehydrogenase

NADP+

NADPH + H+

2 GSH

2 H2O

H2O2

O2·

Glutathione reductase

Glutathione peroxidase

2 GSH

(ROS)

Met Hb

Oxy Hb

Heinz bodies

HO•

Haemolysis

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G6PD deficiency

- **Severity** and **course** of hemolytic episode depend on:
  - G6PD variant
  - Type and duration of oxydative stress
  - Age & Coexisting disease conditions

- Only a **few drugs** with well-documented causal relationship
  - No test available: *in vitro ≠ in vivo*
  - Individual drug metabolism

- Hemolysis most frequently related to the **infection** than to the drug
  - Treat the infection
  - Change the treatment

<table>
<thead>
<tr>
<th>Category of drug</th>
<th>Predictable hemolysis</th>
<th>Possible hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antimalarials</strong></td>
<td>Dapsone</td>
<td>Chloroquine</td>
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<tr>
<td></td>
<td>Primaquine</td>
<td>Quinine</td>
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<td></td>
<td>Pamaquin</td>
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<tr>
<td></td>
<td>Tafenoquine</td>
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<tr>
<td></td>
<td>Methylene blue</td>
<td></td>
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<tr>
<td><strong>Analgesics/Antipyretic</strong></td>
<td>Phenazopyridine</td>
<td>Aspirin (high dose)</td>
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<tr>
<td></td>
<td></td>
<td>Paracetamol (Acetaminophen)</td>
</tr>
<tr>
<td><strong>Antibacterials</strong></td>
<td>Cotrimoxazole</td>
<td>Sulfasalazine</td>
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<td></td>
<td>Sulfadiazine</td>
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<td></td>
<td>Quinolones</td>
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<tr>
<td></td>
<td>Nitrofurantoin</td>
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<tr>
<td><strong>Other</strong></td>
<td>Rasburicase</td>
<td>Chloramphenicol</td>
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<td></td>
<td>Toluidine blue</td>
<td>Isoniazid</td>
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<td></td>
<td>Nitridazole</td>
<td>Ascorbic acid</td>
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<td></td>
<td>Pegloticase</td>
<td>Glibenclamide</td>
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<td></td>
<td></td>
<td>Vitamin K (Menadione)</td>
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<td></td>
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<td>Isosorbide</td>
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<td></td>
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<td>Dinitrate</td>
</tr>
</tbody>
</table>
G6PD deficiency

• Laboratory techniques:
  - Qualitative: MetH reduction test
    ▪ Fails to diagnose heterozygotes with <40% enzyme activity
    ▪ Not reliable for female patients
    ▪ Suitable for emergency screening for G6PD deficiency (eg. administration of rasburicase prior to initiation of chemotherapy)
  - Quantitative: Enzyme levels
    ▪ Should always follow a qualitative test
    ▪ Normal if > 60% (steady-state)
    ▪ Our conclusions:
      - >60%: « Activité suffisante... »
      - <30%: « Possibilité de crise hémolytique... »
      - 30-60%: « Probabilité faible de crise d’hémolyse ».
G6PD deficiency: Pitfalls

• Final G6PD activity should be interpreted in light of the reticulocyte count and/or other RBCs enzyme levels (HK, PK, 6PGD) measured on the same sample
  ➢ Young RBCs and reticulocytes have much higher G6PD activity than mature RBCs
  ➢ Standardisation for the population of RBCs tested: if any doubt, contact the lab.

• Acute hemolytic episode: RBCs with the most severely reduced G6PD activity will have hemolyzed
  ➢ May result in normal or even raised enzyme levels and a false-normal result
  ➢ Test should be repeated 2-3 months after resolution
G6PD deficiency: Management (Prevention)

• **Prevention:**
  - Avoid oxydative stressors

• **Hemolysis:** Make the diagnosis and
  - Stop the drug
  - If severe anemia: Blood transfusion
    - Recommendations cut-off: Hb 7 g/dL
    - If rapid decrease in Hb and hemoglobinuria cut-off: Hb 9 g/dL
  - If acute renal failure: Hemodialysis might be required
NEXT-GENERATION SEQUENCING
**Rare & Very rare anemias**

- **About 90** rare types of anemia have been described.
- **Among them ± 80%** are inherited.
- www.orpha.net

**INTRODUCTION**

- HEMOGLOBINOPATHIES
- MEMBRANE DISORDERS
- ENZYME DEFICIENCIES
- NGS
- CONCLUSION
Rare & Very rare anemias

Clinical Phenotype / Laboratory testing

Transfusion before diagnosis testing
Unsolved cases
Discrepancy between clinical phenotype and diagnosis

Next Generation Sequencing (NGS)
Why Next-Generation Sequencing?

• PCR based methods and Sanger sequencing:
  - Easy and cost-effective for finding the molecular defect in phenotypes where gene implicated is known
  - Eg. G6PD gene, β-thalassemia

• Targeted NGS:
  - Very useful in hemolytic anemias where the conventional testing is not diagnostic
  - Cost-effective and efficient approach for providing rapid and accurate genetic diagnosis
  - One can sequence hundreds of millions of short sequence (200 bp) in a single run and in a short period of time with a low per base cost
Next-Generation Sequencing

Benefits

• More data produced with the same amount of input DNA
• Simultaneous screening of multiple genes in multiple samples
• Identification of new pathogenic variants
• Detection of multigene disorders
• Higher sequencing depth enables higher sensitivity
• Decreased sequencing cost per gene

Challenges

• High complexity of workflow and results
• Huge volume of data to analyze and to store
• Long turnaround time
• Ambiguous results with variants of uncertain significance
NGS to diagnose hereditary hemolytic anemia

• « In house » panel of 4427 genes (mendeliome)
  a. Ataxia (524 genes)
  b. Congenital malformation syndromes (853 genes)
  c. Early onset epileptic encephalopathy (836 genes)
  d. Hereditary Hemolytic Anaemias due to unknown or doubtful origin (44 genes)
  e. Hereditary spastic paraplegia (160 genes)
  f. Neurodevelopmental disorders (1376 genes)
  g. Neuromuscular disorders (535 genes)
  h. Dermatogenetic panel, severe, rare and hereditary genodermatoses (374 genes)
CONCLUSION
Take home messages

• **Hemoglobinopathies**
  - *Thalassemia syndromes*: hemolysis and ineffective erythropoiesis
  - Don’t forget about **unstable Hb variants**
  - **Modified oxygen affinity Hb Variants**: p50 measurement now available at LHUB-ULB

• **Membrane disorders**
  - **Hereditary spherocytosis**: guidance by clinical picture

• **Enzyme deficiencies**
  - **G6PD deficiency**: make the diagnosis to be able to prevent hemolysis as much as possible

• Consider **NGS** in selected patients
• **ERN-EuroBloodNet:** The European Reference Network on Rare Hematological Diseases
http://www.eurobloodnet.eu/
References


Thank you!

This place was a metropolis before the iron shortage.

Without it, the bones had to ration the hemoglobin. Red blood cells are no good without it.

Sorry, kid, that's it.

The great Iron Famine led to the age of Anemia.

We are the survivors. We are-

Hey, looks like they made some diet changes up top. We're all good now.

Oh.

What are you wearing?