Hematology Laboratory for Blood Doping Detection

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Learning objectives

After viewing this presentation, the participant will be able to know and understand:

• how and why disloyal athletes try to increase their circulating hemoglobin mass
• how blood doping can be detected in the hematology laboratory using the Athlete Biological Passport
• which are the main hematological factors which must be considered to avoid false positive accusations
Blood doping

- The attempt to increase the total amount of circulating hemoglobin (and oxygen transport to tissues) through illegal practices
- Used since ‘70s in endurance sports
- Diffused during ‘90s to now
Figure 1. Parameters determining the aerobic capacity. The aerobic capacity, as measured as the maximal O₂ uptake (\(O_2\text{max}\)), depends primarily on the person’s total Hb mass, the maximal cardiac output, and the maximal O₂ extraction in the heart and the skeletal muscles. The total Hb mass results from the blood hemoglobin concentration and the blood volume.
Hypoxia → Increased erythropoietin → Iron Folate B₁₂ → Liver → Blood cells → PT → E → V → Hypoxia
Congenital disorder of oxygen sensing: association of the homozygous Chuvash polycythemia \( VHL \) mutation with thrombosis and vascular abnormalities but not tumors


Adaptation to hypoxia is critical for survival and regulates multiple processes, including erythropoiesis and vasculogenesis. Chuvash polycythemia is a hypoxiainducible regulator characterized by homozygous mutation \((598C\rightarrow T)\) of von Hippel-Lindau gene \((VHL)\), a negative regulator of hypoxia sensing. Although endemic to the Chuvash population of Russia, this mutation occurs worldwide and originates from a single ancient event. That \( VHL \) \(598C\rightarrow T\) homozygosity causes elevated normoxic levels of the transcription factor hypoxia inducible factor-1\(\alpha\) (HIF-1\(\alpha\)), serum erythropoietin and hemoglobin is known, but the disease phenotype has not been documented in a controlled manner. In this matched cohort study, \( VHL \) \(598C\rightarrow T\) homozygosity was associated with vertebral hemangiomas, varicose veins, lower blood pressures, and elevated serum vascular endothelial growth factor (VEGF) concentrations \((P < .0005)\), as well as premature mortality related to cerebral vascular events and peripheral thrombosis. Spino-cerebellar hemangioblastomas, renal carcinomas, and phaeochromocytomas as typical of classical \( VHL \) syndrome were not found, suggesting that overexpression of HIF-1\(\alpha\) and VEGF is not sufficient for tumorigensis. Although hemoglobin-adjusted serum erythropoietin concentrations were approximately 10-fold higher in \( VHL \) \(598C\rightarrow T\) homozygotes than in controls, erythropoietin response to hypoxia was identical. Thus, Chuvash polycythemia is a distinct \( VHL \) syndrome manifested by thrombosis, vascular abnormalities, and intact hypoxic regulation despite increased basal expression of hypoxia-regulated genes. (Blood. 2004;103: 3924-3932)
Autosomal Dominant Erythrocytosis Caused By Increased Sensitivity to Erythropoietin

By Eeva Juvenen, Eero Ikala, Frej Fyhrquist, and Tapani Ruutu

We describe here a family with autosomal dominant erythrocytosis. In in vitro cultures, performed using the methyl cellulose assay, the number of erythroid colonies was normal or marginally increased when a standard concentration of erythropoietin (Epo) was used, but at lower Epo concentrations, the investigated persons formed more colonies than the controls. The difference was generally greater the lower the Epo concentration became. Some erythroid colony growth was seen even in the absence of any added Epo (apart from the minute concentration found in fetal calf serum), a phenomenon not seen in the controls. This finding indicates that the erythrocytosis in this family is caused by hypersensitivity of erythroid progenitors to Epo. The serum Epo concentration was low or low normal in all of the investigated family members, which is in good accordance with hypersensitivity to Epo. The erythrocytosis has not had any obvious effect on the health or life-span of the affected individuals. Many of them have reached an advanced age, and one of the affected family members has won several Olympic gold medals and world championships in endurance sports.

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The propositus, a 53-year-old male, has always been in excellent health. He has won several Olympic gold medals and world championships in cross-country skiing. His Hb levels have been 200 g/L or more since childhood. The laboratory findings at the time of the present investigation are shown in Table 1. The RBC indices were within normal limits. The reticulocyte count was also normal (0.010). The total RBC volume was 4,782 mL or 59.0 mL/kg (normal, 25 to 35), and the 2,3-DPG concentration was slightly reduced (2.9 mmol/L; normal, 3.1 to 5.9). The isoelectric focusing of Hb was normal. A bone marrow (BM) aspirate showed erythroid hyperplasia. The chest x-ray and electrocardiography were normal.
Methods to increase HB mass

- Homologous blood transfusion
- Autologous blood transfusion
- Injection of rHuEPO and derivates
- HIF stabilizers
- Androgen anabolic steroids
- Injection of HBOCs
- Gene doping
Blood transfusion

- 1945: transfusion of 450 ml increases tolerance to hypoxia
- 1960s: first alleged use
- 1968: widespread use after Mexico City
- 1984: Los Angeles USA cycling team
- 2002: winter Olympic Games in Salt Lake City
- 2006: operation Puerto
- 2006: winter Olympic Games in Turin
- 2007: cyclists disqualified for flow-cytometric positivity
- as EPO tests improve, transfusion is back in vogue
Recombinant Erythropoietin, ESAs and biosimilars

- **Late 1980s**: rhu-EPO is a major breakthrough in nephrology and in the whole of medicine
  - EPO is suspected in nearly 20 sudden and unexpected death of European cyclists
  - 1993-1994 and thereafter: large diffusion in Italy and in the world
  - 1998: the Festina scandal (Tour de France)
  - 2000: urine test developed

- **2002**: darbepoietin (NESP) is produced with the additions of carbohydrate chains (longer half-life)
  - NESP found in the urine of Sal Lake City Winter Olympics medals

- **2005**: C.E.R.A. is produced with introduction of PEG polymer into epoetin beta molecule
  - 2008: leading Italian cyclists positive for Methoxy Polyethylene glycol-epoetin beta (MIRCERA) (Tour de France)
  - “copy” epoetins (nonpatented), hematide and other peptide based ESAs
HCT: seasonal variation in cyclists
HCT in elite cross-country skiers

Can blood doping be detected?

- **direct methods**
  - rh-EPO, HIF_stabilizers
  - homologous transfusion
- **indirect methods**
  - Athlete's Biological Passport

<table>
<thead>
<tr>
<th>Date</th>
<th>Cyclist</th>
<th>Banned substance</th>
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<tr>
<td>3 March</td>
<td>Patri Vil (ESP)</td>
<td>Testosterone</td>
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<tr>
<td>11 April</td>
<td>Maximiliano Richeze (ARG)</td>
<td>Stanozolol (steroid)</td>
</tr>
<tr>
<td>28 June</td>
<td>Giovanni Carini (ITA)</td>
<td>EPO</td>
</tr>
<tr>
<td>29 June</td>
<td>Paolo Bossoni (ITA)</td>
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<tr>
<td>5 July</td>
<td>Manuel Beltrán (ESP)</td>
<td>EPO</td>
</tr>
<tr>
<td>8 July</td>
<td>Moisés Duenas (ESP)</td>
<td>EPO</td>
</tr>
<tr>
<td>8 July</td>
<td>Riccardo Ricco (ITA)</td>
<td>MIRCERA</td>
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<td>23 July</td>
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<td>31 July</td>
<td>Maria Moreno (ESP)</td>
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<td>7 October</td>
<td>Leonardo Piegoli (ITA)</td>
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<td>7 October</td>
<td>Stefan Schumacher (GER)</td>
<td>MIRCERA</td>
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<td>12 October 12</td>
<td>Bernhard Kohl (AUT)</td>
<td>MIRCERA</td>
</tr>
<tr>
<td>10 December</td>
<td>Illo Keisse (BEL)</td>
<td>MIRCERA</td>
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rh-EPO direct detection

- urine (and/or blood) analysis by isoelectric focusing and a double blotting procedure
Problems of rh-EPO direct detection

- time window: urine detection only for 1-2 days
- microdosages
- proteases and dilution are used to cheat
- interpretation can be difficult
- new biosimilar products
- new types of ESA
Direct detection of homologous transfusion

- Minor populations of antigen-positive and negative erythrocytes
- DNA identification techniques
Autologous doping transfusion

- No direct method available
- Research studies in progress
  - red cell biochemical changes due to storage
  - altered protein expression, -omics strategies
  - modifications of gradient density
  - microparticles
- Plasticizers in urine

![Diagram of Di-(2-ethylhexyl)phthalate (DEHP)](image)
Indirect blood doping detection

• Blood cell count
  • useful for all types of blood doping
  • used for targeting suspect athletes
  • the laboratory hematologist’s competence

• Longitudinale blood parameters' profile
  (Athlete's Hematological Passport)
  • hemoglobin
  • reticulocyte count
  • OFF score

• Total HB mass
The Athlete Biological Passport (Hematology)

- Identification of simple hematological abnormalities caused by blood doping using INDIVIDUAL REFERENCE VALUE (intraindividual differences).
- Hematological stability permits the longitudinal evaluation of unexpected changes in blood results in a single subject.
- The monitored sequence of results within a structured follow-up program of in and out-of-competitions controls (blood profile).
The OFF score

- to detect the post-administration phase characterized by still high hemoglobin and low reticulocytes (inhibition)
- calculated as:

\[
\text{OFF score: } Hb-60\sqrt{\text{ret}\%}
\]

- high value: >112-123 ....
- low value (associated with reticulocytosis)
Effect of rEPO

- Typical EPO doping protocol:
  - 6 weeks micro-dosing, e.g. 10 units/kg intravenously EOD
  - some athletes may start with higher doses, e.g. 20-40 units/kg, during the first 1-2 weeks (called “loading phase”) and have missed tests during that period
  - athletes may provide diluted urine to escape the EPO test or have access to designer EPOs

- an increase of about 10-15% in the red blood cell mass is expected, e.g. from 1000g to 1150 g.
Effect of rEPO

- Six weeks trial:

Day 0

10 UI/kg IV EOD

Week 6

competition
Effect of rEPO

- Six weeks trial:

Day 0

Week 6

Hb

14.5 g/dL

16.0 g/dL
Effect of rEPO

- Six weeks trial:

Day 0: 1.0 %
Week 6: 0.4 %
Effect of rEPO

- Six weeks trial:

Day 0

Week 6

OFF-score

85

75

95

105

120
Effect of blood transfusion

- Typical blood transfusion protocol
  - Protocol starts 3 months (90 days) before an important competition
  - Day 0: withdrawal of 1 blood bag (450 ml)
  - Day 30: reinfusion of 1, then withdrawal of 2 blood bags
  - Day 60: reinfusion of 2 bags, then withdrawal of 3 blood bags
  - Day 90: progressive reinfusion of 3 blood bags

- In stage races, athletes may reinfuse lower amounts, e.g. 100 ml every day during 12 days

- Other protocol requires the collection of blood bags out of season, the separation of plasma and appropriate storage of the RBCs.
Effect of blood transfusion

- Preparation of 3 blood bags:

  - Day 0
  - Day 30
  - Day 60
  - Day 90

Withdrawal of 1 blood bag (400-500 ml)

reinfusion

competition
Effect of blood transfusion

- Preparation of 3 blood bags:

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
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</table>

Hb

14.5 g/dL

13.7 g/dL

16.5 g/dL
Effect of blood transfusion

- Preparation of 3 blood bags:

<table>
<thead>
<tr>
<th>Day 0</th>
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<th>Day 60</th>
<th>Day 90</th>
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</tbody>
</table>

RET%

- Day 0: 1.0%
- Day 30: 1.7%
- Day 60: RET%
- Day 90: 0.4%
Effect of blood transfusion

- Preparation of 3 blood bags:

Day 0

Day 30

Day 60

Day 90

OFF-score

85

55

130
Experimental autologous transfusion

Large variation in Haemoglobin concentration after blood withdrawal and reinfusion.

The OFF score amplifies the changes observed in Haemoglobin and Reticulocytes.

High Reticulocytes paired with low Haemoglobin concentration suggesting hyperproliferative condition after blood withdrawal (samples 4+5).

Low Reticulocytes with high Hb indicating suppressed erythropoiesis after reinfusion of blood (samples 6+7).

Biological passport: Blood Transfusion

The samples 2-10 were taken on a regular base over a period of ~8 weeks.
Expert Panel: The experts, with knowledge in the concerned field, chosen by the Anti-Doping Organization and/or APMU, who are responsible for providing an evaluation of the Passport. For the haematological module, experts will have knowledge in one or more of the fields of clinical hematology (diagnosis of blood pathological conditions), sports medicine or exercise physiology.
Physiological variability? Interaction with exercise, training, competition, disease?
Indirect evidence
Medico-legal challenge
Standardization and robustness
Not an opinion on guilt, but the scientific basis for a legal verdict
Confounding factors

- Quality of the blood sample, pre-analytical and analytical issues
- Blood changes due to physiological factors:
  - exercise
  - altitude
  - others
- Possible medical conditions
In general, the difference between two sets of haematological data obtained from different samples within 24 hours is the product of a number of variables:

1. Intra-individual, short-term, within day variability, including circadian variation. This factor has been calculated to range between 1.9% and 4.9% for haemoglobin (10,14,15); a value of 2.5% is reported for Haematocrit in athletes (16); and between 5.8% (17) and 11% (18) (short-time, between weeks) for Reticulocytes.

2. Analytical within-run and between-run variability: these are respectively 0.4 and 0.7% for Haemoglobin concentration (10), 8.1 and 3.0% for Reticulocytes (18).

3. Inter-instrument variability (19): This factor cannot be evaluated due to the lack of information on the private test, but the type of instrument is known to be an important variable in inter-laboratory analytical variability (20,21).

4. Pre-analytical variability, which includes the peri-analytical conditions such as the effect of exercise or posture (22).
Pre-analytical and analytical quality

- blood collection (timing, posture, tourniquet, activity...)
- transport
- temperature, storage duration, mixing…

**Athlete Biological Passport Documentation Package:** The material produced by the Laboratory and Athlete Passport Management Unit to support an Adverse Passport Finding such as, but not limited to, analytical data, Expert Panel comments, evidence of confounding factors as well as other relevant supporting information.
Individual variation of HCT in athletes
(Schmidt et al, Int J Sports Med)

- 24 hours: 10 % variation (night low)
- 20’ Head down tilt: 8 % decrease
- 1 l saline infusion: 8 % decrease
- VMT exercise: 10 % increase (plasma ↓)
- 10 day competition: 10 % decrease (plasma ↑)
Confounding physiological factors

• Are related to:
  • Plasma volume shifts (HB and OFF score)
    • Long term: training, racing, season, heat
    • Short term: intense exercise, posture, altitude, water ingestion-infusion-loss (hypo/hyperhydration), height ascent, fever, diarrhea…
  • Blood volume changes (HB, OFF, reticulocytes, owing to relative hemodilution and erythropoietic response)
    • Blood loss or donation
    • Training and altitude (long term)
  • RBC mass changes (HB, OFF score and reticulocytes)
    • Drugs (non-ESA)

3.3.4. Expert Panel
The Expert Panel is responsible for:

a. Reviewing Passport data and results from the Adaptive Model provided by the APMU to identify any possible pathological or confounding conditions that may have impacted an Athlete’s results.
«Minor» confounding factors 
(mostly related to plasma volume shifts, 3-5% HB variation)

- Posture, position of the body (standardized)
- Circadian rhythm (HB oscillation around 0.5 g/dl, peak at morning/noon, nadir around midnight)
- Effect of meals
- Seasonal intra-individual variation (3% HCT variation, effects of rest vs training vs racing)
- Climatic, environment, temperature (increase in PV in hot)
- Stress (short-term hemoconcentration?)
- Nutrition and weight regulation
- Smoking, ethnicity
Exercise

- Plasma volume shifts due to training/competition
  - Acute effect $\rightarrow$ hemoconcentration: maximum cycle exercise: $\uparrow$
    10% HCT due to 15% decrease in plasma volume (Schmidt et al, 2000)
  - Long-term effect $\rightarrow$ hemodilution
  - Tapering $\rightarrow$ hemoconcentration

- Hydration status
  - Dehydration (classical justification)
  - Hyperhydration (possible masking)
Plasma expansion and decreasing HB during strenous prolonged effort

Fig. 7 Individual changes in hemoglobin concentrations ([Hb]) in seven riders during the Tour de France 2007. Each line corresponds to one rider. The X-axis represents time of measurement and the Y-axis represents the hemoglobin concentration ([Hb]) in grams/deciliter (g/dL). Day − 1, measurement point is one day before the beginning of the race; Day 12, measurement point is 12 days after the beginning of the race, and Day 19, measurement point is 19 days after the beginning of the race. * Significantly different from Day − 1 (p < 0.05).

Exercise, in terms of ABP

- Heterogeneity of exercise intensity and duration
- Exact timing of blood testing
  - Pre-competition (not available in ADAMS)
  - In-competition (can be before or after the race)
  - Hemodilution (after three days of race)
  - Tapering (before or after a race: hemoconcentration)
- Interaction with a large number of other confounders factors
- Absence or inversion of a typical (physiologically expected) variation
Abnormal increase in HGB in the last days of a major stage race, compared to Morkeberg et al, 2009 (Report by ABP Expert Panel)
Altitude and hypoxia

• Natural altitude, different methods
  • Acute hemoconcentration
  • Long-term increased red cell mass
  • Mild changes of reticulocytes

• Simulated altitude:
  • intermittent normobaric hypoxia has no effect on ABP parameters
Altitude effects in athletes

- Increases endogenous EPO level
- Increases circulating RBC/HB mass
- Increases $V_{O_{2}\text{max}}$ and performance
- The effect depends on height and duration

- Peripheral blood changes:
  - First days:
    slight HB $\uparrow$, retic $\uparrow$, OFF $=$
  - Longer stay:
    HB $=$, retic $\downarrow$, OFF $=/\uparrow$
  - Back to sea-level:
    HB $=$, retic $\downarrow$, OFF $\uparrow$
    (7-14 days)

No changes in sEPO after 2 weeks

- Hematologic variation are very mild after 4 weeks and later
- Possible ABP flags for the OFF score
- Importance of an expert panel
- «It is unlikely that the values outside the ref. range would have been considered a violation».
- Possible masking effect of ESA/transfusion by altitude-induced ABP fluctuations
Tour of Quinghai Lake: 14-day cycling stage race at an average altitude of 2496 m above sea level (1014-4120m).

- Hemodilution occurs despite increased HB mass, in residents and in acclimatized subjects.
- Impact of Exercise is more important than the impact of altitude in terms of ABP parameters.
- It is unlikely that the altitude of training and sporting events will significantly impact the ABP profile of an athlete and cause false positive results.
Male runner, resident in African highlands
Male cyclist, 2200 m resident

Jan, tested at altitude

June, tested at sea level
Medical conditions

• Increased HB
  • Congenital erythrocytosis
  • Polycythemia vera
  • Dehydration (?)

• Increased reticulocytes:
  • Acute blood loss
  • Compensated hemolysis

• Decreased reticulocytes
  • Infections (?)
  • Alcoholic intoxication (?)
  • Anemias and blood diseases
The Athlete’s medical justifications

- Hemorrhoidal or gastric bleeding
- Menstruation (low or high HB), spontaneous abortion
- Surgery, bone fractures, hemorrhages, hematomes
- Dental extractions, blood donation
- Viral, bacterial and parasitic infections (malaria)
- Red cell membrane abnormalities (spherocytosis, stomatocytosis, xerocytosis…)
- Application of leeches
- Exercise-related hemolysis
- Alcoholic intoxication, drug use, diarrhea and vomit
- Hyper/hypothyroidism, other endocrinopathies
- Multiple association (including stress, dehydration, etc.)
Athletes with high HB/OFF often report that their HB is naturally high, while normal (baseline) HBs in their profile ensue from disease.
Hereditary spherocytosis
Drugs

- Glucocorticoids
- Thyroxine
- Ozone therapy
- Iron, supplements, vitamins (athletes justify high values with iron, $B_{12}$, folates)
- Vegetal drugs
- But also side effect of undetected illegal drugs
  - Anabolic steroids
  - Other hormones or pro-hormones (GH, IGF-1)
Further increase during a recent Grand Tour
Erythropoietic effects of glucocorticoids (GC)

- Increase erythroid colony formation and growth and modulate response to EPO and SCF in vitro and in mice
- Regulate the division and maturation of early BFU-E (and thus increase the output and differentiation of CFU-E)
- Polycythemia in Cushing syndrome, efficacy in Diamond-Backfan anemia
- GC are recognised as performance enhancing in competition, while their use in training is not prohibited
- Among other effects, hematologic effects suggest that performance gains are possible not only in competition
- Confounding effects on ABP parameters are certainly possible OOC
- Should glucocorticosteroids be prohibited at all times?
Erythropoietic effects of anabolic steroids (AS)

- men, women, transsexuals
- animal experiments, human pathology (hyperandrogenism, hypogonadism, AAS side effect: polycythemia)
- direct effect on progenitors
- increased synthesis and secretion of EPO
- increased iron absorption and incorporation into red cells (inhibition of hepcidin transcription)
- increase in RBC mass
- increased HB and HCT
- increased reticulocytes
Summary

- Blood doping can be detected by direct methods
  - ESAs with a short detection window
  - homologous transfusion
- Indirect tests, in the form of the longitudinal hematological monitoring, are a basic complement for autologous transfusion and microdose and combined strategies
- The Athlete Biological Passport, based on the assessment of intraindividual variation of hematological parameters, currently is the most effective indirect method
- The interpretation by experts, including laboratory hematologists, is invaluable to avoid false positivity related to analytical and physiological confounding factors
- Interested hematologists can propose themselves to become an ABP expert