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Supplementary Appendix

Haemoglobin electrophoresis and HPLC

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1. **Haemoglobin electrophoresis in cellulose acetate at alkaline pH** (cellogel; pH=8.3 or pH=8.6). At alkaline pH, haemoglobins are negatively charged proteins so they move toward the anode (+), as shown in Figure S1.

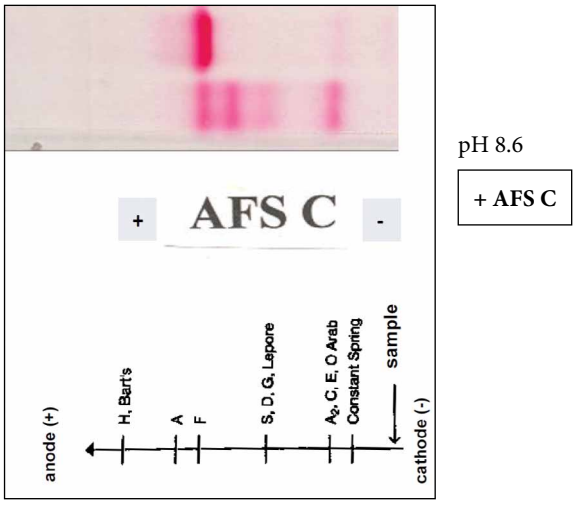


FIGURE S1. Alkaline haemoglobin electrophoresis.

2. **Haemoglobin electrophoresis in agarose citrate at acid pH** (agarose gel, pH=6.0 or pH=6.2 or pH=6.5). At acid pH, haemoglobins are positively charged proteins so they migrate toward the cathode (-), as shown in Figure S2.

   - **At alkaline pH, haemoglobins S, D, G migrate together at the same position. Hb Lepore** (δ-β fusion hybrids) migrates very close to S/G/D. Their distinction is possible with electrophoresis at acid pH, HPLC and sickling test. Three Lepore haemoglobins have been identified on the basis of δ-β crossover: Lepore-Boston (also called Lepore-Washington or Hb Pylos), Lepore-Hollandia, and Lepore-Baltimore. Hb Lepore results in a β-thalassaemia-like condition: heterozygous Hb Lepore resembles thalassaemia minor and the homozygous state results in a thalassaemia major-like condition. **Hb D** has a limited distribution (Punjab region at India-Pakistan border, where its incidence is 3%) and is clinically mild. Hb D heterozygotes are completely asymptomatic; Hb D homozygotes have mild anaemia with many target cells in the blood film or they are asymptomatic. **Hb G** is a rare α chain variant seen in Ghana and in African-Americans (Hb $G^{Philadelphia}$). Hb G is stable and is not associated with haematological abnormalities.
   - There are 6 haemoglobins associated with the **sickling phenomenon** except Hb S (they all have the mutation β6: Glut→Val plus one additional point mutation): Hb C^{Harlem}, Hb $C^{Georgetown}$, Hb $S^{Antilles}$, Hb S^{Oman}, Hb S^{Travis}, and Hb $S^{Providence}$. They are associated with a (+) sickling test and (+) solubility test, but migrate at a different position on alkaline Hb electrophoresis and HPLC. Clinically, these haemoglobins behave as Hb S.
   - **Hb I** (an α chain variant, stable, no symptoms) and a large quantity of Hb Barts (γ4) may give a (+) solubility test. The clinical importance of Hb I is that it migrates at the same position as Hb H in alkaline electrophoresis (fast Hb variant). Hb I is not associ-

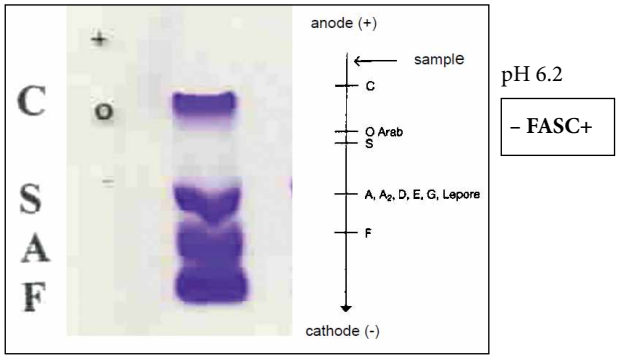


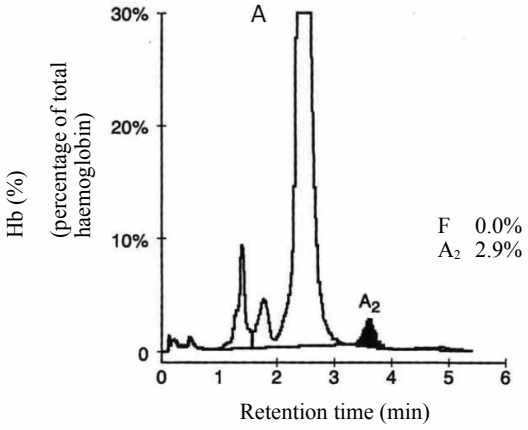
FIGURE S2. Acid haemoglobin electrophoresis.

ated with Hb H inclusions or golf-ball cells. Hb I is found in the Mediterranean littoral and in Africa.

- **Hb O^{Arab}** is rare in the tropics. Hb O is a β haemoglobin variant: Glut → Lys (β121). Hb O is characterised by the formation of denser and more spherical erythrocytes, leading to elevated MCHC in combination with a slight decrease in MCV. The clinical importance of this haemoglobin is that it migrates at the same position as Hb C in alkaline Hb electrophoresis, but they are separated on acid Hb electrophoresis. Haemoglobin O-Arab heterozygotes show no clinical manifestations; homozygotes present with mild haemolysis and splenomegaly of minimal clinical significance, but may develop haemolytic anaemia during infection or severe illness. Importantly, the anaemia caused by combinations of Hb O-Arab with β thalassaemia trait (β+ or β^0) varies from benign to transfusion-dependent, and sickling is enhanced when Hb S and Hb O^{Arab} coexist. Although Hb O^{Arab} is widely distributed, it is mostly detected in Eastern Mediterranean and Middle East populations. The Greek Pomaks, a Muslim population of the mountainous area of Thrace, demonstrate Hb O^{Arab} in impressively high percentage (5.076%), which reaches 27.4% in selected villages (Hb O^{Thrace}).

3. Cation-exchange High Performance Liquid Chromatography (HPLC)

- The normal HPLC pattern is shown in Figure S3.
- Normal values: **HbA2** = 1.9-3.3% and **HbF** = 0-2%



Retention times of important haemoglobins:

Hb F → 1-1.2 min

Hb A → 2.5 min

Hb A2, Hb Lepore, Hb E → 3.3-3.9 min (same retention time)

Hb S, Hb D → 4.5 min (same retention time)

Hb O → 4.8 min

Hb Constant Spring → 5 min

Hb C, Hb G → 5-5.2 min

Hb H (β4), Hb Barts (γ4) → they are eluted very quickly from the column (almost immediately)

Normal values: **HbA2** = 1.9-3.3% and **HbF** = 0-2%

FIGURE S3. Normal HPLC pattern.

Example 1: A 13-year-old girl of Filipino descent, with hypochromia, microcytosis, and many target cells. No history of transfusion and her parents are healthy. Figure S4 shows her HPLC. Diagnosis: Hb E heterozygote (Hb AE).

Example 2: A 33-year-old man from Nigeria with anaemia (Hb 10.0 g/dl, MCV 82 fl), splenomegaly and recurrent leg pain. No history of transfusion. His family history is unknown. Figure S5 shows his HPLC. Diagnosis: Hb SC disease.

J. B. S. Haldane first suggested that that the geographi-

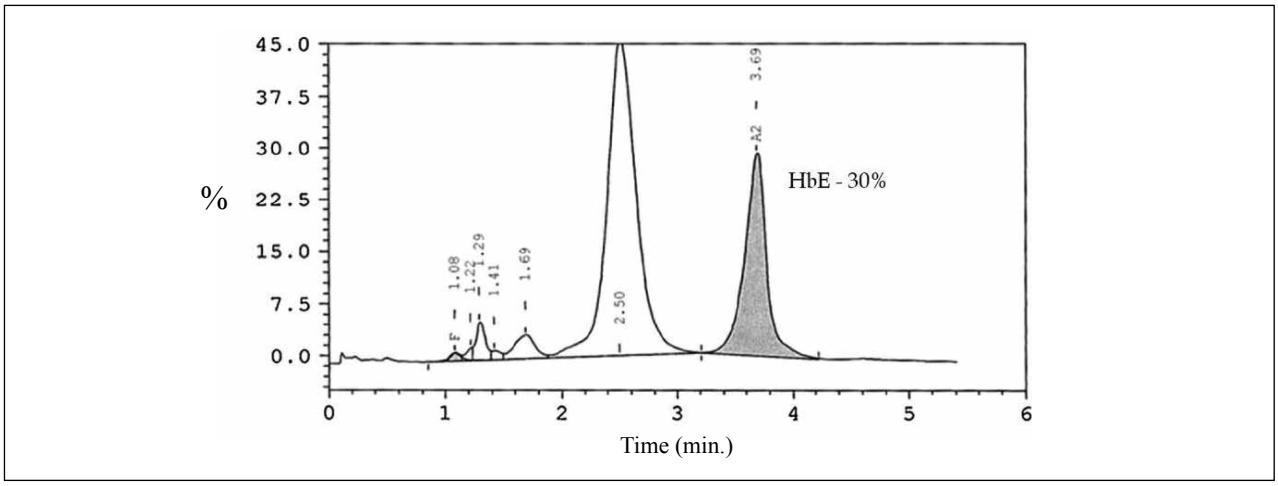


FIGURE S4. HPLC consistent with heterozygous HbE (HbAE).

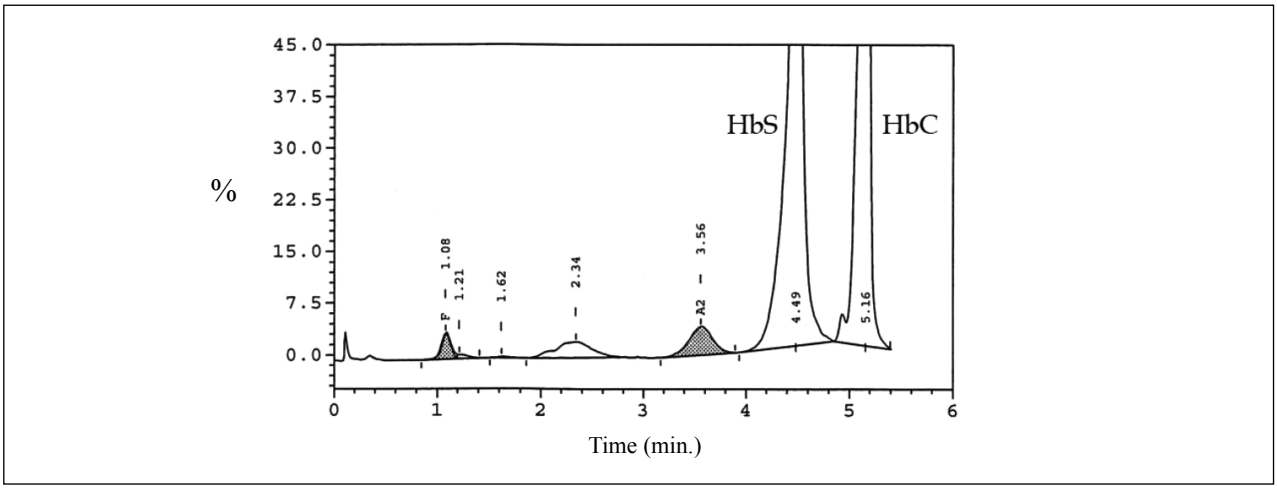


FIGURE S5. HPLC consistent with HbSC disease.

cal co-incidence of malaria and β-thalassaemia major (Cooley's anaemia) could be due to the heterozygotes (β-thalassaemia minor) being at genetic advantage through a partial protection against *P. falciparum*. A relative resistance to malaria was confirmed in Liberian children with thalassaemia minor (β/β^+). Another classic example of what Haldane called **balanced polymorphism** (i.e. heterozygotes are protected against malaria while the harmful genetic effects are restricted to homozygotes) is Hb S. African children who are heterozygous for Hb S are 10 times less likely to develop life-threatening complications of *P. falciparum* infection than those who lack this allele.

Tips:

1. ***Always obtain a family history when haemoglobinopathy or thalassaemia is suspected!***
2. ***The diagnosis of heterozygous β-thalassaemia (β-thalassaemia minor) depends upon finding an increased Hb A2 >3.5%, usually 4-6% (a higher value may be seen in some cases but values of Hb A2 >7% are rare). Hb F is slightly increased in 40-50% of individuals with heterozygous β-thalassaemia (usually up to 3%; in β/β^0 trait up to 5%). In cases of:***
   - ***HbF >5% → consider δβ-thalassaemia carrier (Hb A2 <3%) or HPFH heterozygote (Hb F 5-16%).***
   - ***low Hb A2 (<1.9%) → consider co-inheritance of δ-thalassaemia***
   - ***Hb A2 ≥19% → consider Hb E (Hb E migrates at the same position as HbA2 on alkaline and acid Hb electrophoresis and HPLC).***
3. ***In carriers of sickle cell anaemia (Hb AS), the percentage of Hb S is usually 35-45% (because the rate of Hb S synthesis is slower than Hb A). If:***
   - ***Hb S is <33% → consider S-α thalassaemia co-inheritance.***
   - ***Hb S is ≥50% → consider S-β thalassaemia (also has an increased Hb A2 3.5-5% and HbF 5-10% or more) or sickle cell anaemia and recent blood transfusion.***

I have found the following references of considerable value in preparing this manuscript. Many further references will be found in each of these works.

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