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MILESTONES IN THE HISTORY OF HEMOGLOBIN RESEARCH (In Memory of Professor Titus H.J. Huisman)

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□ Professor Titus H.J. Huisman is best known for his work on hemoglobin (Hb) variants. To date, more than 1,000 Hb variants have been discovered and characterized, of which about one-third were discovered in Titus Huisman's laboratory at the Medical College of Georgia, Augusta, GA, USA. A registry of these Hb variants and other information, a legacy from Professor Huisman, is now available online, at HbVar database (<http://globin.bx.psu.edu/hbvar>). During the last century, major developments in Hb research have been made using physical, chemical, physiological and genetic methods. This review highlights the milestones and key developments in Hb research most relevant to hematologists, and that have impacted our understanding and management of the thalassemias and sickle cell disease.

Keywords Landmarks in sickle cell disease and thalassemia research

INTRODUCTION

The inherited disorders of hemoglobin (Hb), including sickle cell disease and the thalassemias, are the most common 'Mendelian' genetic disorders in the world (1). To date, more than 1,000 Hb variants have been

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discovered and characterized; a registry of these variants and other information, initiated by Titus Huisman, is available online at the database HbVar (<http://globin.bx.psu.edu/hbvar>). The study of these variants established the principle of genotype/phenotype correlation that underlies how molecular genetics impacts our understanding of disease mechanisms, and became a prototype of how to unravel the different factors contributing to complex phenotypes in so-called single gene Mendelian disorders. Hemoglobin research has also led the way in the understanding of gene regulation, and structure-function relationships of macromolecules (2).

Major developments have been made in Hb research using not just genetic approaches, but also physical, chemical, and physiological, particularly in the first half of the last century (2). The list of contributions to the pioneering work is long, and let us not forget that each of these discoveries was only possible through understanding the research and discoveries of earlier pioneers as remarked by Isaac Newton “if I have seen a little further than others, it is because I have stood on the shoulders of giants.” This metaphor is as applicable to mathematics and physics as it is to Hb research (3). Samuel Coleridge (1828) interpreted the metaphor in another way – the dwarf sees further than the giant when he has the giant’s shoulders to mount. These metaphors were said to be derived from Greek mythology in which the blind giant Orion carried his servant Cedalion on his shoulders as depicted in a picture by Nicolas Poussin (1658) (Figure 1).

These milestones in Hb research reflect my own interests and perceptions. I will highlight the key developments most relevant to hematologists, that have impacted the two main Hb disorders, thalassemias and sickle cell disease, in three areas: diagnostics and predictive genetics, biology and pathophysiology, and management.

Hemoglobin Structure

Hemoglobin is the protein in red blood cells that is responsible for the delivery of oxygen from the lungs to the tissues, and the transport of carbon dioxide from the tissues back to the lungs. The human Hb molecule is a tetramer made of two heterodimers, composed of a pair of α -globin-like, and another pair of β -globin-like, polypeptide chains. Each of these globin chains is linked to a heme molecule and binds to oxygen and carbon dioxide, and two other gases, carbon monoxide and nitric oxide. It is the reversible binding to the ferrous iron atom in the globin tetramer that allows Hb to transport these gases in solution in blood (4,5). The three-dimensional structure of the Hb protein was deciphered by Max Perutz using X-ray crystallography, a major discovery for which he was awarded the Nobel Prize for chemistry in 1962 (6). The structural analysis provided an explanation of how Hb functions as an oxygen transporter. Ernie Huehns



FIGURE 1 Nicolas Poussin (1658) – Cedalion (circled) standing on the shoulders of Orion from *Blind Orion Searching for the Rising Sun*.

and others observed that different types of Hb were expressed at different stages of human development, involving two switches, from embryonic to fetal and then fetal to adult (7). In the last 30 years there has been a surge in the progress of understanding the molecular and cellular mechanisms underlying the switch from fetal to adult Hb synthesis. This surge began, perhaps, with the development of recombinant DNA technology and genomics in the early 1970s and 1980s, which led to the identification of a wide range of globin gene mutants that have provided insights on diverse clinical manifestations encountered in patients with thalassemia and sickle cell disease. In addition, evolution of these techniques has enabled development of molecular techniques for prenatal diagnosis (PND) and polymorphism-based population studies, both of which have been applied to many other disorders.

The landmarks over the last century are summarized in Figure 2a, 2b and 2c; discoveries in sickle cell disease and the thalassemias have been mutually beneficial to both disorders. The molecular defect causing sickle cell disease was uncovered more than 60 years ago, while deciphering the molecular defects underlying the thalassemias only became possible in the late 1970s with the emerging techniques and tools of molecular biology and genetics.

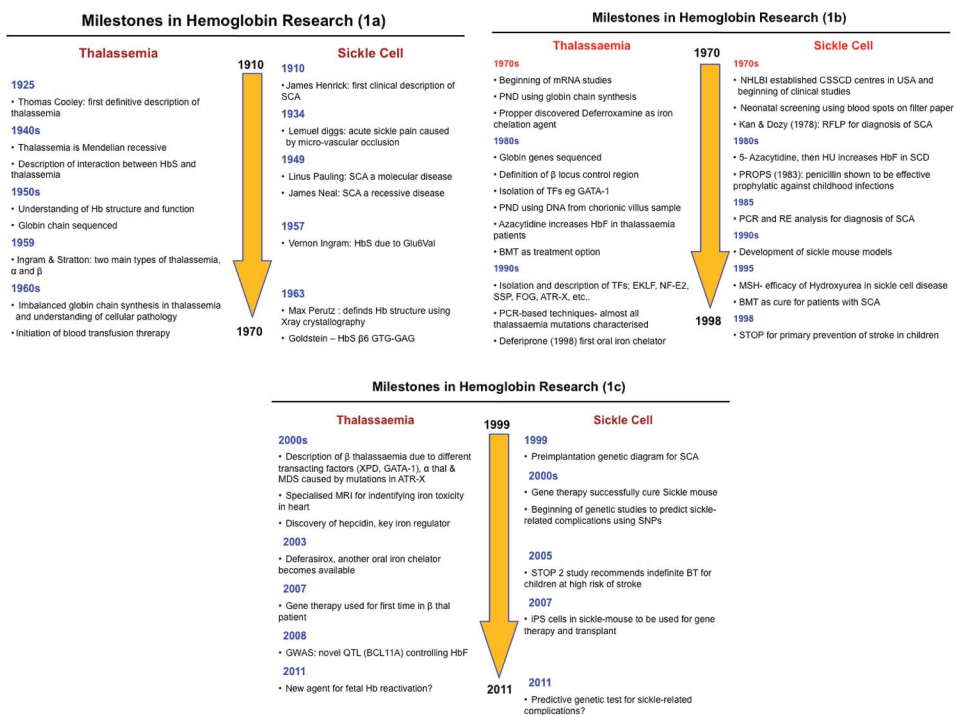


FIGURE 2 Milestones in hemoglobin research. a) 1910 to 1970; b) 1970 to 1998; c) 1999 to 2011.

Setting the Scene: Sickle Cell Disease

In 1910, James Herrick described the pneumonia-like symptoms and the presence of large numbers of peculiar elongated and sickle-shaped red blood corpuscles in a blood sample from a 20-year-old dentistry student from Grenada (8). He surmised that the severe anemia in the patient may be caused by some unrecognized change in the corpuscle itself. In recounting his observation in the Archives of Internal Medicine, Herrick provided the first clinical description of sickle cell disease in the western medical literature. However, there have been earlier medical reports that alluded to some of the complications afflicting black slaves that suggest the presence of sickle cell disease, such as missing spleens on autopsy, severe joint pains, jaundice, and scars suggestive of leg ulcers as far back as the 1800s (9,10). Furthermore, the clinical spectrum of sickle cell disease has also been recognized for centuries in West Africa in the various tribes, accompanied by different traditional medicines such as tattooing and priapism girdle (11,12). There have also been randomized control trials of Neprisan, a traditional medicine in West Africa that had been developed from traditional herbs and roots. After Herrick's report, three others followed within a period of 12 years and all affected were of African descent leading many to believe that only the

black population was susceptible to sickle cell disease (13–15). Once sickle cell disease presented itself as a distinct entity in the western medical literature, and identified as a new disease rather than a variant manifestation of a known condition, many theories behind its pathophysiology were suggested.

In the 1930s, as epidemiology and complications of the disease increased, Diggs and Ching (16) proposed that the painful sickle cell “crises” could be attributed to blockage of the small blood vessels due to the abnormal red cells. In 1949, Linus Pauling and others (17) demonstrated that patients with sickle cell anemia have Hb with an altered charge. The term “molecular disease” was coined, referring to linkage of specific diseases to abnormalities of specific molecules. In 1956, Ingram (18) showed that Hb consists of two identical half molecules in agreement with the X-ray crystallographic evidence from Max Perutz, and that sickle Hb differs from the normal in only a tiny part of the molecule. Soon after in 1957, Ingram (19) showed that the abnormality in sickle Hb was due to a single amino acid substitution (valine for glutamic acid) at position 6 of the β -globin chain. This was then deciphered by Goldstein et al. (20) in 1963 as being caused by a single base change of T>A at codon 6 (GTG β 6GAA).

The 1950s was a period of discovery of the different human Hb and Hb variants (contributors included Herman Lehmann, Phaedon Fessas, and Titus Huisman) (21–25). Diagnostic tools were developed to identify these different Hbs, such as the use of metabisulfite as a reducing agent to confirm the presence of sickle Hb. The sickling test is still used today to confirm a diagnosis of sickle cell disease in some countries (26).

Setting the Scene: Thalassemia

The first definitive description of thalassemia was made in 1925 by Thomas Cooley and his collaborator Pearl Lee (27). The clinical syndrome of severe anemia, hepatosplenomegaly and jaundice in a series of children is almost certainly that of severe β -thalassemia (β -thal), thus clearly separating thalassemia away from the mixed collection of the anemias of infancy noted at that time. It was likely that thalassemia was also being seen in the Mediterranean; similarities of the different anemias encountered in the region did not appear to have alerted the clinicians at that time that thalassemia could be a separate entity in itself (28,29). In the 1940s, progress was made in understanding the genetic basis for thalassemia and it became clear that thalassemia encompasses a complex syndrome inherited in a Mendelian recessive fashion. The mild form of Cooley’s anemia was termed “thalassemia minor” and the severe type described by Cooley as “thalassemia major.” It was around this time that Neel (30) together with the findings of Pauling, suggested that the sickle cell gene is a mutant allele of the Hb A gene; sickle cell anemia is a recessive disease.

While inheritance patterns were being worked out through family studies, rapid progress was made towards elucidation of the Hb chemical structure. The 1950s was a period of discovery of the different types of Hb and Hb variants (31,32); the sequence of the different globin chains was unraveled and rapid progress was made towards an understanding of the structure and function of different Hbs (33), marking the beginning of genotype-phenotype correlation. At the same time, there was a steady accumulation of the inheritance patterns of these different Hb variants, particularly through family studies (34). By the late 1950s, the complete amino acid sequence of the α , β and γ chains had been established. This information, together with the discovery that Hb variants followed a simple Mendelian pattern of inheritance, led Ingram and Stretton (35) to propose that there might be two main types of thalassemia, α and β . The 1960s was a period for formalizing and consolidation for understanding the pathophysiology of thalassemia. Erythrokinetic studies on patients with thalassemia major by Sturgeon and Finch (36) showed a marked degree of ineffective erythropoiesis, implying extensive destruction of red cell precursors in the bone marrow as well as shortened peripheral blood survival. A clue to the basis for the ineffective erythropoiesis was suggested by Phaedon Fessas from Athens in 1963 (37). He could demonstrate irregular inclusion bodies in the bone marrow erythroid precursors of patients with β -thal and suggested that the inclusions are precipitated redundant α chains produced because of the deficiency of β chains in thalassemia cells. However, demonstration of the imbalanced globin chain synthesis was not possible until a landmark development – that of the technique for quantitating and separating α , β , γ and δ chains of Hb *in vitro* from peripheral blood reticulocytes developed by John Clegg and his colleagues, David Weatherall and Michael Naughton (38–40). These *in vitro* studies demonstrated unequivocally that α - and β -thalassemias are disorders characterized by imbalanced globin chain production.

Milestones: 1960–2000

Although a working model of the genetics of the Hb disorders was available and simple techniques for analyzing levels of Hbs A₂ and F, and for detecting Hbs H and Bart's had been developed, progress was frustrating and slow until the 1960s and 1970s, when it became possible to analyze genes directly through mRNA studies. cDNA/DNA hybridization showed the absence of α -globin genes in Hb Bart's hydrops. Around this time, β -thal was also shown to be caused by a deficiency of β -mRNA, describing the first nonsense mutation (41). On the treatment front, intensive blood transfusion for β -thal was recommended, a treatment that is still used today. However, with blood transfusion therapy comes iron overload; several groups of investigators including Richard Propper, Martin Pippard

and colleagues showed that the excess iron leading to iron toxicity could be removed by continuous subcutaneous infusion of the first iron chelating agent, deferoxamine (42,43). In 1970, Roland Scott wrote an article in the Journal of the American Medical Association comparing the disparity in research funding for sickle cell disease and other genetic disorders (44). This prompted the formation of the Association for Sickle Cell Disease in 1971, and comprehensive sickle cell disease centers were established in the USA by the National Heart Lung and Blood Institute (NHLBI), which facilitated the beginning of clinical studies in sickle cell disease.

In the late 1970s/1980s, the development of recombinant DNA techniques made it possible to clone and sequence globin genes and rapid progress was made towards unraveling the molecular basis of the thalassemias. In 1975, with the routine use of real-time ultrasonography for localization of the placenta, sampling of fetal blood became relatively safe, and was utilized for assessing globin chain synthesis for prenatal diagnosis of β -thal, followed a year later for sickle cell anemia (45,46). In 1978, the human β -globin gene was located on chromosome 11. In the same year fetal DNA could be obtained from amniotic fluid cells; Kan and Dozy (47) published a seminal paper on the use of restriction fragment length polymorphism (RFLP) for detection of the sickle mutation on the β -globin gene. They showed that the cleavage site for the restriction enzyme *Hpa*I downstream of the β -globin gene was absent in β chromosomes carrying the sickle mutation. *Hpa*I- β Southern blot hybridization showed two fragments of 7.0 and 7.6 kb for β^A chromosomes, DNA for the sickle mutation produced only 13.0 kb fragments. The β^S gene was thus linked to the *Hpa*I- β RFLP and the strategy was applied in the PND of sickle cell anemia by DNA analysis of amniotic fluid cells. In 1981, Williamson and colleagues showed that it was possible to obtain DNA from chorionic villus samples and in the same issue of The Lancet, Chang and Kan showed that antenatal diagnosis of sickle cell anemia was possible by direct analysis of the sickle mutation (48,49). Linkage of the β^S mutation to the *Hpa*I- β restriction site led to the concept of β haplotypes generated from a panel of different RFLPs. Stuart Orkin and colleagues (50) were quick to capitalize on this concept; using an approach targeting alleles from nine β haplotypes, they identified eight novel β -thal mutations.

The 1980s, a period of recombinant DNA technology, was also a period of considerable effort by several laboratories to identify proteins involved in globin gene regulation and Hb switching. During this period, Forrester *et al.* (51), Dorothy Tuan *et al.* (52) and Mark Groudine *et al.* (53) identified Dnase 1 hypersensitive sites, markers of regulatory activity, upstream of the human β -globin gene cluster. The 1980s/1990s saw the isolation and characterization of several transcription factors controlling globin genes based on the pioneering work of Gary Felsenfeld, Stuart Orkin, Jim Bieker, Doug

Engel, Doug Higgs, George Stam (Stamatoyannopoulos), Frank Grosveld, and others [for reviews see (54,55)]. 1985 was a landmark year, Saiki used an enzymatic approach, polymerase chain reaction (PCR), to amplify the human β -globin gene and identification of the β^S mutation by restriction enzyme analysis (56). Enzymatic amplification marked the beginning of the end of bacterial cloning for sequence analysis of genes, and the beginning of direct sequence analysis. The PCR technology has revolutionized and permeated the whole of DNA diagnostics; Kary Mullis was awarded a Nobel Prize in Chemistry for this invention in 1993. 1987 was another major landmark in Hb research: Frank Grosveld and his group (57) described the dominant control region for the β -globin locus [subsequently renamed β -locus control region (β -LCR)] at the 7th Conference on Hemoglobin Switching]. Elements in the β -LCR critical for regulation of genes in the β -globin gene cluster have been characterized and inserted in gene transfer vectors, making possible the first gene therapy for β -thal. Subsequently, the equivalent of β -LCR has also been shown to exist in the α -globin gene cluster as a collection of multi-species conserved sites (58). The equivalent of the β -LCR has also been shown to exist in other gene systems (59).

Discovery of the LCR prompted many experiments, including many studies in transgenic mice. Data from transgenic mice studies, linking of the human β - or γ -globin genes, individually or together, in different orders (60), and insights from the different natural mutants causing hereditary persistence of fetal Hb (HPFH) (61,62), led to the formulation of the concept of Hb switching that involves a combination of two mechanisms, autonomous gene silencing and gene competition for the repression of one globin gene and activation of another during development (54). In both scenarios, the interaction of *cis*-acting sequencing with *trans*-acting factors is involved.

On the treatment front, bone marrow transplantation was first used to cure β -thal in 1982 (63). The ability of fetal Hb (Hb F, $\alpha_2\gamma_2$) to inhibit sickle Hb polymerization prompted many investigators to find therapies for reactivation or prevention of γ -globin gene repression using pharmacotherapy and gene transfer (64–66). Several independent studies showed that reactivation of Hb F was possible using a number of cytotoxic agents: 5-azacytidine, sodium butyrate, Ara-C, vinblastine, then hydroxyurea (HU). Of these, HU has stood the test of time (67,68) and is now the only agent approved for treatment of sickle cell disease in the USA and Europe. In 1986, the National Institutes of Health (NIH) prophylactic penicillin studies (PROPS) (69) showed that daily penicillin was effective in reducing the incidence of childhood pneumococcal infection in sickle cell disease, and recommended that prophylactic penicillin should routinely be started in infants with sickle cell disease by the age of 3 months. The 1990s was a period of the development of different sickle mouse models (70). Deferiprone, the

first oral iron chelator was licensed in India in 1994, and achieved full marketing authorization in Europe in 2002 (71), and was shown to be clinically beneficial for the treatment of iron overload. In 1998, the stroke prevention in sickle cell anemia (STOP) clinical trials showed that transcranial Doppler ultrasonography, a method of analyzing blood flow in the brain, is an effective screening tool for detecting children at high risk of stroke (72). The trial also showed that blood transfusion was effective in primary stroke prevention, with a 92% reduction in those at high risk as shown by transcranial Doppler velocities.

21st Century: 2000 to the Present Day

By the 2000s, an almost complete spectrum of the α - and β -thal mutations have been identified. Genotype/phenotype correlation indicated the importance of genetic modifiers, clinical and genetic studies provided evidence of *trans*-acting factors controlling different globin genes: α -globin (ATR-X) (73), β -globin (TF11H, GATA1) (74,75), γ -globin (BCL11A) (76,77). Improvements were continually made in different gene transfer vectors; lentivirus, a subclass of retrovirus, was shown to be superior to retrovirus. In 2007, gene therapy was used for the first time in transfusion-dependent β -thal in France. This pilot gene therapy using a lentiviral vector was published in 2010 (78), and considered to be a clinical success. The patient became transfusion-independent and was even started on a phlebotomy program to reduce the iron overload accumulated from the blood transfusion pre gene therapy. In 2007, the group of Tim Townes (79) showed that it was possible to generate pluripotent cells from autologous skin to be used for gene therapy and transplant in mice. Between 2007–2010, genetic studies indicated new genetic loci (*HBS1L-MYB* intergenic region on chromosome 6q, *BCL11A* on chromosome 2p) (76,80,81) and new pathways involving KLF1 in the switch from fetal to adult Hb (82–84). There is a surge in genetic studies (genome-wide and candidate genes) and methods for predicting complications and disease severity in sickle cell disease (85).

CONCLUSIONS

A vast body of knowledge has been accumulated in the last 30 years on the pathophysiology and biology of the Hb disorders. This has led to the development of new agents and new strategies in their management. We stand on the shoulders of the pioneers who went before us to meet other challenges ahead, and these include: (1) making non invasive PND a reality for sickle cell anemia and the thalassemias. It is likely that sequencing of maternal plasma DNA will play an increasingly important role (86,87).

Deep sequencing of DNA should include a comprehensive panel of single nucleotide polymorphisms as predictive diagnostics for delineation of risk factors to facilitate preventive therapy according to the risk profile at birth; (2) develop individually-tailored therapies based on genomic information; (3) develop effective drug therapies for iron chelation, reactivation of fetal Hb in the treatment of β -thal and sickle cell disease. Recent discoveries have suggested some attractive options; for example, knocking down BCL11A; (4) broaden availability of bone marrow transplantation in adults; improve non myeloablative conditioning (88); (5) make gene therapy a reality for β Hb disorders. Continue to improve gene delivery systems: vectors, gene transfer, reduce toxicity of pre transplant conditioning.

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