



The Beautiful World of Haemoglobin

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Haemoglobin (Hb) is the respiratory pigment of man and many other mammals. The molecule captures oxygen in the lungs and transports it within the red blood cells for release in the tissues. It is a fascinating molecule. Although deceptively simple in structure, (Fig. 1) it is intriguingly complex in physiology and genetics. Really, the fully functional molecule is one of the smaller proteins known. It is assembled from four subunits; all proteins and known as globins. Each globin resembles the structure of the simpler oxygen storage molecule, myoglobin, mostly found in muscle. Like myoglobin, each subunit bears a small inorganic iron molecule (haeme) that is the actual oxygen ligand-binding site. The four globins thus have four oxygen binding sites on each Haemoglobin molecule. Although essential in metabolism, body defence and vital regulatory processes, free oxygen is very toxic. It would have wrought havoc in human physiology unless significant biochemical mechanisms for safe oxygen binding and processing such as myoglobin and haemoglobin (and others) had not evolved. In fact, as long as the four globin subunits are dissimilar in pairs, they engage in complex interactions that determine the stability and the efficacy of the whole (Hetero)-tetramer to bind and release oxygen. They indulge in intricate mechanisms that regulate the assembly of the hetero-tetramer from its globin monomers and gracious movements among the globins within the molecule. The subunits co-operate as long as the haemoglobin tetramer is composed of two globins of one biochemical type, and two globins of another type. They are said to be "dissimilar in pairs" such that all physiological haemoglobins must have a pair of alpha (α) globins, while the other pair could be epsilon (ϵ) as in the embryo, gamma (γ) as in the foetus (Hb F or $\alpha_2 \gamma_2$) or beta (β) and minor quantities of delta (δ) as in the adult (Hb A or $\alpha_2 \beta_2$ and Hb A₂ or $\alpha_2 \delta_2$).

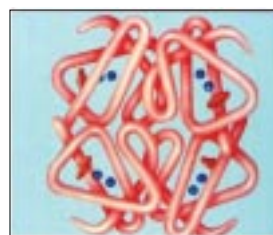


Fig. 1 - The structure of the haemoglobin molecule showing the four globin subunits each having a haem group and attached to each other in the hetero-tetramer.

The genetic control of haemoglobin production i.e. globin bio-synthesis, before and after birth is as complex and demanding as is the physiology of the molecule. Both matters have challenged some of the most leading luminaries in haematology, protein chemistry, physiology and genetics for the large part of the previous century. The molecular details of physiological function and the developmental control of globin gene expression remain fundamental inquiries even in contemporary human biology and medicine. The implications for health and disease are huge.

Congenital disorders of structure or function (or both) known as haemoglobin variants, or of haemoglobin biosynthesis, known as thalassaemia, are among the commonest hereditary disorders of mankind across the entire planet. The most frequent Haemoglobin variant is the Hb S. It is responsible for Sickle Cell Disease among Black and some other peoples. A single nucleotide change occurs in the sequence of the DNA coding for the beta globin gene. It changes the chemical composition (amino acid substitution) of the beta globin subunit. Consequently, the Haemoglobin S molecule assembles into anomalous rods after it unloads the oxygen to the peripheral tissues (deoxygenation). The red blood cells acquire a characteristically abnormal shape (sickle cells) and a shortened life span. Typically, the patients have decreased levels of Haemoglobin in blood (anaemia) and suffer episodes of pain and organ degeneration due to occlusion of blood vessels by the abnormally sickled

red blood cells. A few other Haemoglobin variants have been found in Malta too (Hb F Malta I, Hb Valletta, Hb St. Luke's, Hb Setif and some others). Worldwide, there are over 600 Haemoglobin variants known.

Thalassaemia results in red blood cells that are much smaller than normal (microcytosis), and rapidly destroyed with a shortened life span (haemolysis). The anaemia is due to defective haemoglobinisation of the red blood cells (hypochromia). Since the severe types of thalassaemia found in Malta and the rest of the Mediterranean result from defective beta gene control, it follows, as if it were the "holy grail of Haemoglobin Research" that, if we could understand precisely the biochemical and genetic mechanisms that regulated the physiological developmental transition from embryonic to foetal and subsequently to adult globin biosynthesis, then, we could possibly be able to reverse the perinatal gamma to beta globing gene switch and produce foetal haemoglobin to correct the anaemia of the adult patients. Hb F is a very adequate substitute for the Hb A of adults. The search for therapeutics that could switch Haemoglobin biosynthesis from one gene to another has been pursued for some time in Haemoglobin Research. We have acquired experience with a set of compounds in our clinic but the clinical outcome is not yet completely satisfactory.

As often happens in science, progress in understanding the patho-physiology runs along progress in analytical and related technologies i.e research tools. Undoubtedly, our understanding of Haemoglobin physiology and genetics will gain from the recently published Human Genome Map, just as much as the most modern thoughts and techniques of the time, such as advanced separatory techniques with electrophoresis and chromatography, cell cultures and molecular biology contributed in the past. Often, anecdotal observations on patients, families and populations provide critical clues that push a whole field forward. Above and beyond that which strictly pertains to normal Haemoglobin and Haemoglobinopathies, however, as we look back on the profound knowledge acquired about the assembly and the genetic control of Haemoglobin, we developed paradigms with which to understand other genetic disorders. In particular, we have taken interest in those common complex diseases such as diabetes, heart disease and neuro-degenerative disorders including Parkinson's disease, which are thought to be due to the inheritance of multiple abnormal genes with quantitative effects. It is captivating that many single and multiple gene disorders may have become common in populations because the ancestral genetic abnormality (allele) must have given the carriers of the time (heterozygotes) some selective advantage compared to their contemporaries. Red blood cell parasites such as malaria or leishmaniasis may have been the selective agent in favour of haemoglobinopathies. Although the selective pressure may now be absent from our contemporary human populations, the new ancestral alleles may have become common and carried with them potentially deleterious alleles which happened to be close in the genome with negative impact on body defence mechanisms (inflammation) Sometimes, it looks as if because of discrepancy between the genetic history and the contemporary condition, the human body defences turn upon itself!

From a personal perspective, I was introduced to the challenges of Haemoglobin research in the early seventies during the time that, as a young house-physician at St. Luke's Hospital, I fell under the tutelage of the late Joe Louis Grech who was the Senior Consultant in charge of the Clinical Biochemistry Laboratory at St. Luke's but who also had a great interest in Haematology. We used to spend long hours together setting up the quantification of the minor Haemoglobin with the delta globins, i.e. Hb A₂ which is diagnostic of beta thalassaemia and in separating the blood of many new-born to document the occurrence of the haemoglobin variants already referred to above. Although

there is not any sickle cell disease, the beta thalassaemia is as common in Malta as in other Southern European and Mediterranean peoples (heterozyote carrier rate = 1.2% and there are 36 homozygote patients registered in our clinic).

At that time, Maurice Cauchi had just published in *Nature* together with the Oxford Group about Hb F Malta I, which was found in just fewer than two percent of Maltese newborn. Much later, after I had just returned to the University of Malta from the Medical College of Georgia (Augusta, Georgia, USA), where I had been Assistant and Associate Professor, we could show together with my former colleagues in Augusta that all those who had inherited the Hb F Malta I variant also inherited another variant in the beta globin gene of the same locus and which we then called Hb Valletta. The occurrence of genetic markers like Hb F Malta I and Hb Valletta is the result of genetic events in the course of population movements. Today, we have precise genetic tools for the analysis of certain DNA sequences in the gender determining chromosomes, the X and the Y chromosomes, and the female cognate mitochondrial DNA to correlate with the occurrence of peculiar Haemoglobin variants or indeed many other molecular variants such as blood groups. Together with David Goldstein at University College, London, UK, we have traced the origin of most modern Maltese males, as expected, to Southern Italy and Sicily. It remains to be seen whether we could trace the Hb F Malta I-Hb Valletta locus to an earlier beginning that survived repopulation around the turn of the prior millennium. Joe Louis was also working with William Bannister who had just returned from Oxford as Professor of Physiology on another new Haemoglobin variant which they designated Hb St. Luke's. Traditionally, any new Haemoglobin variant that is discovered is given the name of the place in which it is first described. Later, my group also discovered Hb Setif, Hb Marseilles and a few others among Maltese heterozygotes. Before Cauchi and, Grech and Bannister, Frank Vella had done population level testing for beta thalassaemia among the Maltese.

It can be seen that the University of Malta and St. Luke's Hospital have a long tradition of competitive research on Haemoglobin. As far as I know, the only two research manuscripts published from Malta in the prestigious scientific journal "NATURE" deal with Haemoglobin. The first was the Hb F Malta I paper by Maurice Cauchi in 1969 and the second, which I co-authored, concerned the genetics of Hb F and was written in co-operation with the groups from Malta, Augusta, Pasadena and Los Angeles.

Both Hb F Malta I and Hb St. Luke's influenced my professional career and my personal life. When I finished my training at St. Luke's, Joe Louis Grech and William Bannister suggested that I could continue my studies and read for a Master's degree with the external support of the group in Augusta. The director Titus Huisman later became a prominent guide and mentor for me, an academic colleague on the faculty at Augusta and a great personal friend. We lived and worked in Augusta (golf capital of the world!) for around 13 years. I directed the Laboratory of Haemoglobin Research (Molecular Haematology) in the Veterans' Administration Medical Research Service, which was across the road from the Medical School, in which I had my academic appointment. My project was mainly an epidemiological one and related to diagnostics and experimental profiling. We employed the best analytical and quantitative techniques of the time to determine the types of Hb variants and thalassaemia characteristic of certain populations across the world including Malta. Thereby, comparisons of the biochemical and haematological features, even before the advent of direct DNA studies we could infer the structural organisation and physiological control of the globin genes. The proper description today would be to call it genotype-phenotype correlations or molecular expression profiling.

Hb St. Luke's, which is a variant of the alpha globin gene was found among 0.2% of Maltese newborn. We were apprehensive that the proportion of Hb St. Luke's and related Haemoglobin variants from the UK, Georgia and South Carolina in the USA, was always found below those levels consistent with concurrent ideas about the number of alpha globin genes. Later, we established that the low proportion of Hb St. Luke's and some similar alpha globin variants could be explained by assuming that the normal human alpha globin locus carried duplicated alpha globin genes. Thus, red blood cell precursors (erythroblasts) expressed four alpha globin genes, but the assembly of the Hb St. Luke's tetramers from monomers was defective. Eventually we quantified the number of normal alpha globin genes in the DNA of critical families and confirmed the normal genotype. We also showed, however, that the alpha globin gene number could vary due to the inheritance of alpha globin gene deletions (a thalassaemia), that each alpha globin gene could compensate for the alpha globin deficiency in alpha thalassaemia and that the same mechanism that explained the low proportions of Hb St. Luke's explained the variability in the proportion of other variants including beta globin variants such as Hb S in sickle cell carriers. Furthermore, we could explain the developmental haematology of Sickle Cell Disease, which accounted for discrepancy in the clinical picture on the basis of molecular genetics. Effectively, it resulted in retrospect, that those that we had considered as typical examples of major single gene disorders actually were the product of interaction between multiple genes with quantitative effects.

Today, we use the same model to try to understand the pathogenesis of other complex diseases. We assume that regulatory molecules involved in pathogenesis are also composed of subunits whose biosynthesis is controlled by multiple genes in which DNA sequence variation alters levels of expression and susceptibility or "risk" to disease.

In the course of these many years in the USA and in Malta, there are many anecdotes I can recall, but one stands out in particular. It was a Friday afternoon in the office of Titus Huisman when our close collaborator from Pasadena (Caltech), the late Walter Schroeder faxed to tell us about a graduate seminar he had just attended at which a young post-doc from Yale had shown data pertaining to interruptions in the coding DNA sequence of the globin genes, the exons. He called them intervening sequences or introns. Today we know that almost all human genes are organised in this manner. Indeed, defects at the junctions between exons and introns are among the most common known causes of human genetic disease. Even the most prevalent cause of beta thalassaemia in Malta and the Western Mediterranean is a result of inherited DNA sequence variation (mutation) in the same region (*Fig. 2*) i.e. the junction between the first exon and the first intron of the beta globin gene.

In the case of thalassaemia, we have determined the profile of different DNA mutations in Malta and genotyped all patients. In 1997 we hosted a very successful International Thalassaemia Meeting. Many thalassaemia patients have intractable ankle ulcers because of the severe anaemia. When Dragana Josifova was training in our clinic we evaluated the production and use of extracts from blood platelets in promoting wound healing. The study introduced us to the challenges of wound management and bio-manufacturing. Dragana is now consultant in Genetics Medicine at Guy's Hospital in London. It is gratifying to appreciate the number of graduate students and other

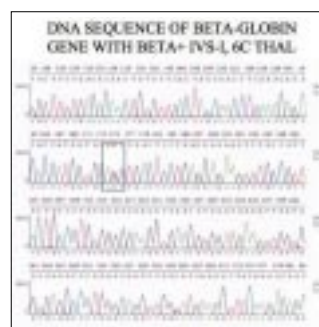


Fig. 2 - DNA sequence of the b globin genes from a Maltese b Thalassaemia heterozygote showing the normal and abnormal nucleotides (*boxed*) overlapping in the read-out.

trainees who passed through my programmes in Augusta and in Malta. These included the first Ph.D.s from our own Faculty of Medicine; Christian Scerri from the Malta Department of Health and the University of Malta, Mohammed Marwan at the Tripoli Biotechnology Research Centre and Connie Bezzina who is now Assistant Professor at the Academic Medical Centre in Amsterdam. Others have conducted undergraduate projects on Haemoglobin or have read for Masters' degrees with us and are now pursuing further studies in other universities or through joint doctoral programmes. They continue to contribute to further develop the programme in Malta.

The main challenges for the future concern the understanding of globin gene control and genetics therapeutics, the bio-manufacturing of genetic therapeutic molecules, the documentation of molecular epidemiology and expression profiling for a number of significant disorders and the further development of genetics services with advanced molecular biology tools.

PROFESSOR ALEX. E. FELICE graduated M.D. and M.Phil. from the University of Malta, subsequently proceeding to the U.S.A. to pursue his doctoral studies. He obtained his Ph.D. from the School of Graduate Studies of the Medical College of Georgia in 1981. In 1986, he was appointed Associate Professor at MCG. In 1992 he was appointed Professor (Biomedical Sciences) at the University of Malta where he teaches Molecular Genetics and Haematology. Here, he directed the establishment of the Thalassaemia and Molecular Genetics services and the development of a Molecular Biotechnology Program. His research in Malta has been, in part, funded by competitive awards of the EU framework and other R & D programmes. He is the author of numerous research manuscripts on the genetics of haemoglobin disorders including thalassaemia and sickle cell disease and human molecular genetics.