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Review Article

The Beautiful World of Human Haemoglobin (revisited)

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Abstract. In this article I sought to tell two stories that were closely tied to each other. One was somewhat personal. It had to do with the course of my professional life, in Malta, the USA, then back to Malta. It involved family, a handful of mentors that shaped my career and many graduate students or trainees. They combined to link with the second objective of this article to recount our contributions to Human Haemoglobinopathy studies. Our research revealed the quantitative effects of genetic co-regulators on complex phenotypes. We assumed that mutations at two to three alleles acting among two to three (globin) loci could act to express a β globin gene variant at any level between as little as 5% or as much as 100% in heterozygotes. The same type of interaction could be seen in α globin and other molecules such as KLF1, the master regulator of Erythropoiesis, and perhaps others with a similar molecular model. The new health and academic professionals that we trained, the new resources in the hospital, the new laboratories and the new International Initiatives promise well for new discoveries to promote expedited diagnoses and new treatments of Rare Disease in general.

1 Preamble

When Prof Giuseppe Di Giovanni asked me to contribute to this second volume of the special issue of XJENZA-ON-LINE to celebrate successful researchers in Malta, I thought of reviewing my research on the genetics and pathophysiology of human Haemoglobin (Hb) largely from a personal perspective. The journal first known as XJENZA was, of course, close to my heart since it was published by the Chamber soon after we had founded it in 1992 (Fig. 1) and I was President (Felice et al., 1996). Patrick Schembri had taken the initiative on the Council, and Angela Xuereb was the first editor of (Xureb, 1996). XJENZA-ON-LINE succeeded the printed version of XJENZA in 2013, with Giuseppe Di Giovanni serving as the editor for a period of 5 years (Di Giovanni, 2013). Subsequently, the role of editor was assumed by Cristiana Sebu, who currently holds the position. The initiative was supported by Peter Serracino Inglot, Mario Tabone and George Pullicino at the MCST. Marion Zammit Mangion, Robert Bort, Paul Debattista, Nicholas Gingell, Christian Scerri, and Kenneth Bartolo were the other members of the founding council.

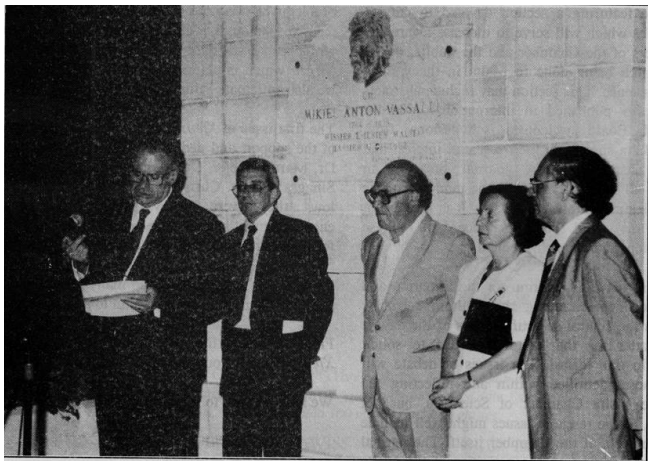

Figure 1: Inaugural event of the MALTA CHAMBER OF SCIENTISTS held on the 29 th of July 1994 on campus of the University of Malta showing left to right his excellency Dr Ugo Mifsud Bonnici then President of Malta addressing the gathering with Prof. Alex Felice, president of the Chamber, The Rev. Professor Peter Serracino Inglott, then Chairman of the Malta Council for Science and Technology and Rector of the University of Malta, Mrs Mifsud Bonnici, and Professor Victor Ferrito, President Elect.

My Story is personal as well as scientific; there was always a bit of Chemistry at home since my father was a chemist of the older tradition and he taught chemistry. Watson and Crick (1953, but see also Pray, 2008) published their manuscript on the structure of the DNA in Nature when I was a young boy starting secondary school-

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ing at the Lyceum in Hamrun. We had exceptional science teachers then. I joined the School of Medicine in 1966, and graduated M.D. in 1971; just over 50 years ago, that we celebrated with family and friends last year. Soon after reading for the Master degree in the Faculty of Medicine and Surgery, Joanna (formerly Nicolas) and I got married and left for our adventure in Augusta, Georgia, at the Medical College of Georgia in the Southeastern USA. Augusta was well located with being barely three hour drive from the State Capital, Atlanta, home of the Braves and the Falcons, the mountains of Tennessee, the old capital Savanah and the golden beaches running from Miami up North to Myrtle beach in Northern Carolina / Virginia. We lived in a mixed society, partly a cosmopolitan environment dominated by a vibrant medical academic community and the military at Fort Gordon and the typical "friendly southerners" Golf was the dominant sport. Augusta still is the Golf capital of the world with its annual Masters' tournament. In the meantime, Joanna joined the Augusta Museum as a docent and later started on her professional experience in librarianship that she later brought back to the library of the University of Malta as Deputy Librarian. Occasionally she joined the team travelling to somewhat obscure places in the forests of Southern Georgia to collect samples from highly informative families that we published. We shared the excitement and tribulations of unnerving weekends to meet deadlines and the interminable wait for the outcomes of submitted manuscripts or grant applications. Even then, though, we shared concerns about the underlying social tensions that came to the fore later. However, we remember odd pleasantries such as being introduced, the first time we went to the Augusta Symphony, as the new couple in the Huisman laboratory "from across the pond" I went through graduate school and an intensive post-doctoral training to be followed by a junior position on the Faculty at MCG then Associate Professor. We returned with a goldmine of experience, the happy memories of establishing a home away from home and bringing up a young family.

I shall give an account of my research on Hb as it developed first as a medical and graduate student at the University of Malta (Felice, 1975) and a trainee with my mentors at St Luke Hospital, later at the NIH funded Comprehensive Sickle Cell Center of the Medical College of Georgia, in Augusta, Georgia, USA (1976 - 1989; Ph.D., 1981) and then back to Malta in 1989. The late Joe Louis Grech was the clinical biochemist at St Luke's. He was specialized in Clinical Pathology with expertise in BioMedicine, mainly Biochemistry. It was the fore-runner then of what we may be calling Genomics Medicine today. He tasted the discovery of the first *Maltese* Hb variants on top of his busy clinical schedule. Joe Louis was an exceptionally fine gentleman, a superb teacher. He led by example. He once told me "young man; you cannot find what you do not look for!" In retrospect, it sounded like what today we know as a *Specific Objective* I often argued with my students that a *specific objective* could not be more than one, maybe two. He had introduced the quantification of the minor Hb component in adult blood, the HbA2 by paper electrophoresis for the diagnosis of the common thalassemia heterozygote, or trait as it is commonly known. Later, he also added the procedure of cellulose acetate electrophoresis for newborn Hb testing. The late William Bannister had returned to Malta from Oxford before I started studying physiology. He had set up a new Laboratory of Protein Chemistry in the Department of Physiology and Biochemistry to research copper bound molecules that were similar in many ways to Hb. They both bound oxygen. With Maurice Cauchi they had just reported the discovery of the Hb F Malta I [or $\alpha_2 G\gamma_2$, 117(G19)His > Arg ; Cauchi et al., 1969] and Hb St Luke [or $(\alpha_1)_2$95(G2)Pro > Argβ_2; Bannister et al., 1972] variants. Willie, as we knew him, was a committed researcher with hardly any interest outside the research laboratory. Although occasionally we disagreed, he also gave me sound advice mostly about threading the treacherous paths to securing research funding in Malta. After my M.D., I researched both Hb variants for my M.Phil. (Felice, 1975, Felice, 1977) that was among the first from the Faculty of Medicine and Surgery. The other was Marie Therese Podesta' now Camilleri Podesta'. With Roger Ellul Micallef, later Rector of the UM, the late Anton Pizzuto and the "Kapillan" of the locality we used to organize collections in isolated villages across Malta and Gozo (Felice et al., 1977) We introduced the practice of signed *informed consent* before testing. In the process, though, with Manuel Agius and George Grima we set up the first Research Ethics Committes.

We never discovered very much then! Even the Hb F Malta I homozygote was elusive, despite the high heterozygote frequency (1.8%) The low proportion of the Hb St Luke variant (< 20% of total Hb) in blood of the adult heterozygotes was intriguing though.

In the course of this research, Hb F Malta I, however, turned out be a most useful biomarker of human γ globin gene expression. It shed light on the structural and functional organization of the globin genome before genome sequencing was widely available. It was found to be uniquely in tight linkage disequilibrium with the β globin variant known as *Hb* Valletta [or $\alpha_2\,\beta_2$87(F3)Thr > Pro ; Felice et al., 1990, Kutlar et al., 1991] Whoever inherited the Hb F Malta I variant also inherited the Hb Valletta

variant on the same chromosome, in cis. With Alex Cammilleri in the course of his M.Sc. (Camilleri, 2018) we found very few instances when one occurred without the other. The recombination rate in this part of the genome must have been very small. This genetic arrangement is still rather unique in human genomics. In the case of the Hb St Luke, the molecular pathophysiology gave us insight into the biochemistry of post-translational assembly of heteropolymers and models of gene expression in Thalassaemia and in Sickle cell Disease (Felice et al., 1977) and later about transcription factors (TF) such as KLF1 (J. Borg, 2010, L. Grech, 2018)

My induction into the Beautiful World of Haemoglobin at the University of Malta was followed by a longish stint on the Faculty, in the Departments of Cell and Molecular Biology and Paediatrics (Paediatric Haematology) of the Medical College of Georgia (MCG) in the School of Medicine, the School of Graduate Studies, and the NIH funded Comprehensive Sickle Cell Center in Augusta. In the process, I inherited the Huisman Laboratory to direct the Haemoglobin Research Program in the Medical Research Service of the adjoining Veterans' Administration Medical Center in Augusta. The group was globally competitive in Hb research. We were deeply engaged with challenging questions about haemoglobin, the structure, the function, the genetics and the patho-physiology. I read for my Ph.D. degree under the tutorship of Titus Huisman, an inspirational man. Together with David Weatherall at Oxford, UK and Walter Schroeder at Caltech, California, USA he was a world leader on Foetal Hb, Sickle cell Disease, and Thalassaemia (Felice, 1986) He trained me in the subtle skills of research project management starting with writing successful competitive grant applications for research funding. Actually, he tore to shreds my first effort at writing a research manuscript for publication telling me "this is philosophy, now go back home and write science" They were among other "pearls of wisdom" from my mentors that later I tried to pass on to my students.

We started with haematology and protein chemistry at the UM and evolved into molecular biology and human genomics at MCG applying a variety of analytical and experimental tools that collectively are nowadays known as "omics" within the larger scope of BioMedicine. Like stamp collectors, we made catalogues and albums of samples that later we turned into biobanks. In the process, we learnt how to "translate" biomedicine between the clinic and the research laboratory. At first, after much correspondence and missed visa deadlines, once in Augusta, I was asked to pursue the initial observation that we had made in the Bannister laboratory about the low and variable expression of the α globin variant Hb St Luke among the heterozygotes from Malta. The issue bore on the genetics of the α globin or α thalassaemia and possible effects on the biosynthesis of HbF in Sickle Cell Disease (SCD) and β thalasaemia as I shall explain below. We thought that understanding the control of globin gene expression and Hb F levels could lead to new treatment of both Thalassaemia and Sickle Cell Disease by turning the perinatal globin switch back in adults who could survive, disease free with HbF instead of HbA or HbS in blood. The data gave insight into competitive post-translational assembly of hetero-dimers between wild type and mutant polypeptides, how this accounted for the quantitative variability of certain Hb variants in heterozygotes and homozygotes, and the effect on Hb F levels in Thalassaemia and Sickle Cell Disease.

On returning to the UM and St Luke Hospital I met up again with Joe Louis Grech and William Bannister. Maurice Cauchi had established himself in Melbourne, Australia though he returned briefly (1992-2003; Cauchi, 2019) We founded a joint project between the Department of Health, then under the direction of Alfred Grech, Chief Government Medical Officer and the University while Edwin Borg Costanzi, a mathematician, was Rector. Alfred Grech secured the support of the World Health Organization for a grant to set up a specialized laboratory for Thalassaemia Testing that was further supported by the University for Hb Research (Felice et al., 1990, Buhagiar et al., 1997) George Hyzler, then John Rizzo Naudi were in the Ministry of Health. Frederick Fenech was Dean of Medicine and Peter Serracino Inglott, a philosopher with extensive interests had succeeded Borg Costanzi as Rector. On campus, we set up joint laboratories for Thalassaemia Testing and Hb Research and a brand new Laboratory of Molecular Genetics. The latter became an advanced diagnostic service for genetics in Malta under the direction of Christian Scerri. Christian was one of the first group of three doctoral graduates who specialized in Pathology and Diagnostic Molecular Genetics. We launched the teaching and applications of modern Molecular Biology in the field of human genomics and genetics medicine. We intended to build on the long tradition that the (Royal) University of Malta had in the field of human haemoglobinipathy. Frank Vella, now retired in Saskatchewan, Canada, where he had been Professor of Biochemistry had pioneered the discovery of new Hb variants in the Far East. Vella was president of the International Union of Biochemistry. The Paediatricians, Manwel Cachia and Tommy Agius Ferrante, their successor Paul Vassalo Agius had considerable experience with Thalassaemia in the clinic. I had many recollections as a trainee in paediatrics. Joe Louis had once told me (if you took my word) that the samples from the Maltese patients of the time were among those that Ceppellini in Milan had

studied to determine the levels of the minor HbA_2 to be pathognomonic of the thalassaemia heterozygote (trait). He was using a cumbersome starch block electrophoresis. Today the test is automated on a High Performance Liquid Chromatograph (HPLC) The test permitted quantification of the reproductive risk among couples to bear homozygote children sick with Thalassaemia Major. In the hospital, first St Luke, then Mater Dei, we set up a specialized Thalassaemia Clinic to serve for clinical excellence in the setting of an academic project. In particular, we sought to document the natural history of Thalassaemia homozygotes that differed in severity of the disease due to the different β globin mutations as genotyped, the Hb F levels, and possibly other "genetic modifiers" We thought that the clinical genomics might suggest alternative therapies with innovative inducers of post-natal HbF (HFIs) or erythropoiesis stimulating agents (ESAs) It continues to thrive under the direction of Christian Scerri and with the recent addition of genetics counselors known as genomic health co-ordinators.

Although the story is personal, it is also that of many mentors, teachers and later younger collaborators who trained with us and joined the new Thalasaemia Project. Soon, Monica Pizzuto, Ruth Galdies and Wilma Cassar joined in the laboratory. Ray Parascandalo, Christian Scerri, Simone Buhagiar and later Dragana Josifova and Mary Rose Caruana joined in the clinic. Ray and Christian later became consultants at St Luke/Mater Dei and Dragana at Guy's Hospital in London. We pioneered a graduate program. Christian, Mohamed Marwan and Connie Bezzina were the first Ph.Ds from the Faculty of Medicine and Surgery ever (C. Scerri, 1998, Bezzina Wettinger et al., 1999, and M. Marwan, 1998). Christian is now Professor and Consultant in Genetics at Mater Dei. Connie, (Bezzina van Imp) now occupies the Chair of Molecular Cardiology at the Amsterdam Medical Center and is elected member of the Royal Academy of Science of the Netherlands. Others followed; Godfrey Grech, Stephanie Bezzina Wettinger, Svetlana Schembri Wismayer (formerly Pulis), Marion Zammit Mangion, Renald Blundel, Steve Bonello, Rosienne Farrugia, Isabel Borg, Joseph Borg, Charmaine Vella, Alex Cammilleri, Seham ElJali and Aisha Benzetoon read for Master degrees and contributed comprehensively to our understanding of Hb Genetics and clinical patho-physiology. The doctoral graduates who worked on a variety of related questions included, Stephanie Bezzina Wettinger, Steve Bonello, Clint Mizzi, (the late) Ali Ashthar, Nikolai Pace, Joseph Borg, Laura Grech, Joanna Vella and Seham el Jali. Most now hold academic or professional positions at the University or Mater Dei Hospital and even elsewhere.

The Thalassaemia Project of Malta was approved and underwritten with a grant from the World Health Organization. We were further supported with the first Maltese EU-funded research award through the new Avicenne Program that Ugo Mifsud Bonnici then Minister of Education had secured in the preparatory phase of EU accession John Rizzo Naudi, who pioneered rare (blood) disease medicine in Malta had succeeded George Hyzler at the Ministry of Health and sustained the project. The Thalassaemia Project developed well with a strong reputation of quality care in a research setting. Furthermore, it led to the development of a newborn testing service with the later addition of hypothyroid testing, the diagnostic molecular genetics laboratory, now under the direction of Christian Scerri and an innovative driver of biobanking even at European level. With Dorita Galea and others from Eurordis, the European network of rare disease patient support groups, we co-founded Eurobiobank, the first pan-European Biobank specialized in Rare Disease Biobank-

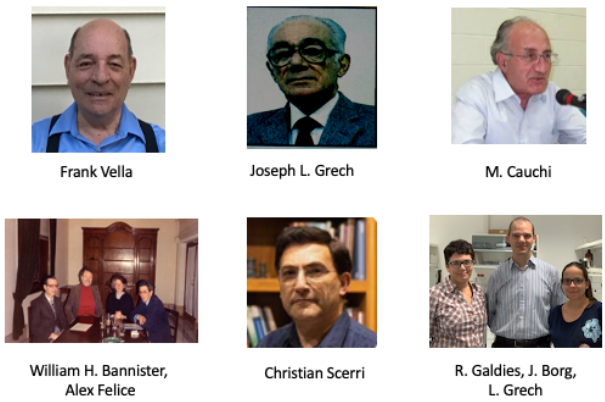

Figure 2: Photos of personages from the past and the present who led the development of research on Haemoglobin and Thalassaemia at the University of Malta showing; a. Professor Frank Vella, who pioneered Thalassaemia testing in Malta and discovered some of the first Hb variants in South East Asia. b. Professor Joe. Louis Grech who directed the Laboratory of Clinical Pathology at St Luke Hospital where he set up Newborn testing for Human Hemoglobinopathies and inspire the further development of the research program. c. Professor Maurice Cauchi reported the discovery of the Hb F Malta I variant and later pursued his career in academic Pathology at Monash University in Melbourne, Australia. d. Professor Janet Rowely (London) Professor WH Bannister and Professor Felice at the signing of the agreement with the World Health Organization to set up the Thalassaemia Project jointly between the Malta Department of Health and the University of Malta. e. Professor Christian A. Scerri now director of the Laboratory of Molecular Genetics. f. Recent graduates and collaborators at the Thalassaemia testing and Haemoglobin research laboratory on campus showing Professor Joseph Borg, Dr. Laura Grech and Ms R Galdies.

ing (Mora et al., 2015) that I currently Chair, to be followed by the European Research Infra-Structure known as BBMRI-ERIC (see https://www.bbmri-eric.eu/) Nikolai Pace is now National Node Director, and Joanna Vella, is a manager. In Malta, we supported the National Alliance for Rare Disease Support. Undoubtedly, the number of graduates and trainees, in particular, those that went on further to develop their own projects and the number of publications were the most satisfactory harvest of the project that endured so long.

Our single over-riding research objective was to understand the mechanisms that regulated the genetic switching of Hb F to Hb A (or γ to β globin gene switching) that occurred physiologically around the time of birth. It is a critical question in human physiology. It could be described as the "holy grail" of Hb research on the assumption that, if one understood the mechanisms of globin gene switching around birth, one could then develop treatments to suppress or revert the switch in order to treat the β(/HbA) haemoglobinopathies, both thalassaemia and SCD (Olivieri et al., 1998, Orkin, 1995). Together with our collaborators in Augusta, Oxford and Rotterdam, we followed two lines of investigation according to the "Augusta Model" In the first instance, as said, like stamp collectors, by exploring the quantitative epidemiology and phenotypes of Hb variants and haemoglobinopathies among families and populations we could infer potential genetic mechanisms. In the second stage, we could put to test laboratory. In the long run, the approach proved successful with the recent the inferred mechanisms using advanced "omics" technologies in the experimental uncovering of the KLF1 locus (Bieker, 2020) as possibly the master regulator of globin gene switching and the biosynthesis of Hb F that may now lead to new treatments (J. Borg, 2010, L. Grech, 2018, 2022) Photos of a few most closely involved in setting up the project are shown in Fig. 2.

The structural organization of the Hb molecule turned out to be deceptively rather simple (see Weatherall et al., 2008 for reviews). It evolved over 450 million years ago since the vertebrate Hb of red blood cells (erythrocytes) and the Myoglobin of the musculature diverged (Vinogradov et al., 2007). It appears that although the evolutionary pressure was primarily to accommodate the requirements for oxygen in the deeper tissues of larger vertebrates, while buffering the accompanying ionic fluxes, the temperature control required by intensive oxidative metabolism may also have contributed selective pressures. The developing foetus and the brain had specific needs. Likely, both differed during development. Conceivably, foetal and adult Hb phenotypes evolved subsequently in synchrony with complex developmental control of gene switching and erythropoiesis (Peschle et al., 1985) Although commonly known as a tetramer of globin sub-units, in my opinion, Hb was better described as a duplex of two heterodimers each made up of one α and one non-α globin sub-units each bound to a heme prosthetic group that in turn bound oxygen. Each $\alpha\beta$ heteroduplex included one α-like and one non-α, or β-like globin, encoded by genes in the HBA(α) and HBG(γ) or HBB(β) loci, respectively. Various Hb molecules were resolved and quantified by physicochemical methods of electrophoresis / chromatography. Developmental regulation of globin genes resulted in the expression of stage specific Hb molecules that accounted for the chemical heterogeneity of Hb lysates from blood samples (Some were acquired, such the HbA1c due to persistent hyperglycemia of diabetes, but the most significant were dependent on developmental control of globin gene expression) (Fig. 3)

Jonxis had observed that unlike that of the adult, a substantial amount of the Hb of the newborn resisted denaturation in alkali (Jonxis et al., 1956) He referred to this fraction as "Foetal Hb" or HbF. Later, Walter Schroeder at Caltech, while sequencing the erythrocyte enzyme Carbonic Anhydrase needed a reference control. He asked for HbF globin from the Huisman Laboratory in Augusta. That actually complicated Schroeder's life because amino-acid 136 of the γ globin gave non-unitary values, as if the protein had been a mixture of two, one with Glycine and the other with Alanine at position 136 (Huisman et al., 1977) I was still at Bannister's lab and had started my postgraduate master's project with the objective of exploring the occurrence of the variant Hb F Malta 1, a variant of the $^{G}\gamma$ globin. Since the prevalence was, uniquely high (1.8%) one of my goals was to find the elusive homozygote. If the assumption of non-allelic duplication of the γ globin gene was correct, then the homozygote ought to have had only the $^{G}\gamma$ variant and the $^{A}\gamma$ wild type globin as was indeed the case. As expected, the quantitative studies and subsequent gene mapping developed a consistent picture of two non-allelic γ globin genes now known as $^{G}\gamma$ and $^{A}\gamma$, on each chromosome as Scroeder and Huisman had predicted. Erythroblasts expressed four (4) γ globin genes, 2 $^{G}\gamma$ and 2 $^{A}\gamma$ Postnatally, as Hb F declined below 1% and Hb A increased, above 95%, the HbF phenotype changed from the high $^{G}\gamma$ typical of the neonate (0.7) to the high $^{A}\gamma$ of the small quantities of HbF in a sub-population of adult erythrocytes (F-erythrocytes) to be found in the adult blood. The Hb F Malta 1 variant declined postnatally faster than the $^{A}\gamma$ globin though it remained detectable (Altay et al., 1977) The Chemical heterogeneity of HbF was shown to be a global phenomenon (Huisman et al., 1977). I was privileged to write Schroeder's obituary that

was accompanied by a chromatograph displaying the separation of the original $^{G}\gamma$ and the $^{A}\gamma$ polypeptides from the same sample that Schroeder had used for amino acid analysis, this time though, on a high resolution HPLC as shown in Fig. 3 (Felice, 1986).

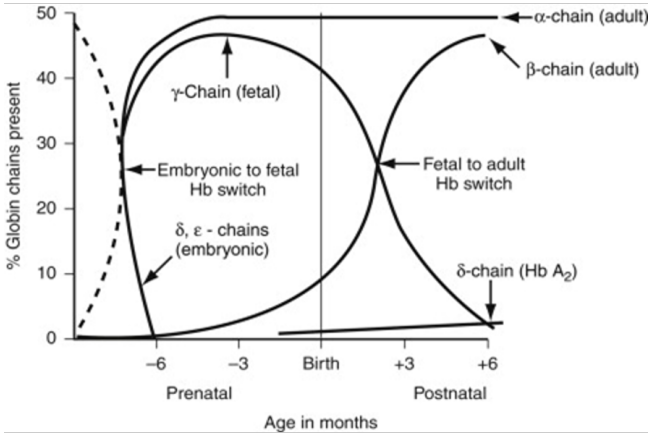

Figure 3: Graphic showing the changes of foetal to adult globin gene expression in the course of human development. It can be seen that two switching events define the transitions in globin / Hb phenotypes. The first occurred in the early embryo from ε and ζ to γ and α. The second occurred around the time of birth from the foetal $G\gamma$ and $A\gamma$ to the adult δ and β globins.

A common polymorphism of the $^{A}\gamma$ globin known as $^{A}\gamma^{T}$ or HbF Sardegna (or $\alpha_2 A\gamma_2$, 75(E19)Ile > Thr) with a frequency of around 15% in Malta and the rest of Southern Europe was also found (Marinucci et al., 1979). It was another useful quantitative biomarker in Thalassaemia research. We observed a few compound/double heterozygotes in whom each β and γ globin gene expressed throughout foetal and adulthood was biochemically marked. The quantitative Hb phenotype served to quantify in vivo the expression of each one of the globin genes during development. As far as I know, this is unique for any locus in the human genome. Residual amounts of Hb F continued to be synthesized throughout adult life and expressed within F-erythrocytes (Lennora et al., 1996). The relative distribution of the HbF among the adult F-erythrocytes varied. Jeanesse Scerri, in her Master project, suggested that it may be under control of the FLVCR1 locus that regulated the cellular distribution of the haeme prosthetic molecule (J. Scerri, 2014, Scerri J., et al., 2022)

Globin Gene Switching was accompanied by partial repression of α globin gene expression and many changes in the structure and metabolism of the erythrocytes that must be tightly maneuvered in stem/progenitor cells (Felice et al., 1979a) However, it seemed that globin gene control could be targeted separately from the differentiation of the erythrocytes for a specific therapeutic benefit.

Like that of many vertebrates, the Hb of human blood was said to be heterogeneous because the phenotype was quantitatively and qualitatively pleiotropic. Seven (7) normal haemoglobin types were physiologically expressed in the course of human development (Fig. 3) They were the embryonic haemoglobins, Hb Gower 1 ($\zeta_2\varepsilon_2$), Hb Portland ($\zeta_2\gamma_2$), and Hb Gower 2 ($\alpha_2\varepsilon_2$), up to 12 weeks of embryonic development, the foetal haemoglobins HbF ($\alpha_2{}^{G}\gamma\gamma_2$) and ($\alpha_2{}^{A}\gamma_2$) up to around the time of birth that was taken over by the adult Hb A ($\alpha_2\beta_2$) with a minor HbA2 ($\alpha_2\delta_2$) making up less than 3.5% in the absence of a β thalassaemia. The heterogeneity reflected the patterns of expression of the α-globin gene locus on human chromosome 16, and, the β-globin gene locus on human chromosome 11 (Fig. 4) Comprehensive reviews can also be found in Weatherall et al., 2008 and an extensive bibliography onthis website.

The α-globin gene locus (Fig. 4) covered a region of around 30kb on the short arm of chromosome 16, contained the embryonic ζ_2 gene, three pseudo genes, $\psi\zeta 1$, $\psi\alpha 2$ and $\psi\alpha 1$ and the two-α globin genes - $\alpha 2$ and $\alpha 1$. Their regulation is extensively described in Higgs et al., 2008. We quantified repression of $\alpha 2$ and $\alpha 1$ accompanying the $\gamma \rightarrow \beta$ transition (Felice A., Huisman THJ., 1997) The non-α or β-globin locus (Fig. 4) was found on the short arm of chromosome 11p15.5 and spanned a region of around 90Kb as described in Fig. 4. The cluster 5' to 3' comprised in this order; the embryonic ε-gene, two foetal genes, G_γ and A_γ a pseudo gene $\psi\beta$, and the adult δ and β genes. Apparently, the nearly balanced biosynthesis of the α and the γ or β globins was autonomous and a deficiency of one led to an excess of inflammatory globin precipitated in the form of haemochromes and free haeme in the erythroblastic islands of the bone marrow (Romano et al., 2022).

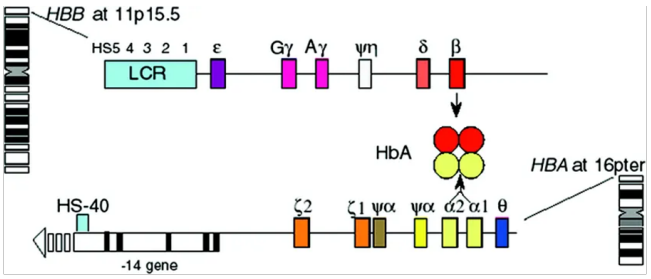

Figure 4: Structural organization of the globin genes. The duplicated α (i.e. α 1 and α 2) were mapped to p16 flanked by an embryonic ζ and an unexpressed τ genes spanning 150Kb. The non-α loci were mapped to p11 in the same order in which they were developmentally expressed i.e. ε, $G\gamma$, $A\gamma$, δ and β. TF binding sites are included among these sequences including for KLF1. A Locus Control Region can be found at the 5' end.

Family studies revealed at least four loci that con-

troled Hb F levels in adults known as co-regulators: HBB (11p15.4/ the XMN1 site; Gilman and Huisman, 1986), HBS1L-MYB (6q23.3; Close et al., 1994) and the BCL11A (2p16.1; Thein et al., 2007) and KLF1 (J. Borg, 2010). Indeed, early clues (Huisman et al., 1975) had inferred important DNA regulatory sequences between the foetal $^{A}\gamma$ globin genes and the adult β globin genes. A poly-pyrimidine rich sequence located here attracted numerous protein complexes that together formed a repressor-like complex. It looped back on itself to silence the foetal gamma globin genes in adulthood (Bank, 2006). Other co-regulators should be discoverable. This may be represented by the "KLF1 Interactome" in our model.

Globin gene expression was controlled alongside the control of Erythropoiesis (Peschle et al., 1985) Two cellular pools governed the definitive, foetal and adult lineage of haematopoiesis. One consisted of a hierarchy of stem and progenitor cells during which a genetic program of lineage specific cellular differentiation was assembled by tightly controlled gene switching events. KLF1 with its very strong promoters had an open configuration and very active at this stage (Heshusius et al., 2022, Herseus et al 2022) It was succeeded by a later terminal phase in bone marrow during which the pro-erythroblasts expressed the determined program of gene expression to differentiate into circulating reticulocytes and erythrocytes. The resting immature progenitors, the BFUe and the CFUe, either spontaneously entered a pathway leading to cell death (apoptosis) or were rescued by Erythropoietin to enter the pathway of erythropoiesis. The KLF1 gene had a closed configuration and inactive beyond this stage (Heshusius et al., 2022) though the protein persisted (Nuez et al., 1995) It involved other TF(s) such as MYB, Tal1, Lmo2, and GATA1 (reviewed in Grech L. 2022) and likely a few others that might assemble into the "KLF1 Interactome" and that we are pursuing in our current research.

The chemical heterogeneity in red cell lysates was the result of developmental changes during foetal and adult development and seen worldwide. Population studies yielded a comprehensive picture of global Hb epidemiology that gave insight into biochemical mechanisms of post-translational protein assembly by interplay between mutations that impaired protein structure and function as in many α and β globin variants including the Hb S of SCD (Perutz et al., 1968). They were commonly due to single point mutations in the exons of the globin genes, or, of genetic regulation as in thalassemia or the Hereditary Persistence of Foetal Hb (HPFH) or both that were associated with quantitative repression of globin gene expression. The most common variants seen among the Maltese are shown by electrophoretic identification and chromatographic (HPLC) quantification (Fig. 5)

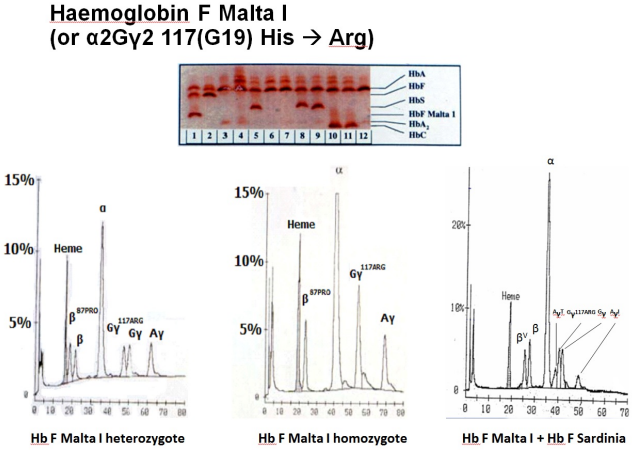

Figure 5: Chemical heterogeneity of Hb phenotypes revealed by iso-electric focusing (top panel) and reverse phase HPLC (bottom panel) of selected Hb variants commonly see among the Maltese. It can be seen that the globin products of the six globin genes active in foetal to adult switching could be uniquely resolved and quantified among Hb F Malta I heterozygotes with or without Hb F Sardegna and the rare Hb F Malta I homozygote.

Two observations were made. The first was that the proportion of β globin variants such as Hb S, Hb C, Hb E, and Hb Leslie among heterozygotes could vary from as little as around 5% to the 50% anticipated (Huisman et al., 1977, Felice et al., 1982, Felice et al., 1978, Felice et al., 1981a, Felice et al., 1981b, Steinberg et al., 1986) Given that they were mutations on one of the two β globin genes on either one of the two parental chromosomes, in trans, one would normally have expected levels of the Hb variants at about 50% of total Hb in the heterozygotes (with one of the two mutated e.g. $[\beta^S/(\beta^A + \beta^S)] = \pm 50\%$) The lower values were accompanied by a corresponding degree of microcytosis (MCV $<$ 80Fl) similar to a thalassaemia, if iron deficiency had been excluded (Steinberg et al., 1986, Felice, 1986) The second observation was that unlike the β globin variants, the α globin variants in association with a microcytosis were associated with higher levels of the α globin variant as in the case of Hb G Philadelphia (Felice et al., 1981b) At that time, I was following up on the discovery of the α globin variant Hb St. Luke that had been reported earlier by Bannister, Grech and their collaborators in Malta and Augusta (Bannister et al., 1972) Like four other variants, Hb Denmark Hill (or $\alpha_2 95(G2)\text{Pro} \rightarrow \text{Alawa}\ \beta_2$; Wiltshire et al., 1972) Hb G Georgia (or $\alpha_2 95(G2)\text{Pro} \rightarrow \text{Thr}\beta_2$; Huisman T. H. J. et al 1970) Hb Rampa (or $\alpha_2 95(G2)\text{Pro} \rightarrow \text{Ser}\beta_2$; de Jong et al., 1971) and Hb Godavari, (or $\alpha_2 95(G2)\text{Pro} \rightarrow \text{Thr}\beta_2$; Wajcman et al., 1998) Hb St Luke resulted from

the amino acid replacement at the α95 position of the α globin. It occurred at the $\alpha_1\beta_1$ interface that held the unlike globins tightly in the heteroduplex with little movement during oxygenation/de-oxygenation cycles. It was less flexible than the symmetrical $\alpha_1\beta_1$ interface and a stronger bond. The five mutations at the same position weakened the $\alpha_1\beta_1$ interface. They resulted in very low levels in the heterozygotes ($5-10\%$) compared to the 25% expected on the basis of a genome with 4 α globin genes active in the erythroblast ($\alpha^X/[\alpha^X\alpha/\alpha\alpha] = 1/4$ or $\pm 25\%$)

When I joined Huisman's group in Augusta, I was given a small laboratory that I modified to explore globin biosynthesis with radio-labeled amino acid precursors and a chromatographic method commonly known as the Clegg columns. I had micro-miniaturized the procedure to ask the question whether some form of thalassaemia was acting, possibly at the post-translational level to account for these observations. If so, the next question was how would the same happen in homozygotes, in particular, the β Thalassaemia Homozygotes with Thalassaemia Major, and, the Hb S homozygotes with Sickle Cell Disease. These experiments are extremely difficult to conduct today because it is nearly impossible to procure radioactively labelled amino acid precursors. Instead, Elaine Fenech is seeking to quantify gene expression with contemporary functional genomics to quantify globin gene expression with mRNA ratios as part of her Master's project.

Several families from Malta and rural Georgia joined our studies by sharing samples and data. We worked with Institutional Review Boards as Research Ethics Committees that were arising following the experience with kidney transplantation and the Helsinki declaration. On reflection, much progress could be done by working closely with patients and families in the community assuming a "unitary value" of health data without the implications of "secondary data" as now entrenched in the GDPR. I thought it needed considerable revision by patients, families and their personal health professionals. The results, partly, summarized in Fig. 6 showed that the proportional level of those β globin variants, with positively charged mutations at the $\alpha_1\beta_1$ interface, was decreased by co-inheritance of an α thalassaemia. The diminished competitivity of the variant β^X globin compared to the wildtype β^A globin for the diminished amounts of the α globin to assemble into $\alpha\beta^X$ or $\alpha\beta^A$ hetero-dimers led to the assembly of fewer heterodimers of the β^X variant compared to the wildtype HbA. In contrast, a β thalassaemia (+) mutation in trans raised the relative quantities of the β^X variant by diminishing the production of the β^A globin in cis. In fact, quantitative and molecular studies such as these allowed us to quantify the in vivo effects of any β^+ thalassaemia mutation (M. Marwan et al., 1999, see also HbVar, Hardison et al., 2002). We saw this in the case of the Hb Valletta variant among the Maltese (C. A. Scerri et al., 1993, †Felice A et al., 2016) Counter-intuitively, a concurrent α thalassaemia raised the proportions of the α globin variant while a β thalassaemia decreased it. Gene mapping and re-sequencing confirmed the conclusion reached with biosynthesis (Felice et al., 1981a, Felice et al., 1981b, Felice et al., 1982).

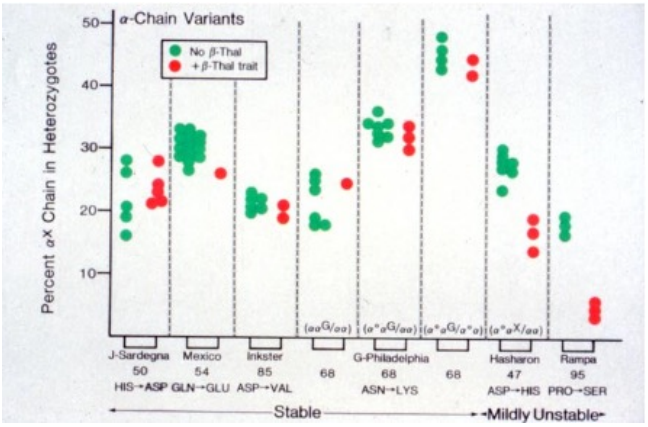

Figure 6: Quantitative epidemiology of selected α globin variants with different mutations in the α or β globins among heterozygotes from Malta and elsewhere with an associated α or β thalassaemia.

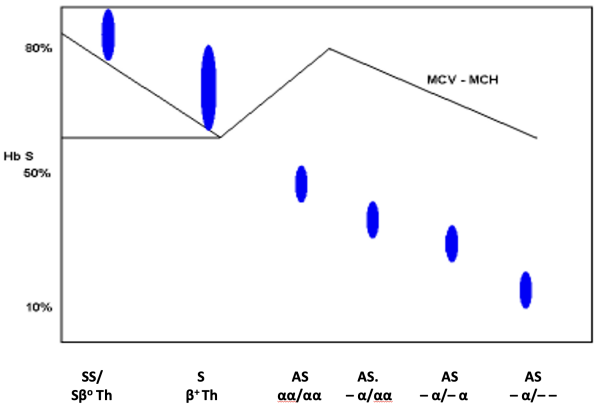

Figure 7: Quantitative epidemiology of selected β globin variants with different mutations in the α or β globins among heterozygotes from Malta and elsewhere with an associated α or β thalassaemia. The data suggested a generalized model by which the level of any heteropolymer with a structure resembling that of Hb could be decreased or increased by interplay between dys-regulatory and dysfunctional mutations affecting competition between pairs of sub-units at the level of post-translational assembly.

We asked whether the conclusions could be generalised to account for the biosynthesis of other proteins with a similar heterodimeric structure by interplay between dysregulatory mutations such as a thalassemia that decreased output, and dysfunctional mutations that decreased stability or assembly of polymeric molecules. Many molecules of body defence mechanism, immunity and inflammation, fell in this category. They had a higher order structure similar to Hb and followed a model that could account for variability in expression in health / disease. Ali Ashtar (2008), Abou Hussein (2009, 2011), Nikolai Pace (2013) and Seham Eljali (1975) have sought to apply the model to Type 2 Diabetes Melitus. It was intriguing to note further that the same mechanisms could account for the variability of the Hb and erythroid phenotype in the KLF1 deficiencies that presented with a ψ thalassaemia (see below) We found KLF1 gene frameworks with mutations in the promoter sequences associated with coding sequence mutations (unpublished observations/manuscript in preparation) The diverse KLF1 frameworks were assembled into various haplotypes/genotypes that we are exploring with respect to corresponding haematological and Hb phenotypes among families with β thalassaemia (Attard, 2020, E. Fenech, 2023).

α-Thalassaemia most commonly arose from deletion(s) of one or both of the two α globin gene in cis ($\alpha\alpha/$) on chromosome 16. Thus, functional erythroblasts with diploid genomes could express any from 4 to 0 α globin genes. It was found most commonly in its milder form with one α globin gene deleted in cis (α^+ Thal or $-\alpha/$) among Africans, or in its more severe form with both α globin genes in cis deleted (α^o or $--/$) in Asians. One of the largest α globin gene deletion assocated with α^o thalassaemia was found in a Black family associated with HbS that also had Hb H disease or the $\alpha/--$ genotype (Felice AE et al 1979) Only a handful of others are known world-wide. Although α globin variants can be found, α Thalassaemia is less common in Southern Europe and the Mediterranean. In Malta, we came across α^+-thalassaemia in the differential diagnosis of microcytosis having excluded the common iron deficiencies, the β thalassaemia heterozygotes and now the new KLF1 deficiencies with a φ thalassaemia. The few significant patients in our clinic with Hb H Disease due to $-\alpha/--$ were Asians though they had relatively quite a milder clinical condition than elsewhere; maybe they ate better here!

However, as indicated above, the haematological and clinical consequences of interplay among homozygotes with Thalassaemia and SCD was significant. Clearly, the co-inheritance of an α thalassaemia had the obvious benefit of decreasing the severity of the imbalanced (α/β) globin biosynthesis in β thalassaemia. Consequently, the dyserythropoiesis due to excessive haeme and α globin was weaker. The effects of an α thalassaemia on the haematological development among the Juvenile SCD that we uncovered together with Graham Serjaent's clinic in Jamaica was more complex (Felice et al., 1987, Sargent G., et al. 1987). It revealed dependencies of the genetic interplay underpinned by the physiological and energetic requirements of early-stage growth in juveniles ($<$ 20 years old) and the insidious onset of an inflammatory siderosis. The same was observed regarding the early stage haematological and clinical sequel of even mild β thalassaemia in Maltese juveniles (Vella, 2018, Benzetoon, 2022, Felice AE et al., manuscript in preparation). Many of the developmental haematological data, when charted resembled those of allosteric enzyme kinetics, the Hb oxygen dissociation curve or the denaturation of nucleic acids maybe reflecting a "Saturation Effect" possibly associated with the bio-energetic requirements of early development and the onset of iron excess over iron deficiency of childhood (Sebu. C., and Felice AE., unpublished observations) These prospective observational studies shall be continued within new international consortia (INHERENT; see below). Pre-conditioning of the bio-energetic balance and control of the underlying inflammation may permit newer HbF inducers (HFIs) and Erythropoiesis Stimulating Agents (ESAs) to act better, particularly in thalassaemia to prevent long term complications.

The sickle haemoglobin (HbS) resulted from a mutation in the β globin gene that substituted glutamic acid, the sixth amino acid of the β globin, to a valine (Perutz et al., 1968) The mutant Hb S moleculae became insoluble and polymerized under hypoxic conditions in the erythrocyte. The rate of polymerization depended on the 40^{th} power of the (intra-cellular) HbS concentration. Repeated cycles of oxygenation and de-oxygenation with accompanying polymerization and de-polymerization of the Hb damaged the erythrocyte membrane. It expressed Phosphatadyl Serine externally and became adhesive to the vascular endothelium. The micro-capillary vasculature was liable to occlusion by adhered sickled cells, localized hypoxia and tissue infarction. HbF was a physiological inhibitor that delayed nucleation of polymerisation. Powars et al., (2005) best described the condition as a chronic haemolytic anaemia punctuated by episodes of vaso-occlusion until the natural history was "catastrophically" altered by a major event such as acute medullary aplasia, or splenic sequestration, stroke, renal failure, or even death. The severity among patients differed markedly with some being barely symptomatic with occasionally a microcytosis or higher HbF while others, albeit less anaemic, spent miserable weeks in extreme pain due to body-wide vaso-occlusive episodes. To

complicate matters, paediatricians were describing a different clinical picture among juveniles than the adult medicine physicians. Together with Graham Sargeant group in Jamaica, we surmised that the common α thalassaemia might decrease the MC(HbS)C, or favour the assembly of Hb F over HbS, or likely both, to decrease the nucleation rate and inhibit intra-erythrocytic polymerization (Stevens et al., 1986, Felice et al., 1987)

Virgil McKee and his wife Kathleen, both paediatricians, joined us from the US Army Medical Corps to take direction of the Paediatric Sickle Cell Clinic that Alex Bruce Tagoe and I had set up in Augusta. Together, we assembled a network of statewide sickle cell clinics to follow the neonates diagnosed by newborn testing and to explore objectively the natural history of SCD. A considerable biomedical data-set accompanied extensive biosynthetic and later genetic analysis served to uncover two important effects of HbF and α thalassaemia on the clinical phenotype of SCD and that could be helpful to evolve new treatments. Deeper engagement with communities in the course of biomedical research and clinical trials has continued to evolve with dynamic eConsent procedures with AI applications on Smart Phones. We have been exploring the added value of a Research Partners' Co-Operative with interested societies and look forward to put them in practice (e.g. see midata.coop).

We made two significant observations. The first was that the rate of post-natal decline of Hb F was considerably delayed in SCD compared to the children with normal Hb type (AA) and even Thalassaemia homozygotes. It reached levels below the 15% at which Hb F was no longer inhibitory of polymerization by the age of 7-10 years. That was approximately around the age that the clinical picture change from paediatric-type to adult-type began to emerge. However, at the time, we could not see an effect of the α thalassaemia on the Hb F/Hb S levels. In retrospect, re-evaluating the data from a different perspective revealed a marginal effect. The second was that, in a dose dependent manner and after HbF had declined below 15% by 7- 10 years of age, the α thalassaemia decreased the MC(HbS)C diminishing the nucleation rate of polymerization and the haemolytic rate. Consequently, although the anaemia was milder, the increased erythrocyte survival increased total Hb / HbS, increasing whole blood viscosity, decreased flow and tissue oxygenation with sporadic infarcts in various organs. Life span improved but quality of life deteriorated due to the effects of the α thalassaemia on whole blood viscosity and the endothelium.

In the meantime, following observations on experimental animal models, Hydroxyurea, well known in paediatric haematology (Dingli et al., 2006) was shown to maintain Hb F levels above the predicted 15% that had emerged from our studies and benefited the SCD children by diminishing physico-chemical nucleation and clinical consequences. The story substantiated the value of newborn testing for expedited diagnoses of rare disease in general and the discovery of new treatments subject to the availability of samples and data with a unitary purpose in Bio-Medicine. Currently, Eurordis and Euro-Biobank strongly argue along the same lines in promoting National Rare Disease Platforms that included the human haemoglobinopoathies within the terms of the relevant European Reference Networks of hospitals and clinics (e.g. Euro-BloodNet)

SCD, like Hb H Disease and Hb E Disease were new clinical challenges in the health system of Malta. Hypermigration in the Central Mediterranean changed the quantitative Hb Epidemiology of Malta and neighboring Southern European countries. The data from Hb Neonatal testing (Galdies et al., 2023) acquired over the last 35 years showed that while the national birth rate declined markedly, the HbS and other African or Eastern Hb variants such as HbE now accounted for nearly one third of all Hb variants that were encountered. A handful of patients with SCD have presented to the clinics. The same must be happening with other, less visible rare disease. They are new clinical challenges in particular in the emergency setting across Europe.

β Thalassaemia became the specific objective of my research after I returned to Malta with the Thalassaemia Project in the late 1980. β thalassaemia arose from partial or complete deficiency of β globin biosynthesis. Unlike deletional α thalassaemia, single nucleotide substitutions or other mutations in the β globin gene gave rise to most common types of β thalassaemia. The HbVar and ITHAgene databases collected details of the mutations and deletions worldwide (Giardine et al., 2007, LeDere et al., 2009, Kountouris et al., 2021) The Thalassaemia International Federation joined patients and families with physicians and experts in biomedicine from across the world. In 1976 we had hosted the International Thalassaemia Meeting that I was privileged to chair. ITHANET was an EU funded IT platform for data sharing INHERENT was a new research network of thalassaemia clinics and laboratories to promote research. These organisations and EURORDIS acted strongly at pan-EU level to promote patient values in health services and research on Rare Disease in general. The National Alliance for Rare Disease Support represented them in Malta. Human haemoglobinopathy, like the Haemophilia community, led to an inclusive model for rare disease management in partnership with patients and families. It created the worldwide context for expedited diagnosis and new treatments.

Classically, β thalasaemia Heterozygotes were not an-

aemic but displayed microcytosis and typically, an elevated Hb A2 ($>$ 3.5%) that was pathognomonic. Actually, silent thalassaemia presented with a normal Hb A2 and the new ψ thalassaemia may have borderline Hb A2 due to KLF1 deficiencies (G. Grech, 2020) The Hb F was occasionally elevated depending on the mutation and the co-regulators. The compound heterozygote with α and β thalassaemia was normocytic but still had an elevated Hb A_2. We identified the β globin mutations that accounted for the thalassaemia of Malta and quantified the allele frequency of each (C. A. Scerri et al., 1993) Collectively they amounted to a heterozygous carrier rate of around 1%. The β^+ IVS-I, 6C, a relatively mild mutation first found among patients from Portugal was the most frequent in Maltese heterozygotes (66%) The β thalassaemia heterozygote, commonly known as thalassemia trait, was physicaly healthy albeit subject to reproductive risk. Older heterozygotes with concurrent cardiometabolic or inflammatory conditions may require closer scrutiny in the context of common MTHFR and folate deficiencies that impacted inflammatory hyperhomocysteinaemia. A formal trial has yet to be reported.

β Thalassaemia homozygotes suffered a severe medullary dyserythropoiesis caused by excessive inflammatory Haeme and precipitation of the excessive α globin as haemochromes on the membrane of the erythroblasts. The excesses inflamed the medullary eco-system of erythroblastic islands assembled around a central macrophage. It resulted in a severe chronic haemolytic anaemia and, disturbed iron traffic giving rise to tissue wide siderosis and a presumed inflammation or hypercoagulability, since infancy. Complications arose in many tissues due to long term hypoxia, siderosis and the underlying inflammation. Hb F in blood lysates (mg/dl) may be elevated by increased biosynthesis or, more often, by preferential survival of the F-erythrocytes with higher HbF due to the heterocellular distribution as explained above.

The Thalassaemia Clinic provided resources to make a genetic diagnosis with counselling of the heterozygotes and couples at risk and for the long-term care of the homozygotes within guidelines of the international research partnerships. In fact, we developed a unique long term prospective clinical cohort to define the natural history of the different β globin mutations, some severe, some mild. Christian Scerri's doctoral project served to define the genetic causes of thalassaemia in Malta and made the initial clinical observation that children with even the mild mutations required treatment as intense as others with the more severe mutations through the juvenile ($<$ 20) years. Mohamed Marwan (1998) conducted similar studies on patients from Libya providing contrasts between the two that could be further compared with related data from our collaborators in S. Italy. Marwan's research further served to quantify the dysfunctional output of the β thalassaemia mutations to improve the classification of disease. Although β^+ IVS-I, 6C mutation diminished β globin output only by two thirds [MC(HbA)C = 3.1pg/RBC] compared to others that abolished it or nearly so, the clinical outcome among juveniles appeared to be the same. As in the case of SCD the severity of the β thalassaemia varied quite markedly. Most patients required life-long transfusion and chelator therapy unless successfully transplanted. Rare, very informative patients were demonstrably homozygotes but without many signs or symptoms. Whole genome sequencing on these can provide much new insight into pathogenesis. Clint Mizzi (Mizzi, 2016) ran a comparative genomic profile between the common mild β+-IVS I, 6C of Malta and the wild type genome that did not reveal additional genomic variation. Perhaps, a similar exersise with the more severe mutations such as the β^o IVS-I, 110A of Cyprus might have yielded a different result. At the genomic level, a comprehensive genotype - phenotype connection classified the severity of the β thalassaemia by virtue of four genetic / molecular parameters. They were;

1. The severity of the β globin gene mutation and the suppression of β globin biosynthesis
2. The co-inheritance of α thalassaemia to diminish the degree of α/non-α imbalance
3. The de-repression of γ globin to compensate for the β globin deficit (and the excessive Haeme)
4. The transmission of co-modifiers that could affect organ-wide complications (e.g., MTHFR$\pm$ / others) in adults

The socio-economic conditions and the quality of care had a direct influence too. Many times, they depended on the availability of national resources. Apart from stem cell and genetic therapies there shall remain a need for improved conservative management with transfusion and chelation and potential molecules that could function as ESAs or HFIs cost effectively world-wide.

In contrast, the clinical classification of β thalassaemia left much to be desired. While the older denominations into minor, intermedia or major was not sufficiently discriminatory and failed to match the genomic classification above, the newer classification reflecting transfusion dependence was subjective and lacked a clear objective, quantifiability suitable for robust Genome Wide Association Studies (GWAS) The clinical genomic prospective study served to establish an objective score calculated from the volume of erythrocyte transfusion, the pretransfusional Hb (G/dL) and BMI (Bugeja, 2008, Vella, 2018, Benzetoon, 2022) The simpler objective quantific-

ation of the early course of the disease should be better suited for GWAS and related research with ESAs and HFIs.

Like others, we had sought to explore the therapeutic value of Hydroxyurea, that had proven useful in SCD. Partly by acting on stem or progenitor cell kinetics and partly, by epigenetically re-architecturing the $\gamma-\beta$ locus, HbF increased and sickling was inhibited. A short clinical trial in Thalassaemia conducted together with students from the Department of Pharmacy (Galea, 2005, Felice et al., 2007) gave disappointing results. Hydroxyurea induced a rapid medullary hypoplasia that we assumed due to the medullary inflammation. Similar outcomes were encountered at other clinical centers. The matter of hypercoagulability and inflammation has plagued clinical thalassaemia research for many years. It has not yet been possible to pin down a secure biomarker that reflected the significant pathophysiology. Neither the comparative genomic review of Mizzi (Mizzi, 2016) nor the protein marker exploration of Vella (Vella, 2018) or the leucocyte transcriptomics of Benzetoon (2022) in association with the innovative objective clinical score have as yet yielded robust biomarkers. The question is important because it seems that ESAs and HFIs action may be obstructed by the underlying inflammation before they could be effective at the globin gene level. Possibly, if Newborn Testing identified all β Thalassaemia and Hb S (SCD) homozygotes at birth, early use of Hydroxyurea or an improved HFI and ESA could sustain high Hb F before the onset of (medullary) inflammation, to prevent long term complications in both conditions. HU may have proven more useful in SCD because the inflammation of SCD acted on the peripheral vasculature rather than the bone marrow as in thalassaemia.

In the meantime, KLF1, a known erythroid transcription factor, emerged as potentially a master regulator of Globin Gene Switching that could have served to be targeted by new ESAs/HFIs to increase HbF for the benefit of SCD and β Thalassaemia. We came across KLF1 while following a family that had been referred to our clinic because of iron resistant microcytosis. The nuclear family, now known as FamF1 had borderline microcytosis, a normal HbA_2 with high Hb F, a phenotype akin to an HPFH, with a normal β globin gene sequence. Joseph Borg took this up first as part of his MSc in the Ithanet project and later for his doctoral project. Ruth Galdies in the testing lab found 6 others with very variable Hb F that were further studied by Laura Grech for her Ph.D. and then followed up by others in the course of our longer term research.

HPFH was thought to derive from errors of globin gene control that sustained continued activity of the γ globin genes beyond the peri-natal period without any haematological impairment. However, whenever deeper studies of globin gene expression were possible, some degree of imbalanced α/non-α biosynthesis emerged; thus, the borderline between HPFH and certain types of β Thalassaemia was somewhat blurred. Furthermore, some types of HPFH expressed Hb F in all erythrocytes (homocellular) or in an expanded population of F-erythrocytes (heterocellular) Some were due to sizable deletions within the $\gamma-\beta$ locus, others were due to meaningful mutations in the $^{G}\gamma$ or the $^{A}\gamma$ promoters. The deletions revealed in cis DNA sequences involved in switching while the promoter mutations revealed TF binding sites (Fig. 4; see Weatherall et al., 2008)

Many more members of the original nuclear family (FamF1) joined the research. GWAS at Sjaak Philipsen's Laboratory in Rotterdam (Erasmus MC) by Joseph and Godfrey Grech led us to a locus on Chromosome 19 that could be responsible for the phenotype, close to the KLF1 TF locus. Sequencing identified a truncation mutation (p.Lys288Ter; rs267607202) It produced a shortened KLF1 protein missing the DNA zinc finger essential for binding to the DNA. The subjects with the higher Hb F had multiple mutations in KLF1 both in cis and in trans. Laura Grech addressed the matter by sequencing the KLF1 of a few hundred patients from our clinic and the biobank with borderline HbA_2 and undiagnosed microcytosis. She found additional mutations in KLF1, some in the promoter and some in the coding sequence and she quantified the functional capabilities of the promoters that were found to be very strong. Many families could now have a new diagnosis, known as ψ-thalassaemia, and a better estimation of risk that they lacked before. This research won us prizes at the second European Biobank Week (Milan 2000) and the biennial Haemoglobin Switching Meeting (Oxford 2018) As in the case of the chemical heterogeneity of the Hb variants that had occupied my interests earlier on, it was concluded that the levels of KLF1 in very early stem or progenitor cells were subject to interplay between rather common dys-regulatory mutations in the (powerful) promoters and dys-functional mutations in coding regions of the gene. It seemed that the KLF1 promoter activity was very strong in the stem/progenitor cellular pool and acted on many erythroid specific loci (Fig. 8) Early-stage de-repression flooded the stem/progenitor cells with KLF1 to occupy and activate promoters of a cognate panel of other loci required for further development of the erythrocyte lineage. They de-repressed the γ globin genes at first followed by the β globin genes perinatally. KLF1 itself was subsequently repressed in the pro-erythroblasts (Herseius et al., 2022). The phenotype of a ψ-thalassaemia or HPFH appeared following the cu-

mulative effects of several mutations. In fact, the highest Hb F among FamF1 was seen in a double heterozygote that depressed the promoter function considerably. It should be possible to mimic it pharmacologically to increase the Hb F of patients with minimal side effects on erythropoiesis and no known parallel effects elsewhere. A broad drug screen and a wider "omics" profiling among additional families with diverse KLF1 genotypes could be useful.

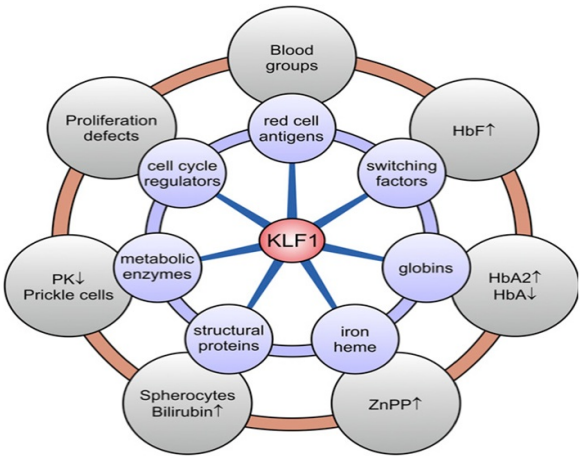

Figure 8: The KLF1 transcription factor now known as master regulator of erythropoiesis and that exhibited remarkable pleiotropy in the pathophysiology of the erythrocyte.

Although considerable headway has been made with cellular and gene therapies, a persistent need for a small molecule therapeutic remained for many patients world-wide. The severity of disease varied and the suitability of clinical settings varied just as much. The ongoing research on physiological mechanisms of globin gene control along the lines reviewed above drives the continued search for a safe and effective one.

As others take over, the way forward is to strengthen what has worked, to embed newborn testing and biobanking for expedited diagnosis and the discovery of new treatments, to look for more effective models, to innovate and improve among the European partnerships with Euro-BioBank/BBMRI-ERIC, the TIF, INHERENT and possibly others.

On reflection, we have uncovered rules governing the assembly of higher order molecules that possibly based on the KLF1 transcription factor regulated the developmental switching of human foetal to adult Hb phenotypes. Further definition of these biochemical and genetic mechanisms may ultimately lead to improved understanding of genome-wide control mechanisms and the design of new pharmacological or genetic treatments for the benefit of patients.

2 Acknowledgements

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