bjh review

Predicting clinical severity in sickle cell anaemia

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Summary

The ability to predict the phenotype of an individual with sickle cell anaemia would allow a reliable prognosis and could guide therapeutic decision making. Some risk factors for individual disease complications are known but are insufficiently precise to use for prognostic purposes; predicting the global disease severity is not yet possible. Genetic association studies, which attempt to link gene polymorphisms with selected disease subphenotypes, may eventually provide useful methods of foretelling the likelihood of certain complications and allow better individualized treatment.

Keywords: single nucleotide polymorphisms, linkage disequilibrium, genetic association, fetal haemoglobin, sickle cell.

'Never make predictions, especially about the future'. Berra Y.

Predicting the phenotype of sickle cell anaemia in the first months of life, or even antenatally, would allow a precise prognosis, permit individualized treatment and avoid unnecessary hazardous interventions. Sickle cell anaemia, a monogenic disorder caused by homozygosity for a single β-globin gene (*HBB*) mutation (HbS; β^6GAG → GTG; Glu → Val; glu6val), behaves clinically as a multigenic trait with exceptional phenotypic variability (Table I). Sickle cell disease encompasses several different genotypes that include sickle cell anaemia, HbSC disease (compound heterozygosity for HbS and HbC genes) and HbS-β-thalassaemia (compound heterozygosity for HbS and β-thalassaemia genes). Most studies of genotype-phenotype relationships have examined individuals with sickle cell anaemia, with or without coincident α-thalassaemia. Understanding the vascular and inflammatory components of the disease pathophysiology provides many loci where the disease phenotype can be impacted by modifying genes (Fig 1). While 'severity scores' have been proposed, none capture satisfactorily the all-embracing severity of disease (Steinberg *et al*, 1973; Odenheimer *et al*, 1987; Bray *et al*, 1994; Miller *et al*, 2000). For example, most clinicians would consider that a child who died during a first episode of sepsis had severe disease. But, how did the pathophysiological consequences of the HbS mutation put this child at risk for sepsis and what would have been the course of disease, if not for the chance encounter with the offending microbe? How should severity be estimated in a 70-year-old patient who survived a stroke, had frequent pain episodes in his youth, is crippled by osteonecrosis and has respiratory insufficiency following repetitive acute chest syndrome? Furthermore, a possible link between the haemolytic features of disease, where sickle erythrocyte destruction leads to high plasma haemoglobin levels that scavenge the vasodilator, nitric oxide (NO), blunting the vasodilatory response and perhaps promoting vaso-occlusion, further hinders the measurement of disease severity (Reiter *et al*, 2002). Forecasting accurately the severity of sickle cell disease may require knowing which genes are associated with its haemolytic and vascular complications and how variants of these genes interact among themselves and with their environment.

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Established predictors of sickle cell disease complications

Fetal haemoglobin

Fetal haemoglobin (HbF) ($\alpha_2\gamma_2$) is the most thoroughly studied genetic modulator of sickle cell anaemia (Table II). Its exclusion from the HbS polymer inhibits HbS polymerization. Among patients with sickle cell anaemia, HbF concentrations vary from 0·1% to 30% with an average of about 8%. Understanding why patients' HbF levels differ so substantially (Dover *et al*, 1987, 1992; Chang *et al*, 1995, 1997; Garner *et al*, 1998, 2002; Ofori-Acquah *et al*, 2004; Wyszynski *et al*, 2004a) may help us to devise better therapeutics to induce HbF (γ-globin gene; *HBG1*, *HBG2*) expression, a goal of β-haemoglobinopathy treatment. Unfortunately, knowing the HbF level of an individual is insufficient to foretell the likely complications. Some patients have devastating disease manifestations with HbF levels near 20%; the oldest patients with sickle cell anaemia – age can be one measure of disease severity – often have very low HbF (Rucknagel *et al*, 1979; Morris *et al*, 1991; Steinberg *et al*, 1995a). These inconsistences, and the differential clinical response among patients achieving similar HbF

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Table I. Some clinical events and common laboratory markers that reflect the phenotypic heterogeneity of sickle cell disease.

Clinical	Laboratory
Pain	Bilirubin
Dactylitis	PCV
Stroke	Erythropoiesis
ACS	Leucocytes
Osteonecrosis	Dense cells
Longevity	HbF/F cells/HbF per F cell
Cholelithiasis	LDH
Renal failure	Creatinine; proteinuria
Proliferative retinopathy	Reticulocytes

Some patients may have all of the complications listed in column 1, while other can have few, or even none of these events.
Some patients die young from disease complications; other survive into the eighth decade. In common laboratory tests, e.g. leucocyte or reticulocyte counts, broad variation can be found among patients. For example, HbF levels can vary by two orders of magnitude; leucocyte counts can be normal or more than twice normal.

levels after treatment with HbF-inducing drugs, may be related to heterogeneity in the cellular distribution of HbF or to the effects of other modifying genes (Dover & Boyer, 1980).

Any increment in HbF was found to increase survival in sickle cell anaemia; patients whose HbF rises most in response to treatment with hydroxyurea also fared best (Platt *et al*, 1991, 1994; Steinberg *et al*, 2003b). A retrospective study of Jamaicans with steady-state HbF levels below 1% compared them with patients having HbF levels between 2·5% and 3·4% and between 4·6% and 5·2%. As expected, packed cell volume (PCV) and mean corpuscular volume (MCV) increased with increasing HbF, but differences in the incidence of painful crises, abdominal crises and the acute chest syndrome were not apparent (Donaldson *et al*, 2001). Earlier studies also found no relationship between HbF and overall severity (Powars *et al*, 1980; Hedo *et al*, 1993). It was suggested that the threshold level of HbF needed to prevent acute clinical events was about 20% while the threshold to prevent organ damage was 10% (Powars *et al*, 1984). The relationship between reduction of most vaso-occlusive complications of sickle cell anaemia (Table I) with higher HbF levels and the lack of a clear reduction of global disease severity may reflect the absence of a reliable severity index.

Beta-globin haplotypes. The HbS gene is found on a genetic background of four major β-globin-like gene cluster haplotypes (Nagel & Labie, 1989). Carriers of the HbS gene on Senegal or Arab-India haplotype usually have the highest HbF level and PCV and the mildest clinical course. Individuals with Bantu (Central African Republic) haplotypes have the lowest HbF level and PCV and the most severe clinical course. Carriers of the Benin haplotype have intermediate features (Nagel & Steinberg, 2001). A C → T polymorphism at position-158 5′ to the $^{G}\gamma$ gene in carriers of Senegal and Arab-India haplotypes is strongly associated with $^{G}\gamma$-globin gene expression and high HbF levels (Nagel *et al*, 1984; Labie *et al*, 1985; Steinberg *et al*, 1995b). Nevertheless, even among carriers of this polymorphism, there is considerable diversity of HbF levels suggesting the actions of additional regulatory elements (Nagel *et al*, 1984; Gilman & Huisman, 1985; Miller *et al*, 1987; Steinberg *et al*, 1995b). A recent study suggested that

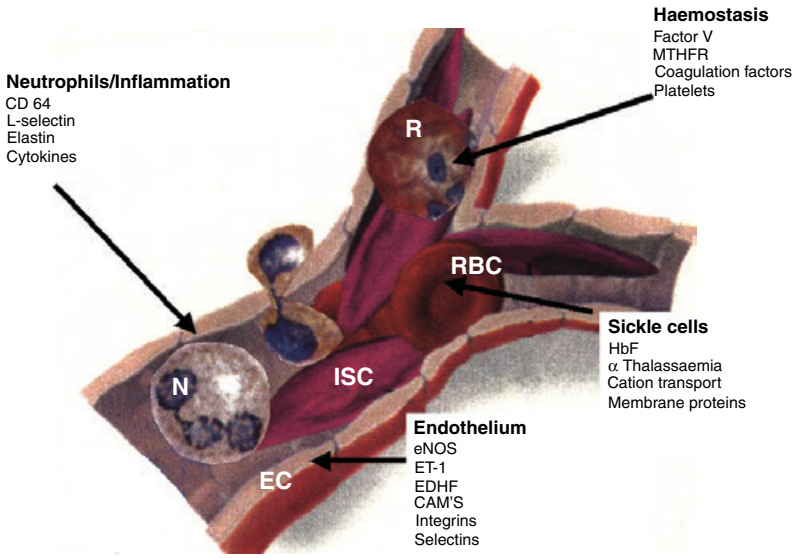

Fig 1. Vaso-occlusion in sickle cell disease is initiated by the presence of the HbS mutation and high concentrations of HbS within the sickle erythrocyte. Circulating sickle cells, a melange of irreversible sickled cells (ISC), discoid sickle cells (RBC) and sickle reticulocytes (R), initiate vaso-occlusion by their interactions with endothelium (EC), granulocytes (G), platelets and each other. Many different proteins in sickle erythrocytes, endothelial cells, leucocytes and in the fluid phase of blood, have the potential to modulate the vaso-occlusive process (Hebbel, 1997).

Table II. Effect of HbF level on some clinical complications of sickle cell disease.

Phenotype	Effect of HbF	Reference
Survival	Protective*	Platt *et al* (1994)
Painful episodes	Protective	Platt *et al* (1991); Bailey *et al* (1992)
Stroke	Unlikely to have major effect unless very high	Bailey *et al* (1992); Ohene-Frempong *et al* (1998); Adekile *et al* (2002)
Osteonecrosis	Protective (limited information)	Hawker *et al* (1982)
Acute chest syndrome	High HbF reduces incidence of ACS in hydroxyurea-treated patients	Charache *et al* (1995); Steinberg *et al* (2003a)); Bailey *et al* (1992)
Proliferative retinopathy	Protective in both HbSC disease and HbSS†	Hayes *et al* (1981); Fox *et al* (1990)
Pulmonary hypertension	No effect	Gladwin *et al* (2004)
Leg ulcers	Protective	Koshy *et al* (1989)
Splenic sequestration	Protective	Bailey *et al* (1992)
Splenic function	Protective	Wali *et al* (2002)
Growth and development	Maintains	Singhal *et al* (1996)
Menarche	Protective	Serjeant *et al* (2001)
Perinatal fetal death	Protective	Morris *et al* (1994)
Priapism	Unclear	Adedeji *et al* (1988); Nolan *et al* (2004)

Heterogeneity among populations of patients with sickle cell anaemia, in the ages of the patients studied and in the numbers of patients in each study sometimes make firm conclusions difficult.
*Protective denotes prolongation of survival or a reduction in the incidence or prevalence of a phenotype with increased HbF.
†HbSS-sickle cell anaemia or homozygosity for the HbS gene.

the β-globin gene cluster haplotype, independent of the HbF level, is a correlate of survival in hydroxyurea-treatment sickle cell anaemia patients (Bakanay *et al*, 2005). How this might occur is unclear; modifying genes might be linked to the β-globin-like cluster although, to date, HbF concentration is the only known *cis*-acting disease modulating factor.

The association of β-globin-like gene cluster haplotype with the severity of sickle cell anaemia must be cautiously interpreted and the prognostic value of knowing the haplotype in an individual is very limited. Studies of several different ethnic groups of patients with sickle cell anaemia with distinct haematological characteristics suggested that the β-globin gene cluster haplotype may be useful as one predictor of disease severity (Labie *et al*, 1985; Hattori *et al*, 1986; Kulozik *et al*, 1986, 1987; Nagel *et al*, 1987; Ragusa *et al*, 1988; Sharon *et al*, 1988; Srinivas *et al*, 1988; Labie *et al*, 1989; Nagel & Labie, 1989; Schroeder *et al*, 1989; Month *et al*, 1990; Ballas *et al*, 1991; Dimovski *et al*, 1991; Powars, 1991a,b; Nagel & Fleming, 1992; Zago *et al*, 1992; Öner *et al*, 1992). In the first studies of Africans and Indian patients, most patients were homozygous for a haplotype and genetically homogeneous compared with their descendants in developed countries studied subsequently. Carriers of the Senegal haplotype appeared to have less severe disease than carriers of other haplotypes but clinical data were limited (Ohene-Frempong & Nkrumah, 1994) The Arab-India haplotype is also associated with milder disease although vaso-occlusive events do occur (Padmos *et al*, 1991; El-Hazmi *et al*, 1992; Adekile & Haider, 1996). In developed countries, where most patients are compound heterozygotes for different haplotypes and genetic admixture is the rule, the effect of haplotype on severity is less clear. In longitudinal studies from the USA, the Senegal haplotype was associated with fewer hospitalizations and painful episodes and had a marginal effect on acute chest syndrome (Powars, 1991a,b). The Bantu haplotype was associated with the highest incidence of organ damage and renal failure and the poorest HbF response to hydroxyurea (Powars *et al*, 1991; Steinberg *et al*, 1997; Bakanay *et al*, 2005).

Genetic regulation of HbF

Fetal haemoglobin expression is a quantitative trait involving complex interactions among chromosome remodelling activities, transcription factors, genes modulating erythropoiesis and elements linked to the β-globin gene cluster, providing ample opportunity for genetic modulation (Tuan *et al*, 1985; Forrester *et al*, 1987; Grosveld *et al*, 1993; Weiss & Orkin, 1995; Tanimoto & Engel, 2000; Blobel & Weiss, 2001).

In a study of siblings with sickle cell disease that included 23 sibling pairs with sickle cell anaemia and six families with HbS-β-thalassaemia, strong correlation was present between siblings for HbF levels before and during hydroxyurea treatment. Also, there was a concordant change in HbF response from baseline to treatment-associated levels (Stein-

berg *et al*, 2003b). As these siblings are likely to share the same parental β-like globin gene clusters with their *cis*-acting regulatory sequences, the results suggested that some elements regulating HbF expression are linked to the β-globin gene cluster. Nevertheless, with rare exceptions, only the 5′ $^{G}\gamma$-globin gene-158 C → T single nucleotide polymorphism (SNP) has been associated with HbF in sickle cell anaemia (Lu & Steinberg, 1996; Plonczynski *et al*, 1997; Ofori-Acquah *et al*, 1999, 2004) suggesting that *trans*-acting regulatory elements exert controls over HbF levels.

The first of these putative elements was the F cell production locus, a quantitative trait locus (QTL) at Xp22 that was associated with F cell number (Thein *et al*, 1994; Chang *et al*, 1995, 1997; Garner *et al*, 1998, 2002). The phenotype of this locus and the $^{G}\gamma$-globin gene-158 C → T polymorphism were estimated to account for about half of the HbF variation in sickle cell anaemia, with the F cell production locus accounting for 35–41% of this variation. Subsequently, a QTL at 6q22·3–23·2 was associated with F cell numbers in a single extended Asian-Indian family. Further study of this region showed that it contained five protein-coding genes, some of which displayed a high degree of alternative splicing, one large, previously uncharacterized gene, *AHI1*, that spanned about 215 kb and several genes that did not appear to be protein coding (Close *et al*, 2004). In an Asian family with β-thalassaemia, a QTL at chromosome 8q appeared to interact with the -158 C → T polymorphism, a result confirmed in a study of twin pairs (Garner *et al*, 2002, 2004). The interaction of the 8q locus with the -158 C → T SNP in regulation of HbF in sickle cell anaemia has not been reported.

More than 180 SNPs in 38 candidate genes that might modulate HbF levels were studied in 280 sickle cell anaemia patients. The strongest association with HbF was found with SNPs near the 6q22·3–23·2 QTL. In an initial analysis, two SNPs were identified in intergenic portions of this QTL and were associated with about a 20% difference in the HbF percentage. When 44 additional SNPs in the genomic region between 136·1 and 137·5 Mb on chromosome 6q were genotyped, 12 SNPs, associated with a 20–30% difference in HbF concentrations were found in the introns of four genes (Wyszynski *et al*, 2004a). These genes were, phosphodiesterase 7 (*PDE7B*), microtubule-associated protein 7 (*MAP7*), mitogen-activated protein kinase 5 (*MAP3K5*) and peroxisomal biogenesis factor 7 (*PEX7*). *PDE7B* is a phosphodiesterase with high affinity and specificity for cAMP (Gardner *et al*, 2000). This pathway was activated in K562 cells and its activation inhibited haemin-induced expression of γ-globin mRNA and decreased transcriptional activity of the γ-globin gene promoter. This suggested that this pathway, independently of MAPK pathways, reduced γ-globin gene expression, an effect opposite to that of the cGMP pathway that increased expression of the γ-globin gene (Ikuta *et al*, 2001; Inoue *et al*, 2004). In K562 cells, the p38-MAPK pathway was associated with the activation of γ-globin gene expression by histone deacetylase inhibitors. PEX7 binds PEX14 (NAPP2) that interacts with the p45 subunit of NF-E2 and with HDAC1; NAPP2 is a possible negative coregulator of NF-E2. Curiously, three of these genes that might potentially effect globin gene expression are in proximity abutting 6q 23·2. Whether or not this has any regulatory importance is unknown. It has been hypothesized that variable expression of a collection of linked genes of functional interdependence may be associated with haematopoietic cell turnover (de Haan *et al*, 2002). Long range linkage disequilibrium (LD), as seen in this QTL, has been reported for other chromosomal regions (Liu & Barker, 1999).

Predictors of the response to hydroxyurea

Hydroxyurea, the sole agent available for specifically treating the complications of sickle cell anaemia, is used in many countries. Its most plausible mechanism of action is its ability to increase HbF levels although it is likely to have other beneficial affects. Hydroxyurea increases HbF in sickle cell anaemia because its cytotoxicity causes erythroid regeneration and perhaps because its metabolism leads to NO-related increases in soluble guanylyl cyclase with an increase of cyclic GMP that augments γ-globin gene expression (Cokic *et al*, 2003). Predicting an individual's HbF response to hydroxyurea treatment would aid in the selection of patients for treatment and reduce toxicity from unfruitful dose escalation. Unfortunately, despite some interesting clues, is not possible to predict presently the HbF response to treatment (Valafar *et al*, 2000).

To identify determinants of HbF regulation, 150 hydroxyurea-treated patients were grouped by quartiles of change in HbF from baseline to 2 years of drug treatment. In the top two quartiles, HbF increased to 18·1% and 8·8%. These patients had the highest baseline neutrophil and reticulocyte counts, and largest treatment-associated decrements in these counts. In the lower two quartiles, 2 year HbF levels (4·2% and 3·9%) and blood counts changed little from baseline. All four quartiles had substantial increases of F cells (erythrocytes containing measurable HbF) in the first year that were maintained for 2 years only in the top three quartiles. Leucocyte and reticulocyte counts decreased initially in all quartiles but drifted back toward baseline levels in the lowest HbF-response quartile. Based on these findings it was suggested that bone marrow ability to withstand hydroxyurea treatment may be important for sustained HbF increases during treatment of sickle cell anaemia (Steinberg *et al*, 1997). In a study of more than 100 children who received maximal hydroxyurea doses, HbF increased to almost 20% and the treatment effects were sustained for 7 years without clinically important toxicity (Ware *et al*, 2002; Zimmerman *et al*, 2004). HbF levels achieved during treatment were associated with baseline HbF level, haemoglobin level, reticulocyte count and leucocyte count.

Hydroxyurea inhibited growth of erythroid burst-forming units (BFU-e) from patients with sickle cell anaemia in a dose-dependent manner and increased HbF in their BFU-e (Yang *et al*, 1997). An *in vitro* cell culture system was established to

evaluate the effects of hydroxyurea on BFU-e colony growth and induction of HbF production in patients who responded well and failed to respond to hydroxyurea treatment. In responders, BFU-e numbers fell more than 20-fold and HbF increased from 5·1% to 19·4%, but there was no change in non-responders. These pilot studies suggested that changes in numbers of BFU-e and HbF after *in vitro* exposure to hydroxyurea may help select patients who will have a favourable response to treatment (Yang *et al*, 1997). S-phase cells were also more numerous in sickle cell anaemia patients that respond to hydroxyurea treatment (Baliga *et al*, 2000).

A total of 226 SNPs in 46 candidate genes with possible roles in HbF regulation and hydroxyurea metabolism were studied in 214 African-American patients with sickle cell anaemia for whom HbF levels were available before treatment and after a stable dose of drug was reached. Included were genes with potential roles in hydroxyurea metabolism, such as *AQP9*, *AGR1*, *ASS*, *PS1*, *SLC14A1*, *LOC57404*, *POR*, *CYP2C9* and *CYP3A5*; cytokines, such as *CSF3*, *CSF2*, *CSF2RB*, *EPO*, *IL1*, *IL3* and *TGF* and genes mediating the effects of hydroxyurea, such as *RRM1*, *RRM2*, *TK1*, *TYMS*, *MPO*, *SOD1*, *ABCB1*. SNPs in cytochrome P450, family 2, subfamily C, polypeptide 9 (*CYP2C9*), a member of the cytochrome P450 family of enzymes that catalyse reactions of drug metabolism, which may have a role in the metabolism of hydroxyurea derivatives, and, aquaporin 9 (*AQP9*), a water-selective membrane channel that stimulates urea transport and permits passage of uncharged solutes, were associated with the HbF response to drug (Wyszynski *et al*, 2004b). *AQP9* and *CYP2CP*, besides the 6q locus, may modulate HbF response to hydroxyurea. Multiple genes are very likely to effect this response so their interactions and the predictive value of their polymorphisms will need to be modelled.

α-*Thalassaemia*

About 30% of patients with sickle cell anaemia have coincidental α-thalassaemia (Steinberg & Embury, 1986) and these compound heterozygotes have less haemolysis, higher PCV, lower MCV and lower reticulocyte counts (Embury *et al*, 1982; Higgs *et al*, 1982; De Ceulaer *et al*, 1983; Steinberg *et al*, 1984). Alpha-thalassaemia reduces the concentration of HbS and HbS polymerization. Clinically, the effect of α-thalassaemia on sickle cell anaemia is not as consistent as the effects of high HbF (Table III). Vaso-occlusive events that are highly dependent on PCV, such as stroke, leg ulcer and splenic function, appear to benefit from the coexistence of α-thalassaemia. The inheritance of α-thalassaemia was associated with a 13%

Table III. Effect of α thalassaemia on some clinical complications of sickle cell anaemia.

Phenotype	Effect of α-thalassaemia	Reference
Survival	Probably little effect	Higgs *et al* (1982); Mears *et al* (1983); Schroeder *et al* (1989); Keclard *et al* (1996); Martinez *et al* (1996); Keclard *et al* (1997); Thomas *et al* (1997); Miller *et al* (2000); Mouele *et al* (2000)
Painful episodes	Protective in HbSC* Permissive† or little effect in HbSS	Platt *et al* (1991); Billett *et al* (1995); Mukherjee *et al* (1998); Powars *et al* (2002)
Stroke	Protective	Miller *et al* (1988); Balkaran *et al* (1992); Adams *et al* (1994); Ohene-Frempong *et al* (1998); Neonato *et al* (2000); Sarnaik and Ballas (2001); Hsu *et al* (2003)
Osteonecrosis	Permissive in HbSS‡ protective in HbSC	Steinberg *et al* (1984); Ballas *et al* (1989); Milner *et al* (1991); Milner *et al* (1993); Powars *et al* (2002)
Acute chest syndrome	Unrelated or protective	Higgs *et al* (1982); Steinberg *et al* (1984); Castro *et al* (1994); Mukherjee *et al* (1998)
Proliferative retinopathy	Unrelated or mild protection	Condon *et al* (1983); Fox *et al* (1993)
Heart	Cardioprotective	Braden *et al* (1996)
Cholelithiasis	Protective	Adekile and Haider (1996); Haider *et al* (1998); Powars *et al* (2002)
Leg ulcer	Protective	Higgs *et al* (1982); Koshy *et al* (1989)
Splenic function	Protective	Adekile *et al* (1996); Wali *et al* (2002)
Growth and development	Unrelated	Singhal *et al* (1996)
Menarche	Unrelated	Serjeant *et al* (2001)
Priapism	Mild protection	Nolan *et al* (2004)
Splenic sequestration	Permissive	De Ceulaer and Serjeant (1991)
HbF	Probably little effect	Embury *et al* (1984)
Albuminuria	Protective	Guasch *et al* (1999)

The effects of α-thalassaemia may differ in sickle cell anaemia and HbSC disease and data on other genotypes of sickle cell disease are very few. Heterogeneity among populations of patients with sickle cell anaemia, in the ages of the patients studied and in the numbers of patients in each study sometimes make firm conclusions difficult.

*Protective denotes a reduction in the incidence or prevalence of a phenotype with α-thalassaemia.

†Permissive denotes an increased incidence or prevalence of a phenotype when α-thalassaemia is present.

‡HbSS-homozygosity for the HbS gene.

prevalence of macroalbuminuria compared with 40% in patients without α-thalassaemia (Guasch *et al*, 1999). Painful episodes, acute chest syndrome and osteonecrosis, complications perhaps more dependent on blood viscosity, are either minimally affected or their prevalence is increased. Coincident α-thalassaemia results in longer erythrocyte lifespan because of the reduction of dense and rigid red cells and the resulting increased PCV and blood viscosity, a consequence of more HbS-containing cells, favours vaso-occlusion. When sickle cell anaemia patients are treated with hydroxyurea, the potentially adverse effects of an increased PCV is likely to be offset by the high HbF concentration, reducing both anaemia and the frequency of vaso-occlusion.

Interactions of α-thalassaemia and HbF. Sickle cell anaemia patients from India with very high HbF levels and F cells of nearly 70% carried the Arab-India haplotype. The frequency of α-thalassaemia was highest in patients with mild disease. Among Saudis and Kuwaitis carrying the Arab-India haplotype and α-thalassaemia, the disease was milder when compared with patients without α-thalassaemia (Padmos *et al*, 1991; El-Hazmi, 1992a,b; Adekile & Haider, 1996). Perhaps, when HbF is exceptionally high, this dominates the increment in PCV caused by α-thalassaemia.

Organ failure was most common in carriers of a Bantu haplotype and least common in Senegal haplotype carriers and α-thalassaemia decreased this risk (Powars, 1991a; Powars *et al*, 1994). In Jamaicans, the absence of α-thalassaemia coupled with a high HbF presaged benign disease (Thomas *et al*, 1997). When phenotypes of sickle cell anaemia were clustered into two or three phenotype groups, neither α-thalassaemia nor the β-globin gene cluster haplotype appeared to influence the clinical events defining the groups (Alexander *et al*, 2004).

Potential predictors of disease severity

The diversity of sickle cell anaemia cannot be explained by HbF and α-globin gene-linked modulation alone. Modifying, or epistatic, genes, which potentially affect the pathogenesis of sickle cell anaemia and modulate the phenotype of disease, have recently become candidates for study. These genes, which include: mediators of inflammation, oxidant injury, NO biology, vasoregulation, cell–cell interaction, blood coagulation, haemostasis, growth factors, cytokines and receptors and transcriptional regulators, act independently of HbS polymerization, the mechanism through which both HbF and α-thalassaemia modulate disease.

Polymorphisms in genes that could modify the phenotype of sickle cell disease

Polymorphisms have been noted in many genes that plausibly effect the phenotype of sickle cell disease (Table IV). 'Candi-

Table IV. Genetic polymorphisms effecting the subphenotypes of sickle cell anaemia.

Subphenotype	Gene/SNP marker*	Effect	Reference
Stroke	*VCAM1*/G1238C	Protective†	Taylor *et al* (2002a)
	VCAM1/T-1594C	Permissive	Hoppe *et al* (2004)
	IL4R/S503P	Permissive	Hoppe *et al* (2004)
	TNFA/G-308S	Protective	Hoppe *et al* (2004)
	LDLR/NcoI +/−	Protective	Hoppe *et al* (2004)
	ADRB2/Q/27E	Protective	Hoppe *et al* (2004)
	AGT/AG repeats	Permissive	Tang *et al* (2001)
	HLA genes	Protective; permissive	see text
Osteonecrosis	*MTHFR*/C677T	Permissive, but questionable	De Castro *et al* (1998); Kutlar *et al* (1998); Zimmerman *et al* (1998); Zimmerman and Ware (1998)
Acute chest syndrome	*NOS3*/T-786C	Permissive	Sharan *et al* (2004)
	NOS1/AAT repeats	Permissive	Sullivan *et al* (2001)
Cholelithiasis	*UGT1A*/promoter repeats	7/7, Permissive	Passon *et al* (2001); Fertrin *et al* (2003)
Priapism	*KL*	Permissive	Nolan *et al* (2004)

National Center for Biotechnology Information (NCBI) gene abbreviations are used.
Only genetic variants with a protective or permissive association with a phenotype and that have been published as complete papers are included in the table. Many more variants have been studied and have shown no associations. Some of these are mentioned in the text.
*The nucleotide or amino acid substitution characterizing the SNP.
†Protective denotes reduction in the incidence or prevalence of a phenotype with a particular SNP; permissive denotes an increased incidence or prevalence of a phenotype when SNP is present.

date gene' studies, seeking associations of SNPs with phenotypes of disease, are in their infancy and when interpreting these studies, the following should be remembered. A candidate gene approach is necessarily limiting and unlikely to examine all genes affecting a phenotype. Studying only candidate genes does not provide a complete picture of the genetic heterogeneity that must account for the complex pathobiology of disease, i.e. the false negative rate from these studies is apt to be high. At this time, whole genome association studies are only beginning. Each candidate gene contains dozens to hundreds of SNPs and picking the 'best' ones to study may be difficult. Non-synonymous coding region SNPs that occur in functionally important regions may alter proteins, thereby affecting a phenotype. Non-coding region SNPs may be even more important as gene regulation is likely to be modulated by small differences in the concentration of regulatory proteins. Unfortunately, besides the basic sequence of promoters, gene splicing elements and 3′ end processing machinery, the regulatory elements of genes may be difficult to identify, although progress is being made (Sabo *et al*, 2004). Likely interactions among genes and between genes and environment are usually neglected but are biologically inescapable. Some phenotypes are difficult to ascertain accurately and their absence at one point in time does not mean that they may not occur later. A small sample size may not permit firm conclusions and unsophisticated analytical methods may cause both false positive and false negative results. The association of a polymorphism with a phenotype may identify LD with the important SNP or gene. An association between a genetic variant and a phenotype need not indicate causality. Comparing the results of genetic marker studies is difficult because geographically separated populations often differ genetically and may have different genetic risk factors.

Painful episodes. Acute painful episodes are the major clinical event in sickle cell disease and are a measure of disease severity and predictor of early death in adults. The rate of painful episodes varies widely among patients; highest pain rates are found in patients with high PCV and low HbF (Platt *et al*, 1991). Nocturnal hypoxia was associated with the frequency of painful episodes (Hargrave *et al*, 2003). We know little else about the causes of the heterogeneity in pain rate and nothing about the genetic basis of this variability.

Stroke. While PCV, leucocyte count and α-thalassaemia are of some use in forecasting the likelihood of stroke, the best predictor is estimating the velocity of blood flow in large cerebral arteries. Transcranial Doppler (TCD) flow of ≥200 cm/s is associated with an increased stroke risk (Adams *et al*, 1992, 1998; Ohene-Frempong *et al*, 1998; Kinney *et al*, 1999). While TCD can predict the likelihood of stroke in sickle cell anaemia, only 10% of individuals with abnormal flow will have a stroke in the following year and a stroke will occur in some individuals with normal blood flow. TCD or peak systolic velocity (Jones *et al*, 2005) are excellent screening methods whose widespread use can prevent stroke by identifying patients who will profit most from prophylactic transfusion. Nevertheless, most transfused patients are unlikely ever to have a stroke even if not transfused, so many individuals are unnecessarily exposed to the hazards of chronic transfusion (Cohen & Porter, 2001). Recent studies have shown that discontinuing transfusion even after several years can be associated with reversion of TCD flow to pretransfusion levels and a stroke (National Heart, Lung, and Blood Institute Clinical Alert; http://www.nlm.nih.gov/databases/alerts/sickle_transfusion.html). Additional prognostic indicators of stroke would therefore be useful. Stroke in sickle cell disease is associated with increased blood pressure, as is stroke in the people without haemoglobinopathies (Pegelow *et al*, 1997). Whether or not antihyperstensive therapy will reduce this risk is unknown.

Most genetic association studies in sickle cell anaemia have examined the stroke phenotype and far less information is available on its many other subphenotypes. A family predisposition to stoke in sickle cell disease suggested that inherited modulation of this phenotype was possible (Driscoll *et al*, 2003). Among the genes associated with stroke in sickle cell anaemia, two alleles of vascular adhesion molecule-1 (*VCAM1*), G1238C in the coding region of Ig domain 5, and, T-1594C, an intronic SNP, had an association with stroke. The coding region SNP was protective while the intronic SNP, predisposed to small vessel stroke (Taylor *et al*, 2002a; Hoppe *et al*, 2004). *VCAM1* is about 19 000 base pairs, has nine exons and nearly 200 SNPs are in the public domain. Clearly, choosing the 'right' SNP is not a trivial task and any association is likely to identify LD.

Six SNPs in the intercellular adhesion molecule-1 and CD 36 genes (*ICAM1*; *CD36*) were not associated with stroke (Taylor *et al*, 2002a). When stroke was subdivided into large and small vessel disease based on imaging studies, SNPs in the interleukin 4 receptor gene (*IL4R*; non-synonymous coding region, S503P) predisposed to large vessel stroke while tumour necrosis factor α gene (*TNFA*; non-coding G308A) and α adrenergic receptor 2 (*ADRB2*; non-synonymous coding region, Q27E) SNPs were protective. In the small vessel stroke group, a low-density lipoprotein receptor (*LDLR*; untranslated region) SNP was protective. Homozygosity for the combination of *TNFA*-308 GG and the *IL4R* 503P heterozygosity was associated with a strong predisposition to large vessel stroke (Hoppe *et al*, 2004). The endothelium and vascular response to regulators may distinguish small from large blood vessels but a continuum must exist, making the pathophysiological basis of these observations enigmatic.

In one study, the prevalence of the C1565T mutant of the platelet glycoprotein IIIa (*ITPG3*) gene was similar in 16 stroke patients compared with controls. In two studies, both involving small numbers of stroke patients, the risk of stroke associated with polymorphisms of the angiotensinogen (*AGT*), a gene implicated in stroke risk in the general population, was equivocal (Tang *et al*, 2001; Romana *et al*, 2004). Other

miscellaneous associations with stroke include the cystathione B synthase gene (*CBS*; 278thr 68 bp insertion) cholesterol ester transfer protein (*CETP*; -628A), and apolipoprotein C III (*APOC3*; -641A) (Hoppe *et al*, 2001; Tang *et al*, 2001; Romana *et al*, 2002). Because of its association with hyperhomocysteinaemia, a recognized risk factor for stroke, the association of the C677T polymorphism in 5,10-methylenetetrahydrofolate reductase (*MTHFR*) was investigated in 48 patients with sickle cell stroke and in 48 controls. The results suggested that this SNP is unlikely to be a risk factor for stroke in this population (Adekile *et al*, 2001). Very small patient numbers call for replication of the results of these and other similar size studies in a larger sample.

Human leucocyte antigen (HLA) genes may be risk factors for vascular disease (Wick *et al*, 1995; Inoue *et al*, 1997; Zimmerman & Ware, 1998; Zou *et al*, 2002). In 22 sickle cell anaemia patients with cerebral infarction, the HLA DRB1*0301 and *0302 alleles increased the risk of stroke and the DRB1*1501 protected from stroke. DQB1*0201, in LD with DRB1*0301, was associated with stroke and DQB1*0602, in LD with DRB1*1501, was protective of stroke. Using the Cooperative Study of Sickle cell Disease (CSSCD) patient database, HLA genotyping was performed in 36 patients with large vessel stroke and 35 with small vessel stroke (Styles *et al*, 2000a; Hoppe *et al*, 2002). A total of 160 patients with a negative magnetic resonance imaging scan served as controls. In the small vessel stroke group, HLA DPB1*0401 was associated with stroke while DPB1*1701 was protective. In the large vessel stroke patients, DPB1*0401 was associated with susceptibility and DPB1*1701 was associated with a trend toward protection. Also, HLA-A*0102 and A*2612 caused susceptibility to stroke and A*3301 was protective of stroke. In a study of seven candidate genes in 42 children (mean age, 7·8 years) with large vessel cerebral artery stenosis, SNPs in the transforming growth factor-β receptor 3 (*TGFBR3*) and adenine cyclase 9 (*ADCY9*) were associated with this phenotype, when compared with 71 controls (mean age 14·8 years) (Driscoll *et al*, 2004). Because of the potential role of blood coagulation, the plasminogen activator inhibitor-1 gene (*PAI1*) has been studied intensively for associations with vascular disease in the general population with variable results; in sickle cell anaemia, it was not associated with stroke (Hoppe *et al*, 2004).

To further define the genetic basis of stroke, the association of SNPs in candidate genes of different functional classes with the likelihood of having a stroke was examined. A total of 113 patients with sickle cell anaemia and a confirmed history of, or incident complete, non-haemorrhagic stroke, documented by imaging studies were compared with 493 control patients. Polymorphisms in four candidate genes, Klotho (*KL*), *TGFBR3*, annexin 2 (*ANXA2*) and bone morphogenetic protein 6 (*BMP6*), were associated with stroke (Steinberg *et al*, 2003c). These genes play roles in the TGF-β/BMP pathway, cell adhesion and NO biology. *KL* (13q12), encodes a membrane protein and regulates many vascular functions including vascular endothelial growth factor expression and NO release by endothelium.

No association with cerebrovascular disease was found when multiple coagulation factors were measured in children transfused for stroke, at risk for stroke and in untransfused controls (Kahn *et al*, 1997; Andrade *et al*, 1998; Liesner *et al*, 1998). The factor V gene R485K polymorphism may have a potential role in stroke and osteonecrosis (Tankut *et al*, 2000). Polymorphisms in low-affinity Fc leucocyte receptors were not associated with stroke (Taylor *et al*, 2002b). Hypoxia-induced cellular activation and release of adhesive and inflammatory mediators might be related to stroke and other vaso-occlusive complications, as suggested by a study of cell adhesion molecules in children with mild sleep hypoxia who had a higher PCV and increased markers of cell adhesion and activation (Setty *et al*, 2003).

To examine the interaction among genes and their variant SNPs and to develop a prognostic model for stroke in sickle cell anaemia, a Bayesian network was developed to analyse 235 SNPs in 80 candidate genes in 1398 unrelated subjects with sickle cell anaemia. SNPs on 11 genes and four clinical variables, including α-thalassaemia and HbF, interacted in a complex network of dependency to modulate the risk of stroke. This network of interactions included three genes, *BMP6*, *TGFBR2*, *TGFBR3* with a functional role in the TGF-β pathway and one gene (*SELP*) associated with stroke in the general population. The model was validated in a different population by predicting the occurrence of stroke in 114 unrelated individuals with 98·2% accuracy, predicting the correct outcome for all seven stroke subjects, and for 105 of 107 non-stroke subjects. This gave a 100% true positive rate and 98·14% true negative rate, and an overall predictive accuracy of 98·2% (Sebastiani *et al*, 2005). As traditional analytical methods are often inadequate for the discovery of the genetic basis of complex traits in large association studies, Bayesian networks are a promising approach. The predictive accuracy of this stroke model is a step toward the development of prognostic tests better able to identify patients at risk for stroke. The presence among the risk factors of genes already associated with stroke in the general population, such as *SELP*, suggests that some genetic factors predisposing to stroke may be shared by both sickle cell anaemia patients and stroke victims in general.

Priapism. The possible association of SNPs in 44 candidate genes with priapism was studied in 148 patients with sickle cell anaemia and incident or a confirmed history of priapism and 529 controls that had not developed priapism. Polymorphisms in *KL* showed an association with priapism by genotypic [rs2249358; OR = 2·6 (1·4–5·5); rs211239; OR = 1·7 (1·2–2·6)] and haplotype analyses (Nolan *et al*, 2004).

Osteonecrosis. The C677T polymorphism in *MTHFR* was found in 36% of 45 adults with sickle cell disease and osteonecrosis but only 13% of 62 control patients (Kutlar *et al*, 1998). Nevertheless, other reports failed to confirm this association (De Castro *et al*, 1998; Zimmerman *et al*, 1998; Zimmerman &

Ware, 1998). The C1565T SNP in plate glycoprotein IIIa (*ITPB3*) and a polymorphism in *PAI1* were not associated with sickle osteonecrosis (Zimmerman & Ware, 1998). In steroid-treated renal allograft recipients, osteonecrosis was associated with the presence of heterozygosity or homozygosity for the 4G/4G allele in the plasminogen activator inhibitor-1 gene (*PAI1*) (Ferrari *et al*, 2002). These studies all examined small numbers of patients and few SNPs.

In a study of 442 patients with sickle cell osteonecrosis of the hip and shoulder and 455 sickle cell disease controls, three to five SNPS in each of 70 candidate genes were examined in an initial screening. Significant associations were observed with SNPS in seven genes, *BMP6*, *TGFBR3*, *TGFBR2*, endothelin-1 (*EDN1*), v-ets erythroblastosis virus E26 oncogene like (*ERG*), *KL* and endothelin converting enzyme-1 (*ECE1*). Evaluation of additional SNPs typed in all seven genes revealed association with many SNPs in and surrounding *KL* and *BMP6*. For *TGFBR2*, *EDN1* and *ERG*, only one additional SNP was associated with osteonecrosis and no additional SNPs were associated in the remaining genes. SNPs in *ANXA2* were also typed because of a previous finding of association between this gene and stroke among patients with sickle cell disease. Of the 18 SNPs typed in *KL*, 10 were significantly associated with osteonecrosis. Most of these SNPs were located in the 20 kb region representing the first half of the first *KL* intron and were in LD. A cluster of viral long terminal repeats (LTRs) within this region suggested that it may have regulatory function. SNPs in *BMP6* and *ANXA2* were also associated with osteonecrosis and disease-associated SNPs tended to be in LD. Two haplotype blocks were defined in *KL*, one in *ANXA2* and one in *BMP6*. For *KL*, there was stronger LD within the blocks in cases compared with controls suggesting that the avascular necrosis cases share a greater proportion of similar haplotypes than controls. For each of these LD blocks, haplotypes were estimated and, for all three genes, the distribution of haplotypes was significantly different in cases and controls (Steinberg *et al*, 2003d; C. T. Baldwin, personal communication). These results may provide insight into the pathogenesis of osteonecrosis in sickle cell disease, help identify individuals at an early age who are at high risk for osteonecrosis and therefore, enable earlier and more effective therapeutic intervention.

Pulmonary hypertension. The development of pulmonary hypertension, a recently appreciated, yet common and severe subphenotype of sickle cell disease (Gladwin *et al*, 2004), is likely to be modulated by the effects of genes that control NO and oxidant radical metabolism, cell–cell interaction, vasculogenesis and vasoreactivity. For example, mutations in bone morphogenetic protein receptor 2 (*BMPR2*) and other genes have been associated with both familial and sporadic pulmonary hypertension (Nichols *et al*, 1997; Machado *et al*, 2001; Ameshima *et al*, 2003). To date, data on the genetics of pulmonary hypertension in sickle cell anaemia are limited. In a study of 45 patients with pulmonary hypertension (tricuspid regurgitant jet of >2·5 m/s) compared with 65 controls, SNPs in *BMPR2* and *ADCY6* were associated with this phenotype (Ashley-Koch *et al*, 2004).

Acute chest syndrome. Increased susceptibility to sickle acute chest syndrome in females only was associated with a T-786C SNP in the endothelial NO synthase gene (*NOS3*) (Sharan *et al*, 2004). Exhaled NO levels were reduced in patients with acute chest syndrome compared with controls and this was associated with the number of AAT repeats in intron 20 of *NOS1* (Sullivan *et al*, 2001).

Prospective and retrospective studies suggest that secretory phospholipase A(2) [sPLA(2)], may be related to the clinical severity of acute chest syndrome and capable of predicting its onset. In a prospective analysis of 21 admissions for painful episodes, each of the six patients who subsequently developed acute chest syndrome had elevated sPLA(2) 24–48 h before its clinical diagnosis (Styles *et al*, 1996, 2000b).

Gallstones. Promoter poylmorphisms in the uridine diphosphate-glucuronosyltransferase 1A (*UGT1A*) gene are associated with unconjugated hyperbilirubinaemia and Gilbert syndrome (homozygosity for the promoter 7/7 TA repeats). Children with sickle cell disease had a significantly higher mean bilirubin level if they carried the 7/7 *UGT1A* genotype compared with the wild type 6/6 or 6/7 genotypes and patients with the 7/7 genotype were more likely to have had a cholecystectomy. This suggested that symptomatic cholelithiasis is more common in carriers of this genotype (Passon *et al*, 2001; Fertrin *et al*, 2003). When treated with hydroxyurea, children with the 6/6 *UGT1A* genotype had normal bilirubin levels compared with individuals with the 6/7 or 7/7 genotypes. A recent study suggested that the 7/7 and 7/8 genotype were risk factors for symptomatic gallstones only in older subjects with sickle cell disease (Haverfield *et al*, 2004). Carriers of the 7/7 genotype had bilirubin levels greater than 51·3 μmol/l despite full-dose hydroxyurea therapy (Heeney *et al*, 2003), suggesting that this SNP may influence the ability of hydroxyurea to prevent gallstone formation.

Leucocytosis. Neutrophils are effectors and modulators of inflammation and tissue damage and activated neutrophils differentially express more than 250 genes (Newburger *et al*, 2000). Circulating granulocytes are increased in patients with sickle cell anaemia but are usually normal in individuals with HbSC disease, a less severe disease genotype (West *et al*, 1992). In sickle cell anaemia, increased granulocyte counts are an adverse risk factor for survival and have been associated with adverse events, such as acute chest syndrome and stroke, in children (Balkaran *et al*, 1992; Platt *et al*, 1994). Animal studies have suggested that the initiation, progression, and resolution of a vaso-occlusive episode resembles ischaemia-reperfusion injury. In transgenic sickle mice, baseline neutrophil counts are elevated nearly threefold while

hypoxia-reoxygenation elicited an increased number of adherent and emigrated leucocytes with evidence of oxidant production in vascular endothelial cells. Anti-P-selectin antibody completely inhibited this inflammatory response, suggesting that leucocyte–endothelium interactions contributed to vaso-occlusive events (Kaul & Hebbel, 2000). Neutrophils may be activated in sickle cell anaemia and activation may also accompany acute painful episodes. Neutrophil CD62L, CD11b, CD66b, CD63 and Fc gamma receptors and plasma levels of lactoferrin, elastase, soluble CD1, CD62L (L-selectin) and interleukin-8 all showed significant differences between control subjects and steady state sickle cell disease patients, with more pronounced changes during painful episodes (Mollapour *et al*, 1998; Lard *et al*, 1999).

Neutrophils from sickle cell disease patients were more adherent to endothelial cells than neutrophils from controls. Patients had increased numbers of neutrophils expressing CD64, suggesting that enhanced adhesion to endothelium could contribute to vaso-occlusive disease (Fadlon *et al*, 1998). Hydroxyurea may also decrease the activation of neutrophils (Saleh *et al*, 1999). The role of leucocytes in mediating tissue injury suggests that differences in neutrophil gene expression or polymorphisms in neutrophil-expressed genes may account for some phenotypic variation in sickle cell disease, however, there are no published studies addressing this issue.

Upregulation of intracellular cyclic adenosine monophosphate dependent protein kinase A (PKA) by epinephrine was found to increase sickle erythrocyte adhesion to endothelium, suggesting that adrenergic hormones may play a role in sickle vaso-occlusion (Zennadi *et al*, 2004). Sickle erythrocyte adherence to laminin appeared to be partially dependent on SNPs in the β-adrenergic receptor gene, *ADRB2*. A coding-region SNP causing an arg16gly substitution appeared to modulate baseline adherence but not epinephrine-stimulated adherence (Eyler *et al*, 2004).

Compound phenotypes. Few studies have combined vaso-occlusive complications to seek associations with polymorphisms in candidate genes. One study of 34 patients with histories of stroke, acute chest syndrome, osteonecrosis and priapism and 63 controls who had not yet had these complications, showed that patients with complications had a significantly higher frequency of the HPA-5b allele compared with controls (Castro *et al*, 2004). In this small study, an individual needed but a single complication to be included and most events were osteonecrosis, with only four individuals having more than a single phenotype.

Predicting disease severity: common genes effecting sickle vaso-occlusion. It is not yet feasible to integrate the many clinical and laboratory abnormalities of sickle cell anaemia into a predictive model that permits an understanding of the interactions among common clinical and laboratory abnormalities. Miller *et al* (2000) used the presence of hand–foot syndrome in infants, leucocyte count and haemoglobin level to predict severe disease outcomes, defined as death, stroke, frequent pain and acute chest syndrome, in patients with sickle cell anaemia and HbS-β^0 thalassaemia.

As genetic data suggested that genes of the TGFβ/BMP pathway, *KL* and *ANXA2* were associated with several vaso-occlusive phenotypes of sickle cell anaemia (Nolan *et al*, 2004; Sebastiani *et al*, 2004), the interaction of vaso-occlusive phenotypes and selected laboratory measurements in sickle cell disease was modelled using Bayesian networks (Sebastiani *et al*, 2004). Clinical and laboratory data from nearly 1500 individuals with sickle cell anaemia, with or without coincident α-thalassaemia, were entered into a network that described the interactions between clinical and laboratory data and their associations with the risk for complications of sickle cell anaemia. The model showed that a complex network of interactions between clinical and laboratory variables underlie common complications of sickle cell anaemia and, ultimately, death. Particularly important was the protective role that α-thalassaemia appeared to play. For example, α-thalassaemia, by decreasing erythrocyte density, reduced haemolysis and was associated with lower levels of bilirubin and an associated decreased risk for priapism. Bilirubin levels may reflect NO availability and NO may be involved in the aetiology of priapism. Alpha thalassaemia was also associated with fewer reticulocytes, which is strongly associated with a decreased risk for acute chest syndrome and osteonecrosis. It was associated with higher level of HbF and a reduction in leucocyte counts with a significant decreased risk for stroke and death. This model can be used to predict the occurrence of certain complications of sickle cell anaemia and early death, given the presence of other disease complications and variations among common laboratory variables. For example, it predicted a 10% risk for stroke at early age and an 11% risk for early death in patients with sickle cell anaemia without α-thalassaemia, compared with a 4% risk for stroke and 1·5% risk for early death for individuals with coincident α-thalassaemia. However, the predictive power of the model is limited, probably reflecting the omission of the genotypic changes that underlie the phenotypes.

Conclusions

Applied genomic medicine is taking its first steps toward aiding the understanding of the pathophysiology of disease, enabling predictive medicine and providing clinically useful pharmacogenomics. The near future should see increased applications in the management of sickle cell anaemia as the genetic basis for its many phenotypes are understood and genotype–phenotype relationships better defined.

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