Chapter 15
Genetic Factors Modifying Sickle Cell Disease Severity

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Abstract Sickle cell disease (SCD) is a monogenic disorder caused by a single base mutation but despite its apparent genetic simplicity, the clinical phenotype is hugely variable. In addition to environmental factors, family and epidemiological studies indicate that genetic variants co-inherited with the sickle mutation have a key role in modifying the disease course. This article provides an overview of the genetic modifiers of SCD known to date. Co-inheritance of α-thalassemia and persistent fetal hemoglobin (HbF) production are established major genetic modifiers but they do not explain the full spectrum of the phenotypic variability of SCD. While characterization of some of the key variants and pathways involved in HbF regulation have provided new therapeutic targets for HbF reactivation, generation of a personalized genetic risk score to inform prognosis and guide management requires a larger panel of genetic modifiers yet to be discovered. Elucidation of new genetic modifiers may also provide an insight into other "druggable" targets for therapeutic intervention.

Keywords Hemoglobin F • GWAS • Genotype/phenotype

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15.1 Introduction

Sickle cell disease (SCD) has been heralded as the first "molecular disease" when Pauling ascribed its basis to the presence of an abnormal hemoglobin in 1949 (Pauling et al. 1949). In 1957, Ingram (1957) described the abnormal hemoglobin as being caused by a single amino acid substitution (glutamic acid changed to valine) at position 6 of the β-globin chain of hemoglobin and in 1963, Goldstein et al. (1963) showed that this arose from a single base change of T>A at codon 6. Figure 15.1 summarizes a timeline of the significant events that have contributed to the understanding of the genetic basis, and management of SCD.

In addition to homozygosity for the β^S allele (HbSS, also referred to as sickle cell anemia, SCA), the syndrome of SCD includes HbSC disease (compound heterozygosity of HbS with HbC, Glu^6 to Lys^6), and HbSβ thalassemia (HbSβ+ or HbSβ0 thalassemia, depending on the type of the β-thalassemia mutation). Generally, the compound heterozygotes have a milder disease than HbSS, but even within each genotypic and ethnic group, a spectrum of clinical variability is the recurring theme. For example, within the HbSS group, at the mild end, patients can be asymptomatic while early mortality, frequent hospital admissions with acute pain episodes, childhood strokes and other end organ damage, typify the severe end of the clinical spectrum.

Both environmental and genetic factors contribute to this clinical variability. The importance of weather changes such as cold and rain as triggers of acute pain have been recognized and reported for many years but the conclusions were not consistent due to logistical difficulties in conducting such studies. Environmental factors also include nutritional state, access to social support, and medical care, all of which influence risk factors such as infections. The impact of environmental factors is demonstrated most graphically on the differences in the natural history and outcomes of SCD between the high- and low-income countries.

Twin studies, based on the concordance and discordance of disease complications and severity, have traditionally been used to assess the relative contributions of genetic and environmental factors in complex disorders such as diabetes and schizophrenia. Since monozygotic twins have identical DNA sequences, variation in their disease course can be attributed largely to the effects of the environment. There are three reports of this kind in SCD; two were limited to single pairs of identical twins, one with HbSS and α-thalassemia, and the other with HbSβ thalassemia (Amin et al. 1991; Joishy et al. 1976). The third study investigated nine pairs of identical twins, six with HbSS and three HbSC, from Jamaica (Weatherall et al. 2005). These twins have been followed for 15 years or more, and as a comparison group for examining degrees of concordance between laboratory parameters, 350 gender- and age-matched sibling pairs were also studied. These studies reported that while the twins showed similarities and concordance in laboratory parameters, and attained height and weight, there was discordance in frequency of acute painful episodes and other clinically critical complications. The conclusion was that environmental factors are of great importance in defining the clinical course of SCD.
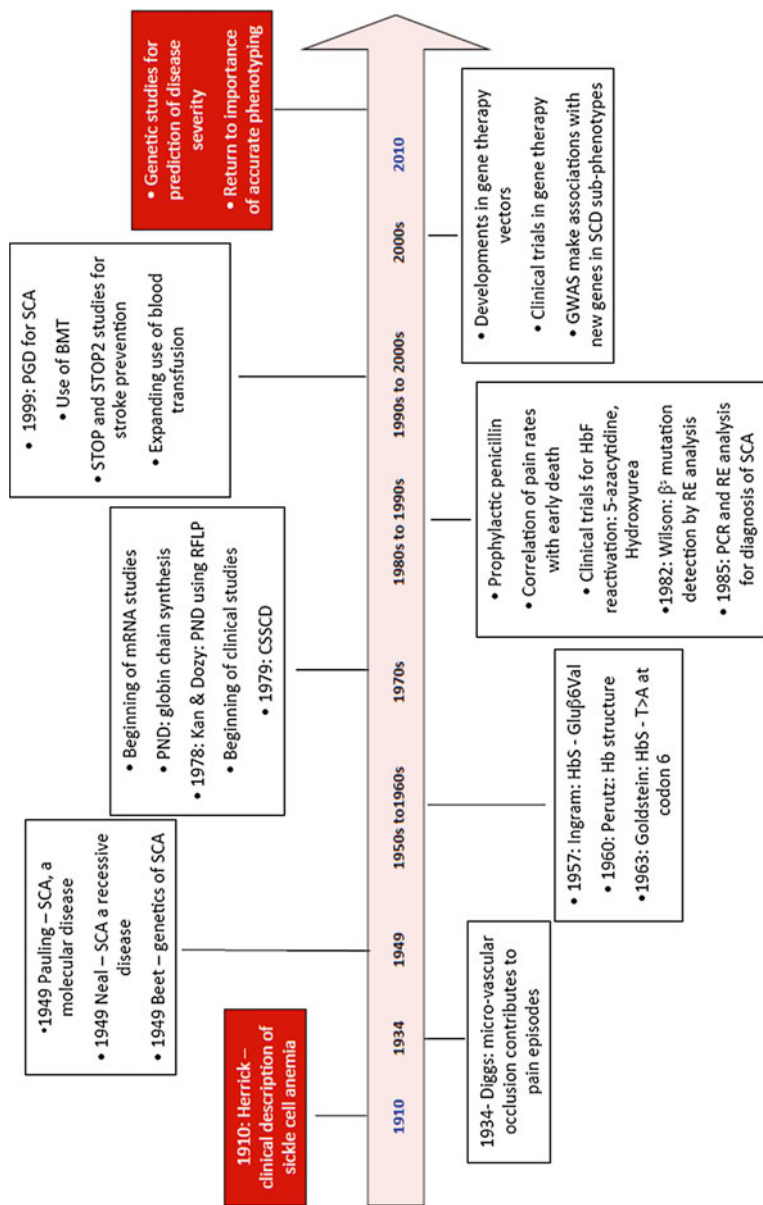
Fig. 15.1 A timeline of significant events that have contributed to the understanding of the genetic basis and management of SCD. *SCA* sickle cell anemia, *mRNA* messenger ribonucleic acid, *PND* prenatal diagnosis, *RFLP* restriction fragment length polymorphism, *CSSCD* co-operative study of sickle cell disease, *PGD* preimplantation genetic diagnosis, *BMT* bone marrow transplantation, *PCR* polymerase chain reaction, *RE* restriction enzyme, *GWAS* genome-wide association study

Family and epidemiological studies indicate that genes co-inherited with the sickle mutation have a key role in modifying the disease course, including higher incidence of stroke (Driscoll et al. 2003) and concordant response to hydroxy-carbamide therapy in siblings (Steinberg et al. 1997). Co-inheritance of α-thalassemia and persistent fetal hemoglobin (HbF) production are established major genetic modifiers of SCD. Numerous candidate gene and genome-wide association studies (GWAS) have defined genetic differences in SCD patients and attempted to identify other genetic variants with particular disease complications. However, the disease complexity has presented immense challenges, and these studies have only provided a small amount of the variation in SCD severity observed in the clinic. Roles for other genetic modifiers of SCD severity have been proposed based on the patho-physiology downstream of the primary event (HbS polymerization), however, the majority of these putative markers have not been replicated.

The clinical diversity of SCD itself presents difficulties for genotype/phenotype correlation studies in terms of accurately defining clinical "sub-phenotypes" (Ballas et al. 2012; Smith-Whitley and Pace 2007; Rees et al. 2010). The clinical implications of a clearer understanding of the genetic variants and mechanisms responsible for the phenotypic variability of SCD are significant. First, the ability to predict disease severity based on a genetic SCD "panel" to facilitate risk stratification of patients: high risk patients might then be followed more intensively, and higher risk therapies (hematopoietic stem cell transplantation, hydroxycarbamide) could be targeted at these patients. Second, new modifying genetic variants might suggest new therapeutic targets for investigation.

We describe the current understanding in terms of determining this phenotypic diversity as well as provide an update of the genetic modifiers of severity in SCD.

15.2   Complications of SCD and Problems in Defining Severity of Phenotypes: Global vs Sub-phenotypes

While full genetic understanding of SCD remains incomplete, the emergence of genome-wide genotyping platforms, next generation sequencing, and rapid advances in genetic research has re-focused some attention back onto phenotyping. Clear and consistent definition of phenotypes is critical to the success of genetic association studies. This is particularly pertinent in SCD where there is profound variety in both the severity and nature of complications. Many of its complications are acute such as recurrent acute pain episodes, acute chest syndrome, priapism and stroke; some are intermittent, leading eventually to chronic complications and organ damage, such as chronic pain, pulmonary hypertension and sickle chronic lung disease, penile dysfunction and cognitive disability. The acute complications vary considerably not only between patients but also in the same patient with time.

Phenotypes can be clinical or laboratory parameters. While laboratory parameters are simple to measure, their values vary with the clinical state of the patient; for

example, lactate dehydrogenase and white cell count which are normally elevated during steady-state, further increase during acute clinical events. Phenotypes are not always consistent or valid, e.g. pulmonary hypertension. Furthermore, some complications are uncommon (e.g. overt strokes) which makes related or "intermediate" traits a preferred endpoint (e.g. imaging results such as raised trans-cranial Doppler (TCD) velocities, or silent infarcts on magnetic resonance imaging).

Many studies focus on *specific* complications of SCD—sometimes described as "*sub-phenotypes*"—i.e. particular end organ damage/failure (e.g. stroke, proteinuria, osteonecrosis, pulmonary hypertension) as separate, individual phenotypic endpoints. *Global* markers of severity—for example mortality—offer the potential for a more cohesive endpoint that may be more informative overall. However, global severity scores have proved difficult to define. Accurate end-point definitions are crucial to enable differentiation of "cases" and "controls". Examples of proposed global severity scores include: (1) a "severity index" based on frequency of acute painful episodes, hospitalization, blood transfusion, infection and specific complications during the previous years starting from the birth of the child (el-Hazmi 1992); (2) the presence of dactylitis in infants, white cell count and hemoglobin (Hb) level to predict severe disease outcomes as defined by death, stroke, frequent pain and acute chest syndrome (Miller et al. 2000); and (3) a global severity score using a Bayesian network model (a "statistical" phenotype) (Sebastiani et al. 2007).

15.3   Genetic Methodologies

Generally, two approaches have been used to locate genetic variants in human disease: linkage analysis and association studies (Hirschhorn and Daly 2005). *Linkage analysis studies* aim to establish linkage between genes that co-segregate with a trait/disease within a family. This technique has been successful in highly penetrative single gene disorders, but has had limited success in many common diseases which comprise complex traits. *Association studies* look for differences in the frequencies of genetic variants between *cases* and *controls* to find genetic variants that are strongly associated with a trait/disease. If a variant is more common in cases than controls, an association is described. Such studies require large sample numbers and until recently have not been feasible due to genotyping cost. Crucially, SNPs identified in pilot studies ("*discovery cohort*") should always be replicated in additional independent populations ("*validation cohort*").

Prerequisites for any genetic association study include: (1) *heritability* (correlation of trait in sibling pairs, good r value); and (2) a clear distinction between *cases* and *controls* (or sufficient variability in a quantitative trait). These criteria present problems in many clinical manifestations of SCD. For example, hospital admissions and duration of stay have used for an objective definition of pain but these measures are influenced by cultural and social factors as well as intermittent illness, such as infections. For convenience, common or "pooled" controls have been used

and this can compromise the analysis by contaminating cases in the controls. *Adequate patient numbers* are essential to allow robust statistical analysis and replication. Again, this presents problems in SCD genetic association studies; most institutions have small numbers of patients (in contrast to hypertensive or diabetic cohorts). Admixture of different ethnic groups is a confounder when different cohorts are pooled for analysis unless population stratification is accounted for prior to association analysis.

Two types of association studies have been utilized in SCD: candidate gene and genome wide association studies (GWAS). *Candidate gene association studies* look for differences in the frequencies of genetic variants in targeted genes between cases and controls, while *GWAS* involve an unbiased scan of the whole human genome (Manolio 2013). Many candidate gene association studies in SCD have been published, but often these associations have not been replicated/validated in independent cohorts. Furthermore, critics of candidate gene studies argue that our limited knowledge of SCD pathophysiology is inadequate to predict functional candidate genes (Manolio 2013). By design, GWAS are more likely to reveal unsuspected interactions as the GWAS approach delivers a "hypothesis free" method that could reveal new genes controlling SCD, and thereby exposing novel pathophysiological pathways.

GWAS will also confirm previous candidate genes if the association is robust (Menzel et al. 2007a; Milton et al. 2012). A case in point is the application of GWAS in the unexpected discovery of *BCL11A* (an oncogene that, hitherto, was not known to have a role in erythropoiesis) as a quantitative trait locus (QTL) controlling HbF (Menzel et al. 2007a; Uda et al. 2008). GWAS also confirmed association of the other two loci—*Xmn*1-*HBG2* (*rs782144*) on chromosome 11p and *HBS1L-MYB* (HMIP) on chromosome 6q—with HbF production, that were previously discovered through candidate gene (Labie et al. 1985) and genetic linkage studies (Craig et al. 1996). Similarly, GWAS confirmed the association between bilirubin level and *UGT1A1* polymorphism in SCD (Milton et al. 2012).

It has also become evident that simpler, "intermediate" phenotypes, such as HbF, that are reproducible and measurable, and disease-related, are much more successful in genetic association in SCD studies than clinical endpoints. Such intermediate endpoints or endo-phenotypes are often quantitative traits; they provide more power in genetic strategies. For example, blood flow velocity in the middle cerebral artery as detected by TCD screening is a biomarker of early cerebrovascular disease in SCD. Studies have shown that chronic blood transfusion therapy at this stage can prevent overt stroke (Adams et al. 1998). In this regard, TCD velocity would be an extremely attractive intermediate phenotype in studies for detecting genetic variants associated with sickle vasculopathy and stroke risk.

Whole genome or whole exome sequencing using next generation sequencing technology in combination with well-defined phenotypes offers the possibility of identifying new genetic variants (Bamshad et al. 2011). GWAS in combination with exome sequencing identified mutations in *GOLGB1* and *ENPP1* with stroke protection in sickle cell anemia (SCA) (Flanagan et al. 2013). In this study, overt stroke was the clinical marker but these variants have yet to be independently validated in a different population group.

15.4 Genetic Modifiers of SCD Severity

Global markers of SCD severity represent the "holy grail" of accurate clinical phenotyping. Multiple attempts at providing scoring systems, and using these for genetic associations have been made. Using the global severity index propounded by El-Hazmi (1992), Nishank identified three *eNOS* gene polymorphisms-*eNOS* 4a/b, *eNOS* 894G>T and *eNOS* -786 T>C associated with SCD severity (Nishank et al. 2013). A GWAS study (Sebastiani et al. 2010) utilized the global severity score devised by the same group (Sebastiani et al. 2007) in over 1200 SCD patients, and replicated in a validation set of samples. Validated SNPs included: *KCNK6* (potassium channel gene) and *TNKS* (telomere length regulator gene).

15.4.1 Modifiers of Global SCD Severity at the Primary Level

The central mechanism underlying the pathophysiology of SCD is the polymerization of deoxygenated HbS and formation of irreversibly sickled erythrocytes that lead to the two hallmarks of the disease—recurrent episodes of vaso-occlusion and pain, and chronic hemolytic anemia. Factors that impact the primary event of the disease process will thus have a global effect on the disease phenotype. They include the causative genotype, co-existing α-thalassemia and the innate ability to produce HbF.

Causative Sickle Genotype

In African-descended populations, HbSS typically accounts for 65–70 %, and HbSC 30–35 % of the cases of SCD, with most of the remainder having HbSβ thalassemia. Other genotypes of SCD have been described, including compound heterozygotes of HbS with HbD, HbO-Arab, but these are rare. While presence of HbS is fundamental to the pathobiology, the likelihood of HbS polymerization and sickling is also highly dependent on the concentration of intra-erythrocytic HbS, as well as the presence of non-HbS hemoglobin (Noguchi et al. 1983). Thus, individuals with HbSS or HbSβ⁰ thalassemia, where the intra-cellular Hb is almost all HbS, tend to have the most severe disease, followed by HbSC and HbSβ⁺ thalassemia. Most studies discussed below consider the homozygous SCD state of HbSS disease only.

HbA ($\alpha_2\beta_2$) or HbA$_2$ ($\alpha_2\delta_2$) do not participate in HbS polymerization. Since the β⁺ thalassemia alleles in Africans are of the milder type with minimal deficit in β globin production, Africans with HbSβ⁺ thalassemia have substantial proportions of intra-erythrocytic HbA and the SCD tends to be very mild. In contrast, individuals with HbSβ⁺ thalassemia in the Mediterranean, have SCD almost as severe as that of HbSS (Serjeant and Serjeant 2001). Subjects with sickle cell trait (HbAS) with HbS of 35–40 %, rarely suffer from symptoms of SCD. Under exceptional circumstances, however, such as intense physical activity and dehydration, the consequent increased intracellular HbS concentration can induce vaso-occlusive pain (Bonham et al. 2010).

The HbS gene is found on a genetic background of four common β-globin haplotypes: Senegal, Benin, Central African Republic (or Bantu), and Arab-Indian. Clinical studies demonstrate variation in SCD severity between the β^S haplotypes, with decreasing severity from the Bantu > Benin > Senegal > Arab-Indian haplotypes. Disease severity correlates inversely with the HbF levels seen in these groups; lowest HbF seen in individuals with Bantu haplotype, and highest HbF in individuals with Arab-Indian haplotype (Nagel et al. 1985, 1987, 1991; Powars 1991; Figueiredo et al. 1996). The differences in clinical severity were ascribed to the difference in HbF levels implicating the *Xmn*1-*HBG2* site which is linked to the Senegal and Arab-Indian β^S haplotype but not to the Bantu haplotype (Labie et al. 1985) (see below for further discussion on modifying effects of HbF on SCD).

Other major genetic factors that influence the primary event of HbS polymerization include the co-inheritance of α-thalassemia and HbF ($\alpha_2\gamma_2$) levels.

Alpha Genotype

About one-third of African-descended patients with SCD have co-existing α-thalassemia (Steinberg and Embury 1986). Most commonly, this is due to the deletion variant ($-\alpha^{3.7}$/); the majority of patients are heterozygous ($\alpha\alpha/-\alpha^{3.7}$) with 3–5 % homozygous for the deletion ($-\alpha^{3.7}/-\alpha^{3.7}$) (Steinberg and Embury 1986; Vasavda et al. 2007). Co-inheritance of α-thalassemia affects SCD red cell phenotype; it reduces intracellular HbS concentration and the propensity of HbS polymerization, reducing the number of irreversibly sickled cells and decreasing hemolysis (Embury et al. 1982; Ballas 2001).

Clinically, co-inherited α-thalassemia protects against complications related to severe hemolysis including pulmonary hypertension, leg ulceration, priapism and albuminuria (Steinberg 2009; Buchanan et al. 2004). Conversely, the increased hematocrit and associated blood viscosity in α-thalassemia predispose to an increased likelihood of developing osteonecrosis, acute chest syndrome (ACS), retinopathy and acute painful vaso-occlusive episodes (Embury et al. 1994). Several studies have also demonstrated association of α-thalassemia with lower TCD measurements and, hence, reduced risk for stroke (Bernaudin et al. 2008; Rees et al. 2009; Flanagan et al. 2011; Cox et al. 2014) while another study could not demonstrate association between α-thalassemia and magnetic resonance angiography (MRA)-defined vasculopathy in paediatric patients with HbSS disease (Thangarajh et al. 2012). The lack of association in the latter study could be related to patient selection. Co-existing α-thalassemia also reduces bilirubin with a quantitative effect that is independent to that of the *UGT1A1* promoter polymorphism (Vasavda et al. 2007). Co-inheritance of α-thalassemia blunts the response to hydroxycarbamide therapy in SCD; this may be explained by its effect on HbF levels and MCV, two key parameters associated with hydroxycarbamide response (Vasavda et al. 2008).

In Jamaicans, the absence of α-thalassemia and higher HbF levels appeared to predict a more benign disease (Thomas et al. 1997), while a subsequent study

reported that α-thalassemia did not promote survival in older Jamaicans with HbSS SCD (Serjeant et al. 2007).

Fetal Hemoglobin

Fetal hemoglobin (HbF, α2γ2) is a major ameliorating factor in SCD. Understanding fetal hemoglobin control and its therapeutic reactivation (pharmacological and genetic approaches) remains a top research priority. HbF reduces the propensity for HbS polymerization and its sequelae in two major ways: (1) the hybrid tetramers ($\alpha_2\gamma\beta^S$) do not partake in HbS polymerization, and (2) the presence of intra-erythocytic HbF dilutes the concentration of HbS (Noguchi et al. 1988). The clinical phenotype of SCD becomes evident within 6 months to 2 years of age as HbF levels decline.

HbF levels impact the "primary" level of disease pathology—HbS polymerization—thus HbF levels have a global beneficial effect. Indeed, in SCD, high HbF levels are a major predictor of survival (Platt et al. 1994), and reduced pain (Platt et al. 1991; Dampier et al. 2004); conversely, low levels of HbF have been associated with increased risk of brain infarcts in young children (Wang et al. 2008). At the sub-phenotype level, there appear to be disparities in its effects on complications such as renal impairment, retinopathy and priapism (Thein 2011; Steinberg and Sebastiani 2012). The failure of HbF to modulate all complications of SCD uniformly in the different reports may be related to the small sample sizes in genetic studies and even smaller numbers of end complications, and to ascertainment of phenotypes.

Update on the Genetic Control of Fetal Hemoglobin (HbF)

Developmental stage-specific expression of the β-like globin genes appears to be governed by two principles: competition for the upstream β-locus control region (LCR), and autonomous silencing of the embryonic and fetal globin genes involving various ubiquitous and erythroid-specific transcription factors (Wilber et al. 2011; Stamatoyannopoulos 2005). Although the fetal globin genes are autonomously silenced in adults, genetic variants lying both within and outside the *HBB* locus lead to natural variation in the level of expression of the fetal globin genes and HbF, of over 20-fold (Thein and Craig 1998). Some of these variants significantly ameliorate the clinical symptoms of the β-hemoglobinopathies. These variants account for 89 % of the quantitative variation but the genetic etiology is complex with no clear Mendelian inheritance patterns (Garner et al. 2000). Three known quantitative trait loci (QTLs) for the common HbF variation in adults include: *Xmn1-HBG2* (*rs782144*) within the β-globin gene cluster on chromosome 11p, *HBS1L-MYB* intergenic region (HMIP) on chromosome 6q23, and *BCL11A* on chromosome 2p16 (Thein and Menzel 2009; Thein et al. 2009; Sankaran et al. 2010).

Variants in the *HBB*, *HMIP* and *BCL11A* loci account for 10–50 % of the variation in HbF levels in adults, healthy or with SCD or β-thalassemia, depending on the population studied (Menzel et al. 2007a; Lettre et al. 2008; Galanello et al. 2009; Bhatnagar et al. 2011; Makani et al. 2011; Badens et al. 2011; Bae et al. 2012; Mtatiro et al. 2014). The remaining variation ('missing heritability') is likely to be accounted for by many loci with relatively small effects, and/or rare variants with significant quantitative effects on γ-globin gene expression that are typically missed.

HBB Cluster on Chromosome 11p

Xmn1-HBG2 (rs782144) in the *HBB* cluster was the first known QTL for HbF and long-implicated by clinical genetic studies (Labie et al. 1985) (see section "Causative Sickle Genotype" above). The differences in clinical severity of SCD were ascribed to the difference in HbF levels implicating the *Xmn1-HBG2* site which is linked to the Senegal and Arab-Indian β^S haplotype but not to the Bantu haplotype (Labie et al. 1985). Recent high resolution genotyping, however, suggests that rs782144 is not likely to be the variant itself, but in tight linkage disequilibrium with causal element(s) that remain to be discovered in the β-globin cluster. In vitro reporter gene assays suggest that Gγ globin promoters isolated from Asian and Senegal chromosomes exert higher transcriptional activity than their counterparts from Benin and Bantu chromosomes (Ofori-Acquah et al. 2001). In particular, the Bantu Gγ promoter is 10 times weaker than the Asian promoter (Ofori-Acquah et al. 2001). However, the association between haplotypes, HbF levels and disease severity in SCD remains somewhat contentious due to the wide variation in HbF levels among individuals of the same haplotype.

BCL11A on Chromosome 2p16

Functional studies in primary human erythroid progenitor cells and transgenic mice demonstrated that BCL11A acts as a repressor of γ-globin gene expression that is effected by SNPs in intron 2 of this gene (Sankaran et al. 2008). Fine-mapping demonstrated that these HbF-associated variants, in particular rs1427407, localized to an enhancer that is erythroid-specific and not functional in lymphoid cells (Bauer et al. 2013). BCL11A does not interact with the γ-globin promoter but occupies discrete regions in the *HBB* complex (Jawaid et al. 2010). The silencing effect of BCL11A involves re-configuration of the *HBB* locus through interaction with GATA-1 and SOX6 that binds the proximal γ globin promoters (Xu et al. 2010, 2013). In a proof-of-principle, *bcl11a* knock-out in sickle mice increased HbF up to 30 %, reversing end-organ damage caused by the SCD (Xu et al. 2011).

HMIP on Chromosome 6q23

High resolution genetic mapping and resequencing refined the 6q QTL to a group of variants in tight linkage disequilibrium (LD) in a 24-kb block between the *HBS1L* and *MYB* gene, referred to as *HMIP-2* (Thein et al. 2007). The causal SNPs are likely to reside in two clusters within the block, at −84 and −71 kb respectively, upstream

of *MYB* (Stadhouders et al. 2014; Menzel et al. 2014). Functional studies in transgenic mice and primary human erythroid cells provide overwhelming evidence that the SNPs at these two regions disrupt binding of key erythroid enhancers affecting long-range interactions with *MYB* and *MYB* expression, providing a functional explanation for the genetic association of the 6q *HBS1L-MYB* intergenic region with HbF and F cell levels (Stadhouders et al. 2012, 2014; Suzuki et al. 2013). A three-base pair (3-bp) deletion in *HMIP-2* -84 region is one functional element in the *MYB* enhancers accounting for increased HbF expression in individuals who have the sentinel SNP rs9399137 that was found to be common in European and Asian populations, although less frequently in African-derived populations (Farrell et al. 2011).

The *HBS1L-MYB* intergenic enhancers do not appear to affect expression of *HBS1L*, the other flanking gene (Stadhouders et al. 2014). *HBS1L* was excluded as having a role in the regulation of HbF and erythropoiesis in a recent report of rare uncharacterized disorders, where whole-exome sequencing revealed mutations in the *HBS1L* gene leading to a loss-of-function in the gene (Sankaran et al. 2013). The individual had normal blood counts and normal HbF levels. Thus, *HMIP-2* is likely to affect HbF and hematopoietic traits via regulation of *MYB. MYB* was also causally implicated by fine-mapping which identified rare missense *MYB* variants associated with HbF production (Galarneau et al. 2010).

MYB expression is also reduced by GATA-1 (Welch et al. 2004) and micro (mi) RNA-15a and -16-1 (Sankaran et al. 2011). Elevated levels of the latter have been proposed as the mechanism for the persistently elevated HbF levels, one of the unique features in infants with trisomy 13 (Huehns et al. 1964). These infants have increased expression of miRNAs 15a and 16-1 produced from an extra copy of the genes encoding miRNAs 15a and 16-1 on the triplicated chromosome 13. A recent study provided evidence that the increased HbF effect is mediated, at least in part, through down-modulation of *MYB* via targeting of its 3′ UTR by the miRNAs 15a and 16-1 (Sankaran et al. 2011).

The *MYB* transcription factor is a key regulator of erythropoiesis, and modulates HbF expression via two mechanisms: (1) indirectly through alteration of the kinetics of erythroid differentiation: low MYB levels accelerate erythroid differentiation leading to release of early erythroid progenitor cells that are still synthesizing predominantly HbF (Jiang et al. 2006), and (2) directly via activation of *KLF1* and other γ-globin repressors (e.g., nuclear receptors TR2/TR4) (Bianchi et al. 2010; Suzuki et al. 2013; Tallack and Perkins 2013).

Modulation of *MYB* expression also provides a functional explanation for the pleiotropic effect of the *HMIP-2* SNPs with other erythroid traits such as red cell count, MCV, MCH, HbA$_2$ levels, and also with platelet and monocyte counts (Menzel et al. 2007b, 2013; Soranzo et al. 2009; van der Harst et al. 2012).

KLF1 on Chromosome 19p13

KLF1 (previously termed *EKLF*), discovered by Jim Bieker in 1993 (Miller and Bieker 1993), re-emerged as a key transcription factor controlling HbF through genetic studies in a Maltese family with β-thalassemia and hereditary persistence of HbF (HPFH). Linkage studies identified a locus for the HPFH that segregated

independently of the *HBB* locus on chromosome 19p13 which encompassed *KLF1* (Borg et al. 2010). Subsequent studies, which included expression profiling of erythroid progenitor cells, confirmed *KLF1* as the γ-globin gene modifier in this family. Family members with HPFH were heterozygous for the nonsense K288X mutation in *KLF1* that disrupted the DNA-binding domain of KLF1, a key erythroid gene regulator. Collective studies have now confirmed that *KLF1* is key in the switch from *HBG* to *HBB* expression; it not only activates *HBB* directly, providing a competitive edge, but also silences the γ-globin genes indirectly via activation of *BCL11A* (Siatecka and Bieker 2011; Zhou et al. 2010; Esteghamat et al. 2013). KLF1 may also play a role in the silencing of embryonic globin gene expression (Viprakasit et al. 2014; Magor et al. 2015).

Although there have been numerous reports of association of *KLF1* variants with increased HbF either as a primary phenotype, or in association with other red cell disorders (Borg et al. 2011), several GWASs of HbF (including ones in SCD patients of African descent) failed to identify common variants (Bhatnagar et al. 2011; Mtatiro et al. 2014).

The emerging network of HbF regulation also includes *SOX6*, chromatin-modeling factor *FOP* and the *NURD* complex, the orphan nuclear receptors TR2/TR4 (part of DRED) and the protein arginine methyltransferase PRMT5, involving DNA methylation and histone deacetylases 1 and 2 epigenetic modifiers. Regulators of the key transcription factors, such as miRNA-15a and 16-1 in *controlling MYB*, could also have a potential role in regulating HbF levels (Suzuki et al. 2014).

15.4.2 *Glucose-6-Phosphate Dehydrogenase Deficiency*

Glucose-6-phosphate dehydrogenase deficiency (G6PD) is common in patients with SCD of African ancestry (Bouanga et al. 1998). There is controversy about the effects of G6PD on TCD velocities, a biomarker for stroke risk in SCD; some studies report that G6PD increases the risk for high cerebral blood flow velocities (Bernaudin et al. 2008; Thangarajh et al. 2012) but others observed no effects (Rees et al. 2009; Cox et al. 2014; Flanagan et al. 2011). These conflicting reports could be related to the methodology used in the assay of the enzyme, or the panel of *G6PD* variants genotyped (Thangarajh et al. 2012; Flanagan et al. 2011). An earlier study showed that G6PD deficiency did *not* influence SCD clinical endpoints including survival, Hb levels, hemolysis, rate of acute pain or acute anemic episodes (Steinberg et al. 1988).

Genetic Modifiers of Organ-Specific Complications

The striking phenomenon in SCD is its clinical diversity. Multiple complications are common in SCD, both acute (frequent pain episodes, acute chest syndrome, strokes) and chronic (pulmonary hypertension, sickle nephropathy, gallstones,

osteonecrosis). The variation in global severity of the disease, as well as the incidence of specific end-organ complications ("sub-phenotypes") in SCD, cannot be explained by these three major genetic modifiers—causative sickle genotype, HbF level and α-globin genotype—alone. While the primary etiology in SCD is HbS polymerization, multiple different (but inter-related) downstream pathological mechanisms contribute to SCD phenotype: hemolysis/heme damage, inflammation, oxidant injury, nitric oxide biology, vaso-regulation, cell adhesion and blood coagulation. These factors have modifying effects independent of HbS polymerization and are likely be multi-genic traits. All of these downstream pathways suggest candidate genes that could plausibly affect the different sickle-related complications. Based on this pathophysiology, researchers have identified candidate genes for ***gene association studies*** related to specific sickle complications or "sub-phenotypes".

Genetic association studies (both candidate gene studies and GWAS) have identified multiple possible genetic associations with SCD complications (Table 15.1).

Acute Pain Episodes

Acute pain episodes (APE) are the hallmark clinical feature in SCD. They are a measure of disease severity and a predictor of early mortality (Platt et al. 1991). Frequency of APE varies widely in SCD patients, with highest pain rates seen in those with high hematocrit and low HbF (Platt et al. 1991). Outwith these associations, there is no concrete further understanding of the genetic basis of APE frequency in SCD. It is probably the complication most affected by environmental factors. A compounding problem with pain studies is the clinical definitions of phenotypes. Nearly all patients with SCD have pain, and it is often difficult to quantitate objectively both frequency and severity of individual APE. Furthermore, the standard treatment for pain in APE is parenteral opioids, and individual response to opioid analgesia is itself related to genetic variability of their metabolism (Ballas 2007), making it harder still to dissect and measure APE accurately. As a result of these complicating features, many genetic studies on pain in SCD are poor, in particular because of lack of clear-cut definitions of *cases* versus *controls* required to make objective associations. Furthermore, some of the studies described are poorly conducted and not corrected for other key modifying factors including genotype and HbF levels. In African American patients and patients from Cameroon, association of HbF with the 3 loci (*BCL11A*, *HBS1L-MYB*, and *Xmn*I-*HBG2*) was accompanied by a corresponding reduction in APEs and hospitalization (Lettre et al. 2008; Wonkam et al. 2014).

Published studies have chosen candidate genes based on APE pathology, itself a complex event involving: red cell deformation, enhancement of white cell adhesion, inflammation, endothelial injury and activation of the coagulation and complement pathways. Examples of studies relating to APE in SCD include genes related to:

- *Oxidative stress*. SCD complications, and notably APE, are associated with oxidative stress. Glutathione S-transferases (GSTs) are a group of enzymes that

Table 15.1 Reported genetic associations with specific SCD sub-phenotypes

SCD sub-phenotype	Gene	References
Acute pain episodes	GSTM1 null genotype	Shiba et al. (2014)
Stroke	*VCAM1*/G1238C	Taylor et al. (2002)
	VCAM1/T1594C	Hoppe et al. (2004)
	IL4R/S503P	Hoppe et al. (2004)
	TNFA/G-308S	Hoppe et al. (2004)
	TNF-α/-308G>A allele	Belisario et al. (2015)
	LDLR/Ncol +/−	Hoppe et al. (2004)
	ADRB2/Q/27E	Hoppe et al. (2004)
	AGT/AG repeats	Tang et al. (2001)
	HLA genes	Styles et al. (2000) and Hoppe et al. (2003)
Osteonecrosis	*MTHFR*/C677T	Zimmerman and Ware (1998)
	IL-1β (-511C>T and +3954C>T)	Vicari et al. (2015)
	BMP6	Baldwin et al. (2005) and Ulug et al. (2009)
Acute chest syndrome	*NOS3*/T-786C	Sharan et al. (2004)
	NOS1/AAT repeats	Sullivan et al. (2001)
	COMMD7	Galarneau et al. (2013)
	HMOX1	Bean et al. (2012)
Gallstones	*UGT1A*/promoter repeats	Passon et al. (2001), Fertrin et al. (2003), and Vasavda et al. (2007)
Priapism	*KL*	Nolan et al. (2005) and Elliott et al. (2007)
Pulmonary hypertension	TGFβ/BMP pathway genes	Ashley-Koch et al. (2008)
	IL-1β (-511C>T and +3954C>T)	Vicari et al. (2015)
	MAPK8 A allele	Zhang et al. (2014)
	eNOS intron 4 VNTR polymorphism	Tantawy et al. (2015)
Leg ulcers	*KL*, *TEK*, TGFβ/BMP pathway genes	Nolan et al. (2006)
	IL-6 (-597G>A and -174G>C) genes	Vicari et al. (2015)
Bacteraemia	TGFβ/BMP pathway genes	Adewoye et al. (2006)
Renal disease	*APOL1*	Ashley-Koch et al. (2011)
Retinopathy	IL-6 (-597G>A and -174G>C) genes	Vicari et al. (2015)
Splenic sequestration	TNFA/-308G>A	Cajado et al. (2011)
	IL-8/-251A>T	Cajado et al. (2011)

Acute pain studies have not been included due to poor quality of the studies

protect against oxidative stress. Shiba found the *GSTM1* null genotype to be associated with increased risk of severe APE in Egyptian SCD patients (Shiba et al. 2014)

- *Vasculopathy*. Vascular endothelial growth factors (VEGF) are known to contribute to the pathogenesis of APE in SCD. A study in Bahrain associated multiple VEGF gene polymorphisms with the risk of APE (Al-Habboubi et al. 2012). Unfortunately, the differences between cases and controls was not clear cut (compared patients with SCD having had a recent APE or not).

- *Thrombosis*. Cystathionine beta-synthase (CBS) enzyme gene mutations are a risk factor for thromboembolic disorders. CBS 844ins68 was three times more frequent among SCD patients with APE (Alves Jacob et al. 2011). Again, there was poor clarification of the difference between "severe" and "mild" individuals with APE.

- *Infections. MBL2* codes for mannose-binding lectin (MBL), and is associated with modifications in the progression of infectious and inflammatory vascular diseases. Using better definitions of APE severity (using APE frequency), *MBL2* polymorphisms have been associated with APE in children with SCD (Oliveira et al. 2009; Mendonça et al. 2010). Unexpectedly, studies have observed no association of *MBL2* variants with susceptibility to infections (Oliveira et al. 2009) (Dossou-Yovo et al. 2009).

Gallstones

Jaundice and a predisposition to gallstones is associated with a variant in the promoter (TA repeats) of uridine diphosphate (UDP)-glucuronosyl-transferase 1A (*UGT1A1*), also referred to as Gilbert's syndrome. Co-inheritance of Gilbert's syndrome with SCD has been shown in multiple populations to increase the risk for developing gallstones (Passon et al. 2001; Fertrin et al. 2003; Vasavda et al. 2007). The influence of *UGT1A1* polymorphism became more evident in patients while on hydroxycarbamide therapy; children with 6/6 *UGT1A1* genotype achieved normal bilirubin levels while children with 6/7 or 7/7 *UGT1A1* genotypes did not (Heeney et al. 2003).

The association of Gilbert's syndrome with gallstones has also been validated in other populations with different hemolytic anemias e.g. hereditary spherocytosis (del Giudice et al. 1999), HbE/β-thalassemia (Premawardhena et al. 2001) and β-thalassemia (Galanello et al. 2001). Thus, the association of *UGT1A1* polymorphisms and gallstones in SCD is a well-replicated phenomenon. GWAS also confirmed the association between bilirubin level and *UGT1A1* polymorphism in SCD (Milton et al. 2012).

The triad of Gilbert's syndrome, SCD and gallstones presents a possible clinical context where genetic information may aid clinical decision-making. More widely in SCD, the role of elective cholecystectomy in asymptomatic gallstones

remains controversial. While one study of SCD patients with asymptomatic gallstones showed significant increased morbidity in patients who were not electively cholecystectomized and subsequently had a symptomatic cholecystectomy (Curro et al. 2007), another study of SCD patients with gallstones demonstrated that the large majority remained asymptomatic over a 13-year follow up period (Attalla et al. 2013).

Thus, the addition of the (*UGT1A1*) genotype to the clinical phenotype of gallstones in SCD presents the question of whether these patients should have elective cholecystectomy.

Sickle Nephropathy

Renal impairment as measured by either proteinuria or glomerular filtration rate (GFR) are common complications of SCD (Sharpe and Thein 2014; Nath and Hebbel 2015), and in some cases sickle renal disease progresses to end-stage renal failure. Renal dysfunction is associated with severity of hemolysis (Becton et al. 2010; Maier-Redelsperger et al. 2010; Day et al. 2012). As a result of this, co-inheritance of α-thalassemia is protective against albuminuria (Nebor et al. 2010a).

The *MYH9-APOL1* locus, an important genetic risk factor for end-stage renal failure in non-SCD populations of African ancestry (Genovese et al. 2010), has also been shown to be associated with sickle cell nephropathy (Ashley-Koch et al. 2011). It is broadly considered that the true association is with *APOL1*, due both to the stronger statistical association with that gene and the lack of identification of causal functional variants in *MYH9*. The original association with *MYH9* has been attributed to the strong linkage disequilibrium between *MYH9* and *APOL1*.

Stroke

A familial predisposition to stroke in HbSS SCD was first identified by Driscoll et al. (2003). This has prompted numerous gene association studies where a variety of associations have been established between multiple genes and stroke—*VCAM1*/G1238C, *VCAM1*/T1594C, *IL4R*/S503P, *TNFA*/G-308S, TNF-α/-308G>A allele, *LDLR*/Ncol +/, *ADRB2*/Q/27E, *AGT*/AG repeats, HLA genes (Hoppe et al. 2004; Taylor et al. 2002; Belisario et al. 2015; Tang et al. 2001; Styles et al. 2000). In some studies, stroke was subdivided into large and small vessel disease based on imaging studies (Hoppe et al. 2004). Of the 38 published SNPs associated with stroke, the effects of α-thalassemia and SNPs in four genes (*ADYC9*, *ANXA2*, *TEK* and *TGFBR3*) could be replicated, although only nominally significant association results were obtained (Flanagan et al. 2011). More recently, GWAS in combination with whole exome sequencing have identified mutations in two genes—*GOLGB1* and *ENPP1*—associated with reduced stroke risk in pediatric patients but, again, this needs validation in independent studies (Flanagan et al. 2013).

Priapism

In males, priapism remains a common manifestation of SCD, found in about 35 % of men (Adeyoju et al. 2002). Independent association studies have identified KLOTHO (*KL*) with priapism in different populations (Nolan et al. 2005; Elliott et al. 2007). Separately, the *TGFβ*/*SMAD* pathway has also been implicated in priapism risk (Elliott et al. 2007).

Osteonecrosis

Osteonecrosis (avascular necrosis of the bone) occurs in about half of all adults in HbSS. Higher hematocrits are a predisposing factor, hence an increased incidence in HbSS patients with co-existing α-thalassemia, and patients with HbSC and HbSβ⁺ thalassemia genotypes. Association with bone morphogenic protein 6 (*BMP6*) have been replicated across populations (Baldwin et al. 2005; Ulug et al. 2009). This relates to TGF-β/SMAD/BMP pathway in bone metabolism. As for BMP6, regulating the activity of the TGF-β pathway to modulate its effects on bone may be possible (Callahan et al. 2002). Studies suggesting that factors in the coagulation pathway may be involved, such as *MTHFR* and platelet adhesion (*HPA-5B* allele), have been inconclusive (Castro et al. 2004; Zimmerman and Ware 1998; Galanello et al. 2001; Kutlar et al. 2001; Andrade et al. 1998).

Leg Ulcers

Leg ulceration varies widely in SCD with much higher prevalence in Jamaican patients than other cohorts (Alexander et al. 2004). This complication is closely associated with hemolysis severity, and therefore co-existing α-thalassemia is protective. Genetic association studies have implicated several genes in the TGF-β/ SMAD/BMP pathway (Nolan et al. 2006). Duffy antigen receptor for chemokines (*DARC*) has also been shown to be associated with persistence of leg ulcers. It was suggested that the relatively higher white cell and neutrophil counts potentiate inflammation in the *Duffy* positive patients (Drasar et al. 2013).

Pulmonary Hypertension

Pulmonary hypertension has been defined in SCD studies using echocardiography, with tricuspid regurgitant jet velocity >2.5 m/s when right heart catheterization is unavailable (when it is defined as mean pulmonary artery pressure ≥25 mmHg and pulmonary capillary wedge pressure ≤15 mmHg). A tricuspid regurgitant jet (TRJ) velocity of >2.5 m/s occurs in about 30 % adults with SCD and is a risk factor for premature death (Gladwin et al. 2004). Pulmonary hypertension is associated with "hemolytic" sickle-complications—renal dysfunction, leg ulceration

and priapism—which suggests a vasculopathy driven by chronic hemolysis underlies pulmonary hypertension, too (Taylor et al. 2008). Association studies have suggested multiple gene associations including: the *TGF-β/BMP* signalling pathway (*ACVRL1*, *BMPR2* and *BMP6*) (Ashley-Koch et al. 2008) and polymorphisms previously implicated in primary idiopathic pulmonary hypertension (Machado et al. 2001).

A more recent multi-center study (Zhang et al. 2014) considered the hypoxic response as contributory to pulmonary hypertension. To identify genes regulated by the hypoxic response and not other effects of chronic anemia, individuals with SCD were compared with patients with Chuvash polycythemia (constitutive upregulation of hypoxia-inducible factors in the absence of anemia or hypoxia). A SNP associated with reduced *MAPK8* expression (encoding a mitogen-activated protein kinase important for apoptosis, T-cell differentiation, and inflammatory responses), correlated with pulmonary hypertension. The association was further confirmed in an independent cohort (Walk-Treatment of Pulmonary Hypertension and Sickle Cell Disease With Sildenafil Therapy (walk-PHaSST) population). The homozygous AA genotype of *rs10857560* was present in all 14 patients with pulmonary hypertension.

Acute Chest Syndrome

Acute chest syndrome (ACS) represents a severe acute manifestation of SCD that is potentially life-threatening. One study showed increased susceptibility to ACS associated with a SNP in endothelial NO synthase gene (*eNOS* or *NOS3*) (Sharan et al. 2004), albeit in female patients only. Separately, low exhaled nitric oxide and a polymorphism in the *NOS1* gene has been implicated in ACS (Sullivan et al. 2001).

Galarneau et al. (2013) performed a gene-centric association study for ACS with individuals from the Cooperative Study of Sickle Cell Disease (CSSCD), with replication in independent cohorts. In the combined analysis, an association was found between ACS and *rs6141803*. This SNP is located 8.2 kb upstream of *COMMD7*, a gene highly expressed in the lung that interacts with nuclear factor-κB signalling.

Another candidate gene is Heme oxygenase-1 (*HMOX1*) which produces the protein HO-1, the rate-limiting enzyme in the catabolism of heme; *HMOX1* might attenuate the severity of APE and hemolysis. Bean et al. (2012) investigated a highly polymorphic (GT)n dinucleotide repeat in the promoter of *HMOX1* and showed that children with two shorter alleles had lower rates of ACS.

Splenic Sequestration

Cajado et al. (2011) identified an association between inflammatory markers TNF-α and IL-8 and splenic sequestration in children with SCD. Specifically, the A allele of the TNF-α -308G>A gene polymorphism was associated with an increased risk

of splenic sequestration; and the T allele of the IL-8 -251A>T gene polymorphism was considered to be a protective factor for splenomegaly.

Infection

Infections are common events in SCD, especially in children. Studies have suggested that the incidence may be modulated by polymorphisms in the *HLA* locus, *MBL2* gene which encodes the mannose binding protein, *MPO* (gene encoding myeloperoxidase), Duffy antigen receptor for chemokines (*DARC*) and *TGF*-β/*BMP* pathway (*BMP6*, *TGFBR3*, *BMPR1A*, *SMAD6* and *SMAD3*) (Costa et al. 2005; Nebor et al. 2010b; Tamouza et al. 2002, 2007; Neonato et al. 1999; Cordero et al. 2009; Al-Ola et al. 2008; Adewoye et al. 2006).

Variable Response to Hydroxycarbamide Therapy

Hydroxycarbamide remains a major treatment option for SCD (Ware 2010; Yawn et al. 2014; National Institutes of Health: National Heart Lung and Blood Institute 2014). Clinical and laboratory response to hydroxycarbamide therapy however, is variable, a main determinant of response appears to be the baseline HbF level. Numerous association studies on HbF response to hydroxycarbamide have been reported, of which the association with baseline HbF levels and *Xmn*1-*HBG2* seems to be the most robust (Ware et al. 2002; Green et al. 2013).

15.5   Conclusion

Although environmental factors are important in determining the clinical outcome of SCD, it is evident that the genetic background of the affected individual imparts a substantial contribution to the clinical severity and response to medication. The attraction of being able to generate a personalized genetic risk score as prognostic marker, and to guide therapeutics, plus the relative ease of genotyping and reducing costs, has been a major driver underlying the recent output of genetic association studies in SCD. But the results are questionable in the majority of these genetic association studies because of lack of replication. Nonetheless, genetic studies have been successful in characterizing some of the key variants and pathways involved in HbF regulation, providing new therapeutic targets for HbF reactivation.

We must continue the quest to discover key modifier genes of SCD as a major research priority. This requires taking advantage of whole genome sequencing and the new genomic platforms, but much larger sample sizes (and therefore multi-center collaborations) are required to tease out small statistical differences. Care must be taken to consider, and appropriately classify, different ethnicities.

Additionally, we must focus on developing rigorous clinical phenotypes and the importance of identification of "cases" and "controls". Clinical researchers need to address the issue of defining and quantifying global sickle severity, as well as precise sub-phenotype definitions. As well as using clinical end points (stroke), it may be useful to use intermediate end points (trans-cranial Doppler velocities) with association studies. Many of the described association studies have highlighted the importance of identification of "cases" and "controls".

Finally, for those variants already identified, we must endeavour to: validate the variants in independent, large populations; identify the causal variants; support the genetic evidence by functional assays or relevant models to uncover the underlying pathogenesis. Understanding of the underlying mechanisms may guide translation of these genetic discoveries into clinical benefit as targeted, novel therapies.

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