Minireview

Genetic basis of heterogeneity and severity in sickle cell disease

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Abstract

Sickle cell disease, a common single gene disorder, has a complex pathophysiology that at its root is initiated by the polymerization of deoxy sickle hemoglobin. Sickle vasoocclusion and hemolytic anemia drive the development of disease complications. In this review, we focus on the genetic modifiers of disease heterogeneity. The phenotypic heterogeneity of disease is only partially explained by genetic variability of fetal hemoglobin gene expression and co-inheritance of α thalassemia. Given the complexity of pathophysiology, many different definitions of severity are possible complicating a full understanding of its genetic foundation. The pathophysiological complexity and the interlocking nature of the biological processes underpinning disease severity are becoming better understood. Nevertheless, useful genetic signatures of severity, regardless of how this is defined, are insufficiently developed to be used for treatment decisions and for counseling.

Keywords: Severity in sickle cell disease, subphenotypes of sickle cell disease, single nucleotide polymorphisms, genome-wide association study, hemolysis, genotype–phenotype correlation

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Introduction

Sickle cell disease, one of the world's commonest single gene disorders, was first described by Herrick in 1910, who linked his patient's symptoms to abnormally shaped erythrocytes in the blood. Pauling and colleagues in 1949 detected abnormal hemoglobin, that has subsequently called sickle hemoglobin (HbS), and was shown by Ingram to contain a valine residue in place of glutamic acid as the 6th amino acid of the β-hemoglobin chain; the mutation was subsequently confirmed as G*A*G to G*T*G in codon 6 (rs334).[1,2]

An understanding of pathophysiology is a prerequisite to appreciating and measuring disease severity. One interpretation of the phenotype of sickle cell disease dichotomizes its pathophysiology into two interrelated branches (Table 1): viscosity-vasoocclusion (Figure 1); hemolysis-endothelial dysfunction (Figure 2). The events depicted within these branches occur simultaneously and neither branch should be considered in isolation from the other. Solely for the purpose of edifying the disease pathophysiology and envisaging the mechanistic basis of disease complications, sickle vasoocclusion and hemolytic anemia can be thought of as discrete pathophysiological entities. Each of these pathophysiologic branches has been associated with certain clinical features.[3] Although clearly an oversimplification of the pathophysiology of sickle cell disease—one also subjected to some criticism—this scheme is useful for understanding the pathobiology, estimating the severity of disease and useful for considering how certain targeted treatments might affect one but not the other pathway. Recent studies have further validated the role of hemolytic anemia as a driver of some pathophysiologic features of sickle cell and other hemolytic anemias.[6–11] As an example of the importance of carefully considering pathophysiology when formulating treatment, a Phase 3 clinical trial focused on reducing the frequency of sickle vasoocclusive events; however, the drug, a Gardos channel inhibitor, was known to ease hemolysis by reducing sickle erythrocyte density. The drug, a cation channel inhibitor, had the anticipated effect of increasing hemoglobin level. A higher hemoglobin level causes increased blood viscosity, unless the additional red cells have a high content of fetal hemoglobin (HbF) which they did not. The primary endpoint of the trial, sickle vasoocclusion, did not improve and might even have worsened, perhaps because of the increased hemoglobin levels. The trial was prematurely terminated.[12]

Sickle cell disease is composed of diverse genotypes (Table 2). Sickle cell anemia denotes homozygosity for the HbS mutation and is the most common, most clinically apparent and best studied form of this disease. Nearly all genetic studies of sickle cell disease have concentrated on this β-globin genotype. Other genotypes of sickle cell

Table 1 Clinical and laboratory features of the vasoocclusive and hemolytic subphenotypes of sickle cell disease.[3–5]

	Viscosity-vasoocclusion	Hemolysis-endothelial dysfunction
Laboratory	Lower LDH Higher PCV	Higher LDH Lower PCV
HbF	High HbF protective	HbF less protective
α Thalassemia	Absence of α thalassemia associated with increased viscosity	α thalassemia protective by reducing MCHC, erythrocyte density and hemolysis
Common clinical complications	1. Acute painful episodes. 2. Acute chest syndrome 3. Osteonecrosis.	1. Leg ulcer 2. Pulmonary hypertension 3. Priapism. 4. Stroke

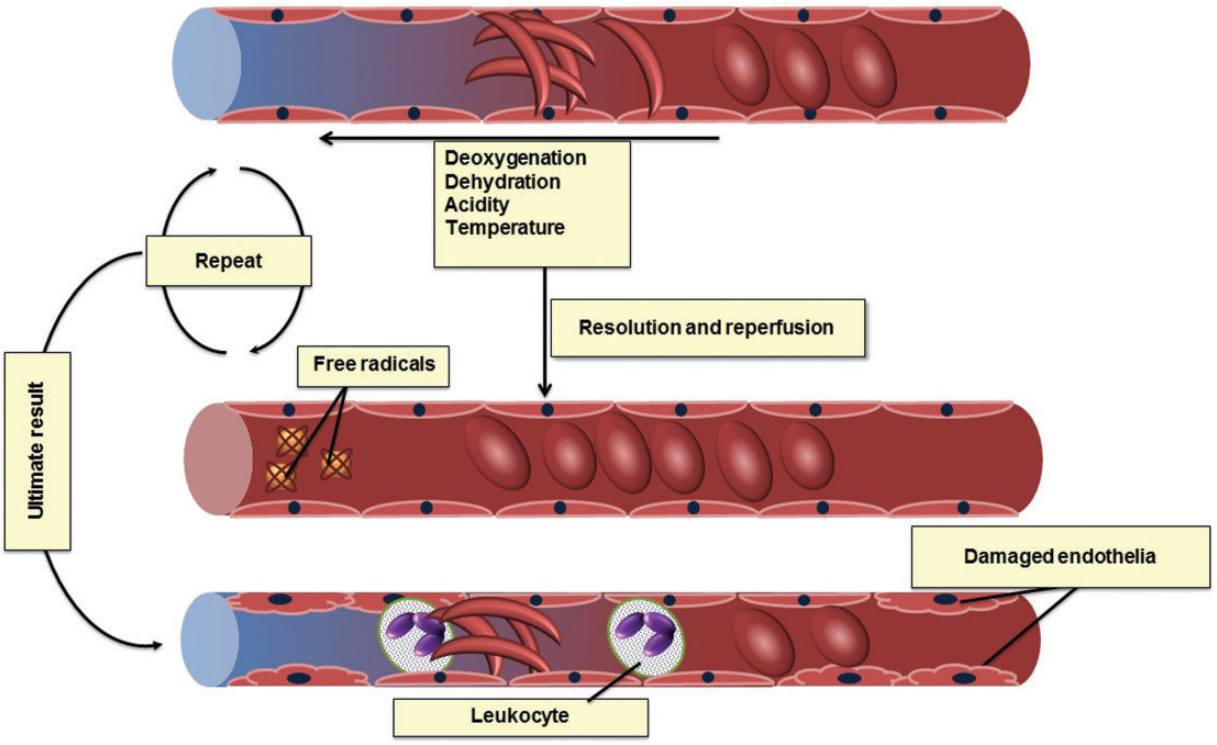

Figure 1 Mechanism of sickle vasoocclusion. Erythrocyte damage and deformation (sickling) occur as a result of polymerization of deoxyHbS and also high concentrations of unpolymerized oxidized HbS, modulated by cellular levels of HbF, erythrocyte cation and water content, pH, temperature, and mechanical stresses that result in membrane damage and eventual failure. Hemolytic anemia and vasoocclusion cause tissue hypoxia. When this occlusion resolved and perfusion is established in the hypoxic tissue, free radicals are produced. These free radicals cause damage to the endothelia making them sticky for RBCs and also for leucocytes. The vascular wall ultimately becomes more vulnerable to occlusion (A color version of this figure is available in the online journal)

disease are due to compound heterozygosity for HbS and other hemoglobin variants like HbC, HbE, and HbD, or the many different genotypes of HbS-β thalassemia. These disorders, with the exception of HbS-β^0 thalassemia, are when considering each genotype, usually less clinically severe than sickle cell anemia. Nevertheless, within each genotype there is great clinical and hematological heterogeneity that is poorly explained by the effects of the other variant hemoglobin. Usually, HbS-β^+ thalassemia is a milder disorder than HbS-β^0 thalassemia but this depends in part on the concentration of HbA found in HbS-β^+ thalassemia. When assessing severity, all sickle cell genotypes should not be lumped together. Only a single genotype should be included in the analysis, otherwise severity differences are likely to be due to the clinical differences among sickle hemoglobinopathies. The clinical and laboratory features of the sickling hemoglobinopathies have been characterized in detail and some of the common complications are shown in Table 3.[1,13]

Genetic association studies

Sickle cell anemia is a prototypical monogenic Mendelian disease but one notorious for its clinical heterogeneity. Some patients are constantly ill and display most of the clinical and laboratory subphenotypes of this disease;

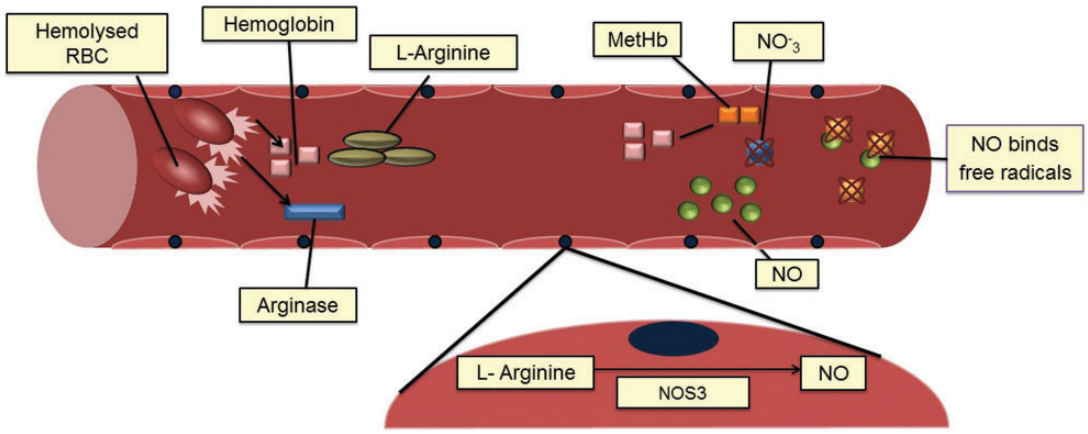

Figure 2 Hemolysis-endothelial dysfunction. With intravascular hemolysis, erythrocytes release hemoglobin and arginase. Arginine is the precursor for NO production by the endothelium via NOS3. Arginase degrades L-arginine, the NOS3 substrate causing reduced NO production. Free plasma hemoglobin interacts with NO producing methemoglobin and nitrate depleting NO. These mechanisms occur during steady state and the amount of intravascular hemolysis varies among patients. An additional mechanism which occurs commonly during vasoocclusive episodes relates to free radicals production with the oxidation of NO. NO depletions will disturb the vasodilatorvasoconstrictor balance, ultimately leading to vasoconstriction of the blood vessels which will complicate the VOC further (A color version of this figure is available in the online journal)

Table 2 Clinical and hematological features of the most common genotypes of sickle cell disease

Genotype	Incidence in African Americans	PCV	Retic	MCV	HbF	% Variant	Severity
Sickle cell anemia (HbS homozygotes)	1/600	25	8	90	5	>90% HbS	4
HbSC disease	1/800	35	3	80	2	50% HbS and HbC	2
S-β^0 thalassemia	1/1500	27	7	82	7	90% HbS	4
S-β^+ thalassemia		38	2	70	2	5%-30% HbA	2
HbSE disease	rare	35	3	75	2	~30% HbE	2
Sickle cell anemia-α thalassemia	30% of all cases	30	6	78	5	>90% HbS	3

Note: Shown are average findings for a young adult for each genotype, in the absence of transfusion or hydroxyurea treatment. Findings in young children will differ. Within any genotype of sickle cell disease, results in an individual patient can vary widely. Severity of disease, rated from most severe (4) to absence of clinical events (0) includes complications related to sickle vasoocclusion and hemolysis. Retic = reticulocyte count. Sickle cell trait is not included among sickle hemoglobinopathies because of its benignity although some complications are well known (1).

others have few overt signs and symptoms. However, it is rare for an affected individual to escape the consequences of HbS polymerization that drives sickle vasoocclusion and the hemolytic anemia that continues relentlessly even when acute events are quiescent. In developed countries, the environment might explain some of the observed heterogeneity of disease but most often, genetic variation among patients, intrinsic, and extrinsic to the sickle erythrocyte, is more likely to account for the bulk of their phenotypic differences.[19] The discovery of this genetic variation is the subject of genotype–phenotype association studies.

Genotype–phenotype association studies require a precise definition of the phenotype. This must be applicable to more than a single patient clinic cohort as individual clinics see small numbers of patients relative to the sample size needed for these studies. Phenotypes that can be expressed as quantitative traits, like HbF, have yielded the most robust and reproducible results. In contrast, sickle cell anemia subphenotypes like disease severity, acute chest syndrome or pain, which do not have well-defined heritability, have yet to be associated with consistent replication of the results of genetic association studies. This does not imply that these and other subphenotypes of disease are not genetically modulated. Many common quantitative traits like height and weight have been associated with dozens of quantitative trait loci or QTL, each one contributing a very small proportion of the variance of the trait. In many studies, the amount of variance explained is less than 10%. To find these associations, tens or hundreds of thousands of people must be studied, a near impossibility in a rare disease like sickle cell anemia. In the absence of assembling cohorts of many thousands of well-phenotyped cases, it is highly unlikely that additional variants with small effects on a subphenotype like acute chest syndrome or sickle cell pain, the most common disease complications, will be found. Even less likely to be discovered are genetic variants modulating the more rare common complications of disease where it is even more difficult to find sufficient numbers of cases to compare with controls.

The relationships between a genetic variant and a disease phenotype is ascertained by comparing the odds of a selected phenotype occurring in carriers of a genetic variant compared with those who do not carry the variant. In most sickle cell disease phenotypes, the definition of cases and controls can be confounded by age. For example, vasoocclusive stroke predominates in children and one might reasonably capture the majority of these strokes by studying adolescents and young adult case and control groups.

Table 3 Some common complications of sickle cell anemia

Complication	Comment
Hyposthenuria	Present in sickle cell trait. Without access to water can lead to dehydration.
Renal Papillary necrosis	Medullary hypertonicity and loss of vasculature. Sometimes NSAID related.[14]
Acute painful episodes	Most common complication in sickle cell disease.
Acute chest syndrome	Second most common complication. Accounts for about 25% of deaths. Often present with or after a painful episode.[15,16]
Pulmonary hypertension	Effect about 30% of patients with sickle cell disease and a major risk factor for near term death.[17]
Priapism	Recurrent ischemic priapism can lead erectile dysfunction.
Leg ulceration	The most common site is the lateral and medial malleoli.[18]
Osteonecrosis	Most common site is the femoral head.

In contrast, the incidence of leg ulcers increases with aging so that young controls might ultimately become cases and confound the distinction of case and control groups. Prerequisites for genetic association studies are evidence that the phenotype examined is heritable and that cases with the phenotype are distinct from controls lacking the phenotype. It is clear that HbF is a heritable trait and at least in the normal population stroke is at least partially a heritable trait; less clear is the heritability of acute painful episodes or acute chest syndrome.

Genetic association studies applied to sickle cell anemia have taken both focused candidate gene and agnostic genome-wide approaches. In the former, variants of candidate genes suspected of being associated with a phenotype and identified using prior knowledge are tested for their association with disease subphenotypes (Table 3). Candidate gene association studies have been criticized for their lack of robustness and replicability.[20] With some exceptions, validation of much of this work is weak.[20,21]

In genome-wide association studies (GWAS), hundreds of thousands and even millions of known genetic variants, usually single nucleotide polymorphisms (SNPs), are queried for the possible association of one or more variants with a subphenotype. Usually when an SNP is associated with a phenotype, the functional locus or a putative mechanism whereby the disease is modified by the variant is unknown. This is because most variants associated with a trait are found in non-coding DNA and are likely to have regulatory functions that can in many instances affect genes far removed from the variant and might even be on a different chromosome. GWAS have been used in sickle cell anemia to study the genetic associations of HbF, bilirubin, cholelithiasis, hemolysis, HbA_2 level, tricuspid regurgitation velocity, stroke, and systemic blood pressure. Genetic association studies are summarized in Table 4.

The results of GWAS of bilirubin and cholelithiasis were robust and identified the well-known *UGT1A* gene family as the major regulator of bilirubin metabolism in African Americans with sickle cell anemia, as it is in other ethnicities. Bilirubin levels increase with hemolysis, and the intensity of hemolytic anemia is a marker of severity in sickle cell disease. Another well-validated result was the association of an SNP in *NPRL3* with hemolysis. This result was initially found in a discovery cohort and the association remained after adjustment for HbF level and the presence of gene deletion α thalassemia. The original result was then replicated using both GWAS and targeted genotyping in three additional cohorts. The *HBA1/HBA2* regulatory elements, hypersensitive sites HS-33, HS-40, and HS-48 are located in introns of *NPRL3*. Perhaps by independently down-regulating expression of *HBA1/HBA2*, variants tagged by this *NPRL3* SNP reduce hemolysis.

GWAS have not validated any of the associations found in candidate gene association studies. This might be due to the stringency needed to accept an association by GWAS where the widely accepted level of significance is 10^{-7} to 10^{-8} allowing for stringent correction of multiple testing. Achieving this level of significance is usually difficult without many thousands of subjects or where the associated SNP has a large effect on the phenotype of interest. It is easy to imagine that complex subphenotypes like pain, acute chest syndrome, and survival are effected by many genes, each with a small effect on the subphenotype, so that very careful experimental design and perhaps a touch of luck are required to achieve a positive result.

Other approaches to mining GWAS data include pathway analysis to discover possible interconnectivity among genes that might influence a subphenotype, inform as to the possible functions of involved genes and help define underlying mechanisms.

As discussed above, genetic association studies rarely detect the functional variant accounting for the phenotype. Association analysis alone cannot substitute for functional and mechanistic studies that are the ultimate test of causation that is needed to validate potential therapeutic targets. Genetic association studies can be used as one means of improving prognosis or identifying regions likely to harbor functional variants.[27] The latter can then be examined functionally and identify promising therapeutic targets.[67–69]

Estimating severity of sickle cell anemia

Determining the severity of sickle cell anemia is difficult and many definitions of severity are possible. Any estimate of severity should control for HbF level, the presence of α thalassemia and age. Discrete clinical features of the disease and laboratory measurements have been used to gauge severity. Patients with HbF ≥8.6% survived longer than those with HbF <8.6%.[22] The tricuspid regurgitant velocity predicted near-term death.[22,70,71] Patients with high rates of pain had shorter survival. A score reflecting the intensity of hemolysis was associated with an increased risk of death.[71,4] To develop a more integrated estimate of severity, a Bayesian network modeled 24 clinical events and

Table 4 Genetic associations with subphenotypes of sickle cell anemia

Subphenotype	Genes	References
Survival	Multiple including *TGFBR3*	22,23–26
Stroke, silent infarction, TCD velocity	Multiple gene identified, *VCAM1, ILR4, ADBR2, HLA, LDLR*, but few have been validated (see text)	27,28–30
Painful episodes	*GCH1*-results reported in abstract only. Biologically plausible. *MBL2* in children, low expression associated with increased pain *PLA2G4A.*	31–34 35
Acute chest syndrome	Many genes have been ''identified'' but no study has been validated. *HMOX1* $(GT)_n$ S/S – Reduce incidence Intergenic region between *DNMT3B –COMMD7* significant in children <age 5 years.	36–39 40 35
Bacteremia/Infection	*MBL2*-contradictory evidence in different populations that that low level protective. Other genes include *CCL5, v*arious HLA alleles, *IGF1R,* TGF-β/SMAD/BMP pathway HLA-E*0101 HLA class 1 Susceptibility for infection in homozygotes HLA-E*0103 HLA class 1 Protection against infection in heterozygotes HLA-DRB1*15 HLA class 2 Protection HLA-DQB1*03 HLA class 2 Increased risk *IGF1*, TGFβ/BMP	41–43 44,41,45
Osteonecrosis	Little evidence for *MTHFR;BMP6*-results validated in 2 different populations	38,46–48
Priapism	*KL, TEK, TGFBR3, AQP1*	49–51
Leg ulcers	TGF-β/SMAD/BMP pathway, *KL*, possibly HLA alleles	51–53
Sickle vasculopathy/TRV velocity	*BMP6, TGFBR3, ACVR1, BMP2*	54
Cholelithiasis	Promoter repeats in *UGT1A1* associated with serum bilirubin	55–57
Renal function/albuminuria/ Glomerular hyperfiltration	*DARC* FY- associated with proteinuria, TGF-β/Smad/BMP pathway, *MYH9, APOL1*	58–61
Multiple subphenotypes	Duffy antigen receptor (*DARC*) No relationship to leg ulcers,? nephropathy, priapism, osteonecrosis, response to opioids	62–64
Hemolysis	*NPRL3 VCAM1, CD36* *NOS3*	65,66

S/S: homozygous for the short GT_n repeat allele for *HMOX1*

laboratory tests to estimate disease severity deriving a score that predicted two-year mortality.[72] In contrast to regression models, which can only represent the dependency of a single outcome variable on one or more predictor variables, a Bayesian network can represent the mutual and hierarchal relationships among many variables using probabilistic rules and thus, in many instances, is better suited to prognostic and diagnostic applications. The network can be used as an unbiased assessment of the clinical severity of patients with sickle cell disease, given any clinical and laboratory profile, and a simple web tool that is freely available has allowed the partial validation of the model in independent studies.[73]

Genetic basis of disease severity

HbF concentration and α thalassemia are the major genetic modifiers of disease but are unlikely to be the only ones.[74,75] They have been discussed extensively and will not be further examined in this review. Their effects on different aspects of disease severity have also been reviewed.

Genetic association studies have used some estimates of severity as phenotypes to understand the genetic basis of their pathophysiology. Using this score derived from Bayesian network modelling as a phenotype, GWAS discovered 40 SNPs that were strongly associated with severity but none met the accepted definition of genome-wide significance. Thirty-two of these SNPs could be analyzed in a small independent cohort but this attempt at replication was only partially successful. Among the replicated associations were SNPs in *KCNK6*, a potassium channel gene but not the Gardos channel that is active in sickle erythrocytes and responsible for the erythrocyte dehydration that impacts the pathophysiology of disease. Other implicated genes had an even more tenuous connection with severity.[21] While in the general population longevity is genetically modulated, survival in sickle cell anemia is likely to be driven by the adverse effects of the disease rather than longevity genes.

As a result of its inhibition of HbS polymerization, HbF is the major modulator of the clinical course of sickle cell anemia. HbF levels are highly heritable trait. The genetic basis of HbF gene regulation has been intensively studied and a substantial portion of the networks regulating HbF gene expression have been defined.[76] HbF has been

associated with protection from some of the complications of sickle cell anemia and in many studies has been associated with increased survival and a reduced incidence of some of the vasoocclusive complications of disease.[76–78] The genetic modulators of HbF, because of the major effect of HbF on HbS polymerization, have been associated with painful episodes of sickle cell anemia,[77,79] one determinant of survival.

Tricuspid regurgitant velocity and pulmonary hypertension are both associated with mortality in sickle cell disease.[70,80,81] Candidate genes associated with tricuspid regurgitant velocity in sickle cell anemia have been reported and some of these genes have been associated with idiopathic pulmonary hypertension in the general population (Table 4).

Sickle cell vascular disease, exemplified by pulmonary hypertension, has been closely linked to the intensity of intravascular hemolysis (Figure 2) and hemolytic anemia has been associated with survival.[4,82] *NPRL3* was associated with hemolysis, perhaps by down-regulating expression of *HBA1/HBA2*, and producing an α-thalassemia-like effect.

Conclusions

The genetic basis of clinical heterogeneity of sickle cell anemia is incompletely understood. In some regions of the world, the environmental impact on the course of disease is likely to be dominant.[83] Understanding the genetic basis of severity will not be simple given the pathophysiological complexity and interlocking nature of the biological processes culminating in a subphenotype. A good deal of work has been done to date but with insufficient progress to permit the use of this information prognostically or therapeutically. In addition to the biological discoveries made during genetic association studies—most vividly illustrated by the totally unexpected association of the role of *BCL11A* in HbF gene expression that has brought us to the cusp of a new approach to HbF induction—the rationale for these studies has been the use of their results for making treatment decisions and for patient counseling.[68,69] The most likely future outcome will be the validation of multiple genetic variants that modulate some of the common subphenotypes of disease. Perhaps with the advent of more careful phenotyping, international collaborations permitting the assembly of larger patient cohorts than possible previously, newer high-throughput genotyping methods, coupled with analytical techniques like Bayesian networks, it might be possible to derive genetic signatures of survival that can be clinically useful.

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DECLARATION OF CONFLICTING INTERESTS

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