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Established and experimental treatments for sickle cell disease

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A B S T R A C T

Sickle cell disease (SCD) is characterized by the presence of sickle hemoglobin (HbS), which has the unique property of polymerizing when deoxygenated. The sickling process is markedly accelerated when intracellular concentration of HbS is increased. Due to the unique dependence of HbS polymerization on its cell concentration, a slight reduction in HbS concentration is likely to have a beneficial effect on the kinetic of polymerization and on the generation of dense, dehydrated red cells. The pathophysiology of acute and chronic clinical manifestations of SCD is strictly related to the hemoglobin cyclic polymerization, to the generation of dense, dehydrated red cells and to the interaction between sickle red cells and abnormal activated vascular endothelial cells.

In the present paper we have reviewed the principal therapeutic strategies and we have explored the future treatment options for sickle cell disease. Therapy of sickle cell disease is based on two major goals. The first one is the decrease in intracellular HbS concentration obtained with agents activating fetal hemoglobin synthesis, such as hydroxyurea (HU) or with erythrocyte-active agents blocking different red cell membrane ion pathways and preventing sickle cell dehydration. The second one is based on therapeutic strategies, which may reduce sickle cell-endothelial adhesive events.

Key words: red cell dehydration, fetal hemoglobin, hydroxyurea, endothelium, cation transport.

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A mutation on the gene for β globin, a subunit of adult hemoglobin A (HbA), is the cause of sickle cell disease (SCD). An adenine (A) to thymine (T) substitution in codon 6 (GAG→GTG) of the β globin gene specifies the insertion of valine in place of glutamic acid in the β-globin chain (β^s, $\beta^{6glu\rightarrow val}$).[1-6] Sickle hemoglobin (HbS) has the unique property of polymerizing when deoxygenated.[1-7] Studies of HbS polymerization kinetics, following deoxygenation, have demonstrated the crucial role of cellular HbS concentration in sickling by showing that the formation of polymers is a high order exponential function of hemoglobin concentration.[1-7] The HbS polymerization is associated with a reduction in cell ion and water content (cell dehydration), increased red cell density and further acceleration of HbS polymerization (Figure 1). These dense, dehydrated erythrocytes are likely to undergo instant polymerization in conditions of mild hypoxia due to their high HbS concentration and they may carry HbS polymers under room air conditions, too.[5]

Pathophysiological studies have shown that the dense, dehydrated red cells play a central role in the acute and chronic clinical manifestations of sickle cell disease, which are based on vaso-occlusion and impaired blood flow, as a consequence of intravascular sickling in capillaries and small vessels (Figure 1). The persistent membrane damage associated with HbS polymerization also favors the generation of distorted rigid cells and contributes to additional vaso-occlusive events and cell destruction in the peripheral circulation (Figure 1).[1-8] These damaged, dense sickle red cells also show a loss of phospholipid asymmetry with externalization of phosphatidylserine (PS), which is believed to play a significant role in promoting macrophage recognition with precocious removal of erythrocytes (erythrophagocytosis), cell apoptosis and activation of coagulation.[9-11] Although the percentage of dense erythrocytes does not predict the severity of the disease, this percentage has been shown to increase prior to or in the first phase of painful crises and decrease thereafter.[1-7] Vaso-occlusive events in the microcirculation are the result of a complex scenario involving interactions between different cell types, including dense, dehydrat-

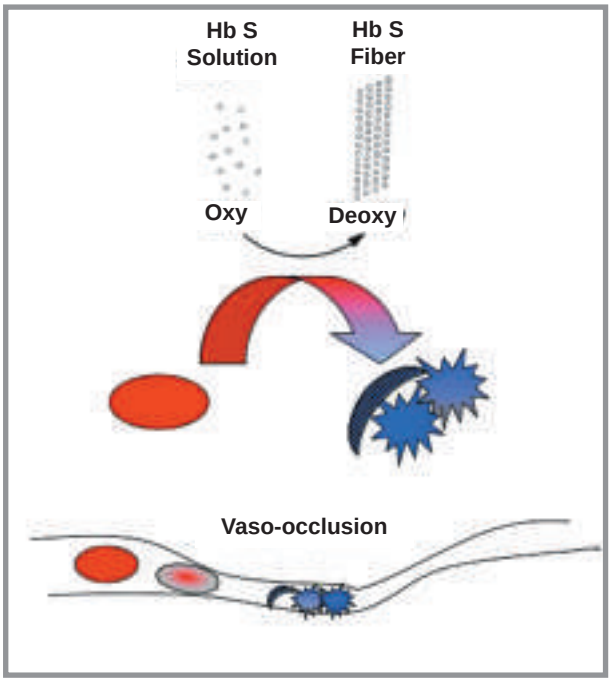

Figure 1. Schematic diagram of pathogenesis of sickle cell disease. The deoxygenation-induced polymerization of hemoglobin S (HbS) leads to erythrocytes morphology changes, generating sickled, dense, dehydrated red cells, which may be trapped into wider diameter vessels. Owing to decreased flexibility and increased tendency to adhere to abnormally activated vascular endothelium, sickled red cells can lead to vaso-occlusion with the concomitant development of pathological conditions such as acute ischemic pain crisis, organ/endothelium damage and acute chest syndrome.

ed sickle cells, reticulocytes, abnormally activated endothelial cells, leukocytes, platelets and plasma factors.[12-14]

The therapeutic strategies for sickle cell disease are based on two major goals. The first one is to decrease the intracellular HbS concentration with agents activating fetal hemoglobin synthesis or with erythrocyte-active agents preventing sickle cell dehydration (Figure 2). The second one is to reduce sickle cell-endothelial adhesive events (Figure 3).

Agents activating fetal hemoglobin: hydroxyurea in sickle cell disease

Studies in human and in animal models for sickle cell disease (SCD) have shown that increasing fetal hemoglobin (HbF) levels significantly decrease hemoglobin S polymerization and sickling (Figure 2). Clinical and epidemiological studies indicate that HbF concentration (F cells) is a crucial determinant of the clinical severity of SCD. In fact, subjects with low HbF concentrations have a more severe clinical presentation, characterized by more frequent painful crises, episodes of acute chest syndrome and an increased mortality, than do patients with higher HbF content.[15-22]

In the last decades, different molecules intended to increase HbF concentration have been studied in sickle cell anemia,[23-29] although hydroxyurea (HU) is the only such drug that has been evaluated in large clinical trials and approved for clinical use.[16-18] Substantial reductions in pain rate, acute chest crises and transfusion requirements have been achieved with hydroxyurea therapy. Recently, Steinberg *et al.* have

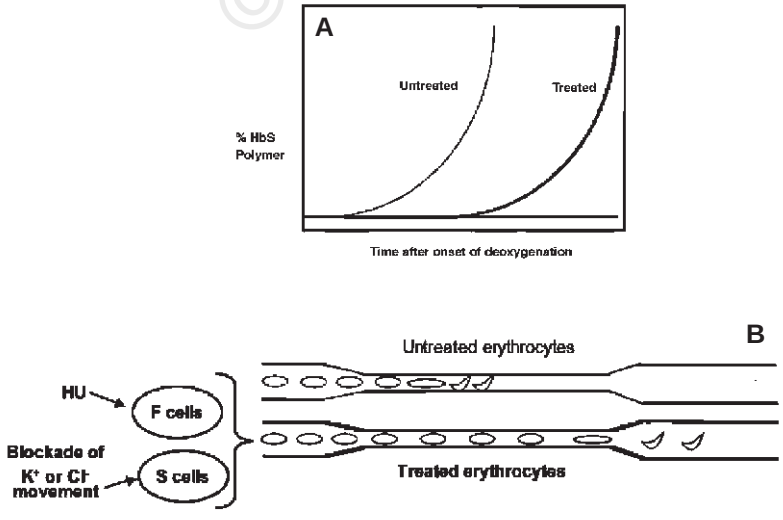

Figure 2. Expected change in delay time for HbS polymerization induced by a hypothetical treatment that reduces HbS concentration and its and its expected effect on capillary sickling and occlusion. A: Schematic simulation of the delay time for polymer formation in an untreated sickle red cell and a treated red cell with reduced HbS concentration. B: The formation of sickle cells and occlusion of a capillary in untreated erythrocytes. The HbS polymerization rate is extremely dependent on HbS concentration. Dilution of HbS obtained by treatment with hydroxyurea (HU), which increase cellular concentration of fetal hemoglobin (F cells), or by increased hydration or lowered dehydration of erythrocytes with the blockade of K+ and Cl- movement, will retard the onset of sickling, eventually to a time where the erythrocytes have passed through the capillaries into wider diameter vessels.

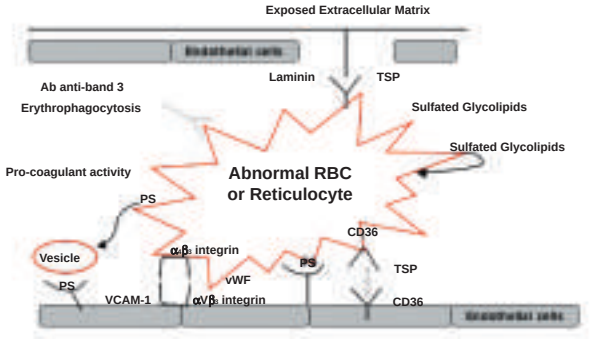

Figure 3. Schematic diagram of possible therapeutic targets for agents that interfere with adherence of sickle red cells (RBC) or reticulocytes to abnormally activated endothelial cells. PS: phosphatidylserine; TSP: thrombospondine; Ab anti-band 3: autoantibodies anti-band 3.; vW: von Willebrand.

showed a 40% reduction in mortality among patients treated with HU, in a long-term (9-year follow-up) study.[17,18] The therapeutic actions of hydroxyurea appear to be mainly based on the effects of increasing HbF concentration and decreased HbS concentration on polymerization and sickling,[15-22,30] but other beneficial effects of HU have recently been described in sickle cell patients.

Accumulating evidence in both animal models for sickle cell disease and in humans with the disease suggest that sickle cell anemia may be considered as a chronic inflammatory disease characterized by acute events further amplifying the inflammatory response.[31–35] HU may affect the inflammatory cascade and adhesion sickle-endothelial cells. In fact, HU treatment reduces the neutrophil count, the release of normal and stress reticulocytes from bone marrow, and the levels of soluble VCAM-1 (Figure 3).[36-42] Beneficial effects of HU treatment are present even before a measurable increase in HbF can be detected, as suggested by the precocious reduction in reticulocyte adhesion receptors $\alpha 4\beta 1$ (VLA-4) and CD36 expression.[42] Moreover, *in vitro* data show that HU may directly affect endothelial cells, which become less available for sickle cell adherence.[43]

Recently, *in vitro* and *in vivo* studies have reported evidences of a new mechanism of action for HU.[44-50] Patients receiving HU treatment show increased levels of nitrite, nitrate and iron nitrosyl hemoglobin, as well as of red cell cGMP content, which may be considered as markers of NO metabolism.[44-51] Nitric oxide (NO) is a potent vasodilator and modulator of inflammatory cascade and is generated by peroxidation of hydroxyurea.[45-47] Cokic *et al.* have recently shown that HU and two other NO-donors increase γ-globin gene expression in erythroid progenitors and this associated with increased cGMP levels, suggesting the presence of an NO-mediated pathway for γ-globin gene induction and related increase in fetal hemoglobin synthesis in erythroid precursors.[52,53]

Finally, another possible therapeutic effect of HU is related to induction of methemoglobin formation, reduce deoxyHbS concentration associated with increase erythropoietin production, which may adjunctively contribute to proliferation of HbF-producing progenitors.[54-56]

Erythrocyte-active agents preventing sickle red cell dehydration

A novel therapeutic strategy in sickle cell disease is based on the reducing polymerization and sickling by reducing HbS concentration inside sickle red cells, throughout the prevention of sickle cell dehydration. One of the distinguish characteristics of sickle cell disease is the presence of dense erythrocytes, formed as a result of cell dehydration and K loss (Figure 2).[57-65] These dense red cells generally have lower HbF content and include both reticulocytes and red cells. Usually, the dense fraction of erythrocytes has the high percentage of irreversible sickle cells (ISCs), cells that maintain their sickle shape even when fully oxygenated. An inverse correlation has been demonstrated between percentage of ICSs and erythrocyte survival.[58] Although the percentage of dense erythrocyte does not predict the severity of the disease, it has been shown to increase prior or in the first phase of the painful crisis and decrease thereafter.[12,57] *In vitro* and *in vivo* studies in animal models for sickle cell disease have suggested a crucial role of dehydrated red cell in the pathogenesis of vaso-occlusive events; in fact, the dense, dehydrated red cells might be easily trapped in postcapillary venules, promoting micro-vascular obstruction.[12,57-64]

Thus, prevention of sickle cell dehydration represents an exciting possible new therapeutic strategy (Figure 2). Studies on sickle cell membrane permeability have shown abnormalities in different specialized membrane-embedded transporters that move cations, anions and water across the erythrocyte membrane (Figure 4).[65-68] In the last two decades, studies on nature and properties of the pathways mediating K loss in sickle cell erythrocytes, have allowed to developed new therapeutic tools to block them.

This strategy was first explored by therapeutic induction of hyponatriemic hypoosmotic state through the administration of 1-desamini-8-D-arginine vasopressin in the setting of very high water intake and severely restricted salt intake.[68] Although the clinical regimen was not reliably sustainable on a long-term

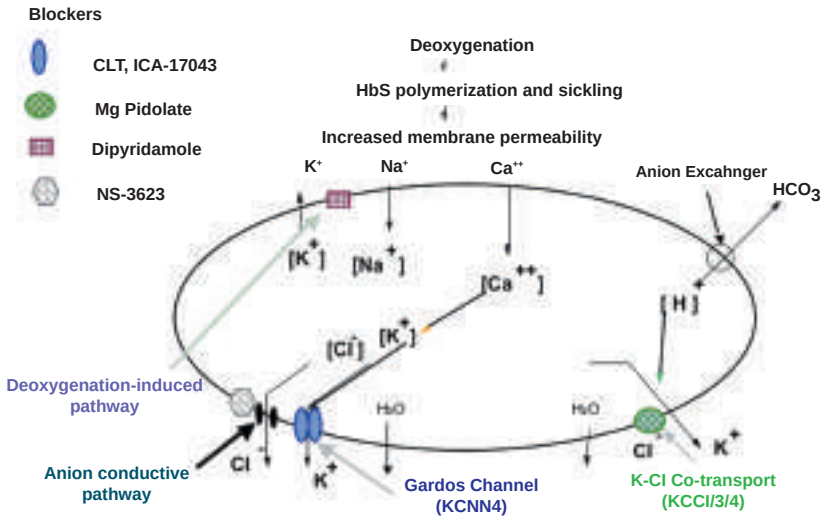

Figure 4. Schematic diagram of the ion transport pathways involved in sickle cell dehydration and action sites of potential therapeutic blockers: Ca^{2+} activated K^+ channel (Gardos channel, KCNN4): Clotrimazole (CLT) and ICA-17043; K-Cl cotransport (KCC1/3/4): Magnesium (Mg) Pidolate; Deoxygenation-induced pathway: Dipyridamole; Anion conductive pathway: NS3623. Deoxygenation induces HbS polymerization and sickling, with associated increased membrane permeability and abnormal function of different ion transport pathways, resulting in K^+, Cl- and water loss and red cell dehydration.

basis, a reduction of sickling was demonstrated *in vitro*.[68] The major pathways for K^+ loss during sickle cell dehydration events are the Ca^{2+}-activated K^+ channel, known as Gardos channel, operating in parallel with the conductive Cl- pathway and the electroneutral K-Cl cotransport (Figure 4).

Ca^{2+}-activated K^+ channel (Gardos channel, KCNN4). Sickle red cells are characterized by increased amounts of calcium, which is functionally and physically sequestered into intracellular vescicles, but maintained in normal concentration in steady-state.[68-74] The cyclic deoxygenation and HbS polymerization has been show to produce transient increase in free intracellular calcium, which is responsible for large K^+ loss with associate Cl- and water loss (Figure 4). This effect is due to activation of a specific Ca-gated K channel that was first described by Gardos.[68-74] *In vitro* activation of the Gardos channel by deoxygenation and inhibition by specific blockers have been demonstrated in sickle cell erythrocytes.[68-78] The event activating the Gardos channel is a stochastic process and is not limited to a particular fraction of susceptible SS red cells delimited by cell age or density. A recent development in the study of Gardos channel has been the description of inhibitory effect of clotrimazole (CLT).[79-83] Based on this evidences, it has been shown that CLT is a specific inhibitor of the Gardos channel and prevents sickle cell dehydration *in vitro*. Subsequently, in a transgenic mouse model for sickle cell disease, oral administration of CLT was reported to specifically block the Gardos channel, increase red cell K content and reduced red cell dehydration. The compound was further tested in human normal and sickle cell volunteers, showing to be a powerful and effective inhibitor of the erythroid Gardos channel and sickle red cell dehydration. Studies on CLT metabolites, with conserved Gardos channel inhibitory power, were used as backbone for a new class of compounds, which possess a more safe drug profile. One of these compounds (ICA-17043) has been shown to have 10-fold greater potency than CLT in blocking the Gardos channel in vitro and in vivo to specifically inhibit Gardos channel and prevent K^+ loss and red cell dehydration.[84] Phase I studies have been reported in normal human subjects and in sickle cell patients, showing significant blockade of the Gardos channel, in absence of any significant side-effects.[85] ICA-17043 is now in Phase II/III clinical trials. Another therapeutic agent, which has been recently shown to modulate the Gardos channel activity, is L-Arginine. Patients with SCD show a state of relative depletion of arginine, which is part of nitric oxide pathway.[86] L-Arginine supplementation of transgenic sickle cell mice resulted in inhibition of erythrocyte Gardos channel activity and amelioration of red cell dehydration.[87]

K-Cl cotransport (KCC1/3/4). Several forms of K-Cl cotransport have been described in various human and mouse tissues. KCC2 expression seems to be limited to brain cells, while human and mouse erythrocytes seem to possess in different and still undeterminated ratios: KCC1, KCC3 and KCC4 isoforms.[88-92] The K-Cl cotransport mediates red cell dehydration in SCD. Studies on K-Cl cotransport function have identified different triggers of activation, such as cell swelling, cell acidification, reduced cell magnesium (Mg) content, membrane oxidative damage and cell age.[84-103] Franco *et al.* have also shown that K-Cl cotransport mainly contributes to dehydration of sickle reticulocytes and that deoxygenation of sickle red cells also stimulates K-Cl cotransport in isotonic solutions at pH 7.4.[104,105] The relative contribution of Gardos channel and K-Cl cotransport in generating dehydrated, dense sickle red cells is a complex and still incompletely known issue.

K-Cl cotransport activity is modulated by red cell Mg content and low Mg levels are associated with abnormal activation of K-Cl cotransport.[84-103] Few studies report a reduction in red cell Mg content in SCD patients.[106-107] Thus, oral Mg supplementation with the aim of increase red cell Mg levels and inhibiting K-Cl cotransport activity may represent a possible therapeutic strategy for ameliorating SCD red cell dehydration. Dietary magnesium supplementation in transgenic sickle cell mice has demonstrated that red cell dehydration can be ameliorate by increasing erythrocyte Mg content.[80,107] Two uncontrolled trails with oral supplementation with Mg pidolate have been carried out in sickle cell patients, showing a reduction in K-Cl cotransport activity, an increase in red cell K and Mg content, an improvement in red cell dehydration and a reduction in the number of painful events.[108,109] A first double-blind, placebo controlled crossover study with Mg pidolate supplementation in sickle cell children did not demonstrate any significant changes in the hematological parameters studied; however the Mg pidolate dosage used was markedly lower than that proposed in the previous studies.[110] Recently, Brousseou *et al.* have shown that infusion of Mg sulfate reduces the length of stay of sickle cell patients hospitalized during vaso-occlusive crises.[111]

Cl- permeability pathway. Studies on conductive Cl- pathway indicate that for red cell dehydration the movement of K must be accompanied by that of chloride (or other monovalent anions) to maintain electroneutrality (Figure 4).[112] Elegant sets of studies demonstrate that movement of K and dehydration via Gardos channel can be blocked if the Cl- conductance pathway is inhibited.[112-115] A specific inhibitor of Cl- conductance has been recently developed (NS3623; Figure 4). NS3623 has been tested in transgenic sickle cell mice and was able to reduce *in vivo* sickle cell dehydration, with a mild echynocytosis at highest dosages.[115] Unfortunately, NS3623 was not further developed for clinical use, in relation to undesirable side effects observed in human subjects.

Deoxygenation-induced Na^+ and K^+ fluxes. Original works by Tosteson and subsequent studies by others have characterized the increased Na^+ and K^+ permeability associated with red cell sickling.[116] When cellular Na^+ is increased, Na-K ATPase pump activation may contribute to sickle cell dehydration (Figure 4).[116-125] The deoxygenation-induced fluxes are inhibited *in vitro* by dipyridamole at concentration achievable *in vivo*.[125]

Anti-adherence therapy in sickle cell disease

Vaso-occlusions are central events in pathophysiology of sickle cell disease acute and chronic organ damage and clinical manifestations. The abnormal adhesive interactions between erythrocyte, reticulocytes, endothelial cells, platelets or soluble mediators may represent a new possible therapeutic target. The end-point of anti-adherence therapy is to interfere with the initialization and/or with the amplification of adhesive events. Although the anti-adherence therapy has been mainly studied during acute painful events, its mechanisms of action are only partially known. RheothRx (Poloxamer 188) is a non ionic surfactant block copolymer that improves microvascular blood flow by lowering viscosity and frictional forces.[126,127] RheothRx has been shown to block hydrophobic adhesive interactions (cell-cell, cell-protein or protein-protein interaction) in blood, resulting in reduction of erythrocytes aggregation and red cells adherence to vascular endothelium, with a hypothesized improvement in microvascular flow.[126,127] Phase II studies have shown a limited favorable effects in treatment of acute pain crises, when associated with HU in sickle cell children.[127] Recent studies on sickle cell-endothelium adhesive mechanism have identified three different interactions which may have particular therapeutic relevance: (i) the integrin $\alpha 4\beta 1$ receptor of fibronectin and the vascular adhesion molecule –1 (VCAM-1); (ii) thrombospondin and/or collagen and receptor CD36, present of the surface of endothelial cells, platelets and reticulocyte-rich subpopulation of normal and sickle erythrocytes; (iii) sulfate glycolipids, which bind thrombospondin, von Willebrand factor multimer and laminin (Figure 3).[126-132] *Ex vivo* and *in vitro* experimental studies have shown that thrombospondin and von Willebrand factor mediated interaction between sickle red cells and endothelium via $\alpha V\beta 3$ integrin, might be blocked by monoclonal antibodies against $\alpha V\beta 3$ integrin receptors.[128] The binding between thrombospondin, von Willebrand factor and laminin that mediate sickle cell-endothelial adherence might be blocked anionic polysaccaride, like high molecular weight dextran sulfate or chondroitin sulfate.[129] The endothelial cells represent another possible therapeutic target. Sulfazosina that inhibit the transcription of nuclear factor NFk-B may interfere with endothelial cell activation. Transgenic sickle cell mice treated with Sulfazosina show a reduction in activated circulating endothelial cells, and in VCAM-1, ICAM and E-selectin vascular wall endothelial expression. In a pilot study, the administration of Sulfazosina to sickle cell patients results in reduction of the endothelial abnormal activation.[130-132]

Therapeutic relevance of nitric oxide (NO) in sickle cell disease

Nitric oxide (NO) is a potent vasodilator and inhibitor of vascular remodeling and also affects the multistep cascade events involved in leukocyte, platelets and endothelial activation. NO is generat-

ed from L-Arginine by endothelial cells via constitutive and inducible nitric oxide synthases.

Recent studies have focused on inhaled NO for the treatment of tissue damage in various ischemic syndromes, including cardiovascular disease, pulmonary hypertension, acute lung distress syndromes. The possible therapeutic role of inhaled NO has been studied in different animal models of lung injury induced by ischemic/reperfusion.[35,136] Inhaled NO prevents leukocyte migration and reduces the permeability of the peripheral microvasculature. In association with surfactant, inhaled NO alleviates alveoli's edema and reduces bronchoalveolar leukocytes and neutrophils infiltration in animal models of ischemic lung injury.[133-143] The beneficial role of inhaled NO in SCD has been recently reported in the treatment of acute vaso-occlusive crisis in a placebo controlled randomized clinical trial, although the mechanism of action in SCD remained unknown.[17] Plasma NO metabolites are decreased in SCD patients during either vaso-occlusive crisis associated with severe pain or acute chest syndrome.[133-143] A decrease in exhaled NO has been reported in sickle cell patients, suggesting a role for NO in the pathogenesis of the pulmonary complications.[140,141] In a transgenic mouse model for sickle cell disease, it has been shown that inhaled NO provides protection during ischemia/reperfusion lung injury, in which endothelial NO production is reduced.[35]

Another possible therapeutic strategy for increase NO production in sickle cell disease is represented by supplementation of L-Arginine.[86,144] Morris CR and co-workers have shown that L-Arginine supplementation alone induces an unexpected decrease in NO metabolite production.[86] In a following pilot study, the Authors observed an in increase in NO metabolite when L-Arginine was co-administrated with HU, suggesting that the combination treatment may have a synergistic effect on NO production.[144] Further studies are currently being planned to test the effects of inhaled NO or NO donors and L-Arginine supplementation in sickle cell disease.

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