Role of Arginase in Sickle Cell Lung Disease and Hemolytic Anemias

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Abstract: Secondary pulmonary hypertension (PH) is a leading cause of mortality and morbidity in patients with hemolytic anemias, including sickle cell disease (SCD) and thalassemia. Asthma is another common co-morbidity in SCD that has been linked to early death. The high frequency of asthma in this population cannot be attributed to genetic predisposition alone, and likely reflects in part, the contribution of overlapping mechanisms shared between these otherwise distinct disorders. There is accumulating evidence that dysregulated arginine metabolism and elevated arginase activity contributes to a number of pulmonary conditions. Patients with hemolytic disorders are at risk for lung complications triggered or worsened by hemolysis-driven release of erythrocyte arginase and low nitric oxide (NO) bioavailability. Inactivation of NO correlates with hemolytic rate and is associated with the erythrocyte release of cell-free hemoglobin, which consumes NO directly, and the simultaneous release of the arginine-metabolizing enzyme arginase, which limits bioavailability of the NO synthase substrate arginine during the process of intravascular hemolysis. Rapid consumption of NO is accelerated further by oxygen radicals in a milieu of oxidative stress common to hemolytic disorders. Once released into circulation, arginase will convert arginine to ornithine, which in turn is the precursor to proline, an amino acid involved in collagen formation, lung fibrosis, airway remodeling and vascular smooth muscle proliferation, common features of pulmonary dysfunction in thalassemia and SCD. Evidence supporting the role of arginase in sickle cell lung disease will be reviewed and relevance to other hemolytic disorders including the thalassemia syndromes will be considered.

Keywords: Arginase, arginine dysregulation, nitric oxide, sickle cell disease, hemolysis, pulmonary hypertension.

COMMON FEATURES AMONG DISTINCT HEMO-GLOBINOPATHIES

The hemoglobinopathies are among the most common genetic disorders in the world. An estimated 5% of the global population are carriers of variants in either α or β globin genes resulting in two major red blood cell disorders, the thalassemia syndromes and sickle cell disease (SCD). These are autosomal recessive conditions that affect over 30 million people worldwide [1-5].

SCD occurs in individuals of African, Caribbean, Mediterranean, Arab and other Middle Eastern descent, and affects more than 70,000 people in the US. Although an accurate account of the global burden of SCD is unknown, recent newborn screening analysis for hemoglobinopathies in the state of California revealed an incidence of 1/393 African American infants born with SCD over an 8.5 year time period [6]. whereas in sub-Saharan Africa, it is estimated that 1-4% of the population is born with this disease [4]. Of interest, 12.5% of sickle-beta thalassemia infants born in California were of Hispanic origin [6].

Genetically, SCD is caused by an amino acid substitution of valine for glutamic acid in the sixth position of the β subunits of hemoglobin. This structural change results in intracellular polymerization of the deoxygenated hemoglobin molecules under hypoxic conditions. Intracellular polymer increases erythrocyte rigidity and ultimately damages and distorts the erythrocyte membrane. This produces a rigid

"sickled" red cell with altered rheological and adhesive properties that becomes entrapped in the microcirculation and gives rise to the vaso-occlusive events characteristic of the disease [7].

Cycles of ischemia and reperfusion produce inflammation associated with increased expression of adhesion molecules on erythrocytes and endothelial cells. Adhesive interactions between erythrocytes, leukocytes, and endothelium, increased levels of circulating inflammatory cytokines, reduced blood flow from increased viscosity and vasoconstriction, hemostatic activation, and endothelial damage are all thought to contribute to obstruction of the microvasculature by sickled erythrocytes [8, 9]. Ultimately these red cell changes initiate a cascade of events that results in episodic vaso-occlusion and subsequent ischemia-reperfusion injury, leading to clinical sequelae [10].

The clinical phenotype of SCD varies widely, and is characterized by anemia, severe pain, and potentially life-threatening complications such as bacterial sepsis, splenic sequestration, acute chest syndrome (pneumonia), stroke and chronic organ damage. These and other manifestations result from chronic hemolysis and intermittent episodes of vascular occlusion that cause tissue injury and organ dysfunction [7, 11, 12].

The thalassemia syndromes are a heterogeneous group of inherited hemoglobin disorders resulting from unbalanced production of the alpha and beta globin subunits of the hemoglobin tetramer [13]. Over 125 different genetic lesions can cause β-thalassemia, which is characterized by decreased (β^+-thalassemia) or absent (B^0-thalassemia) synthesis of the β-globin chain [14]. Thalassemia is most common in

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individuals whose ancestors originated from the Mediterranean region, Africa, southern China, Southeast Asia, and India [1]. An estimated 900,000 births with clinically significant thalassemia disorders are expected in the next 20 years [15]. The clinical spectrum is a consequence of imbalanced globin chain accumulation, resulting in impaired erythropoiesis and hemolytic anemia [16]. The two clinically significant phenotypes of β-thalassemia have been designated thalassemia major and thalassemia intermedia. Thalassemia major is characterized by severe anemia starting during the first year of life, requiring life-long transfusion therapy for survival, while thalassemia intermedia has a later clinical onset with a milder anemia, does not typically require chronic transfusions, and offers a longer life expectancy [16]. Heart failure is the most common cause of death in both forms of the disease [17-19]. Thalassemia heart disease involves mainly left ventricular dysfunction caused by transfusion-induced iron overload. However, recent studies suggest that both thalassemia major and intermedia patients have a unique hemodynamic pattern consistent with right ventricular cardiomyopathy and pulmonary hypertension (PH), in addition to the left ventricular abnormalities [20]. PH in β-thalassemia represents a common, [21-25], yet less well explored complication in the cardiopulmonary spectrum of the disease.

Intravascular or intramedullary hemolysis, chronic anemia, oxidative stress, and a high frequency of PH and other lung complications are common features of both SCD and thalassemia [26, 27]. In particular, secondary PH is emerging as one of the leading causes of mortality and morbidity in patients with hemolytic anemias; [3, 12, 28, 29]; the consequence of complex and multifactorial mechanisms that will be discussed in this review, with a focus on SCD and the role of arginase as an important enzyme contributing to pulmonary disease. However the implications extend to all conditions involving acute or chronic hemolysis (Table **1**) [3, 28, 30-32]. Although PH is the best characterized clinical complication of hemolysis [12, 29, 33, 34], asthma and lung fibrosis are also potential consequences of excess arginase activity and warrant future investigation.

HEMOLYSIS: GLOBAL DISRUPTION OF THE AR-GININE-NITRIC OXIDE PATHWAY

A new disease paradigm involving hemolysis-associated endothelial dysfunction [28, 29] has implication for SCD [29, 35], thalassemia [27, 36], malaria [37-39], and all hemolytic conditions [28]. The process of hemolysis initiates a global disruption of the arginine-NO pathway (Fig. **1**) [26, 28, 40-42]. Under normal conditions, hemoglobin is safely packaged within the erythrocyte plasma membrane, however during hemolysis it is decompartmentalized and released into plasma where it rapidly reacts with and destroys NO [43]. This results in abnormally high NO consumption and the formation of reactive oxygen species, ultimately inhibiting vasodilation. Formation of superoxide from enzymatic oxidases such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [44], xanthine oxidase [45, 46] and uncoupled endothelial NO synthase [41, 47] will also react with and scavenge NO, contributing to a state of NO resistance [47-50]. Consequently, smooth muscle guanylyl cyclase is not activated and vasodilation is inhibited. NO destruction by hemoglobin can also cause further impairment in vascular endothelial function via transcriptional activation of adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, and potent vasoconstrictors such as endothelin-1 [28]. Intravascular hemolysis also has the potential to drive a pro-coagulant state, as NO has properties that inhibits platelet activation, tissue factor expression and thrombin generation [28, 51, 52].

The simultaneous release of erythrocyte arginase during hemolysis [53] will limit the availability of arginine to NOS, contributing to a deficiency of NO. Arginase also redirects the metabolism of L-arginine to L-ornithine and the

Table 1. Hemolytic Disorders Associated with Pulmonary Hypertension

Sickle Cell Disease
Thalassemia syndromes (Intermedia and Major, Hemoglobin H disease etc.)
Paroxysmal Nocturnal Hemoglobinuria
Malaria
Glucose-6-phosphate dehydrogenase deficiency
Hereditary spherocytosis and stomatocytosis (and other red cell membrane disorders)
Hb-Mainz hemolytic anemia (and other unstable hemoglobin variants)
Alloimmune hemolytic anemia
Pyruvate kinase deficiency
Microangiopathic hemolytic anemia (hemolytic uremic syndrome, thrombotic thrombocytopenic purpura)
Evan's syndrome
Hemolysis from mechanical heart valves
Left ventricular assist devices and cardio-pulmonary bypass procedures
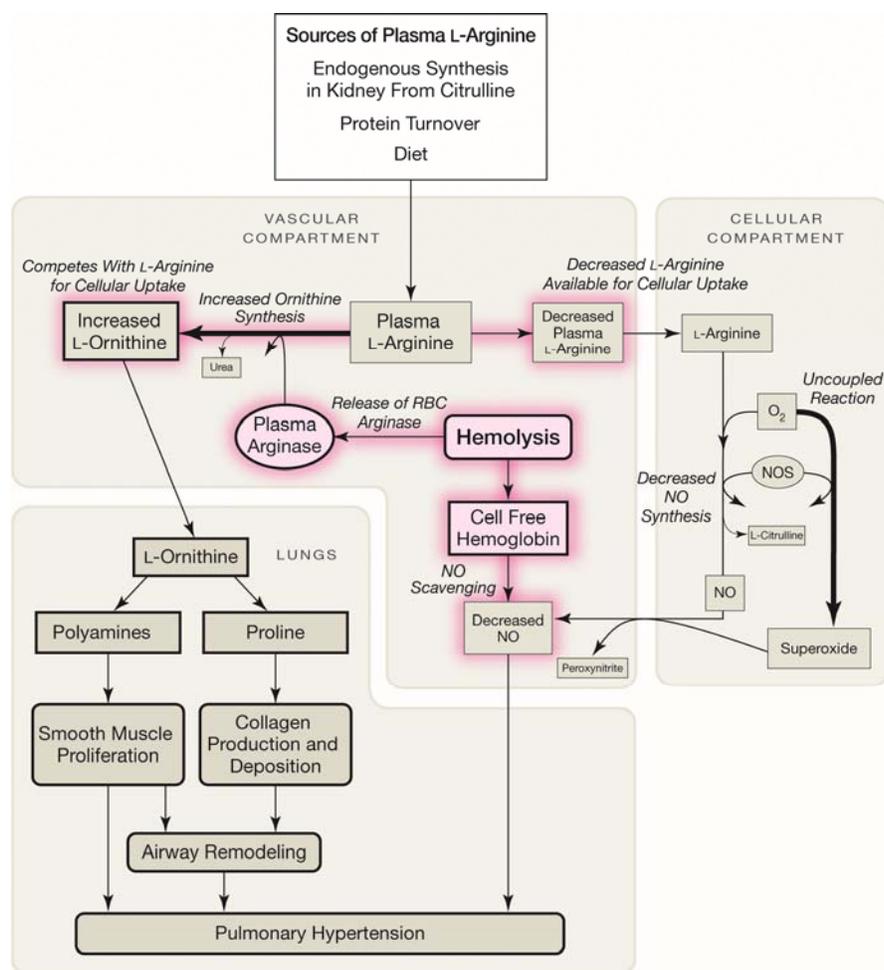
Fig. (1). Altered arginine metabolism in hemolytic disorders. Arginine is synthesized endogenously from citrulline primarily via the intestinal-renal axis. Arginase and nitric oxide synthase (NOS) compete for arginine, their common substrate. In sickle cell disease (SCD), bioavailability of arginine and nitric oxide (NO) are decreased by several mechanisms linked to hemolysis, and similar mechanisms are postulated for thalassemia and other hemolytic disorders. The release of erythrocyte arginase during hemolysis increases plasma arginase levels and shifts arginine metabolism towards ornithine production, decreasing the amount available for NO production. The bioavailability of arginine is further decreased by increased ornithine levels because ornithine and arginine compete for the same transporter system for cellular uptake. Endogenous synthesis of arginine from citrulline may be compromised by renal dysfunction, commonly associated with SCD. Despite an increase in NOS in SCD, NO bioavailability is low due to low substrate availability, NO scavenging by cell-free hemoglobin released during hemolysis, and through reactions with free radicals such as superoxide. Superoxide is elevated in SCD due to low superoxide dismutase activity, high xanthine oxidase activity and potentially as a result of uncoupled NOS in an environment of low arginine and/or tetrahydrobiopterin concentration. Endothelial dysfunction resulting from NO depletion and increased levels of the downstream products of ornithine metabolism (polyamines and proline) likely contribute to the pathogenesis of lung injury, fibrosis and pulmonary hypertension. Reproduced with permission from the American Medical Association [53].

formation of polyamines and L-proline, which are essential for smooth muscle cell growth and collagen synthesis [54, 55]. Therefore, the induction of arginase may also promote aberrant vessel wall remodelling and neointima formation [54], contributing the structural changes that are observed in the lungs of patients with hemoglobinopathies. It is interesting that alterations in the arginine metabolome have been implicated in the pathogenesis of PH in both hemolytic [26, 31, 36, 53, 56-58] and non-hemolytic disorders [59, 60]. By creating a shift towards ornithine metabolism, arginases set off a process that contributes to a proliferative vasculopathy regardless of the initiating trigger [26, 27, 53].

Mechanisms of Arginine Dysregulation

L-Arginine is the only physiologic substrate available to NOS for NO production, and its concentration can be rate-limiting. The low K_m of NO synthase for arginine ($< 10\mu M$) [61]. should prohibit arginine concentration from becoming rate limiting, even when circulating levels are below normal [62-64]. However, rates of NO production vary according to extracellular arginine concentration, even when intracellular arginine levels are in apparent excess, a phenomenon termed the "arginine paradox" [65]. The mechanisms of this paradox are undefined, but likely involve decreased substrate bioavailability, attenuated NO synthesis, increased NO

consumption, and impaired amino acid transport. Arginase activity likely plays a role in the "arginine paradox", particularly in hemolytic disorders. Normal arginine metabolism is impaired in SCD [53] and thalassemia [36, 57] for a variety of reasons that contribute to endothelial dysfunction and PH. Adults with SCD are arginine deficient at steady-state [62, 63], while children have plasma levels that are similar to normal controls. An arginine deficiency develops with age and is influenced by acute events. Plasma arginine concentration decreases significantly in both adults and children during vaso-occlusive crisis (VOC) and acute chest syndrome (ACS), and is associated with low NO metabolite levels [62]. Ultimately, low arginine bioavailability is associated with early mortality in adults with SCD [53]. The role of arginine dysregulation in the thalassemia syndromes is not as well characterized as it is in SCD, however low plasma arginine levels, high plasma arginase activity and a high concentration of the downstream products of arginase metabolites, including ornithine and proline levels have been described together with a high prevalence of PH [27, 36, 57]. It is also of interest to note that lowest plasma arginine concentration is associated with severity of malaria, another hemolytic disorder in which the degree of hypoargininaemia was significantly associated with parasite burden, degree of endothelial dysfunction and cerebral malaria case-fatality in children [37-39]. Erythrocyte arginase release during hemolysis together with the activity of Plasmodium falciparum arginase itself [66, 67] likely contribute to arginine depletion, although the precise mechanisms and contribution of each have not yet been identified.

Increased Arginase Activity

Arginase is an essential enzyme in the urea cycle, responsible for the conversion of arginine to ornithine and urea. The NO synthase (NOS) and arginase enzymes can be expressed simultaneously under a wide variety of inflammatory conditions, resulting in competition for their common substrate [68]. Two forms of arginase have been identified, type 1, a cytosolic enzyme highly expressed in the liver, and type 2, a mitochondrial enzyme found predominantly in the kidney, prostate, testis, and small intestine [68]. Arginase-1 is also present in human red blood cells [69-71]. Plasma arginase activity is elevated in SCD as a consequence of inflammation, liver dysfunction and, most significantly, by the release of erythrocyte arginase during intravascular hemolysis, which has been demonstrated by the strong correlation between plasma arginase levels and cell-free hemoglobin levels and other markers of increased hemolytic rate (Fig. **2**) [53]. In addition, arginase activity is higher in the erythrocytes of patients with thalassemia and SCD compared to normal controls, and strongly correlates to plasma arginase activity (Fig. **3**) [53]. Upregulated expression of arginase-I also results in increased proliferation rates of vascular smooth muscle and endothelial cells [68]. and in this capacity, may further contribute to vasculopathy in addition to its unique role during hemolysis. When arginine is catalyzed to NO, NOS produces the intermediate product N-hydroxy-L arginine (NOHA) [72]. NOHA is a potent arginase inhibitor, reflecting complicated feedback mechanisms in place to maintain homeostasis, with both NOS and arginase playing a regulatory role in NO
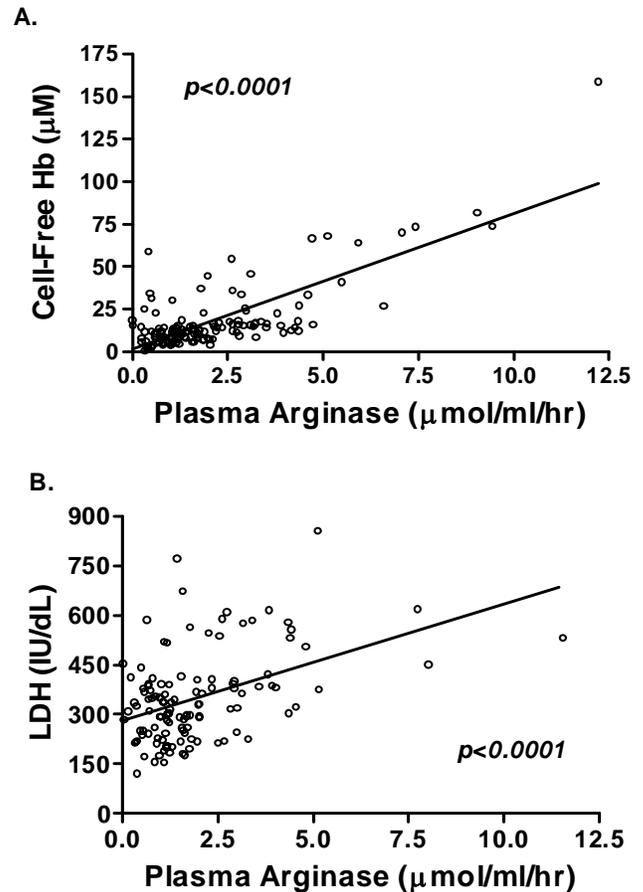
Fig. (2). Association of arginase activity with hemolytic rate. Correlation of plasma arginase activity (μmol/ml/hr) to **A.** cell-free hemoglobin (*Cell-Free Hb*, n=138, *p< 0 .001*) (Reproduced with permission from the American Medical Association [53].) and **B.** serum lactate dehydrogenase levels (*LDH*, n=121, *p<0.001*) in patients with sickle cell disease.

production [68]. As there is only limited arginase-I found in the murine erythrocyte compared to human red blood cells, the major sources of increased arginase activity in the sickle cell mouse [41] originate from cells other than the erythrocyte. It is unfortunately not feasible to extrapolate the contribution of erythrocyte arginase release to complications of hemolysis in SCD from the sickle cell mouse model.

Intracelluar Arginine Transport

Arginase concentration present in the plasma is not reflective of whole body arginase activity, since the arginases are intracellular enzymes that appear in the circulation after cell damage or cell death. The arginine-to-ornithine ratio, which significantly correlates to plasma arginase activity [53] may represent a superior marker of relative arginine bioavailability than arginine concentration alone. Arginine, ornithine and lysine compete for the same arginine transporter system (cationic amino acid transporter, CAT) [73], therefore an arginase-triggered rise in ornithine will further impact arginine transport and bioavailability. Plasma arginine concentration in SCD is approximately 40-50μM at baseline [62], about 50% lower than normal values [62] and well below the K_m for CAT (100-150μM). Even modest fluctuations in extracellular arginine concentration may significantly impact cellular arginine uptake and
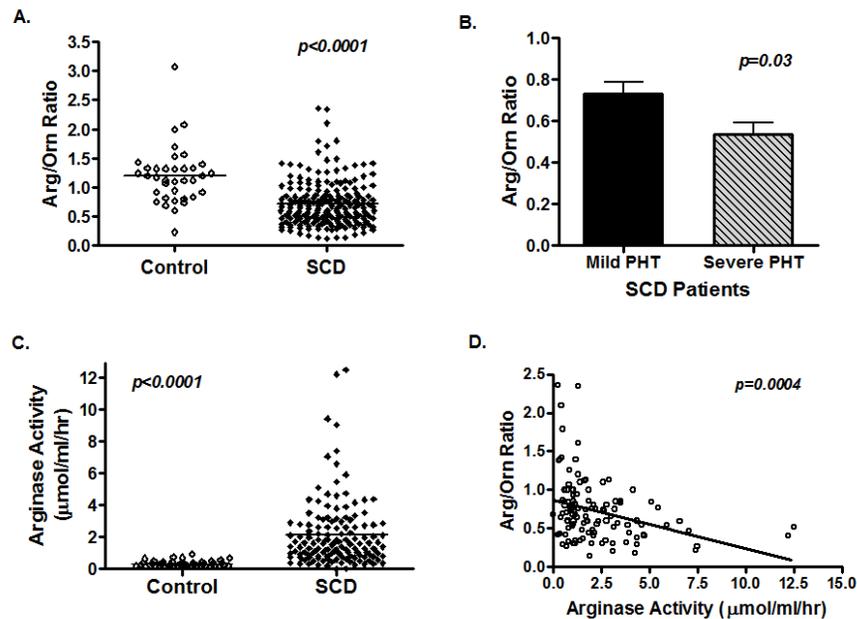
Fig. (3). The arginine-to-ornithine ratio as a surrogate marker for arginase activity, its association with pulmonary hypertension (PHT) and correlation with arginase activity. A. The arginine-to-ornithine ratio (*Arg/Orn*) in normal controls (*Control*, n=36) vs. patients with SCD (n=209). B. Arginine-to-ornithine ratio in sickle cell patients with a tricuspid regurgitant jet velocity (TRV) by echocardiography 2.5-2.9 m/s (*mild PHT*) vs. patients with a TRV ≥ 3.0 m/s (*severe PHT*). C. Plasma arginase activity (μmol/ml/hr) in normal controls (*Control*, n=36) vs. patients with sickle cell disease (*SCD*, n=140). D. Correlation of plasma arginase activity to the arginine-to-ornithine ratio. Reproduced with permission from the American Medical Association [53].

bioavailability. Under such conditions, a higher than expected increase in extracellular L-arginine concentration may be necessary in order to induce a therapeutic effect. A low arginine:ornithine ratio is associated with increased mortality in SCD [53], and severity of PH of various etiologies [60].

Renal Dysfunction

Global arginine bioavailability is diminished further in patients with renal dysfunction through the loss of *de novo* arginine synthesis from citrulline which occurs primarily in the kidney. Renal dysfunction, a common occurrence in SCD [29, 74] will impair the major route for endogenous arginine biosynthesis. Rising creatinine levels correlate strongly to rising citrulline levels suggesting renal insufficiency [53]. Including the impact of renal dysfunction on arginine bioavailability through analysis of the arginine/(ornithine + citrulline) ratio revealed an increased risk of death in SCD patients with low amino acid ratios (Fig. **4**). These findings suggest that adequate arginine bioavailability is critical for survival and provide clinicians with an objective index of disease severity [53]. Recently low global arginine bioavailability ratios have prospectively been linked to increased cardiovascular risk, coronary artery disease and adverse cardiovascular events including death, myocardial infarction and stroke in over 1000 patients undergoing elective cardiac catheterization [75]. This supports a more extensive role for arginine bioavailability in the pathogenesis of vasculopathy independent of genotype.

Endogenous NOS Inhibitors

Low arginine bioavailability may be exacerbated further by the presence of elevated asymmetric dimethylarginine, (ADMA) which is a competitive inhibitor of arginine

transport and all NOS isozymes [76]. High levels of ADMA can also contribute to NOS uncoupling [77]. Circulating ADMA levels are elevated in several conditions of endothelial dysfunction, including SCD [78], and have been implicated in the pathophysiology of systemic and PH [79-82] and risk of early mortality. The most elevated ADMA level occurred in SCD patients with the highest hemolytic rate, and was associated with PH [58, 83] and mortality [83].

ADMA normally produced in the body is hydrolyzed by dimethylarginine dimethylaminohydrolase (DDAH). Homocysteine inhibits DDAH activity. This may represent a mechanism whereby hyperhomocysteinemia, a known risk factor for vascular disease and thrombosis, leads to elevated plasma ADMA levels and decreased NO production in the cardiovascular system through competitive arginine inhibition [81, 82]. Landburg and colleagues recently demonstrated that elevated ADMA levels in patients with SCD did not increase over baseline during VOC [84]. Although they conclude that there is no primary role for ADMA during crisis [84], given that arginine bioavailability decreases significantly during VOC and ACS [62], a rise in the ratio of ADMA-to-arginine may have some impact on global arginine bioavailability and endothelial dysfunction that should be explored further.

Uncoupled Nitric Oxide Synthase

Hemolysis will drive arginine consumption, which will ultimately exacerbate NO sequestration and decrease NO synthesis. Under conditions of hypoxia, high ADMA, low arginine, or low availability of essential NOS cofactors (NADPH and/or tetrahydrobiopterin) [85], NOS will be uncoupled, producing reactive oxygen species in lieu of NO,
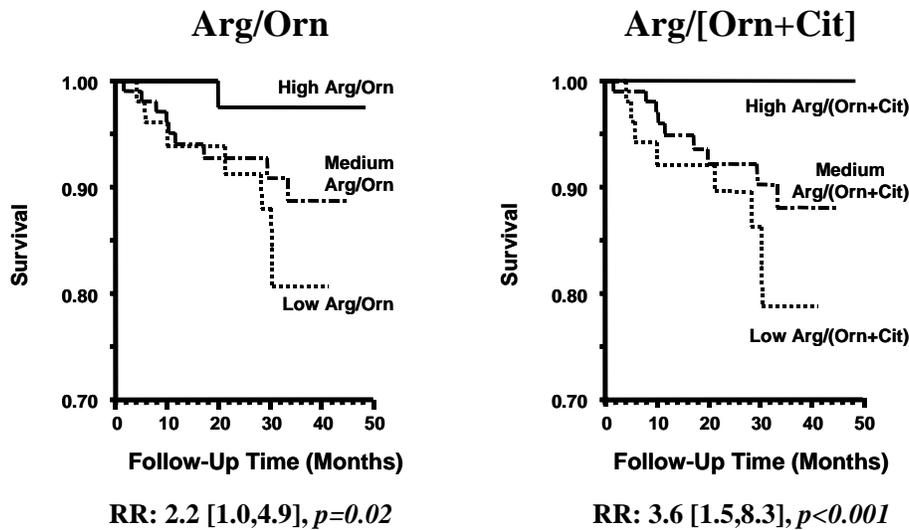
Fig. (4). Association of arginine bioavailability ratios with mortality in sickle cell disease: Kaplan-Meier survival plots. A. Mortality for 3 categories of Arginine-to-Ornithine ratio (Arg/Orn): "High" = upper quartile, > 0.8690; "Medium" = 25th to 75th percentiles, > 0.4385 and ≤ 0.8690; "Low" = lower quartile, ≤ 0.4385. B. Mortality for 3 categories of Arginine-to-(Ornithine + Citrulline) ratio (Arg/[Orn+Cit].): "High" = upper quartile, > 0.6254, "Medium" = 25th to 75th percentiles, > 0.3245 and ≤ 0.6254, "Low" = lower quartile, ≤ 0.3245. (Reproduced with permission from the American Medical Association [53].).

further reducing NO bioavailability and adding the milieu of oxidative stress. An imbalance between eNOS-derived NO and superoxide generation has been established in the hemizygote sickle cell mouse model by Wood and colleagues [86]. These authors were also the first to suggest that abnormal tetrahydrobiopterin function or availability may be yet another mechanism contributing to dysregulation of the arginine-NO pathway in SCD. This is a mechanism now well described in systemic hypertension that has only recently been addressed in PH. Upregulation of NOS would therefore enhance oxidative stress when the local milieu favors NOS uncoupling. Indeed, studies in transgenic sickle cell mice demonstrate that NOS activity is paradoxically increased and uncoupled while NO bioavailability is low [41].

Oxidative Stress

Finally, alterations in the glutathione buffering system common to these hemoglobinopathies [87, 88], may render erythrocytes incapable of handling the increased oxidant burden, thereby predisposing them to hemolysis. Recently we discovered that a depletion of erythrocyte glutamine concentration and aberrations in erythrocyte glutathione metabolism are linked to severity of PH in SCD and biomarkers of hemolytic rate including levels of arginase activity in the plasma [89]. Glutamine, an essential precursor in NADPH biosynthesis, is metabolized to the glutathione substrate glutamate in the process of NADPH production. Glutamine thus plays an antioxidant role through preservation of intracellular NADPH, making it an important amino acid for glutathione homeostasis. Glutamine also serves as a precursor for the *de novo* production of arginine through the citrulline-arginine pathway [90]. Orally ingested glutamine is metabolized to citrulline in the enterocytes, and is subsequently used by the kidneys to synthesize arginine [90, 91]. Glutamine therapy has already demonstrated promise in SCD [92]. Interestingly, oral arginine increased

erythrocyte total glutathione levels in both humans [93, 94] and sickle cell transgenic mice [95], which may improve the redox state of the sickle erythrocyte. Arginine, demonstrating initial promise for treatment of PH in SCD [96], has recently been shown by Kaul and colleagues to improve vascular function in the sickle cell mouse model by ameliorating hemolysis, oxidant stress and the NO resistance state [97]. These findings highlight the complex interactions of hemolysis and oxidative stress, and the close relationship of these two mechanisms in SCD [98]. Long-term benefits of arginine in hemolytic disorders may, however, be limited by the actions of elevated plasma arginase activity, if the dose is not sufficient to overcome the effects of arginase consumption.

Hemolysis-Associated Pulmonary Dysfunction

There is accumulating evidence that dysregulated arginine metabolism and in particular, elevated arginase activity contributes to pulmonary complications in SCD and thalassemia [27, 36, 52, 53, 57, 96, 99]. In SCD, any pulmonary complication will compromise oxygenation and contribute to a vicious cycle of erythrocyte sickling. Derangements of arginine metabolism are also emerging as newly appreciated mechanism in both asthma [99-107] and PH [59, 103] independent of hemolysis, likely the result of inflammatory stimuli and genetic polymorphisms [108-110] that affect arginase induction. Low nitric oxide (NO) bioavailability is one consequence of excess arginase activity. NO is one of the most potent vasodilators known [111] and is essential to vascular homeostasis. It plays an important role in the maintenance of vasomotor tone, limits platelet aggregation and ischemia-reperfusion injury, modulates endothelial proliferation, and has anti-inflammatory properties. Impaired NO bioavailability represents the central feature of endothelial dysfunction, and is a major factor in the pathophysiology of PH [112]. Increased consumption and decreased production of both NO
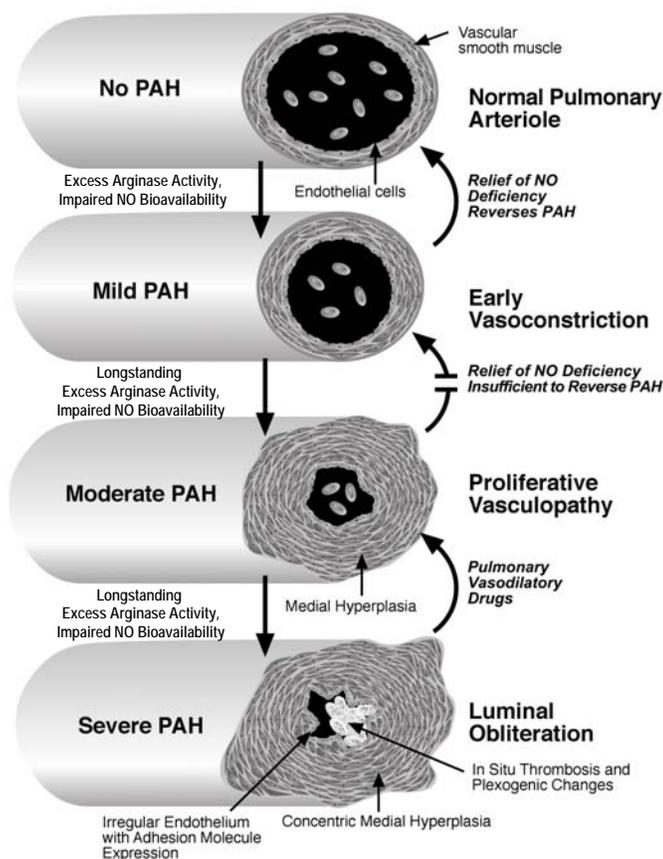
Fig. (5). Progression of pulmonary hypertension in sickle cell disease and thalassemia. In this hypothetical model, impaired NO bioavailability that results from chronic hemolysis and oxidative stress triggers chronic pulmonary vasoconstriction, mildly elevating pulmonary vascular resistance and pulmonary artery pressures. Excess arginase liberated from the erythrocyte during hemolysis consumes arginine, the obligate substrate for NO production, and creates a shift towards ornithine production that contributes to collagen deposition and vascular smooth muscle proliferation. Overabundant thrombin generation contributes to a chronic hypercoagulable state, increases arginase activity, and stimulates polyamine synthesis in vascular smooth muscle cells. As this becomes more long-standing, vascular smooth muscle hyperplasia begins to create a relatively fixed lesion, compounded in later stages by irregular, activated endothelium with expression of adhesion molecules. In situ thrombosis further occludes the vessel lumen, and results in plexogenic changes, further accelerating the progression of the pulmonary artery hypertension. (Modified Figure reproduced with permission [121]).

[43] and arginine contribute [27, 40, 53, 63] to complications associated with hemolysis-associated pulmonary dysfunction [28, 99, 113].

Pulmonary Hypertension

PH is a vascular disorder of the lung in which the pulmonary artery pressure rises above normal levels, compromises oxygenation and right-heart function, and can ultimately become life-threatening [114]. PH is a poorly understood, complex syndrome with many clinical phenotypes. The initial injury leading to pulmonary artery hypertension in different disease states may vary; however, they share a common pathway of vascular remodeling that results in a similar clinical and histopathologic condition. Histopathological findings of autopsy studies in both SCD and thalassemia include plexiform and concentric medial hyperplastic pulmonary vascular lesions, and in situ pulmonary artery thrombosis [115-120], which are common to all forms of PH. PH in SCD and thalassemia is associated with vasoconstriction, vascular smooth muscle proliferation, and irregular endothelium in pulmonary arteries with associated thrombosis. These conditions all contribute to luminal narrowing, and eventual right ventricular failure (Fig. **5**) [121].

Secondary PH is emerging as one of the leading causes of mortality and morbidity in patients with hemolytic anemias [3, 28, 29]. Remarkably, PH develops in over 30% of adults with SCD, is associated with a 50% mortality risk within 2 years of diagnosis (Fig. **6**), and is strongly linked to hemolysis [29]. PH is also a common finding in children with SCD [121-129], yet the implications of this complication for the younger patients are unknown. Studies in both thalassemia intermedia and major demonstrate that adults frequently have undetected PH, with a prevalence of 50-75% reported [18-22, 25], while aggressive transfusion programs provide protection against development of PH [130]. Although the etiology of PH in these
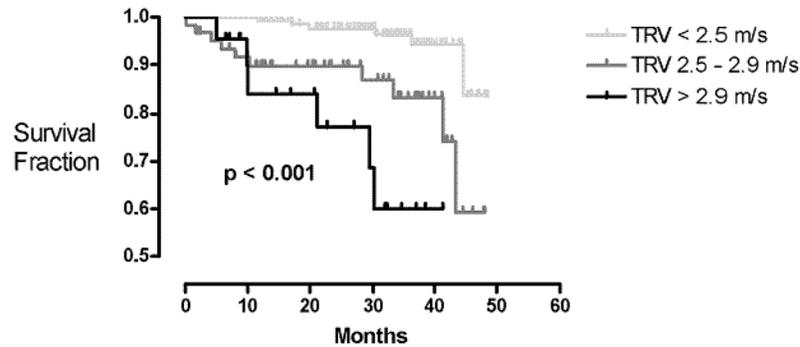
Fig. (6). Survival of patients with SCD according to tricuspid regurgitant jet velocity (TRV). Survival of 247 patients in the NIH SCD cohort is shown from time of screening echocardiogram. Median follow up is 30 months, result significant by logrank test for trend. Significantly greater survival is demonstrated in patients with normal pulmonary artery pressures (indicated by tricuspid regurgitant jet velocity < 2.5 m/sec on Doppler echocardiography) [29].

hemoglobinopathies is complex and multifactorial, it has been definitively linked to hemolytic rate and low NO bioavailability in both human [3, 28, 29, 33, 34] and sickle cell transgenic mouse studies [41, 97]. Endothelial dysfunction is also a result of an impaired arginine-NO pathway, oxidative stress and tissue damage, which enhances hypercoagulability and *in situ* thrombus formation within the pulmonary artery walls, contributing to functional and structural alterations of pulmonary vessels [131]. Oxidative stress is exacerbated by hemolysis [89, 132], the presence of iron overload [133] and through cycles of ischemia-and-reperfusion. Iron overload, present in transfused thalassemia and SCD patients, also affects PH by other mechanisms. It induces interstitial pulmonary fibrosis [134] as well as left and right cardiac hemosiderosis which subsequently results in cardiac dysfunction and affects pulmonary vascular resistance [134]. Chronic anemia and hypoxemia lead to further vasoconstriction and increase of pulmonary vascular resistance. Hypoxia is also shown to alter the von Willebrand factor (vWF) released by endothelial cells in secondary PH, affecting platelet activation and consumption [135]. Of interest, hypoxia has also recently been shown to upregulate arginase II and induce cell proliferation in human pulmonary artery smooth muscle cells [136]. This provides further evidence of the multifaceted role of arginase as a mechanistic trigger in the pathogenesis of PH.

Altered arginase activity within the platelets of patients with SCD likely contribute to pathological platelet activation, and the hypercoagulopathy and vasculopathy in this disorder [137]. High thrombin generation is known to occur in both SCD [138-140] and thalassemia syndromes [141, 142], and contributes to a chronic hypercoaguable state. Thrombin itself increases arginase activity in human endothelial cells [143-145], and will stimulate vascular smooth muscle cell polyamine synthesis by increasing CAT and ornithine decarboxylase gene expression [146], thus playing a role in endothelial dysfunction. Additionally, Villagra and colleagues have recently demonstrated that increased platelet activation in SCD correlates strongly with both PH severity and biomarkers of hemolysis [52]. A mechanistic model emerges here that links coagulation abnormalities to dysregulation of the arginine-NO pathway

in PH that has important implications for hemolytic disorders.

Asthma

The association of PH and arginine metabolites with pulmonary fibrosis and obstructive airway disease that results from airway and interstitial remodeling has not yet been studied in SCD. Asthma is common in SCD [99, 107, 125, 128, 147-155] and thalassemia [156], and is a condition associated with elevated arginase activity [103-105, 107, 109, 110, 157, 158] and an acute arginine [101, 106, 107], and NO deficiency [159-161]. It is likely that dysregulated arginine metabolism and excess production of proline and polyamines contribute to many forms of abnormal pulmonary function in SCD and other hemoglobinopathies [99]. In fact, we may learn a great deal about asthma itself from the "asthma-like condition" that occurs in SCD. There is controversy over the true prevalence of asthma in this population. Although a variable asthma prevalence of 30-70% has been reported in the literature [99, 125, 147-151, 162-164], there are few studies that have systematically and rigorously characterized the "asthma phenotype" in adults and children with SCD. In a retrospective review performed at the Northern California Comprehensive Sickle Cell Center, the presence of obstructive disease (alone or in combination with restrictive disease) interpreted by a pulmonologist was identified in 58% of the 124 adults and children screened by standard pulmonary function testing (PFTs) [99, 125]. This is a much higher prevalence than the expected 18-20% that is described in African Americans in California [165]. We have found that over 50% of SCD patients with obstructive disease in this study also have PH by echocardiography [99, 125]. Again, this is higher than the expected 30% prevalence of PH typically described in adults [29, 166] and children with SCD [121, 125-128].

Onyekwere and colleagues found an inverse correlation of forced expiratory volume in 1 second (FEV_1) with tricuspid regurgitant jet velocity on Doppler echocardiography [127], and we recently identified asthma as a variable associated with PH in children with SCD [125]. This relationship should be evaluated further, since patients with SCD may potentially be at risk for an asthma-

like condition triggered or worsened by hemolysis-driven release of erythrocyte arginase [27, 125], in addition to classic familial asthma [167]. It is unknown whether airway hyperresponsiveness is a contributing factor vs. the result of PH, yet it is possible that the disease co-morbidity is related to a common mechanism. Like PH, asthma is often unrecognized by clinicians. Yet it is treatable co-morbidity known to cause hypoxemia that is associated with stroke [162] and acute chest syndrome [147-149, 151, 154, 162, 163, 168] in addition to PH [125] and early mortality [164, 169]. Given the association of asthma with inflammation, oxidative stress, and hypoxemia, factors known to contribute to a vasculopathy in SCD [27], and the known consequences of these factors on sickle erythrocytes, co-morbid asthma would likely contribute to a vicious cycle of sickling and subsequent complications of SCD [99].

The use of corticosteroids is standard therapy for a moderate to severe acute asthma exacerbation, yet there is controversy around the use of corticosteroids in patients with SCD [170-174], and a potential link to rebound pain [175, 176]. It remains to be determined if this is an issue of insufficient tapering or actually a direct result of steroid use. Controlled clinical trials of standard of care therapies for asthma are critical in order to address these issues. However, until more data is available to guide clinicians, asthma in SCD should be aggressively managed based on established National Institutes of Health Guidelines for asthma management [177-181].

Interstitial Lung Disease

Interstitial lung disease is known to occur in SCD [182, 183], can be identified through the use of high resolution computed tomography [182], and is a condition associated with PH and a high mortality rate [184]. Diffusing capacity of carbon monoxide is a physiologic characteristic most highly correlated with high-resolution computed tomography in patients with idiopathic pulmonary fibrosis [185], and the second most common abnormal finding on pulmonary function tests in a large cohort of sickle cell patients reported on by Klings and colleagues [186]. Yet pulmonary fibrosis is one of the least studied pulmonary complications in SCD. Aberrarations in oxidant:antioxidant balance can lead to a variety of airway diseases, including asthma and pulmonary fibrosis [187], and likely play a contributing role. Hemolysis may also contribute to this complication through altered arginine metabolism and erythrocyte arginase release and a downstream increase in proline and polyamine production. Proline supply can play a crucial role in the development of pulmonary fibrosis. Both arginase I and arginase II have been implicated in the pathogenesis of bleomycin-induced pulmonary fibrosis [188] and together with high proline levels in patients with SCD [53], represents another potentially important pathway contributing to these pulmonary manifestation that warrants further study.

CONCLUSION

Abnormal arginase activity emerges as a recurrent theme in the pathogenesis of a growing number of diverse pulmonary disorders. Cystic fibrosis, chronic obstructive pulmonary disease and various forms of pulmonary fibrosis can be cataloged with asthma and PH mechanistically through an affiliation with arginase excess [103]. Arginase-induced arginine depletion, subsequent decreased NO production, increased NO consumption, NOS uncoupling and ornithine metabolism leading to cell proliferation represents a novel disease paradigm involved in nearly every form of PH. Our data in hemolysis-associated PH has identified erythrocyte arginase release as a significant contributing factor to PH severity and patient mortality in SCD [53]. Elevated arginase activity plays an underappreciated role in both asthma and PH in addition to its contribution in hemolytic disorders, and represents a unique pathway to target with future drug development.

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CONFLICTS OF INTEREST

Claudia R. Morris MD declares no conflicts of interest, but discloses that she is the inventor or co-inventor of several Children's Hospital & Research Center Oakland patent applications, including one for biomarkers of cardiovascular disease, has served on scientific advisory committees for Merck and Icagen, received an educational stipend from INO Therapeutics, and has been a consultant for Biomarin, Gilead Sciences, Inc., and the Clinical Advisors Independent Consulting Group.

ABBREVIATIONS

SCD	=	Sickle cell disease
NO	=	Nitric oxide
NOS	=	NO synthase
PH	=	Pulmonary hypertension
NADPH	=	Nicotinamide adenine dinucleotide phosphate
VCAM-1	=	Vascular cell adhesion molecule-1
VOC	=	Vaso-occlusive crisis
ACS	=	Acute chest syndrome
NOHA	=	N-hydroxy-L arginine
CAT	=	Cationic amino acid transporter
ADMA	=	Asymmetric dimethylarginine
DDAH	=	Dimethylarginine dimethylaminohydrolase

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