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Lactate dehydrogenase to carboxyhemoglobin ratio as a biomarker of heme release to heme processing is associated with higher tricuspid regurgitant jet velocity and early death in sickle cell disease

To the Editor:

Chronic hemolysis is a hallmark of sickle cell disease (SCD). Prior estimates of intravascular hemolysis as a proportion of the total hemolytic rate ranged from less than 10% to more than 30%. Chronic intravascular hemolysis is associated with severe vasculopathic complications including pulmonary hypertension and early mortality in SCD.[1] Intravascular hemolysis leads to release of cellular free hemoglobin and heme. Normally, these are scavenged by respectively haptoglobin and hemopexin. However, in SCD patients the availability of scavenging proteins in plasma is typically decreased because of rapid consumption by the prodigious amount of intravascular hemolysis. Both cellular free hemoglobin and heme, devoid of anti-oxidant buffering mechanisms normally present in red cells,[2] are recognized as erythroid damage-associated molecular pattern molecules (e-DAMPs) and contribute to development of chronic vasculopathy, platelet activation and pulmonary hypertension in SCD. Intravascular heme release is associated with elevated levels of serum lactate dehydrogenase (LDH).[1] All heme metabolized either via extravascular hemolysis or cleared from the plasma via scavenger proteins, will lead to endogenous carbon monoxide (CO) production by heme-oxygenase 1 (HO-1). In contrast to heme, CO has many protective effects including inhibition of polymerization of sickle hemoglobin, increased red blood cell hydration, anti-oxidative and anti-inflammatory effects.[3] Theoretically, the balance of detrimental intravascular hemolysis to beneficial CO production (total hemolysis) could be a prognostic biomarker in SCD. Because the vast majority of endogenous CO is cleared by exhalation, end-alveolar CO (EACO) in morning first breath is an accepted biomarker of endogenously produced CO, and thereby an accepted proxy marker of overall hemolysis. Before exhalation, CO is transported primarily as the conjugate carboxyhemoglobin (HbCO).

Here we explore whether plasma HbCO can be a convenient surrogate marker of endogenous CO production. In addition, we hypothesize that the ratio of LDH to CO may give an estimate of the relative contribution of intravascular heme release to total heme processing and relates to clinical endpoints, and therefore could be a new readily available biomarker of interest in SCD.

We investigated the relationship between EACO to HbCO plasma levels (n = 37; cohort [A]). After establishing a good correlation, we evaluated the relation of the ratio of LDH/HbCO to echocardiography parameters and mortality rates in adults with SCD at the NIH, Bethesda, USA (n = 157; cohort [B]). [NCT01547793; NCT0001648].

A thorough description of study population, trial conduct and statistical analysis is provided in supplemental section 1.

An overview of the cohorts' demographics, hematological and hemolytic parameters is provided in Table S1.

We confirmed the expected correlation among surrogate biomarkers of intravascular heme release (LDH, AST).

We identified a clear positive correlation of HbCO with EACO (r = 0.66, 95%CI [0.36, 0.81]) (Figure S1). However, in both cohorts, there was no consistent correlation of EACO or HbCO with either LDH or AST). On the other hand, in cohort (A), both EACO and HbCO were significantly, positively correlated with absolute reticulocyte counts (Table S2). Additionally, we explored variability in CO production in individual patients (Figure S2). The observations suggest that every patient has a finite HbCO range with minor variation over time and not significantly associated with changes in LDH in this limited dataset. Thus, the serum level of LDH (known to reflect intravascular hemolysis and other tissue injury, but not extravascular hemolysis) appears to be heterogeneous among SCD patients and does not directly represent total hemolysis as represented by HbCO. This interpretation led us to formulate that the fraction of total hemolysis that is intravascular might more directly correlate with vascular health outcomes.

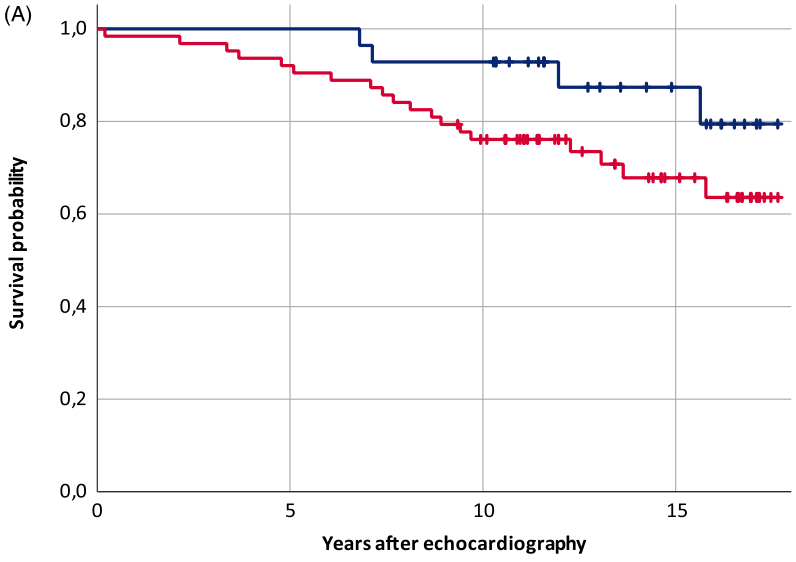
Next, we investigated whether an increased fraction of intravascular hemolysis, that is an increased ratio of LDH to HbCO, was related to clinical endpoints (n = 92 patients; 86 HbSS, three HbSβ^+, three HbSC; median age 30 years IQR 22; 40). Previously, tricuspid regurgitation velocity (TRV) ≥3.0 m/s was associated with the highest risk of development of pulmonary hypertension and increased mortality in SCD. Patients in this high-risk category (n = 13) had a significantly higher LDH/HbCO ratio compared to the other patients ($p = 0.02$) (Figure S3A). There was no significant difference in LDH values between groups. Individual median LDH/HbCO ratios and TRV were significantly correlated (r = 0.38, 95% CI [0.12, 0.60]. However, LDH and TRV were not correlated (r = 0.22, 95% CI [−0.10, 0.52]) (Figure S3B). Of interest, a cutoff value of LD/HbCO ratio of 1200 could be used to exclude patients with high risk TRV values: all patients (n = 25) with LDH/HbCO ratio < 1200 had a TRV <3.0 m/s. The LDH/HbCO ratio was greater than 1200 in 15/16 patients (94%) with catheterization-proven pulmonary hypertension. (supplemental section 2).

Median follow-up was 12.1 years (IQR 10.3, 16.2). During follow-up, 25% (23/91) of the patients died (median age of death 42 years IQR 35, 58); 48% (11/23) were initially diagnosed with pulmonary hypertension based on pulmonary artery catheterization.

All-cause mortality predictability by LDH/HbCO ratio and LDH was analyzed in a Cox proportional hazard model (Table S3). The LDH/HbCO ratio was significantly associated with mortality in the unadjusted analysis ($p < 0.01$). Age, CRP and ferritin were previously reported to be related to early-mortality in SCD. In our cohort age ($p = 0.03$), CRP ($p = 0.05$) and ferritin ($p < 0.01$) were associated with mortality, as was TRV ($p < 0.01$). In the multivariate-adjusted analysis including age, CRP and ferritin, LDH/HbCO ratios remained significantly associated with all-cause mortality ($p = 0.02$). There was a clear trend toward association between LDH and all-cause mortality in the unadjusted analysis ($p = 0.10$), however, it did not reach statistical significance in our cohort. Kaplan–Meier survival probabilities for patients stratified by LDH/HbCO ratios greater than and less than 1200 are shown in Figure 1.

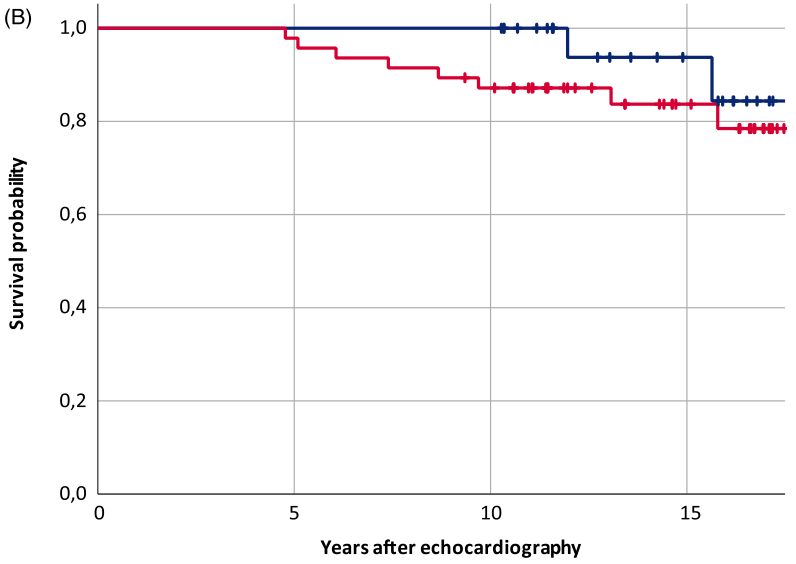
In summary, the LDH/HbCO ratio predicts survival probability independent of age, ferritin and CRP.

The products of intravascular red cell lysis have shown to be of major importance in the pathophysiology of vasculopathy in SCD. Infusion of high dose hemoglobin or heme in SCD mice induces lethal vaso-occlusive events.[4] In contrast, low-dose heme and subsequent induction of HO-1 and production of the gasotransmitter CO is protective against vaso-occlusion in SCD mice.[4] Therefore, it is biologically plausible that the LDH/HbCO ratio, representing an increase in the amount of CO, as product of heme processing, relative to the



No. at risk

LD/HbCO < 1,200	28	28	26	11
LD/HbCO > 1,200	63	58	46	18



No. at risk

LD/HbCO < 1,200	25	25	25	10
LD/HbCO > 1,200	47	46	40	17

FIGURE 1 Survival analysis. Kaplan–Meier estimates of overall survival for patients with LD/HbCO ratios >1200 and patients with LD/HbCO ratios <1200. Panel A Survival probability of all patients independent of tricuspid regurgitation velocity (TRV). In the group patients with LD/HbCO values <1200 5-year overall survival (OS) was 100%, 10-year OS 92.9% and 15-year OS 88.0%. In the group patients with LD/HbCO values >1200 5-year OS was 92.1%, 10-year OS 76.0% and 15-year OS 69.1%. Panel B. Survival probability in the group of patients with TRV values <3.0 m/s. In the group patients with LD/HbCO values <1200 5-year and 10-year OS was 100%, 15-year OS was 94.4%. In the group patients with LD/HbCO values >1200 5-year OS was 97.9%, 10-year OS 87.1% and 15-year OS 84.1%

amount of intravascular heme release, provides useful information on characterization of hemolysis in SCD and its vasculotoxicity.

Excess cell free hemoglobin and heme, not cleared from the circulation via scavenger proteins, are excreted in the urine. Indeed, hemoglobinuria is detectable in many patients with SCD, and associated with biomarkers of intravascular hemolysis. This urinary excretion of heme somewhat confounds the interpretation of CO release as an indication of total hemolysis. However, the amount of excreted heme is limited compared to total red cell turnover. Therefore, we consider that HbCO is generally reflective of the total amount of CO production due to adaptive heme catabolism.

Serum LDH has been the most widely used marker of intravascular hemolysis associated with catheterization-proven pulmonary hypertension or elevated TRV in SCD cohorts. Elevated TRV in SCD was significantly associated with mortality in a recent meta-analysis, but an inconsistent association was observed between LDH and mortality.[5] Notably, the current cohort on whom we have CO, LDH and mortality data is a subset with less statistical power than some of the prior datasets. As such, this present study may be underpowered to replicate prior LDH-mortality associations. Although all patients diagnosed with SCD were eligible for enrollment in these trials our cohort was enriched for SCD patients at risk for vasculopathic complications and/or early death. In this relatively small cohort, we show that the ratio of LDH to HbCO is superior to predict either elevated TRV or mortality as compared to LDH alone. These results suggest that the LDH/HbCO ratio may enhance the utility of LDH to identify patients at risk for vasculopathic complications and early death in SCD.

One limitation of our study is the presentation of HbCO values in relation to other hemolytic parameters in two cohorts that differ in regard to patient selection and size. The EACO cohort (A) was prospective and smaller; and, the HbCO cohort (B) was larger but retrospective and, therefore, may be biased toward a more acutely or chronically affected subpopulation for whom HbCO data were available. Altogether, HbCO results must be considered exploratory and in need of additional validation. The results here do not invalidate previously published correlations of serum LDH from larger studies with greater statistical power. Our new results highlight the potentially greater strength of the LDH/HbCO ratio.

In conclusion, a ratio of two readily available clinical laboratory markers, LDH and HbCO, representative of the intravascular fraction of total hemolytic rate, is promising as biomarker in SCD. Increased LDH/HbCO ratios are strongly associated with increased risk on pulmonary hypertension, the pathophysiologic complication of intravascular hemolysis, and all-cause mortality. And thereby may add to individual risk prediction in SCD patients. We suggest inclusion of these markers in future prospective trials in SCD for validation.

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CONFLICT OF INTEREST

C.P.M.: Novartis, Global Blood Therapeutic, Emmaus, Forma, Roche, CSL Behring, Chiesi. BlueBird Bio: end point adjudicator. G.J.K.: Novartis, Global Blood Therapeutics: research funding and consultancy. Bayer: research funding. CSL Behring: current employment. E.J.B.: Novartis: research funding and consultancy. Agios: research funding and Membership advisory committee. Pfizer, RR Mechatronics: research funding.

AUTHOR CONTRIBUTIONS

A.J.V. analyzed and interpreted the data and wrote the manuscript; C.P.M. participated in the writing of the protocols, enrollment of subjects and analyzed data; L.M. participated in the writing of the protocols, enrollment of subjects and collection of the data; J.H.B. wrote clinical trial and laboratory protocols, participated in subject enrollment, collected and analyzed data (all EACO data); G.J.K. wrote clinical trial and laboratory protocols, supervised subject enrollment and laboratory data collection; E.J.B. interpreted the data, collected and analyzed data. All authors revised the paper critically and approved of the submitted version.

DATA AVAILABILITY STATEMENT

The aggregated data and analyses that support the findings of this study are available upon request from the corresponding author. Other data related to the patients are curated at the NIH, NHLBI, Sickle Cell Branch.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Multiple thrombosis in a patient with Gardos channelopathy and a new *KCNN4* mutation

To the Editor:

Dehydrated hereditary stomatocytosis (DHS) is an autosomal dominant hemolytic disease characterized by red blood cell dehydration due to increased membrane permeability to cations. This default in permeability leads to cell volume dysregulation and morphologic abnormalities. Note, DHS is characterized by heterogeneous phenotypic presentation, varying from hydrops foetalis to mild anemia or asymptomatic well-compensated hemolysis.[1] Two subtypes have been molecularly defined, DHS1 due to *PIEZO1* gain-of-function mutations (representing 90% of all cases) and DHS2 (also named Gardos channelopathy) due to *KCNN4* gain-of-function mutations. Gardos channelopathy is a very rare subtype with less than a dozen families reported so far. So, *KCNN4* encodes the Potassium Calcium-Activated Channel Subfamily N Member 4 in which gain of function mutations result in increased sensitivity to calcium influx, leading to KCl loss and cell dehydration.[1–4] Previous reports highlighted differences between the two forms of DHS. Although iron overload can be observed in both entities, perinatal edema and post-splenectomy thrombosis have mostly been reported in DHS1 so far, while severe anemia appears to be a frequent manifestation of DHS2. Only a few *KCNN4* mutations have been identified to date: V282M, V282E, S314P, R352H and V369_Lys373del, whereas more than 40 different mutations have been reported in *PIEZO1*. Here, we report on a patient with Gardos channelopathy syndrome, which harbored a novel *KCNN4* mutation and suffered from several episodes of venous and arterial thrombosis after splenectomy.

The proband is a 49-year-old man referred to our center for undiagnosed hemolytic anemia. He had no family history of anemia or hemolysis. Hemolysis was first diagnosed at 1 month of age, in a context of infectious disease. The patient had a severe anemia (Hb = 3.7 g/dl) requiring transfusion. He then suffered from several hemolytic crises during childhood, generally related to infectious episodes. An abdominal painful crisis at age 10 led to a splenectomy. After splenectomy, hemoglobin stabilized between 8 and 9 g/dl; hemolysis persisted with undetectable haptoglobin levels and high levels of plasma hemoglobin and free heme, and platelet count increased to 700 000–800 000/μl. At age 30, he was investigated for hyperferritinemia (3193 μg/L). Liver biopsy showed iron overload and portal and periportal fibrosis. The *HFE* gene sequencing was negative. Phlebotomy was then initiated. During the same year, he underwent cholecystectomy for gallstones, which was complicated by catheter-related thrombosis in the left arm. Fluindione (vitamin K antagonist used in Europe, same family as warfarin) was introduced for a period of 3 months. Shortly after the withdrawal of fluindione, he developed facial hemiparesia, revealing an ischemic stroke due to middle cerebral artery thrombosis. Extensive thrombosis investigations showed normal results (absence of FII G20210A and FV Leiden mutations; normal levels of antithrombin III, homocysteinemia, proteins S and C; absence of anticardiolipin antibodies) and there was no family history of thrombosis. Fluindione was reintroduced. Two months later, he was hospitalized for erysipelas complicated by left peroneal vein thrombosis. The INR at admission was 1.92. Despite treatment with Low Molecular Weight Heparin and increased doses of fluindione, thrombosis extended to the left femoral vein. Within 3 days, platelet count increased from 700 000/μl to 2 000 000/μl. Hydroxyurea was introduced but rapidly replaced by anagrelide because it was ineffective. Bone marrow study, performed while the patient was under hydroxyurea treatment, showed a rich marrow with marked signs of dyserythropoiesis, as already described in Gardos patients[3]: there were numerous binucleated erythroblasts, very rare intercytoplasmic bridges and internuclear bridges, but no evidence of dysgranulopoiesis or dysmegacaryopoiesis. Under anagrelide treatment, platelet count decreased to 800 000/μl. Since then, under a combined treatment with fluindione and anagrelide and despite the fact that he had several episodes of erysipelas during the last 19 years, he had no subsequent thrombotic event. Platelet count remained between 300 000 and

ABBREVIATIONS: DHS, dehydrated stomatocytosis; ET, essential thrombocythemia; Lcw, liter cell water; NGS, next generation sequencing; RBC, red blood cell; VKA, vitamin K antagonist; VUS, variant of uncertain significance.