

6-1-2015

Dysregulated Arginine Metabolism and Cardiopulmonary Dysfunction in Patients with Thalassaemia

Claudia Morris

Hae-Young Kim
New York Medical College

Elizabeth Klings

John Wood

John B. Porter

See next page for additional authors

Follow this and additional works at: https://touro scholar.touro.edu/nymc_fac_pubs



Part of the [Medicine and Health Sciences Commons](#)

Recommended Citation

Morris, C., Kim, H., Klings, E., Wood, J., Porter, J. B., Trachtenberg, F., Kuypers, F., Vichinsky, E., & Kuypers, F. (2015). Dysregulated Arginine Metabolism and Cardiopulmonary Dysfunction in Patients with Thalassaemia. *British Journal of Haematology*, 169 (6), 887-898. <https://doi.org/10.1111/bjh.13452>

This Article is brought to you for free and open access by the Faculty at Touro Scholar. It has been accepted for inclusion in NYMC Faculty Publications by an authorized administrator of Touro Scholar. For more information, please contact touro.scholar@touro.edu.

Authors

Claudia Morris, Hae-Young Kim, Elizabeth Klings, John Wood, John B. Porter, Felicia Trachtenberg, Frans Kuypers, Elliott P Vichinsky, and Frans A Kuypers

Author(s) ORCID Identifier:

<http://orcid.org/0000-0001-7916-6625>



Published in final edited form as:

Br J Haematol. 2015 June ; 169(6): 887–898. doi:10.1111/bjh.13452.

Dysregulated Arginine Metabolism and Cardiopulmonary Dysfunction in Patients with Thalassaemia

Claudia R. Morris, MD¹, Hae-Young Kim, MS, DrPh², Elizabeth S. Klings, MD³, John Wood, MD⁴, John B. Porter, MD⁵, Felicia Trachtenberg, PhD², Nancy Sweeters, RN, PNP⁶, Nancy F Olivieri, MD⁷, Janet L Kwiatkowski, MD, MSCE⁸, Lisa Virzi², Kathryn Hassell, MD⁹, Ali Taher, MD¹⁰, Ellis J Neufeld, MD, PhD¹¹, Alexis A. Thompson, MD¹², Sandra Larkin¹³, Jung H. Suh, PhD¹³, Elliott P Vichinsky, MD⁶, Frans A. Kuypers, PhD¹³, and on behalf of the Thalassaemia Clinical Research Network¹⁴

¹ Department of Pediatrics, Division of Emergency Medicine, Emory University School of Medicine, Emory Children's Center for Cystic Fibrosis and Airways Disease Research, Atlanta, GA

²New England Research Institutes Watertown, MA

Address for Correspondence: Claudia R. Morris MD Associate Professor of Pediatrics and Emergency Medicine Emory-Children's Center for Cystic Fibrosis and Airways Disease Research Emory University School of Medicine 1760 Haygood Drive, NE W458 Atlanta, GA 30329 Telephone: (404); 727-5500 Fax: 404-785-7989 claudia.r.morris@emory.edu.

¹⁴This is publication number 34 of the Thalassaemia Clinical Research Network (TCRN). A list of TCRN member institutions and staff appears in Appendix 1.

Abstract Publication: An abstract of this work was presented at the American Society of Hematology for their annual meeting held December 2012, in Atlanta GA.

Authorship Contributions

All authors take responsibility for the work reported, provided substantial contributions to the conception and design, acquisition of data, analysis and interpretation of the data. All authors contributed to the draft or critical revision of this manuscript, and provided final approval for the version to be published. Specifically, CM: Designed research questions, wrote study protocol, analysed and interpreted data and wrote/revised the manuscript. HYK: Assisted with data analysis, statistical design and writing of the manuscript. ESK: Assisted with interpretation of the cardiopulmonary data (particularly pulmonary function testing and echocardiography) and contributed to the writing and critical revision of the manuscript. JW: Assisted with interpretation of the data, particularly the cardiopulmonary data (Doppler echocardiography), and contributed to the writing and critical revision of the manuscript. JBP: Assisted with patient enrollment and writing/revision of the manuscript. FT: Assisted with data analysis, statistical design and critical review of the manuscript. NS: Assisted with patient enrollment, data collection and manuscript review. NFO: Assisted with patient enrollment and writing/revision of the manuscript. JLK: Assisted with patient enrollment and writing/revision of the manuscript. LV: Assisted with protocol design, execution and compliance, and critical review of the manuscript. KH: Assisted with interpretation of data and critical review/revision of the manuscript. AT: Assisted with patient enrollment, interpretation of the data and writing/revision of the manuscript. EJN: Assisted with data interpretation, patient enrollment and writing/revision of the manuscript. AAT: Assisted with patient enrollment and critical review of the manuscript. SL: Performed analyses of biological samples, assisted with interpretation of the data and critical review of the manuscript. JHS: Performed analyses of biological samples, assisted with interpretation of the data and critical review of the manuscript. EPV: Assisted with study design, data interpretation, patient enrollment and review of the manuscript. FAK: Assisted with study design, performed analyses of biological samples, assisted with interpretation of the data and critical review of the manuscript.

DISCLOSURES

All authors declare no conflicts of interest relevant to this study. Claudia R. Morris, MD, is the inventor or co-inventor of several Children's Hospital & Research Center Oakland patents/patent-pending applications that include biomarkers of cardiovascular disease related to arginine bioavailability, is an inventor of an Emory University School of Medicine patent application for a nutritional supplement, is a consultant for Pfizer, NourishLife, LLC and Endeavor Therapeutics, and has received research support from MAST Therapeutics. Ellis Neufeld, MD has received research support from Ferrokin Biosciences, Inc, and from Novartis. Ali T. Taher, MD has received research support from Novartis. John Wood, MD has received research support from Ferrokin Biosciences, Inc, and from Novartis. Elizabeth Klings and Elliott Vichinsky are consultants for Pfizer. Elliott Vichinsky has also received support from Bayer and MAST Therapeutics. Frans Kuypers is consultant for Bayer, RadioRx, GBT, Teruma and Cellgene and has received support from Bayer, GBT, Teruma, CellGene through rblab. All other authors report no disclosures, conflicts of interest or relationships with industry.

³ The Pulmonary Center, Boston University School of Medicine, Boston, MA

⁴ Children's Hospital of Los Angeles, Los Angeles, CA

⁵ University College London, London, UK

⁶ Hematology/Oncology, UCSF Benioff Children's Hospital Oakland, Oakland, CA

⁷ Toronto General Hospital, Toronto, ON, Canada

⁸ Children's Hospital of Philadelphia, Philadelphia, PA

⁹ Division of Blood Diseases and Resources, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

¹⁰ American University of Beirut, Beirut, Lebanon

¹¹ Children's Hospital Boston, Boston, MA

¹² Hematology, Oncology & Stem Cell Transplant, Children's Memorial Hospital, Chicago, IL

¹³ Children's Hospital Oakland Research Institute, Oakland, CA

Abstract

Pulmonary hypertension (PH) commonly develops in thalassaemia syndromes, but is poorly characterized. The goal of this study was to provide a comprehensive description of the cardiopulmonary and biological profile of patients with thalassaemia at risk for PH. A case-control study of thalassaemia patients at high versus low PH-risk was performed. A single cross-sectional measurement for variables reflecting cardiopulmonary status and biological pathophysiology were obtained, including Doppler-echocardiography, 6-minute-walk-test, Borg Dyspnea Score, New York Heart Association functional class, cardiac magnetic resonance imaging (MRI), chest-computerized tomography, pulmonary function testing and laboratory analyses targeting mechanism of coagulation, inflammation, haemolysis, adhesion and the arginine-nitric oxide pathway. Twenty-seven thalassaemia patients were evaluated, 14 with an elevated tricuspid-regurgitant-jet-velocity (TRV) ≥ 2.5 m/s. Patients with increased TRV had a higher frequency of splenectomy, and significantly larger right atrial size, left atrial volume and left septal-wall thickness on echocardiography and/or MRI, with elevated biomarkers of abnormal coagulation, lactate dehydrogenase levels and arginase concentration, and lower arginine-bioavailability compared to low-risk patients. Arginase concentration correlated significantly to several echocardiography/MRI parameters of cardiovascular function in addition to global-arginine-bioavailability and biomarkers of haemolytic rate, including lactate dehydrogenase, haemoglobin and bilirubin. Thalassaemia patients with a TRV ≥ 2.5 m/s have additional echocardiography and cardiac-MRI parameters suggestive of right and left-sided cardiac dysfunction. In addition, low arginine bioavailability may contribute to cardiopulmonary dysfunction in β -thalassaemia.

Keywords

Arginase; β -thalassaemia; global arginine bioavailability ratio; haemolysis; pulmonary hypertension

INTRODUCTION

Cardiovascular complications are among the leading causes of mortality and morbidity in β -thalassaemia (Anthi, *et al* 2013, Farmakis and Aessopos 2011, Morris and Vichinsky 2010). It has been postulated that haemoglobinopathies, along with human immunodeficiency virus infection and schistosomiasis, may be the most common causes of pulmonary hypertension (PH) worldwide (Machado and Farber 2013), given the high prevalence of these diseases globally. In particular, PH develops frequently in patients with haemolytic anemias (Aessopos, *et al* 2005, Farmakis and Aessopos 2011, Morris 2008), including β -thalassaemia. An elevated tricuspid-regurgitant-jet-velocity (TRV) ≥ 2.5 m/s on Doppler echocardiography (echo) is a common finding in patients with thalassaemia (Morris, *et al* 2011) and can identify those at increased risk for PH, although a right heart catheterization (RHC) is required to confirm a PH diagnosis. In patients with sickle cell disease (SCD), another haemolytic anaemia commonly associated with PH, RHC-defined PH is found in 6-11% of patients and is associated with a high mortality risk (Fonseca, *et al* 2012, Gladwin 2011, Mehari, *et al* 2012, Parent, *et al* 2011). An elevated TRV does not carry the same short-term mortality risk in thalassaemia patients that is observed in SCD (Morris, *et al* 2011), however the long-term consequences of PH in β -thalassaemia remain unknown, routine screening is not yet common practice, at-risk patients are not well characterized and few patients receive PH therapy (Morris and Gladwin 2011, Morris and Vichinsky 2010). Pathophysiological changes of PH have been demonstrated postmortem on autopsy studies in thalassaemia intermedia (TI) (Sonakul, *et al* 1988), and a recent multi-centre study using RHC to screen β -thalassaemia patients with a TRV ≥ 3.2 m/s revealed a 2.1% prevalence of PH among 1309 Italian thalassaemia patients screened by echocardiography (Derchi, *et al* 2014). There was a 5-fold higher prevalence of PH in TI patients compared to thalassaemia major (TM) (Derchi, *et al* 2014), suggesting that the prevalence of PH in TI is similar to that of SCD (Gladwin 2011, Parent, *et al* 2011). However, not all at-risk patients underwent RHC and a TRV >3.2 m/s is a conservative cut-off for screening that may underestimate the true prevalence, particularly since 31 out of 33 thalassaemia patients (94%) who underwent a RHC ultimately had confirmed PH (Derchi, *et al* 2014). Advanced age, splenectomy, transfusion-naivety and an elevated nucleated red blood cell count are associated with PH in β -thalassaemia (Derchi, *et al* 2014, Karimi, *et al* 2011, Morris, *et al* 2011, Morris and Vichinsky 2010, Singer, *et al* 2014, Singer, *et al* 2006). The aetiology of PH is multifactorial, and may include the long-term effects of splenectomy, erythrocyte cell membrane pathology, coagulation abnormalities/thrombosis, reduced nitric oxide (NO) bioavailability, excess arginase activity, platelet activation, oxidative stress, iron overload, chronic haemolysis and the anaemia itself (Aessopos, *et al* 2005, El-Hady, *et al* 2012, Morris 2008, Morris, *et al* 2013a, Morris, *et al* 2007, Morris and Vichinsky 2010, Singer, *et al* 2014, Singer, *et al* 2006). In addition, the process of haemolysis disables the arginine-NO pathway through the simultaneous release of erythrocyte-arginase and cell-free haemoglobin, where both NO and its obligate substrate arginine are rapidly consumed (Morris, *et al* 2005a, Morris, *et al* 2005b, Morris, *et al* 2007, Morris 2008, Reiter, *et al* 2002).

Given the paucity of data available characterizing patients with a thalassaemia syndrome at risk for PH, the Thalassaemia Clinical Research Network (TCRN) performed a case-control study to provide a comprehensive description of the cardiopulmonary and biological profile of thalassaemia patients with an elevated TRV (≥ 2.5 m/s) compared to normal TRV (<2.5 m/s).

METHODS

Subject Recruitment and Clinical Evaluation

The TCRN is a National Institutes of Health-sponsored network of major thalassaemia centres in the US, Canada, UK and Lebanon. All patients were enrolled at participating TCRN centres (Beirut, Boston, Chicago, London, Oakland, Philadelphia and Toronto). Local institutional review boards approved the protocol and written informed consent was obtained from all subjects. Subjects with a thalassaemia syndrome (alpha, beta or E-beta-thalassaemia), confirmed by haemoglobin electrophoresis or by molecular diagnosis, aged ≥ 7 years old with a measurable TRV on Doppler-echocardiography were eligible. Patients undergoing chronic transfusion therapy had evaluations within 7-10 days prior to their next scheduled transfusion. A patient requiring < 8 transfusions/year was characterized as TI.

Study Design

This is a case-control study of thalassaemia patients at risk for PH, defined by a TRV ≥ 2.5 m/s on Doppler-echocardiography compared to patients with a thalassaemia syndrome at lower risk for PH, defined by a TRV <2.5 m/s. A single cross-sectional measurement for variables reflecting cardiopulmonary status and biological pathophysiology were obtained, including vital signs, a 6-minute-walk-test (6MWT) with assessment of Borg Dyspnea Score and New York Heart Association (NYHA) functional class, echo with measurement of TRV (m/s), pulmonary function testing including spirometry, lung volumes and gas exchange capacity of the lungs by single-breath diffusion capacity for carbon monoxide (D_LCO), cardiac magnetic resonance imaging (MRI), chest computerized tomography (CT) scans, routine laboratory measurements and assays for biomarkers of coagulation, inflammation, haemolytic rate, the arginine-NO pathway and adhesion molecules. (See Supplement Table 1 for list of all biological assays measured).

Doppler Echocardiography

Echocardiography was performed according to the American Society of Echocardiography guidelines at the participating institutions and read locally. Eligibility confirmation evaluation of TRV on screening echocardiogram was blindly read centrally at the Brigham and Women's Hospital Cardiovascular Imaging Core Laboratory (Core Lab). Echocardiographers were centrally trained and certified for this protocol. Examinations were recorded on separate tapes for each participant and copies sent to the Core Lab for central analysis.

See supplemental for detailed methods for the 6MWT, pulmonary function testing, MRI and chest CT scans.

Laboratory Studies

Routine laboratory tests (complete blood count, serum chemistry profile and ferritin) were performed in the local laboratories of the participating institutions. The plasma and erythrocyte arginine metabolites were analysed via high pressure liquid chromatography-linked tandem mass spectrometry (LC/MS/MS), as previously described (Morris, *et al*, 2008, Morris, *et al* 2013a). Arginase activity and concentration, biomarkers of haemolysis, coagulation, inflammation and adhesion were measured through standard methods by the Frans Kuypers laboratory at Children's Hospital Oakland Research Institute (Red Blood Cell Laboratory; rbclab.com) and through LabCorp – Esoterix Clinical Trials (Cranford, NJ; www.labcorp.com).

Arginase concentration—Arginase concentration was measured using a double monoclonal sandwich enzyme-linked immunoassay (ELISA) for the quantitative measurement of human liver-type arginase in serum (BioVendor Laboratory Medicine, Inc., Chandler, NC) according to the manufacturer's protocol.

Arginase activity—Arginase activity was determined as the conversion of [^{14}C -guanidino]-L-arginine to [^{14}C]urea, which was converted to $^{14}\text{CO}_2$ by urease and trapped as $\text{Na}_2^{14}\text{CO}_3$ for scintillation counting, as previously described (Morris, *et al* 2005b, Morris, *et al* 2013a).

Statistical Analysis

Descriptive statistics were reported as the number and percentage, or the mean and standard deviation. Differences in categorical variables were tested by Fisher's exact test and differences in continuous variables were tested by t-test and analysis of variance (ANOVA). Correlation analysis and linear regression were used to test for associations between arginase activity/concentration, TRV, laboratory parameters and other covariates of interest. Multiple linear regression analysis with stepwise selection was used to evaluate predictors of arginase activity/concentration. Variables that were significant in the univariate analysis ($P < 0.05$) were included. Log-transformation was used as needed to correct for skew in the data. All analyses were performed at the Data Coordinating Center (New England Research Institutes, Watertown, MA) with SAS statistical software (9.2, SAS Institute, Cary, NC) and R (2.11.1, The R Foundation for Statistical Computing, <http://www.r-project.org/>). P -values < 0.05 were considered statistically significant.

RESULTS

Subject Characteristics

Twenty-seven patients with a thalassaemia syndrome were included in this study. The cohort is notable for a high prevalence of splenectomy, paucity of patients transfused since infancy, a relatively high average pre-transfusion haemoglobin and 66.7% of patients with TM. Patient demographics and clinical characteristics are summarized in Table I. Patients with a history of a high TRV ≥ 2.5 m/s ($n=14$) were prospectively recruited and screened by echocardiography at participating sites. A control group of thalassaemia patients with a normal TRV (< 2.5 m/s, $n=13$) were also recruited for participation. Mean TRV for the TRV

2.5 m/s group was 3.1 ± 0.7 m/s (range: 2.7-5.3 m/s). Splenectomy was common in patients with an elevated TRV 2.5m/s (Odds Ratio=11.1 [95% confidence interval [CI]:1.11-112.0], $p=0.04$).

Cardiopulmonary Evaluations

Cardiopulmonary characteristics of patients with thalassaemia according to TRV categories (TRV<2.5, TRV=2.5-2.8, TRV 2.9 m/s respectively) are summarized in Table II. The concordance correlation coefficient for baseline TRV measurements (local site interpretation compared to Core Lab) was 0.94 (95% CI: 0.90-0.99), which demonstrated an excellent agreement between sites. The concordance correlation coefficient measures the agreement between measurements, to evaluate reproducibility or for inter-rater reliability. Systolic and diastolic blood pressure, heart rate and pulse pressure were similar across TRV categories. Patients with an increased TRV had a higher NYHA functional class and Borg Dyspnea Score, suggestive of increased dyspnea after the 6MWT compared to normal TRV controls.

Echo measurements of right atrial size ($p=0.03$), left atrial size ($p=0.002$), left ventricular (LV) mass ($p=0.03$) and LV septal wall thickness ($p=0.03$) were significantly larger in patients with a TRV 2.9 and TRV=2.5-2.8 m/s compared to patients with a TRV<2.5 m/s and remained significantly different when parameters were indexed to body surface area (BSA). MRI measurements of left atrial volume were significantly higher in patients with TRV 2.5 m/s compared to those with a TRV<2.5 m/s ($p=0.008$). TRV correlated to right atrial size (cm^2) ($r=0.5$, $p=0.01$) by echo, right ($r=0.65$, $p=0.01$) and left atrial volume ($r=0.56$, $p=0.03$) on MRI and creatinine ($r=0.42$, $p=0.03$) but not to other cardiopulmonary parameters or clinical/laboratory biomarkers. In particular there were no associations of TRV with 6-minute walk distance (6MWD, $r=0.13$, $p=0.51$), brain natriuretic factor (BNP, $r=0.01$, $p=0.99$) or biomarkers of haemolytic rate including LDH ($r=0.25$, $p=0.22$). Although there was no significant difference in 6MWD based on TRV categories, the 6MWD paradoxically trended higher in patients with a TRV 2.5m/s. The 6MWD also paradoxically correlated to right atrial size ($r=0.42$, $p=0.03$), as well as D_LCO ($r=0.51$, $p=0.008$) and D_LCO corrected for haemoglobin ($r=0.54$, $p=0.005$). D_LCO was generally low in our cohort of thalassaemia patients (mean % predicted: 68.2 ± 16.3 [range 45-115, $n=27$]), however other measurements of pulmonary function were within the normal range and did not differ significantly by TRV. However, 29.6% (8/27) of patients had an forced expiratory volume in 1 s (FEV_1)% predicted < 80%, but a normal FEV_1 /forced vital capacity (FVC) suggestive of restrictive physiology.

Chest CT scanning of these patients revealed areas of intramedullary haematopoiesis within the vertebrae and ribs consistent with thalassaemia. Approximately 1/3-1/2 of patients had normal lung parenchyma regardless of TRV. The rest had a combination of ground glass infiltrates, mosaic attenuation and/or sub-centimetre pulmonary nodules. The small number of patients in each group limited our ability to determine if a different pattern was observed with increasing TRV. Interestingly, pulmonary artery size, a non-specific finding in PH, was not significantly different between those patients with an elevated TRV compared to those with a normal TRV.

Laboratory Parameters

Routine clinical laboratory tests and biomarkers of haemolysis, arginine bioavailability, coagulation, inflammation and adhesion were analysed and compared by TRV sub-groups (Table III; see Supplemental Table I for list of all analyses). Lactate dehydrogenase (LDH) was significantly higher in patients with an elevated TRV compared to those with a TRV <2.5 m/s ($p=0.03$), as were biomarkers of abnormal coagulation (thrombin-anti-thrombin [TAT] complex, $p=0.04$, and monoclonal prothrombin fragment 1.2 [PF12 mono], $p=0.02$). Plasma arginine concentration and biomarkers of global arginine bioavailability (GAB, plasma arginine/ornithine ratio and arginine/(ornithine+citrulline) ratio) were significantly lower in patients with a TRV ≥ 2.5 m/s compared to those with a normal TRV, while arginase concentration and activity were high (Figure 1).

The relationship between arginase activity/concentration and clinical as well as laboratory markers of disease severity were evaluated to identify potential mechanisms for increased enzymatic activity and associated effects on organ function (Table IV). As expected, arginase activity and concentration strongly correlated to each other ($r=0.74$, $p<0.0001$). Arginase concentration and activity similarly correlated significantly to several echocardiography and cardiac-MRI parameters of cardiovascular function in addition to global arginine bioavailability (Table IV, correlations for arginase concentration reported) and biomarkers of haemolytic rate, including LDH, haemoglobin and bilirubin. These associations were more profound in TI patients (arginase concentration Pearson correlation to plasma arginine ($r=-0.72$, $p=0.04$), plasma arginine/ornithine ratio ($r=-0.82$, $p=0.01$), and plasma arginine/(ornithine+citrulline) ratio ($r=-0.79$, $p=0.02$; $N=9$). No significant association of arginase with alanine transaminase argues against hepatic arginase as a major source of excess arginase concentration in our cohort of patients with thalassaemia.

Although arginase concentration correlated with white blood cell count, no other associations with biomarkers of coagulation or inflammation were identified. Interestingly, arginase activity correlated with several biomarkers of coagulation, including PF12 mono ($r=0.40$, $p=0.04$), TAT complex ($r=0.41$, $p=0.04$) and tissue factor concentration ($r=0.45$, $p=0.02$). Arginase activity also correlated strongly with BNP ($r=0.51$, $p=0.007$), while arginase concentration showed no relationship ($r=0.17$, $p=0.42$). Of the adhesion markers measured, arginase concentration correlated directly with soluble Intercellular Adhesion Molecule 1 ($r=0.41$, $p=0.04$) and inversely with L-selectin concentration ($r=-0.44$, $p=0.03$). In multiple regression analysis, variables significantly associated with arginase concentration ($p<0.05$) were considered in a stepwise selection process. Cardiac index, bilirubin and plasma arginine/ornithine ratio remained significant in the final model with arginase concentration ($R^2=0.68$, $p<0.05$ for all independent variables).

No significant differences were found in biomarkers of inflammation or adhesion molecules across TRV measurements. (See supplemental Table I for specific biomarkers of inflammation and adhesion measured).

DISCUSSION

This study provides a comprehensive cardiopulmonary and laboratory profile of patients with thalassaemia and an elevated TRV ≥ 2.5 m/s at increased risk for PH compared to

patients with a TRV < 2.5 m/s, considered at lower risk for PH. Patients with TRV elevation had a higher frequency of splenectomy and significantly larger right atrial size, left atrial volume and left septal-wall thickness on echocardiography and/or cardiac-MRI, with elevated biomarkers of abnormal coagulation, LDH levels and arginase concentration and lower GAB compared to the TRV < 2.5 m/s group. As expected, NYHA functional class was higher in patients with higher TRVs. However no differences in pulmonary function tests or chest CT scans were identified in patients at high vs. normal TRV. Chest CT scanning was abnormal, however, in over half of the patients evaluated. Pulmonary function tests suggested restrictive lung disease in nearly 30% of the thalassaemia cohort, a high proportion but less frequent than observed in SCD (Klings, *et al* 2006).

Most intriguing from the biological studies was the emergence of arginine dysregulation as a factor strongly associated with cardiopulmonary dysfunction in this thalassaemia cohort. This study demonstrates that low GAB in thalassaemia occurs predominantly in patients with an elevated TRV. We have previously reported low GAB in thalassaemia compared to normal control subjects (Morris, *et al* 2005a), however patients were not differentiated by TRV or cardiopulmonary dysfunction. Of interest, Meloni *et al* (2015) recently published an association of TRV elevation with reduced GAB, together with increased anaemia, cardiac index and diastolic dysfunction in 60 TM patients. We describe, however, the first report to identify excess arginase activity as a key mechanism associated with low GAB in thalassaemia. Reduced GAB and NO depletion represent a common theme in otherwise distinct vasculopathies (Morris 2008) and can result from a number of mechanisms, including haemolysis (Morris, *et al* 2005a, Morris, *et al* 2005b, Morris, *et al* 2003, Morris, *et al* 2008). However, low GAB is associated with coronary artery disease and major adverse cardiovascular events including mortality in patients screened for cardiovascular disease (Tang, *et al* 2009, Wang, *et al* 2009), just as it is associated with degree of arginase activity, PH-risk and mortality in SCD (Cox, *et al* 2011, Morris, *et al* 2005b) and mortality risk in malaria (Omodeo-Sale, *et al* 2010). Hypoxic upregulation of arginase II occurs in human lung endothelial cells, while high shear stress has also been found to regulate the arginase pathway (Pernow and Jung 2013). These events may also contribute to excess arginase activity in some patients. Low GAB may represent a unifying mechanism of cardiovascular dysfunction that is not disease-specific. The “GAB ratio” (GABR) is defined as the ratio of arginine to (ornithine+citrulline), and accounts for levels of the substrate (arginine) and its major catabolic products (ornithine and citrulline) in vivo (Morris, *et al* 2005b, Tang, *et al* 2009). Arginine is the common substrate for both NO-synthases and the arginases. Whether inflammatory or haemolytic in origin, arginase will redirect the metabolism of arginine away from NO to ornithine and the formation of polyamines and proline, which are essential for smooth muscle cell growth and collagen synthesis. By creating a shift towards ornithine metabolism, arginase may contribute to a proliferative vasculopathy common to haemolytic disorders (Morris, *et al* 2005a, Morris 2008, Morris 2014, Morris, *et al* 2005b, Morris, *et al* 2013b) but also common to cardiovascular disease (Erdely, *et al* 2010, Sourij, *et al* 2011, Tang, *et al* 2009), heart failure (Tang, *et al* 2013) and PH (Morris and Gladwin 2011). GABR is also associated with advanced LV diastolic dysfunction, increased severity of right ventricular systolic dysfunction, and poorer long-term adverse clinical outcomes in nonhaemolytic patients with chronic systolic heart failure (Tang, *et al* 2013) and diabetes

(Tripolt, *et al* 2012). Low GAB may be exacerbated further by the presence of asymmetric dimethylarginine (ADMA), an endogenous NO-synthase inhibitor that competes with L-arginine for binding to NO-synthases. Well established as another biomarker of cardiovascular disease and endothelial dysfunction, elevated circulating ADMA levels have been implicated in the pathophysiology of systemic and PH and risk of early mortality (Wang, *et al* 2009). In patients with SCD, elevated ADMA levels were found in patients with the highest haemolytic rate and were also associated with PH-risk and early death (Kato, *et al* 2009). Similarly, high plasma ADMA levels have recently been implicated in the pathogenesis of TRV elevation in children with β -thalassaemia (Mohamed, *et al* 2014). Excess ADMA can also contribute to NO-synthase uncoupling (Morris 2008), which is an additional mechanism of arginine dysregulation that impacts GAB. It is interesting to note that sildenafil treatment increased GAB in thalassaemia patients with an elevated TRV (Morris, *et al* 2013a), and may represent a novel mechanism of action for this Food and Drug Administration-approved PH therapy that warrants further investigation.

It is notable that biomarkers of haemolytic rate, including LDH (Kato, *et al* 2006) and arginase (Morris, *et al* 2005b), were substantially different across TRV categories, however cell-free haemoglobin levels did not differ, and did not correlate strongly to other biomarkers of haemolysis including arginase activity/concentration or LDH as it has in other cohorts (Hill, *et al* 2010, Morris, *et al* 2005b). It is possible that sufficient haptoglobin and/or haemopexin were available in some patients to bind and eliminate cell-free haemoglobin to some degree, a consideration to evaluate in future studies. It is also possible that arginase is endothelial cell-derived rather than from the erythrocyte, in response to shear stress from a high cardiac output associated with anaemia. Although multiple sources of plasma arginase are most likely, this study could not differentiate between arginase isoforms or specific cell-types of origin.

Arginine dysregulation and thrombosis may be bi-directionally linked. Intravascular haemolysis and subsequent NO consumption has the potential to drive a pro-coagulant state, as NO has properties that inhibit platelet activation, tissue factor expression and thrombin generation (Morris 2008). Microparticles from platelets and those produced by erythrocyte fragmentation during haemolysis will also activate the intrinsic phase of blood coagulation and trigger thrombin generation (Donadee, *et al* 2011, Ferru, *et al* 2014, Tantawy, *et al* 2013). Thrombin itself increases arginase activity in human endothelial cells (Yang, *et al* 2006), propagating a cycle of endothelial dysfunction. The correlation of arginase activity, but not concentration, to coagulation abnormalities and BNP is therefore intriguing. Arginase concentration and activity are closely related, but not identical, as arginase activity can be modulated by natural and synthetic inhibitors (Stuehr, *et al* 1991) or activators (Yang, *et al* 2006) that will impact its function independent of concentration.

The paradoxical association of higher 6MWD with rising TRV was unexpected. However thalassaemia patients have a higher than normal cardiac index, which may represent compensation for a more severe chronic anaemia that improves 6MWD, at least initially before cardiopulmonary decompensation in more severe cases. In the setting of increased haemolysis and anaemia, stroke volume increases leading to an increased high cardiac output which may increase the TRV. In addition, treatment trials for PH typically enroll

patients with baseline 6MWDs between 150-450 m (Galie, *et al* 2005). A recent study that reported 6MWD data in the largest cohort of patients with thalassaemia and RHC-defined PH revealed an atypically high baseline 6MWD for patients with PH (472 ± 95 m) (Derchi, *et al* 2014), indicating that this may not be an ideal outcome measure in thalassaemia. In addition, a strong correlation of 6MWD to D_LCO and FEV_1/FVC % predicted may indicate additional pulmonary contributions to 6MWD in this cohort as confounding factors.

Patients with an elevated TRV in this thalassaemia cohort interestingly demonstrated evidence of left-sided cardiac dysfunction with increased left- and right-sided chamber size and volume. RHC data from SCD patients suggests that at least 50% of those with PH have evidence of elevated left- and right-sided pressures. Echocardiographic screening studies reveal that, while systolic dysfunction of the left ventricle and left-sided valvular heart disease occur in SCD, the majority of these patients have LV diastolic dysfunction, now termed congestive heart failure with preserved ejection fraction (Sachdev, *et al* 2007). MRI studies of thalassaemia patients demonstrate increased iron deposition within the left ventricle, most probably related to transfusion-associated iron overload (Wood and Noetzli 2010). This can lead to myocardial fibrosis and decreased LV relaxation with consequent reduced diastolic filling. It is possible that other yet to be identified mechanisms contribute to this process as well, because diastolic dysfunction occurs without co-existent iron deposition in SCD.

Limitations

One major limitation to this and other echocardiography screening studies in thalassaemia is that Doppler-defined risk for PH is not confirmed by RHC, which remains the gold standard to diagnosis PH (Klings, *et al* 2014, McLaughlin, *et al* 2009). In clinical practice, this procedure is not routinely performed in patients with thalassaemia. However, there is no evidence to suggest that patients with thalassaemia demonstrating appropriate clinical indications for RHC should be excluded from general standard of care. As awareness of this complication increases among clinicians who appropriately refer at-risk patients to cardiopulmonary specialists with expertise in PH, this procedure will be recommended, particularly in patients with moderate elevations in their TRV (> 3.0 m/s) despite adequate transfusion. Small sample size is an additional limitation. Although overlap in mechanisms contributing to vasculopathy and PH is expected in all forms of thalassaemia, the pathophysiology of PH is often different in patients with TI compared to TM. Combining these phenotypes is not ideal, but was necessary due to limited availability of subjects and the orphan nature of the disease. In addition, a few TM patients had been transfused since infancy, suggesting that some were initially TI patients who became transfusion dependent over time.

Conclusions

Coagulation abnormalities, haemolysis and arginine dysregulation emerged as major mechanisms associated with an elevated TRV in this cohort of thalassaemia patients. An altered arginine metabolome has been implicated in the pathophysiology PH in a number of settings (Morris and Gladwin 2011). These data provide additional support for its role in PH-risk in thalassaemia. Nearly all human cell-types contain arginase, an intracellular

enzyme released into circulation upon cell damage or cell death, but the greatest source of arginine dysregulation in haemoglobinopathies such as SCD is erythrocyte-derived arginase-I released during haemolysis (Morris, *et al* 2005a, Morris, *et al* 2005b). Although the sources and specific enzyme isoforms remain to be identified in β -thalassaemia, it is intriguing that arginase activity and concentration correlated so strongly to echocardiographic and cardiac-MRI parameters of cardiopulmonary function, a paradigm never previously described. It is possible that this may exist commonly in other myocardial disorders, particularly those associated with low GAB (Erdely, *et al* 2010, Sourij, *et al* 2011, Tang, *et al* 2009, Wang, *et al* 2009) and may provide a novel area to pursue therapeutically.

This study provides further evidence that arginine dysregulation may contribute to cardiopulmonary dysfunction in β -thalassaemia. Arginase inhibition or interventions aimed at restoration of GAB may hold promise for haemolytic disorders. These data may establish novel directions for future research in the thalassaemia syndromes and beyond.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This is publication number 34 of the TCRN. A list of TCRN member institutions and staff appears in Appendix 1. The authors would like to thank the agencies that funded this study. This study was supported by the NIH-NHLBI cooperative agreement U01 HL065238NIH grant and CTSA grant UL1 RR024131, FDA grant 1R01FD003531-04 (to CRM). This research was also supported in part by the Intramural Research Program of the NIH, NHLBI and by the following NIH-NHLBI cooperative agreements: U01-HL65232 and NIH/NCRR UL1-RR-024134 to the Children's Hospital of Philadelphia, U01-HL72291 and by Harvard Catalyst CTSC U-01RR025758 to Children's Hospital, Boston, U01-HL65233 to University Health Network Toronto General Hospital, UL1RR024131-01 to Children's Hospital & Research Center Oakland, U01-HL65244 and CTSC UL1-RR024996 to Weill Medical College of Cornell University, and U01-HL65238 to New England Research Institutes. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NHLBI. The authors would also like to thank the patients with thalassaemia and their families who participated in this study.

APPENDIX 1

The following institutions and researchers contributed to the Thalassemia Clinical Research Network Pulmonary Hypertension data reported in this paper.

Children's Hospital, Boston: Ellis Neufeld, MD, PhD, Principal Investigator, Jennifer Eile, MS, PNP, Research Nurse, Latoya Lashley, Study Coordinator; Satellite: University of Texas Southwestern Medical Center at Dallas, Charles Quinn, MD, MS, Principal Investigator, Deborah Boger, RN, MSN, PNP, Study Coordinator, Leah Adix, Study Coordinator, Brad Cook, Study Coordinator. Weill Medical College of Cornell University: Patricia Giardina, MD, Principal Investigator, Dorothy Kleinert, RN, Research Nurse. The Children's Hospital of Philadelphia: Janet Kwiatkowski, MD, Principal Investigator, Sage Green, Study Coordinator; Satellite: Children's Memorial Hospital, Chicago, IL, Alexis Thompson, MD, Principal Investigator, Janice Beatty, RN, Research Nurse, Diane Calamaras, RN, CPNP, Research Nurse, Pauline Hess, study coordinator. Children's Hospital at Oakland: Claudia Morris, MD, Principal Investigator, Elliott Vichinsky, MD, Principal Investigator, Nancy Sweeters, Study Coordinator. Toronto General Hospital,

Toronto, Ontario, Canada: Nancy F. Olivieri, MD, Principal Investigator, Renata Dzackova, Study Coordinator, Cecilia Kim, Study Coordinator, Vivek Thayalasuthan, Study Coordinator. University College London, John Porter, MD, Principal Investigator, Cindy Bhagwandin, Study Coordinator. American University of Beirut Medical Center: Ali Taher, MD, Principal Investigator, Tannous Fakhry, Study Coordinator. NHLBI oversight, Kathryn Hassell, MD. Data Coordinating Center: New England Research Institutes, Sonja McKinlay, PhD, Principal Investigator, Lisa Virzi, RN, MS, MBA, Project Director, Felicia Trachtenberg, PhD, Senior Statistician.

REFERENCES

- Aessopos A, Farmakis D, Deftereos S, Tsironi M, Tassiopoulos S, Moysakis I, Karagiorga M. Thalassemia heart disease: a comparative evaluation of thalassemia major and thalassemia intermedia. *Chest*. 2005; 127:1523–1530. [PubMed: 15888823]
- Anthi A, Orfanos SE, Armaganidis A. Pulmonary hypertension in beta thalassaemia. *Lancet Respir Med*. 2013; 1:488–496. [PubMed: 24429247]
- Cox SE, Makani J, Komba AN, Soka D, Newton CR, Kirkham FJ, Prentice AM. Global arginine bioavailability in Tanzanian sickle cell anaemia patients at steady-state: a nested case control study of deaths versus survivors. *Br J Haematol*. 2011; 155:522–524. [PubMed: 21595648]
- Derchi G, Galanello R, Bina P, Cappellini MD, Piga A, Lai ME, Quarta A, Casu G, Perrotta S, Pinto V, Musallam KM, Forni GL. Prevalence and risk factors for pulmonary arterial hypertension in a large group of beta-thalassemia patients using right heart catheterization: a Webthal study. *Circulation*. 2014; 129:338–345. [PubMed: 24081970]
- Donadee C, Raat NJ, Kanias T, Tejero J, Lee JS, Kelley EE, Zhao X, Liu C, Reynolds H, Azarov I, Frizzell S, Meyer EM, Donnenberg AD, Qu L, Triulzi D, Kim-Shapiro DB, Gladwin MT. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. *Circulation*. 2011; 124:465–476. [PubMed: 21747051]
- El-Hady SB, Farahat MH, Atfy M, Elhady MA. Nitric oxide metabolites and arginase I levels in beta-thalassemic patients: an Egyptian study. *Ann Hematol*. 2012; 91:1193–1200. [PubMed: 22362120]
- Erdely A, Kepka-Lenhart D, Salmen-Muniz R, Chapman R, Hulderman T, Kashon M, Simeonova PP, Morris SM Jr. Arginase activities and global arginine bioavailability in wild-type and ApoE-deficient mice: responses to high fat and high cholesterol diets. *PLoS One*. 2010; 5:e15253. [PubMed: 21151916]
- Farmakis D, Aessopos A. Pulmonary hypertension associated with hemoglobinopathies: prevalent but overlooked. *Circulation*. 2011; 123:1227–1232. [PubMed: 21422398]
- Ferru E, Pantaleo A, Carta F, Mannu F, Khadjavi A, Gallo V, Ronzoni L, Graziadei G, Cappellini MD, Turrini F. Thalassemic erythrocytes release microparticles loaded with hemichromes by redox activation of p72Syk kinase. *Haematologica*. 2014; 99:570–578. [PubMed: 24038029]
- Fonseca GH, Souza R, Salemi VC, Jardim CV, Gualandro SF. Pulmonary hypertension diagnosed by right heart catheterization in sickle cell disease. *Eur Respir J*. 2012; 39:112–118. [PubMed: 21778170]
- Galie N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, Badesch D, Fleming T, Parpia T, Burgess G, Branzi A, Grimminger F, Kurzyna M, Simonneau G. Sildenafil citrate therapy for pulmonary arterial hypertension. *N Engl J Med*. 2005; 353:2148–2157. [PubMed: 16291984]
- Gladwin MT. Prevalence, risk factors and mortality of pulmonary hypertension defined by right heart catheterization in patients with sickle cell disease. *Expert Rev Hematol*. 2011; 4:593–596. [PubMed: 22077523]
- Hill A, Rother RP, Wang X, Morris SM Jr, Quinn-Senger K, Kelly R, Richards SJ, Bessler M, Bell L, Hillmen P, Gladwin MT. Effect of eculizumab on haemolysis- associated nitric oxide depletion, dyspnoea, and measures of pulmonary hypertension in patients with paroxysmal nocturnal haemoglobinuria. *Br J Haematol*. 2010; 149:414–425. [PubMed: 20230403]

- Karimi M, Musallam KM, Cappellini MD, Daar S, El-Beshlawy A, Belhoul K, Saned MS, Temraz S, Koussa S, Taher AT. Risk factors for pulmonary hypertension in patients with beta thalassemia intermedia. *Eur J Intern Med.* 2011; 22:607–610. [PubMed: 22075289]
- Kato GJ, McGowan V, Machado RF, Little JA, Taylor J.t. Morris CR, Nichols JS, Wang X, Poljakovic M, Morris SM Jr. Gladwin MT. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. *Blood.* 2006; 107:2279–2285. [PubMed: 16291595]
- Kato GJ, Wang Z, Machado RF, Blackwelder WC, Taylor J.G.t. Hazen SL. Endogenous nitric oxide synthase inhibitors in sickle cell disease: abnormal levels and correlations with pulmonary hypertension, desaturation, haemolysis, organ dysfunction and death. *Br J Haematol.* 2009; 145:506–513. [PubMed: 19344390]
- Klings ES, Wyszynski DF, Nolan VG, Steinberg MH. Abnormal Pulmonary Function in Adults with Sickle Cell Anemia. *Am J Respir Crit Care Med.* 2006; 173:1264–1269. [PubMed: 16556694]
- Klings ES, Machado RF, Barst RJ, Morris CR, Mubarak KK, Gordeuk VR, Kato GJ, Ataga KI, Gibbs JS, Castro O, Rosenzweig EB, Sood N, Hsu L, Wilson KC, Telen MJ, Decastro LM, Krishnamurti L, Steinberg MH, Badesch DB, Gladwin MT. An official American Thoracic Society clinical practice guideline: diagnosis, risk stratification, and management of pulmonary hypertension of sickle cell disease. *Am J Respir Crit Care Med.* 2014; 189:727–740. [PubMed: 24628312]
- Machado RF, Farber HW. Pulmonary hypertension associated with chronic hemolytic anemia and other blood disorders. *Clin Chest Med.* 2013; 34:739–752. [PubMed: 24267302]
- McLaughlin VV, Archer SL, Badesch DB, Barst RJ, Farber HW, Lindner JR, Mathier MA, McGoon MD, Park MH, Rosenson RS, Rubin LJ, Tapson VF, Varga J. ACCF/AHA 2009 expert consensus document on pulmonary hypertension a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association developed in collaboration with the American College of Chest Physicians; American Thoracic Society, Inc.; and the Pulmonary Hypertension Association. *J Am Coll Cardiol.* 2009; 53:1573–1619. [PubMed: 19389575]
- Mehari A, Gladwin MT, Tian X, Machado RF, Kato GJ. Mortality in adults with sickle cell disease and pulmonary hypertension. *JAMA.* 2012; 307:1254–1256. [PubMed: 22453563]
- Meloni A, Dettlerich J, Pepe A, Harmatz P, Coates TD, Woods JC. Pulmonary hypertension in well-transfused thalassemia major patients. *Blood Cells Mol Dis.* 2015; 54:189–94. [PubMed: 25488617]
- Mohamed ES, Ibrahim B, Amr D, Noha EK, Mokhtar M. Asymmetric dimethylarginine levels in children with beta-thalassemia and their correlations to tricuspid regurgitant jet velocity. *Pediatr Blood Cancer.* 2014; 61:112–118.
- Morris CR. Mechanisms of vasculopathy in sickle cell disease and thalassemia. *Hematology Am Soc Hematol Educ Program.* 2008; 2008:177–185. [PubMed: 19074078]
- Morris CR. Alterations of the arginine metabolome in sickle cell disease: a growing rationale for arginine therapy. *Hematol Oncol Clin North Am.* 2014; 28:301–321. [PubMed: 24589268]
- Morris, CR.; Gladwin, MT. Pulmonary hypertension in sickle cell disease and thalassemia. In: Peacock, A.; Naeije, R.; Rubin, L., editors. *Pulmonary Circulation.* Third edition. Hodder Arnold; London: 2011. p. 271-287.
- Morris CR, Vichinsky EP. Pulmonary hypertension in thalassemia. *Ann N Y Acad Sci.* 2010; 1202:205–213. [PubMed: 20712794]
- Morris CR, Morris SM Jr. Hagar W, van Warmerdam J, Claster S, Kepka-Lenhart K, Machado L, Kuypers FA, Vichinsky EP. Arginine Therapy: A new treatment for pulmonary hypertension in sickle cell disease? *Am J Respir Crit Care Med.* 2003; 168:63–69. [PubMed: 12626350]
- Morris C, Kuypers F, Kato G, Lavrishia L, Larkin S, Singer T, Vichinsky E. Hemolysis-associated pulmonary hypertension in thalassemia. *An NY Acad Sci.* 2005a; 1054:481–485.
- Morris CR, Kato GJ, Poljakovic M, Wang X, Blackwelder WC, V, S. Hazen SL, Vichinsky EP, Morris SM Jr, Gladwin MT. Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension and mortality in sickle cell disease. *JAMA.* 2005b; 294:81–90. [PubMed: 15998894]

- Morris CR, Vichinsky E, Singer ST. Pulmonary hypertension in thalassemia: Association with hemolysis, arginine metabolism dysregulation and a hypercoagulable state. *Advances in Pulmonary Hypertension*. 2007; 5:31–38.
- Morris CR, Suh JH, Hagar W, Larkin S, Bland DA, Steinberg MH, Vichinsky EP, Shigenaga M, Ames B, Kuypers FA, Klings ES. Erythrocyte Glutamine Depletion, Altered Redox Environment, and Pulmonary Hypertension in Sick Cell Disease. *Blood*. 2008; 140:104–112.
- Morris CR, Kim HY, Trachtenberg F, Wood J, Quinn CT, Sweeters N, Kwiatkowski JL, Thompson AA, Giardina PJ, Boudreaux J, Olivieri NF, Porter JB, Neufeld EJ, Vichinsky EP. Risk factors and mortality associated with an elevated tricuspid regurgitant jet velocity measured by Doppler-echocardiography in thalassemia: a Thalassemia Clinical Research Network report. *Blood*. 2011; 118:3794–3802. [PubMed: 21772051]
- Morris CR, Kim HY, Wood J, Porter JB, Klings ES, Trachtenberg FL, Sweeters N, Olivieri NF, Kwiatkowski JL, Virzi L, Singer ST, Taher A, Neufeld EJ, Thompson AA, Sachdev V, Larkin S, Suh JH, Kuypers FA, Vichinsky EP. Sildenafil therapy in thalassemia patients with Doppler-defined risk of pulmonary hypertension. *Haematologica*. 2013a; 98:1359–1367. [PubMed: 23585527]
- Morris CR, Kuypers FA, Lavrishia L, Ansari M, Sweeters N, Stewart M, Gildengorin G, Neumayr L, Vichinsky EP. A randomized, placebo-controlled trial of arginine therapy for the treatment of children with sickle cell disease hospitalized with vaso-occlusive pain episodes. *Haematologica*. 2013b; 98:1375–1382. [PubMed: 23645695]
- Omodeo-Sale F, Cortelezzi L, Vommaro Z, Scaccabarozzi D, Dondorp AM. Dysregulation of L-arginine metabolism and bioavailability associated to free plasma heme. *Am J Physiol Cell Physiol*. 2010; 299:C148–154. [PubMed: 20357184]
- Parent F, Bachir D, Inamo J, Lionnet F, Driss F, Loko G, Habibi A, Bennani S, Savale L, Adnot S, Maitre B, Yaici A, Hajji L, O'Callaghan DS, Clerson P, Girot R, Galacteros F, Simonneau G. A hemodynamic study of pulmonary hypertension in sickle cell disease. *N Engl J Med*. 2011; 365:44–53. [PubMed: 21732836]
- Pernow J, Jung C. Arginase as a potential target in the treatment of cardiovascular disease: reversal of arginine steal? *Cardiovasc Res*. 2013; 98:334–343. [PubMed: 23417041]
- Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO, Schechter AN, Gladwin MT. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med*. 2002; 8:1383–1389. [PubMed: 12426562]
- Sachdev V, Machado RF, Shizukuda Y, Rao YN, Sidenko S, Ernst I, St Peter M, Coles WA, Rosing DR, Blackwelder WC, Castro O, Kato GJ, Gladwin MT. Diastolic dysfunction is an independent risk factor for death in patients with sickle cell disease. *J Am Coll Cardiol*. 2007; 49:472–479. [PubMed: 17258093]
- Singer ST, Kuypers FA, Styles L, Vichinsky EP, Foote D, Rosenfeld H. Pulmonary hypertension in thalassemia: association with platelet activation and hypercoagulable state. *Am J Hematol*. 2006; 81:670–675. [PubMed: 16795058]
- Singer ST, Kuypers F, Fineman J, Gildengorin G, Larkin S, Sweeters N, Rosenfeld H, Kurio G, Higa A, Jeng M, Huang J, Vichinsky EP. Elevated tricuspid regurgitant jet velocity in subgroups of thalassemia patients: insight into pathophysiology and the effect of splenectomy. *Ann Hematol*. 2014; 93:1139–48. [PubMed: 24577514]
- Sonakul D, Pacharee P, Thakerngpol K. Pathologic findings in 76 autopsy cases of thalassemia. *Birth Defects Orig Artic Ser*. 1988; 23:157–176. [PubMed: 3390538]
- Sourij H, Meinitzer A, Pilz S, Grammer TB, Winkelmann BR, Boehm BO, Marz W. Arginine bioavailability ratios are associated with cardiovascular mortality in patients referred to coronary angiography. *Atherosclerosis*. 2011; 218:220–225. [PubMed: 21632053]
- Stuehr DJ, Kwon N, Nathan CF, Griffith OW, Felman PL, Wiseman J. N-Hydroxyl- L-arginine is an intermediate in the biosynthesis of nitric oxide for L-arginine. *J Biol Chem*. 1991; 266:6259–6263. [PubMed: 1706713]
- Tang WH, Shrestha K, Wang Z, Troughton RW, Klein AL, Hazen SL. Diminished global arginine bioavailability as a metabolic defect in chronic systolic heart failure. *J Card Fail*. 2013; 19:87–93. [PubMed: 23384633]

- Tang WHW, Wang Z, Cho L, Brennan DM, Hanzen SL. Diminished global arginine bioavailability and increased arginine catabolism as metabolic profile of increased cardiovascular risk. *Journal of the American College of Cardiology*. 2009; 53:2061–2067. [PubMed: 19477356]
- Tantawy AA, Adly AA, Ismail EA, Habeeb NM. Flow cytometric assessment of circulating platelet and erythrocytes microparticles in young thalassemia major patients: relation to pulmonary hypertension and aortic wall stiffness. *Eur J Haematol*. 2013; 90:508–518. [PubMed: 23506251]
- Tripolt NJ, Meinitzer A, Eder M, Wascher TC, Pieber TR, Sourij H. Multifactorial risk factor intervention in patients with Type 2 diabetes improves arginine bioavailability ratios. *Diabet Med*. 2012; 29:e365–368. [PubMed: 22803961]
- Wang Z, Tang WH, Cho L, Brennan DM, Hazen SL. Targeted metabolomic evaluation of arginine methylation and cardiovascular risks: potential mechanisms beyond nitric oxide synthase inhibition. *Arterioscler Thromb Vasc Biol*. 2009; 29:1383–1391. [PubMed: 19542023]
- Wood JC, Noetzli L. Cardiovascular MRI in thalassemia major. *Ann N Y Acad Sci*. 2010; 1202:173–179. [PubMed: 20712790]
- Yang L, Lewis CM, Chandrasekharan UM, Kinney CM, Dicorleto PE, Kashyap VS. Arginase activity is increased by thrombin: a mechanism for endothelial dysfunction in arterial thrombosis. *J Am Coll Surg*. 2006; 203:817–826. [PubMed: 17116549]

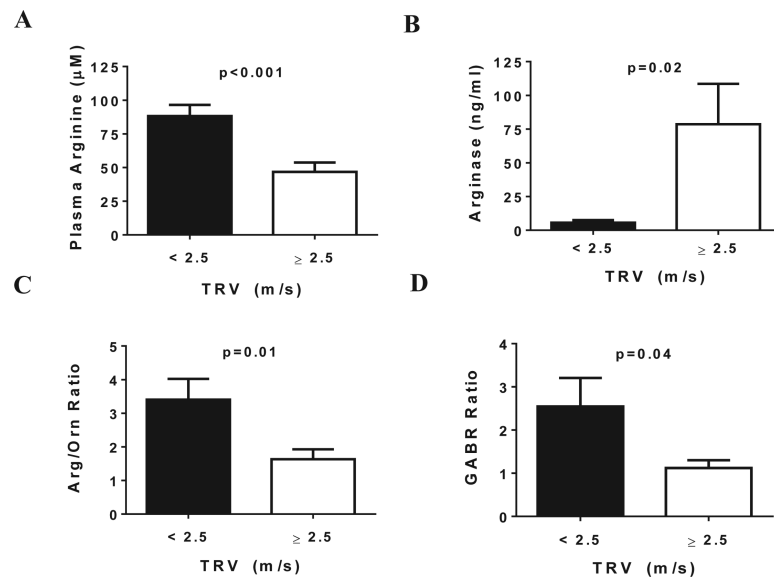


Figure 1. Altered arginine bioavailability in patients with β -thalassaemia and elevation of tricuspid regurgitant jet velocity (TRV)

A. Mean plasma arginine concentration (μM) \pm standard error of the mean (SEM), **B.** mean plasma arginase concentration (ng/ml) \pm SEM, **C.** mean plasma arginine-to-ornithine ratio (Arg/Orn) \pm SEM and **D.** mean plasma global arginine bioavailability ratio (GABR, arginine/[ornithine+citrulline]) \pm SEM in patients with β -thalassaemia with a tricuspid regurgitant-jet-velocity (TRV) <2.5 m/s (n=13) compared to patients with an elevated TRV \geq 2.5 m/s (n=14).

Table I Patient Demographics and Clinical Characteristics

	Total N = 27	TRV<2.5 N=13	TRV>2.5 N=14	p-value
Mean Age, years, (SD)	34.7 (11.7)	33.7 (12.6)	35.6 (11.3)	0.69
Gender, N (%)				
Male	19 (70.4%)	7 (53.9%)	12 (85.7%)	0.10
Female	8 (29.6%)	6 (46.1%)	2 (14.3%)	
Race, N (%)				
White	11 (40.7%)	4 (30.8%)	7 (50.0%)	0.45
Black	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Asian	12 (44.4%)	6 (46.2%)	6 (42.9%)	
Other	4 (14.8%)	3 (23.1%)	1 (7.1%)	
Diagnosis, N (%)				
β-thalassaemia major (TM)	18 (66.7%)	11 (84.6%)	7 (50.0%)	0.10*
Mean transfusions per year	15.3 ± 2.4	15.3 ± 2.6	15.2 ± 2.4	0.94
Pre-transfusion Hb	10.0 ± 0.8	9.9 ± 0.7	10.1 ± 1.1	0.79
Transfused since infancy	11 (40.7%)	8 (61.5%)	3 (21.4%)	0.33
β-thalassaemia intermedia (TI)	9 (33.3%)	2 (15.4%)	7 (50.0%)	
Non-transfusion	5 (18.5%)	1 (7.6%)	4 (28.6%)	>0.99**
< 8 transfusions/year	4 (14.8%)	1 (7.6%)	3 (21.4%)	
Clinical History, N (%)				
Splenectomy	20 (74.1%)	7 (53.9%)	13 (92.9%)	0.03
Hepatitis C	4 (14.8%)	1 (7.7%)	3 (21.4%)	0.60
Smoking in the past year	7 (26.9%)	1 (8.3%)	6 (42.9%)	0.08

Data expressed as number (%) or mean ± SD; p-values compare data from patients with TRV<2.5 m/s vs. TRV ≥ 2.5 m/s

TRV, tricuspid-regurgitant-jet-velocity; SD, standard deviation.

* Compares TRV<2.5 m/s vs. TRV ≥ 2.5 m/s in TM vs. TI

** Compares TRV<2.5 m/s vs. TRV ≥ 2.5 m/s between non-transfused vs. < 8 transfusion/year among TI patients

Table II

Cardiopulmonary Characteristics According to TRV (in m/s)

Variables	TRV<2.5 (n=13)	TRV 2.5-2.8 (N=8)	TRV 2.9 (N=6)	P-value
6MWT, NYHA class and Dyspnea				
6MWT (m)	463.2 (93.4)	504.3 (110.8)	512.3 (107.1)	0.26
NYHA functional class	1.1 (0.3)	1.3 (0.5)	1.8 (0.4)	0.02
Borg Dyspnea Score before walk	0.5 (1.0)	1.8 (2.0)	0.4 (0.8)	0.21
Borg Dyspnea Score after walk	1.0 (1.0)	2.1 (1.9)	2.0 (0.9)	0.05
Oxygen desaturation during/after 6MWT (N, %)	4 (30.8%)	4 (50.0%)	3 (50.0%)	0.44
Echo Parameters				
Local TRV (m/s)	2.1 (0.4)	2.7 (0.0)	3.6 (0.9)	<0.001
Central TRV (m/s)	2.1 (0.4)	2.6 (0.1)	3.2 (0.9)	<0.001
LVEF (%)	64.8 (3.3)	65.4 (2.5)	66.8 (2.4)	0.29
Right atrial size (cm ²)	14.2 (3.3)	16.3 (3.6)	19.2 (3.6)	0.03
Left atrial volume (ml)	41.7 (9.7)	54.4 (12.7)	65.6 (18.5)	0.002
LV end systolic volume (ml)	30.0 (13.5)	37.9 (13.8)	30.5 (5.8)	0.33
LV end diastolic volume (ml)	83.4 (29.3)	109.2 (39.2)	92.5 (19.6)	0.13
LV mass (g)	117.6 (24.7)	154.1 (43.7)	136.3 (35.1)	0.03
LV septal wall thickness	0.8 (0.1)	0.9 (0.1)	0.9 (0.1)	0.02
LV posterior wall thickness	0.8 (0.1)	0.9 (0.1)	0.8 (0.2)	0.07
LV mean wall thickness	0.8 (0.1)	0.9 (0.1)	0.9 (0.1)	0.03
Cardiac Index (l/min/m ²)	2.6 (0.7)	3.7 (1.3)	3.0 (0.9)	0.04
Arterial compliance	1.4 (0.4)	1.3 (0.5)	1.1 (0.2)	0.28
Echo Parameters Indexed to BSA				
Right atrial size (cm ² /m ²)	8.8 (1.8)	9.5 (1.0)	11.3 (2.2)	0.06
Left atrial volume (ml/m ²)	25.2 (5.7)	31.6 (6.0)	38.8 (11.8)	0.004
LV end systolic volume (ml/m ²)	17.8 (6.4)	21.7 (6.8)	17.9 (3.3)	0.32
LV end diastolic volume (ml/m ²)	49.6 (13.3)	62.5 (17.8)	54.5 (12.2)	0.10
LV mass (g/m ²)	70.8 (11.8)	89.8 (24.1)	80.6 (22.8)	0.05
MRI Parameters				
Left atrial volume (ml)	69.0 (14.6)	107.6 (34.9)	100.1 (8.3)	0.008
LV end diastolic volume (ml)	144.5 (48.0)	200.3 (78.4)	157.1 (43.7)	0.12
LV ejection fraction (%)	59.4 (6.3)	58.3 (3.0)	60.6 (1.1)	0.90
LV end systolic volume (ml)	59.2 (23.5)	84.3 (35.0)	60.7 (19.4)	0.17
LV mass (g)	72.8 (29.2)	96.0 (29.1)	87.2 (23.0)	0.11
Right atrial volume (ml)	73.7 (17.6)	113.9 (36.7)	119.3 (17.6)	0.01
Right ventricular end diastolic volume (ml)	138.6 (48.4)	171.6 (59.7)	161.4 (19.0)	0.16
Right ventricular ejection fraction (%)	52.3 (5.5)	53.1 (6.2)	44.9 (10.6)	0.47
Right ventricular end systolic volume (ml)	67.2 (27.4)	80.1 (28.9)	87.5 (18.5)	0.17
Pulmonary Function				

Variables	TRV<2.5 (n=13)	TRV 2.5-2.8 (N=8)	TRV 2.9 (N=6)	P-value
Oxygen saturation on room air before walk (%)	98.0 (1.6)	96.6 (2.0)	97.2 (2.5)	0.13
Oxygen saturation on room air after walk (%)	98.2 (1.6)	95.5 (1.5)	98.2 (2.3)	0.09
D _L CO	13.0 (5.8)	13.9 (11.0)	15.8 (7.2)	0.58
D _L CO % predicted	65.8 (15.1)	68.9 (19.2)	71.8 (16.8)	0.51
D _L CO adjusted for Hb	17.4 (5.4)	20.7 (12.4)	21.3 (6.8)	0.29
FEV ₁ % predicted	82.5 (8.7)	87.4 (13.9)	80.2 (12.5)	0.69
FEV/FVC % predicted	98.1 (6.8)	100.3 (6.9)	93.8 (9.3)	0.82
FEF (25-75) % predicted	84.6 (19.2)	74.0 (31.2)	121.0 (41.4)	0.35
FVLC % predicted	85.8 (10.8)	91.3 (16.0)	83.7 (15.0)	0.67
Total lung capacity % predicted	86.6 (13.9)	88.5 (15.6)	90.5 (6.4)	0.74

Data expressed as number (%) or mean \pm SD; p-values compare data from patients with TRV<2.5 m/s vs. TRV 2.5 m/s

Abbreviations: BSA, body surface area index; CT, computerized tomography; DLCO, diffusing capacity for carbon monoxide; Echo, Doppler-echocardiography; FEF, forced expiratory flow; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; H, haemoglobin; LV, left ventricular; LVEF, left ventricular ejection fraction; MRI, magnetic resonance imaging; NYHA, New York Heart Association; 6MWT, 6-minute walk test; TRV, tricuspid regurgitant jet velocity

Table III

Laboratory Variables According to TRV

Variables	TRV<2.5 (N=13)	TRV 2.5 (N=14)	p-value
Clinical Laboratory Analyses			
WBC (10 ⁹ /l)	10.8 (4.7)	13.8 (4.6)	0.11
Platelet Count (10 ⁹ /l)	478.3 (280.6)	581.2 (257.1)	0.34
Haemoglobin (g/l)	108 (12)	100 (22)	0.25
Haematocrit (%)	32.2 (2.9)	31.3 (6.0)	0.62
Ferritin (µg/l)	3028 (3735)	1636 (1552)	0.51
Bilirubin (µmol/l)	32.5 (18.8)	42.8 (23.9)	0.30
ALT (u/l)	34.3 (24.7)	34.0 (30.5)	0.82
AST (u/l)	33.8 (20.7)	51.4 (32.7)	0.20
Creatinine (µmol/l)	61.9 (17.7)	61.9 (26.5)	0.92
BNP (pg/ml)	23.6 (28.4)	43.5 (51.6)	0.24
Coagulation			
PAI-1 Activity (iu/ml)	13.8 (12.9)	8.7 (4.3)	0.32
PF12 Mono (pmol/l)	124.4 (55.1)	696.9 (1311)	0.02
TAT Complex (ng/ml)	3.3 (2.0)	70.1 (174.0)	0.04
TF (pg/ml)	279.1 (84.9)	296.2 (112.6)	0.71
Haemolysis/Arginine - Nitric Oxide Pathway			
LDH (iu/l)	165.3 (42.5)	236.2 (87.0)	0.03
Arginine (Plasma; µM)	88.2 (30.1)	46.8 (25.8)	0.001
Arginine (erythrocyte; µM)	4.5 (3.0)	4.3 (3.0)	0.78
Arginase concentration (ng/ml)	5.7 (7.0)	78.7 (108.0)	0.003
Arginase activity (u/l)	2.5 (2.6)	7.8 (7.1)	0.05
Plasma Arg/Orn	3.4 (2.2)	1.6 (1.1)	0.01
Plasma Arg/(Orn+Citrulline)	2.5 (2.4)	1.1 (0.7)	0.04
Serum NOx concentration (µM)	31.4 (9.4)	40.9 (17.1)	0.10
VEGF (pg/ml)	842.9 (833.0)	1013 (819.1)	0.23
GSH (RBC; µM)	1807 (860.5)	1501 (799.7)	0.61
Cell Free Haemoglobin (µg/ml)	155.0 (90.8)	182.6 (115.9)	0.58

Data expressed as mean ± standard deviation

Abbreviations: ALT, alanine transaminase; Arg, arginine; AST, aspartate transaminase; BNP, brain natriuretic peptide; GSH, glutathione; LDH, lactate dehydrogenase; NOx, nitric oxide metabolites; Orn, ornithine; PAI1, plasminogen activator inhibitor-1; PF1.2 Mono, monoclonal prothrombin fragment 1.2 ; RBC, red blood cell; TAT, thrombin-anti-thrombin ; TF, tissue factor; VEGF, vascular endothelial growth factor; WBC, white blood cell count.

Table IV

Associations with Arginase Concentration as Measured by Pearson Correlation Coefficient

Variable	<i>r</i>	No. With Data	p-value
Echo Parameters			
Local TRV (m/s)	0.12	26	0.55
Central TRV (m/s)	0.06	26	0.77
Left atrial volume (ml)	0.48	26	0.01
LV end systolic volume (ml)	0.47	26	0.02
LV end diastolic volume (ml)	0.57	26	0.002
LV mass (g)	0.49	26	0.01
LV mean wall thickness	0.36	26	0.07
Cardiac Index (l/min/m ²)	0.75	26	<.0001
Echo Parameters Indexed to BSA			
Right atrial size (cm ² /m ²)	−0.03	25	0.87
Left atrial volume (ml/m ²)	0.46	26	0.02
LV end systolic volume (ml/m ²)	0.56	26	0.003
LV end diastolic volume (ml/m ²)	0.67	26	0.0002
LV mass (g/m ²)	0.48	26	0.01
MRI Parameters			
Left atrial volume (ml)	0.79	14	0.001
LV end systolic volume (ml)	0.53	22	0.01
LV end systolic volume (ml)	0.54	22	0.01
LV mass (g)	0.49	21	0.02
Right atrial volume (ml)	0.79	13	0.001
RV end diastolic volume (ml)	0.32	22	0.14
Clinical Lab Analyses			
WBC (10 ⁹ /l)	0.43	25	0.03
Platelet Count (10 ⁹ /l)	0.32	25	0.12
Haemoglobin (g/l)	−0.46	26	0.02
Bilirubin (μmol/l)	0.50	23	0.02
ALT (u/l)	0.19	26	0.36
AST (u/l)	0.23	16	0.39
Creatinine (μmol/l)	−0.47	26	0.02
Haemolysis			
LDH (iu/l)	0.63	24	0.001
Arginine (plasma, μM)	−0.67	26	0.0002
Arginine (erythrocyte, μM)	0.09	25	0.68
Arginine/Ornithine (plasma)	−0.51	26	0.01
Arginine/(Ornithine+Citruilline)	−0.39	26	0.05
Serum NOx concentration (μM)	0.17	25	0.41

Variable	<i>r</i>	No. With Data	p-value
Cell Free Haemoglobin (µg/ml)	-0.14	26	0.50

Abbreviations: ALT, alanine transaminase; Arg, arginine; AST, aspartate transaminase; LDH, lactate dehydrogenase; LV, left ventricular; NOx, nitric oxide metabolites; Orn, ornithine; RV, right ventricular; TRV, tricuspid regurgitant jet velocity; WBC, white blood cell count. alanine aminotransferase (ALT); aspartate aminotransferase (AST); lactate dehydrogenase (LDH); left