

## References

- Lee S. The value of DNA analysis for antigens of the Kell and Kx blood group systems. *Transfusion* 2007;47:325-395.
- Aygun B, Padmanabhan S, Paley C, Chandrasekaran V. Clinical significance of RBC alloantibodies and autoantibodies in sickle cell patients who received transfusions. *Transfusion* 2002;42:37-43.
- Castro O, Sandler SG, Houston-Yu P, Rana S. Predicting the effect of transfusing only phenotype-matched RBCs to patients with sickle cell disease: theoretical and practical implications. *Transfusion* 2002;42:684-90.
- Vichinsky EP, Earles A, Johnson RA, Hoag MS, Williams A, Lubin B. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood. *N Engl J Med* 1990;322:1617-21.
- Murphy MT, Fraser RH, Goddard JP. Development of a PCR-based diagnostic assay for the determination of KEL genotype in donor blood samples. *Transfus Med* 1996;6:133-7.
- Lee S, Wu X, Son S, Naime D, Reid M, Okubo Y, et al. Point mutations characterize KEL10, the KEL3, KEL4, and KEL21 alleles, and the KEL17 and KEL11 alleles. *Transfusion* 1996;36:490-4.
- Lee S, Wu X, Reid M, Redman C. Molecular basis of the K:6-7 [Js(a+b-)] phenotype in the Kell blood group system. *Transfusion* 1995;35:822-5.
- Rosse WF, Gallagher D, Kinney TR, Castro O, Dosik H, Moehr J, et al. Transfusion and alloimmunization in sickle cell disease. *Blood* 1990;76:1431-7.
- Schonewille H, van de Watering LMG, Loomans DSE, Brand A. Red blood cell alloantibodies after transfusion: factors influencing incidence and specificity. *Transfusion* 2006;46:250-6.
- Moreira Jr G, Bordin JO, Kuroda A, Kerbauy J. Red blood cell alloimmunization in sickle cell disease: the influence of racial and antigenic pattern differences between donors and recipients in Brazil. *Am J Hematol* 1996;52:197-200.
- Murao M, Viana MB. Risk factors for alloimmunization by patients with sickle cell disease. *Braz J Med Biol Res* 2005;38:675-82.

### Association of asymmetric dimethylarginine with sickle cell disease-related pulmonary hypertension

Pulmonary hypertension (PHT) occurs in approximately 30% of adult sickle cell patients and is associated with a high risk of early death. Hemolysis driven reductions in nitric oxide (NO) bioavailability resulting from NO scavenging by cell free hemoglobin and increased arginase activity are of importance in the pathophysiology of SCD related PHT.<sup>1</sup>

Elevated plasma concentrations of asymmetric dimethylarginine (ADMA) contribute to limiting NO bioavailability in SCD.<sup>2</sup> ADMA and symmetric dimethylarginine (SDMA) derive from the irreversible post-translational methylation of arginine residues by protein arginine methyltransferases (PRMT) and are released as free amino acids upon proteolysis. ADMA (but not SDMA) competitively inhibits NO synthase (NOS) enzymes, thereby limiting NO production. ADMA is degraded by dimethylarginine dimethylaminohydrolases (DDAH) whereas SDMA is mainly cleared renally.<sup>3</sup> Elevated plasma ADMA concentrations occur in several forms of PHT and are associated to PHT outcome.<sup>4,5</sup> We investigated whether ADMA concentrations are associated with PHT in SCD.

Serum and EDTA plasma samples were available from adult sickle cell patients consecutively screened for PHT with echocardiography as previously reported.<sup>6</sup> Mild and moderate-severe PHT are defined as tricuspid regurgitant jet flow velocity (TRV) of 2.5-2.9 m/s and TRV $\geq$ 3 m/s respectively, with pulmonary-artery pressures considered

normal in patients with trace or no tricuspid regurgitation (with TRV assigned 1.3 m/s).<sup>1</sup> Plasma concentrations of ADMA, SDMA, amino acids and serum soluble vascular cell adhesion molecule-1 (sVCAM-1) levels were determined as previously described.<sup>2,7</sup> For analysis, HbSS and HbS $\beta^0$ -thalassemia patients were grouped together, as were HbS $\beta^+$ -thalassemia and HbSC patients. *p*-values <0.05 were considered statistically significant (SPSS 12.0.2, SPSS Inc, Chicago, IL, USA). The study was carried out in accordance with the principles of the Declaration of Helsinki.

Two out of 19 PHT patients had moderate-severe PHT. Hydroxyurea use did not differ between patients with and without PHT and no patients used anticoagulation, calcium antagonists, endothelin receptor blockers or sildenafil. Between group comparisons were only per-

**Table 1.** Demographics and laboratory parameters in sickle cell patients with and without pulmonary hypertension.

	HbSS (n=40)/ HbS $\beta^0$ -thalassemia (n=6)		<i>p</i>	HbSC (n=16)/ HbS $\beta^+$ -thalassemia (n=5)	
	PHT	PHT		PHT	PHT
N	28	18		20	1
Age (years)	33 (21-44)	28 (22-52)	0.80	29 (23-39)	41
Male:female	6:22	5:13		10:10	0:1
TRV (m/s)	2.0 (1.3-2.3)	2.7 (2.6-2.8)		2.1 (1.3-2.2)	2.6
sPAP*(mmHg)	21 (12-28)	34 (32-43)		21 (12-25)	31
Hb (mmol/L)	5.7 (5.0-6.2)	4.9 (4.2-5.9)	0.05	7.0 (6.6-7.6)	6.5
HbF (%)	10.6 (6.1-18.3)	5.9 (2.2-14.1)	0.13	1.0 (1.0-2.4)	4
LDH (U/L)	369 (300-515)	575 (388-846)	0.02	231 (202-359)	261
GFR (mL/min)**	151 (120-195)	120 (66-172)	0.10	140 (125-160)	114
ADMA ( $\mu$ mol/L)	0.57 (0.52-0.65)	0.63 (0.58-0.79)	0.01	0.50 (0.45-0.58)	0.48
SDMA ( $\mu$ mol/L)	0.47 (0.42-0.55)	0.51 (0.47-0.83)	0.07	0.46 (0.44-0.56)	0.47
Arginine ( $\mu$ mol/L)	45 (32-56)	46 (41-62)	0.26	67 (57-79)	56
Ornithine (mmol/L)	56 (42-66)	56 (45-75)	0.41	56 (49-62)	44
Citrulline (mmol/L)	23 (16-32)	27 (20-32)	0.66	29 (24-33)	32
Proline (mmol/L)	208 (162-257)	209 (176-234)	0.84	197 (148-247)	257
Arginine/ornithine	0.84 (0.66-1.0)	0.93 (0.72-1.15)	0.45	1.14 (0.86-1.21)	1.1
Arginine/citrulline	1.87 (1.66-2.49)	1.98 (1.43-2.65)	0.84	2.1 (1.6-2.3)	1.5
Arginine/proline	0.23 (0.18-0.31)	0.25 (0.19-0.35)	0.41	0.30 (0.21-0.37)	0.19
sVCAM-1 (ng/mL)	1089 (801-1239)	1542 (1119-1880)	0.007	851 (628-1011)	971

Data are presented as medians with their corresponding inter quartile range. A *p*-value <0.05 is considered statistically significant. \*Right ventricular systolic pressure was estimated based on the modified Bernoulli equation (1) and considered to be equal to the systolic pulmonary artery pressure (sPAP) in absence of right ventricular outflow obstruction. \*\*Glomerular filtration (GFR) rate calculated with Cockcroft and Gault-formula (males: creatinine clearance = 1.23xweight x (140-age)/serum creatinine, females: creatinine clearance = 1.03xweight x (140-age)/serum creatinine).

formed in HbSS/HbS $\beta^0$ -thalassemia patients as only 3 HbSC/HbS $\beta^+$ -thalassemia patients had PHT of whom one had blood samples drawn.

ADMA concentrations in patients without PHT were high compared to previously reported values in healthy race-matched controls. Irrespective of PHT, HbSS/HbS $\beta^0$ -thalassemia patients were characterized by lower hemoglobin, higher LDH, ADMA and sVCAM-1 concentrations than HbSC/HbS $\beta^+$ -thalassemia patients (all  $p < 0.001$ ). ADMA and sVCAM-1 were higher in HbSS/HbS $\beta^0$ -thalassemia patients with PHT than those without PHT, with a significant correlation between ADMA and TRV as well (see correlation studies in *Online Supplementary Table 2*). sVCAM-1 and hemoglobin were significantly correlated to TRV in HbSS/HbS $\beta^0$ -thalassemia patients ( $r_s = 0.49$ ,  $p = 0.002$ ,  $r_s = -0.30$ ,  $p = 0.04$ , respectively). SDMA, but not ADMA, was significantly correlated to GFR ( $r_s = -0.66$ ,  $p < 0.001$ ,  $r_s = -0.08$ ,  $p = 0.60$ , respectively) in HbSS/HbS $\beta^0$ -thalassemia patients. Given the relation between hemolysis and methylarginine concentrations, it is likely that the hemolytic rate is an important determinant of their production in SCD (likely due to the increased protein turn-over in the stress erythropoiesis), also explaining the higher concentrations in HbSS/HbS $\beta^0$ -thalassemia patients. A relative decrease in renal function (generally more evident in HbSS/HbS $\beta^0$ -thalassemia patients) could contribute especially to SDMA elevations. Contributing factors related to the pulmonary vasculature could be shear stress induced PRMT activity<sup>8</sup> and hypoxia induced DDAH downregulation.<sup>9</sup> Although difference in ADMA between patients with and without PHT seems modest, even small increases in extra-cellular ADMA lead to significant intra-cellular NOS inhibition through preferred cellular ADMA uptake over arginine.<sup>3</sup> Indeed, plasma ADMA concentrations  $\geq 0.64 \mu\text{mol/L}$  are associated with strongly reduced pulmonary artery endothelial NOS expression and early death in PHT patients.<sup>5</sup> Based upon the strong correlation of sVCAM-1 to ADMA, it would be interesting to hypothesize that chronic hemolysis induced ADMA elevations significantly contribute to endothelial activation and dysfunction in SCD via NOS inhibition, and that patients with higher ADMA concentrations are more prone to develop a vasculopathy leading to complications such as PHT over time.

Arginase activity (reflected by arginine to ornithine ratios) is elevated in sickle cell patients with moderate-severe PHT but, in agreement with previous studies,<sup>1,10</sup> did not differ between patients with mostly mild PHT and those without PHT. Conceding the fact that we did not determine plasma arginase activity directly, these data suggest that ADMA could play a role of pathophysiological importance at a relatively earlier stage than arginase activity.

The relatively small number of patients needs to be taken into account when interpreting these data and no conclusions can be drawn about HbSC/HbS $\beta^+$ -thalassemia patients. Also, right heart catheterization remains the gold standard diagnostic test for PHT and is recommended in sickle cell patients with moderate-severe PHT detected with echocardiography. However, given the excellent correlation between pulmonary artery pressure and TRV in SCD,<sup>1</sup> and the fact that an elevated TRV is the result of solely left-sided heart disease in only a minority of cases,<sup>11</sup> the lack of right heart catheterization is unlikely to have significantly affected our results. Lastly, our data are largely limited to patients with mild PHT. Nonetheless, mortality is high in these patients<sup>1</sup> and plasma ADMA concentrations were well in

the range associated with death in other forms of PHT.<sup>4,5</sup>

Taken together, our data identify an association of plasma ADMA concentrations to PHT in SCD, possibly identifying a novel factor of importance in its pathophysiology. Also, ADMA induced limitation of NO production may well provide an important new mechanistic link between hemolysis and the characteristic endothelial activation of SCD.

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## References

- Gladwin MT, Sachdev V, Jison ML, Shizukuda Y, Plehn JF, Minter K, et al. Pulmonary hypertension as a risk factor for death in patients with sickle cell disease. *N Engl J Med* 2004;350:886-95.
- Landburg PP, Teerlink T, Muskiet FA, Duits AJ, Schnog JB. Plasma concentrations of asymmetric dimethylarginine, an endogenous nitric oxide synthase inhibitor, are elevated in sickle cell patients but do not increase further during painful crisis. *Am J Hematol* 2008;83:577-9.
- Wilcken DE, Sim AS, Wang J, Wang XL. Asymmetric dimethylarginine (ADMA) in vascular, renal and hepatic disease and the regulatory role of L-arginine on its metabolism. *Mol Gen Metabol* 2007;91:309-17.
- Kielstein JT, Bode-Boger SM, Hesse G, Martens-Lobenhoffer J, Takacs A, Fliser D, et al. Asymmetrical dimethylarginine in idiopathic pulmonary arterial hyper-

tension. *Atheroscler Thromb Vasc Biol* 2005;25:1414-8.

5. Skoro-Sajer N, Mittermayer F, Panzenboeck A, Bonderman D, Sadushi R, Hitsch R, et al. Asymmetric dimethylarginine is increased in chronic thromboembolic pulmonary hypertension. *Am J Resp Crit Care Med* 2007;176:1154-60.
6. van Beers EJ, van Eck-Smit BL, Mac Gillavry MR, van Tuijn CF, van Esser JW, Brandjes DP, et al. Large and medium-sized pulmonary artery obstruction does not play a role of primary importance in the etiology of sickle-cell disease-associated pulmonary hypertension. *Chest* 2008;133:646-52.
7. Schnog JB, Rojer RA, Mac Gillavry MR, Ten Cate H, Brandjes DP, Duits AJ. Steady-state sVCAM-1 serum levels in adults with sickle cell disease. *Ann Hematol* 2003;82:109-13.
8. Osanai T, Saitoh M, Sasaki S, Tomita H, Matsunaga T, Okumura K. Effect of shear stress on asymmetric dimethylarginine release from vascular endothelial cells. *Hypertension* 2003;42:985-90.
9. Arrighoni FI, Vallance P, Haworth SG, Leiper JM. Metabolism of asymmetric dimethylarginines is regulated in the lung developmentally and with pulmonary hypertension induced by hypobaric hypoxia. *Circulation* 2003;107:1195-201.
10. Morris CR, Kato GJ, Poljakovic M, Wang X, Blackwelder WC, Sachdev V, et al. Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. *JAMA* 2005;294:81-90.
11. Sachdev V, Machado RF, Shizukuda Y, Rao YN, Sidenko S, Ernst I, et al. Diastolic dysfunction is an independent risk factor for death in patients with sickle cell disease. *J Am Coll Cardiol* 2007;49:472-9.

**Effect of JAK2 V617F on thrombotic risk in patients with essential thrombocythemia: measuring the uncertain**

Current data about thrombotic risk in ET patients harboring the *JAK2* V617F mutation remain partially inconclusive.<sup>1,2</sup> A systematic literature review of MEDLINE up to February 2008 to identify studies of ET in the *JAK2* era was conducted using the following search algorithm: *JAK2* AND (essential OR thrombocytosis OR thrombocythemia OR thrombosis). All searches were limited to studies of humans published in English. A manual search of abstracts was initially conducted and relevant studies were retrieved in full text. In addition, a manual review of references was carried out to identify any additional relevant articles. To be included in the analysis, studies had to report the prevalence of thrombosis in *JAK2* V617F patients and in wild-type carriers with ET.

Weighted averages were reported as Odds Ratios (ORs) along with their 95% Confidence Intervals (95% CIs) to quantify the effect of *JAK2* positivity on the thrombotic risk in each study. Major thrombotic events were extracted, including strokes and transient ischemic attacks, myocardial infarctions and angina pectoris, peripheral artery occlusion, deep vein thrombosis and pulmonary embolism.

Pooled ORs were calculated according to the Mantel-Haenszel method for fixed effects (FE) and DerSimonian-Laird for random effects (RE). Statistical heterogeneity was measured using the  $\chi^2$  Q test ( $p < 0.10$  is considered representative of significant statistical heterogeneity) and the  $I^2$  statistic, as previously described.<sup>3</sup> To establish the effect of clinical heterogeneity between studies, subgroup analysis was performed. Although the selection of a random- vs. fixed-effects model remains controversial, a fixed-effects model appears more appropriate whenever heterogeneity is limited.

A total of 492 relevant studies were initially retrieved. Among them, 17 studies (see *Online Supplementary*

*Appendix*) met the inclusion criteria. Incidence figures for thrombosis vary from 17% to 43%, and *JAK2* V617F positivity varies from 37% to 71%. A significant association of *JAK2* mutation with thrombosis was evident in half of these studies whereas no such correlation was documented in the remaining studies (Table 1A).

Meta-analysis of 2,905 patients with ET and 778 patients with thrombosis (Table 1B), showed *JAK2* V617F patients have a two-fold risk of developing thrombosis (OR<sub>RE</sub> 1.84, 95%CI 1.40-2.43) with significant heterogeneity between studies ( $I^2=42.5\%$ ).

The statistical heterogeneity reported should be considered a reflection of clinical heterogeneity between different study populations, type (prospective vs. retrospective) and variability in follow-up. Moreover, *JAK2* V617F patients are older at diagnosis, have higher hemoglobin levels, higher leukocyte counts and lower platelet counts.<sup>2,4,6</sup> Leukocytosis is regarded as an additional factor for thrombosis whereas thrombocytosis is not,<sup>5,6</sup> and additional evidence is provided to support the role of granulocytes in MPD-associated thrombosis.<sup>7</sup> The above observations are consistent with the superior effectiveness of hydroxyurea (a non-specific myelosuppressive agent) compared to anagrelide (a platelet-specific cytoreductive agent) in high risk ET<sup>8,9</sup> and supported by the lack of an increased risk of thrombosis associated with extreme thrombocytosis in otherwise low-risk ET.<sup>10</sup> Age is a well-established confounder in thrombotic risk, and cardiovascular risk factors may vary between study groups. However, 2 recent studies have demonstrated that the presence of cardiovascular risk factors does not modify thrombotic risk in patients with ET who experience first-time thrombosis.<sup>11,12</sup> In fact, recurrent thrombosis is again predicted by age (>60 years) and thrombosis history<sup>12</sup> factors with well-established prothrombotic effect in ET.<sup>6,11</sup>

Finally, the allele burden of the mutated *JAK2* gene, the effect of which cannot be estimated, may account for the diversity between studies. Results so far remain contradictory.<sup>2,13</sup>

Given the exaggerating effect of smaller studies, larger

**Table 1A. Characteristics of studies included in the analysis.**

Study	Type	N	JAK2 V617F	Thrombosis	Association of JAK2 V617F with thrombosis
Baxter EJ, 2005	P	51	29 (56.9)	12 (23.5%)	No
Campbell PJ, 2005	P	776	414 (53.3%)	137 (17.7%)	Yes
Wolanskyj AP, 2005	R	150	73 (48.7%)	62 (41.3%)	No
Cheung B, 2006	R	60	29 (48%)	26 (43%)	Yes
Heller PG, 2006	R	50	24 (48%)	12 (24.0%)	Yes
Stevenson WS, 2006	R	27	10 (37%)	7 (25.9%)	No
Alvarez-Larran A, 2007	P	103	44 (42.7%)	22 (21.4%)	No
Finnazzi G, 2007	R	179	103 (57.5%)	47 (26.3%)	Yes
Hsiao HH, 2007	R	53	35 (66%)	17 (32.1%)	Yes
Kittur J, 2007	R	176	96 (54.5)	70 (39.8%)	No
Ohyashiki K, 2007	R	49	31 (63.3%)	11 (22.5%)	Yes
Pemmaraju N, 2007	P	80	38 (47%)	26 (32.5%)	No
Rudzki Z, 2007	R	59	38 (64.4%)	24 (40.7%)	No
Speletas M, 2007	P	111	77 (69.3%)	45 (40.5%)	No
Toyama K, 2007	P	82	58 (70.7%)	16 (19.5%)	Yes
Vannucchi AM, 2007	R	639	382 (59.8%)	188 (29.4%)	Yes
Antonioni E, 2008	R	260	165 (63.5%)	56 (21%)	No

The squares and lines show the estimated odds ratios and their 95% CIs. The size of each square is proportional to the amount of information (weight) available in the subgroup. Overall estimates are shown by a diamond, with the width representing the 95% CI.