

Our recent work on *Tmprss6*<sup>+/-</sup> mice complements the results of Finberg et al.<sup>1</sup> *Tmprss6*<sup>+/-</sup> mice develop normally and are phenotypically indistinguishable from wild-type littermates. At 3 weeks of age they have lower hemoglobin (Hb), red blood cell (RBC) counts, and mean corpuscular volume (MCV; Figure 1A) and higher hepcidin and Id1 levels compared with wild-type (Figure 1B). Adult *Tmprss6*<sup>+/-</sup> mice have Hb and RBC similar to wild-type but reduced MCV, as reported.<sup>1</sup> Transferrin saturation, which shows a trend toward reduction in 8-week-old animals in the previous study,<sup>1</sup> is significantly reduced in our study. *Tmprss6*<sup>+/-</sup> mice of this age show mildly elevated Id1 and hepcidin production, inappropriately high for their low levels of transferrin saturation (Figure 1B-C). C-reactive protein mRNA levels are similar in *Tmprss6*<sup>+/-</sup> and wild-type mice (data not shown), ruling out a potential contribution of inflammation to hepcidin expression. In agreement with the observation of a more severe anemia in young versus adult patients in a large iron-refractory iron deficiency anemia (IRIDA) pedigree,<sup>6</sup> our results show that young *Tmprss6*<sup>+/-</sup> mice, which have increased iron demands, are indeed more iron-deficient than adult mice.

Female mice at 18.5 gestational days that have high iron demands, have lower liver iron concentration (LIC) than wild-type, even if hematologic parameters are similar.<sup>1</sup> To assess the effect of iron restriction, we fed 4-week-old animals of both genotypes with normal or iron-deficient diet for 4 weeks. The iron-deficient diet induced a more pronounced decrease of transferrin saturation in *Tmprss6*<sup>+/-</sup> than in wild-type mice (Figure 1C) and a trend toward a more severe microcytic anemia, although the difference reached statistical significance only for MCV (Figure 1A). However, as in pregnant mice,<sup>1</sup> *Hamp* was strongly down-regulated in both genotypes (Figure 1B), suggesting that iron deficiency overrides the SMAD pathway activation observed in *Tmprss6*-haploinsufficient mice.

We have not measured LIC that was found decreased in 8-week-old *Tmprss6*<sup>+/-</sup> mice.<sup>1</sup> However, transferrin receptor 1 (TfR1) mRNA, a LIC indirect measure, is significantly increased only in adult *Tmprss6*<sup>+/-</sup> mice fed a standard diet (Figure 1B). Likely high basal *TfR1* mRNA levels in young wild-type mice that have high iron requests and in adult wild-type animals on an iron-deficient diet blunt the difference with *Tmprss6*<sup>+/-</sup>.

In conclusion, systemic iron homeostasis is mildly compromised in *Tmprss6*-haploinsufficient mice, which are prone to iron deficiency when iron demands are high for body growth and erythropoiesis expansion or when dietary iron is restricted. In humans, genomewide association studies show that common *TMPRSS6* variants influence iron parameters, Hb, and erythrocyte traits.<sup>7-10</sup> All these findings suggest that susceptibility to iron deficiency may be modulated by *TMPRSS6* mutations even at the heterozygous state.

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## To the editor:

### Pulmonary hypertension and NO in sickle cell

We are honored that our work merits the attention of Dr Bunn and colleagues<sup>1</sup> but disagree with some of their conclusions. Traditional risk factors for vaso-occlusive pain crisis, such as leukocytosis and high hemoglobin levels, incompletely predict vasculopathic events and

mortality in adult patients with sickle cell disease (SCD). We maintain that the evidence supports the following propositions:

**(1) Pulmonary hypertension (PH) is common in adults with SCD and is associated with a high risk of death.**

The tricuspid regurgitation jet velocity (TRV) is a useful noninvasive screening tool for suspected PH; however, the diagnosis requires right heart catheterization. In epidemiologic studies, Doppler-estimated right ventricular systolic pressure more than 2 standard deviations (SD) above the mean (TRV > 2.5 m/s) is common in adults with SCD (approximate 30% prevalence) and is associated with a 9.2 to 15.9 risk ratio (RR) for early death.<sup>2-4</sup> Similarly, NT-proBNP, a biomarker released from cardiomyocytes under pressure stress, is elevated in 30% of SCD patients and a value of 160 pg/mL and above identifies a subgroup with a 19.5% absolute increase in risk of death in the National Institutes of Health (NIH) and Multicenter Study of Hydroxyurea (MSH) cohorts.<sup>5</sup>

Based on these studies, a TRV less than 2.5 m/s or NT-proBNP less than 160 pg/mL are considered normal screening values associated with a low risk of death in SCD.<sup>2</sup> Furthermore, we should agree that a TRV of 3 m/s or higher is more than 3 SD above the mean, present in 10% of SCD adults, and is associated with a high RR of death (> 10).<sup>6</sup> We recommend that standard of care for these patients include clinical evaluation for PH risk factors and right heart catheterization.

The intermediate group (TRV 2.5-2.9 m/s; > 2 SD above mean) remains a source of controversy. In adults with SCD, this group overall appears to have decreased exercise capacity and increased mortality (RR of 4.4;  $P < .001$ ). Refinement of risk definition in this group has great potential to advance the field.

A recent French study of PH in SCD appropriately highlights the limitations of the echocardiogram; the diagnosis of PH requires direct measurement of mean pulmonary artery pressure (mPAP) with PH defined as mPAP 25 mmHg or higher at rest. This study also validates our observations: even after excluding adult SCD patients with greater SCD disease severity, 6% of adults had mPAP 25 mmHg or higher, which meets the consensus definition of PH and is 3 SD above the mean. Furthermore, their patients with confirmed PH had more severe hemolytic anemia and, in a 2-year follow-up, deaths occurred only in the PH group.

### **(2) Hemolytic anemia impairs NO signaling and is a major and attributable risk factor for the development of clinical subphenotypes of SCD.**

In humans, impairment in NO signaling, directly measured via venous occlusion strain-gauge plethysmography, correlates with increased levels of plasma hemoglobin or its surrogate LDH.<sup>7</sup> This relationship between increasing plasma hemoglobin levels and direct measures of decreasing NO-dependent blood flow or low NO bioavailability has been confirmed in many studies in disparate diseases, such as malaria<sup>8</sup> and paroxysmal nocturnal hemoglobinuria.<sup>9</sup> In patients with SCD, the development of vasculopathic complications—such as PH, priapism, and leg ulceration—are associated with markers of hemolysis.<sup>10,11</sup> More than 18 cohort studies have consistently associated the severity of hemolytic anemia with these complications and risk of death, including the NIH-PH Screening,<sup>2</sup> Duke University,<sup>4</sup> University of North Carolina,<sup>3</sup> Multi-center Study of Hydroxyurea,<sup>5</sup> Pulmonary Hypertension and the Hypoxic Response in Sickle Cell Disease,<sup>12</sup> and Cooperative Study of Sickle Cell Disease cohorts,<sup>13</sup> a recently published Greek study,<sup>14</sup> and our unpublished analysis of the Walk-PHASST screening cohort (July 7, 2010).

All mouse models of hemolysis studied to date develop spontaneous PH and right heart failure, including the Berkeley sickle cell mouse,<sup>15,16</sup> the spherocytosis mouse,<sup>17</sup> and the allo-immune hemolysis mouse.<sup>15</sup> Pathologic evaluations in these models find no chronic thrombosis in the pulmonary vasculature; rather, a functional impairment in NO signaling driven by NO scavenging

by plasma hemoglobin. NO depletion, PH, and systemic hypertension have been reported in animal studies by inducing intravascular hemolysis or by infusions of hemoglobin or hemolysate.<sup>18</sup>

Hemolytic anemia does not occur in isolation. Priapism is more frequent in SCD than in other hemolytic diseases, demonstrating the independent contributions of sickle vaso-occlusion and hemolysis-related pathology, analogous to the independent contributions of smoking and high cholesterol to the development of atherosclerosis.

### **(3) Failure of NO-based therapies in early clinical trials does not negate this mechanism of disease.**

Less than 10% of all clinical trials are successful. The SCD field has seen failures of drugs targeting accepted mechanisms, such as poloxamer 188 to improve red cell rheology, senicapoc to inhibit the Gardos channel and to reduce HbS polymerization, and steroids to reduce inflammation. We cannot give up on clinical trials in SCD PH; rather, we need to improve trial designs and explore the insights into biology we have learned from all investigations.

What is crucial for the vitality of future hemoglobinopathy research is that we unite to critically analyze and learn from each trial, maintain the funding and infrastructure of the SCD research groups, and encourage the next generation of physician-scientists interested in SCD research.

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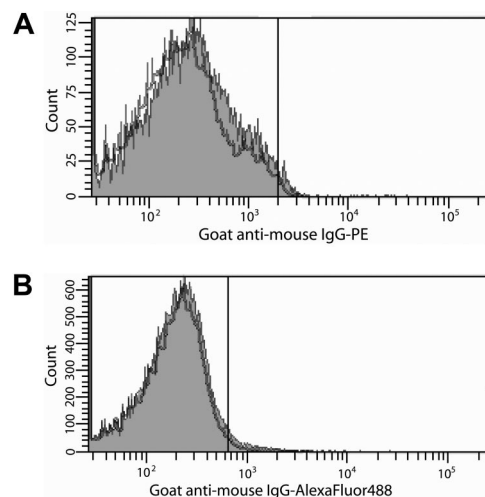
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## To the editor:

### No evidence for tissue factor on platelets

Blood coagulation is initiated *in vivo* by formation of a tissue factor (TF)/factor VIIa (FVIIa) complex.<sup>1</sup> Under physiologic conditions, TF is sequestered from blood and coagulation is initiated only after vascular injury. This paradigm has been challenged by studies suggesting that platelets express active TF after *de novo* synthesis,<sup>2,3</sup> transfer from monocytes,<sup>4</sup> or release from  $\alpha$ -granules.<sup>5</sup> In other studies,<sup>6,7</sup> however, the existence of platelet TF could not be demonstrated.

To resolve this ongoing controversy, experiments were performed with specific, inhibitory anti-TF antibodies<sup>8</sup> using well-defined TF antigen and activity assays.<sup>6,8</sup> Flow cytometric analyses indicated that no TF was expressed by protease-activated receptor (PAR)1- and PAR4-activated platelets (1.6%  $\pm$  1.2%) compared with unactivated platelets (1.5%  $\pm$  0.6%; Figure 1A) despite maximal  $\alpha$ -granule release (> 98% P-selectin-positive platelets), consistent with previous studies demonstrating a lack of platelet TF expression after A23187-induced platelet activation and  $\alpha$ -granule release.<sup>6</sup> Consistent with studies performed in whole blood,<sup>6,7</sup> no TF antigen (< 0.2pM) was detected by immunoassay<sup>8</sup> after prolonged stimulation of platelets with lipopolysaccharide (LPS). In contrast, LPS-stimulated THP-1 cells expressed 6.6 plus or minus 2.0pmol/L TF/10<sup>6</sup> cells. These observations were confirmed in 2 TF-dependent activity-based assays.<sup>6</sup> No factor Xa (FXa) (< 0.1pmol/L FXa/second) was generated by extrinsic FXase using unstimulated or stimulated platelets as a possible TF source. Similarly, no clot was formed in a plasma-based clotting assay (> 999 seconds). In contrast, FXa generation (1.6pmol/L FXa/second) and clot formation (~ 71 seconds) were observed using LPS-stimulated THP-1 cells, but not unstimulated cells. Both were TF-dependent as an inhibitory anti-TF antibody<sup>8</sup> decreased the rate of FXa generation to approximately 0.5pmol/L FXa/second and prolonged the clot time to 255 seconds, which corresponds to approximately 90% inhibition of activity. These observations contradict studies suggesting that platelets synthesize and express



**Figure 1. Search for TF on platelets by flow cytometry.** (A) Washed platelets were activated with PAR1 (100 $\mu$ M) and PAR4 (500 $\mu$ M) agonist peptides (2 hours, 37°C). (B) Platelet-rich plasma was incubated with THP-1 cells in the presence or absence of 250 ng/mL LPS (4 hours, 37°C). For all experiments, TF expression on platelets was determined by immunostaining with an anti-TF antibody<sup>8</sup> (0.5 $\mu$ M) in 20mM Hepes, 0.15 M NaCl (pH 7.4) containing 10  $\mu$ g/mL human Fc, followed by either (A) goat anti-mouse IgG-PE (1:10 dilution) or (B) goat anti-mouse IgG-Alexa Fluor 488 (1:400 dilution) in 10% goat serum. Platelets (10 000) were analyzed by flow cytometry using a Becton Dickinson LSR II flow cytometer. The positive analyses regions were defined such that approximately 2% of the platelets stained with secondary antibodies alone were positive. The gray histograms depict anti-TF antibody staining of platelets activated with PAR peptides (A) or incubated with THP-1 cells and LPS (B). The black histograms depict immunostaining of unactivated platelets (A) or platelets incubated with THP-1 cells alone (B). The data shown are representative of 2 experiments performed in triplicate.

TF *de novo* in a time-dependent manner in response to platelet activation<sup>2,3</sup> and preclude the notion that TF is stored in and released from platelet  $\alpha$ -granules.<sup>5</sup>