Prevalence of reduced fibrinogen binding to platelets in a population of Thoroughbreds

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Objective—To measure the frequency and magnitude of reduced fibrinogen binding in a population of horses from a Thoroughbred breeding farm.

Animals—444 Thoroughbred horses, 1 to 27 years old, including 316 females, 72 geldings, and 56 sexually intact males.

Procedures—Blood was collected from horses into tubes containing acid citrate dextrose adenine, and washed platelets were examined by use of flow cytometry for their ability to bind fibrinogen.

Results—Data regarding fibrinogen binding to activated platelets were normally distributed, with nearly identical amounts of variation regardless of sex. In 3 horses, fibrinogen binding to platelets was reduced from 67.6% to 83.4%, compared with normal platelets, which indicated an inability of platelets to aggregate in response to thrombin (0.1 U/mL).

Conclusions and Clinical Relevance—Platelet fibrinogen binding of the affected horses identified in this study was characteristic of a reported heritable bleeding disorder in which the reduction in fibrinogen binding correlated with prolonged bleeding times in template bleeding assays. The bleeding disorder is distinct from Glanzmann thrombasthenia, in which platelets fail to bind fibrinogen because of lack of αIIb-β3 integrin on their surface. The prevalence of affected horses within the small sample population studied here (0.7% [n = 3]) is considerably higher than the prevalence of bleeding disorders within more genetically diverse groups. (*Am J Vet Res* 2007;68:716–721)

Platelets are the primary cellular mediators of hemostasis and, when activated in response to vascular injury, initiate the production of thrombin. Thrombin activates fibrinogen, which polymerizes to form the fibrin meshwork that blocks the injured vessel. Platelets play an active role in this meshwork by binding fibrinogen through αIIb β3 integrins on their surfaces. Substantial reduction of fibrinogen binding to platelet surfaces can result in a poorly stabilized fibrin meshwork and lead to increased risk of bleeding. Two heritable platelet function defects have been identified in horses, both of which are characterized by reduced fibrinogen binding.

Glanzmann thrombasthenia is a heritable bleeding disorder that results from mutations of genes that encode either the integrin subunit αIIb or β3. Clinically, Glanzmann thrombasthenia has been diagnosed in 4 horses of different breeds, including a Standardbred,[1] an Oldenbourg filly,[a] a Thoroughbred cross, and a Quarter Horse.[2] Genetic analysis of the latter 2 horses revealed a single guanine to cytosine substitution in exon 2 of the αIIb gene for which the Thoroughbred cross was homozygous and the Quarter Horse was a compound heterozygote.[3] The resulting amino acid change causes reduced surface expression of αIIb β3 in platelets, absence of clot retraction, and failure to bind fibrinogen in response to multiple platelet agonists including ADP, collagen, and platelet activating factor. Such horses are at increased risk of bleeding even during nonstrenuous activity.

A second defect in fibrinogen binding has been identified in a Thoroughbred mare and its offspring and is distinct from Glanzmann thrombasthenia in that fibrinogen binding is reduced instead of completely eliminated and fibrinogen binding is observed in response to platelet activation by thrombin.[4,5] Platelets from the 2 horses bind normal amounts of fibrinogen in response to ADP, which leads to activation of the αIIb β3 integrin and aggregation through 3 ADP receptors: P2Y1, P2Y12, and P2X1. However, platelets from horses with this disorder have reduced prothrombinase activity and a delayed response to collagen as measured by use of aggregometry, both of which are character-

Abbreviations

PG	Prostaglandin
vWF	von Willebrand factor
uPA	Urokinase plasminogen activator
QPD	Quebec platelet disorder

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istic of defective granule secretion. The 2 horses have prolonged bleeding times, and the mare has had severe bleeding incidents in response to trauma.[4]

Both disorders can be identified by the amount of fibrinogen bound by platelets that have been stimulated with thrombin. Platelets with Glanzmann thrombasthenia fail to bind fibrinogen,[6] whereas those from horses with the second disorder[5] have reduced capacity to bind fibrinogen in response to thrombin. The purpose of the study reported here was to estimate the prevalence of abnormal fibrinogen binding by platelets in response to thrombin in a population of 444 Thoroughbreds.

Materials and Methods

Animals—Four healthy mares (horses 1 to 4), of ages 7, 21, 24, and 24 years, from the Center for Equine Health at the University of California, Davis, were used to compare fibrinogen binding by platelets from fresh blood with fibrinogen binding by platelets from blood stored overnight. Horses enrolled in the prevalence study were from a Thoroughbred breeding farm in California and included mares, geldings, and stallions of ages 1 to 27 years (median, 6 years). Studies were conducted between May 11, 2005, and February 17, 2006.

Platelet preparation—Blood samples were collected under institutionally approved protocols into evacuated tubes that contained acid citrate dextrose adenine (15% final concentration) and shipped in insulated containers to the University of California, Davis, for delivery within 24 hours. On arrival, blood was warmed to 37°C for 30 minutes prior to centrifugation in 17 × 120-mm conical polypropylene tubes at 200 × *g* for 15 minutes at room temperature (25°C). Platelet-rich plasma was transferred to 17 × 100-mm, round-bottom, polypropylene tubes; 10 µg of PGE_1/mL (final concentration)[b] was added; and the platelet-rich plasma was centrifuged at 400 × *g* for 15 minutes at room temperature. Platelet pellets were then suspended in 10 mL of Tyrode-HEPES (12mM $NaHCO_3$, 138mM NaCl, 2.9mM KCl, 10mM HEPES, and 5 µg of PGE_1/mL; pH, 7.2), centrifuged, and suspended in Tyrode-HEPES at 1×10^8 cells/mL. Calcium chloride was added to platelets in 5 equal aliquots at 5-minute intervals from a 10mM stock in Tyrode-HEPES to achieve final concentrations of 2mM $CaCl_2$ and 5×10^7 cells/mL. All cell counts were determined with an automated blood counter.[c]

Fibrinogen binding assay—Washed platelets were incubated with Alexa 488–labeled fibrinogen (3 µg/mL)[d] for 1 minute at room temperature prior to activation. Platelets were activated with 0.1 U of thrombin[e]/mL and evaluated via flow cytometry after incubation for 30 minutes at 37°C.[f] Forward and side scatter voltages were set to detect machine noise, which was removed during subsequent analyses. The FL1 detector was set to 500 V to prevent saturation of the detector by emission from the labeled fibrinogen. Platelet quality was monitored by measuring fibrinogen binding to resting platelets for each sample. Ten horses from which platelets bound an amount of fibrinogen that approximated the defined batch mean were routinely sampled to ensure consistency of the assay.

Sampling criteria—For each batch of samples tested, the mean fluorescence value was calculated, and each sample value was expressed as the percentage difference from the batch mean. This approach was taken to account for among-batch variation. Platelets from horses for which the mean fluorescence value deviated from the batch mean by > 1 SD were tested multiple times (2 to 4) on the basis of a horse's availability for repeated sampling.

Statistical analysis—In the storage study, each horse was sampled in triplicate, means and SDs were calculated, and differences were compared by use of paired *t* tests. In the prevalence study, age and sex distributions of horse fluorescence values were summarized as means and SD and compared by use of 1-way ANOVA

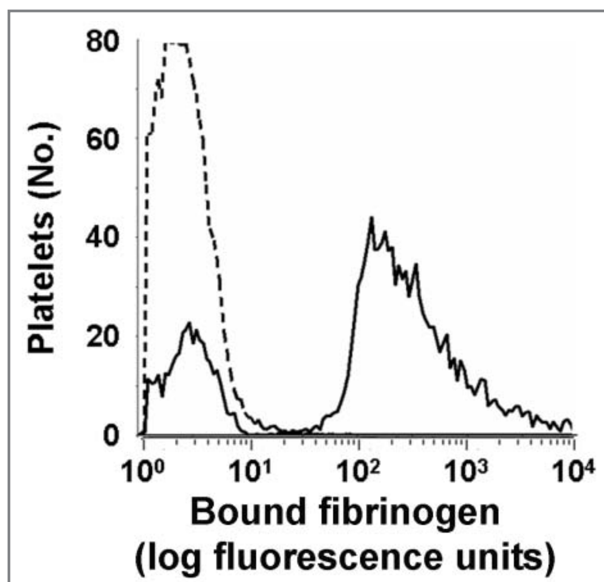

Figure 1—Distribution of platelets with bound labeled fibrinogen from a healthy Thoroughbred before (dotted line) and after (solid line) activation of platelets with thrombin (0.1 U/mL).

Table 1—Fibrinogen binding by fresh and stored platelets from 4 control Thoroughbred horses.

	Normalized fibrinogen binding (%)			Relative fluorescence intensity		
Horse	Fresh	Stored	*P*	Fresh	Stored	*P*
1	17.2 ± 10.1	2.7 ± 20.5	0.332	1,056 ± 42.9	962.9 ± 222.6	0.516
2	–20.9 ± 13.2	–8.0 ± 12.4	0.283	718.9 ± 150.4	857.2 ± 98.3	0.253
3	–2.4 ± 11.9	2.2 ± 9.1	0.620	832.8 ± 68.1	870.6 ± 198.1	0.770
4	6.1 ± 15.3	3.1 ± 4.2	0.759	916.2 ± 200.5	873.0 ± 146.9	0.778

Values are reported as mean ± SD.

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in which deviation of variances was assessed by use of the Levene test; for both tests, $P < 0.05$ was considered significant. The mean percentage difference of fibrinogen binding to platelets was calculated for horses tested multiple times. The fibrinogen binding data from the population set were analyzed via 2 approaches because no threshold-cutoff value for fluorescence values has been determined in prior studies. First, a threshold of 80% less than the mean value for the first batch or the mean of sequential samplings was used. This arbitrary threshold was based on 2 reported clinical cases (with mean ± SD values equal to –84.3 ± 14.6% and –82.8 ± 7.9%)[5] and evidence from human Glanzmann thrombasthenia.[6] Second, 99% left-sided reference intervals were calculated with the cutoff determined as the 90% lower limit for the first percentile of the reference interval. The choice of a left-sided interval assumed that only values lower than the mean were considered abnormal. Reference interval calculations were performed with a parametric (normal approximation) percentile method.[7,g] Deviation from normality of variables within the populations was determined by the Kolmogorov-Smirnov test.

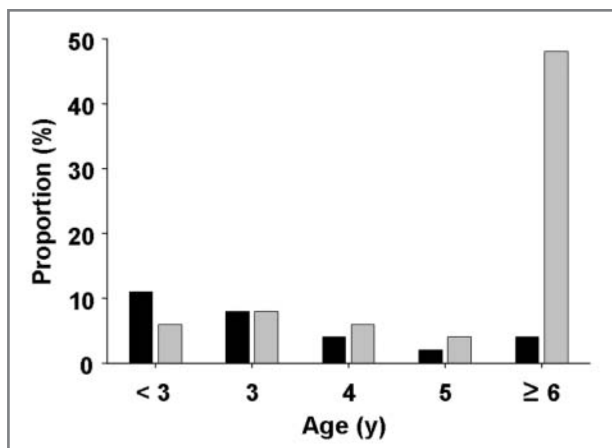

Figure 2—Age distribution of male (sexually intact and gelded [black bars]) and female (gray bars) Thoroughbreds that were examined for diminished fibrinogen binding to platelets.

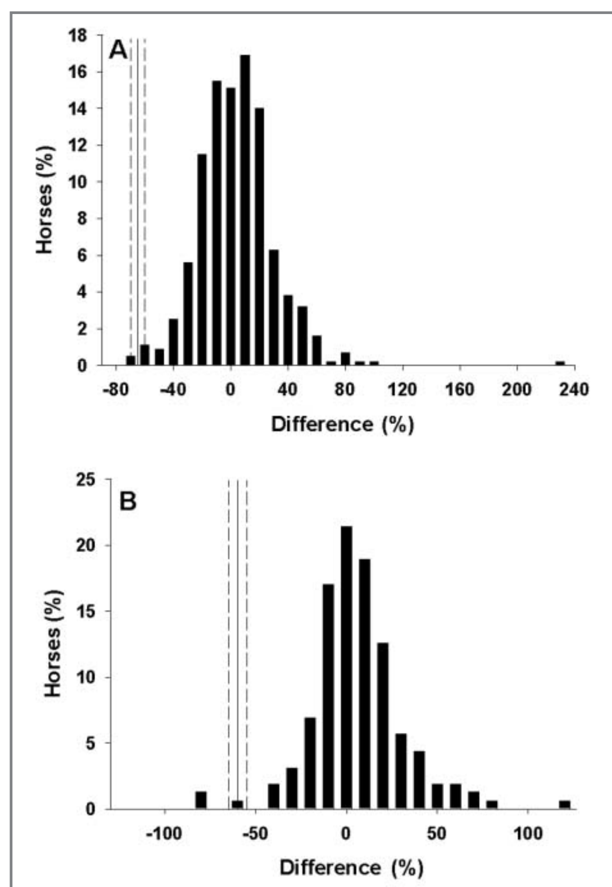

Figure 3—Distribution (percentage of sampled population) of Thoroughbreds with various percentage differences in fibrinogen binding to platelets stimulated with thrombin, compared with mean value for the population. Vertical solid line and dashed lines indicate lower limit and 95% confidence interval used to identify abnormal results. A—Percentage difference, compared with mean value for all 444 Thoroughbreds in the study. B—Mean percentage difference, compared with mean value for 159 Thoroughbreds that were tested multiple times.

Results

Activation of washed platelets from clinically normal horses resulted in fluorescent intensities 300 to 500 times those measured for resting control platelets. This increase in fluorescent intensity was measured for individual platelets by use of flow cytometry and visualized for an entire platelet population as a severe leftward shift in the intensity profile by approximately 80% in the platelet population (**Figure 1**). On the basis of evaluation of normalized fibrinogen binding and mean fluorescence intensity, there were no apparent differences for the fibrinogen binding capacities of fresh versus stored platelets (**Table 1**).

Blood samples were collected from 316 females, 72 geldings, and 56 sexually intact males, with ages that ranged from 1 to 27 years, and shipped overnight from

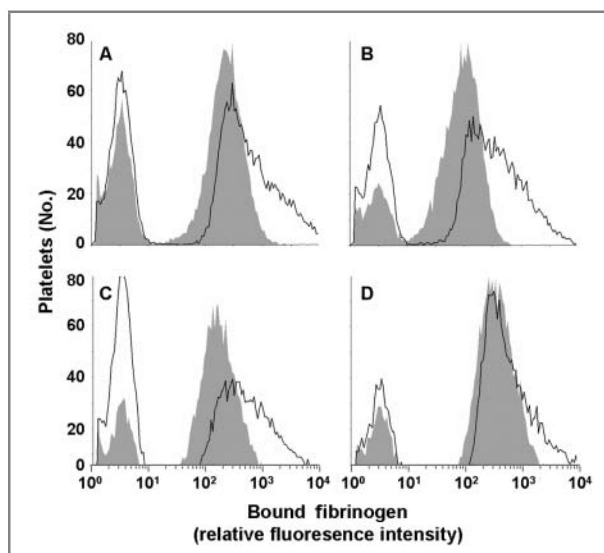

Figure 4—Distribution of platelets with bound labeled fibrinogen from a healthy Thoroughbred (solid line) and a Thoroughbred with reduced fibrinogen binding (shaded area) after activation of platelets with thrombin (0.1 U/mL). A—Index case (horse with reduced fibrinogen binding[5]). B, C, and D—Horses A, B, and C, respectively, of the present study.

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the breeding farm in batches (10 to 20 samples/shipment). The sex-based age distribution was skewed to younger ages for males (median, 2 years) and older ages for the females (median, 6 years; **Figure 2**). Comparison of mean fluorescence values for the first sampling (n = 444) revealed no significant difference among age and sex groups (P = 0.783 and P = 0.573, respectively). In addition, the variance did not differ among age and sex groups (P = 0.803 and P = 0.763, respectively).

Two subsequent analyses were performed on the basis of data from the first sampling of all horses in the study and mean data from the 159 horses that were sampled multiple times (n = 103 with 2 samples, 44 with 3 samples, and 12 with 4 samples; **Figure 3**). The means for these sample populations were –0.9% (range, –79.9% to 222.6%) and –0.7% (range, –83.4% to 111.0%), respectively. No evidence was found for deviation from normality in the populations used for these analyses (P = 0.074 and P = 0.256, respectively). In 1 instance, a horse had platelets that bound approximately twice the amount of fibrinogen as the mean for the entire distribution on the first screening. However, platelets from that horse bound normal amounts of fibrinogen when the test was repeated, suggesting that the platelets were not abnormal.

The lower limit of normal fibrinogen binding for all horses in the study and the mean values from the 159 horses sampled multiple times were –65.1% (90% confidence interval, –68.8% to –61.3%) and –58.4% (90% confidence interval, –64.2% to –52.7%), respectively. Six possible affected horses were identified on the basis of the first sample results by use of these limits, 3 of which were excluded after a single additional round of screening. Platelets from 3 remaining horses (a 17-year mare, a 3-year filly, and an 11-year mare; horses A, B, and C, respectively) had mean ± SD fibrinogen binding values of –83.4 ± 7.5% (horse A, 4 samplings), –81.2 ± 1.8% (horse B, 2 samplings), and –67.6 ± 10.7% (horse C, 3 samplings).

Fibrinogen binding in the former 2 of these horses was nearly identical to that of the previously identified index case (horse A, P = 0.918; horse B, P = 0.795).[5] As was apparent for the previously identified index case, the fibrinogen binding profiles of platelets from the horses in the present study were characterized by a leftward shift in distribution that was less than that observed for control platelets and a symmetric distribution that lacked a tail or skew towards higher intensities as occurred when control platelets were activated (**Figure 4**). Fibrinogen binding to platelets from horse C was also similar to the index case (P = 0.185), but differed in regards to a slightly greater leftward shift of the activated platelet population and a slight skew to higher fluorescent intensities suggestive of greater fibrin formation.

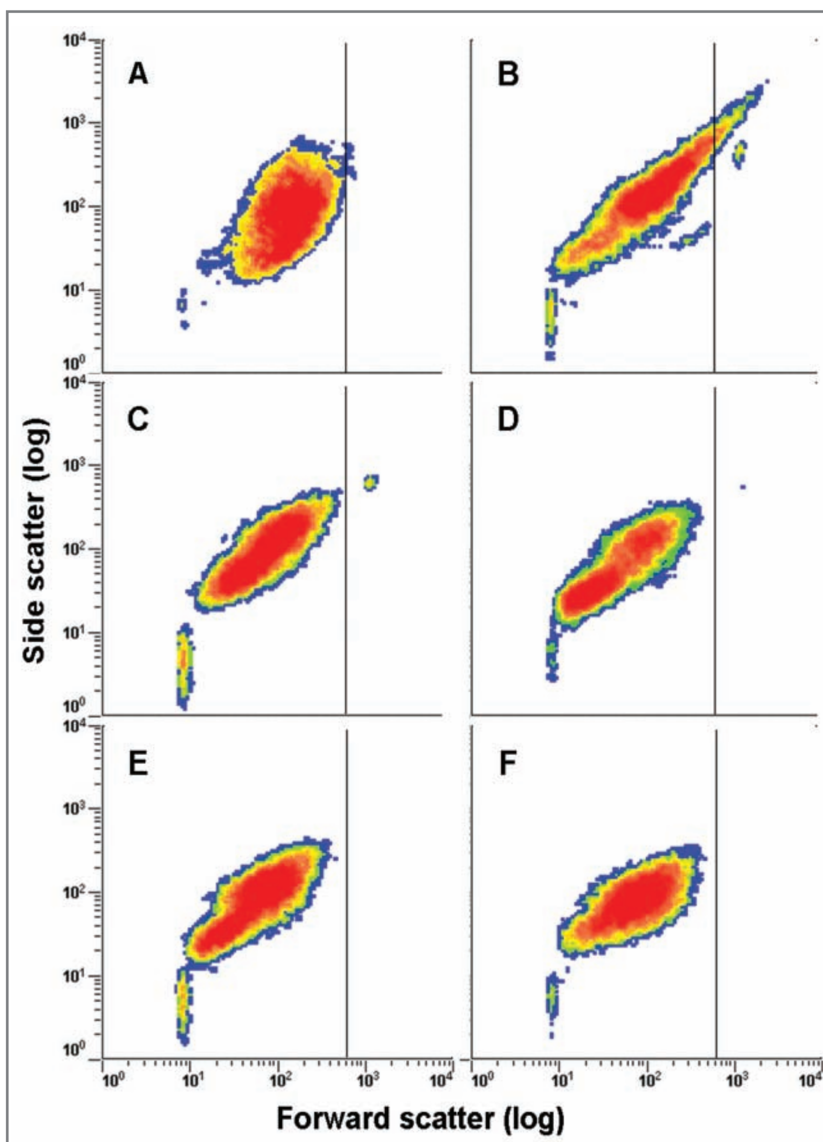

Figure 5—Plots of flow cytometric side scatter versus forward scatter (log values) of platelets of Thoroughbreds. Distributions are color coded according to a logarithmic scale corresponding to the number of platelets at each position in the diagram; blue indicates the minimum number of platelets, and red indicates the maximum. Vertical lines indicate the size limit for nonactivated platelets. A—Nonactivated platelets from a clinically normal horse. B—Activated platelets from a clinically normal horse. C—Activated platelets from the index case. D—Activated platelets from horse A. E—Activated platelets from horse B. F—Activated platelets from horse C.

The skew of the fluorescent distribution to higher intensities resulted from the formation of platelet-fibrinogen aggregates and was indicated by the differences in scatter distributions between resting and activated control platelets (**Figure 5**). Forward scatter directly correlated with the size of platelet-aggregate particles, and platelets from the previously identified index case failed to form an elongated distribution characteristic of aggregates. In addition to reduced fibrinogen bind-

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ing, platelets from horses A, B, and C failed to produce an extensive aggregate distribution that was larger in size than the resting platelet distribution.

Because a defined control population did not exist at the breeding farm, the effects of storage and shipping on these results were difficult to assess. Measurements of fibrinogen binding for both the first sampling of all the horses in the study and for horses sampled multiple times were distributed normally, which was consistent with random uncertainties in the measurements. Although systematic uncertainties arising from shipping could not be discounted, the mean fluorescence intensity of the fibrinogen bound to platelets from the breeding farm population (993.8 ± 364.3 arbitrary fluorescent units; n = 668 measurements) did not differ significantly from that of stored blood from the 4 control horses (horse 1, $P = 0.883$; horse 2, $P = 0.517$; horse 3, $P = 0.559$; and horse 4, $P = 0.556$). Therefore, there was not a clear effect of shipping or processing on the outcome of this study.

Discussion

In this study, fibrinogen binding to platelets from a population of 444 Thoroughbred horses was used to establish a threshold between normal and reduced fibrinogen binding in response to low concentrations of thrombin. There were no readily apparent effects of shipping and processing the blood on the outcome of the assay. Although the use of the reversible inhibitor PGE_1 alters platelet function during processing, it was used in decreasing concentrations at each step of processing and was absent during the final processing step and subsequent activation reactions. Therefore, shipping and processing the samples appear to have had small and nonbiased effects on the results.

Application of the derived limits to the study population led to the identification of 3 (0.7%) horses that consistently had fibrinogen binding less than the threshold value. Prevalence of reduced fibrinogen binding in the limited population of this study was remarkably high in comparison to that of well-characterized bleeding diatheses in humans. In the United States, hemophilia A occurs in the human population with a prevalence of 1:5,000 to 1:10,000.[8,9] Manifestation of this bleeding disorder requires the inheritance of a single defective allele and occurs considerably more frequently than autosomal recessive disorders. For instance, the inability to produce vWF, which is required for platelet adhesion under conditions of high shear-rate, is an autosomal recessive disorder that occurs with a frequency of 3 to 5 cases/million humans of the general population.[10] These individuals are at risk for clinically severe bleeding. However, individuals with reduced vWF production or the production of dysfunctional vWF have been identified with a prevalence of approximately 1% in the general human population.[11] These individuals are at a reduced risk for chronic, severe bleeding incidents and may or may not have prolonged bleeding times, but are, nevertheless, at risk of bleeding during trauma or major surgery.[12]

None of the affected horses identified in the present study had known histories of severe chronic bleeding, and template bleeding times were not measured. However, fibrinogen binding to platelets from these horses was identical to that of a Thoroughbred mare and its affected offspring with a secretion defect that was reported by our laboratory group.[5] Both of the horses have prolonged bleeding times, and the mare was originally identified because of severe bleeding resulting from trauma (pin firing).[4] Neither of the horses originated at the breeding farm of the present study, and it remains unclear whether the horses described in this study had the same platelet function defect. Defects in secretion and the function of αIIb β3 integrins are well-characterized mechanisms leading to reduced fibrinogen binding in response to thrombin. Platelets from humans with Glanzmann thrombasthenia do not bind fibrinogen in response to agonists because they do not express αIIb β3 integrins.[13-15] As was found with the previously reported cases,[5] platelets from affected horses in the present study bound small amounts of fibrinogen in response to thrombin, which is not characteristic of Glanzmann thrombasthenia. Therefore, the affected horses in this study may have had a secretion defect, which could result from either a defect in α-granule contents or signal transduction.

Platelet α-granules contain several proteins, including fibrinogen, factor V, and uPA, that affect fibrinogen polymerization following platelet activation. Quebec platelet disorder is a well-characterized human bleeding diathesis in which these contents are absent as a result of overexpression of uPA, which causes proteolysis of other granule contents.[16] The absence of factor V in platelets in humans with QPD results in complete loss of prothrombinase activity that is required for production of thrombin and subsequent activation of fibrinogen polymerization.[17] In addition to reduced fibrinogen polymerization, increased secretion of uPA by QPD-affected platelets leads to increased plasmin activation and fibrinolysis. Bleeding incidents in patients with QPD can be managed by treatment with antifibrinolytic agents, such as ε-aminocaproic acid, which was found to resolve severe bleeding in the index case horse.[4]

In mice, deletions of genes for the Akt family of serine-threonine kinases (also designated protein kinase B) result in defective secretion of platelet α-granules and dense granules.[18] The Akt is a family of 3 isoforms, 2 of which (Akt1 and Akt2) are present in human and mouse platelets and are activated by stimulation of protease activated receptors in response to thrombin.[18,19] Similar to the horses reported in the present study, platelets from mice with an $Akt1^{+/-}$ $Akt2^{-/-}$ genotype have an approximately 80% reduction in fibrinogen binding.[18] These mice do not have prolonged tail vein bleeding times, but do not form stable thrombi in response to ferric chloride–induced injury of the carotid artery. These results have been interpreted as resulting from different effects of shear rate on the thrombi formed in these different locations. Although $Akt2^{-/-}$ mice have normal growth and development, they have diminished ability to decrease blood glucose concentration in response to insulin, which ultimately affects the hepatic and skeletal muscle systems.[20] Platelets from $Akt1^{-/-}$ mice do not have reduced fibrinogen binding, but these mice have prolonged tail vein bleeding times and significantly delayed platelet aggregation in response to thrombin as

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measured by use of optical aggregometry.[21] In contrast to Akt $2^{-/-}$ mice, Akt $1^{-/-}$ mice have impaired growth and development that persists into adulthood, but normal glucose metabolism.[22,23]

Activation of Akt in platelets involves 3 connected signal transduction pathways, mutations in any of which could potentially lead to the observed phenotype. These signal pathways regulate phosphorylation of the highly conserved regulatory and catalytic-kinase domains of Akt isoforms. Catalytically active Akt is phosphorylated on the regulatory and kinase domains.[24] In human platelets, protease activated receptor activation leads to phosphorylation of the regulatory domain by 3-phosphoinositide dependent kinase 1, whereas the catalytic domain can be phosphorylated by either 3-phosphoinositide dependent kinase 2 or through a protein kinase C–dependent pathway.[19]

Although the genetic factors leading to reduced fibrinogen binding in horses remain unclear, results of the present study indicated that a defect in this measure of platelet function occurred with a relatively high frequency in the sampled Thoroughbred population and increased the number of identified horses to 5. As has been found for Glanzmann thrombasthenia in horses, reduced fibrinogen binding may cross breed lines.[1,2,a] In a manner similar to individuals with von Willebrand disease, horses with reduced fibrinogen binding frequently lack observable clinical signs, but have a platelet phenotype that has been associated in some cases with prolonged bleeding times[5] and, in 1 horse, severe bleeding incidents because of trauma.[4]

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