A mutation in the canine Kindlin-3 gene associated with increased bleeding risk and susceptibility to infections

Mary K. Boudreaux[1]; K. Jane Wardrop[2]; Veronika Kiklevich[3]; Peter Felsburg[4]; Kevin Snekvik[5]

[1]Department of Pathobiology, Auburn University, Auburn, Alabama, USA; [2]Department of Veterinary Clinical Sciences, Washington State University, Pullman, Washington, USA; [3]San Antonio Veterinary Referral Specialists, San Antonio, Texas, USA; [4]Department of Clinical Studies-PHL, University of Pennsylvania, Philadelphia, Pennsylvania, USA; [5]Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, USA

Dear Sirs,

Leukocyte adhesion deficiency III (LAD-III) is a rare disorder characterised by failure of activation of β1, β2, and β3 -type integrins expressed on haematopoietic cells. Affected individuals have a bleeding diathesis similar to that observed with Glanzmann thrombasthenia and also have persistently high leukocyte counts and are susceptible to infections. Reports have suggested that LAD-III in people is caused by a mutation near a splice site within *CalDAG-GEFI* that results in absence of expression of CalDAG-GEFI protein (1, 2). CalDAG-GEFI is a guanine nucleotide exchange factor that facilitates the exchange of GDP/GTP by Rap1b, a GTPase involved in signalling and activation of integrins (3). Supportive evidence of the LAD-III phenotype has been provided by *CalDAG-GEFI* knock-out mice models that also have mildly elevated leukocyte counts and impaired platelet aggregation and neutrophil adhesion (4). While *CalDAG-GEFI* knock-out mice do not experience spontaneous haemorrhage or susceptibility to infections, this is presumed to be due to their location in a protected environment. Two large animal models (dogs and cattle) of CalDAG-GEFI deficiency or dysfunction have been reported (5, 6). In these reports, four different mutations in *CalDAG-GEFI* are described, two of which result in the appearance of premature stop codons and presumed lack of protein expression secondary to nonsense mediated decay. In one model, CalDAG-GEFI protein is expressed but protein function is impaired. In contrast to what had been described in people and the knock-out mouse model, affected animals only experience a bleeding diathesis; affected animals do not have persistently elevated leukocyte counts and are not prone to development of infections. Recently, several reports have implicated mutations in *KINDLIN3* in people as cause for an LAD-III phenotype (7–9). Investigators were unable to document that the previously reported mutation in *CalDAG-GEFI* in people resulted in abnormal CalDAG-GEFI expression or was causative for an LAD-III phenotype. The genes encoding CalDAG-GEFI and Kindlin-3 are 0.5 Mb apart on chromosome 11 in people and investigators concluded that the mutation identified in *CalDAG-GEFI* is an insignificant polymorphism in linkage disequilibrium with the mutation identified in *KINDLIN3* (7). While these findings explain the discrepancy between the reports in people and the large animal models regarding CalDAG-GEFI absence or dysfunction, the discrepancies, including elevated white counts and leukocyte dysfunction, in the knock-out mouse model are still unexplained. Spontaneous *KINDLIN3* mutations had not yet been reported in large

Table 1: Complete blood count findings over a five-year period in a German Shepherd dog with A 12-base pair insertion in the coding region for KINDLIN3 and presumptive LAD-III.

Age	WBC	Neut	Band	Lymph	Mono	Eos	Platelets
9 mo	39,000	ND	ND	ND	ND	ND	98,000
12 mo	38,400	23,424	0	9,216	1,536	4,224	133,000
13 mo	37,900	22,361	0	11,749	2,653	1,137	101,000
1 yr 11 mo	61,200	41,616	0	7,344	8,568	3,672	112,000
4 yr 1 mo	29,300	18,166	0	6,153	586	4,395	96,000
4 yr 2 mo	38,900	28,397	0	3,501	4,668	2,334	170,000
4 yr 6 mo	24,100	15,183	0	2,892	1,446	4,579	199,000
4 yr 9 mo	101,800	87,548	3,054	3,054	7,126	1,018	adequate
4 yr 9 mo	91,500	84,180	915	5,490	0	915	adequate
4 yr 10 mo	27,800	20,016	0	3,614	3,058	1,112	140,000
4 yr 11 mo	187,440	151,826	7,498	3,749	22,493	1,874	39,000
4 yr 11 mo	178,200	156,816	7,128	1,782	12,474	0	39,000
4 yr 11 mo	116,000	90,480	6,960	3,480	15,080	0	79,000
5 yr	75,400	64,844	0	754	5,278	4,524	adequate
5 yr	70,857	55,977	0	3,543	7,794	3,543	79,000
5 yr 1 mo	71,500	54,340	0	6,435	7,150	3,575	429,000
5 yr 1 mo	75,000	54,750	0	7,500	12,750	0	192,000
5 yr 4 mo	39,500	30,415	0	3,555	3,950	1,580	151,000

ND, not determined; WBC, white blood cell count/μl; Neut, neutrophils/μl; Lymph, lymphocytes/μl; Mono, monocytes/μl; Eos, eosinophils/μl. Reference Intervals (per microliter): WBC 5,800 to 11,700; Neutrophils 3,000 to 7,100; Band Neutrophils 0 to 300; Lymphocytes 1,100 to 5,100; Monocytes 0 to 800; Eosinophils 0 to 100; Platelets 157,000 to 394,000.

Correspondence to:
Mary K. Boudreaux, DVM, PhD
166 Greene Hall, Department of Pathobiology
College of Veterinary Medicine, Auburn University
Alabama 36849–5519, USA
Tel.: +1 334 844 2692, Fax: +1 334 844 2652
E-mail: boudrmk@auburn.edu

Received: August 17, 2009
Accepted after major revision: October 15, 2009
Prepublished online: November 13, 2009

doi:10.1160/TH09-09-0571
Thromb Haemost 2010; 103: 475–477

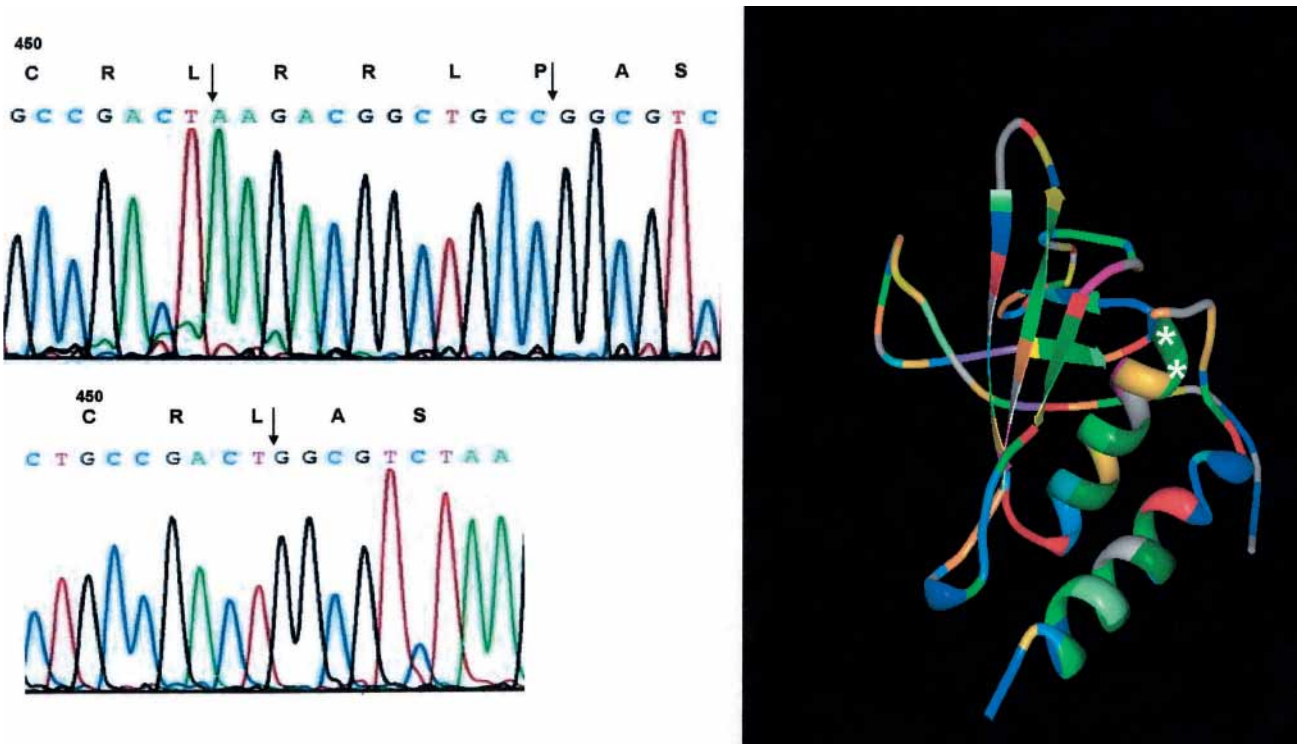

Figure 1: Kindlin-3 gene sequences and model depicting location of 12-base pair insertion. Gene sequences encoding a portion of the Kindlin-3 pleckstrin-homology (PH) domain of the F2 FERM subdomain obtained from affected dog (upper sequence) and normal dog (lower sequence). The arrows indicate the location of the 12-base pair insertion. The two asterixes within the model demarcate the leucine and alanine that flank the location of the insertion near one end of an alpha helix within the PH domain. The terminal alpha helix depicted in the figure is distal to the PH domain. The model was obtained from the NCBI protein data bank (PDB). "Solution structure of the PH domain of Kindlin-3 from human" Li H., Sato M., Koshiba S., Watanabe S., Harada T., Kigawa T., Yokoyama S.

animal models for comparison to findings in people or *KINDLIN3* knock-out mouse models which essentially mimic findings in people (10).

A male German Shepherd dog presented at the Washington State University College of Veterinary Medicine on numerous occasions starting at six months of age with complaints of lameness, abnormal bleeding, and infections, including deep pyoderma and pododermatitis, gingivitis, and cellulitis, often accompanied by fever. Radiographs documented lesions within long bones of the front legs as well as ununited anconeal and coronoid processes. The affected dog developed profuse haemorrhage after a laceration on the lip and was euthanised at six years of age. A male sibling of the affected dog bled to death at three years of age.

Assays of primary and secondary haemostasis and complete blood counts (CBC) were performed on multiple occasions. Assays of haemostasis included buccal mucosal bleeding times (BMBT), coagulation screening assays (PT, APTT), vWF antigen concentration, factor VIII-C activity, platelet number, platelet aggregation, and clot retraction. The BMBT was consistently prolonged and was usually greater than 8 minutes (min) (reference 2.5 to 4.5 min). Coagulation screening assays were within reference intervals as were vWF antigen (122%) and factor VIII-C (115%). Platelet aggregation studies indicated a delayed reaction and diminished overall response to ADP and collagen when compared to a normal control. Clot retraction was also impaired. CBCs were performed 18 times over a five-year period (▶Table 1) during which time platelet numbers ranged from 39,000/µl to 429,000/µl (reference interval 157,000 to 394,000/µl) and white cell counts ranged from 24,000 to 187,440/ul (reference interval 5,800 to 11,700/µl). Flow cytometry studies indicated normal levels of CD41 (GPIIb) and CD61 (GPIIIa) on platelets and CD11a, CD11b, CD11c and CD18 on leukocytes. DNA was isolated from whole blood obtained from the affected dog as well as from normal controls, including several normal German Shepherds, and subjected to PCR. DNA sequences of coding regions for CalDAG-GEFI, GPIIb, and GPIIIa were determined to be comparable to sequences obtained from the canine genome. These findings ruled out the presence of a CalDAG-GEFI disorder, Glanzmann thrombasthenia, or LAD-I. Recent reports implicating *KINDLIN3* mutations in people as cause for LAD-III and the similarity of the phenotypes when comparing the affected dog to affected children prompted the evaluation of *KINDLIN3* five years after the dog was euthanised. A 12-base pair insertion was identified in the coding region for *KINDLIN3* in the affected dog but not in the ca-

nine genome sequence or the control dog sequences. This mutation is predicted to result in the insertion of amino acids RRLP within an alpha helix located in the Kindlin-3 pleckstrin-homology domain of the F2 band 4.1, ezrin, radixin, moesin (FERM) subdomain (▶Fig. 1). Because the dog was no longer available for study, further studies could not be conducted.

Kindlin-3 binds to membrane distal motifs within beta-1 and beta-3 tails and is thought to enhance Talin-induced inside-out signalling mediated by binding of Talin to membrane proximal beta-1 and beta-3 tail motifs (10, 11). The F3 subdomains of the FERM domains of Kindlin-3 and Talin are highly homologous and are thought to be involved in beta tail binding. Kindlin-3 null murine platelets failed to spread on immobilised fibrinogen when activated by thrombin; these finding suggest that Kindlin-3 also plays a role in integrin outside-in signalling(10). Kindlin-3 null murine neutrophils were unable to adhere and arrest on activated endothelial cells, further demonstrating the global effects of Kindlin-3 absence on platelet and leukocyte function (11).

This report describes for the first time the association of a spontaneous mutation in *KINDLIN3* with an LAD-III phenotype in a large animal model. The persistently elevated leukocyte counts, chronic infections, and bleeding tendencies for the life span of the affected dog were very similar to the clinical signs reported in affected children. The mutation likely results in either absence of protein synthesis or marked impairment of protein function, particularly with respect to mediating integrin activation. Further studies are required to document the specific effects of this mutation on leukocyte function *in vitro*. This model, coupled with the information provided by the CalDAG-GEFI large animal models, suggest that while Kindlin-3 is important for integrin signalling in multiple haematopoietic cells, CalDAG-GEFI is primarily critical for integrin signalling within platelets. Spontaneous mutations in large animal models provide unique opportunities to evaluate pathways critical for cell function.

References

1. Pasvolsky R, Feigelson SW, Kilic SS, et al. A LAD-III syndrome is associated with defective expression of the Rap-1 activator CalDAG-GEFI in lymphocytes, neutrophils, and platelets. J Exp Med 2007; 204: 1571–1582.
2. Kilic SS, Etzioni A. The clinical spectrum of Leukocyte Adhesion Deficiency (LAD) III due to defective CalDAG-GEFI. J Clin Immunol 2009; 29: 117–122.
3. Crittenden JR, Bergmeier W, Zhang Y, et al. CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. Nature Medicine 2004; 10: 982–986.
4. Bergmeier W, Goerge T, Wang HW, et al. Mice lacking the signaling molecule CalDAG-GEFI represent a model for leukocyte adhesion deficiency type III. J Clin Invest 2007; 117: 1699–1707.
5. Boudreaux MK, Catalfamo JL, Klok M. Calcium-diacylglycerol guanine nucleotide exchange factor I gene mutations associated with loss of function in canine platelets. Translational Res 2007; 150: 81–92.
6. Boudreaux MK, Schmutz S, French P. Calcium diacylglycerol guanine nucleotide exchange factor I (CalDAG-GEFI) gene mutations in a thrombopathic Simmental calf. Vet Pathol 2007; 44: 932–945.
7. Kuijpers TW, van de Vijver E, Weterman MAJ, et al. LAD1/variant syndrome is caused by mutations in FERMT3. Blood 2009; 113: 4740–4746.
8. Svensson L, Howarth K, McDowall A, et al. Leukocyte adhesion deficiency-III is caused by mutations in *KINDLIN3* affecting integrin activation. Nature Med 2009; 15: 306–312.
9. Malinin NL, Zhang L, Choi J, et al. A point mutation in *KINDLIN3* ablates activation of three integrin subfamilies in humans. Nature Medicine 2009; 15: 313–318.
10. Moser M, Nieswandt B, Ussar S, et al. Kindlin-3 is essential for integrin activation and platelet aggregation. Nature Medicine 2008; 14: 325–330.
11. Moser M, Bauer M, Schmid S, et al. Kindlin-3 is required for β2 integrin-mediated leukocyte adhesion to endothelial cells. Nature Med 2009; 15: 300–305.