Published in final edited form as:

J Thromb Haemost. 2008 September ; 6(9): 1608–1610. doi:10.1111/j.1538-7836.2008.03068.x.

Exclusion of ABCA-1 as a candidate gene for canine Scott syndrome

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The collapse of phospholipid asymmetry across membrane bilayers mediates the physiologic processes of cell activation and apoptosis [1,2], whereas a failure to regulate transbilayer lipid movement is associated with a variety of pathologic conditions [3]. The terms ‘flippase’, ‘floppase’, and ‘scramblase’ refer to membrane transporter activities responsible for inward, outward, and bidirectional lipid movement; however, the biologic basis and regulation of these activities is not yet fully understood [4,5]. Scott syndrome (OMIM: 262890), a rare hemostatic defect characterized by an inability of stimulated platelets to externalize phosphatidylserine (PS), provides a unique tool to study cell membrane lipid handling. Heterozygosity for a novel missense mutation in the lipid translocator, adenosine triphosphate binding cassette 1 (*ABCA1*), was recently described in a Scott syndrome patient [6]. A paucity of cases, however, hinders genetic and platelet function studies to further define how this mutant protein contributes to expression of the Scott platelet phenotype. Spontaneous mutations of *ABCA1* and targeted disruption of the murine gene produce the clinical phenotype Tangier disease (OMIM: 205400) primarily characterized by a lack of plasma high-density lipoprotein (HDL) cholesterol and accumulation of lipid within reticuloendothelial cells [7,8]. To further clarify a role for *ABCA1* in stimulated platelet PS movement, we looked for evidence of *ABCA1* deficiency in platelets from Scott syndrome German shepherd dogs (GSD) [9] and performed linkage analyses to test for association of the canine Scott platelet phenotype with genetic markers within and flanking the *ABCA1* locus. Canine Scott platelets display the hallmarks of their human counterpart e.g. normal aggregation, but a failure to generate prothrombinase activity and a lack of PS externalization and microparticle release in response to physiologic (thrombin plus collagen) or pharmacologic (ionophore) agonists [10].

The presence of ABCA1 protein in Scott GSD platelets was confirmed in Western blots of platelet lysates [11] (Fig. 1A) probed with an affinity-purified rabbit polyclonal antibody [ABCA1 antibody (ab14146), Abcam] that recognizes a peptide sequence of human ABCA1 with 100% homology to dog, mouse, and rat ABCA1. The free cholesterol content of Scott ($n = 5$) and control dog ($n = 8$) platelets were similar, with mean values of 14.3 μg cholesterol per 10^8 platelets and 12.2 μg cholesterol per 10^8 platelets, respectively. As Tangier platelets demonstrate impaired collagen-induced aggregation [12], we compared the aggregation of Scott and control dog platelets post-stimulation with collagen (12 μg per mL) and throughout a dosage range of the GPVI ligand, convulxin [CVX (0–30 nM)]. We found no significant difference ($P = 0.13$) in mean maximal collagen-induced aggregation of Scott GSD platelet-rich plasma ($n = 5$; 75% aggregation) vs. control dogs ($n = 8$; 80% aggregation), and the CVX response of Scott and control dog platelets was essentially the same (Fig. 1B). Additional

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Disclosure of Conflict of Interests The authors state that they have no conflict of interest.

manifestations of platelet dysfunction in Tangier patients include decreased reactivity to low dose thrombin and defective granule formation and release [7,12,13]. We therefore evaluated Scott GSD platelets in flow cytometry experiments to assess various granule-dependent responses. We found no significant differences ($P > 0.1$) between Scott GSD ($n = 5$) and control dogs ($n = 8$) in mean percentages of CD62P positive platelets (denoting P-selectin expression) post-thrombin (0.5 U per mL) stimulation (Scott GSD = 70.3%; control = 62.5%) or CVX (10 nM) stimulation (Scott GSD = 53%; control = 48.8%), mean mepacrine uptake (denoting dense granule content [14]) (Scott GSD = 68.8% positive; control = 75%) and mean CD61 positive leukocytes (denoting platelet-leukocyte aggregate formation [15]) post-thrombin (2 U per mL) stimulation (Scott GSD = 12.2%; control = 10.2%). In contrast, the mean percentage Annexin V positive platelets (denoting PS externalization) in response to combined stimulation with thrombin (0.5 U per mL) plus CVX (10 nM) was significantly reduced ($P < 0.0001$) for Scott platelets (Scott GSD = 5.6%; control = 41%). Although species differences in platelet physiology limit direct comparisons, the pattern of activation response for Scott GSD differs from human and murine Tangier platelets, indicating that the ABCA1 protein within Scott GSD platelets is functional.

Phenotyping of the study pedigree (Fig. 1C) for linkage analyses was based on residual serum prothrombin [9,16]. Abnormal platelet function of affected GSD ($n = 22$) in this screening test was confirmed by flow cytometric findings of diminished PS externalization and microvesiculation post-calcium ionophore. The GSD were genotyped for an intragenic canine *ABCA1* (Gene ID: 481651) microsatellite and 10 additional markers [17] spanning CFA11 (chromosomal location of canine *ABCA1*) at 5–10 cM intervals. The power of these analyses to identify a disease gene was assessed by simulating 40 000 replicate data sets using the SLINK function of LINKAGE[18]; conditional on the observed pedigree, autosomal recessive inheritance, and variation of recombination rate between marker and disease locus (θ) along a gradient from 0 to 0.1. These simulations predicted approximately 80% power to identify a disease locus at cutoff LOD = 2, for markers within $\theta = 0.05$ from the disease locus. We found that two point and multipoint linkage analyses, calculated using SUPERLINK v. 1.6 (<http://bioinfo.cs.technion.ac.il/superlink/>), failed to demonstrate an association between canine Scott syndrome and the *ABCA1* locus [LOD <1 (Fig. 1C)].

The lipid transporter ABCA1 plays a central role in the process of reverse cholesterol transport [8]. The protein mediates lipidation of the carrier protein ApoA-1 at both the plasma membrane and within endocytic vesicles [19]. A defect of vesicular trafficking has been invoked as a common mechanism underlying the spectrum of impaired platelet reactivity demonstrated by *ABCA1* mutant (Tangier) platelets [12]. Our series of experiments in Scott GSD, however, failed to demonstrate any of the manifestations of granule dysfunction expressed by Tangier patients and *ABCA1* knockout mice. In addition to normal ABCA1 protein expression in platelets, genetic analyses revealed no linkage between *ABCA1* and the canine Scott phenotype. While our findings do not identify the canine disease gene, we conclude that this gene is distinct from *ABCA1*. The defective gene product appears to play a critical role in facilitating movement of PS; however, disruption of this pathway does not affect other parameters of normal platelet response to thrombin and collagen stimulation.

Acknowledgments

This work was funded in part by Cornell University's Vertebrate Genomics Center and the National Science Foundation. Some genotype data were obtained through a submission to the NHLBI Mammalian Genotyping Service (HV4814).

References

1. Bevers EM, Comfurius P, Dekkers DW, Zwaal RF. Lipid translocation across the plasma membrane of mammalian cells. *Biochim Biophys Acta* 1999;1439:317–30. [PubMed: 10446420]
2. Martinez MC, Freyssinet JM. Deciphering the plasma membrane hallmarks of apoptotic cells: phosphatidylserine transverse redistribution and calcium entry. *BMC Cell Biol* 2001;2:20. [PubMed: 11701087]
3. Zwaal RF, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci* 2005;62:971–88. [PubMed: 15761668]
4. Kol MA, de Kruij B, de Kroon AI. Phospholipid flip-flop in biogenic membranes: what is needed to connect opposite sides. *Semin Cell Dev Biol* 2002;13:163–70. [PubMed: 12137736]
5. Sims PJ, Wiedmer T. Unraveling the mysteries of phospholipid scrambling. *Thromb Haemost* 2001;86:266–75. [PubMed: 11487015]
6. Albrecht C, McVey JH, Elliott JL, Sardini A, Kasza I, Mumford AD, Naoumova RP, Tuddenham EG, Szabo K, Higgins CF. A novel missense mutation in ABCA1 results in altered protein trafficking and reduced phosphatidylserine translocation in a patient with Scott syndrome. *Blood* 2005;106:542–9. [PubMed: 15790791]
7. Orso E, Broccardo C, Kaminski WE, Bottcher A, Liebisch G, Drobnik W, Gotz A, Chambenoit O, Diederich W, Langmann T, Spruss T, Luciani MF, Rothe G, Lackner KJ, Chimini G, Schmitz G. Transport of lipids from golgi to plasma membrane is defective in tangier disease patients and Abc1-deficient mice. *Nat Genet* 2000;24:192–6. [PubMed: 10655069]
8. Zarubica A, Trompier D, Chimini G. ABCA1, from pathology to membrane function. *Pflugers Arch* 2007;453:569–79. [PubMed: 16858612]
9. Brooks MB, Catalfamo JL, Brown HA, Ivanova P, Lovaglio J. A hereditary bleeding disorder of dogs caused by a lack of platelet procoagulant activity. *Blood* 2002;99:2434–41. [PubMed: 11895776]
10. Toti F, Satta N, Fressinaud E, Meyer D, Freyssinet JM. Scott syndrome, characterized by impaired transmembrane migration of procoagulant phosphatidylserine and hemorrhagic complications, is an inherited disorder. *Blood* 1996;87:1409–15. [PubMed: 8608230]
11. Boudreaux MK, Catalfamo JL, Klok M. Calcium-diacylglycerol guanine nucleotide exchange factor I gene mutations associated with loss of function in canine platelets. *Transl Res* 2007;150:81–92. [PubMed: 17656327]
12. Nofer JR, Herminghaus G, Brodde M, Morgenstern E, Rust S, Engel T, Seedorf U, Assmann G, Bluethmann H, Kehrel BE. Impaired platelet activation in familial high density lipoprotein deficiency (Tangier disease). *J Biol Chem* 2004;279:34032–7. [PubMed: 15163665]
13. Schmitz G, Schambeck CM. Molecular defects in the ABCA1 pathway affect platelet function. *Pathophysiol Haemost Thromb* 2006;35:166–74. [PubMed: 16855366]
14. Gordon N, Thom J, Cole C, Baker R. Rapid detection of hereditary and acquired platelet storage pool deficiency by flow cytometry. *Br J Haematol* 1995;89:117–23. [PubMed: 7833250]
15. Klinkhardt U, Harder S. Flow cytometric measurement of platelet-leukocyte aggregates: a possible target to monitor platelet function? *Semin Thromb Hemost* 2005;31:400–3. [PubMed: 16149016]
16. Weiss HJ, Vivic WJ, Lages BA, Rogers J. Isolated deficiency of platelet procoagulant activity. *Am J Med* 1979;67:206–13. [PubMed: 572637]
17. Clark LA, Tsai KL, Steiner JM, Williams DA, Guerra T, Ostrander EA, Galibert F, Murphy KE. Chromosome-specific microsatellite multiplex sets for linkage studies in the domestic dog. *Genomics* 2004;84:550–4. [PubMed: 15498461]
18. Ott J. Computer-simulation methods in human linkage analysis. *Proc Natl Acad Sci U S A* 1989;86:4175–8. [PubMed: 2726769]
19. Neufeld EB, Stonik JA, Demosky SJ Jr, Knapper CL, Combs CA, Cooney A, Comly M, Dwyer N, Blanchette-Mackie J, Remaley AT, Santamarina-Fojo S, Brewer HB Jr. The ABCA1 transporter modulates late endocytic trafficking: insights from the correction of the genetic defect in Tangier disease. *J Biol Chem* 2004;279:15571–8. [PubMed: 14747463]

Fig. 1.

Platelet expression of ABCA1, aggregation response to convulxin stimulation, and *ABCA1* linkage analyses in Scott German shepherd dogs (GSD). (A) Western blot analyses of ABCA1 in platelet lysates. Platelet proteins were solubilized in Laemmli buffer, separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4–15% gels), and transferred to nitrocellulose for hybridization with an ABCA-1 antibody. Each lane contains 40 μ g of platelet protein. Lanes 1 & 6 = human control, Lanes 2 & 5 = canine controls, Lanes 3 & 4 = Scott GSD. (B) Platelet aggregation in response to convulxin stimulation. Washed platelets (300×10^9 per L) were stimulated with convulxin through a dosage range of 0–30 nM. The graph displays mean % aggregation for Scott GSD ($n = 3$) and control dogs ($n = 4$). (C) Pedigree of GSD segregating Scott syndrome used for linkage analyses. Solid symbols represent GSD with high residual serum prothrombin and impaired platelet PS externalization, open symbols represent GSD with normal residual serum prothrombin, shaded symbols represent GSD with no samples for testing. Straight lines connect parents and offspring, curved lines connect individual dogs appearing more than once in the pedigree. Inset table displays the maximum two point LOD scores (for disease allele frequency of 0.25) and associated recombination rates (θ) obtained for microsatellites located within and 10 cM flanking canine *ABCA1*.