

## **Gene of the Issue: *ANO6* and Scott Syndrome**

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Scott Syndrome is a very rare inhibited bleeding disorder characterised by an isolated deficit in procoagulant activity in platelets and other blood cells, caused by a lack of phosphatidylserine (PS) exposure following activation.<sup>1,2</sup> It results from mutations in *ANO6*, which encodes the phospholipid scramblase protein, TMEM16F.<sup>3</sup>

### **Platelet PS exposure in coagulation**

Negatively charged phospholipids – particularly PS, but also phosphatidylethanolamine (PE) – facilitate Ca<sup>2+</sup>-dependent binding of the  $\gamma$ -carboxyglutamate (Gla) domains of vitamin K-dependent coagulation factors (factors II [prothrombin], VII, IX and X) to phospholipid membranes.<sup>4</sup> This promotes the formation of the tenase and prothrombinase complexes on PS-exposing platelets. PS-exposing platelets also enhance the activities of these complexes by nearly 1000-fold.<sup>5</sup> Platelet PS exposure therefore plays a central role in coagulation.

### **Scott Syndrome**

Patients present with a mild-to-moderate bleeding phenotype, suffering from post-operative or post-partum haemorrhage but without easy bruising or excessive bleeding from superficial cuts.<sup>2,6,7</sup> Only 6 cases of Scott Syndrome have been reported to date,<sup>2,6-9</sup> though it may be underdiagnosed due to its relatively mild clinical presentation until a significant haemostatic challenge and lack of routine diagnostic test. Scott Syndrome patients have prolonged prothrombin times and high residual plasma prothrombin levels, but a definitive diagnosis requires measurement of platelet PS exposure by flow cytometry. After diagnosis, management involves appropriate platelet transfusions during procedures to prevent excessive blood loss.<sup>2,10</sup>

The original patient, Mrs Scott (MS), had deficient platelet procoagulant activity but all other platelet functions, including adhesion, aggregation and secretion, were normal.<sup>2</sup> The description of a second patient (FS) and her family indicated that Scott Syndrome was a genetic condition following an autosomal recessive inheritance pattern.<sup>6</sup> FS and her two older sisters, who had both died from post-partum haemorrhage, were suggested to be homozygous for the Scott mutation. Platelets from two of FS' asymptomatic children displayed intermediate procoagulant activity between those of their mother and controls, suggesting they were heterozygous for the Scott mutation. MS' asymptomatic mother, father and son also showed intermediate platelet prothrombinase activity,<sup>11</sup> again indicating an autosomal recessive trait. It may be a coincidence that all of the small number of Scott patients described to date are female, but the 3 patients known to have had children (MS, FS, VW<sup>7</sup>) all suffered with post-partum haemorrhage, perhaps illustrating the particular importance of platelet-based coagulation post-partum.

### **Identification of ANO6**

Scott Syndrome platelets were originally described as lacking platelet factor 3, leading to reduced factor Va and Xa binding to the surface of activated platelets<sup>12</sup> and consequent

reduced subendothelial fibrin deposition.<sup>13</sup> Platelet factor 3 was later identified as the exposure of the negatively-charged phospholipid PS on the surface of activated platelets.<sup>1</sup> Studies on resealed RBC ghosts showed that the Scott Syndrome defect was in an intrinsic membrane component responsible for PS exposure.<sup>14</sup>

PS is normally restricted to the inner leaflet of the plasma membrane by inward 'flippase' activity (Fig. 1). Procoagulant platelet stimuli, such as dual stimulation with collagen and thrombin, or a Ca<sup>2+</sup> ionophore, trigger a large, sustained increase in cytosolic Ca<sup>2+</sup>. This inhibits flippase activity and activates a 'scramblase' activity – bidirectional, non-selective movement of phospholipids, leading to loss of membrane asymmetry. Flippase activity is unaffected in Scott Syndrome patients but the scramblase activity is defective.<sup>15,16</sup> As a result, PS exposure is almost completely abolished in Scott Syndrome platelets following stimulation.<sup>1</sup> Platelet microparticle release, which requires PS exposure, is also abolished.<sup>17</sup> Procoagulant platelets resemble necrotic cells, with a diluted cytoplasm, few remaining organelles and rapid swelling into large 'balloon' -like structures.<sup>18,19</sup> Procoagulant ballooning is also diminished in Scott Syndrome platelets.<sup>20,21</sup>

Scott Syndrome red blood cells (RBCs) also display defective PS exposure, microparticle release and echinocytosis.<sup>14</sup> Immortalised Scott Syndrome B-lymphocytes also have impaired PS exposure and procoagulant activity.<sup>22</sup>

Two proteins were previously proposed to be the phospholipid scramblase: PSLCR1 and ABCA1. PSLCR1 was initially described as a putative scramblase in RBCs.<sup>23</sup> However, PSLCR1 mRNA and protein<sup>24</sup> levels, and later sequence,<sup>25</sup> were all normal in Scott Syndrome patients (MS and FS). Furthermore, PSLCR1<sup>-/-</sup> mice have normal haemostasis and platelet and RBC PS exposure,<sup>26</sup> excluding PSLCR1.

A missense mutation in the *ABCA1* gene was identified in a third Scott patient (VW) (c.6064G>A (*ABCA1* R1925Q))<sup>27</sup> which reduced its expression in lymphocytes. This mutation, however, has not been found in any other Scott Syndrome patients and *ABCA1* is

now known to play a role in cholesterol efflux.<sup>28</sup> *ABCA1* mutations cause Tangier disease, patients of which expose normal platelet PS.<sup>29,30</sup>

TMEM16F was eventually identified as the scramblase protein following a cell-based screen in which TMEM16F mutants displayed constitutive PS exposure.<sup>3</sup> *ANO6*, which encodes TMEM16F, was first described *in silico* in 2004.<sup>31</sup> It is a member of the anoctamin family, so called as they were predicted to encode proteins with 8 transmembrane domains. The publication of the mouse TMEM16F structure, however, confirmed that it has 10 transmembrane domains.<sup>32</sup> The properties of the *ANO6* gene and TMEM16F protein are listed in Table 1. Four Scott Syndrome patients have been sequenced, each with mutations in *ANO6* (Table 2)<sup>3,33,34</sup>. Notably, each known mutation results in a complete absence of expression of TMEM16F in patients. It is not known whether other mutations exist that affect TMEM16F function or reduce rather than abolish its expression, but it is plausible that asymptomatic individuals carry variants of the TMEM16F protein that affect procoagulant scramblase activity.

Scott Syndrome platelets show additional differences beyond TMEM16F. A proteomic screen of a Scott Syndrome patient's platelets (VW) found differential expression of 134 proteins, with aquaporin 1 expression being particularly strongly upregulated, and increased protein phosphorylation following stimulation.<sup>35</sup> Further differences in tyrosine phosphorylation have also been determined between Scott Syndrome platelets (MS) and controls.<sup>16</sup> The *ABCA1* mutation in VW<sup>27</sup> shows that other mutations are present that may contribute to the clinical presentation.

### **Animal models of Scott Syndrome**

Several animal models support the identification of *ANO6* mutations as causative of Scott Syndrome. Four different viable *Ano6*<sup>-/-</sup> mouse knockout lines have been described<sup>20,36-38</sup>. Their phenotypes slightly differ, but, as in Scott Syndrome platelets, agonist-induced PS

exposure is substantially reduced. Where measured, arterial thrombosis is also inhibited. The reduction in PS exposure is not complete, suggesting that another scramblase or mechanism of PS exposure may exist but plays a much lesser role in thrombosis. It is particularly striking that the bleeding phenotype of the four viable *Ano6*<sup>-/-</sup> mouse lines differ considerably ranging from no difference in tail bleeding time compared to wild type mice,<sup>37</sup> most similar to Scott Syndrome patients, to significantly prolonged bleeding times<sup>20,38</sup>. A canine Scott syndrome also occurs in German Shepherd dogs,<sup>39</sup> also due to a mutation in *ANO6*.<sup>40</sup> In these Scott Syndrome dogs, bleeding time is normal.

*Ano6*<sup>-/-</sup> mouse models have implicated TMEM16F in many other processes including pain behaviour, mucus production, bone mineralisation and preventing immune cell overactivation.<sup>41-44</sup> TMEM16F is also implicated in entry of enveloped viruses, including Ebola and HIV.<sup>45,46</sup> Due to the small number of Scott Syndrome patients it is not known if TMEM16F plays similar roles in human physiology.

Overall, although Scott Syndrome is a very rare bleeding disorder, its description has contributed to the understanding of platelet procoagulant activity and highlights the importance of platelet-based coagulation in acute haemostatic challenges and thrombosis.

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**Figure 1: TMEM16F in platelet procoagulant activity.** (A) In unactivated platelets, phosphatidylserine (PS) is restricted to the inner leaflet of the plasma membrane by flippase activity. The scramblase, TMEM16F (encoded by *ANO6*), is not active. In response to a high, sustained increase in cytosolic  $Ca^{2+}$  concentration, the flippase is inactivated and TMEM16F is activated. TMEM16F catalyses bidirectional movement of PS and other phospholipids, resulting in loss of plasma membrane asymmetry. (B) shows the response at a cellular level. Strong stimuli, such as collagen + thrombin or  $Ca^{2+}$  ionophores, trigger PS exposure, which promotes thrombin generation, rapid swelling (sometimes called ‘ballooning’) and release of PS-exposing microparticles. Each of these responses is deficient in platelets from patients with Scott Syndrome.

**Table 1: Details of *ANO6* and its protein product, TMEM16F**

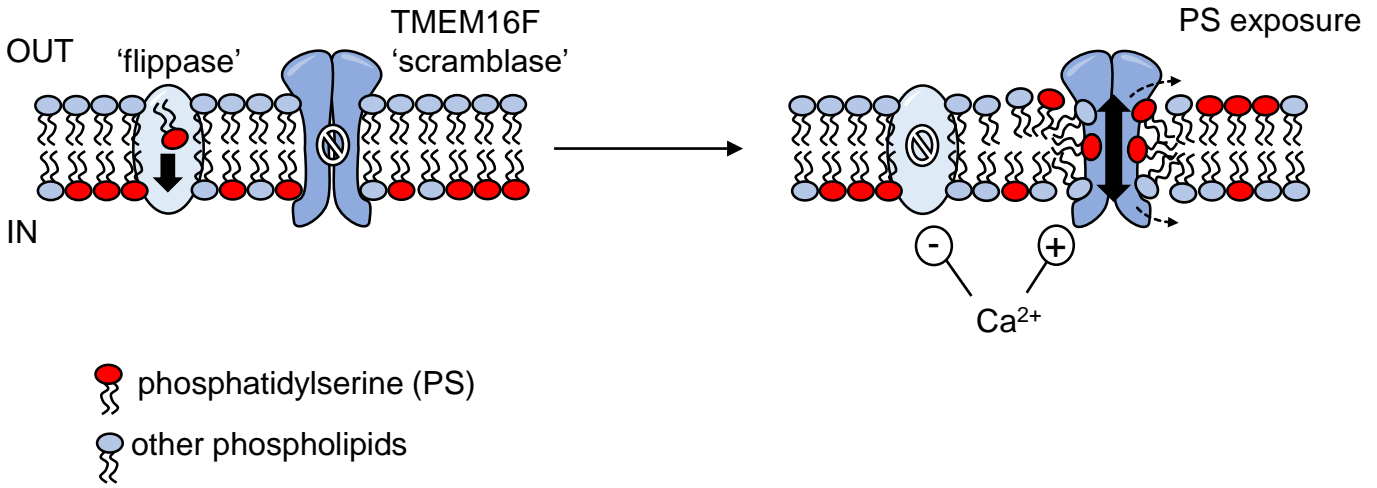
	Human	Mouse	Dog
Gene name	ANO6	Ano6	ANO6

Location	12q12 Chromosome 12	Chromosome 15	Chromosome 27
Base pairs	224,418	183,908	184,366
Exons	20	20	22
	<b>TMEM16F</b>	<b>Tmem16f</b>	<b>TMEM16F</b>
Amino acids	910	911	910
Transmembrane domains	10	10	10*

\* presumed, based on sequence similarity

Patient	References	ANO6 Mutation(s)
<b>MS</b>	Original patient description: Weiss <i>et al.</i> , 1979 (Ref 2) Sequencing: Suzuki <i>et al.</i> , 2010 (Ref 3)	<ul style="list-style-type: none"> <li>• Homozygous G&gt;T mutation at a splice acceptor site in intron 12. Predicted to lead to the skipping of exon 13 and a frameshift in exon 14 causing premature termination.</li> <li>• Each of MS' asymptomatic parents were heterozygous for the G&gt;T mutation.</li> </ul>
<b>VW</b>	Original patient description: Parry <i>et al.</i> , 1980 (Ref 8) Diagnosis of Scott Syndrome: Munnix <i>et al.</i> , 2003 (Ref 5) Sequencing: Castoldi <i>et al.</i> , 2011 (Ref 28)	<ul style="list-style-type: none"> <li>• VW was a compound heterozygote with 2 mutations in ANO6. <ul style="list-style-type: none"> <li>○ A G&gt;A mutation at the first nucleotide of intron 6 disrupting the donor splice site consensus sequence, which leads to the skipping of exon 6; predicted to cause in-frame deletion of 38 amino acids at the N-terminal cytoplasmic tail.</li> <li>○ An insertion in exon 11 (c.1219insT), predicted to cause a frame shift and premature termination.</li> </ul> </li> </ul>
<b>FS</b>	Original patient description: Toti <i>et al.</i> , 1996 (Ref 4)	<ul style="list-style-type: none"> <li>• No sequence available</li> </ul>
<b>2 siblings</b>	Original patient description: Halliez <i>et al.</i> , 2015 (Ref 6) Sequencing: Boisseau <i>et al.</i> , 2016 (Ref 27)	<ul style="list-style-type: none"> <li>• Both siblings were compound heterozygous <ul style="list-style-type: none"> <li>○ A nonsense variant c.889C&gt;T in exon 8 leading to premature termination.</li> <li>○ A deletion of exons 1-10 on the other allele.</li> </ul> </li> </ul>
<b>Brazilian patient</b>	Original patient description: Flores-Nascimento <i>et al.</i> , 2012 (Ref 7)	<ul style="list-style-type: none"> <li>• No sequence available</li> </ul>
<b>German Shepherd Dogs (GSDs)</b>	Original Description: Brooks <i>et al.</i> , 2012 (Ref 34) Sequencing: Brooks <i>et al.</i> , 2015 (Ref 35)	<ul style="list-style-type: none"> <li>• A G&gt;A mutation at the exon 16 splice donor site.</li> <li>• All affected GSDs were homozygous (AA), and all carriers were heterozygous (AG).</li> </ul>

**Table 2: Known ANO6 mutations in patients with Scott Syndrome**, in order of first report. German Shepherd Dogs with Scott Syndrome are included for comparison. For all mutations so far described, the effect is likely to be complete loss of TMEM16F protein expression.

**A****B**