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Whole genome sequencing identifies novel candidate genetic variants in canine stomatocytosis

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ABSTRACT

Stomatocytosis is a rare spectrum of red blood cell (RBC) disorders. In humans, stomatocytosis is typically caused by genetic changes in specific ion exchange and transport genes. Stomatocytosis has been identified in dogs, however the underlying genetic causes are unknown. Recently, stomatocytosis was reported in a Beagle and Australian Cattle Dog for the first time. Here, whole-genome sequencing (WGS) of these dogs was undertaken to identify candidate genetic variants driving or impacting stomatocytosis. Cases were compared to WGS of 119 controls of several breeds and > 1,000 dogs from public and private datasets. Candidate genes were identified, including genes linked to stomatocytosis in humans: *SPTB* and *KCNN4*. Notably, each case carried a different homozygous intronic SNP in *SPTB* only 24 bases apart (Beagle – chr8:39,194,923; ACD – chr8:39,194,947; CanFam3.1), which were not homozygous in other dogs. Variants with predicted deleterious impact in additional ion transport-related genes were also identified: *SLC8A3*, *DYSF*, *SLC12A8*, *INPP5E*, *SLC1A1*, and a novel *SLC41A3* genetic change carried by the Australian Cattle Dog. Human and mouse scRNAseq and proteomics data indicate that these candidate genes are expressed in RBCs or their immature precursors. Taken together, these genetic data obtained from spontaneous stomatocytosis in a non-human species provide novel insights and candidate genes for evaluation of rare red cell disorders in humans.

1. Introduction

Erythrocyte cell volume is regulated by the transport of cations pumped across the plasma membrane to maintain osmotic potential and keep the cell correctly hydrated (Flatt and Bruce, 2009). In humans, erythrocytes have high intracellular potassium (K^+) concentration and low intracellular sodium (Na^+) concentration compared to plasma, and this cation gradient is created by an ATP-dependent Na^+/K^+ pump counteracting the passive outward movement of potassium (Andolfo et al., 2016). Genetic variants altering basal red cell membrane cation permeability result in a leak that deregulates cellular volume (Flatt and Bruce, 2018). This deregulation can lead to stomatocytosis (STOM) in humans, which is often characterized by abnormal 'cup-shaped' erythrocyte morphology (Flatt and Bruce, 2009). Although canine immature RBCs (reticulocytes) have abundant membrane Na^+/K^+ –ATPase activity, this is lost in most dogs during red cell maturation, hence canine erythrocytes contain low K^+ and high Na^+ concentrations (Clauvel de Mendonca et al., 1970; Inaba and Maede, 1986; Richhardt et al., 1979; Parker, 1992).

Stomatocytosis is associated with increased cellular fragility and intravascular hemolysis, along with an increase in circulating reticulocytes (reticulocytosis). In humans, common clinical symptoms include fatigue, pallor, jaundice, splenomegaly, and gallstone formation (Andolfo et al., 2018).

Stomatocytosis is classified into two main forms: dehydrated and overhydrated, with dehydrated being the most common form, occurring in approximately 1 in 50,000 human births (Delaunay et al., 1999; Badens and Guizouarn, 2016). However, stomatocytosis incidence may be underestimated, as it may remain undiagnosed and can be confused with more common hereditary red cell disorders such as hereditary

Abbreviations: RBC, red blood cell; WGS, whole genome sequencing; STOM, stomatocytosis; DBVD, Dog Biomedial Variant Database; DGP, Dog Genome Project; AHT, Animal Health Trust; OMIM, Online Inheritance in Man; MSigDB, Molecular Signatures Database; GWAS, genome wide association study.

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spherocytosis (Andolfo et al., 2016).

Monogenic inheritance in people with erythrocyte defects accounts for 69 % of cases, while at least 15 % arise from multi-locus inheritance (Andolfo, 2021). Some of the genes linked to stomatocytosis in humans are involved in ion exchange or transport of Ca^{2+}, K^{+}, Na^{+}, and Cl^{-}. For example, hereditary dehydrated stomatocytosis in humans is commonly caused by autosomal dominant genetic changes in ion channel genes *PIEZO1* or *KCNN4* (Risinger and Kalfa, 2020; Albuisson, 2013; Nakahara, 2023; Andolfo, 2018). Overhydrated hereditary stomatocytosis is linked to genetic changes in anion transport genes *SLC4A1*; *RHAG*, and *SLC2A1* (GLUT1) (Risinger and Kalfa, 2020; Bruce, 2005).

Rarely, stomatocytosis can be caused by genetic changes in lipid metabolism genes, such as *ABCG5* and *ABCG8*, which encode sterol transporters (Andolfo et al., 2018).

Hereditary stomatocytosis has been reported in some dog breeds, namely Alaskan Malamutes (Subden et al., 1972; Sande et al., 1982; Pinkerton et al., 1974), Drentse Patrijshonds (Slappendel et al., 1991; Slappendel et al., 1994), and Standard Schnauzers (Bonfanti et al., 2004; Shmukler, 2012). Similar to humans, canine stomatocytosis is phenotypically heterogeneous, suggesting multiple etiologies exist with different underlying genetic modifiers. Notably, some cases have been discovered incidentally after routine hematology and blood smear analysis, with no associated clinical signs. Autosomal recessive inheritance is suspected in Alaskan Malamutes, but modifiers and multigenic models could not be ruled out (Subden et al., 1972). Inheritance in Schnauzers appears to be more complex, given pedigree analysis and affected individuals of intermediate phenotypes (Shmukler, 2012). A candidate gene analysis of the affected Schnauzers did not reveal any coding changes associated with stomatocytosis in the genes examined (*SLC4A1*; *RHAG*, or *SLC2A1*), and stomatin expression was not altered (Shmukler, 2012; Paltrinieri et al., 2007).

Recently, stomatocytosis was described in a Beagle and Australian Cattle Dog (ACD) for the first time (Castillo and Williams, 2021). Both cases reported an asymptomatic decrease in red blood cell (RBC) count and changes in red cell distribution width parameters, including marked abnormalities in volume distribution of the RBC population. Morphologic examination of peripheral blood films identified variable numbers of stomatocytes, knizocytes, mild anisocytosis, mild macrocytosis, and mild polychromasia.

To explore underlying genetic causes of stomatocytosis in the Beagle and Australian Cattle Dog, whole genome sequencing (WGS) of these two cases was undertaken.

2. Methods

2.1. Sample Collection & Ethics

Blood surplus to clinical requirements from the two affected dogs had been archived (-20C) with informed owner consent. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) with an RNAse digestion step. DNA was cleaned and concentrated using DNA Clean and Concentrator-5 (Zymo Resesarch). DNA underwent PCR-free library preparation and Illumina 150-bp paired end sequencing at 30x coverage.

Anonymised and unpublished canine 30x WGS data from 119 dogs of several breeds, generated with informed owner consent in previous projects, was also utilized in joint genotyping to improve variant calling and downstream variant filtration.

2.2. WGS sequencing

QC was conducted with FastQC (v0.11.7). Reads were trimmed with Sickle (v1.33), aligned to CanFam3.1 with BWA (v0.7.12-r1039), and duplicates marked with Picard (v2.18.4). Variants were called in individual samples, followed by joint genotyping in GATK4 (4.1.7.0).

2.3. Annotation resources

Variants were annotated with SNPEff (v4.3 t) and Variant Effect Predictor (VEP v104.3), which includes variant impact predictions (Cingolani, 2012; Cingolani, 2012; McLaren, 2016). Sorting Intolerant from Tolerant (SIFT) scores integrated from VEP provided predictions of deleteriousness. SIFT calculates conservation value and scaled probability for each position to predict whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids (Kumar et al., 2009). Relatedly, 'disruptive' variants are a SNPEff variant category predicted to have moderate impact. Specifically, a disruptive inframe deletion is defined as, 'One codon is changed and one or more codons are deleted (e.g.: A deletion of size multiple of three, not at codon boundary)'.

Biomart (biomaRt v2.40.5) was used to integrate canine, human, and mouse IDs and GO Terms. RefSeq gene summaries were downloaded from UCSC GoldenPath (https://hgdownload.soe.ucsc.edu/goldenPath/; accessed on 16 Sept 2020) (Karolchik, 2004). UniProt gene summaries were downloaded from https://www.uniprot.org/ (accessed on 16 Sept 2020) (UniProt, 2023). Human GWAS associations with mean corpuscular volume and erythrocyte measurement were downloaded from the NHGRI-EBI GWAS Catalog (Supplementary Table 2; accessed on 16 Feb 2023) (Sollis, 2023). Stomatocytosis genes were curated from the literature, OMIM (accessed on 22 Oct 2021) (Online Mendelian Inheritance in Man, xxxx), GSEA MSigDB (v2023.1.Hs, Human Gene Set: HP_STOMATOCYTOSIS, M36538, HP:0004446; accessed on 16 Feb 2023) (Subramanian, 2005) and KEGG (H01978, H01979; accessed on 16 Feb 2023) (Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2023). Stomatocytosis variants in human KCNN4 were downloaded from ClinVar (accessed on 30 Mar 2023) (Landrum, 2018).

2.4. Other public & private canine WGS datasets

Variants called from canine WGS were obtained for > 1,000 dogs in public and private datasets. This included the Dog Biomedical Variant Database (DBVD, n = 590) (Jagannathan et al., 2019), Dog Genome Project (DGP, n = 722) (Plassais, 2019), and Animal Health Trust's / Kennel Club Genetics Centre's Give a Dog a Genome (AHT, n = 200; ENA PRJEB33007). These datasets each used the CanFam3.1 assembly.

2.5. Discordance analyses

PLINK (v1.9) (Purcell, 2007; Chang, 2015) was used to extract and count genotypes from VCFs from WGS of > 1,000 dogs included in the aforementioned public and private datasets (AHT, DBVD, DGP). Genotypes of cases were compared jointly and independently to these other dogs and the 119 controls, which included examination of homozygous and heterozygous variants. Discordance threshold required genotypes carried by cases to be found in ≤ 5 controls and an average ≤ 5 dogs across public and private databases (AHT, DBVD, DGP). This low but non-zero threshold was set to allow for the possibilities that dogs in the external datasets may have had stomatocytosis, or that a genetic change impacting stomatocytosis may not be fully penetrant.

2.6. Regulatory Site analysis

Tomtom (v5.5.2) was used to conduct motif analysis on the genomic sequence of SPTB intron 12 containing candidate stomatocytosis variants (chr8:3,919,4903 – chr8:39,194,967) (Gupta et al., 2007). The motif database used was the 'JASPAR core 2022 for vertebrates'. Human scRNAseq data containing erythroid cells (Dominguez Conde, 2022) was accessed via The Human Cell Atlas (Regev, 2017) to determine gene expression of possible SPTB regulators identified in the motif analysis.

To assess regulatory evidence available for candidate noncoding variants in erythrocyte genes, UCSC liftover was used to obtain the corresponding human (hg38) coordinates (Kent, 2002). UCSC

regulatory tracks (accessed on 16 Aug 2023) provided evidence from ENCODE (Consortium, 2012; Consortium, 2011; Consortium, 2020) and GeneHancer (Fishilevich, 2017).

2.7. Protein modelling

Three-dimensional (3-D) modelling of SLC8A3 was undertaken using the 3-D structure obtained through Phyre2 based on the canine protein sequence (Kelley et al., 2015). The PyMOL Molecular Graphics System (Version 2.4.0, Schrödinger, PyMOL) was used to model the effect of the Asn823Lys amino acid change.

2.8. GWAS / PLINK

VCFs were imported into PLINK (v1.9) (Purcell, 2007; Chang, 2015). Logistic regression was conducted on stomatocytosis cases compared to the 119 controls using 100 k maxT permutations. Recessive model statistics were also calculated with 100 k maxT permutations.

2.9. Shared dominant & recessive scans

Strict discordance analyses were conducted where both stomatocytosis cases shared the same genotype, while no other dog (controls, AHT, DBVD, DGP) carried the same genotype. For the dominant scan, where both cases were heterozygous, discordance threshold required no control or dog from public and private datasets to carry the variant (either in the heterozygous or homozygous state).

2.10. Visualizations

Gene schematics were generated with Integrative Genomics Viewer (IGV) (Robinson, 2011; Thorvaldsdottir et al., 2013; Robinson et al., 2017). Visualizations generated in R (v3.6.2) were: the Manhattan plot using qqman (Turner, 2018), genotype stacked bar plots using ggplot2 (Wickham, 2016), protein schematics using drawProteins (Brennan, 2018), and the Circos plot was generated using circlize (Gu et al., 2014). Protein-protein networks were visualized using STRING (v12.0) (Szklarczyk, 2023). Multiz alignments of 100 vertebrate species were generated using the UCSC genome browser (accessed on 27 Sept 2024) (Kent, 2002).

2.11. Data Sharing Statement

WGS of both stomatocytosis cases can be found in the European Nucleotide Archive (ENA study: ERP166774; samples: ERS22550814 and ERS22550815). Genotype data of candidate variants may be found in a data supplement available with the online version of this article.

3. Results

Genetic variants identified in stomatocytosis cases were compared to WGS variant call sets (CanFam3.1) of 119 control dogs and > 1,000 dogs from public and private datasets (Fig. 1, Supplementary Table 1). This included the Dog Biomedical Variant Database (DBVD, n = 590) (Jagannathan et al., 2019), Dog Genome Project (DGP, n = 722) (Plassais, 2019), and Animal Health Trust's Give a Dog a Genome (AHT, n = 200). Because stomatocytosis may be caused by different genetic changes in each case, cases were considered independently as well as jointly.

To aid canine stomatocytosis candidate gene identification, variants were annotated using a variety of resources. This included variant consequence and impact prediction, the functions of corresponding genes, and biological pathway information. This also included integration of gene associations with erythrocyte measurement and mean corpuscular volume from > 100 human genome-wide association studies (GWAS) (Supplementary Table 2).

3.1. Stomatocytosis cases do not carry coding variants consistent with single-variant monogenic inheritance in genes with known links to stomatocytosis

Forty-nine genes linked to stomatocytosis were curated from the literature, Online Mendelian Inheritance in Man (OMIM), Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Molecular Signatures Database (MSigDB) (Supplementary Table 3). Coding variants in curated genes called from canine stomatocytosis cases were examined (Supplementary Table 4). Corresponding genotypes were always found in at least one or more control dogs or dogs from the public and private datasets. These results raise several possibilities regarding the mode of inheritance or causal variant(s), including the possibilities that in one or both cases the causal variant is noncoding, is coding but not fully penetrant, is in a gene not currently linked to stomatocytosis in humans, or the condition is multi-locus / polygenic.

3.2. Homozygous intronic variants in stomatocytosis gene SPTB found in both canine stomatocytosis cases

Noncoding variants within 5 kb of curated stomatocytosis genes were examined (Supplementary Table 5). Notably, both cases contained a different homozygous SNV only 24 bp apart in intron 12 of *SPTB*. The quality of both base calls was very high (QUAL > 1,000), and each SNV was detected in all sequencing reads of the corresponding affected dog (Beagle − chr8:39194923 A = 29 reads; ACD − chr8:39194947C = 39 reads) (Fig. 1B, Table 1), Supplementary Table 6). These SNVs were not homozygous in any other canine data examined (Supplementary Table 7). Moreover, these variants were rarely found even in the heterozygous state in other dogs (DBVD AF = 0.000868). Motif analysis weakly suggests this intronic region may contain regulatory binding sites (Supplementary Fig. 1), but neither canine ChIP-seq nor ATAC-seq data are available, and this intronic region is not conserved with humans, so supporting analyses are not possible.

3.3. Candidate canine stomatocytosis deleterious missense variant in *KCNN4*

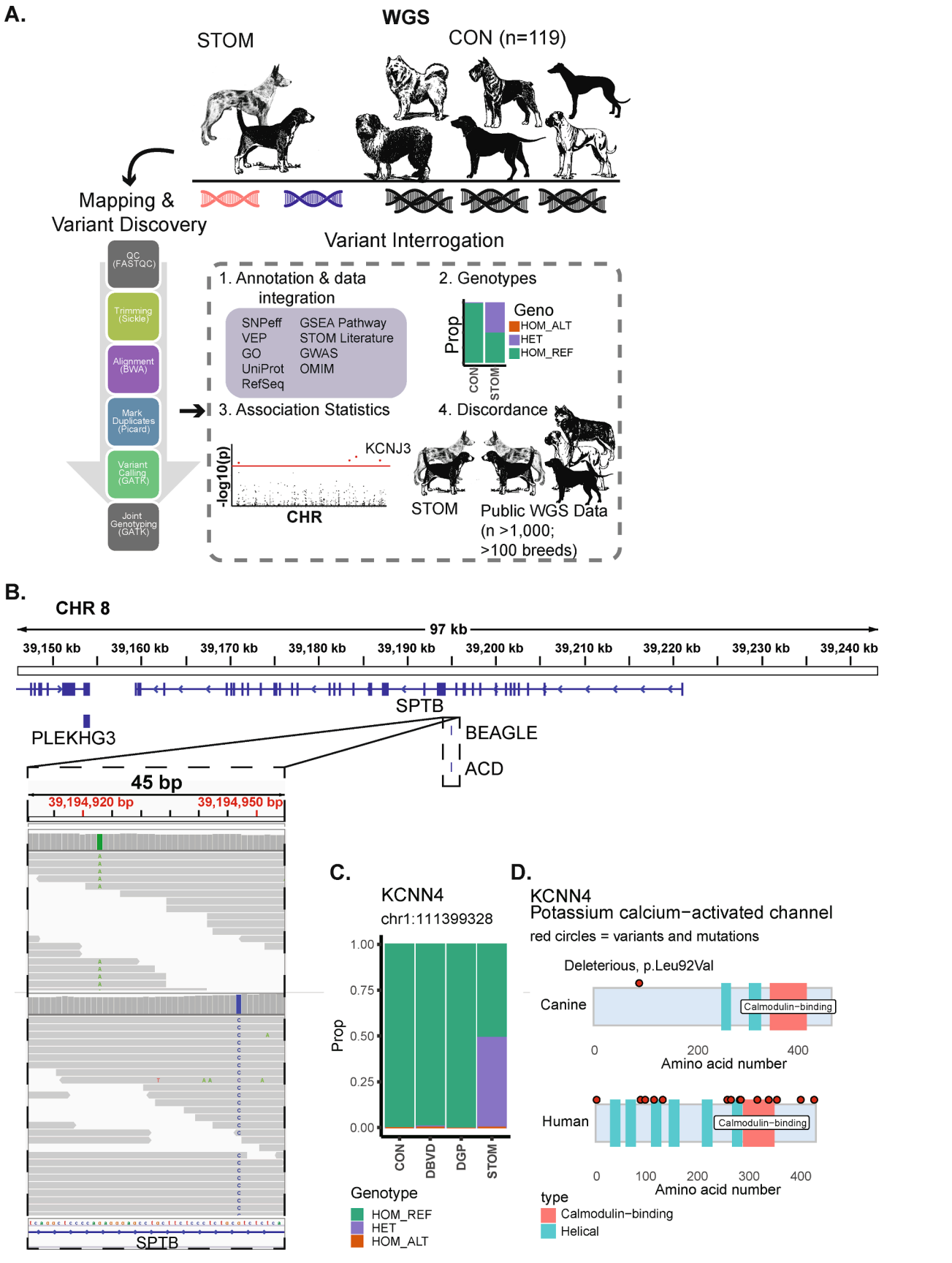
The possibility was examined that causal variants may reside in genes with known links to stomatocytosis but require additional modifiers or not be completely penetrant. Variants called in canine stomatocytosis cases within 5 kb of known human stomatocytosis genes were included if the corresponding genotype was found in ≤ 5 of the 119 controls and mean ≤ 5 in public and private datasets containing more than 1,000 dogs.

The Beagle with stomatocytosis carries a rare heterozygous variant in *KCNN4* predicted to be deleterious (SIFT = 0.02) (chr1:111,399,328, c.274C > G, p.Leu92Val) (Fig. 1C-1D, Table 1, Supplementary Table 6). This variant was never homozygous for the alternate allele in any canine data examined. The variant was heterozygous in one control dog and rare in public datasets (DBVD AF = 0.0085; DGP AF = 0.0028) (Fig. 1C). The heterozygotes are from several breeds, indicating this rare variant is not simply a variant distinctive of Beagles (Supplementary Table 7).

3.4. Deleterious and disruptive variants in novel candidate stomatocytosis ion channel and transport genes

To explore the possibility that stomatocytosis is caused or modified by variants in genes not commonly linked with the disorder in humans, a discordance analysis was conducted comparing predicted deleterious and disruptive variants found in each case to control dogs and dogs from public and private datasets.

Notably, both cases carry a different homozygous deleterious or disruptive variant in *SLC8A3*, consistent with homozygous recessive inheritance, which is never homozygous in any other dog (Fig. 2A, Table 1, Supplementary Tables 6-7). The Australian Cattle Dog carries a



(caption on next page)

Fig. 1. Study overview & variants in canine cases within genes linked to human stomatocytosis. **A.** Study Overview – WGS on canine stomatocytosis cases and controls. Variant interrogation to identify candidate drivers or modifiers of canine stomatocytosis: 1. Annotation and data integration from a variety of sources, including VEP for consequence and impact prediction, UniProt and RefSeq for gene functions, and genetic associations with mean corpuscular volume and erythrocyte measurement from > 100 human GWAS studies. 2. Variants called in canine stomatocytosis cases were compared to the genotypes of 119 controls and > 1,000 dogs from public and private datasets in genes linked to human stomatocytosis or erythrocyte and ion exchange related genes. 3. GWAS showed significant association of loci with canine stomatocytosis. 4. Discordance analysis of variant genotypes shared between cases, which were never observed in controls or dogs in the public and private datasets. **B.** Schematic of the *SPTB* gene labelled with HOM ALT intronic variants found in the Beagle (chr8:39,194,923) and Australian Cattle Dog (chr8:39,194,947) with stomatocytosis, which were not homozygous in any other dog. The 45 bp excerpt shows a pileup of the sequencing reads at this locus (Beagle, top track; ACD, bottom track). Note, full depth coverage is not shown. **C.** Genotype stacked bar plot of a deleterious missense variant in *KCNN4*, which is never homozygous in any dog, heterozygous in the Beagle with stomatocytosis, and extremely rare in controls and dogs from public and private datasets. **D.** Protein schematics of KCNN4, canine (top), human (bottom). The p.Leu92Val variant found in the Beagle with stomatocytosis is indicated. Stomatocytosis variants in human KCNN4 from ClinVar are also indicated (G3E, M88R, R97S, V114L, L131I, V256M, G263S, L280Q, V282M, V282E, S314P, A336V, R352H, T398M, Q424H).

disruptive 3 bp deletion in the coding region of SLC8A3 (c.1343_1345delTCA, p.Phe448_Thr449delinsSer) that encodes the calx-beta 1 domain, which is a calcium-binding domain essential for ion exchange (Fig. 2B, Supplementary Table 2A). This variant is neither homozygous nor heterozygous in any other dog data examined, indicating it is either an extremely rare variant or a novel genetic change. The amino acids spanning this deletion are highly conserved across vertebrates, with F448 being completely conserved in all 100 species examined, and T449 showing conservation in all except stickleback, Atlantic cod, and lamprey (Supplementary Figs. 2-3). The homozygous missense variant in the same gene in the affected Beagle (chr8:43,782,435, c.2469C > A, p.Asn823Lys) is predicted to be deleterious, never homozygous in any other dog, and is extremely rare even in the heterozygous state, with heterozygosity observed only in a single Pomeranian. Protein modelling of the predicted canine protein structure in PYMOL showed Asn823 lies in the 9th transmembrane helix and has polar contact with three amino acids of the same alpha-helix (Ala819, Ser820 and Ser827), and amino acids Asp803 and Ser807 on the 8th transmembrane helix (S8). Asn823Lys is predicted to have a deleterious effect, leading to the loss of all hydrogen bonds with S8 and with Ala819 and Ser820, while creating a new polar contact with Ser143 on the 2nd transmembrane helix (Fig. 2C).

Both the Beagle and Australian Cattle Dog were also homozygous for a rare deleterious variant in the key calcium ion sensor *DYSF* (chr17:51,074,518, rs851039220, SIFT = 0), which amongst control dogs and dogs from public datasets was only found to be homozygous in a single American Hairless Terrier (Fig. 2D, Table 1, Supplementary Tables 6-7). This variant causes a p.Arg1433His amino acid change in a C2 calcium-dependent phospholipid binding domain (Fig. 2E).

Finally, a small number of additional variants predicted to be deleterious in genes with functional roles involving ion sensing, exchange, or transport were identified (Fig. 2, Table 1, Supplementary Tables 6-7). The Beagle was homozygous for a rare deleterious variant in *SLC12A8* (chr33:28,093,777, rs852823683, SIFT = 0.03). Consistent with a recessive model, the variant was never homozygous in the unaffected dogs. The Australian Cattle Dog additionally carries deleterious variants in *INPP5E* (chr9:49,062,279, rs851552394, SIFT = 0), *SLC1A1* (chr1:92,964,307, rs850990814, SIFT = 0.04), and a novel genetic change in *SLC41A3* (chr20:195,027, SIFT = 0) not seen in any other dog.

Together, these deleterious variants may be novel causal drivers or modifiers of the canine stomatocytosis phenotype.

3.5. Noncoding variants in novel candidate erythrocyte genes

A discordance analysis was also performed to identify candidate noncoding variants in genes not previously implicated in human stomatocytosis. In the absence of canine ChIP-seq or ATAC-seq data, noncoding variants lack the same level of supporting evidence as coding variants, and therefore stricter criteria were implemented. Variants were required to reside within 5 kb of genes with functional roles in erythrocytes. Criteria for homozygous variants in cases required that no unaffected dogs were homozygous. Criteria for variants heterozygous in cases required no other dogs were homozygous and ≤ 1 dog from the combined controls, public, and private datasets could be heterozygous.

Eleven candidate noncoding variants were identified, several of which reside in or around genes associated with erythrocyte-related phenotypes in GWAS, such as mean spheric corpuscular volume (Supplementary Fig. 4, Supplementary Table 8). Some of these variants have regulatory evidence at the corresponding chromosomal position in humans, including candidates in *ADD2*, *EPAS1*, *NCKAP1L*, *RHCE*, and *ZFP36L1* (Supplementary Table 9).

Notably, the affected Beagle carried candidate variants in both *ADD1* (chr3:61,426,241) and *ADD2* (chr10:69,092,241), which links the Spectrin (SPTB) cytoskeleton to the plasma membrane. Moreover, the affected Beagle also carried a homozygous variant in *RHCE* (chr2:74,468,272), as well as a novel genetic change or ultra-rare variant not found in any other dog in *NCKAP1L* (chr27:838,892).

3.6. The KCNJ3 region associates with canine stomatocytosis

To further explore the possibility of shared genetic modifiers or drivers underlying canine stomatocytosis in the Beagle and Australian Cattle Dog, logistic regression was conducted on the WGS data comparing cases to the 119 controls. Four loci significantly associated with canine stomatocytosis, including one locus in the *KCNJ3* region (EMP2 p = 0.0325; Supplementary Fig. 5A, Table 1, Supplementary Table 10). This region contains variants shared by both STOM cases (chr36:1,341,625, chr36:1,445,919) as well as additional rare variants found in either the affected Beagle (chr36:1,443,327; DBVD AF = 0.0028) or the Australian Cattle Dog (chr36:1,420,941, chr36:1,435,448, chr36:1,465,946, chr36:1,518,090; DBVD AFs = 0.0034——0.00086), suggesting that variants in the *KCNJ3* region may collectively impact stomatocytosis (Supplementary Fig. 5B-3C, Table 1, Supplementary Tables 7 and 11).

3.7. Dominant and recessive scans identify candidate variants shared between stomatocytosis cases

Given the affected dogs are from different breeds, it is unlikely stomatocytosis is caused by only a single driver shared by both cases, though still possible, and they may also share disease modifiers. Therefore, fully discordant dominant and recessive scans were conducted. Variants with identical genotypes in both cases, which were absent in unaffected dogs, were identified. The recessive scan showed both STOM cases have a homozygous 5 bp intronic deletion in *RYR2*, which is not homozygous in any unaffected dog (Supplementary Fig. 5D, Supplementary Table 12). Similarly, the dominant scan showed both STOM cases have a heterozygous 1 bp intronic insertion in *PLS1* that is never found in any unaffected dog (Supplementary Fig. 5E, Supplementary Table 13).

3.8. No evidence of variants involved in lipid metabolism genes

Stomatocytosis in rare instances can be caused by genetic changes in lipid metabolism genes (Andolfo et al., 2018). This may underlie stomatocytosis in Drentse Patrijshonds (Slappendel et al., 1991; Slappendel

Table 1
Candidate canine stomatocytosis genes and variants. Gene functions are provided by RefSeq and UniProt. Human GWAS associations provided by NHGRI-EBI GWAS Catalog (Sollis, 2023). (Editable version of this table uploaded separately).

GENE	CHROM	POS	ID	TYPE	EFFECT	HGVS P	STOM	GENO	FUNCTION	Human GWAS
Genes linked to stomatocytosis in humans										
SPTB	8	39,194,923	.	SNP	intron		BEAGLE	HOM	Major constituent of the cytoskeletal network underlying the erythrocyte plasma membrane. Spectrin functions in stability of erythrocyte membranes, and mutations in this gene have been associated with spherocytosis type 2, hereditary elliptocytosis, and neonatal hemolytic anemia.	Mean corpuscular hemoglobin concentration, reticulocyte count, Chagas cardiomyopathy, red blood cell distribution width, Mean spheric corpuscular volume
SPTB	8	39,194,947	.	SNP	intron		ACD	HOM		
KCNN4	1	111,399,328	.	SNP	missense	p.Leu92Val	BEAGLE	HET	Forms a voltage-independent potassium channel activated by intracellular calcium. Activation is followed by membrane hyperpolarization which promotes calcium influx.	Red blood cell count, Mean corpuscular hemoglobin concentration, hemoglobin measurement, mean corpuscular volume, erythrocyte count, reticulocyte count
Deleterious & Disruptive variants in ion-related genes										
SLC8A3	8	43,782,435	.	SNP	missense	p.Asn823Lys	BEAGLE	HOM	Mediates the electrogenic exchange of Ca(2 +) against Na(+) ions across the cell membrane, contributing to the regulation of cytoplasmic Ca(2 +) levels, Ca(2 +)-dependent cellular processes, and cellular Ca(2 +) homeostasis.	peripheral arterial disease
SLC8A3	8	43,901,708	.	DEL (−3)	disruptive inframe deletion	p. Phe448_Thr449delinsSer	ACD	HOM		
DYSF	17	51,074,518	rs851039220	SNP	missense	p.Arg1402His; p. Arg1433His	BOTH	HOM	Key calcium ion sensor. Contains C2 domains that play a role in calcium-mediated membrane fusion events, suggesting that it may also be involved in membrane regeneration and repair.	PR interval, pulse pressure measurement, heart failure
SLC1A1	1	92,964,307	rs850990814	SNP	missense	p.Arg280His	ACD	HOM	Functions as a symporter that transports one amino acid molecule together with two or three Na(+) ions and one proton, in parallel with the counter-transport of one K (+) ion. Mediates Cl(−) flux that is not coupled to amino acid transport; this avoids the	Ischemic stroke

(*continued on next page*)

Table 1 (*continued*)

GENE	CHROM	POS	ID	TYPE	EFFECT	HGVS P	STOM	GENO	FUNCTION	Human GWAS
									accumulation of negative charges due to aspartate and Na(+) symport.	
SLC41A3	20	195,027	.	SNP	missense	p.Pro282Thr; p.Pro262Thr	ACD	HET	Ubiquitously expressed Na(+)/Mg(2 +) ion exchanger located in the plasma membrane, of which little is known.	
SLC12A8	33	28,093,777	rs852823683	SNP	missense	p.Gly634Ser	BEAGLE	HOM	Cation/chloride cotransporter	Mean spheric corpuscular volume, reticulocyte count, reticulocyte measurement
INPP5E	9	49,062,279	rs851552394	SNP	missense	p.Arg81Trp	ACD	HOM	Mobilizes intracellular calcium and acts as a second messenger mediating cell responses to various stimulation.	A1C measurement, red blood cell distribution width
Non-coding variants in erythroctye-related genes										
ADD1	3	61,426,241		SNP	intron		BEAGLE	HET	Membrane-cytoskeleton-associated protein that promotes the assembly of the spectrin-actin network. Binds to calmodulin. GO: erythrocyte differentiation	
ADD2	10	69,092,241		SNP	intron		BEAGLE	HOM	Binds to the erythrocyte membrane receptor SLC2A1/GLUT1 and provides a link between the spectrin cytoskeleton to the plasma membrane.	
CDK6	14	18,404,991		SNP	intron		ACD	HET	Required for erythroid proliferation. Mice lacking the encoded protein exhibit hematopoietic defects, including reduced number of erythrocytes. GO: regulation of erythrocyte differentiation.	Red blood cell count, Mean corpuscular hemoglobin, Mean corpuscular volume, Mean spheric corpuscular volume
EPAS1	10	48,605,947 48,553,571		SNP	intron		BEAGLE	HOM	Transcription factor induced when oxygen levels fall. Mutations in this gene are associated with erythrocytosis familial type 4. GO: erythrocyte differentiation	
ITGAD	6	16,812,460	rs851634536	SNP	intron		BEAGLE	HOM	Clears senescent erythrocytes from the blood.	Mean corpuscular hemoglobin
NCKAP1L	27	838,892		SNP	intron		BEAGLE	HET	Essential hematopoietic-	

(*continued on next page*)

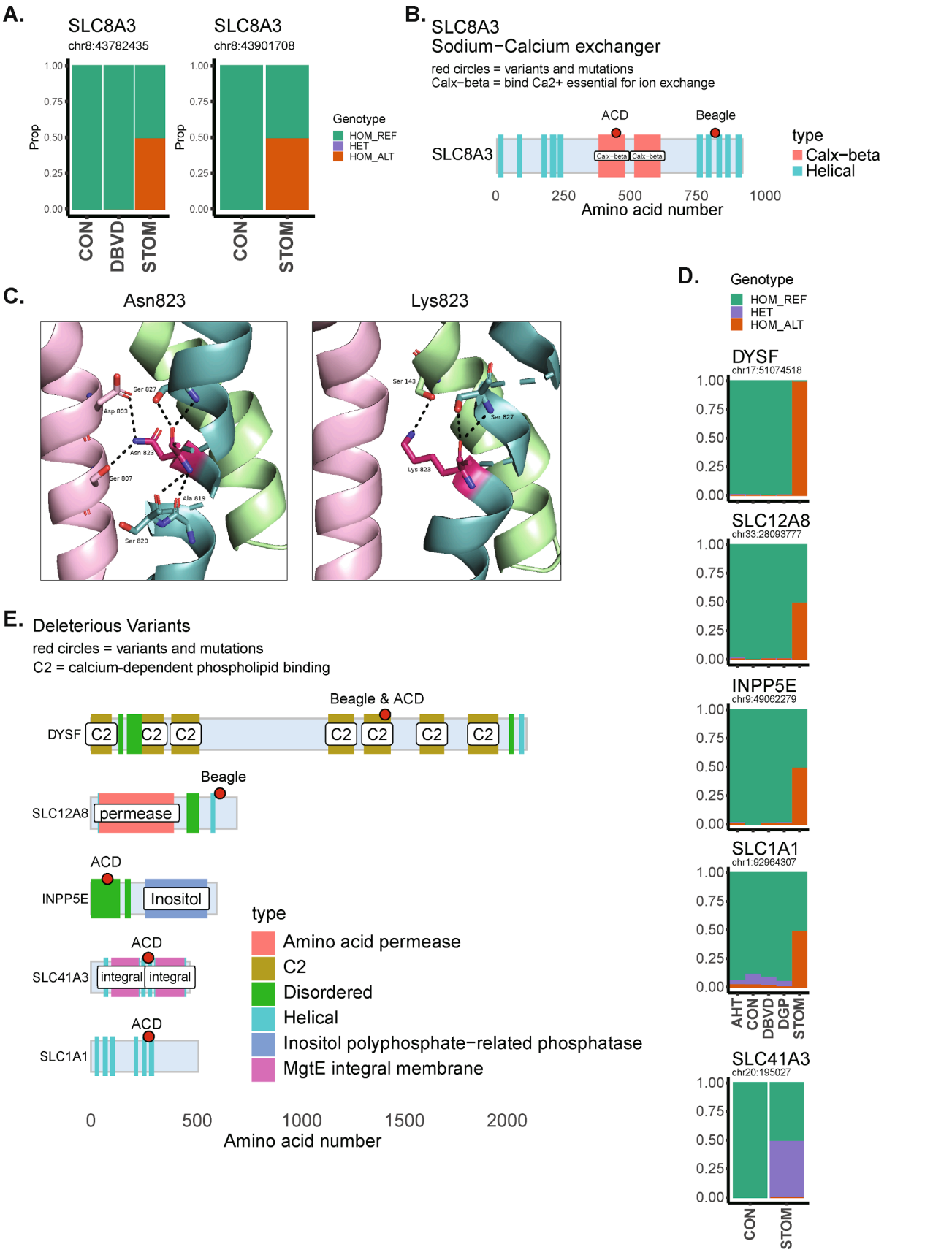
Table 1 (*continued*)

GENE	CHROM	POS	ID	TYPE	EFFECT	HGVS P	STOM	GENO	FUNCTION	Human GWAS
									specific regulator of the actin cytoskeleton. Controls erythrocyte membrane stability. GO: erythrocyte homeostasis, positive regulation of erythrocyte differentiation, erythrocyte development	
RHCE	2	74,468,272		SNP	intron		BEAGLE	HOM	Part of an oligomeric complex likely to have a transport or channel function in the erythrocyte membrane	
SOX6	21	39,120,172		SNP	intron		BEAGLE	HOM	Transcription factor that plays a key role in erythrocyte differentiation and development.	Mean spheric corpuscular volume, Mean corpuscular volume, Mean corpuscular hemoglobin concentration, Mean corpuscular hemoglobin
ZFP36L1	8	42,675,057		DEL (−1)	upstream		ACD	HOM	Negatively regulates hematopoietic/ erythroid cell differentiation.	Mean spheric corpuscular volume
GWAS Association of KCNJ3 and rare variants in region										
KCNJ3	36	1,341,625		SNP	intergenic region		BOTH	HOM/ HET	Inward rectifier potassium channel characterized by a greater tendency to allow potassium to flow into the cell rather than out of it.	
KCNJ3	36	1,420,941	.	SNP	intron		ACD	HET		
KCNJ3	36	1,435,448	.	SNP	intron		ACD	HET		
KCNJ3	36	1,443,327	.	SNP	intron		BEAGLE	HOM		
KCNJ3	36	1,445,919	.	DEL (−105)	intron		BOTH	HOM/ HET		
KCNJ3	36	1,465,946	.	SNP	intron		ACD	HET		
KCNJ3	36	1,518,090	.	SNP	intergenic region		ACD	HET		
Discordant Dominant and Recessive Scan of variants shared in cases										
RYR2	4	2,473,540	.	DEL (−5)	intron		BOTH	HOM	Calcium channel that mediates the release of Ca(2 +). Required for cellular calcium ion homeostasis.	diastolic blood pressure, systolic blood pressure, PR interval, aortic measurement
PLS1	23	38,257,613	.	INS (1)	intron		BOTH	HET	Actin-bundling protein in the absence of calcium.	Red blood cell count, Mean corpuscular hemoglobin, QRS duration, mean corpuscular volume

et al., 1994). Therefore, lipid metabolism genes linked to stomatocytosis in humans were independently examined in canine cases (Supplementary Table 14). Lipid metabolism genes with no known link to stomatocytosis were also examined. No strong lipid metabolism candidates emerged, as the genotypes present in cases were either commonly found in the public data as a whole, or the variants were common in the respective breeds for each affected case (Supplementary Table 15-16).

3.9. Canine stomatocytosis candidate gene expression

Discordance and statistical analyses identified candidate genes in canine stomatocytosis (Fig. 3A). Expression in RBCs at the transcript or protein level would further support these candidates as drivers or modifiers of the disorder. While these data are not available for canines, public human and mouse single-cell RNA sequencing (scRNA-seq) data and liquid chromatography–mass spectrometry (LC–MS) data were examined (Dominguez Conde, 2022; Bryk and Wisniewski, 2017;



(caption on next page)

Fig. 2. Deleterious and disruptive variants in novel candidate stomatocytosis ion channel and transport genes. **A.** Genotype stacked bar plots of *SLC8A3* homozygous alternate variants called in the Beagle (left) and Australian Cattle dog (right), which are not found to be homozygous in any other dog. Davison Canine Controls (CON, n = 119), Dog Biomedical Variant Database (DBVD, n = 590), Dog Genome Project (DGP, n = 722), and Give a Dog a Genome (AHT, n = 200). **B.** Protein schematic of SLC8A3 indicating the location of amino acid changes in stomatocytosis cases corresponding to variants in A. Note, the disruptive deletion in the Australian Cattle Dog p.Phe448_Thr449delinsSer is in the calx-beta 1 domain, which is a calcium binding domain essential for ion exchange. **C.** 3-D structure of SLC8A3 canine protein sequence. PyMOL was used to model the effect of the Asn823Lys amino acid change found in the Beagle. Left: Asn823 lies in the 9th transmembrane helix (S9) and shows polar contact with three amino acids of the same alpha-helix (Ala819, Ser820 and Ser827), and amino acids Asp803 and Ser807 on the 8th transmembrane helix (S8). Right: Asn823Lys shows the loss of all hydrogen bonds with S8 and with Ala819 and Ser820, while creating a new polar contact with Ser143 on the 2nd transmembrane helix. **D.** Genotype stacked bar plots of deleterious variants in ion-related genes found in cases, for which the corresponding genotypes are rarely or never found in controls or dogs from public and private datasets. **E.** Protein schematics indicating amino acid changes in stomatocytosis cases corresponding to variants in D. Note, the homozygous p.Arg1433His amino acid change found in both canine stomatocytosis cases in the key calcium ion sensor gene *DYSF* is in a C2 calcium-dependent phospholipid binding domain.

Ayturk, 2020).

Erythrocyte and erythroid (immature RBCs) scRNA-seq data were examined from the Human Cell Atlas (Regev, 2017). All candidate genes showed expression in human erythroids or mouse erythrocytes (Fig. 3A). Additionally, human erythrocyte proteomics detected the presence of SPTB, KCNN4, SLC41A3, ADD1, ADD2, NCKAP1L, and RHCE protein (Fig. 3A).

Notably, some of these candidate genes interact. Visualization of functional protein association networks for each case shows links between candidates (Fig. 3B). This includes RYR2 and KCNJ3 in both dogs, as well as the extension to KCNN4 in the Beagle. Network visualization also highlights the links between *SPTB*, *ADD1*, *ADD2*, and *DYSF* in the Beagle.

4. Discussion

Stomatocytosis has been recently reported in a Beagle and an Australian Cattle Dog for the first time (Castillo and Williams, 2021). We conducted WGS of these dogs. As sibling and parent data were not available for the two affected dogs, we compared the data to WGS from > 1,000 other dogs to identify variants in genes that may drive or influence the disorder.

Cases did not carry coding variants consistent with single-variant monogenic inheritance in genes linked to stomatocytosis in humans. This is consistent with stomatocytosis in Schnauzers, where likewise, no coding changes in known stomatocytosis genes have been detected (Shmukler, 2012; Paltrinieri et al., 2007).

This raised several possibilities which were systematically examined, including that causal variants are noncoding, the existence of a monogenic coding variant that is not fully penetrant, multigenic / multi-locus, or causal variant(s) in gene(s) not currently linked to stomatocytosis in humans.

Association statistics and discordant analyses identified candidate driver or modifier genes for canine stomatocytosis, including candidate variants in known stomatocytosis genes – *SPTB* and *KCNN4*. Notably, both affected dogs carried different homozygous intronic SNPs in erythrocytic spectrin beta (*SPTB*) only 24 bp apart. These variants were not homozygous in any other dog. Spectrin comprises a major constituent of the erythrocyte cytoskeleton and therefore its stability. The normal erythrocyte biconcave shape, and the deformability which allows red blood cells to flow through narrow capillaries is critically dependent on lipid-protein and protein–protein interactions in the intracellular cytoskeleton (Andolfo et al., 2016). Spectrin is in the MSigDB gene set for stomatocytosis, and genetic changes in this gene have been associated with conditions related to stomatocytosis, including spherocytosis and elliptocytosis (Perrotta, 2009; Harper, 2013). *KCNN4* encodes a potassium channel that is activated by intracellular calcium. Genetic changes in this gene can alter Ca^{2+} sensitivity of the K^+ channel, leading to stomatocytosis. In humans, the clinical phenotype can greatly vary between carriers, ranging from mild to severe anemia, or even no documented sign of anemia for parents who also carry a *KCNN4* genetic variant (Allegrini, 2022). Recognizing the clinical heterogeneity of stomatocytosis even when the same *KCNN4* genetic variant is present, the heterozygous variant (chr1:111,399,328) predicted to have a deleterious impact on *KCNN4*, which was detected in the Beagle and found at very low frequency in external datasets, does not preclude the variant being causal in the affected Beagle, especially considering the relatively mild clinical signs noted in both affected dogs.

Like several of the genes linked to stomatocytosis in humans are involved in ion exchange or transport, several of the canine stomatocytosis candidates are in ion-related genes and were predicted by SNPEff or VEP to be deleterious. This included variants in *SLC8A3*, *DYSF*, *INPP5E*, *SLC12A8*, *SLC1A1*, and *SLC41A3*.

Both *SLC8A3* and *DYSF* are particularly notable, given that both affected dogs contain homozygous deleterious or disruptive variants in these genes, consistent with autosomal recessive inheritance. This included a variant predicted to alter contacts between transmembranes, a disruptive deletion in a calcium binding domain essential for ion exchange, and an amino acid change in a C2 calcium-dependent phospholipid binding domain. *SLC8A3* encodes a Na^+/Ca^{2+} exchange protein involved in maintaining Ca^{2+} homeostasis in a wide variety of cell types. DYSF is a key calcium ion sensor involved in calcium-triggered synaptic vesicle-plasma membrane fusion.

Candidate genes *SLC12A8*, *INPP5E*, and *SLC1A1* have additionally been associated with red blood cell phenotypes (Table 1, Supplementary Table 2). SLC12A8 is a cation cotransporter, and variants in this gene have been associated with mean spherical corpuscular volume, reticulocyte measurement, and reticulocyte count in human GWAS (Vuckovic, 2020; Grozio, 2019). *INPP5E* is associated with red blood cell distribution width in human GWAS and mobilizes intracellular calcium and acts as a second messenger mediating cell responses to various stimulation (Vuckovic, 2020). Notably, expression of *SLC1A1* has been associated with RBC count and hemoglobin measurement in patients with renal tumours (Ergün et al., 2019). SLC1A1 functions as a symporter that transports an amino acid with sodium ions in parallel with the countertransport of a potassium ion. This protein also mediates chloride ion flux to prevent the accumulation of negative charges due to sodium ion symport. The novel genetic variant in *SLC41A3* (chr20:195,027) carried by the affected Australian Cattle Dog was not seen in any other dog. SLC41A3 is a ubiquitously expressed sodium/magnesium ion exchanger located in the plasma membrane, of which little else is known (Fleig et al., 2013).

A stringent discordance analysis of noncoding variants identified candidates in several erythrocyte genes associated with erythrocyte-related phenotypes in human GWAS, such as mean spheric corpuscular volume (Supplementary Table 8). Notably, the affected Beagle carried candidate variants in interacting genes *ADD1* and *ADD2*, which links the Spectrin (SPTB) cytoskeleton to the plasma membrane. Moreover, the Beagle also carried a homozygous variant in *RHCE*, a gene which has transport function in the erythrocyte membrane, as well as a novel genetic variant in *NCKAP1L*, a gene which is an essential hematopoietic-specific regulator of the actin cytoskeleton.

Lastly, while it is unlikely stomatocytosis is caused by the same driver in both affected dogs, such occurrences have been identified in canines with other phenotypes. For example, a homozygous variant in *PRCD* shows complete concordance with progressive rod-cone

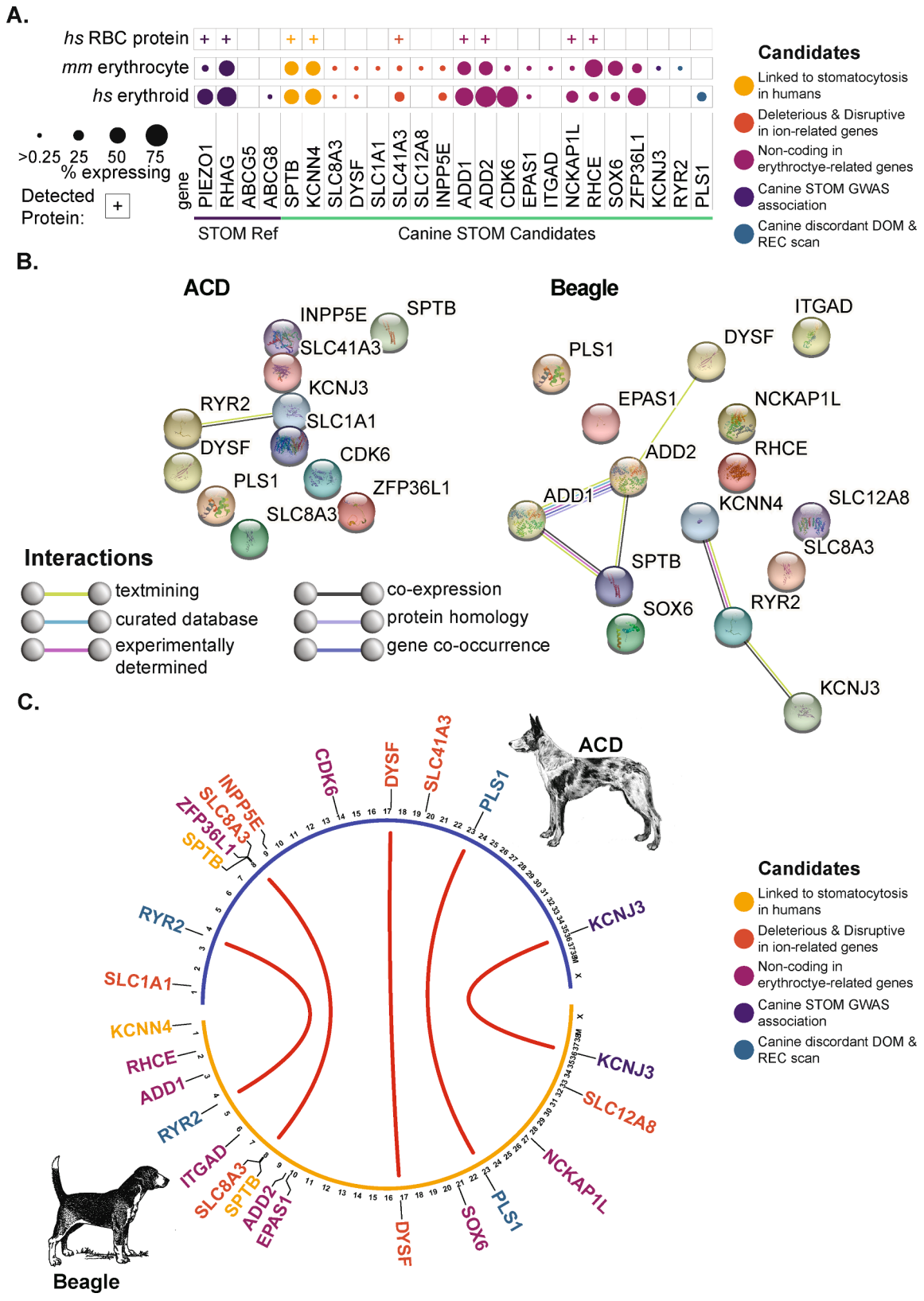


Fig. 3. Candidate canine stomatocytosis genes. **A.** Dot plot indicating detection of expression of candidate stomatocytosis genes in human erythroid scRNA-seq, mouse RBC scRNA-seq, and human RBC proteomics. Detected expression of non-candidate genes linked to stomatocytosis in humans are shown for reference (STOM Ref) – *PIEZO1*, *RHAG*, *ABCG5*, and *ABCG8*. **B.** STRING network of candidate canine stomatocytosis genes showing interacting genes. **C.** Circos plot summarizing candidate canine stomatocytosis genes. Red links indicate candidate genes shared between cases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

degeneration in multiple dog breeds (Zangerl, 2006). Moreover, variants shared between the two affected dogs may modify the disorder, even if such variants do not drive stomatocytosis. When exploring variants shared by the affected dogs, logistic regression showed an association of the *KCNJ3* region, the recessive scan showed both cases have an intronic deletion in *RYR2*, and the dominant scan identified both dogs carried an intronic insertion in *PLS1* not found in any unaffected dog. KCNJ3 is a potassium ion channel characterized by potassium outflow from cells. RYR2 is a calcium channel that mediates the release of Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm. PLS1 plays a role in actin-bundling in the absence of calcium.

To examine evidence for the roles of these genes in the physiological activity of normal red blood cells, publicly available transcriptomic and proteomic data were examined. Because RBCs are denucleated before entering the bloodstream, scRNAseq data of erythroids (immature nucleated RBCs) was examined in addition to mature RBCs. Genes ubiquitously expressed in erythrocytes and linked to stomatocytosis in humans were used as a reference for comparison to candidates. However, scRNAseq has only a limited ability to accurately gauge what proportion of RBCs express a given gene, due to gene dropout. This is exemplified with the reference gene *ABCG8*, which showed only minimal detected expression in human erythroids and no detected expression in mouse erythrocytes, and the reference *ABCG5* showed no detection in either single-cell dataset. Moreover, scRNAseq of erythroids only captures a snapshot of these immature cells and does not necessarily reflect scaled protein abundance of the corresponding genes of mature erythrocytes. Lastly, expression of these genes in humans and mice does not guarantee expression in canines. Together, therefore, while these analyses provided at least some evidence of expression of candidate genes in species where data is available, accurate abundance in mature canine erythrocytes cannot be determined by these analyses.

4.1. Limitations

Overall, several novel and plausible candidate canine stomatocytosis genes were identified in this study. However, no information is available about the parents or siblings to help refine these candidates. Moreover, this does not exhaustively represent the full range of variation that may be contributing to the disorder in these animals, such as structural variants. Furthermore, to emphasize the importance at the gene-level, rather than individual variants, VEP and SNPEff annotations of negative impact are only predictive, and rather than being causal, a given candidate variant may instead be genetically linked to a causal variant. As additional reference canine genomes, canine WGS datasets, and associated annotation resources become available, further filtration will be possible to narrow down candidate variants.

It is also important to note that the erythrocyte phenotypes of dogs in public and private datasets are not available, though WGS was undertaken in these dogs, particularly those in the DBVD, often as part of case-control studies for various diseases. Additionally, given that stomatocytosis is difficult to diagnose, and is often misdiagnosed (Flatt and Bruce, 2009), we cannot rule out that one or more dogs in the public and private datasets may have stomatocytosis. Lastly, the datasets used for comparison (AHT, DBVD, DGP) are of very different origins, so it is unlikely any of these dogs are represented more than once, but this possibility also cannot be completely ruled out.

4.2. Future work

Multi-gene panel testing improves diagnosis and management of patients with hereditary anemias (Russo, 2018). Currently, only ~ 50 genes are linked with stomatocytosis in humans, whereas the related condition spherocytosis has > 300 associated pathogenic genes (Liu, 2020). In recent studies of patients with suspicion of hereditary anemias or erythrocyte defects, 16–35 % remained undiagnosed (Andolfo, 2021; Russo, 2018).

'One Health' is defined by the World Health Organisation as an integrated, unifying approach to balance and optimize the health of people, animals and the environment – recognising that work in one species may be beneficial to understanding the health of another. Here, the One Health approach recognizes the potential for shared genes and pathways in human and canine stomatocytosis, such that candidate genes identified in this canine study may not only be appropriate for future examination in canine stomatocytosis cases, but could also be considered in cases of human stomatocytosis where variants in known stomatocytosis genes have not been detected.

Novelty Statement

This is the first time the genetics of a Beagle and Australian Cattle Dog with stomatocytosis have been examined, and the first time that any canine with stomatocytosis has been whole-genome sequenced. This study has identified novel candidate stomatocytosis genes, which may warrant evaluation in human stomatocytosis. At a time when genetic testing and precision medicine are achievable in clinical practice and improving patient diagnoses and management, this One Health initiative could aid both human and canine genetic diagnostics for stomatocytosis.

CRediT authorship contribution statement

M.D. Wallace: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **S. Falcone:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **D. Castillo:** Writing – review & editing, Resources, Investigation, Conceptualization. **T.L. Williams:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **L.J. Davison:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2025.149314.

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