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# Canine junctional epidermolysis bullosa due to a novel mutation in *LAMA3* with severe upper respiratory involvement

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## Abstract

**Background ––—**Junctional epidermolysis bullosa (JEB) is a group of congenital blistering skin diseases characterized by clefting through the lamina lucida of the basement membrane zone.

**Objectives ––—**To characterize the clinical and morphological features of a congenital mechanobullous disease in a litter of puppies with severe upper respiratory involvement, and to identify an associated genetic variant.

**Animals ––—**Five of eight puppies in an Australian cattle dog cross-bred litter showed signs of skin fragility. Three were stillborn and one died at one month of age. The two surviving puppies were presented with blistering skin disease and severe respiratory distress. Additionally, one unaffected sibling was examined, and blood was obtained for genetic testing.

**Methods and materials ––—**Post-mortem examination, histopathological evaluation and electron microscopy were performed. Whole genome sequencing (WGS) of one affected puppy was compared to a database of 522 dogs of 55 different breeds for variant analysis. Sanger sequencing of one additional affected and one unaffected sibling confirmed the variant.

**Results ––—**Clinically, severe mucocutaneous ulcers occurred in frictional areas with claw sloughing. Histopathological results revealed subepidermal clefts and electron microscopy confirmed the split in the lamina lucida. Post-mortem examination documented extensive pharyngeal and laryngeal lesions with granulation tissue and fibrinous exudate obscuring the airway. Moderate tracheal hypoplasia contributed. The WGS revealed a novel missense variant in the laminin α3-chain XP_537297.2p(Asp2867Val), with an autosomal recessive mode of inheritance.

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**Conflicts of Interest:** No conflicts of interest have been declared.

**Conclusions and clinical relevance ––**A novel variant caused a generalized and severe phenotype of JEB with an unique clinical presentation of upper-airway obstruction.

## Introduction

Junctional epidermolysis bullosa (JEB) is a group of congenital blistering skin diseases characterized by clefting through the lamina lucida of the basement membrane zone (BMZ).[1] As such, individuals affected with JEB suffer with an early onset skin and mucosal (mucocutaneous) fragility resulting in blisters, deep erosions and ulcers. Depending on the nature of the mutation, a variety of extracutaneous clinical features also may develop.[1] This manuscript describes JEB due to a novel variant in a litter of crossbred puppies, which developed mucocutaneous blistering, nail loss and enamel defects, as well as exuberant granulation tissue of the larynx and trachea resulting in severe respiratory distress.

## Clinical findings

In a litter of eight Australian cattle dog cross-bred puppies from an asymptomatic dam and unknown sire, five puppies were reported to show signs of skin fragility (pedigree shown in figure 4a). Three puppies were stillborn and skin fragility was identified in two of these puppies during revival attempts. Three live puppies also showed a blistering skin disease with congenital onset, increased respiratory effort and a failure to thrive. One of these three puppies appeared to exhibit a more severe phenotype and died at two months of age. The surviving two affected puppies (one male, one female) were presented at four months of age and are described in this investigation. Two puppies (both females) remained asymptomatic, one of which was examined for this study.

The two examined affected puppies showed stunted growth, weighing 50% less than their unaffected sibling. The second unaffected puppy was not available for examination. Puppies showed increased respiratory rate (>40/min) with prominent inspiratory stridor and the owners reported frequent choking while eating and drinking. Deep erosions, ulcers and haemorrhagic crusts were present on concave pinnae and in high friction areas such as footpads, digits and distal limb pressure points (Figure 1). Nearly all nails were lost and claw-fold ulcers were observed. Small haemorrhagic vesicles, deep erosions, scars and milia were found on the ventral abdomen and groin (Figure 1). Extensive ulcers and scarring in the oral cavity resulted in tongue rolling (Figure 1). Enamel hypoplasia was prominent (Figure 2). Ulcers affected the mucocutaneous junctions of lips, genitalia and anus, and not the eyelids. Congenital EB, with autosomal recessive inheritance, was suspected due to the early onset, mechanobullous pattern of epithelial sloughing and the pedigree analysis (Figure 4a). Due to lesion severity, breathing difficulty and a guarded prognosis, both puppies subsequently were euthanized. With owner consent, tissue and DNA samples for further diagnostics were collected ante- (one puppy) or post-mortem (one puppy). A DNA sample also was collected from the examined, normal, healthy sibling.

## Post-mortem findings

A complete post-mortem examination (female) confirmed skin and mucocutaneous junction lesions described clinically. Additionally, multifocal to regional ulcers affected the oral mucosa, dorsal and ventral tongue, and gingiva, being more severe on soft palate, pharynx and larynx (Figure 2). Ulcers often were raised due to granulation tissue and thick matts of a tan fibrinous exudate, which in the larynx narrowed the glottis by approximately 70% and obscured the epiglottis (Figure 2). Choanal narrowing was mild and bordered by granulating ulcers. The trachea was moderately narrowed along most of its length (tracheal hypoplasia) (Figure 2). Enamel hypoplasia was extensive on incisor, premolar and molar teeth (Figure 2). Lesions were not observed in the gastrointestinal tract, eyes or skeletal muscle.

## Histopathological evaluation and transmission electron microscopy

Surgical biopsy (male) and post-mortem samples (female) of skin and mucosa confirmed subepithelial clefting at ulcer margins (Figure 3). Basal cells were intact in clefted epithelium. In Case 2, clefts occurred commonly in skin, oropharyngeal and laryngeal lesions, and were minimal in the trachea. Intact vesicles lacked inflammation, and ulcers had mixed neutrophilic inflammation and fibrin exudation. Granulation tissue was thick in laryngeal ulcers (Figure 3) and mild-to-moderate in skin other mucosal sites. The trachea lumen was narrowed from cartilage ring collapse (Figure 3) with overriding ends in association with folding of the mucosa and a short dorsal tracheal ligament. Periodic acid Schiff staining in both cases revealed a thin positive band attached to the submucosa at the base of clefts, consistent with retention of the lamina densa and clefting more superficially. Transmission electron microscopy of lip mucosa identified the lamina densa and anchoring fibrils attached to the submucosa at the base of clefts (Figure 3) and confirmed clefting through the lamina lucida.

## Whole genome sequencing

Genomic DNA (EDTA blood and skin tissue) was isolated from the two affected and one healthy puppy. Whole genome sequencing (WGS) was performed on one affected puppy, and variant calls were compared to a database of 522 dogs of 55 different breeds (details in Supporting information, Appendix S1). Filtering of WGS data to include only variants unique to the affected dog resulted in a final dataset of 466 high- and moderate-impact biological variants in 65 genes (Table S1). A homozygous missense variant at XM_537297.6c:8615T>A in *laminin α3* (*LAMA3*) was prioritised because of the potential impact on the BMZ and matching clinical, histological and ultrastructural phenotype. This variant also was absent from a second database of whole genome sequences containing data from 582 dogs from 126 breeds and eight wolves (including six Australian cattle dogs) assembled by the Dog Biomedical Variant Database Consortium.[2] Further evaluation with Sanger sequencing using primers developed for the *LAMA3* variant confirmed its presence in the additional affected and one unaffected siblings. Both affected puppies were homozygous for the *LAMA3* variant, while the unaffected sibling was a heterozygote (Figure 4). The missense *LAMA3* variant was located on chromosome 7:64427161 (ENSCAFG00000018173)T>A (CanFam 3.1 assembly), and predicted a change in the

highly conserved LG domain of laminin α3 (LAMA3), XP_537297.2p(Asp2867Val) (Figure 4). The FASTQ files for the WGS data are freely available under the reference PRJNA687247 at https://www.ncbi.nlm.nih.gov/sra/PRJNA687247. *In silico* protein prediction of the C-terminus of LAMA3, showing the LG1–3 domains, suggested significant alteration in the protein structure (Figure 4).

## Discussion

This report describes a novel autosomal recessive missense variant in the laminin α3-chain causing JEB with a unique clinical presentation. Severe upper respiratory tract lesions developed in addition to the typical mucocutaneous, mechanobullous blister formation, nail loss and enamel defects recognized previously in people and dogs with JEB (OMIM entry 226700; OMIA entry 002269–9615 and 061677–9615).[1,3] Indeed, the excessive granulation tissue and strictures in the larynx and trachea occluded the airway lumen by 70% and led to devastating respiratory signs and frequent choking in the affected puppies. Using the current human EB guidelines, the JEB described herein would be classified as generalized and severe.[4] Although, excessive granulation tissue, especially periungual, occurs in people with severe JEB phenotype, the degree of laryngeal and tracheal changes in the puppies exceeded that described in these people, and did not match any canine JEB cases described previously.[3,5] Interestingly, JEB with devastating progressive tissue granulation affecting the nail beds, larynx and upper respiratory tract, leading to severe respiratory compromise and the need for a permanent tracheostoma, has been recognized in the Punjabi people in India under the name of a laryngo-onycho-cutaneous syndrome (LOC) (OMIM entry 245660). In addition to the excessive granulation tissue and respiratory distress, people with LOC syndrome exhibit some skin fragility, enamel hypoplasia, milia formation and onycholysis/onychodystrophy; features also seen in the affected puppies.[4,6] However, the variant causing the LOC syndrome is a nonsense mutation near the N-terminus of the α3A isoform[6], while a missense mutation close to the C-terminus, within a highly conserved laminin globular domain 3 (LG3), was found in the affected puppies. This variant displaces a hydrophilic aspartate, an amino acid frequently present in protein-binding sites, with a larger hydrophobic valine; thus, potentially interfering not just with the binding abilities and conformation of the α3 chain, but also with its signalling properties. As proper conformation of α3 chain is required for correct assembly of the α3, β3 and γ2 chains into a functional laminin 332, which then connects with other BMZ proteins, such change could diminish binding properties of the affected protein.[7] Specifically, the tertiary, triangle-like structure of LG1–3 domains of the α3 chain is critical for proper binding of the γ2 chain tail, which then – all together – facilitate binding with integrins.[8,9] While the clinical and microscopic evidence of skin fragility and defect within the lamina lucida in the puppies would support this hypothesis, further studies focusing on the laminin 332 expression and functional studies would be required to confirm it. Besides their role in maintaining the integrity of BMZ, LG domains also have been shown to have crucial signalling function and an important role in wound healing.[10,11] Multiple human JEB subtypes are known to exhibit exuberant granulation tissue, especially in orofacial and periungual regions with time.[1] The severe, debilitating proliferative changes in the puppies' larynx and trachea early in their lives were unique compared to most human JEB subtypes. Indeed, breathing difficulties

were noted as early as six weeks of age and showed gradual progression. Interestingly, LG1–3 domains were shown to play an important role in wound healing by binding growth factors (GFs) via heparin-binding domains,[10,11] one suspected signalling sequence being only four amino acids away from the identified mutation in these puppies (Jun Ishihara, Imperial College London, UK, personal communication). The mechanism of how this variant could have affected healing and the exuberant granulation tissue formation remains unknown.

Herein, we characterized a new recessive form of canine JEB associated with a missense mutation in the *LAMA3* gene, predicting a change in the highly conserved LG3 domain of laminin α3 chain, XP_537297.2p(Asp2867Val). This variant was associated with a previously undescribed phenotype characterized by skin fragility, nail and enamel abnormalities, in addition to exuberant granulation tissue and scarring formation in larynx and trachea. This publication demonstrates the importance of clinical, histopathological and ultrastructural observations, which not only support the findings of the WGS, but also are important for disease classification.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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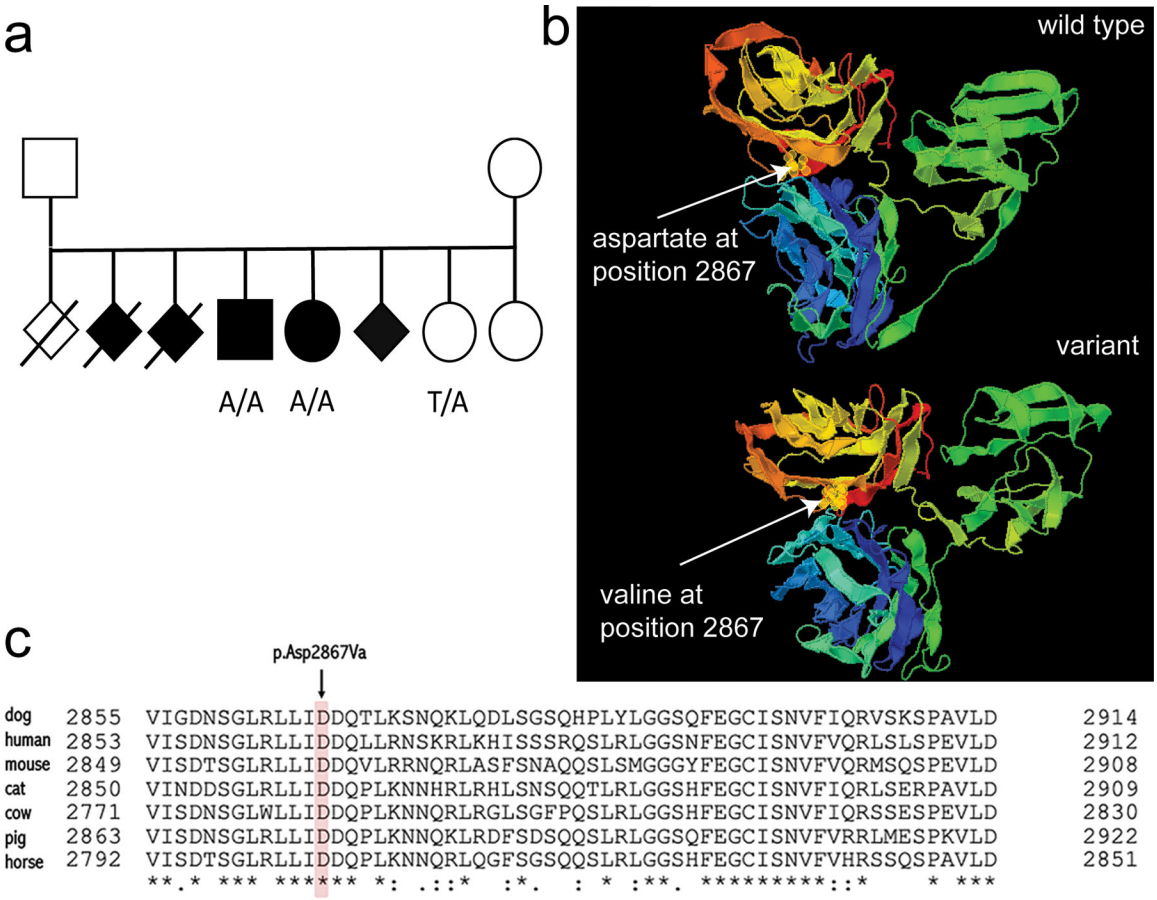


**Figure 1. Clinical presentation of junctional epidermolysis bullosa-affected Australian cattle dog cross-bred puppies.**

(a) Both puppies lost nearly all their claws. Mucocutaneous fragility resulted in deep erosions and ulcers, especially in areas of pressure and friction such as (b) footpads, (c) pinnae, (d) inguinal region, (e) oral cavity and (f) genitalia. (d, e) Repeated cycles of blistering and attempted healing resulted in scarring, (d) milia and (e) adhesions such as the depicted tongue rolling.

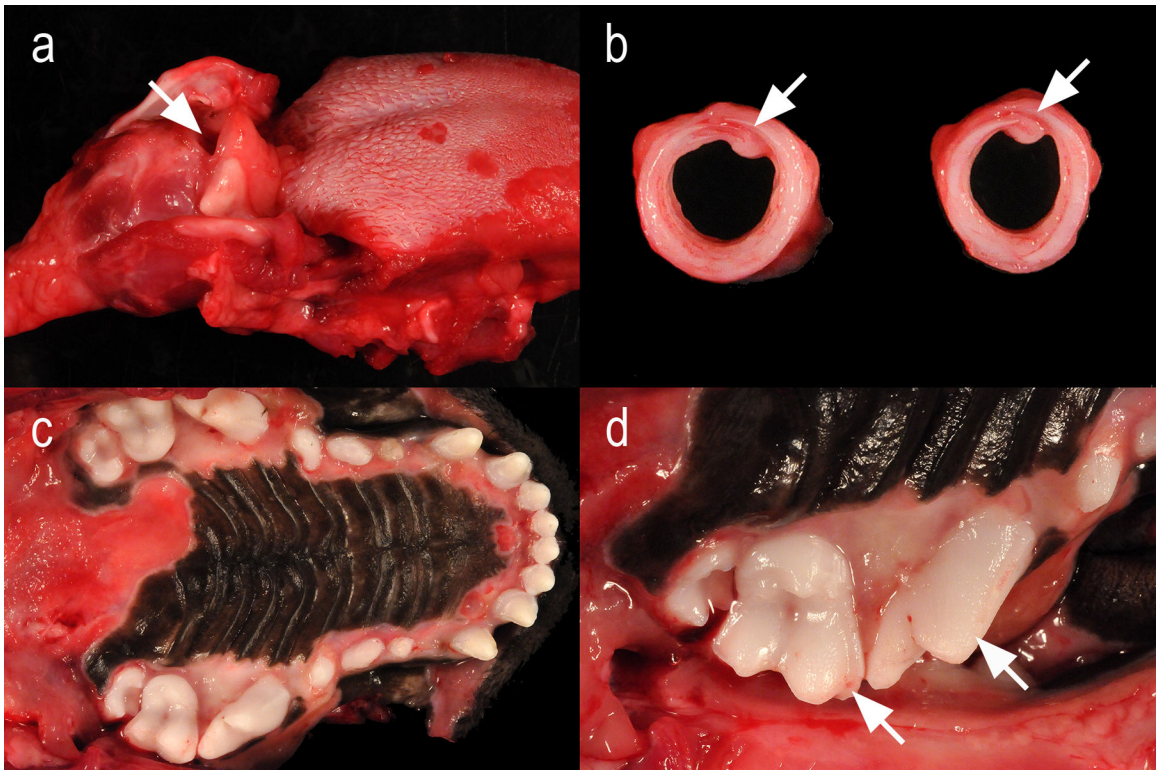


**Figure 2. Post-mortem findings in a female Australian cattle dog cross-bred puppy with junctional epidermolysis bullosa.**

(a) Ulcers are multifocal to regional on the tongue and larynx. Granulation tissue and fibrin exudate obscure the epiglottis and occlude nearly 70% of the glottis (arrow).

(b) Tracheal lumen is moderately narrowed (hypoplastic trachea) with overlapping ends of cartilage rings (arrows) and in-folding of the dorsal tracheal mucosa.

(c) Ulcers are multifocal to regional on the gingiva bordering the teeth and on the hard and soft palate.

(d) Enamel hypoplasia is extensive on the teeth and prominent on the molars (arrows).

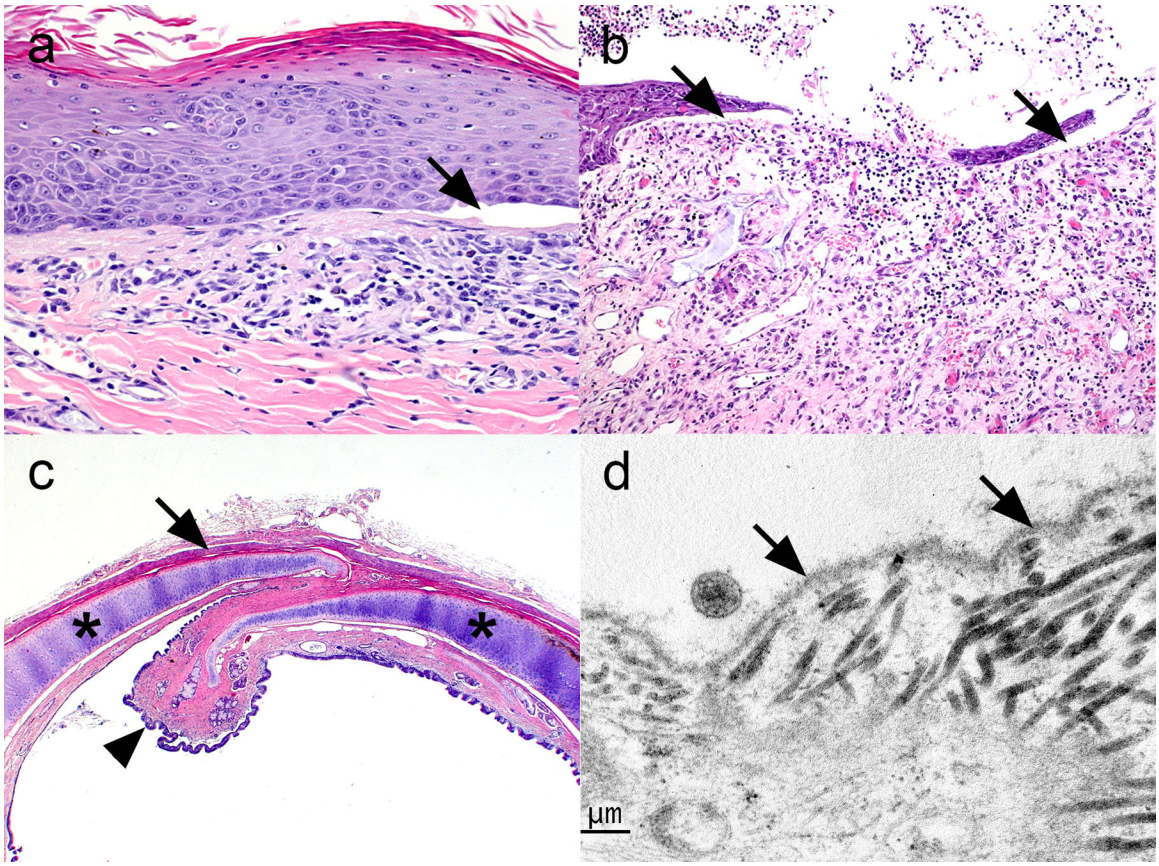


**Figure 3. Histopathological and transmission electron microscopy (TEM) findings in a female Australian cattle dog cross-bred puppy with junctional epidermolysis bullosa.**

(a) Ear, haired skin; a subepidermal cleft (arrow) is present with mild-to-moderate granulation tissue at its base. Haematoxylin & eosin, ×20.

(b) Larynx; subepidermal clefts (arrows) are adjacent to ulcers and prominent granulation tissue. H&E, ×10.

(c) Trachea; the dorsal portion of a cross-section of the narrowed trachea demonstrates a collapsed cartilage ring (asterisks) with overlapping cartilage ends, folded mucosa (arrowhead), and a thin elongated dorsal tracheal ligament (arrow). H&E, ×1.25.

(d) Lip mucosa; TEM of the base of a cleft reveals a retained lamina densa (arrows) and intact anchoring fibrils, which confirms clefting through the lamina lucida. ×30,000.

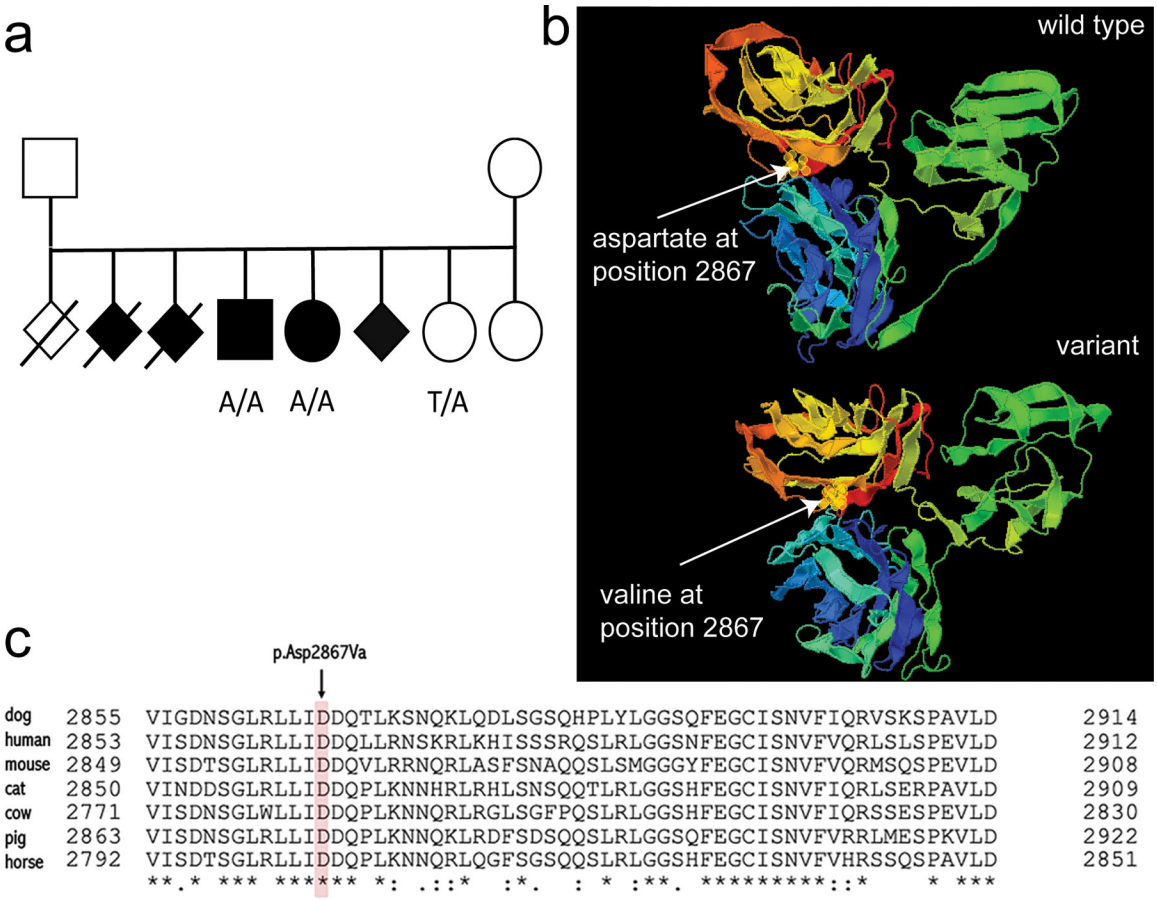


**Figure 4. Pedigree analysis of five Australian cattle dog cross-bred puppies affected with signs of skin fragility from an asymptomatic dam and unknown sire, Laminin α3 (LAMA3) laminin globular (LG) 3 (LG3) domain alignment and predicted effect of the genetic variant on the protein structure.**

(a) Pedigree of the affected puppies. The sire is unknown and, therefore, genetic relatedness between dam and sire cannot be excluded. Squares, males; circles, females; diamonds, unknown sex. Affected individuals are indicated by the solid colour filled symbols and genotypes of the two tested puppies indicate the homozygous variant XM_537297.6c:8615T>A in *laminin α3* (*LAMA3*). Three puppies were stillborn and revival attempt caused skin fragility in two of the three.

(b) Modelling of the wild-type (WT) and the variant laminin α3-chain LG domains 1–3. The predicted change of XM_537297.6c:8615T>A is computationally modelled and shows tertiary structure change. Position 2867 contains the amino acid aspartate in the WT protein and valine in the variant. The variant position is located within the LG3 close to the C-terminus, affecting the structure of the LG1–3 tandem. The N-terminus is shown in dark blue and the C-terminus is red.

(c) Multiple-species alignment of the LG3 amino acid sequence. Highlighted in red, the aspartate at position 2867 is located in a highly conserved region in multiple species. [UniProt Website, Entry E2RPP1; Available online: https://www.uniprot.org/uniprot/E2RPP1 (accessed on 10 December 2020).]