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Genetic investigation of inherited skin diseases in cats and dogs

PhD Thesis submitted by

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Abstract

The skin is the largest organ in the body and performs many vital functions. Its structure and integrity can be compromised by various external and internal factors. One such disruption can result from the consequences of deleterious genetic variants, leading to skin disease. In humans, over 1,500 monogenic diseases with skin involvement have been described. In veterinary medicine, skin problems are one of the most common reasons for a visit in a veterinary practice and genetic factors are included in the wide range of causes of skin diseases. The number of known heritable skin diseases in cats and dogs is significantly lower compared to humans, however, it is rapidly increasing.

Within my PhD thesis I investigated 16 inherited skin diseases in cats and dogs with the aim of identifying the underlying genetic defects. According to their clinical manifestation, these can be grouped into distinct disease categories. My research includes skin diseases classified as cornification disorders, blistering disorders, connective tissue disorders, and skin appendage disorders. In the group of cornification disorders, there were four new breed-specific types of ichthyoses caused by variants in *ABHD5* (Golden Retriever), *KRT1* (Shar Pei), *KRT10* (Chihuahua), and *SDR9C7* (Chihuahua). Moreover, footpad hyperkeratosis in a Rottweiler caused by a variant in *DSG1*, and Darier disease in a Shih Tzu caused by a variant in *ATP2A2* were included among the studied cornification disorders. Several forms of epidermolysis bullosa were investigated belonging to the group of blistering disorders: epidermolysis bullosa simplex in a Welsh Corgi (Cardigan) caused by a variant in *KRT5*, dystrophic epidermolysis bullosa in neonatal Basset Hounds caused by a variant in *COL7A1*, and junctional epidermolysis bullosa in two unrelated domestic shorthair cats and in a litter of Australian Shepherds caused by variants in *COL17A1* and *LAMB3*, respectively. I investigated various forms of Ehlers-Danlos syndrome within the group of connective tissue disorders. In a family of domestic shorthair cats, dermatosparaxis Ehlers-Danlos syndrome was caused by a variant in *ADAMTS2*. Classical Ehlers-Danlos syndrome in a Chihuahua was caused by a variant in *COL5A2*. The same form of the syndrome was diagnosed in a Bengal, a domestic shorthair cat, and two sibling Bombays, all resulting from three independent variants in *COL5A1*. Two of the studied skin disorders affected skin appendages, namely hair shaft dysplasia in two unrelated domestic shorthair cats caused by individual variants in *DSG4*, and sebaceous gland dysplasia in two domestic shorthair kittens caused by a variant in *SOAT1*. And lastly, I investigated acrodermatitis enteropathica, a miscellaneous disorder in a litter of Turkish Vans. The zinc deficiency disorder manifested with severe signs of skin disease and was caused by a variant in *SLC39A4*.

The results from this PhD thesis highlight the potential of next-generation sequencing technologies to identify underlying pathogenic variants in inherited skin diseases in cats and dogs. Knowledge about the disease-causing gene and understanding of the underlying pathomechanisms often resulted in a definitive diagnosis for the patient, which in turn could lead to a more accurate prognosis, thoughtful breeding recommendations, better management of the disease, and guidance for targeted therapy. Such a precision medicine approach in veterinary medicine is not only beneficial for the animals but also enhances knowledge of rare skin diseases that can be transferred to human medicine, demonstrating the value of a One Health approach.

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Introduction

Rare diseases are a global health priority. Although, as the name implies, the prevalence of each rare disease is low, the total amount of individuals suffering from a rare disease is substantial. Estimates show that approximately 5% of the human population might be affected by a rare disease. Currently, over 6,000 rare diseases are reported, the vast majority having genetic causes [1,2].

In veterinary medicine, diseases or problems affecting the skin are among the most common causes of a visit to the veterinary practice [3]. In both humans and animals, the causes of skin diseases are diverse and range from infections, allergic reactions, environmental factors to genetic alterations. Inherited skin diseases, termed genodermatoses, fall under the umbrella of rare diseases [3,4]. The majority of genetic skin diseases are caused by a single gene defect and can be grouped into different categories [5,6]. In the following sections, I will describe selected groups of genodermatoses and their characteristics in more detail after a brief introduction about the physiological functions of mammalian skin.

Skin function and structure

The skin is the largest organ in the body and performs many vital functions. Most importantly, it acts as a mechanical barrier between the organism and the environment by protecting against physical, chemical, and biological damage, as well as preventing trans-epidermal water loss. Beyond its protective role, the skin helps regulate body temperature, plays a role in immune defense, and serves as an important sensory organ [7,8]. Structurally, the skin consists of three layers – the epidermis on the surface, the dermis in the middle, and the deepest layer called hypodermis or subcutaneous tissue – and the adnexa, which comprise hair follicles and various glands (Figure 1) [9].

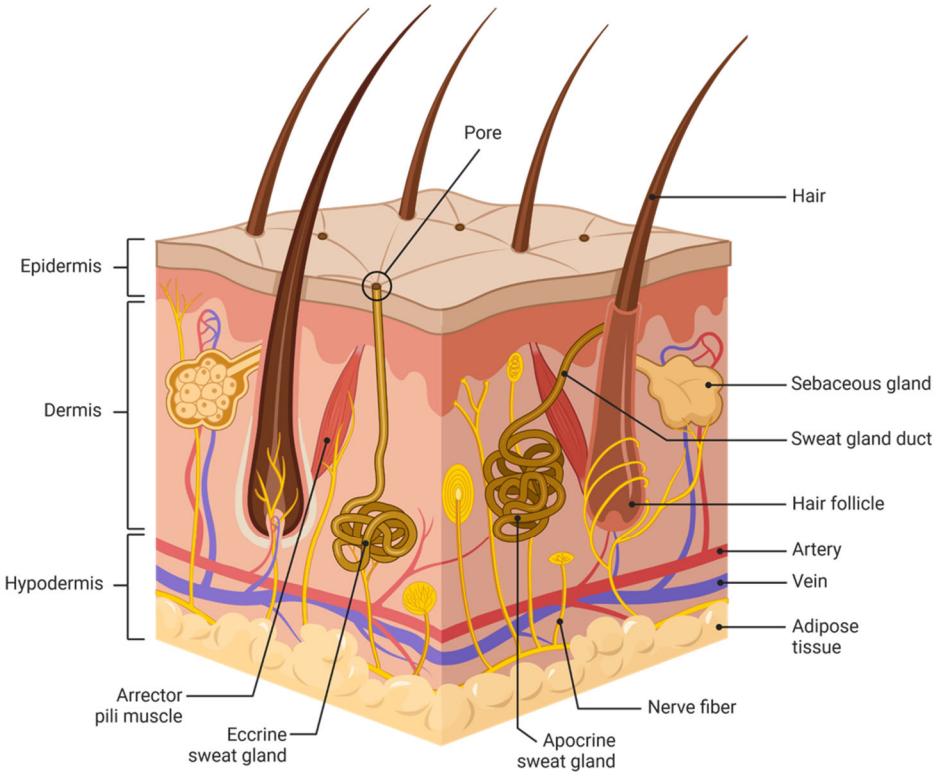

Figure 1. Schematic representation of human skin. The three different layers of the skin, as well as structures belonging to the skin appendages, are indicated. Adapted from “Anatomy of the Skin” by BioRender (2023) [10].

Epidermis

The outermost layer of the skin is the epidermis, a stratified squamous epithelium. Keratinocytes are the main cell type of the epidermis. Alongside them, there are pigment-producing melanocytes, antigen-presenting Langerhans cells, and Merkel cells that are specifically involved in tactile sensitivity. The epidermis is structurally organized into distinct

layers, according to keratinocyte morphology and position. From bottom to top, these include the basal layer (stratum basale), the spinous layer (stratum spinosum), the granular layer (stratum granulosum), and the cornified layer (stratum corneum) (Figure 2). In areas of thick skin, such as the palms and soles, the clear layer (stratum lucidum) is additionally located between the granular and the cornified layer.

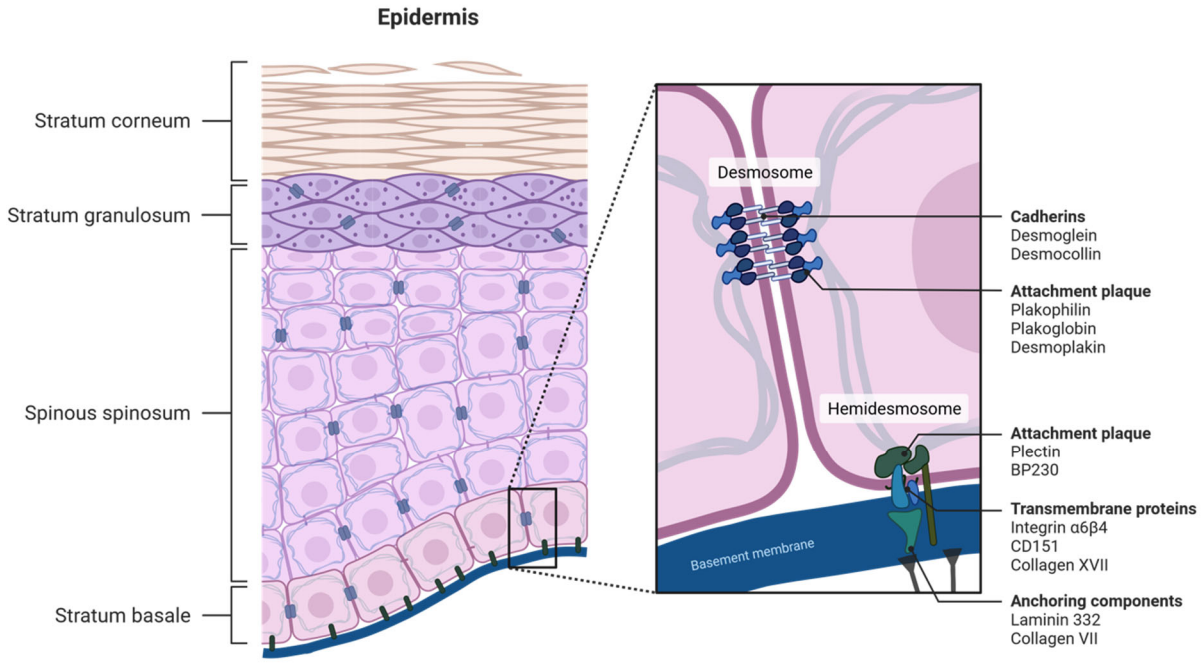

Figure 2. Schematic representation of the epidermis and its distinct layers on the left. The picture on the right provides a detailed view of a desmosome, one of the most important epidermal intercellular junctions, as well as the basement membrane zone with a hemidesmosome, which anchors the epidermis to the underlying dermis. Adapted from “Desmosomal Protein Distribution in the Epidermis” by BioRender (2023) [10].

The basal layer is composed of proliferating keratinocytes. As their progeny move upwards through the epidermal layers, they terminate proliferation and start the differentiation process. On top of the basal layer is the spinous layer. The name derives from the cell connectors between the keratinocytes, giving the cells a spiny appearance. The granular layer is characterized by keratinocytes containing intracellular keratohyalin granules that are involved in the cornification process. The cornified layer is the most superficial layer of the epidermis. It is composed of flat, terminally differentiated keratinocytes, so-called corneocytes, that have lost their nuclei and cell organelles. The outermost cells are eventually shed into the environment through a process called desquamation. The epidermis is a self-renewing tissue with a perfect balance between proliferation, differentiation, and desquamation [7–9]. Intercellular junctions play a crucial role in ensuring epidermal integrity and facilitating intercellular communication, with desmosomes being central for mechanical strength. These

macromolecular complexes anchor keratin filaments to the cell membrane and connect adjacent keratinocytes, enabling the cells to resist trauma. The basement membrane zone acts as the interface between the epidermis and the underlying dermis. In this region, the hemidesmosomes are the key structures that anchor the two skin layers together (Figure 2) [8,9,11].

Dermis

The dermis, situated between the epidermis and the subcutaneous tissue, is a fibrous connective tissue that is rich in extracellular matrix components. It is divided into two layers: the superficial papillary dermis that interfaces with the epidermis, and the more profound reticular dermis. Collagen is the main component of the dermis and provides tensile strength, while elastic fibers give the skin its elasticity and resilience. Non-collagenous glycoproteins help maintain skin structure and facilitate cell-matrix interactions, and proteoglycan and glycosaminoglycan molecules provide the skin with hydration. The dermis also contains a variety of cells including fibroblasts (responsible for extracellular matrix production), lymphocytes and mast cells, endothelial cells, and nerve cells [8,9,12].

Hypodermis

The hypodermis, also known as the subcutaneous tissue or subcutis, is the deepest layer of the skin. It primarily consists of adipose tissue and has important functions such as insulation, energy storage, and cushioning against mechanical trauma. This skin layer also contains collagenous tissue and blood vessels. The presence and thickness of the hypodermis varies based on the specific region of the body [8,9].

Appendages

The skin appendages include eccrine and apocrine sweat glands, hair follicles, and sebaceous glands. They are ectodermally derived structures contributing to the overall function of the skin. They originate from epidermal buds, which grow down into the dermis during embryonic development. In some species, eccrine sweat glands contribute to body temperature maintenance, with a secretory coil deep in the dermis and the intraepidermal duct excreting the sweat onto the skin surface. Apocrine sweat glands are primarily involved in scent release and their duct opens into the hair follicles [9]. Hair follicles vary considerably in size and shape from species to species and location on the body. The process of hair growth in these follicles occurs in a cyclical manner involving phases of growth (anagen), regression (catagen), and rest (telogen). Each follicle functions as an independent unit, undergoing self-renewal during

each hair cycle to continuously produce new hair [9,13]. Sebaceous glands are typically associated with hair follicles and secret sebum, a waxy substance with the major function to lubricate the hair and skin, making them impermeable to water [14].

Genodermatoses

Monogenic skin diseases are termed genodermatoses, derived from the Greek words ‘genos’ for origin, and ‘derma’ referring to skin. This heterogeneous group includes diseases that are either strictly limited to the skin or include systemic features. The latter have been reported to account for the majority of genodermatoses [5,15]. The clinical presentation of genodermatoses is extremely diverse. Diseases are typically grouped according to their clinical manifestations; however, it is worth noting that the severity and presentation of clinical signs can be remarkably variable not only across these groups but also within them. Additionally, the clinical signs presented in the different conditions can overlap [6,16].

It has been shown that numerous human genetic diseases, including genodermatoses, have homologous counterparts in animals [17,18]. In humans, currently over 1,500 Mendelian disorders with skin involvement are known [15,19]. In cats and dogs, fewer heritable skin diseases have a defined genetic cause, however, the number is rapidly increasing [18,20].

The categories of genodermatoses described below represent a selection, which is based on the phenotypes studied during my PhD. Thus, it does not include a complete listing of the defined groups.

Cornification disorders

Cornification is a highly complex process by which the epidermal cells undergo terminal differentiation from basal keratinocytes to highly specialized corneocytes [21]. This results in the formation of the skin barrier, protecting the body from environmental harm and preventing trans-epidermal water loss [22,23].

Many genes are involved in this finely orchestrated process and an alteration in any step can lead to disease through disturbed cornification and subsequent disruption of barrier function [21]. The variety of involved genes is also reflected in the heterogenous clinical presentation of resulting disorders of cornification (DOC). However, they all have some common clinical features including dry, hyperkeratotic skin, and scaling. These result from the reaction of the epidermis and its attempt to repair the disrupted barrier by increased proliferation of basal cells [22,24].

DOC are often grouped based on their clinical manifestations and specific aspects of cornification that are disrupted. One large heterogenous group is ichthyosis with generalized scaly and hyperkeratotic skin that includes both non-syndromic and syndromic forms. In non-syndromic forms, the phenotypic manifestation of the disease is limited to the skin whereas

syndromic ichthyoses additionally involve other organs. Ichthyoses are histologically further categorized as non-epidermolytic or epidermolytic, the latter characterized by vacuoles and lysis of keratinocytes [21]. In humans, at least 69 different genes have been described in different forms of ichthyosis [25,26]. Another group of DOC is palmoplantar keratoderma, caused by defective genes specifically expressed in palms and soles. It must be mentioned that these groups are not exclusive, as palmoplantar keratoderma can exist as an isolated disorder but also in various forms of ichthyosis [6,27]. Additional groups of DOC include Darier disease and Hailey-Hailey disease [24].

Various canine and feline DOC are known. Usually, DOC are congenital, however, onset at a later age, as in hereditary footpad hyperkeratosis in the Kromfohrländer and Irish Terrier, has been observed [22,28,29]. The spectrum of clinical signs for DOC is broad, from the relatively mild scaling seen in *PNPLA1*-associated Golden Retriever ichthyosis to the severe form of *SLC27A4*-associated syndromic ichthyosis in Great Danes, which resulted in euthanasia of the affected puppies [30,31]. Ichthyosiform disorders have strong breed predilections; however, spontaneous mutations can arise in any breed or mixed-breed animals (Table 1) [21].

Table 1. Selected cornification disorders in cats and dogs for which the genes harboring candidate variants have been identified.

Phenotype	**Species**	**Breed**	**Associated gene**	**MOI**[1]	**OMIA#** [20]
Ichthyosis	Dog	German Shepherd Dog	*ASPRV1*	AD	002099
	Dog	American Bulldog American Bully	*NIPAL4*	AR	001980
	Dog	Golden Retriever	*PNPLA1*	AR	001588
	Dog	Great Dane	*SLC27A4*	AR	001973
	Dog	Jack Russell Terrier	*TGM1*	AR	000546
Epidermolytic hyperkeratosis	Dog	Norfolk Terrier	*KRT10*	AR	001415
Darier disease	Dog	Irish Terrier	*ATP2A2*	AD	002265
Verrucous epidermal keratinocytic nevi	Cat Dog	Domestic shorthair cat Chihuahua Chihuahua cross Labrador Retriever	*NSDHL*	XSD	002117

Footpad hyperkeratosis	Dog	Irish Terrier Kromfohrländer	*FAM83G*	AR	001327
	Dog	Dogue de Bordeaux	*KRT16*	AR	002088
Congenital keratoconjunctivitis sicca and ichthyosiform dermatosis	Dog	Cavalier King Charles Spaniel	*FAM83H*	AR	001683
Hereditary nasal hyperkeratosis	Dog	Greyhound Labrador Retriever	*SUV39H2*	AR	001373
Ectodermal dysplasia/skin fragility syndrome	Dog	Chesapeake Bay Retriever Golden Retriever	*PKP1*	AR	001864

[1]MOI = mode of inheritance: AD = autosomal dominant, AR = autosomal recessive, XSD = X-linked semi-dominant

Blistering disorders

Blistering disorders are characterized by structural anomalies that reduce skin integrity and its resilience to mechanical stress. The main group of blistering disorders is epidermolysis bullosa (EB) with typical clinical signs of erosions and blisters resulting from dermo-epidermal tissue separation caused by minimal trauma. Additional blistering disorders, including erosive or hyperkeratotic conditions, are categorized under the broader term 'other disorders with skin fragility'. In these, blisters are only a minor part of the clinical picture and are often not seen because skin cleavage is very superficial [32–35].

Four primary types of EB are identified, classified according to the level at which skin cleavage occurs [32]. The first is EB simplex (EBS) with an intraepidermal level of cleavage, resulting from defective intermediate filament components of the basal keratinocytes [36]. Junctional EB (JEB) is the second type with cleavage within the basement membrane zone, caused by defective macromolecules of the hemidesmosomes [11]. The third type is dystrophic EB (DEB) with cleavage in the uppermost part of the dermis, due to a defect in the anchoring fibrils [35,37]. The fourth and last type is Kindler EB (KEB), a rare syndromic disorder with multiple sites of cleavage [32,37].

In humans, pathogenic variants in at least 16 different genes have been associated with the different types of EB and 23 additional genes have been described in other skin fragility disorders [32].

In both cats and dogs, hereditary EB has been known for decades. However, molecular studies are rare, as reflected by the fact that pathogenic variants in only four genes have been

identified so far (Table 2) [38–44]. The clinical presentation of feline and canine EB is similar to that of human patients, with skin blistering and erosions appearing shortly after birth. However, lesions predominantly appear in hairless areas such as the muzzle, oral cavity, or paw pads. It has been hypothesized that hair has a protective effect by acting as a mechanical barrier and anchoring the epidermis to the dermis [38]. Due to the severity of the lesions, challenges in management, and often poor prognosis, many of the affected animals are euthanized or die within the first months of life [38,40,41,44].

Table 2. Selected blistering disorders in cats and dogs for which the genes harboring candidate variants have been identified.

Phenotype	Species	Breed	Associated gene	MOI[1]	OMIA# [20]
Epidermolysis bullosa simplex	Cat	Domestic shorthair cat	*KRT14*	AR	002281
	Dog	Eurasian	*PLEC*	AR	002080
Junctional epidermolysis bullosa	Dog	Australian Cattle Dog cross German Pointer	*LAMA3*	AR	001677
Dystrophic epidermolysis bullosa	Dog	Central Asia Shepherd Dog Golden Retriever	*COL7A1*	AR	000341
Epidermolytic hyperkeratosis	Dog	Norfolk Terrier	*KRT10*	AR	001415
Ectodermal dysplasia/skin fragility syndrome	Dog	Chesapeake Bay Retriever Golden Retriever	*PKP1*	AR	001864

[1]MOI = mode of inheritance: AR = autosomal recessive

Connective tissue disorders

Connective tissue, the most abundant tissue in the body, is divided into three major types: soft connective tissue (embedding organs and organ parts), hard connective tissue (bone and cartilage), and blood. This tissue comprises a variety of cells and fibers set within a matrix of collagen, elastic fibers, glycoproteins, and glycosaminoglycans. Heritable connective tissue disorders result from genetic defects that disturb the assembly and/or homeostasis of this extracellular matrix [45].

Connective tissue disorders can be classified into two major groups – elastinopathies and collagenopathies – based on the primary component of connective tissue involved. Marfan syndrome, pseudoxanthoma elasticum, and cutis laxa syndromes belong to the

elastinopathies, while the most common collagenopathies include Ehlers-Danlos syndrome (EDS), osteogenesis imperfecta, and Alport syndrome [45].

EDS is the largest group among these connective tissue disorders. The extended 2017 EDS classification has described 14 different types of EDS, for 13 of which the molecular cause is known [46]. The diagnosis of an EDS type is based on the combination of clinical signs and complementary molecular analysis. Common clinical features of the heterogeneous EDS group are soft and hyperextensible skin, easy bruising, and joint hypermobility. The molecular basis of many EDS types is linked to defects in genes that code either for fibrillar collagen or for the enzymes that facilitate its posttranslational modification. At least 20 different genes harboring disease-causing variants have been identified in human EDS [46,47].

EDS is also seen in cats and dogs, where it constitutes the majority of hereditary connective tissue diseases with skin involvement (Table 3). The typical hyperextensible and fragile skin is also observed in these species, whereas joint laxity is less prevalent compared to human EDS patients [48].

Table 3. Selected connective tissue disorders in cats and dogs for which the genes harboring candidate variants have been identified.

Phenotype	**Species**	**Breed**	**Associated gene**	**MOI**[1]	**OMIA#** [20]
Dermatosparaxis Ehlers-Danlos syndrome	Dog	Alapaha Blue Blood Bulldog Louisiana Catahoula Leopard Dog Dobermann American Pit Bull Terrier	*ADAMTS2*	AR	000328
Classical Ehlers-Danlos syndrome	Cat Dog	Domestic shorthair cat Labrador Retriever Mixed breed	*COL5A1*	AD	002165
Classic-like Ehlers-Danlos syndrome	Dog	Mixed breed	*TNXB*	AR	002203
Geleophysic dysplasia	Dog	Beagle	*ADAMTSL2*	AR	001509
Wrinkled skin	Dog	Shar Pei	*HAS2*	CPX	001561

[1]MOI = mode of inheritance: AR = autosomal recessive, AD, autosomal dominant, CPX = complex

Skin appendage disorders

Skin appendages are affected in a variety of disorders that differ greatly in their clinical appearance and underlying causes. Some concern specific appendages such as sweat

glands, sebaceous glands, or hair follicles. On the other hand, many developmental disorders like ectodermal dysplasia typically affect two or more appendages [14,49–51]. Accordingly, the clinical manifestations range from purely cosmetic problems in isolated hair disorders to life-threatening conditions in anhidrotic ectodermal dysplasia due to decreased sweating and thus heat intolerance [51,52].

The hair follicle (HF) stands out as one of the most complex epidermal appendages. This micro-organ has self-renewal ability undergoing hair cycles throughout life. Recent advances in molecular genetics have led to the identification of numerous genes expressed in HFs and variants in some of these genes have been shown to underlie hereditary hair diseases in humans [52].

Disorders and variation associated with skin appendages have been reported in both cats and dogs (Table 4). Interestingly, genetic variations associated with the development of hair follicles and other skin appendages can result not only in diseases but also breed-specific traits that are specifically selected by breeders. For example, curly hair in various dog and cat breeds is associated with variants in the *KRT71* gene, while the unique hair morphology of the Cornish Rex breed is due to a specific variant in the *LPAR6* gene [53–58].

Table 4. Selected skin appendage disorders in cats and dogs for which the genes harboring candidate variants have been identified.

Phenotype	**Species**	**Breed**	**Associated gene**	**MOI**[1]	**OMIA#** [20]
Hypotrichosis	Cat	Lykoi	*HR*	AR	002229
Hypotrichosis	Dog	American Hairless Terrier Deerhound	*SGK3*	AR	001279
Hypotrichosis, with thymic aplasia	Cat	Sacred Birman	*FOXN1*	AR	001949
Hypotrichosis, with whiskers short and curled	Cat	Sphynx	*KRT71*	AR	001583
Curly/wolly coat	Cat	Cornish Rex German Rex	*LPAR6*	AR	001684
Curly coat	Dog	many breeds	*KRT71*	AR	000245
	Cat	Devon Rex	*KRT71*	AR	001581
	Cat	Selkik Rex	*KRT71*	AID	001712

Hair, long	Cat	Maine Coon Ragdoll Norwegian Forest Cat	*FGF5*	AR	000439
	Dog	many breeds			
Furnishings (moustache and bushy eyebrows)	Dog	many breeds	*RSPO2*	AD	001531
Anhidrotic ectodermal dysplasia	Dog	German Shepherd Dog Mixed breed Dachshund	*EDA*	XLR	000543
Ectodermal dysplasia	Dog	Chinese Crested Dog Xoloitzcuintli Peruvian Hairless Dog	*FOXI3*	AID	000323
Ectodermal dysplasia/skin fragility syndrome	Dog	Chesapeake Bay Retriever Golden Retriever	*PKP1*	AR	001864

[1]MOI = mode of inheritance: AR = autosomal recessive, AID = autosomal incomplete dominant, AD = autosomal dominant, XLR = X-linked recessive

Aim and hypothesis of the thesis

The general aim of this thesis was to identify new genetic variants causative for inherited skin diseases in cats and dogs. It was hypothesized that the investigated diseases follow a simple Mendelian inheritance pattern with causative variants identifiable through positional cloning methods and whole genome sequencing approaches.

The following disorders were investigated:

Cats:

- Acrodermatitis enteropathica in Turkish Vans
- Hair shaft dystrophy in two unrelated domestic shorthair cats
- Ehlers-Danlos syndrome in a Bengal, a domestic shorthair cat, and two Bombays
- Sebaceous gland dysplasia in domestic shorthair cats
- Ehlers-Danlos syndrome in a domestic shorthair cat family
- Junctional epidermolysis bullosa in two unrelated domestic shorthair cats

Dogs:

- Footpad hyperkeratosis in a Rottweiler
- Junctional epidermolysis bullosa in Australian Shepherds
- Dystrophic epidermolysis bullosa in Basset Hounds
- Ichthyosis in Golden Retrievers
- Ehlers-Danlos syndrome in a Chihuahua
- Epidermolysis bullosa simplex in a Welsh Corgi (Cardigan)
- Non-epidermolytic ichthyosis in a Shar Pei
- Darier disease in a Shih Tzu
- Non-epidermolytic ichthyosis in a Chihuahua
- Epidermolytic ichthyosis in a Chihuahua

Results

A missense variant in *SLC39A4* in a litter of Turkish Van cats with acrodermatitis enteropathica

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Article

A Missense Variant in *SLC39A4* in a Litter of Turkish Van Cats with Acrodermatitis Enteropathica

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Abstract: In a litter of Turkish Van cats, three out of six kittens developed severe signs of skin disease, diarrhea, and systemic signs of stunted growth at 6 weeks of age. Massive secondary infections of the skin lesions evolved. Histopathological examinations showed a mild to moderate hyperplastic epidermis, covered by a thick layer of laminar to compact, mostly parakeratotic keratin. The dermis was infiltrated with moderate amounts of lymphocytes and plasma cells. Due to the severity of the clinical signs, one affected kitten died and the other two had to be euthanized. We sequenced the genome of one affected kitten and compared the data to 54 control genomes. A search for private variants in the two candidate genes for the observed phenotype, *MKLN1* and *SLC39A4*, revealed a single protein-changing variant, *SLC39A4*:c.1057G>C or p.Gly353Arg. The solute carrier family 39 member 4 gene (*SLC39A4*) encodes an intestinal zinc transporter required for the uptake of dietary zinc. The variant is predicted to change a highly conserved glycine residue within the first transmembrane domain, which most likely leads to a loss of function. The genotypes of the index family showed the expected co-segregation with the phenotype and the mutant allele was absent from 173 unrelated control cats. Together with the knowledge on the effects of *SLC39A4* variants in other species, these data suggest *SLC39A4*:c.1057G>C as candidate causative genetic variant for the phenotype in the investigated kittens. In line with the human phenotype, we propose to designate this disease acrodermatitis enteropathica (AE).

Keywords: *Felis catus*; whole genome sequencing; dermatology; genodermatosis; zinc

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1. Introduction

Lethal acrodermatitis (LAD) is a monogenic autosomal recessive disease in Bull Terriers and Miniature Bull Terriers (OMIA 002146-9615). It is characterized by skin lesions on the feet and face, diarrhea, bronchopneumonia, and a failure to thrive [1–3]. Some studies also observed a decreased plasma zinc level in LAD-affected dogs [2,4]. Oral or parenteral supplementation of zinc, however, did not improve the clinical signs of these dogs [1]. LAD in dogs is caused by a splice defect in the *MKLN1* gene [5]. This gene encodes the muskelin 1 protein, which is, intracellularly, widely expressed and is discussed to be involved in several functions, including cell adhesion, morphology, spreading, and intracellular transport processes [6–16]. To date, the exact pathogenesis of LAD has not been elucidated [5].

Acrodermatitis enteropathica (AE) is a related phenotype, which is also inherited as an autosomal recessive trait. It has been described in humans (OMIM # 201100) and cattle (OMIA 000593-9913) [17,18]. The bovine disease was termed lethal trait A46, bovine

hereditary zinc deficiency or Adema disease. Clinical findings were rapidly associated with low plasma zinc levels [18–22].

Zinc ions (Zn^{2+}) are essential for many biological processes. More than 300 enzymes need zinc as an essential co-factor, and approximately 2800 human proteins are potentially zinc-binding. In these metalloproteins, the binding of zinc is needed either because the metal ion is involved in the catalytic mechanism, or because it stabilizes the protein tertiary or quaternary structure [23,24]. In zinc-deficient individuals, the clinical manifestations are correspondingly diverse.

AE in humans and cattle is caused by variants in the *SCL39A4* gene, encoding the solute carrier family 39 member 4, an intestinal zinc transporter that has also been termed ZIP4 [25–28]. The loss of function of this transporter leads to a severe systemic zinc deficiency. Untreated, the disease ends fatally. However, as there is another, non-saturable transport pathway besides the high-affinity SLC39A4 transporter, the zinc deficit in AE patients can be successfully treated with sufficient supplementation of oral zinc [18,19,21,28–34].

In the present study, we investigated a litter of Turkish Van cats with skin lesions and additional clinical signs of zinc deficiency. The goal of the study was to characterize the clinical and histopathological phenotype and to identify the underlying causative genetic variant.

2. Materials and Methods

2.1. Animal Selection

This study included a Turkish Van cat family with 12 animals and 173 genetically diverse control cats from other breeds. Genomic DNA was isolated from ETDA blood samples with the Maxwell RSC Whole Blood Kit using a Maxwell RSC instrument (Promega, Dübendorf, Switzerland).

2.2. Histopathological Examinations

Skin biopsies (6 mm punch and wedge incision) were taken under general anesthesia from Case 1 and 2 at the time of presentation at 10 weeks of age, when Case 1 was euthanized. Further biopsies were taken from Case 3 at the time of euthanasia, two weeks after the initial presentation, at 12 weeks of age. Areas biopsied included the paw pads, claw folds, axilla, inguinal region, and concave pinnae. From the euthanized kittens, tissue samples were also taken from muco-cutaneous junctions, the thoracic esophagus, stomach, and small intestine. The samples were fixed in 10% neutral buffered formalin and routinely processed including staining with hematoxylin and eosin (HE) and periodic acid Schiff (PAS).

2.3. Whole Genome Sequencing

An Illumina TruSeq PCR-free DNA library with ~400 bp insert size of an AE affected Turkish Van cat was prepared. We collected 188 million 2 × 150 bp paired-end reads or 20.7× coverage on a NovaSeq 6000 instrument. The reads were mapped to the FelCat9.0 cat reference genome assembly and aligned as described [35]. The sequence data were submitted to the European Nucleotide Archive with the study accession PRJEB7401 and sample accession SAMEA8609184.

2.4. Variant Calling

Variant calling was performed as described [35]. The SnpEff software was used to predict the functional effects of the called variants [36] together with NCBI annotation release 104 for the FelCat9.0 genome reference assembly. For variant filtering, we used 54 control genomes (Supplementary Table S1). The control genomes were derived from 29 purebred cats of 8 different breeds and 25 random-bred domestic cats.

2.5. Gene Analysis

Numbering within the feline *SLC39A4* gene corresponds to the NCBI RefSeq accession numbers XM_004000173.3 (mRNA) and XP_004000222.2 (protein). We performed a multiple species comparison of orthologous SLC39A4 amino acid sequences with the accessions NP_570901.2 (*Homo sapiens*), XP_001157597.3 (*Pan troglodytes*), XP_001098635.2 (*Macaca mulatta*), XP_005628372.1 (*Canis lupus familiaris*), NP_001039532.1 (*Bos taurus*), NP_082340.1 (*Mus musculus*), and NP_001071137.1 (*Rattus norvegicus*). Protein alignments were either directly taken from the precomputed NCBI HomoloGene database [37] or performed with blastp on the NCBI BLAST server [38].

2.6. Sanger Sequencing

Sanger sequencing of PCR amplicons was used to confirm the candidate variant *SCL39A4*:c.1057G>C and to genotype cats. With the primers 5′-GGA TGG GGC TTT AAG GGT TA-3′ (Primer F) and 5′-GCT GAC CTT GGG TGT CAA GT-3′ (Primer R) a 334 bp PCR product was amplified from genomic DNA using AmpliTaqGold360Mastermix (Thermo Fisher Scientific, Waltham, MA, USA). After treatment with shrimp alkaline phosphatase and exonuclease I, PCR amplicons were sequenced on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific). The Sequencher 5.1 software was used to analyze the Sanger sequences (GeneCodes, Ann Arbor, MI, USA).

3. Results

3.1. Family Anamnesis, Clinical Examinations and Histopathology

A 10 week-old Turkish Van litter was referred due to a 4 week history of rapidly progressing, severe dermatological and systemic disease in three out of six kittens. The phenotype distribution was suggestive of a monogenic autosomal recessive mode of inheritance (Figure 1).

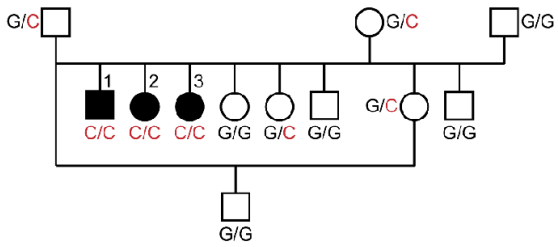

Figure 1. Pedigree of the investigated Turkish Van family. Squares represent males and circles females. The three affected kittens are numbered and indicated by filled symbols. Genotypes at the *SCL39A4*:c.1057G>C variant are indicated.

All kittens had a normal development and growth until 6 weeks of age. The mother was healthy. Starting from 6–8 weeks of age, the three affected kittens displayed similar systemic and dermatological signs of varying severity. The affected cats showed growth retardation and developed diarrhea. Dermatological signs included widespread and severe scaling, alopecia, and moist dermatitis as well as numerous erosions and focal ulcerations. Lesions had a predominantly ventral and distal distribution, most severely in friction areas but also around mucocutaneous junctions, concave pinnae and paw pads (Figure 2). Furthermore, there was a widespread and severe secondary pyoderma. Interdigital crusts and erosions were present in clawfolds, but the claws were normal. Several paw pads had splitting lesions in a horizontal direction across the complete pad and erosions or ulcerations in the junction between paw pad and skin (Figure 2d). There were no visible oral lesions and teeth were normal. Systemic signs included anorexia, fever, dehydration, decreased body weight (on average 40% less than unaffected siblings). Thoracic auscultation, abdominal and lymph node palpation were all unremarkable. Prior to referral, the affected kittens had tested negative for Demodex gatoi and for dermatophytosis via a

PCR test. Clinical signs had not improved upon treatment with systemic amoxicillin and prednisolone for 5 days. Complete blood counts were unremarkable.

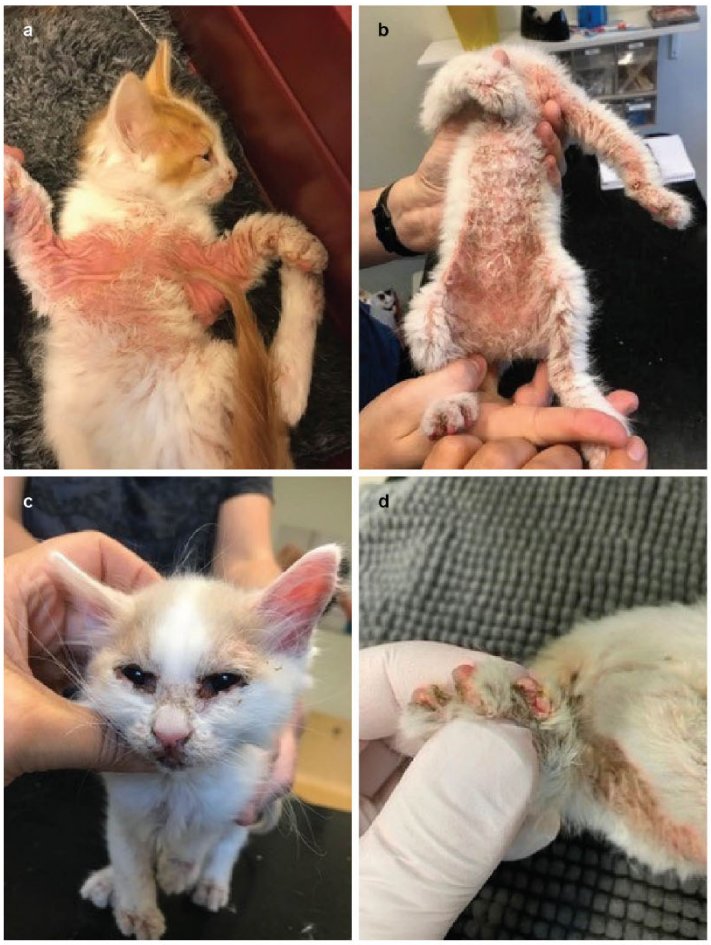

Figure 2. Clinical phenotype of cats affected with AE. (**a**) Skin disease with erythema and beginning alopecia on the ventrum. (**b**) More severe presentation with erythema, crusting, scaling, and alopecia on the ventral and distal aspects. (**c**) Characteristic skin lesions in the face and the ear pinnae. (**d**) Interdigital erosions.

The histological findings were similar in all samples from the three kittens. The epidermis was mildly to moderately hyperplastic, and the granular cell layer was thin to absent. Few apoptotic cells were present in the superficial epidermis in some samples. The epidermis was diffusely covered by variable amounts of laminar to compact, parakeratotic keratin, characterized by retained nuclei in the stratum corneum. In between the corneocytes, numerous cocci and/or yeasts were present. Multifocally, the biopsies were covered by large serocellular crusts, composed of corneocytes, proteinaceous material, degenerate inflammatory cells, and abundant cocci. In the epidermis, underneath some of the crusts, spongiosis and leukocyte exocytosis was present. In the superficial dermis, a mild perivascular infiltrate composed of mast cells, few lymphocytes and rare plasma cells was noted. In the biopsies of one kitten, there were also some eosinophils. Multifocally, the epidermis was ulcerated and there were abundant inflammatory cells underneath the ulcer (Figure 3). Findings in non-haired skin from the paw pads were similar (Figure 3b).

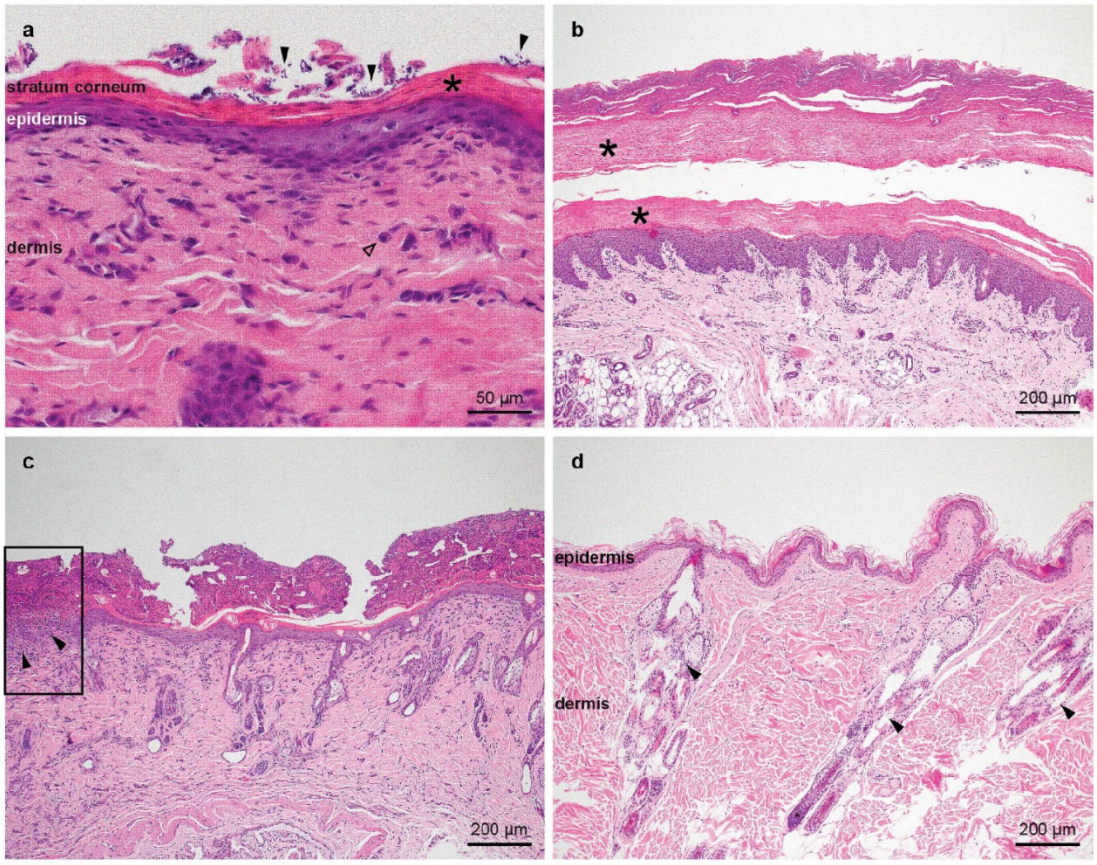

Figure 3. Histopathological images of representative skin biopsies, hematoxillin and eosin stain. (**a**) The epidermis is mildly hyperplastic and covered by compact parakeratotic keratin, characterized by the presence of retained nuclei in the stratum corneum (asterisk). There are numerous yeasts in between the corneocytes, indicated by the filled arrows. There is a mild perivascular infiltrate in the superficial dermis composed mostly of mast cells (empty arrow). (**b**) The epidermis of the paw pad is mildly hyperplastic and covered by a thick layer of compact parakeratotic keratin (asterisks). (**c**) The epidermis is mildly hyperplastic and covered by a thin layer of compact parakeratotic keratin. The stratum corneum is covered by a thick serocellular crust, composed of corneocytes, proteinaceous material, degenerate inflammatory cells, and abundant cocci. In the rectangle on the left edge of the image, an ulcer is present, characterized by the lack of the epidermis and abundant inflammatory cells in the superficial dermis underneath the ulcer (filled arrows). (**d**) Skin of a healthy cat as control showing the epidermis of normal thickness covered by basketweave orthokeratotic keratin together with the dermis and the adnexa (filled arrows).

Two affected kittens were euthanized due to rapidly progressing and severe generalized disease at 10 (Case 1) and 12 (Case 3) weeks of age. The third affected kitten, Case 2, appeared relatively bright, playful and gained weight, until 12 weeks of age, when it suddenly and rapidly deteriorated and died before it could be euthanized. The three unaffected siblings have continued to grow and develop into healthy adult individuals.

3.2. Genetic Analysis

In order to characterize the underlying causative genetic variant, we sequenced the genome of Case 2 at 20.7× coverage and searched for homozygous variants in *MKLN1* and *SLC39A4*, the two candidate genes for the observed phenotype. We filtered for private

variants, which were exclusively present in the affected cat and absent from the genomes of 54 other cats (Table 1 and Supplementary Tables S1 and S2).

Table 1. Results of variant filtering in the affected Turkish Van cat against 54 control genomes. Only homozygous variants are reported.

Filtering Step	Variants
all variants in the affected cat	5,518,410
private variants	56,054
protein-changing private variants	161
protein-changing private variants in *MKLN1* or *SLC39A4*	1

This analysis identified a single homozygous private protein-changing candidate variant in *SLC39A4*. The variant can be designated as ChrF2:85,320,523C>G (FelCat9.0). It was a missense variant, XM_004000173.3:c.1057G>C, predicted to change a highly conserved glycine residue in the first transmembrane domain of the zinc transporter, XP_004000222.2:p.(Gly353Arg).

We confirmed the presence of the *SLC39A4* missense variant by Sanger sequencing (Figure 4). Genotypes at the variant perfectly co-segregated with the phenotype in the available family members. All three affected cats were homozygous. The parents and two close relatives carried the mutant allele in a heterozygous state. All other non-affected cats were homozygous for the wildtype allele (Figure 1).

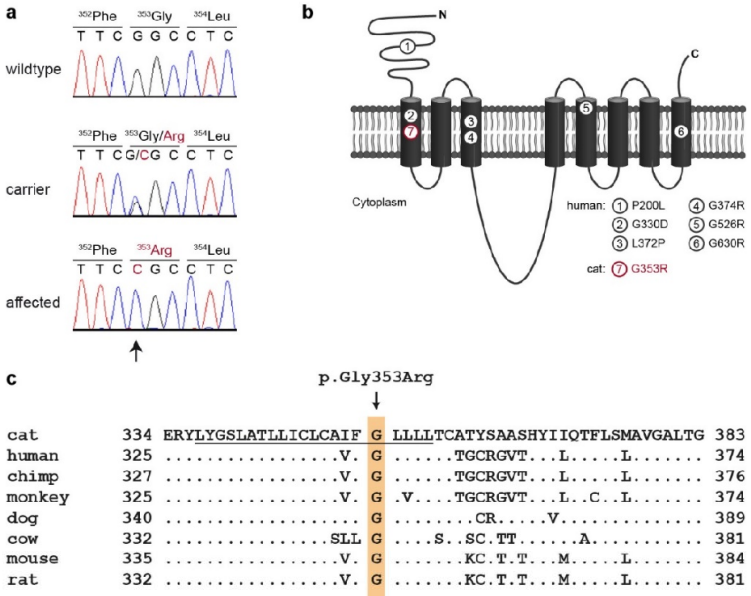

Figure 4. Details of the *SLC39A4*:c.1057G>C, p.Gly353Arg variant. (**a**) Representative electropherograms of three cats with different genotypes are shown. The variable position is indicated by an arrow and the amino acid translations are shown. (**b**) Model of SLC39A4 membrane topology, modified from [39]. The positions of six functionally characterized missense variants identified in human AE patients are indicated. The position of the feline p.Gly353Arg variant identified in the investigated cat is indicated in red. (**c**) Multiple-species alignment of the SLC39A4 amino acid sequences in the region harboring the p.Gly353Arg variant. The variant affects a highly conserved glycine residue. The sequence of the first transmembrane domain is underlined.

We determined the genotypes at *SLC39A4*:c.1057G>C for the index family as well as 173 other cats. The mutant allele was exclusively detected in the Turkish Van family and not in cats from other breeds. All non-affected cats were either heterozygous or homozygous for the wildtype allele (Table 2).

Table 2. Genotype–phenotype association of the *SCL39A4*:c.1057G>C variant with AE.

Cats	G/G	G/C	C/C
Cases ($n = 3$)	-	-	3
Controls, Turkish Van cats from index family ($n = 9$)	5	4	-
Controls, other breeds ($n = 173$)	173	-	-

4. Discussion

The characteristic clinical signs and the early age of onset in the three affected cats described in this study strongly suggested the presence of an inherited disease. The clinical phenotype mostly resembled that of lethal acrodermatosis (LAD), previously described in Bull Terriers and Miniature Bull Terriers, and acrodermatitis enteropathica (AE), which is known in humans and was also described in cattle under the name lethal trait A46, bovine hereditary zinc deficiency, or Adema disease [1–3,17,18]. The histopathological findings are unique and have not been reported in cats before. They strikingly resembled the findings seen in lethal acrodermatitis in Bull terriers and miniature Bull terriers [1–5] or other disorders associated with impaired zinc absorption, such as canine zinc responsive dermatosis [40,41] or bovine hereditary zinc deficiency [17,18]. Another disorder with diffuse parakeratosis described in dogs and very rarely in cats is superficial necrolytic dermatosis; however, this disorder is associated with either hepatic disease or hepatic or pancreatic neoplasia and seen in much older individuals. Thallium toxicosis also presents with severe parakeratotic hyperkeratosis but is very unlikely in such young kittens and is nowadays rare, since thallium was banned as rodenticide [40,41]. A last differential would have been a generalized form of non-epidermolytic ichthyosis with secondary infection. However, in generalized ichthyosis, the stratum corneum is usually orthokeratotic [40,41].

The genome of one affected cat was sequenced and filtered for private variants in the two candidate genes for both differential diagnoses. The analysis revealed a private missense variant in the *SLC39A4* gene encoding the solute carrier family 39 member 4, a zinc transporter with eight transmembrane domains, which is responsible for the saturable zinc uptake in the small intestine [29,39]. Loss of function of SLC39A4 leads to a severe zinc deficiency, which ends fatally if untreated. The fact that there is another, unsaturable intestinal zinc transport pathway enables the treatment of the deficiency with oral supplementation of large doses of zinc [18,19,21,28–34].

Missense variants in transmembrane domains were previously described to impair the function of SLC39A4 [39]. So far, one variant in the first transmembrane domain has been reported in a human patient [26]. The identified missense variant in the affected cats changes a highly conserved glycine residue in the first transmembrane domain. We assume that the change in the uncharged glycine to the positively charged arginine is intolerable for the integration of the protein in the membrane, thus causing a complete loss of function of the transporter. This hypothesis is further supported by the fact that four of the known pathogenic human variants involve an exchange of a glycine within a transmembrane domain to a charged amino acid.

We have to caution that we did not experimentally confirm the loss of function of SLC39A4 on the protein level. While the comparative data from human and cattle clearly suggest that *SLC39A4*:c.1057G>C is a very likely candidate causative variant for the observed skin lesions in the affected cats, further experiments are required to obtain definitive proof for this hypothesis.

5. Conclusions

In this study, we provide the first report of acrodermatitis enteropathica in cats. The identification of a candidate causative variant, *SLC39A4*:c.1057G>C, enables genetic testing to prevent future breeding of affected cats. For affected kittens, the genetic test also offers the chance to initiate adequate therapy, since AE is treatable with oral zinc supplementation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/genes12091309/s1, Table S1: Accession numbers of 55 cat genome sequences. Table S2: Private variants in the sequenced Turkish Van cat with acrodermatitis enteropathica (AE).

Author Contributions: Conceptualization, S.Å. and T.L.; investigation, S.K., R.C., M.W. and S.Å.; data curation, V.J.; writing—original draft, S.K., R.C., M.W., S.Å. and T.L.; writing—review and editing, S.K., R.C., M.W., V.J., S.Å. and T.L.; supervision, S.Å. and T.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The affected Turkish Van cats in this study were privately owned and skin biopsies and blood samples for diagnostic purposes were collected with the consent of their owners. The collection of blood samples from healthy cats was approved by the "Cantonal Committee for Animal Experiments" (Canton of Bern; permit 71/19).

Data Availability Statement: The genome sequence data were submitted to the European Nucleotide Archive (ENA). All accession numbers are listed in Supplementary Table S1.

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Conflicts of Interest: The authors declare no conflict of interest.

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Independent *DSG4* frameshift variants in cats with hair shaft dystrophy

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ORIGINAL ARTICLE

Independent *DSG4* frameshift variants in cats with hair shaft dystrophy

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Abstract

Investigations of hereditary phenotypes in spontaneous mutants may help to better understand the physiological functions of the altered genes. We investigated two unrelated domestic shorthair cats with bulbous swellings of the hair shafts. The clinical, histopathological, and ultrastructural features were similar to those in mice with lanceolate hair phenotype caused by loss-of-function variants in *Dsg4* encoding desmoglein 4. We sequenced the genomes from both affected cats and compared the data of each affected cat to 61 control genomes. A search for private homozygous variants in the *DSG4* candidate gene revealed independent frameshift variants in each case, c.76del or p.Ile26fsLeu*4 in case no. 1 and c.1777del or p.His593Thrfs*23 in case no. 2. DSG4 is a transmembrane glycoprotein located primarily in the extracellular part of desmosomes, a complex of adhesion molecules responsible for connecting the keratin intermediate filaments of neighbouring epithelial cells. Desmosomes are essential for normal hair shaft formation. Both identified *DSG4* variants in the affected cats lead to premature stop codons and truncate major parts of the open-reading frame. We assume that this leads to a complete loss of DSG4 function, resulting in an incorrect formation of the desmosomes and causing the development of defective hair shafts. Together with the knowledge on the effects of *DSG4* variants in other species, our data suggest that the identified *DSG4* variants cause the hair shaft dystrophy. To the best of our knowledge, this study represents the first report of pathogenic *DSG4* variants in domestic animals.

Keywords *Felis catus* · Whole-genome sequencing · Dermatology · Genodermatosis · Alopecia · Lanceolate · Hair shaft dysplasia

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Introduction

Hair is composed of terminally differentiated dead keratinocytes. It is characteristic for terrestrial mammals, and involved in various functions such as physical protection, thermal regulation, or sensory perception. The hair follicle, a complex miniorgan residing in the dermal layer of the skin, is responsible for the formation of hair (Schneider et al. 2009). The hair shaft consists of the hair cuticle, the cortex, and the innermost medulla. It is surrounded by the inner root sheath, the companion layer, and the outer root sheath (Cheng and Bayliss 2008; Shimomura and Christiano 2010; Welle and Wiener 2016).

Significant progress has been made in identifying numerous genes expressed in the hair follicle (Shimomura and Christiano 2010; Wiener et al. 2020). A malfunctioning hair follicle, e.g., due to a genetic defect, represents one of several possible causes for hair loss resulting in alopecia or hypotrichosis (Cheng and Bayliss 2008; Schneider et al. 2009; Shimomura and Christiano 2010; Welle and Wiener 2016; Ahmed et al. 2019).

Targeted or spontaneous mutant mice provided many valuable resources for dermatological research (Sundberg 1994; Sundberg and King 1996, 2000; Nakamura et al. 2001; Chen et al. 2008). An ethylnitrosourea induced and, subsequently, a spontaneous mouse mutant with irregular hair shafts characterized by focal deformities and pronounced enlargement at the apex, resembling a lance head, were termed lanceolate hair (*lah* and *lah-J*) (Montagutelli et al. 1996; Sundberg et al. 2000). The causative genetic variant for the *lah* mouse mutant was identified in the *Dsg4* gene encoding desmoglein 4 (Kljuic et al. 2003). Several additional spontaneous allelic mutants with the same phenotype have been discovered in mice (Berry and Sundberg, unpublished data). A related phenotype in humans, localized autosomal recessive hypotrichosis (LAH, OMIM #607903), is due to genetic variants in the human ortholog, *DSG4* (Kljuic et al. 2003). Finally, a spontaneous lanceolate hair phenotype due to a *Dsg4* variant also exists in rats (Jahoda et al. 2004).

We previously described the clinical and histopathological findings in a litter of cats with alopecia and hair shaft irregularities that closely resembled the phenotype of *Dsg4*-deficient *lah* mice (Rostaher et al. 2021). In the present study, we investigated one additional unrelated case of a similar hair shaft dystrophy and searched for the underlying causative genetic variants in the previously reported cats.

Methods

Animal selection

This study included two unrelated domestic shorthair (DSH) cats affected by hair shaft dystrophy, and 61 genetically diverse control cats. The 63 sequenced cats included 34 purebred cats from 11 different breeds and 29 randombred domestic cats (Table S1). Genomic DNA was isolated from ETDA blood samples with the Maxwell RSC Whole Blood Kit using a Maxwell RSC instrument (Promega, Dübendorf, Switzerland).

Histopathological examinations

Skin biopsies from alopecic skin were taken under general anesthesia according to standard procedures. The biopsies were processed routinely and stained with hematoxylin and eosin, prior to histological evaluation.

Whole-genome sequencing

Illumina TruSeq PCR-free DNA libraries with ~ 300 bp insert size of both affected cats were prepared and sequenced at 24 × coverage (case no. 1) and 21 × coverage (case no. 2) on a NovaSeq 6000 instrument. The reads were mapped to the FelCat9.0 cat reference genome assembly as described (Jagannathan et al. 2019). The sequence data were submitted to the European Nucleotide Archive with the study accession PRJEB7401 and sample accessions SAMEA7853384 (case no. 1) and SAMEA7853385 (case no. 2).

Variant calling and filtering

Variant calling was performed as described (Jagannathan et al. 2019). The SnpEff software was used together with NCBI annotation release 104 of the FelCat9.0 genome reference assembly to predict the functional effects of the called variants (Cingolani et al. 2012). For variant filtering, we used 61 control genomes (Table S1). Filtering was done with a self-written C + + program on a modified vcf-file containing genotype calls of the 2 affected cats and the 61 control cats, and SnpEff predicted functional effects of all variants.

Gene analysis

Numbering within the feline *DSG4* gene corresponds to the NCBI RefSeq accession numbers XM_019815116.1 (mRNA) and XP_019670675.1 (protein).

Sanger sequencing

Sanger sequencing of PCR amplicons was used to confirm the candidate variants *DSG4*:c.76del and *DSG4*:c.1777del and to genotype cats. We used forward and reverse primers together with the AmpliTaqGold360Mastermix (Thermo Fisher Scientific, Waltham, MA, USA) to amplify a PCR product from genomic DNA (Table 1). After treatment with shrimp alkaline phosphatase and exonuclease I, PCR amplicons were sequenced on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific). The Sequencher 5.1 software was used to analyze the Sanger sequences (GeneCodes, Ann Arbor, MI, USA).

Results

Family anamnesis, clinical examinations, and histopathology

Case no. 1 was a female random-bred DSH cat, born in 2019 on a farm in Switzerland. An unusual generalized moderate-to-severe hypotrichosis was observed at the first check-up examination at 3 months of age. The cat was otherwise in good general health. The owner reported that a maternal half-sibling of this cat had been born naked and died within a few days after birth. The hair loss in case no. 1 symmetrically affected the convex pinnae, parts of the face, the back, and the legs including the dorsal paws (Fig. 1a, b). The cat was mildly pruritic. Dermatophytosis and infestation with ectoparasites as underlying cause for the alopecia were excluded clinically and by treatment with Fluralaner (Bravecto(R), MSD Animal Health GmbH, Switzerland) and topical enilconazole (Imaverol(R), Covetrus AG, Switzerland). The trichogram revealed broken and split hair shafts. The hair shafts presented with severe

Table 1 Details of the primers used for Sanger sequencing

Variant	Primer name	Primer sequence (5′ to 3′)	Amplicon length (bp)	T_M (°C)
DSG4:c.76del	Primer F1	GGAGGGCAAAGAGCCTGTAT	362	60
	Primer R1	TGGGTTTGCCATTGCTATTT		
DSG4:c.1777del	Primer F2	GACGGCTGAGCAGCTTTTAT	445	60
	Primer R2	GCCCTTATTAGCCCCATTGT		

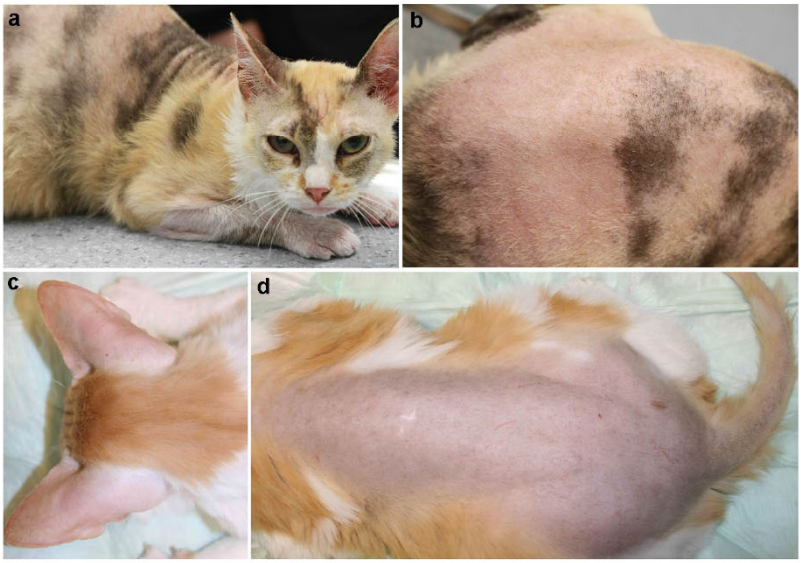

Fig. 1 Clinical phenotype of the affected cats with hypotrichosis and alopecia on the convex pinnae, parts of the face, the back, and the legs. **a**, **b** Case no. 1. **c**, **d** Case no. 2

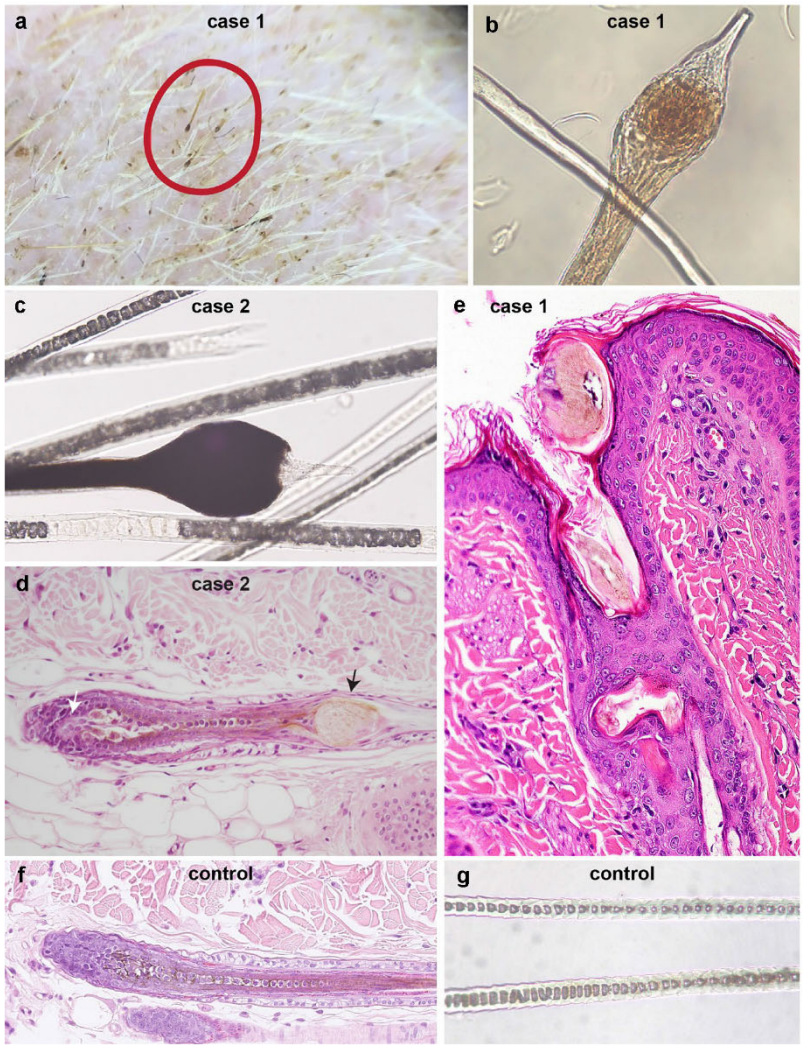

Fig. 2 Clinical and histological features of the lanceolate hair shafts and follicles of cases no. 1 and no. 2 (**a**–**e**). **a** The Dermascope image shows hair with irregular thickenings of the hair shafts (red circle). **b**–**c** Trichogram of hair with lance-head shaped distal ends. **d** The histological image of an anagen hair follicle shows a bulbous or lance-head shaped swelling of the already fully cornified hair shaft (black arrow) just distal to the melanogenic zone at the border of inferior portion and isthmus. This is associated with individual cells or small cell clusters in the precortex and premedulla (white arrow) that are enlarged, rounded, and have abundant pale cytoplasm, suggesting that they are undergoing degenerative changes. **e** The bulbous swelling of the hair shaft is located close to the orifice. The cortex of the hair shaft is fragmented and broken hair shaft fragments are oriented horizontally. **f** Histology of a feline control hair follicle. **g** Trichogram of a feline control hair

bulbous swellings. These swellings were present anywhere in the hair shaft but most often close to the tip (Fig. 2a, b).

In 2010, a 1.5 year old male castrated DSH cat (case no. 2) from a local shelter in Germany was presented with progressive alopecia of the dorsum, the plantar, and palmar surfaces of the limbs, the convex pinnae, and most of the face, as previously published (Fig. 1c, d) (Rostaher et al. 2021). In addition to body hairs being affected, the vibrissae were short and broken. Macroscopic evaluation of the skin surface and microscopic examination of trichograms revealed many short, broken hair shafts, some of which had bulbous or lance-head shaped ends (Fig. 2c). All of the cat's littermates showed similar skin lesions.

The histopathological evaluation revealed that the number and size of the hair follicles was normal and the presence of hair shafts in all hair follicles. Most hair follicles were in anagen. Numerous, but not all hair shafts were dystrophic. The dystrophy was characterized by severe well circumscribed thickening of the hair shafts starting above the melanogenic zone (Fig. 2d). In addition, there was loss of a ladder-like pattern of pigment distribution. In Fig. 2e, the swelling of the hair shaft was present more distally in the infundibulum. This reflects most likely the outgrowing of the bulbous swelling and represents the lance-head shaped hair tip. Additionally, the dystrophy was characterized by an irregular outer contour of the hair shafts, fragmentation

Table 2 Results of variant filtering in the affected cats against 61 control genomes

Filtering step	Variants case no. 1	Variants case no. 2
All variants	5,955,464	6,162,272
Private variants	26,179	26,624
Protein-changing private variants	76	93
Protein-changing private variants in *DSG4*	1	1

Only homozygous variants are reported

within the cortex and the cuticle, and dense eosinophilic cornified material that surrounded the hair shafts. In some infundibula, the fragmented hair shafts were oriented horizontally. Furunculosis was present in one biopsy. Sebaceous and sweat glands were normal. The epidermis was moderately hyperplastic and covered by a small amount of laminar orthokeratotic keratin (Fig. 2e).

Genetic analysis

Given the results of the clinical and histopathological analysis, *DSG4* was considered the primary functional candidate gene for the observed phenotype in both cases. To characterize the underlying causative genetic variant, we sequenced the genome of the affected cats and searched for private variants in *DSG4*. Since the cats were not known to be related, we hypothesized that their phenotypes were due to independent pathogenic variants. We therefore searched for private variants in each of the affected cats' genomes individually by comparing them to the genomes of 61 other cats (Table 2, Table S1, Table S2).

This analysis identified a single homozygous private protein-changing candidate variant in *DSG4* in each of the cases. The two variants can be designated as ChrD3:55,315,010del (FelCat9.0) for case no. 1 and ChrD3:55,336,127del (FelCat9.0) for case no. 2. Both variants represented 1 bp deletions, causing frameshifts and resulting in premature stop codons (Table 3). The variants truncate 98% and 43% of the open-reading frame, respectively.

We confirmed the presence of the *DSG4* frameshift variants by Sanger sequencing (Fig. 3) and genotyped a cohort of 46 unaffected cats from different breeds for both variants. The affected cats carried one frameshift allele in a homozygous state and were homozygous wild-type at the variant of the other case. All other non-affected cats were homozygous for the wild-type allele at both variants (Table 4).

Table 3 Variant designations of the identified *DSG4* frameshift deletions

Cats	HGVS variant designations		
	Genomic (FelCat9.0)	mRNA (XM_019815116.1)	Protein (XP_019670675.1)
Case no. 1	ChrD3:55,315,010del	c.76del	p.Ile26Leufs*4
Case no. 2	ChrD3:55,336,127del	c.1777del	p.His593Thrfs*23

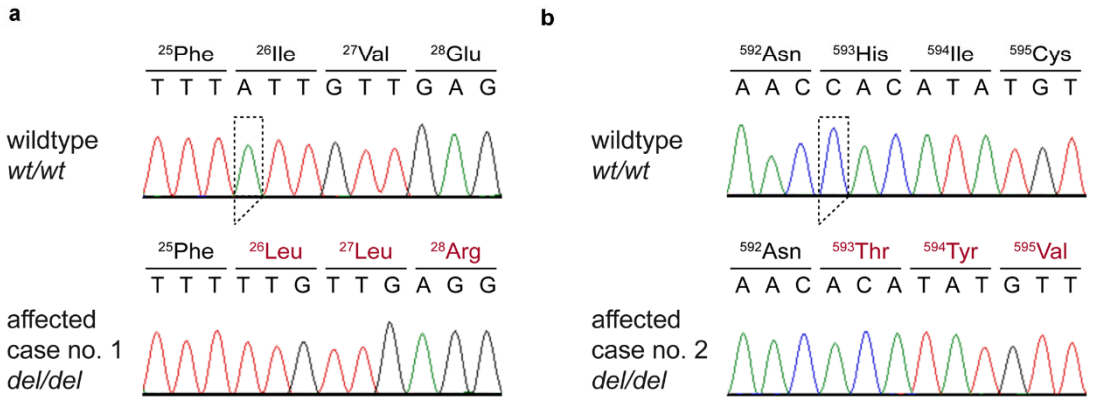

Fig. 3 Details of the **a** *DSG4*:c.76del and **b** *DSG4*:c.1777del variants. Representative electropherograms of a control and the affected cats are shown. The amino acid translations of the wild-type and mutant alleles are indicated

Table 4 Genotype–phenotype association of the *DSG4* variants with hair shaft dystrophy in cats

Cats	c.76del			c.1777del		
	wt/wt	wt/del	del/del	wt/wt	wt/del	del/del
Case no. 1	–	–	1	1	–	–
Case no. 2	1	–	–	–	–	1
Controls (n = 46)	46	–	–	46	–	–

Discussion

In this study, we investigated two unrelated DSH cats affected with hair shaft dystrophy. One of the two investigated cats (case no. 2) was part of a previous study about the clinical, histological, and ultrastructural features of the disease, which revealed significant similarity to $Dsg4^{lah}$ and $Dsg4^{lahJ}$ mutant mice (Rostaher et al. 2021). The other case (case no. 1) was an unrelated DSH cat that presented with a similar clinical and histopathological phenotype.

We sequenced the genomes of both cats and filtered for private homozygous variants in our primary functional candidate gene *DSG4*. This analysis identified independent private variants in each of the cases. Both variants represented frameshift deletions predicted to result in true null alleles. The finding of independent loss-of-function variants in the same gene in cats with comparable phenotype represents a very strong evidence for the causality of these variants.

DSG4 has been identified as the fourth and last member of the desmoglein family (Whittock and Bower 2003). In human anagen hair follicles, *DSG4* is expressed in the precortex, cortex, and lower cuticle of the hair shaft. It is additionally expressed in the inner root sheath cuticle above the bulb region (Bazzi et al. 2006). As component of the desmosomes, *DSG4* is involved in linking the intracellular network of keratins of a trichocyte to the intermediate filaments of the neighbouring trichocytes. This intra- and inter-cellular network of keratins and desmosomes provides the hair shaft with its tensile strength and elasticity (Bazzi et al. 2009). It also plays a central role in coordinating cellular dynamics in the lower hair follicle during the switch from proliferation to differentiation (Kljuic et al. 2003). The mutant cats had a prominent hair shaft defect characterized by a bulbous swelling distal to the melanogenic zone. This probably leads to an increased fragility and the formation of a lance-head shaped hair tip (Rostaher et al. 2021).

Variants in *DSG4* have previously been described in mice and rats with lanceolate hair phenotype, and in human patients with localized autosomal recessive hypotrichosis, as well as monilethrix-like hypotrichosis (Kljuic et al. 2003; Jahoda et al. 2004; Moss et al. 2004; Nagasaka et al. 2004; Rafiq et al. 2004; Messenger et al. 2005; John et al. 2006; Schaffer et al. 2006; Shimomura et al. 2006; Zlotogorski et al. 2006; Wajid et al. 2007). We assume that loss of *DSG4* function leads to an incorrect formation of the desmosomes resulting in impaired adhesion between neighbouring trichocytes and a defective hair shaft formation in the affected cats.

In summary, we identified two likely disease-causing variants in *DSG4* in cats with a recessive hair shaft dystrophy. The hair phenotype of the affected cats was comparable to the phenotype in lanceolate hair mice. Our findings therefore corroborate the relevance of desmoglein 4 for correct hair shaft formation and the known genotype–phenotype correlations from other species. The affected cats were homozygous for the mutant alleles, indicating that inbreeding contributed to the observed genodermatoses. To the best of our knowledge, this study represents the first report of pathogenic *DSG4* variants in domestic animals.

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Author contributions Conceptualization: TL and AR; investigation: SK, AR, SR, and MW; data curation: VJ; writing—original draft: SK, AR, SR, MW, and TL; writing—review and editing: SK, AR, SR, VJ, JS, MW, and TL; supervision: TL. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials The genome sequence data were submitted to the European Nucleotide Archive (ENA). All accession numbers are listed in Table S1.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The affected cats in this study were privately owned and skin biopsies and blood samples for diagnostic purposes were collected with the consent of their owners. The collection of blood samples from healthy control cats was approved by the "Cantonal Committee for Animal Experiments" (Canton of Bern; permit 71/19).

Consent to participate Not applicable.

Consent for publication Not applicable.

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Independent *COL5A1* variants in cats with Ehlers-Danlos syndrome

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Independent *COL5A1* Variants in Cats with Ehlers-Danlos Syndrome

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Abstract: We investigated four cats with similar clinical skin-related signs strongly suggestive of Ehlers-Danlos syndrome (EDS). Cases no. 1 and 4 were unrelated and the remaining two cases, no. 2 and 3, were reportedly siblings. Histopathological changes were characterized by severely altered dermal collagen fibers. Transmission electron microscopy in one case demonstrated abnormalities in the collagen fibril organization and structure. The genomes of the two unrelated affected cats and one of the affected siblings were sequenced and individually compared to 54 feline control genomes. We searched for private protein changing variants in known human EDS candidate genes and identified three independent heterozygous *COL5A1* variants. *COL5A1* is a well-characterized candidate gene for classical EDS. It encodes the proα1 chain of type V collagen, which is needed for correct collagen fibril formation and the integrity of the skin. The identified variants in *COL5A1* are c.112_118+15del or r.spl?, c.3514A>T or p.(Lys1172*), and c.3066del or p.(Gly1023Valfs*50) for cases no. 1, 2&3, and 4, respectively. They presumably all lead to nonsense-mediated mRNA decay, which results in haploinsufficiency of *COL5A1* and causes the alterations of the connective tissue. The whole genome sequencing approach used in this study enables a refinement of the diagnosis for the affected cats as classical EDS. It further illustrates the potential of such experiments as a precision medicine approach in animals with inherited diseases.

Keywords: *Felis catus*; dermatology; genodermatosis; skin; precision medicine; animal model

1. Introduction

Ehlers-Danlos syndromes (EDS) are heritable disorders of connective tissue that share several clinical features, such as skin hyperextensibility, joint hypermobility, abnormal wound healing, and easy bruising [1]. Based on clinical findings and the mode of inheritance,

different subtypes are recognized in humans, caused by alterations in 20 different genes [1–3] (Supplementary Table S1). One subtype is classical EDS, which is caused by defects in type V collagen and results from pathogenic variants in *COL5A1* or *COL5A2* [2,4–9]. Type V collagen is a major regulator of collagen fibril diameter and has a critical role during the early process of collagen fibril nucleation [1]. Targeted and spontaneous animal models for EDS have recently been reviewed [10].

Spontaneous cases of EDS have been previously described in cats as cutaneous asthenia or dermatosparaxis (OMIA 000327-9685; [10–23]). A candidate causative variant in *COL5A1* has been identified in one cat. The affected cat carried a heterozygous single base pair deletion, c.3420del, resulting in a frameshift and premature stop codon [24].

In this study, we characterized the clinical and histopathological phenotype of four cats with clinical signs of classical EDS and performed genetic analyses to search for the causative genetic variants.

2. Materials and Methods

2.1. Animal Selection

This study included four EDS-affected cats, one Bengal cat (case no. 1), two presumably related shelter cats reported to be Bombay with unknown lineage (cases no. 2 and 3), and one domestic shorthair cat (case no. 4). For case no. 4, we additionally had samples of two unaffected siblings and both parents. Samples from 54 additional genetically diverse control cats of the Vetsuisse Biobank were used for the whole genome sequencing data analysis (Supplementary Table S2).

2.2. Clinical and Histopathological Examinations

All cats underwent clinical examination. For case no. 4, hematological and biochemical parameters were measured, and ELISA tests for Feline Leukemia Virus (antigens), Feline Immuno-deficiency Virus (antibodies), and Feline Coronavirus (antibodies) were performed (IDEXX Laboratories, Kornwestheim, Germany). Furthermore, a skin intradermal allergy test (Artuvetrin Haut Test, Nextmune, Leipzig, Germany) and a serum allergy test (IDEXX Laboratories, Kornwestheim, Germany) were carried out.

Skin biopsies were obtained from cases no. 1, 2, and 4. The biopsies were fixed in 10% buffered formalin and processed routinely. The slides were stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Masson's Trichrome (MT), prior to histopathological examination.

2.3. Transmission Electron Microscopy (TEM)

Skin punch biopsies from case no. 4 and the skin punch biopsy of an age-matched control cat were fixed in a 2.5% glutaraldehyde/0.1 M cacodylate buffer solution, then washed in 0.1 M cacodylate buffer (cacodylic acid sodium salt trihydrate; Merck KGaA, Darmstadt, Germany), and afterwards contrasted for 4 h in 1% osmium tetroxide (Polysciences Europe GmbH, Hirschberg, Germany). Samples were then dehydrated in an ascending series of ethanol and embedded in a mixture of Epon 812 (epoxy resin), dodecenylsuccinic anhydride (plasticizer), methylnadic anhydride (hardener), DMP 30 (catalyst) (all: Merck KGaA, Darmstadt, Germany), and polymerized at 60 °C for 5 days. Semi- and ultrathin sections were cut with an ultra-microtome Reichert Ultracut S (Leica, Wetzlar, Germany). Semithin sections (0.5 µm) were stained with 1% Toluidine blue solution (Merck KGaA, Darmstadt, Germany). From the semi-thin sections, representative areas for ultrastructural analysis were selected by light microscopy (Axioimager, Zeiss, Oberkochen, Germany). Ultrathin (80 nm) sections were mounted on copper-grids (Agar Scientific Ltd., Stansted, Essex, UK), contrasted with lead citrate and UranyLess (Electron Microscopy Sciences, Hatfield, PA, USA), and examined with a transmission electron microscope (CM12; Zeiss, Oberkochen, Germany).

2.4. DNA Extraction

Genomic DNA was isolated from EDTA blood using the Maxwell® RSC Whole Blood DNA Kit with the Maxwell® RSC instrument (Promega, Dübendorf, Switzerland).

2.5. Whole-Genome Sequencing and Variant Calling

Illumina TruSeq PCR-free DNA libraries were prepared for cases no. 1, 2, and 4 and sequenced on a NovaSeq 6000 instrument. The sequence data were submitted to the European Nucleotide Archive with the study accession PRJEB7401 and sample accessions SAMEA5885921 (case no. 1), SAMEA5885927 (case no. 2), and SAMEA7853381 (case no. 4). Mapping and alignment were performed as described [25]. Variant calling was performed using GATK HaplotypeCaller [26] in gVCF mode as described [25]. To predict the functional effects of the called variants, SnpEff version 4.3t software [27] together with NCBI annotation release 104 for the felCat9.0 genome reference assembly was used. For variant filtering, we used 54 control genomes (Supplementary Table S2).

2.6. Gene Analysis

We used the felCat9.0 reference genome assembly and NCBI annotation release 104. Numbering within the feline *COL5A1* gene corresponds to the NCBI RefSeq accession numbers XM_023242950.1 (mRNA) and XP_023098718.1 (protein).

2.7. PCR and Sanger Sequencing

Candidate variants were confirmed and genotyped either by direct Sanger sequencing of PCR amplicons or fragment length analysis of PCR products on a 5200 Fragment Analyzer (Agilent, Basel, Switzerland). PCR products were amplified from genomic DNA using AmpliTaq Gold 360 Mastermix (Thermo Fisher Scientific, Reinach, Switzerland) together with a forward and reverse primer (Supplementary Table S3). After treatment with exonuclease I and alkaline phosphatase, amplicons were sequenced on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific). Sanger sequences were analyzed using the Sequencher 5.1 software (GeneCodes, Ann Arbor, MI, USA).

3. Results

3.1. Clinical History and Examination

Case no. 1 was a 1-year-old purebred Bengal cat with a history of multiple repetitive skin lesions after minimal trauma occurring since the age of 6 months. At the time of presentation, a scarring alopecic lesion of about 5 × 5 cm was visible on the dorsal neck, which resulted from a previous trauma. Furthermore, the skin was diffusely thinner and much more elastic compared to the skin of a healthy young Bengal cat. In addition, the patient presented with multiple small skin tears distributed over the entire trunk/back area (Figure 1). The cat had no pruritus and there were no signs of active inflammation. Skin cytology, skin scrapings, and the trichogram were unremarkable.

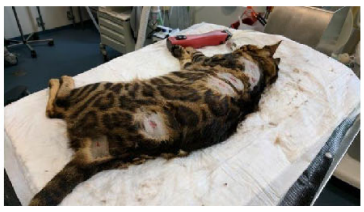

Figure 1. Clinical image of the EDS-affected cat no. 1. Multiple small skin tears were distributed over the entire back.

Cases no. 2 and 3, presumably related female spayed Bombay cats, were presented for multiple wounds in the hyperextensible skin predominantly at the neck, base of ear, and shoulders (Figure 2a). The wounds occurred after self-trauma or exuberant play. Case no. 2

additionally showed spasms of the cutaneous trunci followed by frantic self-mutilation since adoption at 8 weeks of age. At 11 months of age, case no. 2 also experienced multiple partial and grand mal seizures necessitating phenobarbital therapy. Case no. 3 experienced a grade 3 patellar luxation at 18 months of age presumed to be related to joint hypermobility due to EDS. Excessive facial skin folds were apparent (Figure 2b).

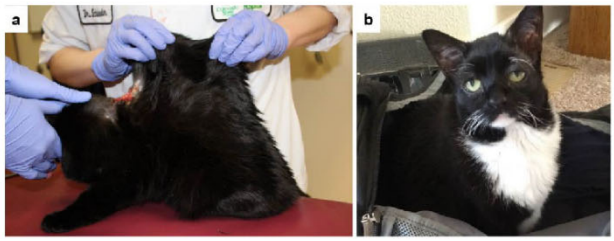

Figure 2. Clinical images of the EDS-affected cats no. 2 and 3. (**a**) Severe hyperextensibility of the dorsal skin. The skin extensibility index was 19% [20]. Note an extensive laceration on the back. (**b**) Excessive skin tissue is visible on the face of case no. 3.

Case no. 4 was a 2-year-old male castrated indoor DSH cat that was examined for recurrent laceration wounds on the neck and shoulders since adoption (one year before), and moderate head and neck pruritus (observed all year-round with exacerbation in June and August). Hematology and biochemistry (including basal cortisol) were normal, and Feline Leukemia Virus, Feline Immunodeficiency Virus, and Feline Coronavirus were negative one year before referral. On physical examination, a 1 cm scar on the ventral neck and a 0.5 cm crust above the right eye were noted. The skin extensibility index was 22% (Figure 3a). No joint laxity, hernias, ocular, or heart abnormalities were found. Neither ecto- and endoparasite treatment of both cats, nor an eight-week elimination diet improved the pruritus. Over the course of the disease, the cat developed new lesions due to pruritus when treated only with oral antihistamines (Fenistil, GlaxoSmithKline Consumer Healthcare GmbH & Co. KG, Munich, Germany) (Figure 3b,c). With short courses of systemic prednisolone (injections of 2 mg/kg, or orally 0.6 mg/kg once daily, as needed), the scratching stopped, and the lesions healed. Systemic medication was withdrawn as indicated prior to biopsy sampling. A welfare score (8; 0–21) was not suggestive of a psychogenic induced pruritus [28]. Based on all the above and since the patient fulfilled Favrot's criteria [29], which help to establish the diagnosis of atopic dermatitis, a feline atopic skin syndrome (FASS) was diagnosed and a concomitant collagen disorder leading to lacerations due to pruritus (traumatic splitting) was suspected [30]. A skin intradermal allergy test as well as a serum allergy test were negative and could not identify the triggering agents of FASS. The owner declined antiallergic therapies other than antihistamines, and the cat was euthanized 2.5 years after the first presentation due to a very large laceration wound and deterioration, despite surgical and emergency treatment.

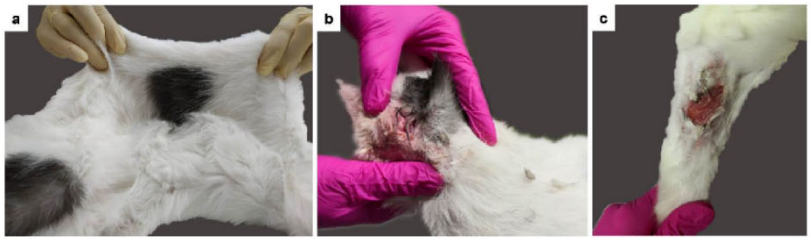

Figure 3. Clinical images of the EDS-affected cat no. 4. (**a**) The abnormal extensibility of the dorsal skin is clearly visible. The skin extensibility index was 22%. (**b**,**c**) Lacerations of the skin on the head and the leg.

Two unaffected siblings and both parents of the patient were also clinically evaluated. They did not have any history compatible with allergy and no abnormality was noted. Their skin extensibility indices were normal (father: 15.6%, mother: 6.6%, brother 17.6%, sister: 10.7%).

3.2. Histopathological Examination

The pathological changes in the skin biopsies were characterized by severely altered dermal collagen fibers and a slightly thinner epidermis. Changes were more severe in the superficial and mid dermis but were also present in the deep dermis. The abnormal collagen fibers were haphazardly arranged, shortened, and sometimes curled. They were uneven in length and width. Many fibers were very wispy and the interfibrillar spaces were widened. Multifocally small areas of hemorrhage were seen (Figure 4).

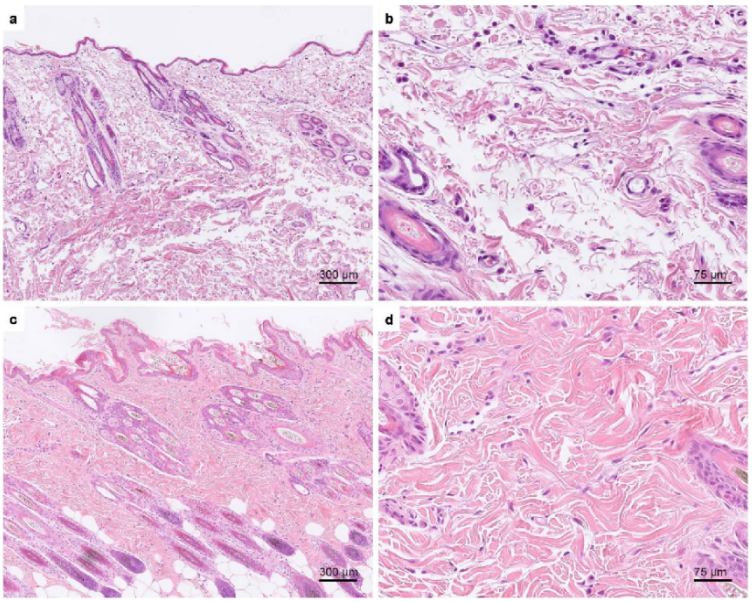

Figure 4. Hematoxylin and eosin-stained skin biopsies of case no. 4 and an age-matched control. (**a**,**b**) Histopathological changes seen in the affected cat. The epidermis appears thinner than in the skin biopsies of the age-matched control cat (**c**,**d**). (**a**) The dermal collagen fibers are haphazardly arranged, shortened, and sometimes curled. They were uneven in length and width. Many fibers are very wispy and the interfibrillar spaces are widened. (**b**) Higher magnification of the dermal changes seen in the affected cat. (**c**) Normal skin of an age-matched control cat. The dermal collagen fibers are much thicker and longer and the interfibrillar spaces are smaller than in the affected cat. (**d**) Higher magnification of normal skin.

3.3. Ultrastructural Examination

TEM analysis of case no. 4 skin connective tissue revealed abnormalities in the collagen fibril organization and structure. Abnormal findings from the reticular part of the dermis were loosely packed and disorganized collagen fibers (Figure 5b,d). Individual collagen fibers contained curled fibrils (Figure 5b,d). Higher magnifications showed cross-sections of collagen fibrils with different diameters and irregularly outlined fibrils within a fiber (Figure 5f,h). In contrast, the connective tissue of an age-matched control cat contained densely packed collagen fibers with parallel-aligned fibril organization (Figure 5a,c). Cross-sections of fibrils showed uniform diameters and regularly shaped, almost round outlines (Figure 5e,g).

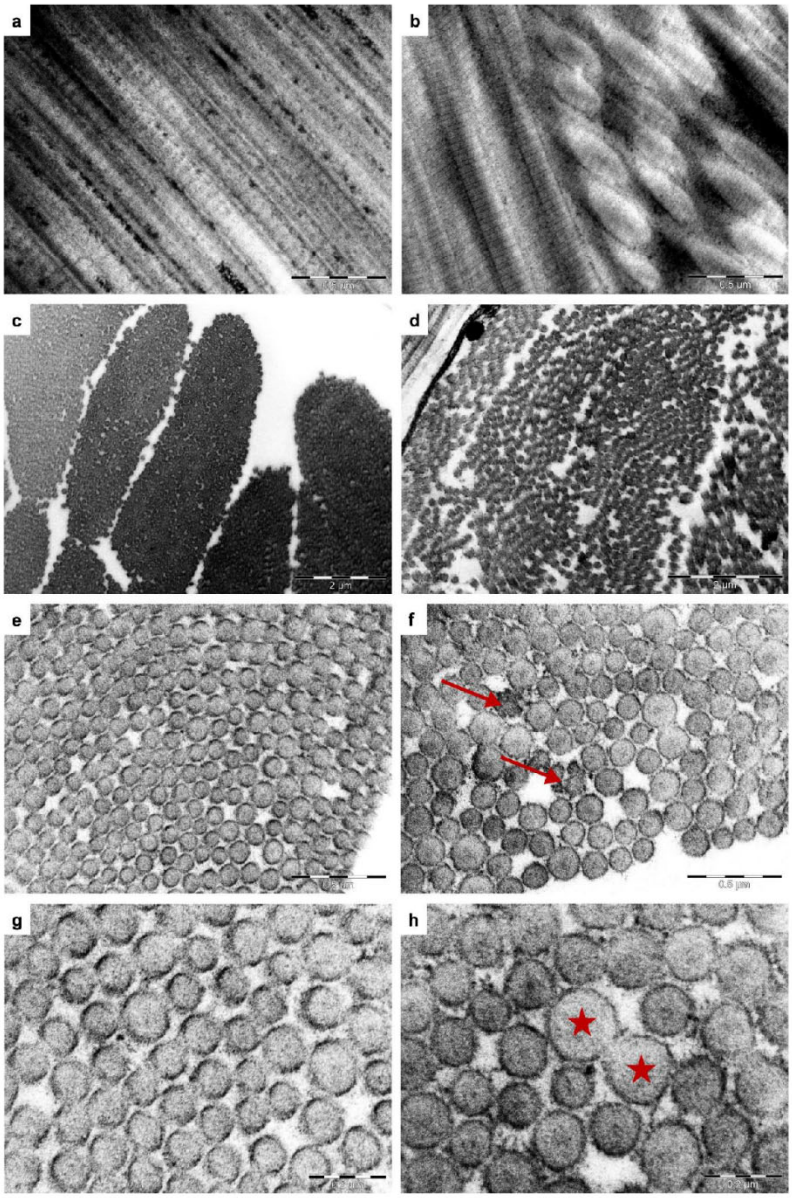

Figure 5. Ultrastructural morphology of the skin from one of the affected cats (case no. 4) and an age-matched control cat. (**a**,**c**,**e**,**g**) Reticular connective tissue of a normal cat's skin (control cat). (**a**) Longitudinal section of parallel-aligned collagen fibrils (×53.000). (**c**) Collagen fibers are composed of densely packed fibrils (cross-section, ×11.500). (**e**,**g**) Details of cross-sectioned collagen fibers. Consistent diameters of collagen fibrils with regularly shaped and almost round outlines (×53.000, ×110.000). (**b**,**d**,**f**,**h**) Representative collagen fibril abnormalities observed in the reticular dermis of case no. 4 cat. (**b**) Longitudinal sections show disordered, curled fibrils (×53.000). (**d**) Collagen fibers are loosely packed (cross-section, ×11.500). (**f**) Collagen fibrils with irregular outlines (arrows, ×53.000) and variable diameters (**h**). (**h**) Asterisks indicate fibrils with a diameter almost twice as large as surrounding fibrils with a normal diameter (×110.000).

3.4. Genetic Analysis

To identify the causative genetic variants, the genomes of three affected cats (cases no. 1, 2, and 4) were sequenced. Case no. 3 was presumably related to case no. 2 and assumed to share the same pathogenic variant. Since the other cats were not known to be related, we hypothesized that their phenotypes were due to independent pathogenic variants. We therefore performed an individual variant filtering in each of the affected cats by comparing them to the genomes of 54 control cats. We focused our search on private protein-changing variants in the 20 known EDS candidate genes (Tables 1, S1, S2 and S4).

Table 1. Results of variant filtering in the affected cats against 54 control genomes.

Filtering Step	Variants Case No. 1		Variants Case No. 2		Variants Case No. 4	
	hom	het	hom	het	hom	het
all variants	5,758,034	9,091,644	4,612,879	8,593,879	4,949,250	8,310,846
private variants	44,007	938,018	6242	194,315	11,606	168,540
protein-changing private variants	91	2901	15	880	51	775
in 20 known EDS candidate genes	0	1	0	1	0	3

In case no. 1, we identified one heterozygous private protein-changing variant within an EDS candidate gene (Figure 6a). The variant, *COL5A1*:c.112_118+15del, is a 22 bp deletion that removes the exon1/intron1 boundary (Table 2, Figure 6b). The presence of the variant was confirmed by PCR and fragment length analysis.

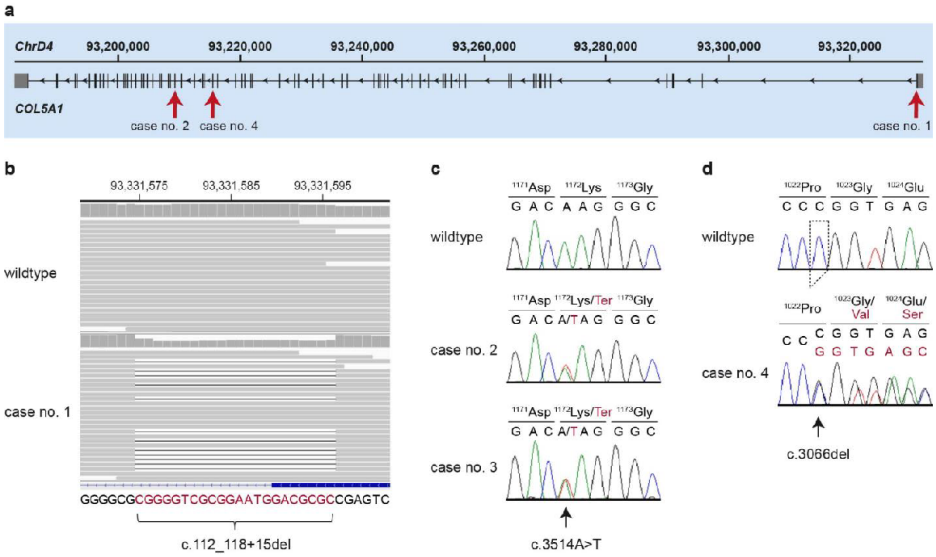

Figure 6. Details of the *COL5A1* variants. (**a**) Overview of the major transcript isoform (XM_023242950.1) of the *COL5A1* gene with the intronic and exonic regions. The positions of all three identified variants are indicated by arrows. (**b**) Integrative Genomics Viewer (IGV) screenshot showing the short-read alignments of a control and the EDS-affected cat (case no. 1) at the position of the deletion. The heterozygous deletion is visible in the short-read alignments and the reduced coverage at the deleted bases. The sequence at the bottom represents the sequence of the coding strand in 3′ to 5′ orientation. The deleted bases are indicated in red. (**c**) Representative electropherograms of a control and two EDS-affected cats (case no. 2 and 3) are shown. The variable position is indicated by an arrow and the amino acid translations are given. Mutant alleles are indicated in red. (**d**) Representative electropherograms of a control and an EDS-affected cat (case no. 4) are shown. The amino acid translations of the wildtype and mutant alleles are indicated.

Table 2. Variant designations of the identified *COL5A1* variants according to Human Genome Variation Society (HGVS) nomenclature.

Cats	HGVS Variant Designations		
	Genomic (felCat9.0)	**mRNA (XM_023242950.1)**	**Protein (XP_023098718.1)**
case no. 1	ChrD4:93,331,577_93,331,598del	c.112_118+15del	r.spl?
cases no. 2 and 3	ChrD4:93,209,345T>A	c.3514A>T	p.(Lys1172*)
case no. 4	ChrD4:93,215,496del	c.3066del	p.(Gly1023Valfs*50)

In case no. 2, we identified another single candidate variant, a heterozygous nonsense variant in *COL5A1*, predicted to truncate 36% of the coding sequence (Table 2). We confirmed identical heterozygous genotypes in cases no. 2 and 3 by Sanger sequencing (Figure 6a,c).

In case no. 4, we identified three heterozygous variants in known EDS candidate genes, a frameshift deletion in *COL5A1* and two missense variants in *TNXB*. The targeted genotyping of all three variants in the complete family comprising both parents and two additional unaffected littermates excluded the *TNXB* variants (Supplementary Figure S1). The remaining variant, *COL5A1*:c.3066del, is a one base pair deletion, resulting in a frameshift and predicted to truncate 44% of the open reading frame (Table 2, Figure 6a,d). The genotyping results in the family further revealed that *COL5A1*:c.3066del represented a de novo variant as the mutant allele was absent from the leukocyte DNA of both parents.

4. Discussion

In this study, we describe four cats with classical EDS. All cats were characterized by similar clinical skin-related symptoms strongly suggestive of EDS [2]. Histopathology of the examined skin biopsies was also suggestive of EDS. In addition, TEM on case no. 4 identified abnormal morphological features indicative of EDS, such as variable diameters of collagen fibrils, disorganized, distorted collagen fibers, and irregularly outlined fibrils, which have been reported in the literature to be present in most patients with a genetically confirmed diagnosis of EDS [31–33].

Whole genome sequencing was performed to confirm the suspected clinical and histological diagnosis and to identify the underlying causative genetic variants in all four cats. We identified three independent heterozygous private protein-changing variants in *COL5A1* in the four investigated affected cats. A de novo mutation event was experimentally confirmed for case no. 4. We assume that the pathogenic *COL5A1* variants in the other affected cats were also due to recent de novo mutation events. For cases no. 2 and 3, the most likely scenario was a germline mutation in one of their parents. These results resemble previously described findings in a cat and three dogs affected by EDS due to de novo mutations in *COL5A1* [24,34].

COL5A1 encodes the proα1 chain of type V collagen, which together with type I collagen forms heterotypical fibrils. Type V collagen is a major regulator of collagen fibrillogenesis, and the reduction in collagen V expression results in fewer collagen I fibrils with increased diameters [35,36]. Correct fibril formation is required for the integrity of the skin [1].

In humans, the functional loss of one *COL5A1* allele is the most commonly reported molecular mechanism in classical EDS [36,37]. As type V procollagen molecules cannot accommodate more than a single proα2(V) chain, the reduction in available proα1(V) chains results in the production of about half the normal amount of type V collagen [38]. We assume that all three newly reported feline variants lead to nonsense-mediated mRNA decay, which results in a haploinsufficiency of *COL5A1* and causes the alterations of the connective tissue.

Case no. 2 experienced epilepsy and presumptive hyperesthesia signs. Notably, an EDS-affected Burmese cat was described as experiencing prolonged episodes of disturbing vocalizations that were considered to be partial seizures. Upon necropsy, this cat was found

to have flattened cerebral gyri [14]. Epilepsy has been noted in human patients with EDS and is associated with many structural brain defects, including periventricular heterotopia in patients with *COL5A1* variants [39,40]. EDS-associated small fiber polyneuropathy in human patients manifests as acute, unpredictable burning pain, muscle cramping, and also compulsive scratching due to severe episodic itch [41]. Given comparable observations in human EDS patients and the earlier reported EDS-affected Burmese cat, it is reasonable to suspect that epilepsy and hyperesthesia were likely part of the EDS phenotype in case no 2.

Case no. 4 was diagnosed with EDS and concurrent FASS. Diagnosis of FASS can be difficult since it is a clinical diagnosis, and no diagnostic tests exist to diagnose or exclude FASS. Allergy tests such as serology and intradermal testing only help to identify the triggering agents to perform allergen specific immunotherapy. However, FASS cases have sometimes been described to have negative allergy testing results [30]. Furthermore, the cat was presented with head and neck pruritus. This is a skin reaction pattern that is consistent with the diagnosis of FASS after excluding other possible causes, as performed in our case [30]. Therefore, based on the clinical presentation and the fact that the cat showed a seasonal exacerbation, as well as the response to histamines (partial) and steroids (complete), we concluded that the itchiness has an underlying allergic cause. A neurological component of the itch therefore seemed unlikely, but it could not be completely excluded without further diagnostic imaging or necropsy, which was declined by the owner.

EDS-affected pets with other concurrent diseases inducing self-trauma (such as itchiness in case no. 4 due to FASS) have exacerbated symptoms that might lead to euthanasia. A faster whole genome sequencing-based diagnosis of the primary collagen genetic disease might lead to a better overall management of the patients, with the potential of decreasing the euthanasia rates of such complicated cases. A precise classification of EDS relies on molecular confirmation with identification of a causative variant [2]. This study illustrates the potential of whole genome sequencing as a precision medicine approach in animals with inherited diseases.

Consensus criteria for the interpretation of genetic variants in domestic animals have not yet been defined. However, such criteria have been established for human patients and they have proven highly useful to provide a standardized classification reflecting the strength of evidence for pathogenicity [42]. When we apply the human guidelines to the investigated cats, all three reported *COL5A1* variants would be classified as pathogenic according to the ACMG/AMP criteria [42]. All three reported variants represent null variants (very strong evidence of pathogenicity, PVS1), are absent from controls (moderate evidence of pathogenicity, PM2), and the patients' phenotypes are highly specific for a disease with a single genetic etiology (supporting evidence of pathogenicity, PP4). For case no. 4 we have the confirmed de novo mutation event and family segregation data as additional criteria for pathogenicity.

5. Conclusions

We characterized four cats with EDS and identified three independent causal variants in the *COL5A1* gene. De novo mutation events leading to haploinsufficiency of *COL5A1* appear to represent an important molecular etiology in feline EDS.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/genes13050797/s1, Figure S1: Pedigree with genotypes from the family of case no. 4. Table S1: Candidate Genes; Table S2: Public Genomes; Table S3: Primer Sequences; Table S4: Private Variants.

Author Contributions: Conceptualization, T.L., N.A., N.M.F. and S.K. (Sabine Kaessmeyer); investigation, S.K. (Sarah Kiener), N.A., J.S., F.L., S.S., M.W., N.M.F. and S.K. (Sabine Kaessmeyer); data curation, V.J.; visualization, S.K. (Sarah Kiener), N.A., J.S., M.W., N.M.F. and S.K. (Sabine Kaessmeyer); writing—original draft preparation, S.K. (Sarah Kiener), N.A., M.W., N.M.F. and S.K. (Sabine Kaessmeyer); writing—review and editing, S.K. (Sarah Kiener), N.A., J.S., P.-K.H., F.L., V.J., C.S., S.S., M.W., U.M., T.L., N.M.F. and S.K. (Sabine Kaessmeyer). All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal procedures were performed according to local regulations. The "Cantonal Committee for Animal Experiments" approved the collection of blood samples from cats (Canton of Bern; permit 71/19).

Informed Consent Statement: The owners of the cats in this study gave informed consent to use samples and data for research.

Data Availability Statement: The accessions for the sequence data reported in this study are listed in Table S2.

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Conflicts of Interest: The authors declare no conflict of interest.

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SOAT1 missense variant in two cats with sebaceous gland dysplasia

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ORIGINAL ARTICLE

SOAT1 missense variant in two cats with sebaceous gland dysplasia

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Abstract

Spontaneously arisen hereditary diseases in domestic animals provide an excellent opportunity to study the physiological functions of the altered genes. We investigated two 4-month-old sibling domestic short haired kittens with dry dark debris around the eyes, nose, and ears, dark crusting on the legs and a thin poor hair coat. Skin biopsies revealed abnormal sebaceous gland morphology with lack of normal sebocyte arrangement and differentiation. Hair follicles had a distorted silhouette, interpreted as a change secondary to the observed sebaceous gland dysplasia. Whole genome sequencing on both affected kittens and 65 genetically diverse feline genomes was performed. Filtering for variants that were present in both kittens but absent from the control genomes revealed a homozygous missense variant in *SOAT1*, encoding sterol O-acyltransferase 1. The protein is localized in the endoplasmic reticulum and catalyzes the formation of cholesteryl esters, an essential component of sebum and meibum. The identified *SOAT1*:c.1531G > A variant is predicted to change a highly conserved glycine residue within the last transmembrane domain of SOAT1, p.Gly511Arg. In mice, variants in *Soat1* or complete knockout of the gene lead to the "hair interior defect" (hid) or abnormal Meibomian glands, respectively. *SOAT1*:c.1531G > A represents a plausible candidate variant for the observed sebaceous gland dysplasia in both kittens of this study. The variant was not present in 10 additional cats with a similar clinical and histopathological phenotype suggesting genetic heterogeneity. *SOAT1* variants should be considered as potential cause in hereditary sebaceous gland dysplasias of humans and domestic animals.

Keywords *Felis catus* · Cat · Dermatology · Genodermatosis · Animal model · Precision medicine · Veterinary medicine

Introduction

Sebaceous glands are small exocrine glands and produce sebum, which is a complex mixture of lipids. Sebum composition differs between species, most likely due to species-specific functional requirements (Picardo et al. 2009). Most sebaceous glands are associated with hair follicles where they constitute a crucial component of the pilosebaceous unit. Sebum is released by holocrine secretion into the follicular canal (Montagna 1967; Geueke and Niemann 2021). The secretion promotes skin barrier function, contributes to proper hair follicle growth and homeostasis, serves as hydrophobic shield for the hair coat, and plays a dynamic role in thermoregulation (Zouboulis 2010; Shamloul and Khachemoune 2021a; Zouboulis et al. 2022). Sebum fulfills additional functions such as eccrine emulsification, synthesis of cytokines, chemokines, interleukins, pheromone and fatty acids, acid mantle formation, and hormone production (Shamloul and Khachemoune 2021a). Glands are larger and

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more numerous on the face, external auditory canal, and anogenital surfaces.

Meibomian glands are modified sebaceous glands on the palpebral border, which secrete meibum into the tear fluid to prevent its evaporation and protect the ocular surface (Montagna 1967; Shamloul and Khachemoune 2021a). Meibum has a unique composition of neutral lipids different from sebum (Butovich 2017).

Abnormal sebaceous gland activity has been implicated in a number of medical conditions and defective sebaceous glands have been linked to a variety of skin disorders (Shamloul and Khachemoune 2021b; Geueke and Niemann 2021).

Numerous mouse mutants with abnormal sebaceous glands have been reported (Ehrmann and Schneider 2016). However, it often remains unclear whether the observed phenotypes are a direct consequence of aberrant sebaceous gland development and/or sebaceous gland activity or rather unspecific secondary changes of other more general skin and hair follicle defects (Geueke and Niemann 2021).

In cats, reports about primary sebaceous gland disorders are rare (Scott 1989; de Sepibus et al. 2004). Sebaceous adenitis in cats is usually associated with mural folliculitis and either of unknown cause or associated with an internal malignancy (Scott et al. 1995; Pascal-Tenorio et al. 1997; Gross et al. 2001; Rottenberg et al. 2004; Singh et al. 2010; Linek et al. 2015; Kasabali et al. 2017).

Yager et al. reported ten kittens with a congenital dermatosis and abnormal sebaceous gland morphology (Yager et al. 2012). To the best of our knowledge, so far, no causal genetic variants for sebaceous gland related pathologies in domestic animals have been reported in the scientific literature.

The aim of the present study was to characterize the clinical and histopathological features of two cat siblings with striking skin abnormalities and to investigate a possible underlying genetic defect.

Materials and methods

Ethics statement

The cats in this study were privately owned and skin biopsies and blood samples for diagnostic purposes were collected with the consent of their owners. The collection of blood samples was approved by the Cantonal Committee for Animal Experiments (Canton of Bern; permit BE71/19). All animal experiments were done in accordance with local laws and regulations.

Clinical and histopathological examinations

A physical examination of the two index cases was performed by the attending veterinarians. Two to four 4–6 mm skin punch biopsies per cat were taken and routinely processed for histopathology. Hematoxylin and eosin (H&E) stained slides were reviewed by board certified veterinary pathologists (B.G.M., V.K.A., J.A.Y.). A full necropsy was performed after euthanasia.

Animal selection for genetic analyses

The study included a total of 728 cats. Two of them represent the index cases for the sebaceous gland dysplasia phenotype described in this study. During the course of this study, we investigated samples from ten additional cats with similar clinical and histopathological phenotypes. Information on all 12 cases diagnosed with sebaceous gland dysplasia is compiled in Table S1. The remaining 716 cats represent a genetically diverse convenience cohort from the Vetsuisse Biobank. No consistent phenotype information on these cats is available and they were considered population controls.

DNA extraction

Genomic DNA was extracted from EDTA blood, native tissue samples, or formalin-fixed paraffin-embedded (FFPE) tissue samples. Extractions were performed with the Maxwell® RSC Whole Blood DNA Kit, Maxwell® RSC PureFood GMO and Authentication Kit, or Maxwell® RSC DNA FFPE Kit, respectively, on a Maxwell® RSC 48 instrument (Promega, Dübendorf, Switzerland).

Whole genome sequencing and variant calling

The genome of both affected kittens was sequenced at 20×coverage using PCR free libraries on an Illumina NovaSeq 6000 instrument. The sequencing data was mapped to the genome reference and variant calling was done as described before (Jagannathan et al. 2019; Kiener et al. 2021). Here, we used the cat genome reference assembly F.catus_Fca126_mat1.0 and NCBI Annotation Release 105 (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Felis_catus/105/). For the filtering of private variants, we used previously obtained genome sequences from 65 genetically diverse cats (Table S2). All sequence data were deposited at the European Nucleotide Archive and accession numbers are listed in Table S2.

Targeted genotyping

We used Sanger sequencing to genotype the candidate variant (XM_011291017.4:c.1531G > A). PCR

amplification, subsequent sequencing and data analysis was performed as described (Kiener et al. 2021). The primer sequences used for this experiment are given in Table S3.

Results

Clinical history

Five kittens born from a feral mother were presented at a shelter for first examination at approximately 4 months of age. Two kittens, one male and one female, had similar skin lesions of dry dark brown to black debris around ears, eyes, nares and dark crusting along legs associated with partial alopecia (Fig. 1A). The remaining hair coat was thin, in poor condition, and easily epilated. Other skin surface areas were covered with the same brown material to a lesser degree. The lesions persisted, progressed and failed to completely resolve over a time period of 4 months despite multiple attempted treatments (shampoo bathing, antibiotics, food trial, olive oil bathing, Revolution®, Convenia®, terramycin, terbinafine, famciclovir). Shampoo bathing and famciclovir seemed to have helped the most, but still resulted in very little improvement. Concurrent dermatophytosis (*M. canis*) developed and was treated when skin biopsy was elected. Both affected kittens were euthanized due to persistent skin problems and unlikelihood of adoption. The mother and the other littermates did not have skin lesions.

Histopathological examination

The main pattern observed was the generalized abnormal sebaceous gland morphology identified in both primary skin biopsies and post mortem samples of skin (Fig. 2A, B). Some glands were proliferative with reserve cell predominance, increased mitotic activity and apoptosis (Fig. 2B). The apoptosis was not associated with satellitosis. There was an almost total absence of normal differentiation from reserve cells to mature lipid laden sebocytes. The number of mature sebocytes was decreased. Peri-isthmus lymphocytic infiltrates were present around some sebaceous glands with minimal to absent follicular mural involvement. Most hair follicles were in anagen phase. There was evidence of follicular dysplasia with wavy contours and few misshapen anagen bulbs. Dark brown to black discoloration of follicular keratin was visible, as well as frequent misshapen and malacic hair shafts, some of which were mineralized. Superficial perivascular inflammation was very minimal to absent and likely secondary to superficial infections. The epidermis showed moderate to marked orthokeratotic hyperkeratosis and was variably acanthotic. These findings led to a final diagnosis of diffuse sebaceous gland dysplasia with follicular dysplasia, hair shaft malacia, and moderate to marked hyperkeratosis.

Genetic analysis

We performed whole genome sequencing on both affected kittens and compared the data to 65 genomes from genetically diverse cats. Hierarchical filtering steps were applied to identify a candidate causative variant for the sebaceous

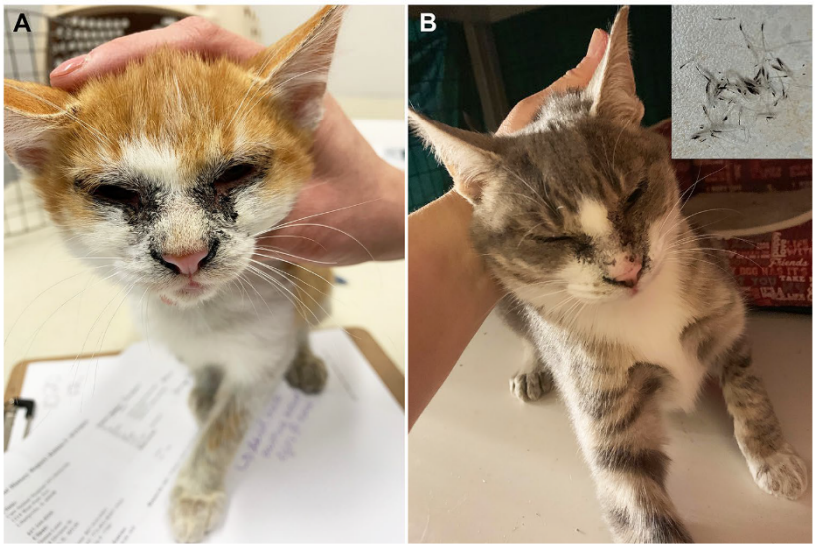

Fig. 1 Clinical phenotype of affected kittens. **A** Case 2, bilateral symmetrical scaling with black debris, hair clumping and hypotrichosis periocular and surrounding the nostrils and legs. **B** Case 1, similar changes to case 2, however, less florid. Insert top right: note easily epilated clump of hairs with scaling and black debris

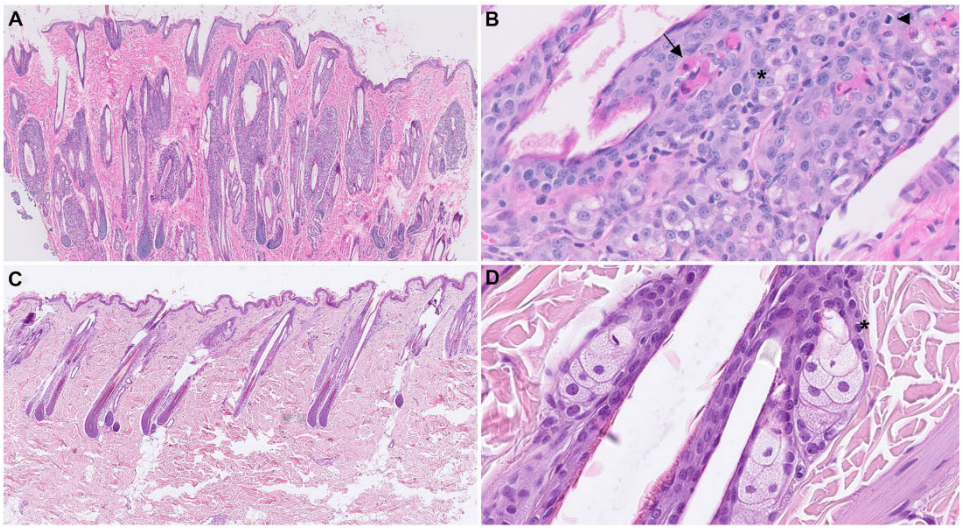

Fig. 2 Histopathological phenotype of an affected kitten (case 2). **A** Case 2, variably prominent sebaceous glands with abnormal morphology, follicular dysplasia and basket weave hyperkeratosis, H&E 20x. **B** Case 2, abnormal sebaceous gland arrangement and differentiation characterized by increased number of reserve cells (asterisk), apoptotic cells (arrow) and mitotic activity (arrowhead), H&E 400x. **C** Age matched control, normal pilosebaceous morphology with well differentiated sebaceous glands at the isthmus level, H&E 20x. **D** Normal sebaceous gland maturation from reserve cell (asterisk) to mature sebocytes, H&E 400x

gland dysplasia phenotype. Of all variants present in both kittens, we filtered for shared private variants, i.e. variants that were present in both kittens but absent in the 65 control genomes that we used. We subsequently filtered these for protein-changing variants with a SnpEff impact of "high" or "moderate." The final step prioritized the variants for functional candidate genes. These filtering steps identified a clear top candidate variant that can be designated as ChrF1:20,914,140G > A with respect to the F.catus_Fca126_mat1.0 genome reference assembly (Table 1, Tables S2, S4).

The identified variant represented a missense variant in the *SOAT1* gene, XM_011291017.4:c.1531G > A. It is predicted to change an evolutionarily conserved glycine residue in the last transmembrane domain of sterol O-acyltransferase 1, XP_011289319.1:p.(Gly511Arg) (Fig. 3). The effect of the amino acid exchange was classified as deleterious or pathogenic by the variant impact predictors PredictSNP (87% probability, Bendl et al. 2014), Provean (score − 6.610, Choi and Chan 2015), and MutPred2 (score 0.767, Pejaver et al. 2020).

We confirmed the presence of the c.1531G > A variant in genomic DNA of both affected kittens by Sanger sequencing. Both kittens were homozygous for the mutant allele, consistent with an autosomal recessive mode of inheritance. We further genotyped archived samples from 10 additional cats with a similar clinical and histopathological phenotype, as well as 716 genetically diverse population controls from the Vetsuisse Biobank, all of which were homozygous wildtype (Table 2).

Discussion

The two kittens investigated in this study displayed a distinct phenotype, which we refer to as sebaceous gland dysplasia. This unique constellation of clinical and histopathological changes was initially described in ten

Table 1 Summary of the variant filtering steps in both kittens affected with sebaceous gland dysplasia

Filtering step	Homozygous	Heterozygous
Shared variants in both affected kittens	3,944,823	3,181,925
Shared private variants in both affected kittens	4006	34,891
Shared private and protein-changing variants	16	157
In plausible functional candidate genes	1	0

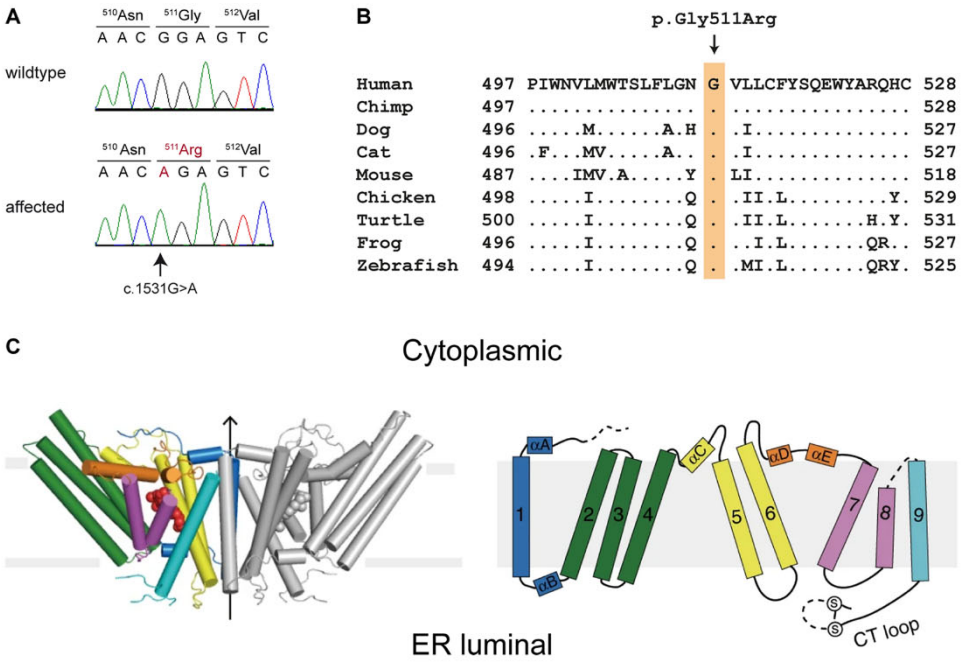

Fig. 3 Details of the *SOAT1*: c.1531G>A, p.Gly511Arg variant. **A** Electropherograms with the amino acid translations of two cats with different genotypes. The arrow indicates the single nucleotide change. **B** Multi-species sequence alignment of the last transmembrane domain of SOAT1 harboring the p.Gly511Arg variant. **C** Structural model of a SOAT1 dimer in side view (left image) and the topological model of SOAT1 (right image). The full functional enzyme consists of a dimer of dimers (images from Guan et al. 2020)

Table 2 Genotype–phenotype association at the *SOAT1*:c.1531G>A variant with sebaceous gland dysplasia

Cats	G/G	G/A	A/A
Affected kittens (littermates), index cases ($n=2$)	–	–	2
Unrelated affected cats ($n=10$)	10	–	–
Unrelated control cats ($n=716$)	716	–	–

unrelated kittens (Yager et al. 2012). In accordance with this initial study, lesions developed at a very young age pointing towards a congenital disorder. Interestingly, a clinical presentation of progressive hypotrichosis/alopecia rather than scale was the predominant feature in the previously reported kittens (Yager et al. 2012). In addition to hypotrichosis/alopecia, the two index cases of the present study had a bold black debris covering the skin surface, which we interpreted as adherent oxidized sebum and keratin. Histopathological findings were unique with diffusely abnormal sebaceous gland morphology. The characteristic histopathology included disrupted sebaceous gland maturation with reduction of mature sebocytes, increased number of undifferentiated reserve cells and presence of apoptotic cells. In contrast to the ten cases reported previously (Yager et al. 2012), sebaceous glands looked proliferative in some areas and vacuolated sebocytes with prominent eosinophilic globules were not as obvious.

The pedigree with two out of five littermates being affected, the early onset and the phenotypic characteristics suggested a genetic cause. We therefore, investigated the genomes of the affected kittens and searched for plausible candidate variants. The whole-genome sequencing data of both kittens identified 173 protein-changing variants that were exclusively present and shared in these 2 cats, but absent from 65 control genomes. We prioritized genes based on the clinical and histologic findings, and one variant was of particular interest. It was a homozygous missense variant in *SOAT1*, encoding sterol O-acyltransferase 1. This intracellular protein is localized in the membrane of the endoplasmic reticulum, where it catalyzes the formation of cholesteryl esters from cholesterol and long chain fatty acyl-CoA. It thus maintains the ratio of free cholesterol and cholesteryl esters in the cells (Wu et al. 2010). Cholesteryl esters are stored in lipid droplets within the cell or transported to other tissues (Guan et al. 2020). They are important components

of sebum and meibum and fulfill essential functions in the lipid envelope of the epidermis (Butovich 2017).

The mature SOAT1 protein contains nine transmembrane domains and is incorporated into the ER membrane as a dimer of dimers. The central cavity is formed by the six transmembrane helices 4–9, and the transmembrane helices 1, 6 and 9 are involved in dimer assembly (Guan et al. 2020; Long et al. 2020). The identified variant in the affected kittens from this study is predicted to change a highly conserved glycine residue in the ninth and last transmembrane domain, p.Gly511Arg. We hypothesize that this compromises the enzymatic activity.

In a genetically engineered *Soat1*-null mouse model (Meiner et al. 1996), the predominant phenotype is Meibomian gland dysfunction. $Soat^{-/-}$ mice had notably smaller eye openings and very thick meibum with lipid-like debris around the eye openings. The Meibomian lipids showed cholesterol instead of cholesteryl esters as the dominant lipid. Abnormalities in their skin and fur coat were not visible (Butovich et al. 2021). However, the spontaneous "hair interior defect" (hid) caused by a 6.8 kb deletion in *Soat1* was reported in the AKR/J mouse (Trigg 1972; Wu et al. 2010). Characteristics of the hid-phenotype included hair with deficiency in projections of cortex cells and low levels of trichohyalin (Wu et al. 2010).

In the affected kittens from this study, the Meibomian glands were not examined. However, on clinical pictures, the eye openings were very small, possibly due to ocular surface irritation, similar to those of *Soat1*-null mice (Butovich et al. 2021). The results of both mouse models support our hypothesis that the identified feline *SOAT1* variant might cause the observed phenotype through a direct impact of defective SOAT1 on sebaceous gland development and/or function. Alternatively, an additional indirect effect of this variant has to be considered, as in vitro studies of *SOAT1*-knockdown cells showed a reduced expression of cholesterol metabolism genes and fatty acid biosynthesis genes (Zhu et al. 2021). Deficiency of *Scd1* encoding stearoyl-coenzyme A desaturase 1 also results in aberrant sebaceous and/or Meibomian glands in mice (Ehrmann and Schneider 2016).

Unfortunately, both kittens from our study were euthanized shortly after presentation and before the genetic investigations were completed. Therefore, no suitable samples for functional follow-up such as an analysis of the lipid content of the skin and tear fluid were available.

Since the clinical and histopathological features of the cat siblings from this study were similar to those described by Yager et al. (2012), we genotyped ten additional cases with similar phenotypic changes including one cat from the Yager et al. (2012) study for the identified variant. All ten additional cats were homozygous for the wildtype allele at the *SOAT1*:c.1531G>A variant. As already previously proposed, the term sebaceous gland dysplasia does not represent a homogeneous condition, but rather the phenotypic expression of more than one disorder of sebaceous gland development (Yager et al. 2012). Our genotyping results support this statement and suggest genetic heterogeneity of this phenotype.

In conclusion, we describe the clinical and histopathological findings of two kittens with sebaceous gland dysplasia. Whole-genome sequencing revealed a homozygous candidate variant in *SOAT1*, p.Gly511Arg, as a potential and highly plausible underlying defect. Further studies are required to evaluate the exact functional impact of the variant. Our results propose a new candidate gene for sebaceous gland dysplasia phenotypes, which might be relevant for future unsolved cases in veterinary and human medicine.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00438-023-02020-6.

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Author contributions Conceptualization, VKA and TL; investigation, SK, BM and VKA; data curation, VJ; writing—original draft, SK, BM, VKA and TL; writing—review and editing, SK, BM, VKA, MW, JAY, VJ and TL; supervision, TL All authors have read and agreed to the published version of the manuscript.

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Data availability The genome sequence data were submitted to the European Nucleotide Archive (ENA). All accession numbers are listed in Table S2.

Declarations

Conflict of interest The authors declare no conflict of interest.

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Identification of an *ADAMTS2* frameshift variant in a cat family with Ehlers-Danlos syndrome

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Investigation

Identification of an *ADAMTS2* frameshift variant in a cat family with Ehlers–Danlos syndrome

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Abstract

We investigated 4 European domestic shorthair kittens with skin lesions consistent with the dermatosparaxis type of the Ehlers–Danlos syndrome, a connective tissue disorder. The kittens were sired by the same tomcat but were born by 3 different mothers. The kittens had easily torn skin resulting in nonhealing skin wounds. Both clinically and histologically, the skin showed thin epidermis in addition to inflammatory changes. Changes in collagen fibers were visible in electron micrographs. The complete genome of an affected kitten was sequenced. A one base pair duplication leading to a frameshift in the candidate gene *ADAMTS2* was identified, p.(Ser235fs*3). All 4 affected cats carried the frameshift duplication in a homozygous state. Genotypes at this variant showed perfect cosegregation with the autosomal recessive Ehlers–Danlos syndrome phenotype in the available family. The mutant allele did not occur in 48 unrelated control cats. *ADAMTS2* loss-of-function variants cause autosomal recessive forms of Ehlers–Danlos syndrome in humans, mice, dogs, cattle, and sheep. The available evidence from our investigation together with the functional knowledge on *ADAMTS2* in other species allows to classify the identified *ADAMTS2* variant as pathogenic and most likely causative variant for the observed Ehlers–Danlos syndrome.

Keywords: skin, dermatology, dermatosparaxis, veterinary medicine, *Felis catus*, whole genome sequence, precision medicine

Introduction

The Ehlers–Danlos syndrome (EDS, *Fibrodysplasia elastica*) represents a group of hereditary disorders associated with defects in collagen biosynthesis (Malfait *et al.* 2017). This heterogenous group of connective tissue disorders is named after Edvard Ehlers and Henri-Alexandre Danlos who independently described human patients with the syndrome in detail at the beginning of the 19th century (Parapia and Jackson 2008).

Signs may vary between species and the different types of EDS, e.g. joint hypermobility is primarily observed in humans. However, a universally occurring feature is the hyperelasticity of the skin and the resulting tendency to skin tears. One type of EDS, called dermatosparaxis EDS (dEDS), was first observed, and its biochemical background was described in detail in cattle (Lapière *et al.* 1971) (OMIA 000328-9913). The connective tissue shows alterations of its normal structure due to a deficiency of the enzyme procollagen peptidase, which catalyzes the formation of type 1 procollagen (Lapière and Nusgens 1993). The structurally abnormal dermal collagen shows decreased strength; therefore, skin wounds can already be caused by minimal trauma like handling or even normal activity (Counts *et al.* 1980; Crosaz *et al.* 2013; Hargis and Myers 2017). Histologically, dermatosparaxis skin shows variations in terms of the caliber of collagen fibers, which are irregular, undirected, and loosely arranged (Colige *et al.* 2004; van Damme *et al.* 2016). Variants in the ADAMTS2 gene are known to cause dermatosparaxis in humans (Colige *et al.* 2004; van Damme *et al.* 2016) as well as in cattle (Colige *et al.* 1999), sheep (Zhou *et al.* 2012; Monteagudo *et al.* 2015; Joller *et al.* 2017), and dogs (Jaffey *et al.* 2019). The ADAMTS2 gene encodes ADAM metallopeptidase with thrombospondin type 1 motif 2, also termed procollagen I N-proteinase, which cleaves the propeptides of procollagen type I and II (Wang *et al.* 2003). The role of different ADAMTS proteases for normal collagen biosynthesis and in dEDS has been reported (Le Goff *et al.* 2006).

Different forms of EDS are known to occur in humans (Malfait *et al.* 2017, 2020) as well as in several animal species, including cattle (Hanset and Lapiere 1974; Carty *et al.* 2016; Jacinto *et al.* 2020), dog (Bauer *et al.* 2019; Jaffey *et al.* 2019; Kiener *et al.* 2022b), sheep (Joller *et al.* 2017), cat (Counts *et al.* 1980; Weingart *et al.* 2014; Spycher *et al.* 2018), horse (Eßer *et al.* 1999), and mink (Hegreberg

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1975). Diagnosis is mainly based on the clinical appearance of the affected animal, histopathological examination of the collagen fibrils, and genetic analyses. In domestic cats, until now, only one gene, *COL5A1*, was reported to be involved in autosomal-dominant EDS (Spycher *et al.* 2018; Kiener *et al.* 2022a). Herein. we report the results of a comprehensive clinical, pathological and genetic analysis of dEDS in a cat family.

Materials and methods

Ethics statement

All cats in this study were privately owned. The index case, a deceased kitten, was transferred to the Institute of Veterinary Pathology of the Justus Liebig University Giessen for diagnostic purposes. The other 3 affected kittens were examined and treated at the Small Animal Clinic of the Justus Liebig University Giessen. All animals in this study were examined with the consent of the owner and handled according to good ethical standards. The "Cantonal Committee for Animal Experiments" (Canton of Bern; permit 71/19) and the Regional Council of Gießen (reference number 19 c 20 15 h 02 Gi 19/1 KTV 22/2020) approved the collection of samples from control cats.

Animals

A group of free roaming farm cats (European domestic shorthair) is presented here. Initially, one female kitten with skin lesions resembling the appearance of dermatosparaxis was found dead. Later, 3 additional affected kittens were observed in 2 subsequent litters. All 3 litters that produced affected kittens were apparently from the same sire (tomcat). Subsequently, as many cats as possible ($n = 27$) from this semi-feral population, including mothers, littermates, and the tomcat, were captured for sampling and visual inspection. Despite the free roaming lifestyle of the cats, the farmer and owner of the cats was able to provide information about the kinship of the population.

Clinical and pathological examination

Standard clinical and pathological examinations were done. Necropsy was performed on all affected kittens, and representative organ samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (HE). Additionally, histochemical stains were performed on the skin, included periodic acid–Schiff reaction (PAS) and Masson trichrome stain. The skin of one affected kitten was examined by transmission electron microscopy. For this purpose, skin samples were fixed with 1.5% glutaraldehyde and 1.5% formaldehyde (freshly made from paraformaldehyde) in 0.15 M HEPES buffer. For epoxy resin embedding, cells were postfixed in 1% osmium tetroxide in aqua bidest, stained in half-saturated watery uranyl acetate, dehydrated in an ascending ethanol series, and finally embedded in Agar 100 (Agar scientific Ltd., UK). Ultrathin sections were cut using an ultramicrotome (Reichert Ultracut E, Leica) and examined with a transmission electron microscope (Zeiss EM 902). Digital images were captured with a slow-scan 2 K CCD camera (TRS, Tröndle, Moorenweis, Germany).

DNA extraction

For the purpose of whole genome sequencing, genomic DNA was isolated from muscle tissue of the deceased kitten using a Maxwell RSC Tissue DNA Kit and a Maxwell RSC instrument (Promega, Dübendorf, Switzerland). For genotyping, DNA extraction from buccal swaps (sterile transport swabs; COPAN Italia SpA, Brescia, Italy) was executed using the Gentra Puregene Tissue Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions.

Whole genome sequencing, variant calling, and variant filtering

An Illumina TruSeq PCR-free DNA library with ~330 bp insert size of the deceased affected cat was prepared and sequenced on a NovaSeq 6000 instrument with 23× coverage (Illumina, San Diego, CA, USA). The sequence data were submitted to the European Nucleotide Archive with the study accession PRJEB7401 and sample accession SAMEA7376282. Mapping and alignment to the F.catus Fca126 mat1.0 reference genome assembly were performed as described (Jagannathan *et al.* 2019). Variant calling was performed using GATK HaplotypeCaller (McKenna *et al.* 2010) in gVCF mode as described (Jagannathan *et al.* 2019). Functional effects of the called variants were predicted with the SnpEff version 4.3t software (Cingolani *et al.* 2012) together with NCBI annotation release 105 for the F.catus_Fca126_mat1.0 genome reference assembly.

For variant filtering, we used 77 control genomes (Supplementary Table 1). A hard filtering strategy was employed, which required either a homozygous alternate (1/1) or heterozygous (0/1) genotype call in the affected kitten, while the 77 control cats were required to have either a homozygous reference (0/0) or missing (./.) genotype call in the vcf-file (Supplementary Table 2). Variants in 20 known functional candidate genes for EDS obtained from Kiener *et al.* 2022b were prioritized.

Genotyping by Sanger sequencing

The *ADAMTS2* variant was genotyped by Sanger sequencing of PCR amplicons [XM_023254116.2:c.698dup or ChrA1:90,995,621dup (F.catus_Fca126_mat1.0 assembly)]. A forward primer 5′-TTCAATGTACCTGGCAAGCC-3′ and a reverse primer 5′-ATGCTGCAGATGGTGACTAC-3′ were designed with the software Primer3 (Untergasser *et al.* 2012) to produce a fragment with a size of 169 bp (wild type) or 170 bp (mutant) with standard PCR conditions. Purified PCR products were sent to LGC Genomics GmbH, Berlin (Germany), for Sanger sequencing, using the reverse primer. A similar approach was used to genotype the *COL1A2*:XM_003982764.6:c.2384G > A variant, using 5′-TCCCTAGAGCTGCCATTGAT-3′ and 5′- GAGGCAAGGTTGTTTG GCTA-3′ as forward and reverse primers, respectively (152 bp fragment size).

Parentage testing

A DNA profile, based on 16 microsatellite markers, for parentage verification was commissioned from Laboklin GmbH & Co KG (Bad Kissingen, Germany). It was carried out with genomic DNA from the 3 mother cats, the 4 affected kittens, and the presumed father.

Results

Clinical and pathological findings

The initial case, a deceased female kitten of unknown age was in good body condition (weight: 1 kg). Body and tissues were affected by moderate postmortem changes. In addition to moderate anemia, the skin was markedly thin and soft and was easily torn. Large portions of the head, as well as the left side of the neck, exhibited extensive alopecia and severe multifocal ulcerative and purulent dermatitis, occasionally accompanied by partially detachable dark brown crusts up to 1 cm thick (Fig. 1a). Additionally, there was a prolapse of the rectum (Fig. 1b) as

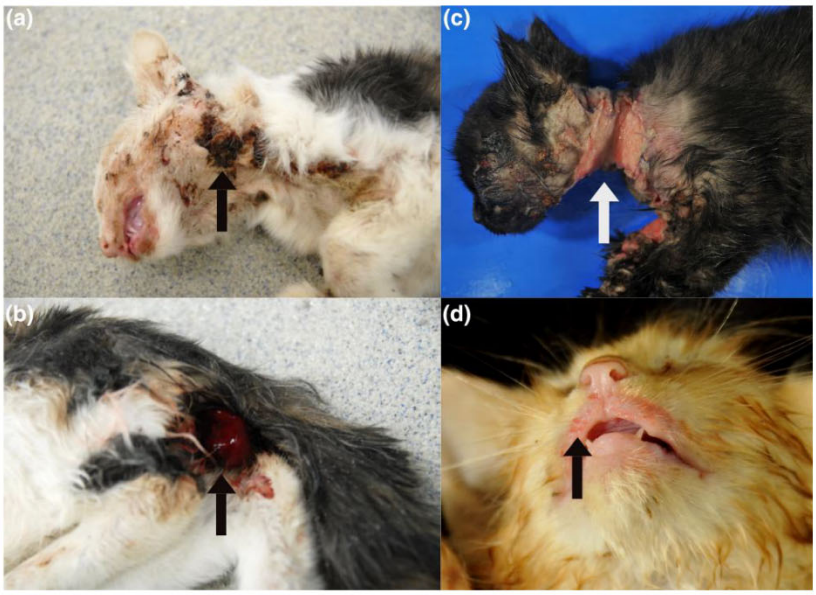

Fig. 1. Gross condition of the deceased affected kittens (a and b initial case; c and d from second and third litter). a) Almost the entire head and the left neck showed extensive alopecia and severe multifocal ulcerative and purulent dermatitis occasionally accompanied by barky, dark-brown crust formation (arrow). The oral mucosa was moderately anemic. b) A rectal prolapse was also present in the initial case (arrow). c) After surgical treatment: severe loss of the epidermis especially in the cranial body regions (head, neck, forelimbs) with severe ulcerative partly crustose dermatitis and fragile skin, that tore at light touch (arrow); d) Kitten with a milder course, gross lesions were found exclusively on the head (temples and mucocutaneous junctions) with mild to moderate ulceration and crusting (arrow).

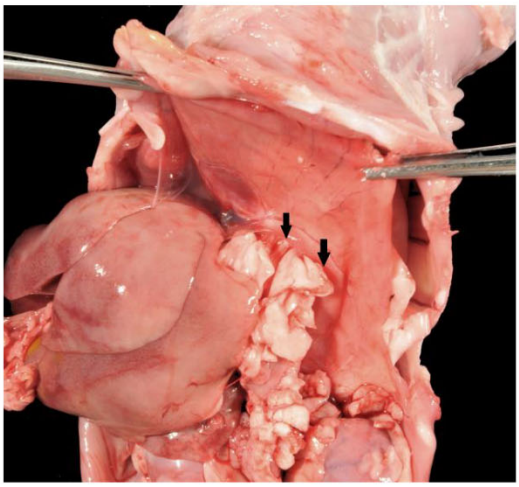

Fig. 2. Abdominal cavity (lower and middle part of the figure) with a diaphragmatic hernia (arrows). The stomach and large parts of the omentum majus passed through this defect into the thoracic cavity, which is located behind the diaphragm in the upper part of the figure.

well as an invagination in the colon involving 3 cm of the large intestine with venous infarction of the invaginated part (intussusceptum). A diaphragmatic hernia, through which the stomach and large portions of the omentum majus entered the thoracic cavity (Fig. 2), was also observed. The urea concentration in the aqueous humor was 20 mmol/L (reference value: 5.0–11.3 mmol/L).

Three additional affected kittens from the following litters showed similar dermatological lesions as the first kitten (Fig. 1c and d). During handling, the skin was easily torn, and preexisting wounds were exacerbated even by gentle manipulation. Wounds in different stages and sizes were present. The head, ventral neck and front legs, and axillar region were most severely affected in all 3 cats; distribution of the lesions was more or less symmetrical. In addition, these kittens showed significantly reduced growth compared to their unaffected littermates. One of the cats was euthanized at first presentation due to an impaired general condition. In 2 of the 3 kittens, a symptomatic therapy with topical wound care and systemic anti-infective treatment were attempted, but due to progressive deterioration, both cats were humanely euthanized 5 days and 33 days after start of therapy, respectively.

Histological examination revealed that the skin of the initial case was multifocally affected by both a mild to severe chronic pyogranulomatous and an acute ulcerative and suppurative dermatitis accompanied by serocellular crust formation, which contained bacteria (Fig. 3a). Adjacent to the ulcerative lesions, cleft-formation at the dermo-epithelial junction was often observed (Fig. 3a). The PAS reaction revealed that the basement membrane zone formed the floor of the cleft (Fig. 3b). The collagen fibers stained uniformly blue with Masson Trichrome stain (Fig. 3c) and showed a loose arrangement around the clefts. In the unaffected skin epidermis, dermis and adnexa were present and the collagen fibers were arranged physiologically. The invagination in the colon was accompanied by a moderate to severe chronic suppurative colitis characterized by a moderate to high amount of mononuclear cells infiltrating the intussusceptum, while the part of the colon containing the invaginated part (intussuscipiens) was infiltrated with macrophages and neutrophil granulocytes.

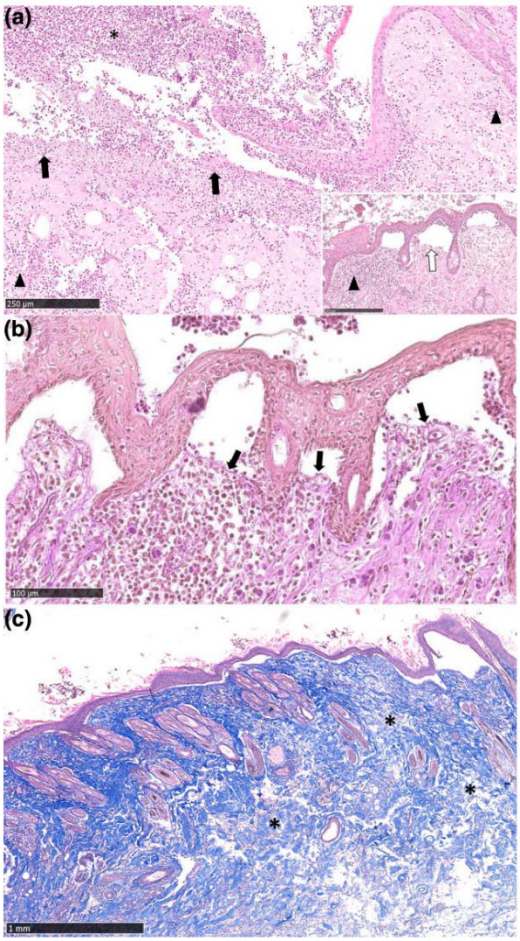

Fig. 3. Skin of the head of an affected kitten. a) HE stain: severe chronic pyogranulomatous (arrowhead) and severe acute ulcerative and suppurative dermatitis (solid arrows) accompanied by serocellular crusts which contained bacteria (asterisk). Cleft-formation (open arrows) was observed at the dermo-epithelial junction adjacent to the ulcerative lesions. b) PAS reaction: cleft-formation at the dermo-epithelial junction. The basement membrane zone (arrows) formed the floor of the cleft. c) Masson trichrome stain: collagen fibers were stained uniformly blue and loosely arranged (asterisks) in the area of the clefts.

Electron microscopy of the skin of one of the affected kittens showed severe abnormalities in the collagen fibers. The longitudinal section showed electron-loose parts framed by electron-dense filaments suggesting an "empty-tube" appearance. Cross section of collagen fibers showed electron dense ribbon-like structures up to 250 nm in diameter (Fig. 4).

Genetic analyses

The genome of one affected cat was sequenced at 23× coverage. Genome sequences from 77 cats representing 14 breeds and 35 random-bred individuals and one of unknown origin were used as controls. Filtering for private protein-changing variants in the affected cat identified 2 potentially pathogenic variants in known

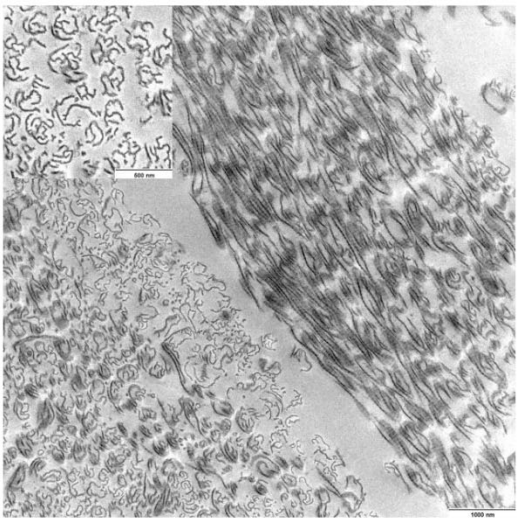

Fig. 4. Longitudinal and cross section of collagen fibers (affected kitten): thin ribbon-like electron-dense fibrils appear disordered with an electron-lucent central area (hollow appearance).

Table 1. Results of variant filtering in the affected cat against 77 control genomes.

Filtering step	Heterozygous	Homozygous
All variants in the sequenced cat	6,011,674	5,983,799
Private variants	70,995	20,434
Protein-changing private variants	353	81
Protein-changing private variants in functional candidate genes	1	1

EDS candidate genes, a heterozygous missense variant in *COL1A2*, and a homozygous frameshift variant in the *ADAMTS2* gene (Table 1; Supplementary Table 2). Genotyping of cats from the pedigree excluded the *COL5A1* variant as the genotypes did not co-segregate with the EDS phenotype, and 4 unaffected cats were homozygous for the mutant allele (Supplementary Table 3). The *COL1A2* variant was XM_003982764.5:c.2384G>A or XP_003982813.1:p.(Arg795Gln).

Visual inspection of the short-read alignments in IGV (Robinson *et al.* 2011) indicated a homozygous insertion of a single base pair in exon 4 of the 22 annotated exons of the known candidate gene *ADAMTS2* (Fig. 5). This variant can be designated as XM_023254116.2:c.698dup or XP_023109884.2:p.(Ser235Glnfs*4). It truncates nearly 80% of the wild type *ADAMTS2* open reading frame. The genomic variant designation is ChrA1:90,995,621dup (F.catus_Fca126_mat1.0).

The *ADAMTS2* variant was confirmed via PCR and follow-up Sanger sequencing. All available cats ($n = 31$) were genotyped for the variant (Fig. 6; Supplementary Table 3). All 4 affected kittens were homozygous for the mutant allele. Twenty cats were heterozygous, including the parents of affected kittens as well as some of their littermates. The remaining 7 cats were homozygous for the wild type allele. Microsatellite-based parentage testing confirmed the paternity of the suspected tomcat for all 3 litters, in which the 4 affected kittens occurred (Supplementary Table 4).

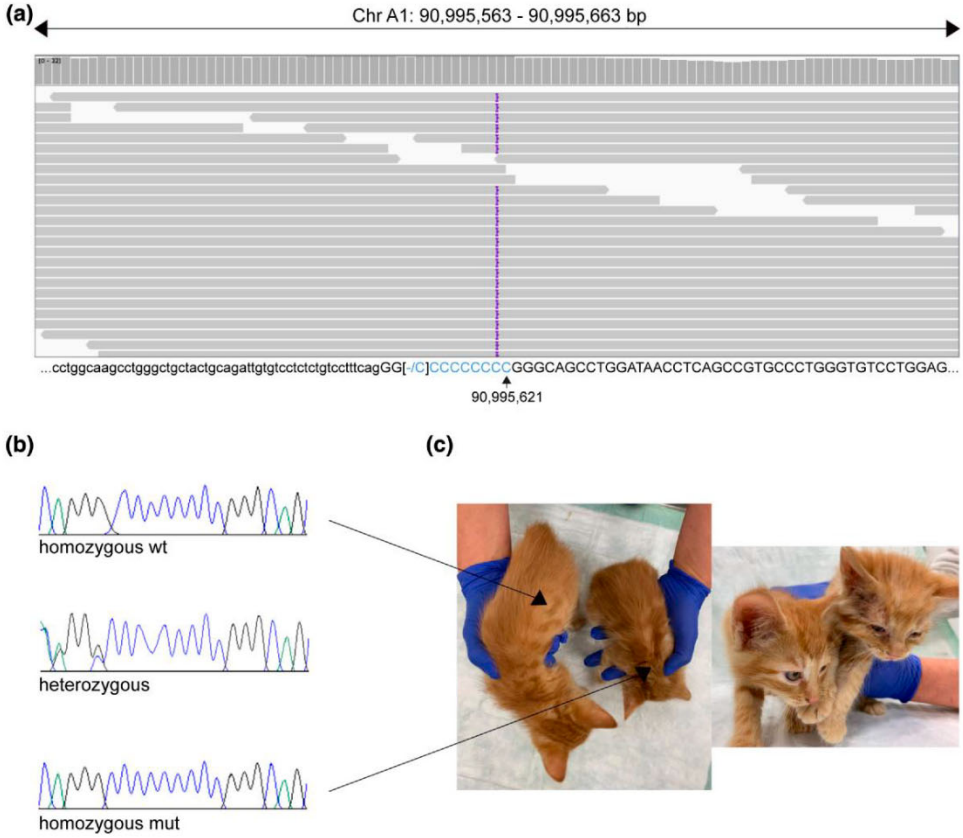

Fig. 5. The EDS-associated ADAMTS2 variant on chromosome A1. a) Integrative Genome Viewer (IGV) screenshot of the affected cat's sequence data indicates a one base pair insertion within a poly C stretch. In the IGV alignment, the insertion/duplication is at the left end of this C-stretch. However, according to the 3′-rule of HGVS, the variant is annotated as ChrA1:90,995,621dup. Coordinates refer to the F.catus_Fca126_mat1.0 assembly. Lower case letters indicate intronic, uppercase letters indicate exonic bases. b) Sanger electropherograms of an unaffected (top), a heterozygous (middle), and an affected cat homozygous for the mutant allele (bottom). Please note that Sanger sequencing was conducted using a reverse primer. Therefore, overlapping electropherogram peaks appear to the left of the heterozygous insertion/duplication. c) Phenotype of a healthy kitten (left) and an EDS-affected sibling showing typical skin lesions and growth retardation (right).

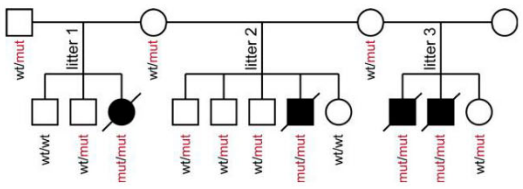

Fig. 6. Pedigree of cat family comprising 3 litters with affected kittens, all sired by the same father. Litters 1–3 are consistently numbered in Supplementary Tables 3 and 4. Males are shown as squares and females as circles. Open symbols indicate unaffected cats, which may be heterozygous carriers of the ADAMTS2:c.698dup variant as stated in the individuals' genotypes. All affected individuals, homozygous for the ADAMTS2 frameshift duplication, are deceased and indicated by filled strikethrough symbols.

Discussion

EDS in humans is known to occur in 13 different subtypes including the autosomal recessive dEDS caused by *ADAMTS2* variants (Malfait *et al.* 2017). So far, in domestic cats only classical EDS (cEDS) caused by autosomal dominant *COL5A1* variants has been characterized at the molecular level (Spycher *et al.* 2018; Kiener *et al.* 2022a).

In this study, we describe a dEDS phenotype in domestic cats due to autosomal recessive loss of function in the *ADAMTS2* gene by a comprehensive clinical, pathological and genetic analysis in a cat family. *ADAMTS2* loss-of-function variants cause autosomal recessive forms of EDS in humans, mice, dogs, cattle, and sheep but have so far not been reported in domestic cats.

During the gross and histological examination of the initial case (first deceased kitten), the skin appeared easily torn. Almost the entire head area and the left side of the neck showed focal alopecia and severe ulcerative purulent dermatitis with serocellular crusts. Similar clinical findings were present in the dermatological examination of the other 3 kittens, with the exception that fresh wounds with less crusting and without secondary pyoderma predominated. In all affected cats, the head, neck and front legs/axilla were most severely affected, which probably resulted from physiological friction and strain to the skin in these body regions. These dermatological findings were consistent with the presence of collagen dysplasia

(dermatosparaxia) in other species [overview given by (Vroman *et al.* 2021)]. For example, in hereditary equine regional dermal asthenia, body sites exposed to stress or pressure are most prone to similar lesions (Rashmir-Raven 2013). Comparable dermatological phenotypes can also be observed when caused by variants in ADAMTS2, such as in dogs (Jaffey *et al.* 2022). In previously reported cats with EDS, in which the molecular cause was not identified, skin fragility and predisposition to skin tears was also described as the main clinical finding (Crosaz *et al.* 2013; Hansen *et al.* 2015). Normal handling or even the normal activity of the animal may lead to skin injuries (Counts *et al.* 1980; Crosaz *et al.* 2013; Hargis and Myers 2017).

Hypermobility of the joints, as described for examples in humans and dogs with EDS, has not been described in cats (Mauldin and Peters-Kennedy 2015) similar to the present cases. Histologic examination of the skin showed no abnormalities except for focal loose arrangement of collagen fibers and cleft formation. This might result from the severe ulceration and inflammation and has to be differentiated from subepidermal blistering diseases. Lack of joint hypermobility and variation regarding the caliber of the collagen fibers with irregular, undirected, and loose arrangement have been described as typical for dEDS (Gross *et al.* 2008). Apart from multifocal loose arrangement of collagen fibers, the skin of the necropsied cat showed a regular anatomical morphology; however, histologic findings may vary in cats with collagen dysplasia ranging from no dermal changes up to a thinner dermis with fine collagen fibers separated by an increased amount of ground substance. Normal collagen fibers stain uniformly blue with Masson trichrome stain as in this case, whereas abnormal fibers have segmental red staining areas that are birefringent under polarized light (Butler 1975; Holbrook *et al.* 1980; Sequeira *et al.* 1999; Crosaz *et al.* 2013; Mauldin and Peters-Kennedy 2015).

Due to the postmortem changes in most of the affected kittens, the skin of only one cat was examined by electron microscopy and revealed empty tube appearance of collagen fibers typical for EDS. The inflammatory skin lesions were likely due to secondary infections and not primarily associated with dEDS. The same applies in all likelihood also for the follicular hyperplasia of the mesentric lymph nodes. A hernia diaphragmatica (Fig. 2), also observed in one of the present cases, has been previously described in cats with collagen dysplasia (Benitah *et al.* 2004). It is also possible that the rectal prolapse (Fig. 1b) represented a consequence of the collagen disturbances due to dermatospraxis EDS but might also have resulted from the colo-colic intussusception. The accompanied chronic purulent colitis suggested the presence of a bacterial infection and might have been the cause for intussusception (Uzal *et al.* 2016). Hernia diaphragmatica or rectal prolapse or any other clinical sign except the cutaneous lesions was not present in other kitten affected by EDS.

Different variants within the *ADAMTS2* were already proven to be causative for cases of dermatospraxis EDS in humans (van Damme *et al.* 2016), sheep (Zhou *et al.* 2012; Monteagudo *et al.* 2015; Joller *et al.* 2017), cattle (Colige *et al.* 1999), and dogs (Jaffey *et al.* 2019, 2022). The human ClinVar database lists NM_014244.4(*ADAMTS2*):c.691del as a pathogenic variant. This variant also introduces a frameshift at a position comparable to the feline c.698dup variant. The feline *ADAMTS2* frameshift variant detected herein therefore represents a highly plausible candidate variant for the EDS phenotype in the affected cats. The causality of the *ADAMTS2* frameshift variant is further supported by the perfect cosegregation of genotypes with phenotypes in an extended pedigree with 31 cats, of which 4 were affected.

When we apply the ACMG/AMP consensus criteria for human diagnostics (Richards *et al.* 2015) to the feline *ADAMTS2*: c.698dup frameshift variant, we have one very strong evidence for pathogenicity (null variant in a gene, where loss of function is a known mechanism of disease, PVS1), one moderate criterion (mutant allele is absent from 77 control genomes, PM2), and one supporting evidence (cosegregation with disease in multiple affected members, PP1). Taken together, these 3 lines of evidence allow to classify *ADAMTS2*:c.698dup as pathogenic.

The autosomal recessive disorder analyzed herein phenotypically resembles an EDS form that Hansen *et al.* (2015) already described for a case in Burmese cats (Hansen *et al.* 2015). No molecular genetic analysis was reported in that case. In contrast, different previously identified variants in the *COL5A1* gene were involved in autosomal dominant cEDS cases in cats (Spycher *et al.* 2018; Kiener *et al.* 2022a). Similar to EDS in humans, there are different types of this syndrome in animals that show locus heterogeneity and different modes of inheritance (Malfait *et al.* 2017).

Our analysis suggests that inbreeding within a population of free-roaming farm cats has provoked the outbreak of a lethal recessive disease. The genome of the sequenced case did not show a particularly high level of homozygous variant calls. Nonetheless, the results of our study are in agreement with a more representative study reporting 19% of UK nonpedigree cats with a higher than expected content of homozygous genome regions due to recent inbreeding events (Irving McGrath *et al.* 2021). Potential health risks due to inbreeding should be kept in mind when managing free-roaming cat populations.

Conclusion

In summary, we describe the *ADAMTS2*:c.698dup frameshift variant as a highly plausible candidate causative variant for dEDS, an autosomal recessive form of EDS in cats. Similar *ADAMTS2* variants have been reported in humans, cattle, sheep, and dogs with dEDS. The functional knowledge from other species and the perfect cosegregation of the genotypes with the phenotype in a medium sized cat family support the causality of the detected *ADAMTS2*:c.698dup variant. Our findings enable genetic testing that can be used to detect healthy carriers and to eradicate this potentially lethal disease from the cat population.

Data availability

The whole genome sequence data from this study is publicly available from ENA (European Nucleotide Archive). The accessions are listed in Supplementary Table 1. Supplementary Material provided at figshare: https://doi.org/10.25387/g3.22809347.

Supplemental material available at G3 online.

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Conflicts of interest

The authors declare no conflict of interest.

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Independent *COL17A1* Variants in Cats with Junctional Epidermolysis Bullosa

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Abstract: Epidermolysis bullosa (EB), characterized by defective adhesion of the epidermis to the dermis, is a heterogeneous disease with many subtypes in human patients and domestic animals. We investigated two unrelated cats with recurring erosions and ulcers on ear pinnae, oral mucosa, and paw pads that were suggestive of EB. Histopathology confirmed the diagnosis of EB in both cats. Case 1 was severe and had to be euthanized at 5 months of age. Case 2 had a milder course and was alive at 11 years of age at the time of writing. Whole genome sequencing of both affected cats revealed independent homozygous variants in *COL17A1* encoding the collagen type XVII alpha 1 chain. Loss of function variants in *COL17A1* lead to junctional epidermolysis bullosa (JEB) in human patients. The identified splice site variant in case 1, c.3019+1del, was predicted to lead to a complete deficiency in collagen type XVII. Case 2 had a splice region variant, c.769+5G>A. Assessment of the functional impact of this variant on the transcript level demonstrated partial aberrant splicing with residual expression of wildtype transcript. Thus, the molecular analyses provided a plausible explanation of the difference in clinical severity between the two cases and allowed the refinement of the diagnosis in the affected cats to JEB. This study highlights the complexity of EB in animals and contributes to a better understanding of the genotype-phenotype correlation in *COL17A1*-related JEB.

Keywords: *Felis catus*; WGS; dermatology; skin; JEB; splicing; precision medicine; animal model

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1. Introduction

Epidermolysis bullosa (EB) represents a group of rare genetic disorders characterized by loss of dermoepidermal integrity (blistering) in haired skin and mucous membranes in response to friction or trauma [1,2]. The prevalence of inherited forms of EB in humans ranges from 19 to 67 per million people in different countries [3]. The prevalence in veterinary medicine is unknown due to its rarity and lack of large database registries. In human medicine, EB was first described by Koebner in 1886 [4], followed by veterinary medicine, in the 1970s in sheep and dogs [5,6], then in the 1980s in horses and cattle [7,8] and in 1990s in cats [9,10]. Pathogenic variants affecting 16 distinct genes result in four main classical EB types: EB simplex (EBS), characterized by basal or suprabasal clefting; junctional EB

(JEB), characterized by clefting within the lamina lucida of the basal membrane zone (BMZ); dystrophic EB (DEB), characterized by separation within or below the lamina densa of the BMZ; and Kindler syndrome (KS). characterized by poikiloderma, trauma-induced skin blistering, mucosal inflammation, and photosensitivity [3]. EB types are further subclassified into more than 30 subtypes, according to their clinical severity (severe, intermediate, and localized, with/without extracutaneous involvement) and the underlying molecular defect [1,3]. Related to these (sub)types, several phenotypes have been described, with complications ranging from localized skin blistering, deranged wound healing, sepsis, blindness, dehydration, and gastrointestinal problems (e.g., malnutrition, esophageal stenosis) to death. In humans, the prognosis of EB varies depending on the subtype of the disorder. While some patients have normal life expectancies, others can be at major risk of death during the first few years of life [11]. Mortality related to EB may occur at different ages. It is common in early infancy in cases of severe JEB in humans [11], while in severe recessive DEB, it is more common in young adulthood [12]. Likewise, animals are euthanized or die during the first months of life. Reports of survival for months or years in animals with EB are rare [13].

Treatment options for patients with EB are limited. Allogeneic stem cell transplantation (SCT) has been proposed as a therapeutic approach, but the experience is still limited [11,14]. Therefore, the primary aim of the treatment is to protect the skin from unnecessary trauma and prevent complications, which includes nutritional support, pain management, infection control, and promotion of healing [11].

In contrast to humans, where more than 1000 variants in at least 16 genes have been associated with EB [1], molecular studies are rarely reported in domestic animals. EBS was associated with variants in *PLEC* in a litter of dogs [15], *KRT5* in cattle [16,17] and a dog [18], and *KRT14* in a cat [19]. DEB was associated with variants in *COL7A1* in cattle [20] and dogs [21]. JEB was associated with variants in all three genes encoding subunits of laminin 332, *LAMA3* in dogs [22], horses [23] and cattle [24], *LAMB3* in dogs [25], and *LAMC2* in horses [26], sheep [27], and cattle [28].

In human JEB patients, variants in the *COL17A1* gene encoding the three identical subunits of collagen type XVII often result in relatively mild phenotypes [29]. In a single dog with a severe JEB phenotype, collagen XVII deficiency was demonstrated utilizing immunofluorescence, but the underlying genetics was not investigated [30]. Collagen type XVII, also known as BP180/BPAG2, is a type-II-oriented transmembrane collagen composed of 3 identical 180 kDa α-chains [31]. It is one of the hemidesmosomal components of basal keratinocytes. Collagen type XVII links keratin intermediate filaments to the underlying dermis via plectin, BP230, laminin 332, and collagen type VII [32].

This study documents two spontaneous JEB cases in cats with independent variants in the *COL17A1* gene, leading to clinical phenotypes of different severity.

2. Materials and Methods

2.1. Ethics Statement

All cats in this study were privately owned. Skin biopsies and blood samples for diagnostic purposes were collected with the consent of their owners. The collection of blood samples from control animals for WGS analyses was approved by the Cantonal Committee for Animal Experiments (Canton of Bern, Switzerland; permit BE94/2022). A skin sample for transcript analysis from another control cat was collected post-mortem. All animal experiments were done in accordance with local laws and regulations.

2.2. Histopathological Examinations

Punch biopsies of the skin lesions from haired skin, paw pads, and oral mucosa were collected and submitted for histopathological examination. Biopsies were fixed in 10% neutral-buffered formalin, routinely processed, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff reagent (PAS).

2.3. Animals for Genetic Analyses

The study included two unrelated domestic shorthair cats diagnosed with epidermolysis bullosa (index cases). Blood samples of both cases and a skin sample of case 2 were available. Additionally, a skin sample from an EB-unaffected unrelated control cat was collected for transcript analysis. This was a 13-year-old domestic shorthair cat that was euthanized due to metastatic mammary carcinoma.

2.4. DNA Isolation and Whole Genome Sequencing

DNA was isolated from EDTA blood on a Maxwell RSC 48 instrument using the Maxwell RSC Whole Blood DNA Kit (Promega, Dübendorf, Switzerland). The genomes of both affected cats were sequenced at 32× (case 1) and 30× (case 2) coverage using PCR-free libraries with ~400 bp insert size on an Illumina NovaSeq 6000 instrument (Next Generation Sequencing Platform of the University of Bern). Paired-end 2 × 150 bp reads in fastq files were processed into binary alignment map (bam-file) with respect to the F.catus_Fca126_mat1.0 genome reference assembly (GCF_018350175.1), and single nucleotide variants and small indels were called using GATK HaplotypeCaller [33]. The SnpEff software v5.0e [34] was used together with NCBI annotation release 105 for functional effect prediction of the called variants. The accession numbers of the sequence data of both cases were deposited in the European Nucleotide Archive and are listed in Table S1.

2.5. Variant Filtering

We individually filtered for private homozygous or heterozygous variants in both index cases by comparing their genomes to a control cohort comprising 82 publicly available WGS data from genetically diverse cats (Table S1). The allele frequency threshold in the control cohort was set to 0. In a second step, protein-changing variants were filtered, considering variants with a SnpEff predicted impact of "high" or "moderate", as well as "low" impact variants affecting the splice region [34]. In a last step, we filtered for protein-changing variants in 16 known EB candidate genes [35].

2.6. Targeted Genotyping

The candidate variants detected by Illumina WGS of case 1 and case 2 were validated using Sanger sequencing. PCR products were amplified from genomic DNA using AmpliTaqGold360Mastermix (Thermo Fisher Scientific, Waltham, MA, USA) with forward and reverse primers (Table S2) and standard PCR protocols with 30 cycles. The purified amplicons were sequenced on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific, Reinach, Switzerland) and analyzed with the Sequencher 5.1 software (Gene-Codes, Ann Arbor, MI, USA).

2.7. RNA Extraction and RT-PCR

RNA was extracted from skin samples of case 2 and an unaffected control cat using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). Tissue processing, RNA extraction, and reverse transcription into cDNA were performed as previously described using the SuperScript IV Reverse Transcriptase kit (Thermo Fisher Scientific) [36]. A 518 bp PCR product was amplified with AmpliTaqGold360Mastermix (Thermo Fisher Scientific) from cDNA using a forward primer spanning exon boundaries 7 and 8, and a reverse primer spanning exon boundaries 12 and 13 of the *COL17A1* gene (Table S2). PCR amplicons were then Sanger sequenced and analyzed as described above.

2.8. Bioinformatic Prediction of Functional Effects on Splicing

The SpliceAI web server was used for the prediction of the functional impact of genomic variants on splicing [37,38]. This tool assesses the impact of human variants and uses the human genome reference sequence and data on human transcripts. The sequence of the feline 5′-splice site after exon 45 of the *COL17A1* gene is perfectly conserved with the corresponding human sequence (positions −3 up to +10 of the splice site). A prediction for

the splice site following exon 10 could not be performed as feline and human sequences are not highly conserved.

3. Results

3.1. Clinical and Histopathological Phenotype Characterization

Case 1: A 3-week-old, female, intact American Shorthair cat was presented to an animal emergency room after being found outside by a good Samaritan. The kitten was surrendered to a rescue organization and hospitalized for nutritional support, treatment of hypothermia and dehydration, and multifocal areas of integumentary crusting and hypotrichosis (Figure 1a). On intake, a skin fungal culture was performed due to suspicion of cutaneous dermatophytosis. The kitten was started on empirical amoxicillin (Amoxi-Drops, Zoetis, Parsippany-Troy Hills, NJ, USA; 11 mg/kg PO twice daily) and topical miconazole (MicaVed Lotion 1%, Vedco, St. Joseph, MO, USA; daily). Empricial antihelmintic treatment consisted of a five-day course of fenbendazole (Panacur, Merck, Rathway, NJ, USA; 50 mg/kg PO daily). After 1-week of hospitalization, the kitten was brighter, eating vigorously, and interactive, but the integumentary lesions did not resolve. On physical examination, the kitten had bilateral mucoid ocular discharge and ceruminous exudate in the ear canals. The kitten also had hair loss with adherent scale and crust around the eyes, the dorsal muzzle, and dorsal cranium. The nailbeds were erythematous. Ulcers were present on the tongue and hard palate. The epidermis of the dorsal head easily sloughed with gentle pressure during cleaning (positive Nikolsky sign). Topical silver sulfadiazine cream (Ascend Laboratories, LLC, Parsippany, NJ, USA) was applied to the resulting ulcers on the dorsal head to facilitate re-epithelialization and to prevent secondary infections. Amoxicillin was also discontinued, and amoxicillin trihydrate/clavulanate potassium (Clavamox, Zoetis; 25 mg/kg PO twice daily) and itraconazole (Itrafungol, Elanco, Greenfield, IN, USA; 11 mg/kg PO daily) were started in its place.

Figure 1. Details of the clinical phenotype of case 1. (**a**) Case 1 on day of initial presentation. (**b**) Erosions, ulcers, and crusts on the tongue and (**c**) lips. (**d**) Blistering vesicles with absence of nails on the paws. (**e**,**f**) Erosions, ulcers, and crusts on the periocular region and ear pinnae.

The haired skin ulcers healed; however the oral ulcers were static (Figure 1b,c). The kitten was weaned onto commercial kitten canned food (Purina® Pro Plan® Veterinary Diets Kitten Canned Formula, Nestle Purina, St. Louis, MO, USA), and began to show signs of dysphagia (dropping food, chewing on one side of the mouth). A second course of Clavamox and itraconazole were given and a confirmatory dermatophyte PCR was negative. The kitten remained hospitalized for approximately 4-weeks before being discharged to a foster home.

Onychomadesis was noted at approximately 9-weeks of age, when the kitten was seen by an emergency clinician for a swollen right front paw (Figure 1d). The kitten began to exhibit avoidance behavior and gait abnormalities associated with its foot lesions. On examination, no nails were observed on any of the paws and the right forepaw had swelling with blistering lesions in the nail bed. A second dermatophyte PCR was submitted and a third course of itraconazole and Clavamox were initiated with the addition of topical chlorhexidine, acetic acid, and ketoconazole wipes (Mal-A-Ket Wipes, Dechra, Northwich, UK), an application of selamectin (Revolution, Zoetis), and an Elizabethan collar to prevent self-traumatization. The kitten was treated with compounded buprenorphine (Covetrus® Roadrunner Pharmacy, Phoenix, AZ, USA, 0.01 mg/kg oral transmucosal, three times daily) intermittently for analgesic support during nursing care.

The second dermatophyte culture and PCR were negative, and slow improvements in the skin and paw lesions were noted. A recheck examination performed 2-weeks later, however, revealed the oral, nail bed, and integumentary lesions to be static. Red-tinged vesicles were observed at each nail bed instead of mature nails. Persistent ulcerations were still present on the hard palate, at the lip commissures, on the tongue, and on the concave surface of both pinnae (Figure 1e,f). The kitten was admitted to the hospital again with a restricted environment to prevent self-harm and was offered only paper litter and soft food. A Calicivirus PCR using an oropharyngeal and conjunctival swab and SNAP FIV/FeLV Combo Test (IDEXX, Westbrook, ME, USA) were performed at this time and were negative. A complete blood count and biochemistry panel also revealed no significant findings.

In addition to collecting punch biopsy samples for histopathology, tissue samples were also collected for a second Calicivirus PCR and systemic mycoses PCR, which were both negative. Skin cytology of the lesions, prior to biopsy collection, were negative for microorganisms, but an aerobic culture and sensitivity panel grew a secondary methicillin-resistant, coagulase-negative Staphylococcus species, which was susceptible to and treated with clindamycin (ClindaCure, Vedco; 17 mg/kg PO twice daily).

Histopathologic examination of the haired skin from the pinna and limb revealed subepidermal clefts with mild dermal inflammation and crusts containing cocci (Figure 2). The mucosa of the tongue was detached and demonstrated regional coagulative necrosis.

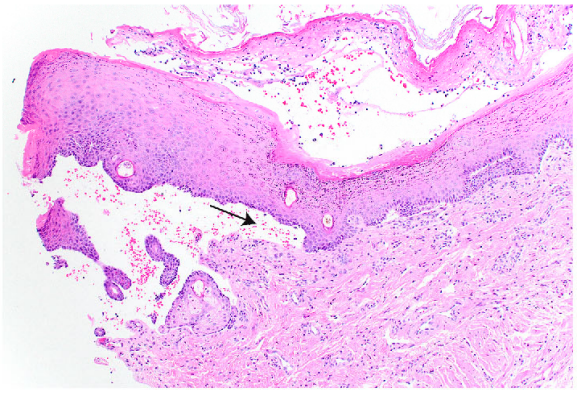

Figure 2. Histopathological findings in a skin biopsy from case 1. Subepidermal clefting is visible (arrow). H&E staining.

Due to the poor prognosis, the low potential for adoption, and the continuous development of painful ulcers on the tongue and extremities, the patient was ultimately euthanized. Requests for a necropsy were denied by the rescue organization in charge of the patient's care.

Case 2: A 4-month-old male, intact, European Shorthair outdoor cat was referred for onychomadesis and skin and oral erosions. The condition had not responded to cefalexin (Cefacat, Biokema, Crissier, Switzerland; 25 mg/kg twice daily for 2 weeks) and prednisolone (2 mg/kg daily for 10 days). The cat was fed on a complete commercial diet and was otherwise in good general condition. On physical examination, multifocal erosions and crusts, mostly on the lips, hard palate, and ear pinnae (Figure 3a–d) were noted. Several claws were either dystrophic (Figure 3e) or missing, and the associated nail beds were eroded and covered by dark brown crusty material (Figure 3f). Additionally, some paw pads showed focal erosions with re-epithelization (Figure 3g). The affected feet were painful upon manipulation. Cytological evaluation of impression smears acquired from the pinnae and feet did not reveal any micro-organisms. Wood's lamp examination and a fungal culture from skin scrapings were negative for dermatophytes. The complete blood count and biochemical results were all within normal limits, and the cat tested negative for FIV, FeLV, and Calicivirus. Based on the clinical data, a diagnosis of hereditary EB was suspected.

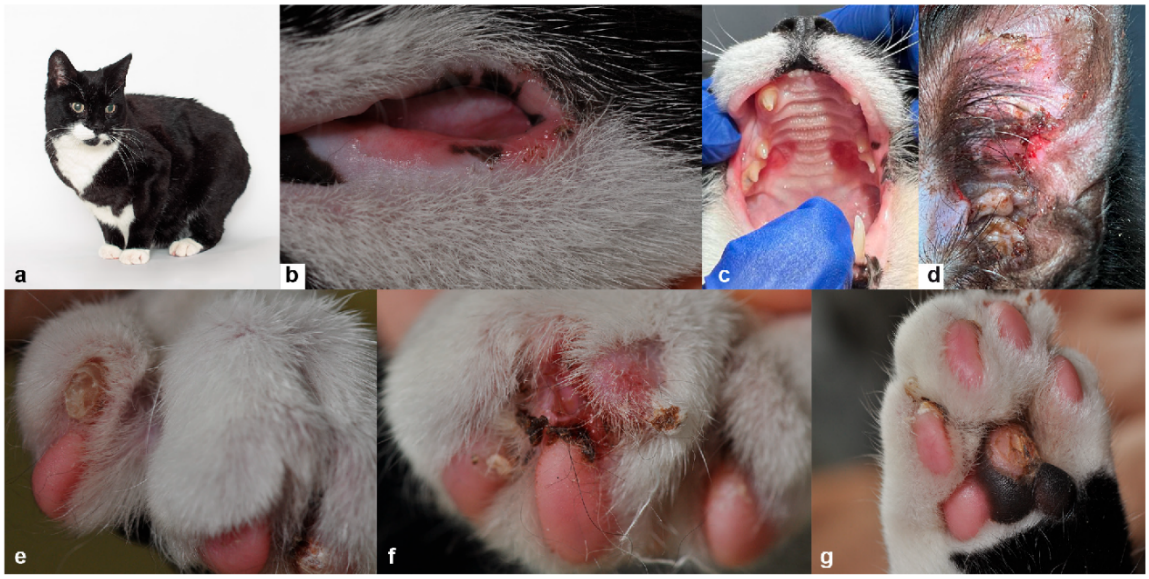

Figure 3. Details of the clinical phenotype of case 2. (**a**) Case 2 at 11 years of age. (**b**) Erosions and crusts on the lips, (**c**) hard palate and (**d**) ear pinnae. (**e**–**g**) Claws were dystrophic, and associated nail beds were eroded and covered with dark brown crusts.

Histopathological features included multifocal subepidermal clefts and vesicles (Figure 4a) with a minimal presence of erythrocytes and inflammatory cells. The periodic acid-Schiff (PAS) staining positive basement membrane was clearly visible only on the dermal side (Figure 4b). The basal cells in areas with clefts showed variable condensed nuclei and hypereosinophilic cytoplasm, and the dermis was unremarkable.

The patient was eventually adopted by one of the authors (AR) at the age of 1 year. At the time of writing, the cat was still alive at the age of 11 years. Its JEB phenotype was associated with a good long-term outcome, as the clinical signs diminished in severity over time. The oral ulcers, although still present, reduced approximately 20% in size and the patient showed onychomadesis only in single claws every 2–3 months. The perioral,

pinnal, and paw pad lesions have been re-occurring on average 1–3 times per year within the observation period.

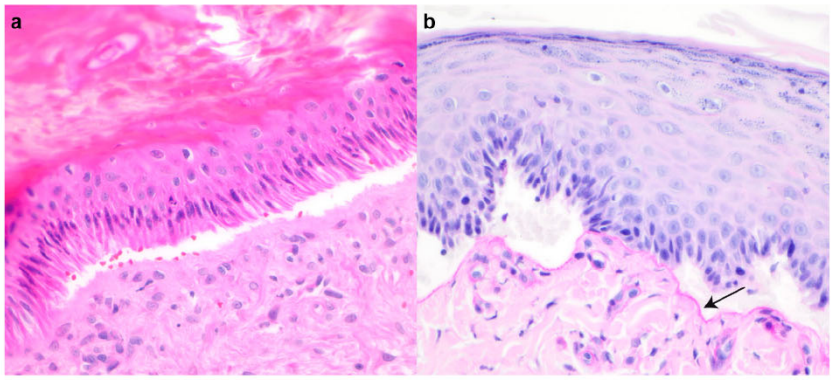

Figure 4. Histopathological findings in case 2. (**a**) Subepidermal cleft formation with low number of erythrocytes within the cleft. H&E staining. (**b**) Basal membrane (arrow) visible at the dermal side of the cleft. PAS staining.

3.2. Genetic Analysis

We individually compared the genome sequence data of case 1 and case 2 to 82 genetically diverse control genomes and searched for plausible causative variants. Several filtering steps were performed. Heterozygous variants were considered for a potential dominant mode of inheritance and homozygous variants for a potential recessive mode of inheritance. We only retained case-specific private variants that were absent from all control cats. The last automated filtering step identified protein-changing private variants. Finally, a subjective inspection of the variants prioritized variants in 16 known EB candidate genes [35], based on the results of the clinical and pathological examinations (Tables 1 and S3).

Table 1. Variants detected by whole genome sequencing of the two affected cats.

Filtering Step	Variants Case 1		Variants Case 2	
	Het	Hom	Het	Hom
All variants	6,364,334	5,790,564	6,976,390	5,784,208
Private variants (allele frequency of 0 in control cohort)	61,931	13,136	88,531	16,633
Protein-changing private variants	369	83	573	90
Protein-changing variants in 16 candidate genes	0	1	1	1

The analyses identified a single candidate variant in case 1, but two possible variants in case 2. Case 2 had a heterozygous variant in *LAMA3*, which is a known candidate gene for EB. However, *LAMA3*-related forms of EB are recessively inherited, which would be incompatible with a mono-allelic *LAMA3* variant. Furthermore, three different protein impact prediction software tools classified the *LAMA3* variant as neutral or unknown (SNPs&GO, PON-2P, PredictSNP [39–41]. This prompted us to exclude the *LAMA3* variant as potential candidate for the EB phenotype in case 2.

The final result of the WGS analyses yielded very clear top candidate causative variants for both cases. Both identified variants were present in a homozygous state and located in the *COL17A1* gene (Figure 5). The variant in case 1 affected a canonical splice site; the variant in case 2 affected a splice region (Table 2).

The *COL17A1*:c.3019+1del variant in case 1 was predicted to disrupt the 5′-splice site of intron 45 with a probability of 99% [37]. As the cat was already euthanized at the time of the genetic investigation, no samples were available to investigate the predicted splice defect at the mRNA level.

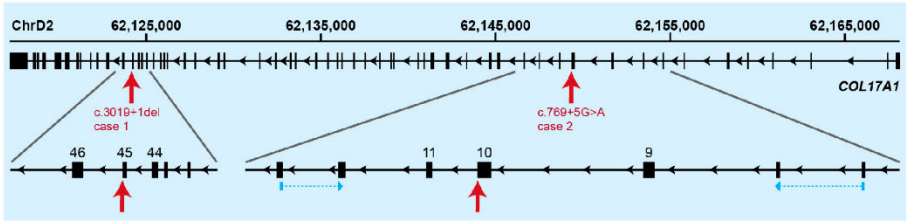

Figure 5. Overview of the genomic organization of the *COL17A1* gene. Exon numbering refers to the transcript with accession number XM_006938156.5. The positions of the variants of both investigated cats are indicated by red arrows. Enlarged details of the gene around both variants with the relevant exons are shown. Locations of the RT-PCR primers for cDNA analysis of the c.769+5G>A variant are indicated by blue arrows.

Table 2. Variant designations of the identified *COL17A1* variants according to Human Genome Variation Society (HGVS) nomenclature.

Cat	HGVS-g [1]	HGVS-c [2]	HGVS-r [3]	HGVS-p [4]
	NC_058378.1	XM_006938156.5	XM_006938156.5	XP_006938218.3
Case 1	ChrD2:62,124,169del	c.3019+1del	r.spl?	n.a.
Case 2	ChrD2:62,149,308C>T	c.769+5G>A	r.[=,r.769_770insguacaug]	p.([=,p.Val257Glyfs*82])

[1] g = linear genomic reference sequence; [2] c = coding DNA reference sequence; [3] r = RNA reference sequence (transcript); [4] p = protein reference sequence. * is the HGVS approved abbreviation for a termination codon.

The *COL17A1*:c.769+5G>A variant in case 2 affected the 5′-splice site of intron 10. We assessed the effects on splicing at the transcript level using RNA isolated from a skin biopsy. RT-PCR revealed that two different transcripts were expressed in the skin of case 2: the wildtype transcript and a mutant transcript with 7 nucleotides from intron 10 added to the normal exon 10, XM_006938156.5:r.769_770insguacaug (Figure 6). The 7 nucleotide insertion leads to a frameshift and introduction of a premature stop codon, truncating 1158 codons or 77.5% of the wild type *COL17A1* open reading frame.

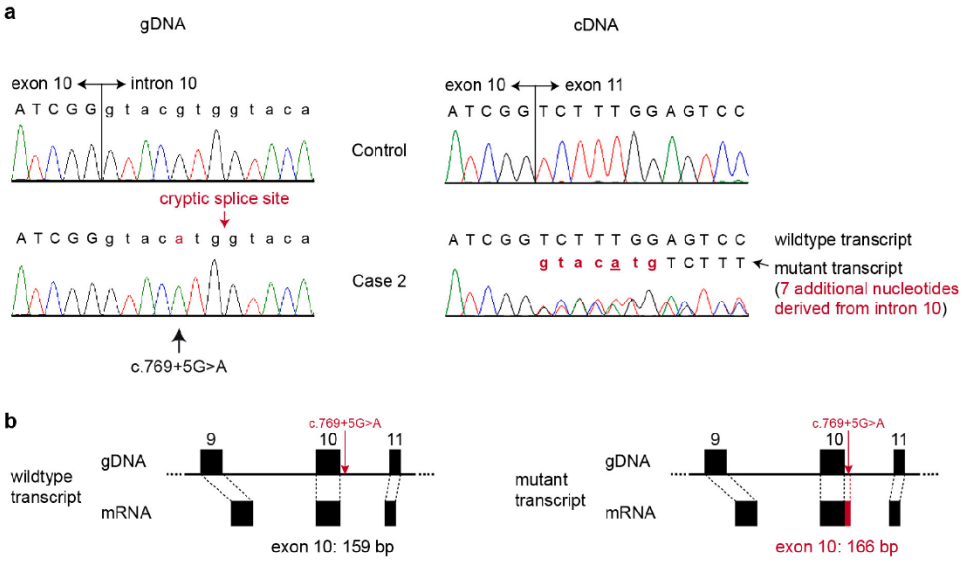

Figure 6. Effect of the *COL17A1*:c.769+5G>A variant in case 2 on splicing (**a**) Sanger electropherograms of the c.769+5G>A variant of case 2 and a control cat. The homozygous single nucleotide exchange at

the genomic DNA level is indicated by a black arrow. At the cDNA level, the variant leads to partial aberrant splicing, resulting in the simultaneous expression of wildtype transcript and a mutant transcript with an additional 7 nucleotides derived from the beginning of intron 10. (**b**) Schematic representation of exons 9, 10, and 11 of the *COL17A1* gene and the wildtype and mutant transcripts. The c.769+5G>A variant is indicated at the genomic DNA level by a red arrow. The resulting wildtype and mutant transcripts are displayed.

4. Discussion

In this study, we investigated two unrelated cats with clinical and histopathological signs of EB. The clinical lesions in each cat were remarkably similar (cutaneous and oral ulcers, epidermal sloughing with minor trauma, and sloughing of the nails). Histopathology revealed an impaired ability of the epidermis to adhere to the dermis, which resulted in separation at the dermoepidermal junction. These features were not sufficient to subtype the EB; however, the PAS stain revealed the basement membrane on the dermal side of the cleft. This narrowed the potential subtypes to EBS and JEB. Case 1 was euthanized due to the severity of the oral lesions; the lesions in case 2 were not as severe. Cat 2 is alive at the time of the writing and under the direct care of a veterinarian.

EB is a rare disease also in the feline species, with few cases reported at the clinicopathological level. Previously reported cases were characterized by onychomadesis and localized or generalized skin and oral ulcerations, resulting in either mild or severe phenotypes, as seen in our cases [9,13,19,42]. A *KRT14* variant was identified in a single cat with EBS [19]. In another study involving two cats diagnosed with JEB, *LAMC2* and *LAMB3* were identified as possible candidate genes by indirect immunofluorescence, but the underlying genetics was not reported [13].

Whole-genome sequence analyses of the two affected cats described herein identified two independent variants in *COL17A1*, a well-known functional candidate gene for JEB. The variant in case 1 was a homozygous single base-pair deletion at a canonical GT 5′-splice site, c.3019+1del. Unfortunately, no samples were available to assess the effect of the deletion at the transcript level. However, variants affecting the +1G at the 5′-splice site of other genes were previously described as pathogenic or likely pathogenic, most likely leading to a complete loss of gene function in dogs and humans with other inherited diseases [43–45]. These results corroborate an important function for the G at the +1 position of a canonical 5′-splice site. We hypothesize that the c.3016+1G>A variant in case 1 resulted in a complete loss of *COL17A1* function.

The second case had a homozygous single base pair exchange 5 nucleotides after the exon 10/intron 11 boundary, c.769+5G>A. A corresponding splice region variant at the +5 position has recently been described in the *CYB5R3* gene in a cat with methemoglobinemia [46]. The G at position +5 of the 5′-splice site occurs in 78% of human 5′-splice sites and normally forms a base-pair with a cytosine from the U1 snRNA [47]. Apart from the canonical GU dinucleotide, the +5 position exhibits the strongest sequence conservation in the intronic 5′-splice site, suggesting its important role in mRNA splicing [48].

The identified variant in case 2 resulted in an aberrant mRNA transcript; however, a wildtype transcript was also expressed. This provides a plausible explanation for the difference in phenotype severity between the two cases. We hypothesize that the presence of some functional protein in case 2 preserves residual collagen type XVII function, whereas in case 1, we suspect that no functional protein was expressed, resulting in the observed severe clinical phenotype.

The identification of two independent *COL17A1* variants in two unrelated cats with an EB phenotype provides strong support for the claimed causality of these two variants. However, our short read sequencing approach did not have 100% sensitivity to detect all variants. Large structural variants were not investigated in this study. Our analysis was limited to the genomic DNA level in case 1. In case 2, we identified a genetic variant at the genomic DNA level and confirmed its functional consequences at the transcript level. We did not experimentally confirm the predicted complete or partial protein deficiency in collagen type XVII in both cats.

To the best of our knowledge, we report the first two pathogenic *COL17A1* variants and their genotype-phenotype correlation in domestic animals. One earlier report characterized an autosomal recessive JEB in German Shorthaired Pointers with documented absence of collagen type XVII protein expression [30]. However, the molecular genetics in these dogs was not investigated.

5. Conclusions

We characterized two cats with JEB and identified two independent homozygous causal variants in the *COL17A1* gene. The splice site variant c.3019+1del in case 1 was predicted to lead to a complete deficiency in collagen type XVII and was associated with a severe clinical phenotype. The splice region variant c.769+5G>A in case 2 resulted in partial aberrant splicing with residual expression of wildtype transcript, which likely is responsible for the milder clinical phenotype in this cat.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes14101835/s1, Table S1: Accession numbers of 84 cat genome sequences; Table S2: Details of the primers used for PCR and Sanger sequencing; Table S3: Protein changing private variants in case 1 and case 2.

Author Contributions: Conceptualization, T.L., E.A.M., C.Y. and A.R.; investigation, S.K., H.T., D.R., P.G., S.S., A.L., E.A.M., C.Y. and A.R.; data curation, V.J.; writing—original draft preparation, S.K., H.T., D.R., T.L., C.Y. and A.R.; writing—review and editing, S.K., H.T., D.R., P.G., S.S., A.L., V.J., T.L., E.A.M., C.Y. and A.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The diagnostic examinations did not constitute an experimental study but were part of a clinical veterinary cases and therefore did not require official or institutional ethical approval. The collection of blood samples was approved by the Cantonal Committee for Animal Experiments (Canton of Bern; permit BE94/2022).

Informed Consent Statement: Written consent for the use of samples and data for research purposes was obtained from the owners of the cats in this study.

Data Availability Statement: The accession numbers of the sequence data that are reported in this study are listed in Table S1.

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Conflicts of Interest: The authors declare no conflict of interest.

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A *DSG1* frameshift variant in a Rottweiler dog with footpad hyperkeratosis

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A *DSG1* Frameshift Variant in a Rottweiler Dog with Footpad Hyperkeratosis

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Abstract: A single male Rottweiler dog with severe footpad hyperkeratosis starting at an age of eight weeks was investigated. The hyperkeratosis was initially restricted to the footpads. The footpad lesions caused severe discomfort to the dog and had to be trimmed under anesthesia every 8–10 weeks. Histologically, the epidermis showed papillated villous projections of dense keratin in the stratum corneum. Starting at eight months of age, the patient additionally developed signs consistent with atopic dermatitis and recurrent bacterial skin and ear infections. Crusted hyperkeratotic plaques developed at sites of infection. We sequenced the genome of the affected dog and compared the data to 655 control genomes. A search for variants in 32 candidate genes associated with human palmoplantar keratoderma (PPK) revealed a single private protein-changing variant in the affected dog. This was located in the *DSG1* gene encoding desmoglein 1. Heterozygous monoallelic *DSG1* variants have been reported in human patients with striate palmoplantar keratoderma I (SPPK1), while biallelic *DSG1* loss of function variants in humans lead to a more pronounced condition termed severe dermatitis, multiple allergies, and metabolic wasting (SAM) syndrome. The identified canine variant, *DSG1*:c.2541_2545delGGGCT, leads to a frameshift and truncates about 20% of the coding sequence. The affected dog was homozygous for the mutant allele. The comparative data on desmoglein 1 function in humans suggest that the identified *DSG1* variant may have caused the footpad hyperkeratosis and predisposition for allergies and skin infections in the affected dog.

Keywords: *Canis lupus familiaris*; whole-genome sequence; animal model; genodermatosis; skin; dermatology; keratinocyte; SAM syndrome; precision medicine

1. Introduction

The skin forms an essential barrier against the environment. In humans, the soles of the feet and the palms of the hands are covered by the specially structured palmoplantar epidermis, which has to bear the strongest mechanical forces of the entire skin. Genodermatoses characterized by altered structural and junctional proteins of these specialized regions comprise the palmoplantar keratodermas (PPK), a diverse group of inherited disorders collectively characterized by excessive or abnormal thickening of the palmoplantar skin. Variants in at least 32 genes have been shown to cause different forms of isolated or syndromic PPK in humans (Table 1) [1,2].

Table 1. Overview of genetic causes of human palmoplantar keratodermas (PPK).

Gene	Phenotype	Inheritance [a]	Ref.
AAGAB	Palmoplantar keratoderma, punctate type IA; PPKP1A	AD	[3,4]
AQP5	Palmoplantar keratoderma, Bothnian type	AD	[5]
CARD5	Pityriasis rubra pilaris	AD	[6]
COL14A1	Palmoplantar keratoderma, punctate type IB; PPKP1B	AD	[7]
CTSC	Papillon-Lefevre syndrome	AR	[8]
DSG1	Palmoplantar keratoderma I, striate, focal, or diffuse; PPKS1	AD	[9]
DSP	Palmoplantar keratoderma II, striate, focal, or diffuse; PPKS2	AD	[10]
ENPP1	Cole disease	AD	[11]
FAM83G	Palmoplantar keratoderma and exuberant scalp hair.	AR	[12]
GJA1	Palmoplantar keratoderma with congenital alopecia	AD	[13]
GJB2	Keratoderma, palmoplantar, with deafness	AD	[14]
GJB3	Erythrokeratodermia variabilis et progressiva 1	AD or AR	[15]
GJB4	Erythrokeratodermia variabilis et progressiva 2	AD	[16]
GJB6	Ectodermal dysplasia 2, Clouston type	AD	[17]
JUP	Naxos disease	AR	[18]
KANK2	Palmoplantar keratoderma and woolly hair	AR	[19]
KRT1	Palmoplantar keratoderma, epidermolytic or nonepidermolytic	AD	[20]
KRT6A	Pachyonychia congenita 3	AD	[21]
KRT6B	Pachyonychia congenita 4	AD	[22]
KRT6C	Palmoplantar keratoderma, nonepidermolytic, focal or diffuse	AD	[23]
KRT9	Palmoplantar keratoderma, epidermolytic	AD	[24]
KRT16	Palmoplantar keratoderma, nonepidermolytic, focal 1, FNEPPK1	AD	[25,26]
KRT17	Pachyonychia congenita 2	AD	[26]
LOR	Vohwinkel syndrome with ichthyosis	AD	[27]
POMP	Keratosis linearis with ichthyosis congenita and sclerosing keratoderma	AR	[28]
SASH1	Cancer, alopecia, pigment dyscrasia, onychodystrophy, and keratoderma	AR	[29]
SERPINB7	Palmoplantar keratoderma, Nagashima type; PPKN	AR	[30]
SLURP1	Meleda disease	AR	[31]
TAT	Tyrosinemia, type II	AR	[32]
TGM1	Ichthyosis, congenital, autosomal recessive 1	AR	[33]
TRPV3	Palmoplantar keratoderma, nonepidermolytic, focal 2; FNEPPK2	AD	[34]
WNT10A	Schöpf–Schulz–Passarge syndrome	AR	[35]

[a] AD: autosomal dominant; AR: autosomal recessive.

Footpad hyperkeratosis in dogs is a genetically heterogenous group of inherited diseases corresponding to human PPK. So far, causative genetic variants for two different forms of canine footpad hyperkeratosis have been reported. Hereditary footpad hyperkeratosis (HFH) in Irish Terriers and Kromfohrländer dogs is caused by a variant in the *FAM83G* gene [36] encoding a protein involved in BMP and WNT signaling [37–39]. The syndromic HFH phenotype is characterized by an orthokeratotic hyperplasia of the footpad epidermis in combination with an irregular hair morphology. *FAM83G* variants have also been described in human patients with PPK and exuberant scalp hair [12] and the wooly mouse mutant [40]. A *KRT16* frameshift variant has been reported in Dogues de Bordeaux with focal nonepidermolytic footpad hyperkatosis [41]. Interestingly, this disease is inherited as an autosomal recessive trait, while the human forms of *KRT16*-associated focal nonepidermolytic PPK typically are inherited as autosomal dominant traits [25,26].

This study was initiated after an owner reported a juvenile male Rottweiler dog suffering from footpad hyperkeratosis. The goal of the study was to characterize the clinical and histopathological phenotype and to identify a possible underlying causative genetic defect.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments were performed according to local regulations. The dog in this study is privately owned and was examined with the consent of the owner. The "Cantonal Committee for Animal Experiments" approved the collection of blood samples (Canton of Bern; permit 75/16).

2.2. Animal Selection

A male Rottweiler dog with footpad hyperkeratosis was investigated. Footpad biopsies were collected at initial presentation to rule out infectious and inflammatory causes of hyperkeratosis. The clinical presentation was inconsistent with other causes of secondary hyperkeratosis. An EDTA blood sample was collected for genomic DNA isolation. Additionally, we used 15 blood samples from other Rottweilers, which had been donated to the Vetsuisse Biobank. They represented population controls without reports of footpad hyperkeratosis. The photo of the control Rottweiler, shown in Figure 1B, represents a stock photo from the University of Pennsylvania (UPENN) veterinary hospital. This dog was not genotyped. The biopsy of the control dog, shown in Figure 2A, originates from a six-month-old healthy Beagle that was part of another IACUC-approved study at the UPENN School of Veterinary Medicine.

2.3. Histopathological Examinations

Two 6 mm punch biopsies from the footpads were obtained under general anesthesia. The samples were fixed in 10% neutral buffered formalin and routinely processed, including staining with hematoxylin and eosin.

2.4. DNA Extraction

Genomic DNA was isolated from EDTA blood with the Maxwell RSC Whole Blood Kit using a Maxwell RSC instrument (Promega, Dübendorf, Switzerland).

2.5. Whole-Genome Sequencing

An Illumina TruSeq PCR-free DNA library with ~500 bp insert size of the affected dog (RO015) was prepared. We collected 329 million 2 × 150 bp paired-end reads on a NovaSeq 6000 instrument (37x coverage). Mapping and alignment were performed as described [42]. The sequence data were deposited under the study accession PRJEB16012 and the sample accession SAMEA6249501 at the European Nucleotide Archive.

2.6. Variant Calling

Variant calling was performed using GATK HaplotypeCaller [43] in gVCF mode as described [42]. To predict the functional effects of the called variants, SnpEff [44] software together with NCBI annotation release 105 for the CanFam3.1 genome reference assembly was used. For variant filtering we used 655 control genomes (Table S1).

2.7. Gene Analysis

We used the CanFam3.1 dog reference genome assembly and NCBI annotation release 105. Numbering within the canine *DSG1* gene corresponds to the NCBI RefSeq accession numbers NM_001002939.1 (mRNA) and NP_001002939.1 (protein).

2.8. Sanger Sequencing

The *DSG1*:c.2541_2545delGGGCT variant was genotyped by direct Sanger sequencing of PCR amplicons. A 402 bp (or 397 bp in case of the mutant allele) PCR product was amplified from genomic DNA using AmpliTaqGold360Mastermix (Thermo Fisher Scientific, Waltham, MA, USA) together with primers 5′-GAG CAC TGA ACC GAT TTG CC -3′ (Primer F) and 5′- GGC ATA GTC AAA GAG GTG GGT-3′ (Primer R). After treatment with exonuclease I and alkaline phosphatase, amplicons were sequenced on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific). Sanger sequences were analyzed using the Sequencher 5.1 software (GeneCodes, Ann Arbor, MI, USA).

3. Results

3.1. Clinical Examination

A six-month-old male intact Rottweiler dog presented for evaluation of thick, rapidly growing footpads and discomfort (shifting stance, unwilling to stand for long periods). An unusual appearance of the pads (described as "dryness") had been first noted by the owners when the dog was obtained at eight weeks of age. At the time of the initial presentation, the patient was otherwise healthy with no other systemic or dermatologic signs. On examination, all pads on all feet were markedly thickened by dense mounds of adherent keratin (Figure 1). The digital pads and metatarsal/metacarpal pads were the most severely affected. Fissures and mobile keratin ridges were present along the edges of the pads. There was mild diffuse scale over the trunk, which was considered to be within normal limits.

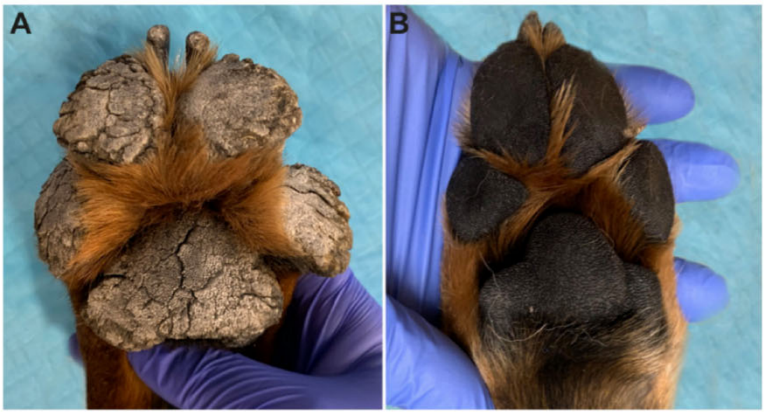

Figure 1. Clinical phenotype at six months of age. (**A**) Marked expansion of the footpads by thick projections of dense keratin in the affected Rottweiler. The adjacent haired skin appears unaffected on this image. (**B**) Footpads of a normal six-month-old control Rottweiler.

Regular application of moisturizers (urea), keratolytic (propylene glycol), and keratoplastic agents (salicylic acid/sulfur) and regular home trimming was recommended. The patient was unable to tolerate oral retinoids (isotretinoin). Initially, physical trimming of footpads under general anesthesia was performed every 4–6 months. However, the frequency by which this was required increased over time, and by the third year of life, it was performed every 8–10 weeks. To address significant discomfort, the patient was also started on gabapentin and codeine for pain control.

Additionally, between 8–12 months of age, the patient developed mild nonseasonal pruritus and recurrent ear infections. The patient was placed on an isoxazoline (Bravecto®) for parasite control. A 10-week strict hydrolyzed protein (Royal Canin Ultamino®) diet trial was performed without improvement, and the patient was presumptively diagnosed with atopic dermatitis. At one year of age, the patient was started on 0.5 mg/kg Oclacitinib (Apoquel®) for control of pruritus.

Despite control of pruritus, the patient continued to develop recurrent ear infections and intermittent episodes of bacterial skin infection. Superficial bacterial infections developed most frequently in areas of heavy wear (elbows, lateral hocks) but were also found occasionally on the trunk, neck, and glabrous areas. At sites of infection, hyperkeratotic plaques with superficial crusting were noted (Figure S1). Infections were managed with topical antiseptics and systemic culture-based antibiotics when indicated.

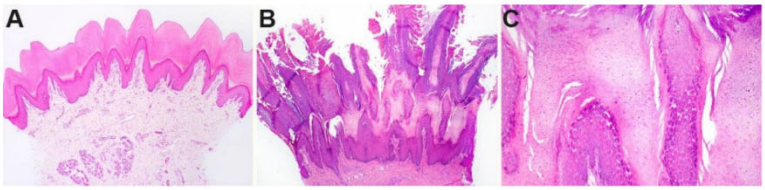

Figure 2. Histopathological phenotype of footpad skin. (**A**) Footpad skin of a normal six-month-old control dog. (**B**) In a biopsy taken from the affected dog at six months of age, a dense proliferation of the stratum corneum (outermost anucleated layer of the skin) markedly expands the epidermis. The stratum corneum is arranged in papillated villous projections of dense keratin. (**C**) Higher magnification of the stratum granulosum and stratum corneum of the affected dog shows an expansion of the granular cell layer.

3.2. Histopathological Findings

Histologically, the stratum corneum was markedly expanded by villous projections of orthokeratotic hyperkeratosis. The subtending granular layer of the epidermis was mildly hyperplastic. The samples had no significant inflammation (Figure 2).

3.3. Genetic Analysis

We sequenced the genome of the affected dog and searched for homozygous and heterozygous variants in 32 known candidate genes (Table 1) that were not present in the genome sequences of 647 control dogs and 8 wolves (Table 2, Table S2).

Table 2. Results of variant filtering in the affected Rottweiler dog against 655 control genomes.

Filtering Step	Homozygous Variants	Heterozygous Variants
All variants in the affected Rottweiler	3,310,269	2,516,875
Private variants	842	3290
Protein-changing private variants	4	25
Private variants in known candidate genes	1	0

This analysis identified a single homozygous private protein-changing variant in *DSG1*, a known candidate gene for palmoplantar keratoderma in humans [9]. The variant, a 5 bp deletion, can be designated as Chr7:58,163,636_58,163,640del5 (CanFam3.1 assembly). It is a frameshift variant, NM_001002939.1:c.2541_2545delGGGCT, predicted to truncate 207 amino acids from the C-terminus of the wildtype DSG1 protein, NP_001002939.1:p.(Gly848Trpfs*2). We did not investigate whether any mutant protein is expressed or whether the premature stop codon caused by the frameshift deletion leads to nonsense-mediated decay of the transcript.

We confirmed the presence of the 5 bp coding deletion in *DSG1* by Sanger sequencing and genotyped 15 control Rottweiler dogs. The case was homozygous for the mutant allele, while none of the 15 control dogs carried this allele (Figure 3).

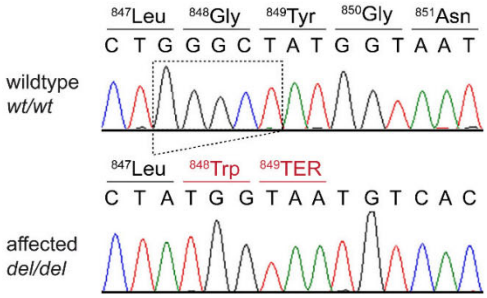

Figure 3. Details of the *DSG1*:c.2541_2545delGGGCT variant. Representative electropherograms of a control and the affected dog are shown. The amino acid translations of the wild-type and mutant alleles are indicated.

4. Discussion

In this study, we identified a homozygous *DSG1*:c.2541_2545delGGGCT frameshift variant in a Rottweiler dog with severe footpad hyperkeratosis. *DSG1* encodes desmoglein 1, a calcium-binding transmembrane glycoprotein of the cadherin family. Desmoglein 1 represents a major component of desmosomes that mediates cell–cell adhesion between keratinocytes in the upper layers of the epidermis [45]. Intact desmosomes are essential to maintain the skin barrier function [45]. Desmoglein 1 also represents the major autoantigen in human pemphigus foliaceus [46].

Variants in human *DSG1* cause striate palmoplantar keratoderma I (SPPK1). It is interesting to note that SPKK1 in humans is an autosomal dominant phenotype with the pathogenic variants being present in a heterozygous state in affected individuals [9,47]. In humans, SPKK1 is caused by haploinsufficiency of desmoglein 1 [9,47]. The investigated dog of our study was homozygous for a presumed null allele of *DSG1* and unlikely to express any functional desmoglein 1. In humans, a rare syndromic form of PPK referred to as SAM syndrome has been reported in patients with biallelic *DSG1* loss-of-function variants [48]. Subsequent studies of further human patients with biallelic *DSG1* variants confirmed the dermatitis and multiple allergies but failed to replicate the reported malabsorption and metabolic wasting [49,50]. The clinical presentation of the affected dog in our study, including the development of atopic dermatitis and hyperkeratotic lesions at sites of bacterial skin infection, resembles the phenotype of human SAM syndrome patients without the metabolic wasting, similar to what has been reported in several human cases [49,50].

The histopathological alterations in the footpad skin of the affected Rottweiler were comparable to the changes seen in footpad hyperkeratosis of *FAM83G* mutant Irish Terriers and Kromfohrländer dogs [36] and *KRT16* mutant Dogues de Bordeaux [41]. Thus, the histopathology cannot predict the specific underlying genetic defect. The clinical phenotype of the studied Rottweiler was more severe than in the previously described canine inherited footpad hyperkeratoses [36,41] and required periodical trimming of the excessively hyperkeratotic footpads. In addition to the severely affected footpads, the *DSG1* mutant Rottweiler also had an allergic skin disease and was prone to repeated bacterial skin infections. Such features have not been reported in *FAM83G* mutant Irish Terriers or Kromfohrländer dogs [36] or in *KRT16* mutant Dogues de Bordeaux [41].

Unfortunately, we did not have access to the parents of the affected dog or any other heterozygous dog. It would be interesting to investigate whether heterozygous dogs have completely normal footpads or whether they exhibit a mild phenotype that might go unnoticed by their owners.

In summary, we identified a Rottweiler dog with severe footpad hyperkeratosis that clinically and genetically resembled human SAM syndrome without metabolic wasting. To the best of our knowledge, this dog represents the first nonhuman patient with a spontaneous *DSG1* gene defect.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/4/469/s1, Figure S1: Hyperkeratotic plaque at the elbow of the affected dog. Table S1: Accession numbers of 648 dog and 8 wolf genome sequences; Table S2: Private variants in the sequenced Rottweiler with footpad hyperkeratosis.

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LAMB3 missense variant in Australian Shepherd dogs with junctional epidermolysis bullosa

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LAMB3 Missense Variant in Australian Shepherd Dogs with Junctional Epidermolysis Bullosa

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Abstract: In a highly inbred Australian Shepherd litter, three of the five puppies developed widespread ulcers of the skin, footpads, and oral mucosa within the first weeks of life. Histopathological examinations demonstrated clefting of the epidermis from the underlying dermis within or just below the basement membrane, which led to a tentative diagnosis of junctional epidermolysis bullosa (JEB) with autosomal recessive inheritance. Endoscopy in one affected dog also demonstrated separation between the epithelium and underlying tissue in the gastrointestinal tract. As a result of the severity of the clinical signs, all three dogs had to be euthanized. We sequenced the genome of one affected puppy and compared the data to 73 control genomes. A search for private variants in 37 known candidate genes for skin fragility phenotypes revealed a single protein-changing variant, *LAMB3*:c.1174T>C, or p.Cys392Arg. The variant was predicted to change a conserved cysteine in the laminin β3 subunit of the heterotrimeric laminin-322, which mediates the binding of the epidermal basement membrane to the underlying dermis. Loss-of-function variants in the human *LAMB3* gene lead to recessive forms of JEB. We confirmed the expected co-segregation of the genotypes in the Australian Shepherd family. The mutant allele was homozygous in two genotyped cases and heterozygous in three non-affected close relatives. It was not found in 242 other controls from the Australian Shepherd breed, nor in more than 600 other controls. These data suggest that *LAMB3*:c.1174T>C represents the causative variant. To the best of our knowledge, this study represents the first report of a *LAMB3*-related JEB in domestic animals.

Keywords: dog; *Canis lupus familiaris*; whole genome sequence; wgs; dermatology; genodermatosis; skin; laminin; precision medicine

1. Introduction

When a human or animal, usually at or soon after birth, develops erosions and epithelial sloughing on the mucosae, areas of friction, and extremities, a genetic disorder of skin fragility is to be considered. A consensus reclassification of skin fragility disorders was published recently, which separates those that affect the basement membrane itself or the basal keratinocytes (i.e., hereditary epidermolysis bullosa (EB) variants) from others, in which the separation occurs more superficially in

the epidermis [1]. In this reclassification, four main categories of inherited "classical" EB are proposed, which reflect the differences in the level of cleavage in the basement membrane zone [1]. Also included in this reclassification are four new categories of epidermal disorders of skin fragility associated with 20 possibly mutated genes, namely: peeling skin disorders, erosive skin fragility disorders, keratinopathic ichthyoses, and pachyonychia congenita [1]. Finally, a single syndromic connected tissue disorder with (dermal) skin fragility associated with *PLOD3* variants and a lysyl hydroxylase-3 deficiency was also included in this group of diseases [1]. All of the known 37 candidate genes for these human diseases are summarized in Table 1.

Table 1. Consensus reclassification of epidermolysis bullosa and other disorders with epidermal fragility and their known functional candidate genes, as of 2020 [1].

Disorder	Level of Cleavage	Gene	Protein	Inheritance [1]
Classical Epidermolysis Bullosa (EB)				
EB simplex (EBS)	Basal epidermal	*CD151*	CD151 molecule (Raph blood group)	AR
		DST	dystonin	AR
		EXPH5	exophilin 5	AR
		KLHL24	kelch like family member 24	AD
		KRT5	keratin 5	AD, AR
		KRT14	keratin 14	AD, AR
		PLEC	plectin	AR
Junctional EB (JEB)	Junctional	*COL17A1*	collagen type XVII, α 1 chain	AR
		ITGA3	integrin subunit α 3	AR
		ITGA6	integrin subunit α 6	AR
		ITGB4	integrin subunit β 4	AR
		LAMA3	laminin subunit α 3	AR
		LAMB3	laminin subunit β 3	AR
		LAMC2	laminin subunit γ 2	AR
Dystrophic EB (DEB)	Dermal	*COL7A1*	collagen type VII, α 1 chain	AD, AR
Kindler EB	Mixed	*FERMT1*	fermitin family homolog 1	AR
Other Disorders with Skin Fragility				
Peeling skin disorders	Intraepidermal	*CAST*	calpastatin	AR
		CSTA	cystatin A	AR
		CTSB	cystatin B	AR
		DSG1	desmoglein 1	AR
		FLG2	filaggrin family member 2	AR
		SERPINB8	serpin family B member 8	AR
		SPINK5	serine peptidase inhibitor Kazal type 5	AR
Erosive skin fragility disorders	Intraepidermal	*DSC3*	desmocollin 3	AR
		DSG3	desmoglein 3	AR
		DSP	desmoplakin	AR
		JUP	junction plakoglobin	AR
		PKP1	plakophilin 1	AR
Keratinopathic ichthyoses	Intraepidermal	*KRT1*	keratin 1	AD
		KRT2	keratin 2	AD
		KRT10	keratin 10	AD, AR
Pachyonychia congenita	Intraepidermal	*KRT6A*	keratin 6A	AD
		KRT6B	keratin 6B	AD
		KRT6C	keratin 6C	AD
		KRT16	keratin 16	AD
		KRT17	keratin 17	AD
Syndromic connective tissue disorder with skin fragility	Dermal	*PLOD3*	procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	AR

[1] AD—autosomal dominant; AR—autosomal recessive.

In domestic dogs, only two other epidermal disorders of skin fragility have been reported, namely: epidermolytic ichthyosis associated with a *KRT10* variant in Norfolk terriers [2], and ectodermal dysplasia/skin fragility syndrome with a *PKP1* variant in Chesapeake Bay Retrievers [3] (Table S1). In contrast, cases of hereditary EB have been recognized for decades, and the causative genetic variants have now been characterized in three canine, one feline, two equine, two ovine, and five bovine EB variants (Table S1).

In dogs, there is at least one example for each of the three main subtypes of classical EB in which the genetic defect has been reported, namley: a *PLEC* variant in the EB simplex of Eurasier dogs in the USA [4]; a *LAMA3* variant in the junctional EB of German Shorthaired Pointers in France [5]; and *COL7A1* variants in the dystrophic EB (mild) in Golden Retrievers, also in France [6], or severe in Central Asian Shepherds [7].

Laminin-332, a rod-like heterotrimer composed of the laminin α3, β3, and γ2 chains, is a critical component of hemidesmosomes, adhesion complexes that attach the basal epidermal keratinocytes to the underlying dermal connective tissue [8–10]. The prominent role of laminin-332 for skin integrity stems from its ability to link two important molecules—one in the epidermis and the other in the dermis. Via its carboxy-terminus, laminin α3 binds to the external domains of the integrin α6β4 that protrude from the basal keratinocytes. At the other end of the laminin trimer, the amino-terminal domains of the laminin β3 and γ2 chains bind to the NC1 amino-terminus of the superficial dermal collagen type VII [11].

Genetic variants in the *LAMA3*, *LAMB3,* and *LAMC2* genes that encode the laminin α3, β3, and γ2 chains are causative for the intermediate and severe forms of junctional EB (JEB), not only in humans [1], but also in animals (Table S1). Variants in any one of these genes can lead to a similar phenotype, as the abnormal expression or function of either of the three individual laminin chains is expected to impair the assembly or the function of the entire laminin-332. A good example of this phenomenon is the near identical phenotype exhibited by American Saddlebred horses with severe JEB associated with a *LAMA3* variant [12], and that found in Belgian, Breton, Comtois, and Italian draft horses caused by a *LAMC2* variant [13–15].

While JEB subsets associated with *LAMB3* variants are common in humans [16–18], they have not yet been reported in animals. So far, an abnormal epidermal expression of laminin β3—without investigation of the underlying molecular genetics—has only been shown in a single cat exhibiting a phenotype of mild EB [19].

Herein, we report a missense variant in *LAMB3,* which we believe is causative of a JEB phenotype with intermediate severity in a litter of Australian Shepherds in Ontario, Canada. Of clinical interest is the demonstration, for the first time or so it seems, of intestinal epithelial sloughing in a case of animal JEB.

2. Materials and Methods

2.1. Ethics Statement

The affected Australian Shepherds in this study were privately owned, and skin and biopsy samples were collected with the consent of their owners. The collection of all other blood samples was approved by the "Cantonal Committee for Animal Experiments" (Canton of Bern; permits 75/16 and 71/19).

2.2. Animal Selection

This study included 247 Australian Shepherds. Genomic DNA was either isolated from EDTA blood samples with the Maxwell RSC Whole Blood Kit, or from formalin-fixed paraffin-embedded (FFPE) tissue samples with the Maxwell RSC DNA FFPE Kit using a Maxwell RSC instrument (Promega, Dübendorf, Switzerland).

2.3. Histopathological Examinations

Skin punch biopsies (8 mm) were obtained under general anesthesia. The samples were fixed in 10% neutral buffered formalin and routinely processed, including staining with hematoxylin and eosin.

2.4. Whole Genome Sequencing

An Illumina TruSeq PCR-free DNA library with ~400 bp insert size of a JEB affected Australian Shepherd was prepared. We collected 175 million 2 × 150 bp paired-end reads or 18.9× coverage

on a NovaSeq 6000 instrument. The reads were mapped to the dog reference genome assembly CanFam3.1 and were aligned as previously described [20]. The sequence data were submitted to the European Nucleotide Archive, with study accession number PRJEB16012 and sample accession number SAMEA6862980.

2.5. *Variant Calling*

Variant calling was performed as previously described [20]. To predict the functional effects of the called variants, SnpEff software [21], together with NCBI annotation release 105 for the CanFam 3.1 genome reference assembly, was used. For variant filtering, we used 73 control genomes (Table S2).

2.6. *Gene Analysis*

We used the dog reference genome assembly CanFam3.1 and NCBI annotation release 105. Numbering within the canine *LAMB3* gene corresponds to the NCBI RefSeq accession numbers XM_014115071.2 (mRNA) and XP_013970546.1 (protein). For a multiple species comparison of the *LAMB3* amino acid sequences, we used the following accessions: NP_000219.2 (*Homo sapiens*), NP_001075065.1 (*Bos taurus*), XP_023496552.1 (*Equus caballus*), NP_001264857.1 (*Mus musculus*), NP_001094311.1 (*Rattus norvegicus*), XP_425827.3 (*Gallus gallus*), XP_002933550.2 (*Xenopus tropicalis*), and XP_700808.6 (*Danio rerio*).

2.7. *Sanger Sequencing*

To confirm the candidate variant *LAMB3*:c.1174T>C, and to genotype all of the dogs in this study, Sanger Sequencing was used. A 403 bp PCR product was amplified from genomic DNA using AmpliTaqGold360Mastermix (Thermo Fisher Scientific, Waltham, MA, USA) and primers 5′-TCT TGT GCC AAG CAC TGT TC-3′ (Primer F) and 5′-GGC ATA GGT GAG TCC CGT AA-3′ (Primer R). A smaller PCR product of 153 bp size was amplified for FFPE-derived DNA with primers 5′-GGT GGC TGC TTT TCT GTC TC-3′ (Primer F) and 5′-GGT GAG TCC CGT AAA TCC TG-3′ (Primer R). After treatment with shrimp alkaline phosphatase and exonuclease I, PCR amplicons were sequenced on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific). Sanger sequences were analyzed using the Sequencher 5.1 software (GeneCodes, Ann Arbor, MI, USA).

3. Results

3.1. *Family Anamnesis, Clinical Examinations, and Histopathology*

Three Australian Shepherd puppies with severe skin lesions were identified in a highly inbred litter resulting from a father–daughter mating. The litter consisted of three affected and two non-affected puppies that were born out of normal parents. The pedigree relationships were suggestive for a monogenic autosomal recessive inherited disease (Figure 1).

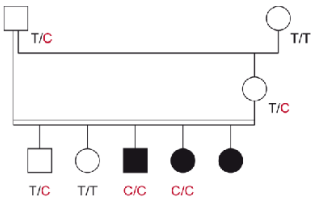

Figure 1. Pedigree of the investigated Australian Shepherd family. Squares represent males and circles represent females. The three affected puppies are indicated by the filled symbols. Note that the father of the litter was simultaneously the maternal grandfather. A close inbreeding loop greatly increases the risk for recessive hereditary defects. Genotypes at the *LAMB3*:c.1174T>C variant are indicated for all animals, from which a DNA sample is available (see Section 3.2).

At the time of their first presentation to the breeder's veterinarian for vaccination at 7 weeks of age, the three affected puppies were noted to have ulcers in the mouth, inner pinnae, and abdomen. The puppies reportedly also had marked lymph node enlargement. The average weight of the affected puppies was half that of their unaffected siblings.

At 17 weeks of age, one of the affected dogs, a blue merle with copper intact female was presented to the dermatologist for evaluation of severe ulceration of both the oral cavity and haired skin. Ulcers were located on the gingival and buccal mucosa, tongue, and hard and soft palates (Figure 2a). The concave pinnae, bilaterally, were also ulcerated, oozing, and covered with exudate (Figure 2b), but the otoscopic examination only revealed mild erythema in the ear canal. Several footpads, either digital or central, were also ulcerated (Figure 2c), and four claws were missing or misshapen. Erosions and ulcers were covered by thick crusts on the elbows, hocks, and the tip of the tail. The vulva and anus were grossly normal.

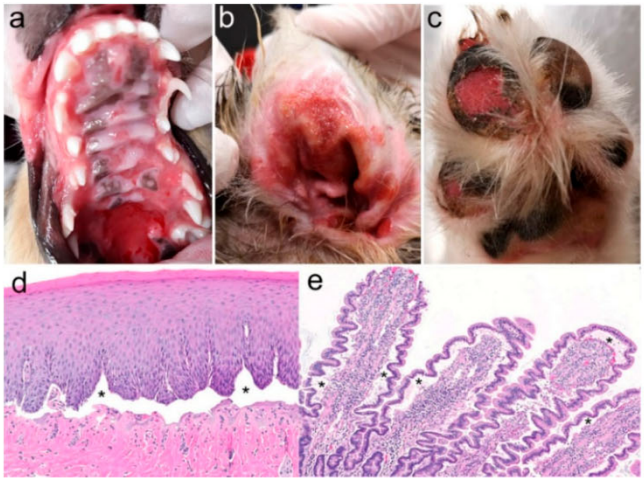

Figure 2. Clinical and histopathological phenotype. (**a**) Severe coalescing ulcers on the gingiva and hard and soft palate, (**b**) concave pinna (**c**) and footpads. Biopsy samples collected from the (**d**) oral cavity and (**e**) duodenum revealed widespread separation of the epithelium from the underlying connective tissue (asterisks).

Thoracic auscultation, abdominal, and lymph node palpation were all unremarkable. Blood was collected for a complete blood count and a serum chemistry panel, and the most relevant changes were a mild regenerative anemia (hemoglobin: 129 (reference range: 134–207 g/L); reticulocytes: 118 (10–110 k/μL)) and hypoproteinemia (total proteins: 46 (55–75 g/L); albumin: 23 (27–39 g/L); globulins: 23 (24–40 g/L)). To determine if these abnormal changes were due to digestive ulcers, an upper gastrointestinal endoscopy was performed under general anesthesia, two weeks after the original admission to the specialty clinic. The esophagus appeared normal, and the stomach and duodenum were hyperrhemic but did not show a visible loss of epithelium; endoscopic biopsies were nevertheless collected from the stomach and duodenum. During this general anesthesia, punch skin biopsies were collected from the concave pinnae, footpads, and oral cavity (hard palate, buccal mucosa, and tongue).

Microscopically, the skin and mucosal biopsy samples all exhibited limited-to-widespread epidermal detachment (Figure 2d), and ulcers were covered with serocellular crusts; inflammation was sparse in non-ulcerated areas. In some sections (as in Figure 2d), the basement membrane could be discerned at the base of the clefts, thus suggesting the diagnosis of JEB. The endoscopic biopsies from the stomach (pyloric and nonpyloric areas) and duodenum all showed mild-to-moderate inflammation with lymphocytes, plasma cells, and eosinophils, with a detachment of the epithelium from the

underlying lamina propria (Figure 2e). Because of the severity of the lesions, the dog was euthanized at 7.5 months of age.

The medical records of the two other affected puppies were also reviewed. A blue merle with copper intact male puppy was noted to have ulcers on the tongue, gingiva, soft palate, pharynx, tonsils, and larynx. Skin ulcers were found on the concave pinnae and pressure points of one elbow, one hock, and both stifles. Because of the worsening lesions, this puppy was euthanized at 16 weeks of age, with biopsy samples of the tongue, soft palate ear, and footpad collected post-mortem. As for the samples obtained from the littermate described above, microscopic lesions consisted of subepidermal vesicles leading to dermo-epidermal separation, ulceration, and granulation tissue.

The third affected puppy, a blue merle female, had been euthanized at 5 months of age because of severe gingival, labial, oropharyngeal, and esophageal ulceration. The dog had crusts on the chin, ulcers and crusts on the concave pinnae and footpads, and exudate at the base of multiple claws; samples for histopathology were not collected.

Finally, both the sire and dam, as well as the two healthy siblings, were examined by veterinarians, and they were deemed to be free of skin lesions.

3.2. Genetic Analysis

In order to characterize the underlying causative genetic variant, we sequenced the genome of one affected dog at 18.9× coverage and searched for homozygous variants in the 37 genes known to cause human skin fragility (Table 1), which were exclusively present in the affected dog and absent from the genomes of 73 other dogs (Table 2, Tables S2 and S3).

Table 2. Results of variant filtering in the affected Australian Shepherd dog against 73 control genomes. Only homozygous variants are reported.

Filtering Step	Variants
All variants in the affected dog	3,111,811
Private variants	11,754
Protein-changing private variants	54
Protein-changing private variants in 37 candidate genes	1

This analysis identified a single homozygous private protein-changing variant in *LAMB3*, a known candidate gene for JEB in humans [1]. The variant can be designated as Chr7:8,286,613A>G (CanFam3.1 assembly). It is a missense variant, XM_014115071.2:c.1174T>C, predicted to change a highly conserved cysteine residue in the third EGF-like domain of laminin β3, XP_013970546.1:p.(Cys392Arg).

We confirmed the presence of the *LAMB3* missense variant by Sanger sequencing (Figure 3). The mutant allele showed the expected co-segregation with JEB in the available family. The two available DNA samples from the JEB affected puppies carried the mutant allele in a homozygous state, while their parents were heterozygous, as expected for obligate carriers (Figure 1).

We determined the genotypes at *LAMB3*:c.1174T>C in a cohort comprising 247 Australian Shepherd dogs, including the index family. The mutant *LAMB3* allele was not detected in the homozygous state in any of the 245 non-affected Australian Shepherd dogs or 663 dogs from other breeds. Three of these dogs, all members of the index family, carried the mutant *LAMB3* allele in a heterozygous state (Table 3).

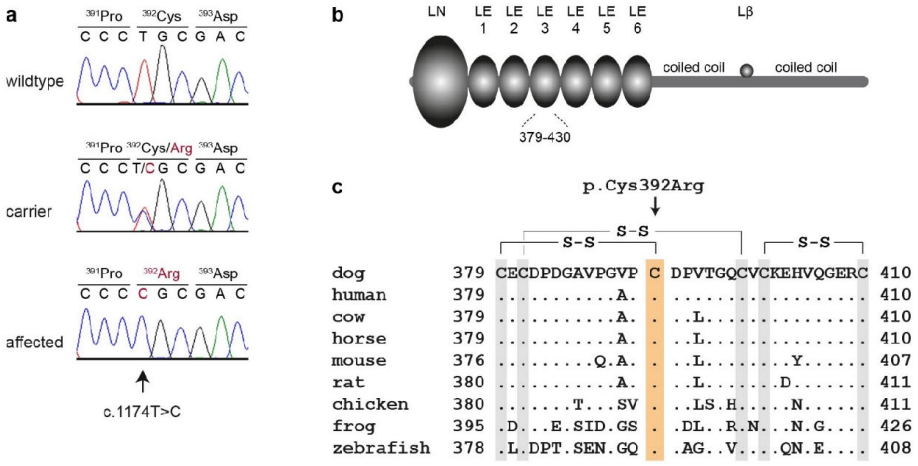

Figure 3. Details of the *LAMB3*:c.1174T>C, p.Cys392Arg variant. (**a**) Representative electropherograms of three dogs with different genotypes are shown. The variable position is indicated by an arrow, and the amino acid translations are shown. (**b**) Domain organization of the 1172 amino acid laminin β3 precursor [8]. The N-terminus consists of a globular domain (LN), followed by six laminin EGF-like (LE) domains. These N-terminal domains are located in the basement membrane and may be involved in binding to collagen VII. The C-terminal half of laminin β3 participates in two coiled-coil domains that mediate trimerization with the α3 and γ2 chains in the laminin-332 heterotrimer. The small Lβ domain mediates the binding of agrin. (**c**) Multiple-species alignment of the beginning of the LE3 domain harboring the p.Cys392Arg variant. The variant affects a highly conserved cysteine residue that forms a disulfide bridge with Cys-379 [22]. Note that all six cysteine residues in this region contribute to disulfide bonds, and are strictly conserved across vertebrates.

Table 3. Genotype-phenotype association of the *LAMB3*:c.1174T>C variant with JEB.

Dogs	T/T	T/C	C/C
Cases (n = 2) [1]	-	-	2
Controls, Australian Shepherd dogs (n = 245)	242	3	-
Controls, other breeds (n = 663) [1]	663	-	-

[1] These genotypes were derived from 590 genome sequences reported in the literature [20], and the 73 control genomes used in this study.

4. Discussion

In the affected Australian Shepherds described in this study, the age of lesion onset, as well as the presence of ulceration in the oral cavity and pressure points on the limbs with a loss of claws, all suggested the clinical diagnosis of a skin fragility disorder, of which EB is the most representative disease group in domestic animals and humans (Table S1). Because of the resembling phenotypes, clinical signs cannot alone reliably permit differentiation between the three main subtypes of animal EB (simplex, junctional, and dystrophic). For a more precise diagnosis, the specific location of the dermo-epidermal separation must be determined, for example, with a periodic acid Schiff (PAS) stain to visualize the glycoproteins in the basement membrane lamina densa [7], single or double antigen immunomapping [23], or transmission electron microscopy [4]. In this case, the routine histopathology enabled the visualization of the basement membrane delineating the contour of dermal imprints of the epidermal ridges, thus establishing that clefting occurred in a supra-lamina densa manner; this confirmed the diagnosis of JEB.

There is only one other occurrence of JEB in the canine species [5,23,24]. In the early 1990s, JEB was first discovered in German Shorthaired Pointers in the French Alps. The clinical signs were indistinguishable from those present in the Australian Shepherds described herein. Both the Pointer and Australian Shepherd puppies exhibited the first clinical signs weeks after, and not at, birth. In both cases, lesions consisted of ulcerative skin lesions affecting the inner (medial and concave) pinnae, footpads, and at pressure points of the extremities [23,24]. Shedding of the claws was also reported [24]. Of interest is that dental enamel abnormalities, a common finding in human JEB [1], were not recognized in either the German Shorthaired Pointers or the Australian Shepherds described in this study. A unique finding seen in one of the three Australian Shepherd puppies was the endoscopic observation of duodenal hyperrhemia, which was found on the histopathology to be associated with an extensive detachment of the digestive epithelium from its underlying connective tissue. Unfortunately, as ulceration of the duodenum was not seen during endoscopy, we cannot rule out that the digestive epithelial detachment seen on the histopathology might have been artifactual. Nevertheless, the *LAMA3, LAMB3,* and *LAMC2* genes, which encode the three laminin-332 chains, are all expressed in the small intestine [25]. As a result, based on our hypothesis, that the *LAMB3* missense variant affects the adhesive function of the laminin-332, it is conceivable that any trauma to the small intestine during the endoscopic biopsy process could result in a forced epithelial separation from the lamina propria, a phenomenon that normally does not happen to that extent in healthy individuals. To our knowledge, such a lesion has never been reported in a case of animal EB, and these are findings seen more often in the severe generalized than intermediate variants of human JEB; they are typically not found in localized JEB [26].

In this study, we identified a homozygous missense variant, *LAMB3*:p.Cys392Arg, as a candidate causative variant for a new JEB in Australian Shepherd dogs. *LAMB3* encodes the laminin β3 chain, which, together with the α3 and γ2 chains, forms the heterotrimer laminin-332. Laminin β3 has two coiled-coil domains for the heterotrimer formation with the α3 and γ2 chains at its C-terminal end. The N-terminus consists of a globular domain (LN) and six laminin-type epidermal growth factor-like (LE) repeats [8,22]. The LE domains have conserved disulfide bonds, which may be important for the tertiary structure of these domains [27,28]. The LN and LE domains form a short arm in the cross-shaped laminin-332 heterotrimer, and mediate binding to type VII collagen in hemidesmosomes, which are necessary for the stable association between the epithelium and the stroma underneath [8,11].

The p.Cys392Arg variant changes one of the highly conserved cysteine residues in the third LE domain, which prevents the formation of the disulfide bond between Cys-392 and Cys-379. We hypothesize that this may lead to a change in the tertiary structure of laminin β3, and impair the binding of laminin-322 to collagen type VII in hemidesmosomes. Further experiments at the protein level are required in order to confirm this putative pathomechanism.

With this description, we now have two variants of canine intermediate JEB due to variants in related genes (*LAMA3* in German Shorthaired Pointers and *LAMB3* in Australian Shepherds) encoding the laminin α3 and β3 chains that assemble with the γ2 chain to form the laminin-332 heterotrimer. In both of these breeds, the variants are predicted to result in some residual protein function (Australian Shepherds), or in the secretion of some normal laminin-332 trimers (German Shorthaired Pointers) [5], which may explain the similar absence of lesions at birth and the intermediate clinical phenotype.

In humans, the specific variants and their consequences at the mRNA and protein levels contribute to the spectrum of severity encountered in different subtypes of EB [10]. Severe forms of JEB are associated with nonsense, frameshift, or out-of-frame splicing variants that result in nonfunctional or complete loss of the protein. Intermediate JEB occurs when a laminin chain is mutated, but the LM-332 heterotrimer can still form, which is often the case for missense variants [16]. Missense variants affecting cysteine residues in the LE domains, *LAMB3*:p.Cys355Arg and p.Cys433Trp, have been reported in human patients with intermediate JEB [16–19]. The clinical phenotype observed in the investigated dogs homozygous for p.Cys392Arg can also be classified as JEB of intermediate severity, and corresponds well to the human spectrum of genotype–phenotype correlations.

5. Conclusions

We characterized a new recessive form of JEB in Australian Shepherd dogs. A precision medicine approach identified a missense variant in the *LAMB3* gene, c.1174T>C or p.Cys392Arg as likely candidate causative variant. Our data enable genetic testing to avoid the unintentional breeding of further affected dogs and provide the first spontaneous large animal model for JEB due to altered laminin β3.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/9/1055/s1: Table S1, genetic variants causing epidermolysis bullosa or skin fragility disorders in animals. Table S2, accession numbers of 74 dog genome sequences. Table S3, private variants in a JEB affected Australian Shepherd dog.

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Conflicts of Interest: The authors declare no conflict of interest.

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A *COL7A1* variant in a litter of neonatal Basset Hounds with dystrophic epidermolysis bullosa

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A *COL7A1* Variant in a Litter of Neonatal Basset Hounds with Dystrophic Epidermolysis Bullosa

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Abstract: We investigated three neonatal Basset Hound littermates with lesions consistent with epidermolysis bullosa (EB), a group of genetic blistering diseases. A clinically normal bitch was bred to her grandfather by artificial insemination. Out of a litter of seven puppies, two affected puppies died and one was euthanized, with these puppies being submitted for diagnostic necropsy. All had multiple bullae and ulcers involving the nasal planum and paw pads, as well as sloughing claws; one puppy also had oral and esophageal ulcers. The complete genome of one affected puppy was sequenced, and 37 known EB candidate genes were assessed. We found a candidate causative variant in *COL7A1*, which encodes the collagen VII alpha 1 chain. The variant is a complex rearrangement involving duplication of a 107 bp region harboring a frameshift deletion of 7 bp. The variant is predicted to truncate more than 75% of the open reading frame, p.(Val677Serfs*11). Targeted genotyping of this duplication confirmed that all three affected puppies were homozygous for the duplication, whereas 12 unaffected Basset Hounds did not carry the duplication. This variant was also not seen in the genomes of more than 600 dogs of other breeds. *COL7A1* variants have been identified in humans and dogs with dystrophic epidermolysis bullosa (DEB). The identified *COL7A1* variant therefore most likely represents the causative variant and allows the refinement of the preliminary EB diagnosis to DEB.

Keywords: *Canis lupus familiaris*; whole genome sequence; skin; dermatology; genodermatosis; collagen VII; precision medicine

1. Introduction

Epidermolysis bullosa (EB) is a heterogeneous group of genetic disorders associated with skin fragility and blistering, as well as secondary to minor mechanical trauma [1]. Four categories, or "classical types", of EB have been described in humans, broadly distinguished by the location of blistering in the skin [1]. Epidermolysis bullosa simplex (EBS) causes blistering in basal or suprabasal keratinocytes; junctional epidermolysis bullosa (JEB) causes blistering at the lamina lucida of the basement membrane zone; dystrophic epidermolysis bullosa (DEB) causes blistering in the superficial dermis; and, in Kindler epidermolysis bullosa (KEB), lesions are present in multiple levels of the basement membrane zone, and affected individuals have clinical features not seen in the other types [2].

Further sub-classification in humans describes the phenotype, mode of inheritance, targeted protein and relative expression, the gene involved, and the genetic variant present, with variant specifics if

available [1,2]. A recent reclassification of human DEB categorizes phenotypes according to recessive, dominant, and compound heterozygous allelic variation, however, certain clinical presentations are associated with more than one pattern of inheritance [1]. Thus far, all types of DEB in humans have been attributed to variants of the *COL7A1* gene, which codes for the collagen VII alpha 1 chain, although a recent case describes a DEB-like phenotype found in an individual with a variant in *PLOD3*, which encodes for lysyl-hydroxylase 3 [1,3,4]. A human DEB variant database has documented 659 variants of *COL7A1* [5]. Recessive forms of DEB may be more severe than dominant types, but there is considerable phenotypic overlap between the two [1,6–8].

In dogs, cases of putative EBS, JEB, and DEB have been described, although fewer subtypes and genetic variants than in humans have been recognized [9–18]. A recent case report of recessive DEB in two neonatal Central Asian Shepherd siblings found a homozygous nonsense variant of *COL7A1* that resulted in a premature stop codon, likely preventing the production of functional protein [14] (OMIA 000341-9615). A mild, recessive form of DEB in a family of Golden Retrievers was identified, and genetic analysis found a homozygous missense variant, p.Gly1906Ser, in *COL7A1* [15,16]. Another report of putative DEB in an Akita Inu was diagnosed via observation of the periodic acid-Schiff (PAS)-positive basement membrane at the roof of the blister, separation of the tissues beneath the lamina densa, and decreased anchoring fibrils on electron microscopy. The causative genetic variant was not reported [17]. Herein, we describe cases of DEB in a single litter of neonatal Basset Hounds, a breed in which, to our knowledge, this condition has not yet been reported.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments were performed according to local regulations. Puppies in this study were privately owned and deceased animals were submitted for diagnostic necropsy. The Cantonal Committee for Animal Experiments approved the collection of blood samples for the 12 normal Basset Hounds (Canton of Bern; permit 75/16).

2.2. Animal Selection

A clinically normal, approximately two-and-a-half-year-old Basset Hound bitch was bred to her maternal grandfather. He had been deceased for over 20 years, and the veterinarian reported that the last of 5 straws of frozen semen were used for this breeding.

An unrelated, stillborn, neonatal puppy with grossly normal skin was the control for electron microscopy. Control blood samples were from 12 clinically normal Basset Hounds in the Vetsuisse Biobank.

2.3. Necropsy, Histopathology, and Electron Microscopy

All pathological specimens were evaluated by a board-certified veterinary pathologist (D.S.R.) and a veterinary pathologist in training (T.M.G.). A full suite of tissues from all 3, and skin from the control puppy, were immersed in 10% neutral buffered formalin and processed for routine histopathology. Skin sections were routinely stained with hematoxylin and eosin, gram, and periodic acid–Schiff. A smaller subset of tissues were saved from all three affected puppies and were stored at −20 °C for DNA extraction. For transmission electron microscopy (TEM), sections of skin from the euthanized puppy were collected and immediately suspended in 3% glutaraldehyde. Skin from the normal puppy was suspended in modified Karvosky fixative. Samples were rinsed with 0.1 M sodium cacodylate buffer, and post-fixed in 1.5% potassium ferrocyanide and 2% osmium tetroxide in water. Samples were stained with T-O-T-O, followed by lead aspartate, and were dehydrated in a graded series of acetone (10%, 30%, 50%, 70%, 90%, 95%, 100%) for 10–15 min each. They were then infiltrated with araldite resin, ultrathin sectioned, and placed on TEM grids. Images were taken in a FEI Helios Nanolab 650 Scanning Electron Microscope in STEM mode.

2.4. DNA Extraction

Genomic DNA was either isolated from frozen liver tissue with a Maxwell RSC Tissue DNA Kit or from ETDA blood samples with the Maxwell RSC Whole Blood Kit using a Maxwell RSC instrument (Promega, Dübendorf, Switzerland).

2.5. Whole Genome Sequencing

An Illumina TruSeq PCR-free DNA library with ≈400 bp insert size of an affected Basset Hound puppy was prepared. We collected 263 million 2 × 150 bp paired-end reads on a NovaSeq 6000 instrument (30× coverage). The reads were mapped to the dog reference genome assembly CanFam3.1 as previously described [19]. The sequence data were deposited under the study accession PRJEB16012 and the sample accession SAMEA6862953 at the European Nucleotide Archive.

2.6. Variant Calling

Variant calling was performed using GATK HaplotypeCaller [20] in gVCF mode as described [19]. To predict the functional effects of the called variants, we used SnpEff [21] software together with NCBI annotation release 105 for the CanFam3.1 genome reference assembly. For variant filtering, we used 73 control genomes (Table S1). Four of the 73 control genomes were derived from Basset Hounds. The same Basset Hounds were also included in the 12 Basset Hound controls for targeted genotyping. Numbering within the canine *COL7A1* gene corresponds to the NCBI RefSeq accession numbers NM_001002980.1 (mRNA) and NP_001002980.1 (protein).

2.7. Sanger Sequencing and Genotyping

The *COL7A1*:[c.2028_2034;c.1993_2050+56dup] variant was genotyped by direct Sanger sequencing of PCR amplicons (Figure S1). A 389 bp (or 489 bp in case of the mutant allele) PCR product was amplified from genomic DNA using AmpliTaqGold360Mastermix (Thermo Fisher Scientific, Waltham, MA, USA) together with primers 5′-GTG GGA GGG CTA TAG GGA AG-3′ (Primer F) and 5′-AAA GGA GGC CAA AGG AGA AA-3′ (Primer R). After treatment with exonuclease I and alkaline phosphatase, amplicons were sequenced on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Sanger sequences were analyzed using the Sequencher 5.1 software (GeneCodes, Ann Arbor, MI, USA). A 5200 Fragment Analyzer was used for the sizing and visualization of PCR products (Agilent, Santa Clara, CA, USA).

3. Results

3.1. Clinical History and Necropsy Findings

Out of seven puppies, one was stillborn with an abdominal wall defect and protruding intestines; another died at 12 h of age following weakness and dyspnea. Of the remaining puppies, two were born with erosions of the skin on the muzzle. Within two days after birth, these two and one additional puppy were noted with blisters on the paw pads, multiple sloughed nails, and crusts around the ear canals. Two such affected puppies died naturally during the night, between the second and third day of life, were frozen near the time of death, and submitted for routine necropsy approximately 3 months later. The third was humanely euthanized between 48–72 h after birth and submitted for necropsy approximately 1 week after death. The two remaining puppies of the litter were clinically normal at three months of age.

Grossly, the euthanized puppy had bullae up to 8 mm in diameter affecting the nasal planum, dorsal muzzle, and paw pads (digital, metacarpal, and metatarsal) from all four limbs (Figure 1). Several nails were sloughed. There were multifocal to coalescing areas of ulcerations on the tongue and lips. The other two affected puppies had similar gross lesions, including bullae and ulceration of the nasal planum and dorsal muzzle, and of the paw pads. Both had multiple sloughed claws. The gross

diagnosis was multifocal, cutaneous, and mucocutaneous bullous dermatopathy with ulceration, consistent with a severe form of EB.

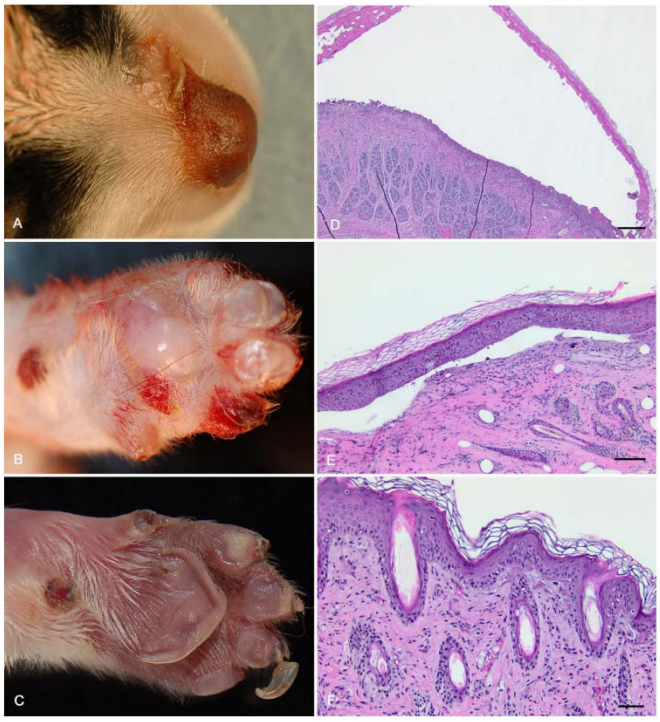

Figure 1. Gross necropsy with histopathology of three puppies with epidermolysis bullosa (EB). (**A**–**C**) All three dogs had bullae of the nasal planum and dorsal muzzle, and two had areas of ulceration. (**D**,**E**) Microscopic exam of the euthanized puppy found sub-basilar bullae. Scale bars = 500 µm. (**F**) In adjacent, less affected areas, there was subepidermal clefting with accumulation of proteinaceous fluid and a small amount of necrotic debris. There was also mild perivascular lymphoplasmacytic dermatitis. Scale bar = 50 µm.

3.2. Histologic Findings

All puppies had similar microscopic changes. Most prominently, all three had sub-basilar bullae in the paw pads and nasal planum (Figure 1). In adjacent, less affected areas, there was clefting at the dermal–epidermal junction with accumulation of proteinaceous fluid. Sections stained with PAS found positively stained material at both the roof and floor of the bullae. Ruptured bullae were associated with granulation tissue, lymphoplasmacytic and neutrophilic inflammation, and many surface-associated Gram-positive and Gram-negative bacteria. The euthanized puppy also had severe lingual and esophageal ulceration, with granulation tissue and surface-associated bacteria. All other organs were microscopically normal.

3.3. Electron Microscopy

While many structures of the basement membrane zone were identified, inadequate preservation (autolysis) precluded identification of the specific location of clefting, as well as abnormalities within structures such as hemidesmosomes and anchoring fibrils. Images are available in the Supplementary Materials section (Figure S2).

3.4. Genetic Analysis

We sequenced the genome of one affected puppy at 30× coverage and searched for private homozygous and heterozygous variants in 37 known candidate genes (Table S2) that were not present in the genome sequences of 73 control dogs (Table 1, Table S3).

Table 1. Results of variant filtering in an affected Basset Hound and 73 control genomes.

Filtering Step	Homozygous Variants	Heterozygous Variants
All variants	3,198,983	2,768,869
Private variants	18,820	80,057
Protein-changing private variants	49	174
Protein-changing private variants in 37 candidate genes	3	0

The automated analysis identified three homozygous protein-changing variants in *COL7A1*, a known candidate for DEB in humans and dogs. The GATK software called three independent smaller variants within a stretch of 11 nucleotides. However, visual inspection of the short-read alignments revealed that these three variants were not correctly called (Figure 2). They are part of a single complex duplication event spanning parts of exon 15 and intron 15 of the *COL7A1* gene, starting at position 40,524,267 and ending at 40,524,380 on chromosome 20 (CanFam3.1 assembly). The variant can be designated as NM_001002980.1:[c.2028_2034del; c.1993_2050+56dup]. The variant causes a frameshift. Assuming the formation of a transcript, in which the mutant exon 15 is spliced to the unchanged exon 16 without inclusion of duplicated sequences, this frameshift is predicted to truncate 2260 amino acids (76%) from the C-terminus of the wildtype COL7A1 protein NP_01002980.1:p.(Val677Serfs*11). We did not investigate whether any mutant protein is expressed or whether the complex rearrangement leads to nonsense mediated decay or altered splicing of the mutant transcript.

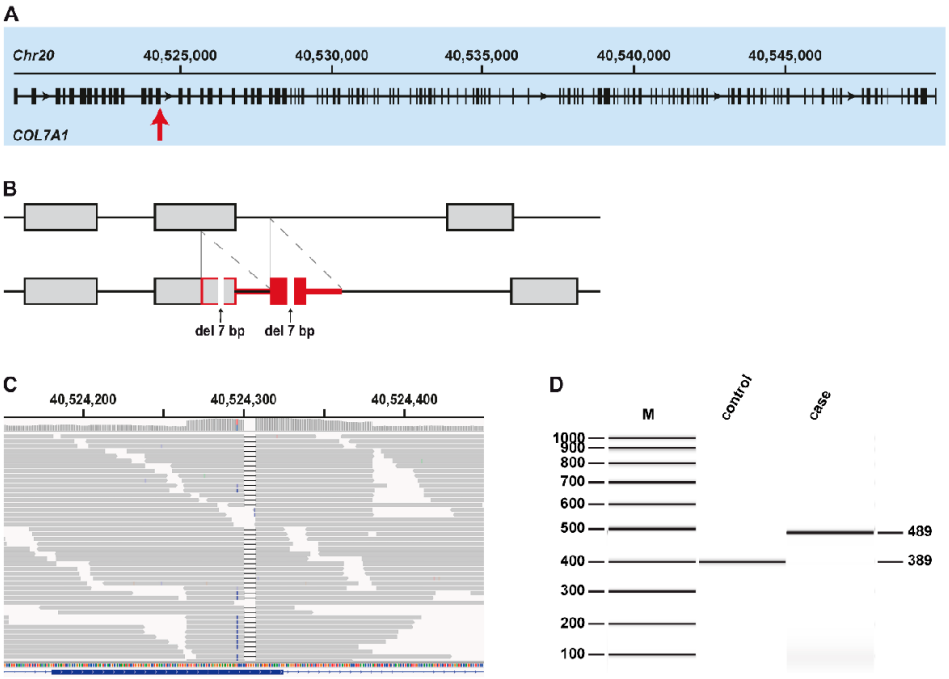

Figure 2. Details of the complex duplication in *COL7A1*. (**A**) Overview of the *COL7A1* gene with the intronic and exonic regions. The major transcript isoform (NM_001002980.1) is encoded by 118 exons.

The position of the variant is indicated by an arrow. (**B**) Schematic illustration of the variant, where the exons are displayed with boxes and the introns with lines in between. The vertical grey lines indicate the position of the duplicated region, which is framed in red in the mutant allele at the bottom. The oblique dashed lines indicate the position of the actual duplication, which is colored solid red. The arrows indicate the deletion of seven nucleotides in both copies of the duplicated sequence. (**C**) Integrative Genomics Viewer (IGV) screenshot showing the short-read alignments of the dystrophic epidermolysis bullosa (DEB)-affected puppy at the position of the duplication. The increased coverage at the top is characteristic for a duplication. The deletion in both copies of the duplicated sequence is visible in the short-read alignments. (**D**) Fragment analyzer gel image with the PCR amplicons of a healthy control Basset Hound and a DEB-affected Basset Hound, indicating the insertion of 100 nucleotides in the mutant allele.

We confirmed the exact sequence of the variant in *COL7A1* by Sanger sequencing in one of the affected puppies (Figure S1) and genotyped all three affected puppies and an additional 12 control Basset Hounds for this variant. All three affected puppies were homozygous for the mutant allele, while none of the 12 control Basset Hounds carried this allele. The duplication was also absent from the 73 control genomes used in variant filtering and another 588 publicly available genomes from other dog breeds and wolves reported in the study by [19].

4. Discussion

A bullous dermatopathy was diagnosed in three neonatal Basset Hound puppies from a single litter. Given the constellation of gross and microscopic lesions, age of onset, and multiple instances of inbreeding in the pedigree, EB was our top differential diagnosis. Our finding of a homozygous, candidate-causative variant in *COL7A1* likely leading to a lack of collagen VII production in all three puppies is compatible with DEB, as this gene has multiple documented variants known to cause human DEB [1–8], and is found in two other canine cases of DEB [14–16].

The genome of one puppy was sequenced and assessed for homozygous and heterozygous genetic variants of candidate genes known to cause EB. We identified a homozygous variant of *COL7A1* in this puppy, and later confirmed that the other two were homozygous for the same variant, and that it was not seen in 12 control Basset Hounds nor in more than 600 dogs from other breeds. We were unable to obtain samples from the non-affected close relatives to confirm the co-segregation of the variant with the DEB phenotype in the family. The genotype distribution and the family history with non-affected parents and a close inbreeding loop strongly suggested autosomal recessive inheritance.

The identified variant involves a complex duplication event in an early exon that likely renders the gene inactive via a frameshift and resultant premature termination codon (PTC). Thus, we suspect that the collagen VII alpha 1 chain, the protein product of *COL7A1*, is not produced in affected puppies. While this specific variant appears to be unique to this family of puppies, a nonsense variant that resulted in PTC and absence of functional collagen VII alpha 1 chains caused similar lesions in Central Asian Shepherd dogs diagnosed with recessive DEB [14].

Autosomal recessive DEB is associated with PTC-producing variants of *COL7A1* in humans. Human *COL7A1* variants leading to PTCs were identified across multiple locations in the *COL7A1* gene in patients with autosomal recessive DEB (RDEB; OMIM #226600) [1,8]. Homozygous PTCs or compound heterozygous PTCs can lead to a lack of COL7A1 protein in humans, and the clinical picture includes severe blistering and erosions with onset at birth, pseudosindactyly, and contracted joints [8]. However, Varki et al. discuss instances in which an individual with PTCs on both alleles of *COL7A1* have a mild phenotype [8]. One such case was determined to be due to in-frame exon skipping through aberrant splicing [8,22]. In the investigated Basset Hound puppies, the presence of a homozygous frameshift and PTC with severe clinical signs and onset at birth are consistent with absence of a functional COL7A1 protein. Collagen VII is the main component of U-shaped structures called anchoring fibrils that help adhere the epidermis to the dermis by attaching to the lamina densa at

both ends, with the middle extending into the dermis, entrapping interstitial collagen [23]. Variants that cause defects in, or in this case lack of collagen VII, compromise the stability of the epidermal–dermal junction, and it follows that areas subject to the most friction in nursing puppies—paw pads, esophagus, tongue, and the nasal planum—will be most affected, as seen in the puppies studied here [23].

Sub-classification of EB can utilize periodic acid–Schiff staining patterns and ultrastructural morphology of the basement membrane zone [4,9,24]. In this case, neither yielded definitive results—perhaps due to deposition of fibrin on the edges of advanced bullae (producing staining on both sides of the bullae) and post-mortem decomposition (sub-optimal preservation of membranes, filaments, and fibrils). When electron microscopy is used for diagnosis in humans, guidelines suggest that freshly induced blisters are sampled, as lesions more than 1 h old may produce ambiguous results [24].

Our study thus shows the value of a precision medicine approach and the molecular genetic characterization of dogs with suspected inherited disease. Whole genome sequencing is becoming increasingly efficient at identifying causative pathogenic variants, enabling precise diagnosis even when no fresh samples for a sophisticated histological investigation are available. Knowledge of the presumed causative genetic defect will enable genetic testing and the detection of healthy carriers to avoid the unintentional breeding of further affected dogs.

5. Conclusions

We found a homozygous complex duplication in *COL7A1* likely leading to the absence of functional COL7A1 protein in three neonatal, sibling Basset Hounds with severe and extensive blistering of the paw pads and nasal planum, nail loss, and tongue and esophageal involvement consistent with DEB. There are only a few documented cases of DEB in dogs, and to our knowledge, this is the first instance described in Basset Hounds.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/12/1458/s1: Figure S1: Genomic sequences of the wildtype and mutant alleles. Figure S2: EM images for one affected puppy, and a normal control. Table S1: Known functional candidate genes for epidermolysis bullosa and other skin fragility disorders. Table S2: Accession numbers of 74 dog genome sequences. Table S3: Private variants in a DEB-affected Basset Hound.

Author Contributions: Conceptualization, T.M.G., S.K., D.S.R., and T.L.; Data curation, T.M.G., S.K., V.J., D.S.R., and T.L.; Investigation, T.M.G., S.K., D.S.R., and T.L.; Methodology, T.M.G., S.K., V.J., D.S.R., and T.L.; Supervision, D.S.R. and T.L.; Writing—original draft, T.M.G. and S.K.; Writing—review and editing, T.M.G., S.K., V.J., D.S.R., and T.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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ABHD5 frameshift deletion in Golden Retrievers with ichthyosis

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Investigation

ABHD5 frameshift deletion in Golden Retrievers with ichthyosis

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Abstract

Ichthyoses are hereditary skin disorders characterized by the formation of scales and defects in the outermost layer of the epidermis. In dogs, at least six different breed-specific ichthyoses including a relatively common *PNPLA1*-related autosomal recessive ichthyosis in Golden Retrievers are known. In this study, we investigated 14 Golden Retrievers with scales that were not homozygous for the mutant *PNPLA1* allele suggesting a genetically distinct new form of ichthyosis. Histopathological examinations showed lamellar, orthokeratotic hyperkeratosis, and mildly hyperplastic epidermis that led to the diagnosis of a nonepidermolytic ichthyosis. Combined linkage and homozygosity mapping in 14 cases and 30 nonaffected family members delimited a critical interval of ~12.7 Mb on chromosome 23. Whole-genome sequencing of an affected dog revealed a single protein-changing variant within this region that was not present in 795 control genomes. The identified variant is a 14 bp deletion in the *ABHD5* gene (c.1006_1019del), leading to a frameshift and altering the last 14 codons p.(Asp336Serfs*6). The genotypes at this variant showed perfect cosegregation with the ichthyosis phenotype in a large family comprising 14 cases and 72 controls. *ABHD5* encodes an acyltransferase required for lipid metabolism. In humans, variants in *ABHD5* cause Chanarin-Dorfman syndrome, a neutral lipid storage disease with ichthyosis. Our data in dogs together with the knowledge on the effects of *ABHD5* variants in humans strongly suggest *ABHD5*:c.1006_1019del as candidate causative genetic variant for a new canine form of ichthyosis, which we propose to designate as Golden Retriever ichthyosis type 2 (ICH2).

Keywords: *Canis lupus familiaris*; dog; dermatology; genodermatosis; metabolism; lipid storage disorder; animal model; precision medicine; veterinary medicine

Introduction

Ichthyoses comprise a heterogeneous group of cornification disorders characterized by generalized dry and scaly skin. Various genetic defects have been described, all disrupting the skin barrier and leading to hyperkeratosis and scaling of the skin (Oji *et al.* 2010; Schmuth *et al.* 2013). The classification of ichthyoses distinguishes between syndromic and nonsyndromic forms. Nonsyndromic ichthyoses refer to those with the phenotypic manifestation of the disease limited to the skin whereas syndromic ichthyoses additionally involve other organs. Ichthyoses can be further subdivided into epidermolytic and nonepidermolytic ichthyoses. This differentiation is light microscopy based. Epidermolytic ichthyoses are accompanied by epidermolytic hyperkeratosis at the ultrastructural level. Typical findings are intracellular vacuolization and formation of small intraepidermal blisters (Oji *et al.* 2010).

In humans, at least 67 different genes have been described to be related with different forms of ichthyosis (Uitto *et al.* 2020). In dogs, several breed-specific ichthyoses have been described. However, in only six of the canine ichthyoses, the underlying genetic defect has been identified (Mauldin 2013; Leeb *et al.* 2017). A heterozygous missense variant in *ASPRV1* caused an autosomal dominant form of nonepidermolytic ichthyosis in a German Shepherd (Bauer *et al.* 2017; OMIA 002099-9615). A mild epidermolytic ichthyosis in Norfolk terriers is caused by a splice-site variant in *KRT10* (Credille *et al.* 2005; OMIA 001415-9615). In American Bulldogs, a frameshift deletion in *NIPAL4* was found to cause autosomal recessive congenital ichthyosis (Casal *et al.* 2017; OMIA 001980-9615). A splice site variant in *SLC27A4* was described in Great Danes with a clinically severe, autosomal recessive syndromic ichthyosis (Metzger *et al.* 2015; OMIA 001973-9615). An autosomal recessive nonepidermolytic ichthyosis in Jack Russell Terriers is caused by a LINE-1 insertion into the *TGM1* gene (Credille *et al.* 2009; OMIA 000546-9615). Probably the most common canine ichthyosis is an autosomal recessive ichthyosis in

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Golden Retrievers (OMIA 001588-9615). The first case report is from 2004 (Hall and Yager 2004) and the disease phenotype has been well characterized in the following years (Guaguère *et al.* 2007, 2009; Cadiergues *et al.* 2008; Mauldin *et al.* 2008). The underlying genetic defect is a homozygous insertion-deletion (indel) variant in *PNPLA1* encoding patatin like phosphatase domain containing 1. The protein plays a key role in lipid organization and metabolism of the epidermal barrier and the defective protein in the affected Golden Retrievers causes malformation of the intercellular stratum corneum lipid layer and abnormal desquamation (Grall *et al.* 2012).

In this study, we investigated 14 Golden Retrievers with clinical and histopathological signs of nonepidermolytic ichthyosis. Despite the phenotypic similarity to the *PNPLA1*-related ichthyosis, none of these dogs carried the mutant *PNPLA1* allele in a homozygous state. In the present study, we therefore aimed to characterize this presumably new form of inherited ichthyosis and to unravel the causative genetic variant. We propose to term this specific phenotype Golden Retriever ichthyosis type 2 (ICH2, OMIA 002368-9615).

Materials and methods

Ethics statement

The Golden Retrievers in this study were privately owned and skin biopsies and blood samples for diagnostic purposes were collected with the consent of their owners. The collection of blood samples was approved by the *Cantonal Committee for Animal Experiments* (Canton of Bern; permit BE71/19). All animal experiments were done in accordance with local laws and regulations.

Clinical and histopathological examinations

A physical examination was performed by the attending veterinarians. Two to four 4–6 mm skin punch biopsies per dog were taken and routinely processed for histopathology. Hematoxylin and eosin (H&E) stained slides were reviewed by board certified veterinary pathologists (DJW and EAM).

Animal selection for genetic analyses

This study included 482 Golden Retrievers. They comprised 86 closely related dogs including 14 ICH2 affected and 72 unaffected relatives originating from North America. The remaining 396 dogs were Golden Retrievers of European origin from the Vetsuisse Biobank.

DNA extraction

Genomic DNA was extracted from EDTA blood using the Maxwell® RSC Whole Blood DNA with the Maxwell® RSC instrument (Promega, Dübendorf, Switzerland).

Linkage analysis and homozygosity mapping

Genotype data for 44 Golden Retrievers comprising dogs from 7 litters and their parents were obtained with 220 k Illumina CanineHD BeadChips by Geneseek/Neogen (Supplementary File S1, 11 unaffected parents, 14 affected, and 19 unaffected offspring). For all dogs, the call rate was >95%. Using PLINK v1.9 (Purcell *et al.* 2007), markers on the sex chromosomes or with unknown positions were removed. We further removed markers that had missing genotypes in any of the 44 dogs, Mendel errors, or a minor allele frequency <0.01. The final pruned dataset contained 110,720 markers and was organized into four separate subfamilies comprising between 5 and 23 dogs. To analyze the data for parametric linkage, an autosomal recessive inheritance model with full penetrance, a disease allele frequency of 0.55 and the Merlin software (Abecasis *et al.* 2002) were applied.

For homozygosity mapping, the genotype data for the 14 ICH2 affected dogs were used. Markers with call rates <100% and markers on the sex chromosomes were excluded. The –homozyg-group option in PLINK was used on a final dataset of 198,921 markers to search for extended regions of homozygosity. The output intervals were matched against the intervals from linkage analysis in Excel spreadsheets to find overlapping regions (Supplementary Table S1). A tped-file containing the markers on chromosome 23 was visually inspected in an Excel spreadsheet to precisely delimit the homozygous shared haplotype in the cases (Supplementary Table S1). All positions correspond to the CanFam3.1 reference genome assembly.

Whole-genome sequencing

An Illumina TruSeq PCR-free library with an insert size of ~330 bp was prepared from one ICH2 affected dog and sequenced at 26x coverage on an Illumina NovaSeq 6000 instrument. The reads were mapped to the dog CanFam3.1 reference genome assembly as previously described (Jagannathan *et al.* 2019). The sequence data were deposited under study accession PRJEB16012 and sample accession SAMEA8797074 at the European Nucleotide Archive.

Variant calling and variant filtering

Variant calling was performed using GATK HaplotypeCaller (McKenna *et al.* 2010) in gVCF mode as described (Jagannathan *et al.* 2019). To predict the functional effects of the called variants, the SnpEff software (Cingolani *et al.* 2012), together with NCBI annotation release 105 for the CanFam 3.1 genome reference assembly was used. For filtering of private variants, we used 795 control genomes (Supplementary Table S2). Numbering within the canine *ABHD5* gene corresponds to the NCBI RefSeq accession numbers XM_542689.5 (mRNA) and XP_542689.2 (protein).

Targeted genotyping

We used Sanger sequencing to confirm the candidate variant *ABHD5*:c.1006_1019del. A 389 bp (or 375 bp in case of the mutant allele) PCR product was amplified from genomic DNA using AmpliTaqGold360Mastermix (Thermo Fisher Scientific, Waltham, MA, USA) and primers 5′-CTG CTG GCC CTG TCA TTA GT-3′ (Primer F) and 5′-CAG GCT CTC TCT CCC ACA TT-3′ (Primer R). After treatment with exonuclease I and alkaline phosphatase, we sequenced the amplicons in both directions on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific Corporation, Waltham, MA, USA). Sanger sequences were analyzed with the Sequencher 5.1 software (Gene Codes Corporation, Ann Arbor, MI, USA). Subsequently, targeted genotyping of dogs was performed by fragment length analysis of PCR products on a 5200 Fragment Analyzer (Agilent, Basel, Switzerland).

The *PNPLA1*:c.1445_1447delinsTACTACTA variant causing another form of ichthyosis in Golden Retrievers (Grall *et al.* 2012) was genotyped by Sanger sequencing of PCR amplicons as described above. A 300 bp PCR product (or 305 bp in case of the mutant allele) was amplified with the primers 5′-GGC CCT GAT AGT GAA GGA CA-3′ (Primer F) and 5′-TCC TAA CAC CTG CTC CTG CT-3′ (Primer R). The reverse primer was used for sequencing.

Results

Clinical description

ICH2 affected dogs had large white to gray and powdery to adherent scale throughout the hair coat. The abdominal skin was mildly hyperpigmented. Thick white scale was adherent to the concave surface of the pinnae (Figure 1).

Histopathological examination

The skin biopsies of affected dogs showed moderate epidermal hyperplasia without dermal inflammation. The main change was expansion of the stratum corneum by laminar orthokeratotic hyperkeratosis. Numerous keratinocytes in the granular layer contained perinuclear clear spaces (Figure 2). The clinical signs together with the histopathological findings led to the diagnosis of a nonepidermolytic ichthyosis.

Genetic analysis

The 14 available cases belonged to seven different litters, each with unaffected parents. Both male and female dogs were affected. The pedigree showed multiple inbreeding loops and was strongly suggestive for a monogenic autosomal recessive inheritance (Figure 3).

By combining linkage analysis and homozygosity mapping, we determined the critical interval for the disease-causing genetic variant. A single ~12.7 Mb segment on chromosome 23 showed both linkage in the families and shared homozygous genotypes in all 14 available cases (Figure 4A). The LOD score of the linked interval on chromosome 23 was 2.75 (Table 1, Supplementary Figure S1). The exact coordinates of the critical interval were Chr23:1–12,734,912 (Supplementary Table S1).

The genome of one affected dog was sequenced at 26x coverage. Assuming that the disease allele is rare in the dog population, we filtered for private homozygous variants that were absent from 795 control genomes (Supplementary Table S2). This analysis identified 32 private homozygous variants in the critical interval, of which only one was predicted to be protein changing (Table 2, Supplementary Table S3).

The identified candidate variant was a 14 bp deletion in the last exon of *ABHD5*, XM_542689.5:c.1006_1019del (Figure 4, B and C). The formal genomic designation of the variant is Chr23:2,587,000–2,587,013del (CanFam3.1). It causes a frameshift, resulting in a premature stop codon and altering the last 14 codons of the open reading frame, XP_542689.2:p.(Asp336Serfs*6).

We confirmed the presence of this variant in the index case by Sanger sequencing and genotyped all 85 relatives. The genotypes at the variant showed perfect cosegregation with the ICH2 phenotype. All 14 available cases were homozygous for the deletion. The 11 parents of the 14 cases who represent obligate carriers were all heterozygous. The remaining 61 unaffected relatives were either heterozygous or homozygous for the wildtype allele. The mutant allele was absent from 396 Golden Retrievers of European origin (Table 3, Supplementary Table S4).

Discussion

In this study, we investigated 14 Golden Retrievers with a new form of ichthyosis termed ICH2 that so far appears to be limited to dogs from North America. Histopathology classified ICH2 as nonepidermolytic ichthyosis. The 14 available cases came from seven different litters, all of which were related. The clinical and histological presentation of ICH2-affected dogs strongly

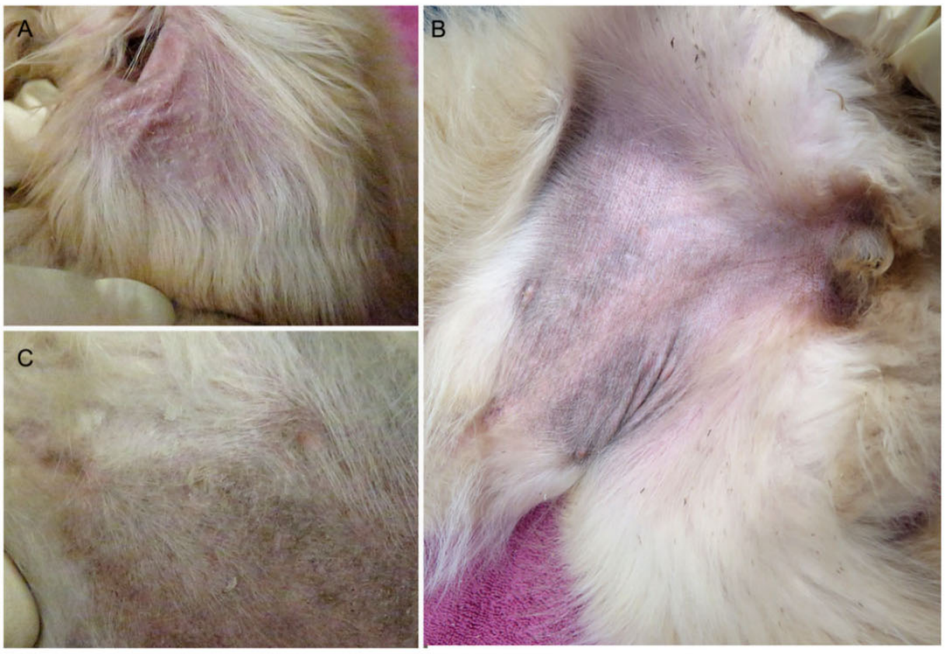

Figure 1 Clinical images from an 11-week-old Golden Retriever with ICH2. (A) Adherent scales on the inner pinna. (B) Thick scales on the ventral thorax and (C) abdominal hyperpigmentation.

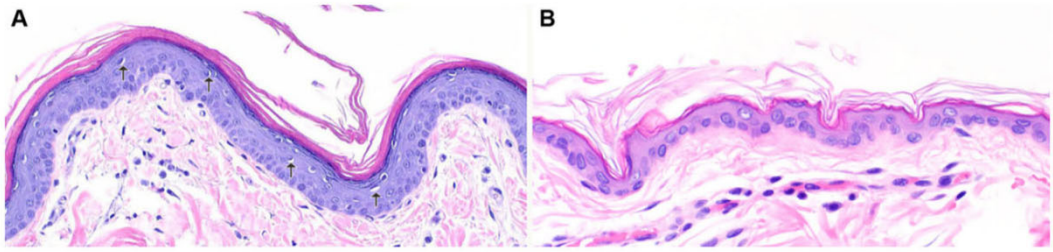

Figure 2 Histopathology of a Golden Retriever with ICH2. (A) Skin biopsy from an affected dog reveals marked thickening of the epidermis with expansion of the stratum corneum by laminar orthokeratotic hyperkeratosis. Numerous keratinocytes have perinuclear clear spaces (arrows). (B) Normal skin from an unaffected Golden Retriever. H&E 200X.

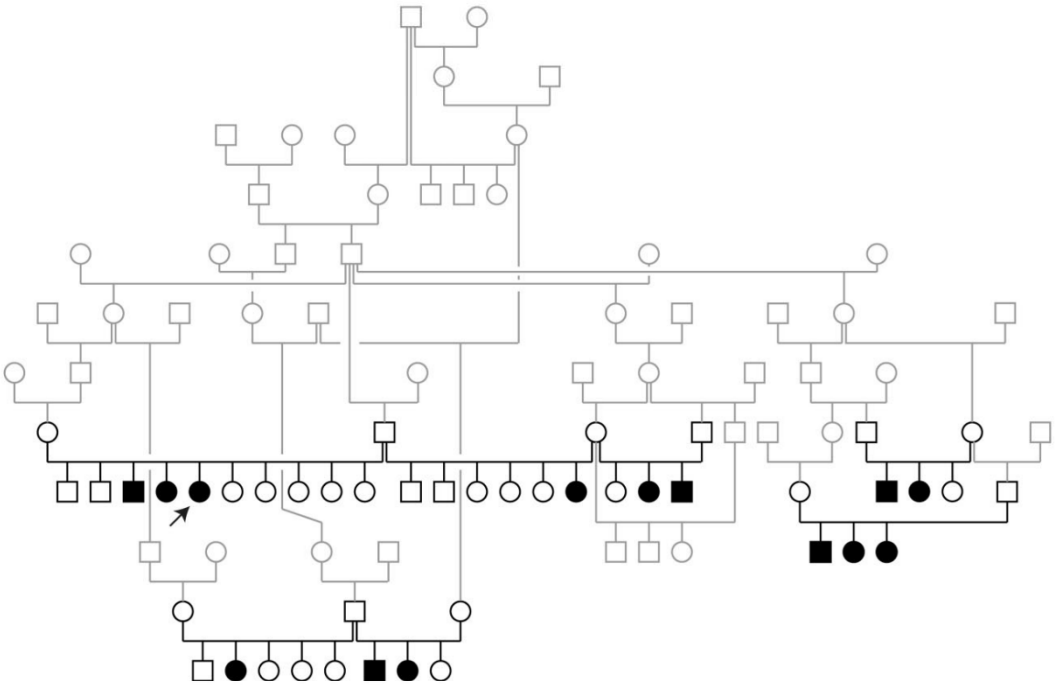

Figure 3 Pedigree of the investigated Golden Retriever family. Squares represent males and circles represent females. The affected dogs are indicated by filled symbols. Note the multiple inbreeding loops within this pedigree. All affected dogs have shared common ancestors in their maternal and paternal lineages. The 44 dogs that were genotyped on microarrays and used for the linkage analysis are indicated in black and constitute four separate subfamilies. The other dogs are shown in gray. The arrow indicates the dog that was used for whole-genome sequencing.

resembled that of the well-known *PNPLA1*-related ichthyosis in Golden Retrievers (Grall *et al.* 2012). Experienced breeders reported more severe and adherent scaling in ICH2-affected Golden Retrievers. Histologically, the epidermis in ICH2-affected dogs is thicker than in dogs with the *PNPLA1*-related ichthyosis dogs and this may correspond with a more severe barrier defect. ICH2 cases tend to have more keratinocytes with perinuclear clear spaces than dogs with the *PNPLA1*-related ichthyosis.

Using a hypothesis-free positional approach we delimited a ~12.7 Mb interval or roughly 0.5% of the 2.4 Gb dog genome for the ICH2 locus. Contrasting the genome sequence of an affected dog against 795 control genomes identified a single private homozygous coding variant within the critical interval, a 14 bp deletion in the last exon of the *ABHD5* gene. *ABHD5* encodes α-β hydrolase domain containing 5 also known under the alias name CGI-58. This protein is involved in the biosynthesis of major components of the skin barrier formation, especially ω-O-acylceramide (Ghosh *et al.* 2008; Ohno *et al.* 2018). Studies in *Abhd5*$^{-/-}$ knockout mice showed drastically reduced triglyceride (TG) hydrolase activity in the epidermis. The defective epidermal TG catabolism led to an impaired synthesis of acylceramides and defective formation of the corneocyte lipid envelope resulting in a dysfunctional permeability barrier of the skin (Radner *et al.* 2010). ABHD5 also activates adipose triglyceride lipase (ATGL), which catalyzes the initial step of lipolysis converting TG to diglycerides (DG) (Zimmermann *et al.* 2004; Lass *et al.* 2006).

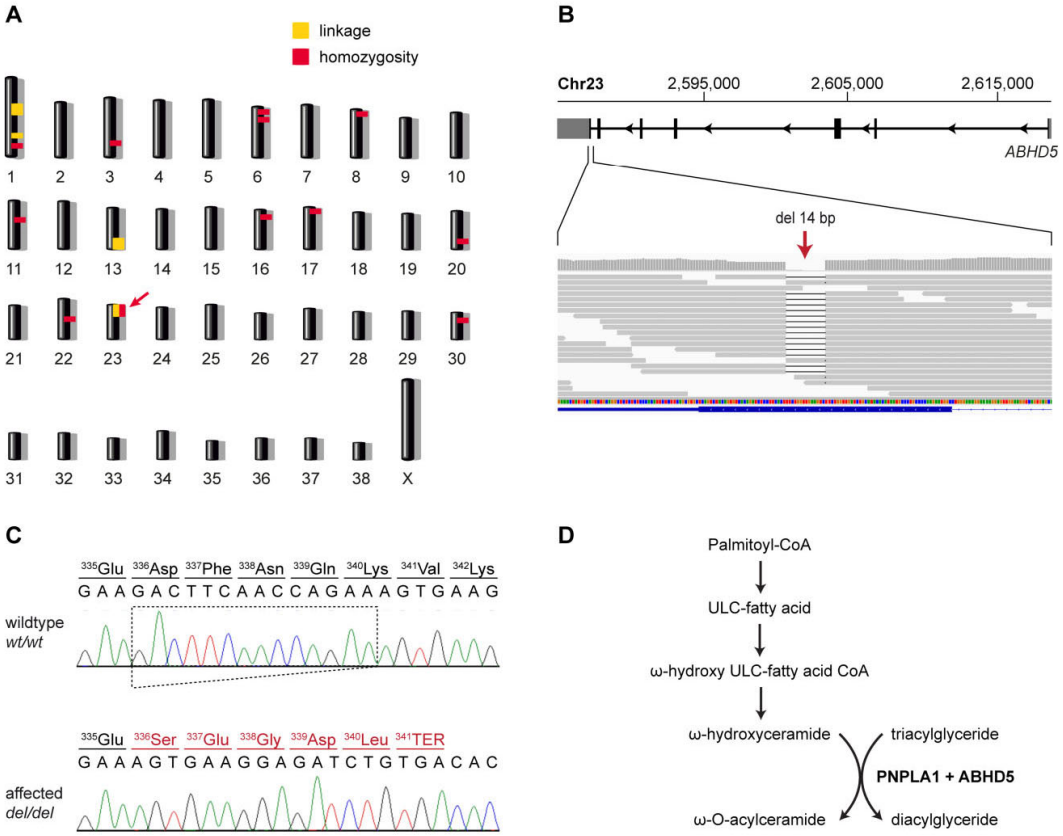

Figure 4 Mapping of the ICH2 locus and details of the *ABHD5*:c.1006_1019del variant. (A) Combined linkage and homozygosity mapping revealed a single overlapping region on chromosome 23 indicated by an arrow. (B) Integrative Genomics Viewer (IGV) screenshot showing the position of the deletion in the short-read alignments of the ICH2 affected dog. (C) Sanger sequencing confirmed the detected 14 bp frameshift deletion. The altered reading frame and the premature stop codon are indicated in red. (D) Metabolic pathway for the synthesis of ω-O-acylceramide, an essential lipid required to maintain skin barrier function (Haydar Eskiocak *et al.* 2019). In the last step of this pathway, ABHD5 acts as a coactivator of PNPLA1 and may help to provide the required triacylglycerides within the endoplasmic reticulum (Ohno *et al.* 2018). A lack of either ABHD5 or PNPLA1 leads to ichthyosis in humans (Lefevre *et al.* 2001; Grall *et al.* 2012).

Table 1 Results of linkage analysis in 44 dogs from 7 litters and their parents

Chrom	Start (Mb)	Stop (Mb)	Max LOD score	α[a]
1	41.498	48.203	0.43	0.65
1	69.318	69.332	3.46	1
1	105.516	106.520	0.23	0.63
13	57.599	63.242	1.07	0.73
23	0.000	12.822	2.75	1

[a] α indicates an estimated proportion of the four subfamilies that show linkage. The dogs were organized in four subfamilies (see Materials and Methods and Figure 3).

ABHD5 loss-of-function variants in humans were reported to cause Chanarin-Dorfman syndrome (CDS) (OMIM # 275630, Dorfman *et al.* 1974; Chanarin *et al.* 1975; Lefevre *et al.* 2001). CDS is a rare autosomal recessive inherited syndromic form of ichthyosis. In CDS, a nonepidermolytic ichthyosis is often accompanied by hepatic steatosis with hepatomegaly, myopathy, and neurological disorders due to lipid droplet accumulation in various tissues (Dorfman *et al.* 1974; Chanarin *et al.* 1975; Bruno *et al.* 2008).

Table 2 Results of variant filtering in the ICH2 affected Golden Retriever against 795 control genomes

Filtering step	Variants
All variants in the affected Golden Retriever	3,040,308
Private variants	1,110
Private variants in critical interval	32
Private protein-changing variants in critical interval	1

Only homozygous variants are reported.

Table 3 Genotypes at the *ABHD5*: c.1006_1019del variant in Golden Retrievers of North American and European origin

Dogs	wt/wt	wt/del	del/del
ICH2 affected Golden Retrievers ($n=14$)	—	—	14
Obligate carriers for ICH2 ($n=11$)	—	11	—
Unaffected other relatives ($n=61$)	35	26	—
Golden Retrievers of European origin ($n=396$)	396	—	—

More than ten pathogenic human *ABHD5* variants have been described, many of which are affecting the α-β hydrolase domain, lipid binding region, pseudocatalytic domain, or acyltransferase domain (Lefevre *et al.* 2001; Schweiger *et al.* 2009). The *ABHD5*:c.1006_1019del frameshift deletion identified in the affected Golden Retrievers of this study alters the last 14 codons of the open reading frame. They include the last 10 codons of the α-β hydrolase domain (Schweiger *et al.* 2009). A comparable *ABHD5* nonsense variant truncating the last 14 codons of the homologous human sequence was reported in four CDS patients from a consanguineous family (Aggarwal *et al.* 2012). This strongly suggests an essential function of the C-terminal tail of ABHD5 and supports the hypothesis that the observed *ABHD5*:c.1006_1019del frameshift deletion is indeed causative for the ichthyosis in the investigated Golden Retrievers.

In summary, we discovered a new autosomal recessive ichthyosis in Golden Retrievers, which we propose to designate as Golden Retriever ichthyosis type 2 (ICH2). The identified candidate causative variant enables genetic testing to prevent the unintentional breeding of affected puppies. Further studies are warranted to clarify whether the phenotypic changes in the affected dogs are limited to the skin or whether ICH2 also affects other organs like CDS in humans.

Data availability

The genome sequence data were submitted to the European Nucleotide Archive (ENA). All accession numbers are listed in Supplementary Table S2.

Supplementary material is available at *G3* online.

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Conflicts of interest

M.L.C. is affiliated with the PennGen Laboratory, which offers genetic testing for dogs. The other authors declare no conflict of interest.

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A *COL5A2* In-Frame Deletion in a Chihuahua with Ehlers-Danlos Syndrome

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Abstract: Ehlers-Danlos syndrome (EDS) is a group of heterogeneous, rare diseases affecting the connective tissues. The main clinical signs of EDS are skin hyperextensibility, joint hypermobility, and skin fragility. Currently, the classification of EDS in humans distinguishes 13 clinical subtypes associated with variants in 20 different genes, reflecting the heterogeneity of this set of diseases. At present, variants in three of these genes have also been identified in dogs affected by EDS. The purpose of this study was to characterize the clinical and histopathological phenotype of an EDS-affected Chihuahua and to identify the causative genetic variant for the disease. The clinical examination suggested a diagnosis of classical EDS. Skin histopathology revealed an abnormally thin dermis, which is compatible with classical EDS. Whole-genome sequencing identified a heterozygous de novo 27 bp deletion in the *COL5A2* gene, *COL5A2*:c.3388_3414del. The in-frame deletion is predicted to remove 9 amino acids in the triple-helical region of COL5A2. The molecular analysis and identification of a likely pathogenic variant in *COL5A2* confirmed the subtype as a form of classical EDS. This is the first report of a *COL5A2*-related EDS in a dog.

Keywords: *Canis lupus familiaris*; dog; dermatology; genodermatosis; skin; precision medicine; animal model

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1. Introduction

Ehlers-Danlos syndrome (EDS) is a clinically and genetically heterogenous group of heritable connective tissue disorders. Typical clinical signs include joint hypermobility, skin hyperextensibility, and tissue fragility [1]. Different types of EDS can be distinguished according to the underlying pathomechanisms, such as defective primary structure and processing of collagen, collagen folding and cross-linking, structure and function of the myomatrix, glycosaminoglycan biosynthesis, intracellular processes, or complement pathway [2]. In humans, variants in 20 genes have so far been shown to cause different forms of EDS (Table 1) [2].

In dogs, EDS was first described as cutaneous asthenia more than 70 years ago [3]. To date, several reports of dogs with connective tissue disorders, such as EDS have been published [4–17]. However, only for a few of them, the underlying genetic variant has been identified. EDS in a Doberman Pinscher was caused by a homozygous nonsense variant in

ADAMTS2 (OMIA 000328-9615) [18], which encodes the procollagen I N-proteinase that excises the N-propeptide of type I and type II procollagens [19].

Table 1. Overview on genetic causes of human Ehlers-Danlos syndrome (EDS), adapted from [2].

Gene	EDS Type	Inheritance
ADAMTS2	Dermatosparaxis	AR
AEBP1	Classical-like type 2	AR
B3GALT6	Spondylo-dysplastic	AR
B4GALT7	Spondylo-dysplastic	AD
CHST14	Musculocontractural	AR
COL1A1	Classical, Vascular, Artrochalasia	AD
COL1A2	Artrochalasia, Cardiac valvular	AD
COL3A1	Vascular	AD
COL5A1	Classical	AD
COL5A2	Classical	AD
COL12A1	Myopathic	AD
C1R	Periodontal	AD
C1S	Periodontal	AD
DSE	Musculocontractural	AR
FKBP14	Kyphoscoliotic	AR
PRDM5	Brittle cornea syndrome	AR
PLOD1	Kyphoscoliotic	AR
SLC39A13	Spondylo-dysplastic	AR
TNXB	Classical-like	AR
ZNF469	Brittle cornea syndrome	AR

Two compound heterozygous missense variants in *TNXB* were reported in a single mixed-breed dog with EDS. However, the evidence for pathogenicity was extremely weak and it is not fully clear whether this dog really suffered from a *TNXB*-related form of EDS (OMIA 002203-9615) [20]. The encoded tenascin-X is a large extracellular matrix protein, which is an essential regulator of collagen deposition by dermal fibroblasts [21–23].

Two independent variants in *COL5A1* have been identified in a Labrador Retriever and a mixed-breed dog affected with EDS (OMIA 002165-9615) [24]. *COL5A1* encodes the α1 chain of type V collagen, which is important for correct collagen fibrillogenesis [25].

In this study, we investigated the clinical and histopathological phenotype of an EDS-affected Chihuahua and the underlying causative genetic defect.

2. Materials and Methods

2.1. Animal Selection

This study included an EDS-affected Chihuahua and its unaffected parents. For the whole genome sequencing data analysis, we used 783 control dogs from different breeds and 9 wolves (Table S1). The control dogs and wolves had already been used in earlier studies, e.g., [26,27].

2.2. Clinical and Histopathological Examinations

The affected dog and both its parents underwent clinical examination. A piece of skin flap from a trauma-induced wound at the dorsum of the affected dog was fixed in 10% neutral-buffered formalin, processed routinely, and sections were stained with hematoxylin-eosin and saffron (HES).

2.3. DNA Extraction

Genomic DNA was isolated from EDTA blood of the affected dog using the Maxwell® 16 Blood DNA Purification Kit and from buccal cells of both parents using the Maxwell® 16 Buccal Swab LEV DNA Purification Kit, both using the Maxwell® 16 Instrument (Promega, Dübendorf, Switzerland). Genomic DNA was frozen at −20 °C until further use.

2.4. Whole-Genome Sequencing and Variant Calling

An Illumina TruSeq PCR-free DNA library with ~340 bp insert size of the affected dog was prepared and sequenced on a NovaSeq 6000 instrument with 25× coverage (Illumina, San Diego, CA, USA). The sequence data were submitted to the European Nucleotide Archive with the study accession PRJEB16012 and the sample accession SAMEA8797073. Mapping, alignment, and variant calling were performed as described [26]. Private variant filtering was performed with a hard filtering approach requiring the genotype 0/1 for heterozygous or 1/1 for homozygous variants in the affected dog and simultaneously a homozygous reference or missing genotype in the control dogs (0/0 or ./.).

2.5. Gene Analysis

All references within the canine *COL5A2* gene correspond to the NCBI RefSeq accession numbers XM_005640393.3 (mRNA) and XP_005640450.1 (protein). We used the CanFam3.1 reference genome assembly and NCBI annotation release 105.

2.6. PCR and Sanger Sequencing

Sanger sequencing was used to validate the candidate variant *COL5A2*:c.3388_3414del in the affected dog and to genotype both parents. PCR products were amplified from 10 ng genomic DNA using GoTaq® G2 Flexi DNA Polymerase in a total volume of 25 µL including 0.2 µL *Taq* polymerase, 5 µL 5× buffer, 1.5 µL $MgCl_2$ solution at 25 mM (Promega, Madison, WI, USA), 0.5 µL dNTP mix at 10 mM each (MP Biomedicals, Irvine, CA, USA) together with 0.5 µL each forward primer 5′-TAGCGTTCAGGCTTCCACTG-3′ and reverse primer 5′-CTCCAACACCTACGTGAGCC-3′ (primer concentrations were 10 µM). PCR amplification comprised 32 cycles (denaturation 30 s at 94 °C, annealing 40 s at 60 °C, and elongation 40 s at 72 °C) followed by 5 min at 72 °C. Electrophoresis was performed on a 2% agarose gel. After DNA gel extraction using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), amplicons were sequenced on an ABI 3730XL DNA Analyzer (Thermo Fisher Scientific, Reinach, Switzerland). Sanger sequences were analyzed using the Chromas 2.6.6 software (Technelysium, Pty, Ltd., South Brisbane, Australia).

3. Results

3.1. Clinical History and Examination

An 18-month-old spayed female Chihuahua was referred to the dermatology consultation for suspicion of EDS. The first clinical signs were noted on both eyes during puppyhood, at which time the dog was presented to the ophthalmologist with a diagnosis of corneal endothelial dystrophy. Moreover, its skin was hyperextensible (Figure 1a) and abnormalities were noted on the face around the eyes and on the extremities where the skin was fragile and tore easily after scratching or rubbing (Figure 1b–d). Several episodes of wounds on the trunk after minor injuries or scratching were also reported (Figure 1e). The healing process was difficult and long at each time. The dog had presented bilateral inguinal hernia. The dog lived with both parents and its two sisters and was the only one presenting these abnormalities. The dog wore a coat and a protective pet cone all day long in order to protect the skin and prevent any injuries and wound formation (Figure 2). At the time of consultation, the dog was in good general health condition. The skin was hyperextensible and no wounds were noted during clinical examination.

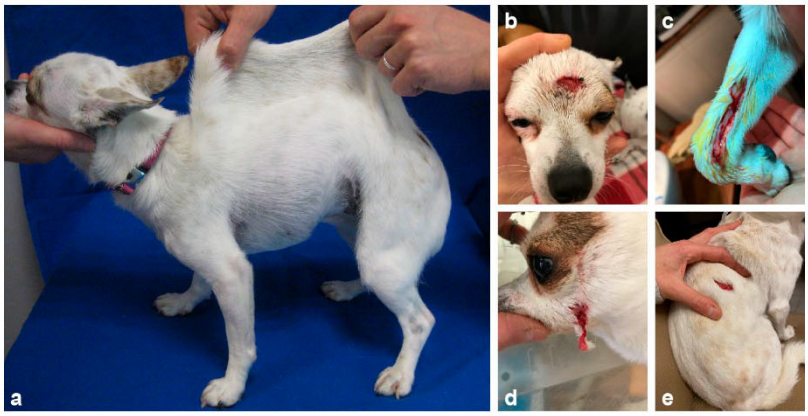

Figure 1. Clinical signs of the classical Ehlers-Danlos-affected Chihuahua dog. (**a**) Skin hyperextensibility over the dorsum. (**b**) Skin laceration resulting from minimal trauma on the head, (**c**) on the forelimb, (**d**) on the lower jaw, and (**e**) on the dorsum.

Figure 2. The dog lives continuously with a protective suit and an Elizabethan collar to prevent wound formation.

3.2. Histopathological Examination

Histopathological examination showed a marked reduction in dermis thickness (Figure 3a). The density of collagen fibers was markedly reduced giving a loose appearance of the dermis. There was moderate variation in collagen fibers size (Figure 3b). The epidermis and hypodermis were unremarkable.

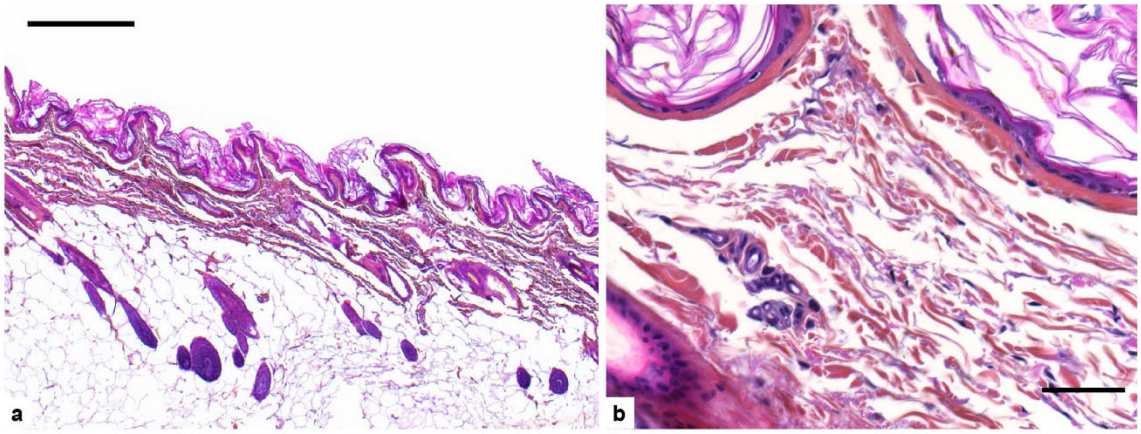

Figure 3. Cutaneous histopathological findings of the dog affected with Ehlers-Danlos syndrome (EDS). (**a**) Dermis is abnormally thin while epidermis and hypodermis are normal. Hematoxylin-eosin and saffron stain (HES), bar 500 µm; (**b**) collagen fibers are moderately uneven in size and their density is markedly reduced. Hematoxylin-eosin and saffron stain (HES), bar 50 µm.

3.3. Genetic Analysis

Whole-genome sequencing and private variant filtering were performed to identify the causative genetic variant. For this analysis, we compared the sequence data of the affected dog to the genomes of 783 control dogs of different breeds and 9 wolves (Table S1). We specifically looked for protein-changing variants in the 20 known EDS candidate genes (Table 1). The outcome of the various filtering steps is summarized in Tables 2 and S2.

Table 2. Results of variant filtering in the affected dog against 792 control genomes.

Filtering Step	Heterozygous Variants	Homozygous Variants
All variants in the affected dog	4,191,411	2,457,482
Private variants	18,956	1258
Protein-changing private variants	104	2
Protein-changing private variants in 20 candidate genes	1	0

We identified a single private protein-changing variant within an EDS candidate gene, *COL5A2*. The variant can be designated as Chr36:30,548,697_30,548,723del. It is a heterozygous in-frame deletion of 27 bases (XM_005640393.3:c.3388_3414del), predicted to remove 9 amino acids, XP_005640450.1:p.(Lys1130_Asp1138del). The presence of the variant in a heterozygous state in the EDS-affected Chihuahua was confirmed by Sanger sequencing. The genotyping results of both parents revealed that *COL5A2*:c.3388_3414del represented a de novo variant as the mutant allele was absent from buccal cell DNA of both parents.

4. Discussion

In this study, we describe a Chihuahua with Ehlers-Danlos syndrome. EDS is a heterogeneous group of diseases affecting connective tissue. In human patients, 13 clinically different EDS subtypes with known causal variants in 20 different genes are recognized. A recently introduced additional clinical subtype has not yet been characterized at the molecular level [2].

In humans, the diagnosis of an EDS subtype is made by identifying clinical signs that can be categorized into major or minor criteria and by complementary molecular analysis. For the so-called classical EDS, the major and minor criteria are listed in Table 3.

Table 3. Classical EDS clinical criteria in humans, adapted from [1].

Major criteria	1. Skin hyperextensibility and atrophic scarring 2. Generalized joint hypermobility
Minor criteria	1. Easy bruising 2. Soft, doughy skin 3. Skin fragility (or traumatic splitting) 4. Molluscoid pseudotumors 5. Subcutaneous spheroids 6. Hernia (or history thereof) 7. Epicanthal folds 8. Complication of joint hypermobility (e.g., sprains, luxation/subluxation, pain, flexible flatfoot) 9. Family history of a first degree relative who meets clinical criteria

Minimal criteria suggestive for classical EDS are the presence of either both major criterions or one major criterion and at least three minor criteria [1]. As this classification of EDS subtypes has been developed in humans, it must be used with caution in animals. Nevertheless, given the clinical, histopathological, and molecular similarities between animal models of EDS and human EDS, it is realistic to use it to define an animal EDS subtype.

The dog described here presented in its clinical history the first major criterion, namely (1) hyperextensibility of the skin and atrophic scarring, as well as three clinical signs listed as minor criteria, namely (1) easy bruising, (3) skin fragility and traumatic splitting, and (6) hernia. Thus, by referring to the human classification, the dog falls into the clinical category of classical EDS. As in humans, clinical diagnosis must be supplemented by molecular analysis. In our case, the genetic analysis confirmed the clinical hypothesis of a classical EDS.

Suspicion of EDS in animals usually leads to performing a skin biopsy and histopathological analysis of the dermis. Classically, histopathological descriptions report a dermis of normal thickness but present disorganized, smaller, curved collagen fibers of variable length and unequal diameter [9,16,24,28,29]. However, these characteristics are not always found and some affected animals show no abnormality on histological analysis. In some cases, a dermis of reduced thickness has been identified in affected animals, notably cats and dogs [5,29], as it is in our case.

Using a whole-genome sequencing approach, we identified a single heterozygous protein-changing variant in a known EDS candidate gene in the investigated EDS-affected Chihuahua. The variant is an in-frame deletion of 27 bases in *COL5A2*, encoding the α2 chain of type V collagen.

Type V collagen is present mainly as heterotrimers of two α1(V) chains (encoded by *COL5A1*) and one α2(V) chain [30,31]. Homotrimers of three α1(V) chains or heterotrimers comprised of an α1(V), α2(V), and α3(V) chain (encoded by *COL5A3*) also exist; however, their physiological function is largely unknown [2,32]. Type V collagen co-assembles with type I collagen into heterotypic fibrils in the extracellular matrix, with type V collagen being crucial for initial fibril formation [25]. Correct fibril formation and integrity play a key role in maintaining the physical properties of skin and other tissues [2].

The three chains of type V collagen are assembled into a triple helix. The sequence of each procollagen chain is characterized by extended Gly-Xaa-Yaa repeats. The presence of glycine (which has no side chain) in every third position permits the formation of the triple-helical structure. The Xaa and Yaa are often proline and hydroxyproline but can be any amino acid [2].

COL5A1-associated EDS mostly results from *COL5A1* haploinsufficiency, as type V procollagen molecules cannot accommodate more than a single proα2(V) chain, and the reduction of available proα1(V) chains results in the production of about half the normal amount of type V collagen [33]. By contrast, proα1(V) chains can form functional homotrimers [34]. Thus, no heterozygous *COL5A2* null alleles have been identified in EDS patients so far [35].

COL5A2-associated EDS is mostly related to structural variants located in the triple helix domain, resulting in the production of mutant proα2(V) chains which are expected to be incorporated in defective type V collagen molecules [35].

In accordance with [35], the *COL5A2* variant identified in the EDS-affected dog from this study is predicted to remove three Gly-Xaa-Yaa triplet repeats and thus induce a structural alteration of the synthesized mutant proα2(V) chains. We assume that this causes aberrant heterotrimer formation and a defective type V collagen structure corresponding to a dominant-negative gain-of-function in the mutant allele.

In domestic animals, only one other *COL5A2* variant, p.Gly789Val, has been reported to cause EDS in Holstein cattle [28].

5. Conclusions

To the best of our knowledge, our study represents the first report of a *COL5A2*-related EDS in dogs. The reported *COL5A2* deletion arose by a de novo mutation event, which strongly supports its causality for the EDS phenotype.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/genes13050934/s1, Table S1: Public Genomes; Table S2: Private Variants.

Author Contributions: Conceptualization, L.C. and T.L.; investigation, S.K., L.C., A.B., N.C.-F. and E.R.-G.; data curation, V.J.; writing—original draft preparation, S.K. and L.C.; writing—review and editing, S.K., L.C., V.J., A.B., N.C.-F., E.R.-G. and T.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval were waived for the investigation of the affected dog in this study as it was performed during clinical and pathological veterinary diagnostics. All animals in this study were examined with the consent of their owners and handled according to good ethical standards. The "Cantonal Committee for Animal Experiments" approved the collection of blood samples from control Chihuahuas (Canton of Bern; permit 71/19).

Informed Consent Statement: The owners of the dogs in this study gave informed consent to use samples and data for research.

Data Availability Statement: The accessions for the sequence data reported in this study are listed in Table S1.

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KRT5 missense variant in a Cardigan Welsh Corgi with epidermolysis bullosa simplex

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SHORT COMMUNICATION

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KRT5 missense variant in a Cardigan Welsh Corgi with epidermolysis bullosa simplex

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Abstract

Epidermolysis bullosa (EB) is a group of blistering disorders that includes several subtypes, classified according to their level of cleavage. Typical clinical signs are blisters and erosions resulting from minimal trauma. The disease has been described in many mammalian species and pathogenic variants in at least 18 different genes have been identified. In the present study, we investigated a Cardigan Welsh Corgi with congenital clinical signs consistent with epidermolysis bullosa. The puppy had blisters and erosions on the paw pads, and the oral mucosa. Histologic examination demonstrated the typical clefting between the dermis and epidermis and confirmed the clinical suspicion. We obtained whole genome sequencing data from the affected puppy and searched for variants in candidate genes known to cause EB. This revealed a heterozygous missense variant, *KRT5*:p.(E476K), affecting the highly conserved KLLEGE motif of keratin 5. The mutant allele in the affected puppy arose owing to a de novo mutation event as it was absent from both unaffected parents. Knowledge of the functional impact of *KRT5* variants in other species together with the demonstration of the de novo mutation event establishes *KRT5*:p.(E476K) as causative variant for the observed EBS.

KEYWORDS
animal model, *Canis lupus familiaris*, dermatology, dog, genodermatosis, precision medicine, skin, veterinary medicine

INTRODUCTION

Epidermolysis bullosa (EB) comprises a heterogeneous group of blistering disorders. They are characterized by blisters and erosions resulting from minimal trauma (Fine et al., 2008; Has et al., 2018). Classification into different types of EB is based on the level of cleavage. These are EB simplex (EBS), with a level of cleavage in the basal layer of the epidermis, junctional EB, with a level of cleavage at the dermo-epidermal junction, and dystrophic EB, with a level of cleavage just below the basal membrane. The fourth category, Kindler EB, with a mixed level of cleavage, is a rare EB type with a very severe clinical phenotype (Has et al., 2020). The human classification is also the basis for the classification of EB types in animals (Medeiros & Riet-Correa, 2015).

Hereditary forms of EB follow a monogenic mode of inheritance with autosomal dominant or autosomal recessive inheritance. So far, a large number of disease causing alleles have been identified in at least 18 genes coding for structural proteins in human skin (Fine et al., 2008; Has et al., 2014; Lemke et al., 2014).

In domestic animals, cases of EB have been described but molecular studies are rare. Causative genetic variants have been identified in dogs, cats, horses, cattle and sheep (Medeiros & Riet-Correa, 2015). In dogs, variants in four different genes, namely *COL7A1*, *LAMA3*, *LAMB3* and *PLEC*, have been described (Baldeschi et al., 2003; Capt et al., 2005; Garcia et al., 2020; Herrmann et al., 2021; Kiener et al., 2020; Mauldin et al., 2017; Niskanen et al., 2017).

The identification of a pathogenic variant and thus the molecular characterization of a suspected inherited disease is of great value as it provides a fast and minimally invasive definitive diagnosis. Further, it offers the opportunity to give breeding recommendations and may even provide guidance for targeted therapy (Leeb et al., 2022). Therefore, the aim of this study was to characterize the clinical and histopathological phenotype of the affected puppy and to identify the underlying genetic variant using a whole genome sequencing approach.

We examined a Cardigan Welsh Corgi puppy with skin blistering and erosions that were noticed shortly after birth. It was one of a litter of three puppies, in which both other littermates and the parents were healthy. The affected dog was smaller than the littermates. Vesicles and ulcers were extensively present in the oral cavity, lips and paw pads (Figure 1). Gentle pressure on the skin near affected areas would result in sloughing of the adjacent epidermis (positive Nikolsky sign; Maity et al., 2020). The puppy was given supplementary nutrition and adopted by a veterinarian at 10 weeks of age. At that time, the lesions in the oral cavity had healed and the dog was able to eat normally. The dog continued to have waxing and waning lesions on the paw pads, ears, axilla and groin. Blisters would arise intermittently, ulcerate and heal with scarring. At 1 year of age, four skin punch biopsies from lesional areas were obtained under general anesthesia. Histopathologic examination revealed intact subepidermal vesicles along with ulcers, granulation tissue and regions of dermal scarring. Small remnants of basal keratinocytes were evident at the margins of the vesicles and scattered basal keratinocytes were apoptotic. The dog was still alive at 17 months of age (at the time of writing this paper).

Based on the characteristic clinical signs and the early age of onset, we suspected an underlying genetic defect and performed a genetic analysis. We therefore collected EDTA blood samples of the complete family and isolated genomic DNA from peripheral leukocytes. The genome of the affected puppy was sequenced at 22× coverage on an Illumina Novaseq 6000 instrument. Mapping and variant calling with respect to the UU_Cfam_GSD_1.0 reference genome assembly were performed as described (Jagannathan et al., 2019). We searched for private variants by comparing the sequencing data of the affected puppy with 563 control genomes and identified 134 heterozygous and six homozygous private

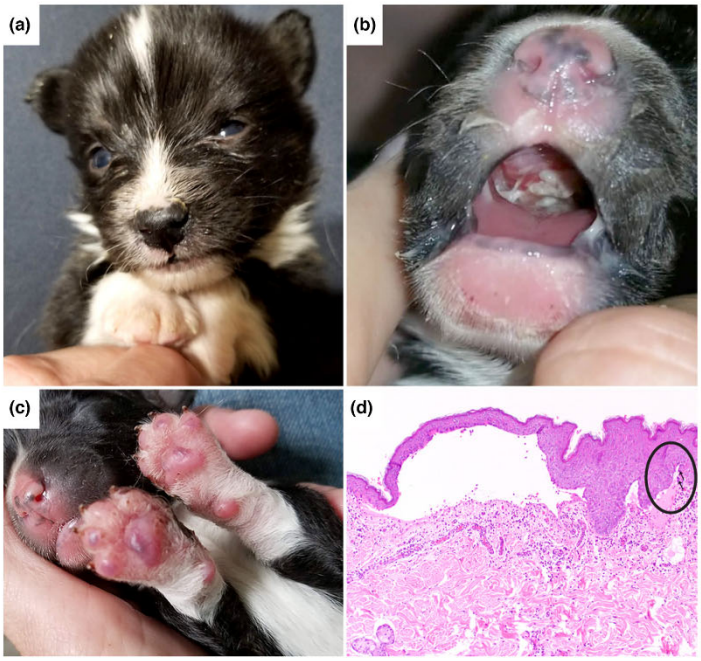

FIGURE 1 Phenotype of the Cardigan Welsh Corgi affected with epidermolysis bullosa. (a) Underweight 12-day-old puppy. (b) Extensive ulcers in the oral cavity. Note that the epidermis sloughs with gentle pressure on the perioral skin. (c) Numerous vesicles on the paw pads. (d) Broad subepidermal vesicle with remnants of basal keratinocytes at the marginal base of the blister (thin arrows in black circle). H&E 10×.

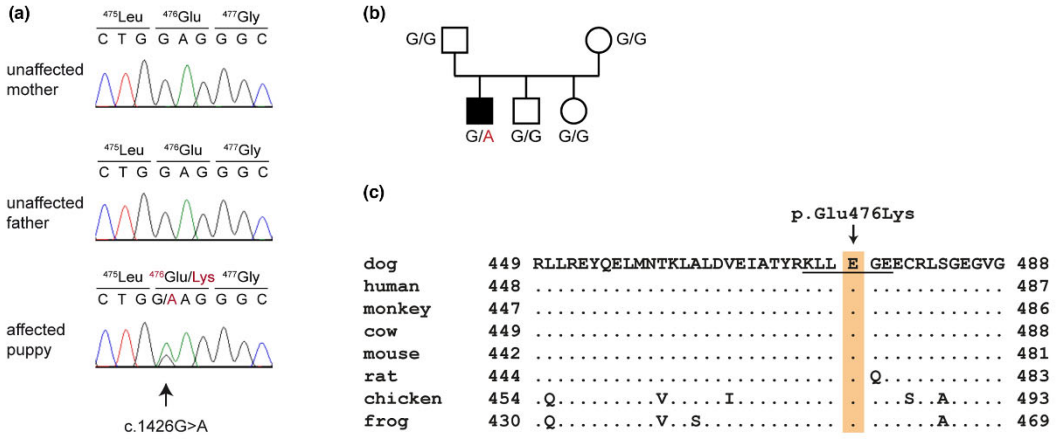

FIGURE 2 Details of the identified variant in *KRT5*. (a) Electropherograms showing the *KRT5*:C.1426G>a missense variant. The variable position is indicated with an arrow and the amino acid translations are shown. (b) Pedigree of the family of the affected dog. Squares represent males and circles represent females. The filled symbol indicates the affected dog. The genotypes for each animal at the position of the identified *KRT5* variant are given. (c) Multiple-species alignment of the KRT5 protein in the region of the p.E476K variant. The variant affects the first glutamate residue of the highly conserved KLLEGE motif (underlined).

protein-changing variants (Tables S1 and S2). These included a heterozygous single nucleotide substitution in *KRT5*, Chr27:44080887C>T (UU_Cfam_GSD_1.0), which is a well-characterized candidate gene for EB. This missense variant, NM_001346035.1:c.1426G>A, is predicted to change a conserved glutamate in keratin 5, NP_001332964.1:p.(E476K). Sanger sequencing confirmed the presence of the variant in the affected dog and showed that it had arisen de novo, as both parents were homozygous for the wild-type allele (Figure 2).

KRT5 encodes keratin 5, which is a type II keratin and expressed in the basal keratinocytes. Together with keratin 14 it builds intermediate filaments which are important for cell structure and stability (Arin, 2009). Both keratins comprise two helical rod domains, which assemble into alpha-helical coiled-coil heterodimers (Fuchs & Cleveland, 1998; Stephens et al., 1997). When this structural framework is disorganized, cells become fragile and tend to rupture under stress (Fuchs & Cleveland, 1998).

The identified heterozygous missense variant E476K affects the first glutamate residue of the KLLEGE motif at the end of the rod domain of keratin 5. This motif is highly conserved and important for filament assembly and stability (Letai et al., 1992, 1993; Müller et al., 2006; Wilson et al., 1992). Previously, variants affecting the first and second glutamate residues of the KLLEGE motif of keratin 5 have been described in humans and cattle with EBS (Ford et al., 2005; García et al., 2011; Lane et al., 1992; Schuilenga-Hut et al., 2003; Stephens et al., 1997; Yasukawa et al., 2006). The human variant E475K, homologous to the canine E476K variant, was reported to cause EBS in three human patients (Schuilenga-Hut et al., 2003; Yasukawa et al., 2006).

Human patients with a E477K variant affecting the second glutamate in the KLLEGE motif may develop a particularly severe and sometimes lethal EBS phenotype. Molecular modeling demonstrated the transition from a negatively charged glutamate to a positively charged lysine on the surface of the 2B domain of keratin 5 that is in direct contact with the 2B domain of keratin 14 (Lalor et al., 2019).

Monoallelic *KRT5* variants leading to the production of an aberrant keratin 5 protein are a common cause for EBS (Has et al., 2020). They act in a dominant negative manner, in which the abnormal protein produced by the mutant allele interferes with the normal protein in the process of keratin filament assembly (Yasukawa et al., 2006). Knowledge of the functional impact of *KRT5* missense variants at this position in humans and cattle, together with the demonstrated de novo mutation event and the absence of the mutant allele from a large number of control dogs, establishes the pathogenicity of the detected heterozygous *KRT5*:p.(E476K) variant according to human standards (Richards et al., 2015). The molecular analysis refined the diagnosis in the affected dog from EB to EBS. To the best of our knowledge, this is the first report of a *KRT5* variant causing EBS in dogs.

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CONFLICT OF INTEREST

The author declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Accession numbers for the whole genome sequence data sequences are given in Table S1.

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SUPPORTING INFORMATION

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A *de novo* variant in the keratin 1 gene (*KRT1*) in a Chinese Shar-Pei dog with severe congenital cornification disorder and non-epidermolytic ichthyosis

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RESEARCH ARTICLE

A *de novo* variant in the keratin 1 gene (*KRT1*) in a Chinese shar-pei dog with severe congenital cornification disorder and non-epidermolytic ichthyosis

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Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Abstract

A 3-months old Chinese shar-pei puppy with ichthyosis was investigated. The dog showed generalized scaling, alopecia and footpad lesions. Histopathological examinations demonstrated a non-epidermolytic hyperkeratosis. The parents of the affected puppy did not show any skin lesions. A trio whole genome sequencing analysis identified a heterozygous *de novo* 3 bp deletion in the *KRT1* gene in the affected dog. This variant, NM_001003392.1:c.567_569del, is predicted to delete a single asparagine from the conserved coil 1A motif within the rod domain of KRT1, NP_001003392.1:p.(Asn190del). Immunohistochemistry demonstrated normal levels of KRT1 expression in the epidermis and follicular epithelia. This might indicate that the variant possibly interferes with keratin dimerization or another function of KRT1. Missense variants affecting the homologous asparagine residue of the human KRT1 cause epidermolytic hyperkeratosis. Histologically, the investigated Chinese shar-pei showed a non-epidermolytic ichthyosis. The finding of a *de novo* variant in an excellent functional candidate gene strongly suggests that KRT1:p.Asn190del caused the ichthyosis phenotype in the affected Chinese shar-pei. To the best of our knowledge, this is the first description of a *KRT1*-related non-epidermolytic ichthyosis in domestic animals.

Introduction

Ichthyoses are a heterogeneous group of hereditary cornification disorders. They are characterized by generalized dry skin, scaling and/or hyperkeratosis. Several genetically distinct forms have been identified in a variety of dog breeds [1]. An epidermolytic form with autosomal recessive inheritance due to a variant in epidermal keratin 10 (*KRT10*) has been documented in the Norfolk terrier [2]. Six other canine ichthyosis forms that are characterized at the molecular level represent non-epidermolytic ichthyoses. A *PNPLA1*-associated autosomal

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recessive form that involves altered glycerophospholipid metabolism has been reported in golden retrievers [3, 4]. A loss of function variant in the *TGM1* gene encoding transglutaminase 1 leads to autosomal recessive ichthyosis in Jack Russell terriers due to calcium dependent cross-linking of peptides (e.g. involucrin, loricrin) involved in forming the cornified envelope [5]. *NIPAL4* (ichtyn) deficiency was reported in ichthyotic American bulldogs [6, 7]. *ABHD5*-related autosomal recessive ichthyosis represents another defect in lipid metabolism that has been reported in golden retrievers [8]. An autosomal dominant form of ichthyosis in a German shepherd dog was caused by a missense variant in *ASPRV1* encoding a protease required for the posttranslational processing of profilaggrin [9]. Finally, an autosomal recessive *SLC27A4*-related severe syndromic form of ichthyosis has been reported in Great Danes [10, 11]. Moreover, cornification disorders suggestive of ichthyosis have been described based on clinical examination and histopathologic changes in soft-coated wheaten terriers, West Highland white terriers, English springer spaniels, Labrador retrievers [1].

While cases of *KRT1*-related ichthyosis have been documented in humans, they have not been reported in dogs to date [12–14]. This investigation documents a congenital cornification disorder in a Chinese shar-pei puppy due to a 3 base pair deletion in the *KRT1* gene.

Materials and methods

Clinical examinations

Clinical evaluation of the patient was performed by a board certified veterinary dermatologist (TN). Skin scrapings and skin cytology were performed, and punch biopsies from the right antebrachium, the neck and right shoulder were collected for histopathologic and immunohistochemical examination. Blood samples from patient and his parents were collected for genetic testing.

Histopathological and immunohistochemical examinations

Submitted formalin fixed punch biopsies were bisected. Four-micron, hematoxylin and eosin-stained paraffin-sections and immunohistochemical stains were evaluated by a board certified veterinary pathologist (VKA).

Immunohistochemistry for KRT1 expression was performed on all three biopsy samples from the patient as well as on sections healthy skin from two Chinese shar-pei dogs (and other breeds (standard poodle, terrier-mix, boxer)). Four micron paraffin sections were collected on "plus" coated slides and air dried at 37˚C overnight and subsequently deparaffinized (xylene: 10 min 2x, followed by 100% ethanol: 1 min 3x, 95% ethanol: 1 min and 70% ethanol: 1min). After quenching of endogenous peroxidase (500 ul 10% sodium azide; 500 ul 30% hydrogen peroxide in 50 ml PBS; 25 min at room temperature), slides were rinsed in PBS 3x and immersed in preheated antigen retrieval solution (1x Dako Target Retrieval Solution; stock solution S1699, pH6); retrieval was performed a pressure cooker for 5 minutes. Slides were cooled down to room temperature, washed in PBS 3x. After exposing slides to 10% horse serum in PBS (15 min) the anti-CK-1 antibody (Clone 4D12B3: sc-65999; Santa Cruz Biotechnology, Inc. Dallas, Texas USA; 1:500 dilution in 10% horse serum in PBS) was applied for 60 min. After three rinses in PBS the following steps were performed: application of ImmPRESS HRP Horse Anti-Mouse IgG Polymer Reagent (Vector Cat.# MP-7402; 30 min), thorough PBS rinses and addition of substrate (Vector, SK-4800). Development was monitored microscopically and reaction was stopped by immersing the slides in Milli-Q/distilled water. Counterstain (Gill's Hematoxylin #2 RICCA, 3536–16; 15–30 s) was stopped by washing slides in running tap water. Slides were then cover-slipped using Shandon-Mount media (Thermo Scientific, 1900331).

Genetic examinations

Animal selection. This study included a total of 22 Chinese shar-peis. They comprised one ichthyosis affected Chinese shar-pei and its unaffected parents. Additional samples from 19 unrelated Chinese shar-peis without clinical signs of ichthyosis from the Vetsuisse Biobank were used as controls.

Whole-genome sequencing. Illumina TruSeq PCR-free libraries with insert sizes of ~330 bp were prepared from the affected dog and both parents. The libraries were sequenced with 2 x 150 bp chemistry on a NovaSeq 6000 instrument. The reads were mapped to the CamFam3.1 reference genome assembly as described [15]. The sequence data were submitted to the European Nucleotide Archive with the study accession PRJEB16012 and sample accessions SAMEA7198604 (affected puppy), SAMEA7198605 (unaffected dam) and SAMEA7198612 (unaffected sire). Variant calling was performed as described [15]. To predict the functional effects of the called variants, the SnpEff software [16] together with NCBI annotation release 105 for the CanFam 3.1 genome reference assembly was used. For variant filtering, we used 793 control genomes derived from 784 dogs and 9 wolves (S1 Table). We applied two different hard filtering approaches for homozygous and heterozygous private variants in the affected dog: In the search for private homozygous variants, we retained only variants with genotype 1/1 in the affected puppy and genotypes 0/0 or ./. in the 793 control genomes. In the search for private heterozygous variants, we retained only variants with genotype 0/1 in the affected puppy and genotypes 0/0 or ./. in the 793 control genomes. Subsequently, the private variants were combined in an Excel-file for further inspection (S2 Table). For functional prioritization, variants with SnpEff impact predictions high or moderate were combined and termed "protein-changing variants".

Confirmation of parentage. To confirm the parentage of the presumed parents and the affected dog, we used the genome sequence data (vcf-file) of the affected dog and its parents. Using PLINK v1.9 we extracted 6,269,532 informative markers distributed over all autosomes and performed a pairwise IBD estimation with the—genome command [17]. The sire-offspring and dam-offspring pairs both had an estimated overall IBD proportion (PI_HAT) of 50% with 0% P(IBD = 0), 100% p(IBD = 1) and 0% p(IBD = 2) as expected for parent-offspring duos.

Gene analysis. We used the dog reference genome assembly CanFam3.1 and NCBI annotation release 105. Numbering within the canine *KRT1* gene corresponds to the NCBI RefSeq accession numbers NM_001003392.1 (mRNA) and NP_001003392.1 (protein). For a multiple species comparison of KRT1 amino acid sequences, we used these accessions: NP_006112.3 (*Homo sapiens*), NP_001104288.1 (*Pan troglodytes*), XP_002687292.1 (*Bos taurus*), NP_032499.2 (*Mus musculus*), NP_001008802.2 (*Rattus norvegicus*). A precomputed multiple species sequence alignment was obtained from the NCBI HomoloGene website (https://www.ncbi.nlm.nih.gov/homologene).

Sanger sequencing. We used Sanger sequencing to confirm the *KRT1*:c.567_569del variant and to perform targeted genotyping of all samples. AmpliTaqGold360Mastermix (Thermo Fisher Scientific, Waltham, MA, USA) and the primers 5'-CCT GGT GGC ATA CAG GAA GT-3' (forward primer) and 5'-CTC GTT CGC ACC CTA GAA AG-3' (reverse primer) were used to amplify a 454 bp product. After treatment with shrimp alkaline phosphatase and exonuclease I, PCR amplicons were sequenced on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific). Sanger sequences were analyzed using the Sequencher 5.1 software (GeneCodes, Ann Arbor, MI, USA).

Ethics statement

All animal experiments were performed according to the local regulations. The dogs in this study were privately owned and skin biopsies and blood samples for diagnostic purposes were

collected with the consent of the owner. The collection of blood samples from healthy dogs was approved by the "Cantonal Committee for Animal Experiments" (Canton of Bern, Switzerland; permit 71/19).

Results

Family anamnesis, clinical examinations, histopathology

A 3-months old male Chinese shar-pei was presented for scaly skin and reduced overall body growth when compared with his 3 littermates, a female and 2 male puppies with clinically normal skin. Dam and sire were in the same household and clinically normal. Administration of Clavamox (ZoetisUS; 62.5 mg twice daily for 10 days) followed by Convenia (ZoetisUS; 80 mg/ml 0.54 ml), prednisone (5 mg twice daily, then once daily) and frequent bathing with Hexa-Chlor-K shampoo (GelnHaven Therapeutics, Schuyler, Oregon) revealed minimal improvement. Terramycin eye ointment (ZoetisUS) had been applied for entropium of the left eye. At time of presentation, the dog appeared bloated and uncomfortable despite eating and consuming normal amounts of water.

At the time of presentation severe generalized scaling and alopecia was noted, with scaling most prominent on the head (Fig 1A), neck (Fig 1B), abdomen (Fig 1C), legs, axillary folds (Fig 1D) and paws. Prominent follicular fronds accompanied surface scaling. The paw pads appeared deformed and hyperkeratotic. Pruritus was not observed. The left eye had an entropium. Skin scrapings for Demodex mites were negative. Skin cytology revealed numerous yeast organisms.

Biopsies from all three locations revealed severe hyperkeratosis, characterized by prominent keratin lamellae overlaying a marked compact layer of keratin (Fig 2A and 2B). The epidermis was markedly acanthotic and most infundibular regions were markedly dilated resulting in narrowing of the interfollicular epidermis (Fig 2A). The follicular lumina were filled with keratin and the infundibular epithelium was hyperplastic. Some perinuclear clearing was most evident in the prominent granular layer with irregularly sized keratohyalin (Fig 2C). Dispersed mast cells and some plasma cells and neutrophils were present in the superficial dermis and the sebaceous glands were prominent. Several small neutrophilic crusts with some cocci were noted entrapped within the thick keratin layer (Fig 2D). In the sample from the shoulder some follicles contained neutrophils in their lumina and the epidermis was covered by parakeratosis. Superficial yeast organisms were not observed in sections stained with periodic acid-Schiff stain. Many hair follicles and remaining hair shafts contained clumped melanin. The following morphologic diagnoses were made: 1) severe acanthosis and superficial and follicular hyperkeratosis suggestive of a cornification disturbance and 2) multifocal neutrophilic pustular dermatitis and neutrophilic luminal folliculitis and 3) melanin pigment clumping indicating dilute hair coat color. The latter was considered an expected incidental finding as the dog had a d^1/d^2 genotype at the *MLPH* gene [18, 19] and was born out of two clinically inconspicuous dilute-colored parents.

Given the overwhelming features of follicular and superficial hyperkeratosis, a hereditary cornification disorder consistent with ichthyosis was considered. Pustules and superficial folliculitis indicated a secondary pyoderma, which, based on skin cytology, was accompanied by a superficial yeast infection.

Genetic analysis

In order to characterize the underlying causative genetic variant we sequenced the genome of the affected puppy at 18.9x coverage and searched for variants in 36 candidate genes for

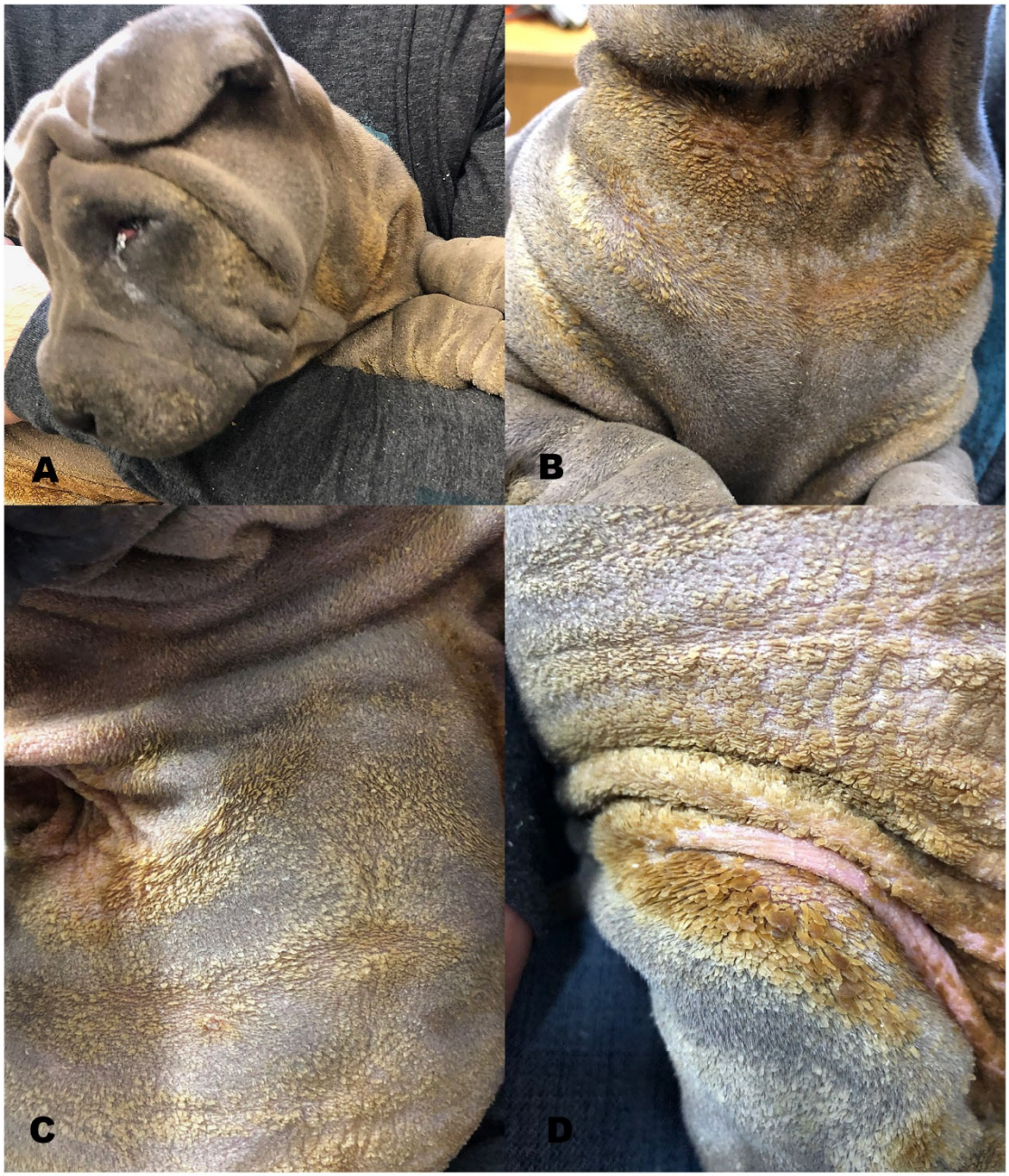

Fig 1. Clinical presentation of affected Chinese shar-pei. Severe generalized alopecia and scaling with marked follicular fronds on (A) head, (B) neck, (C) abdomen and (D) axillary folds.

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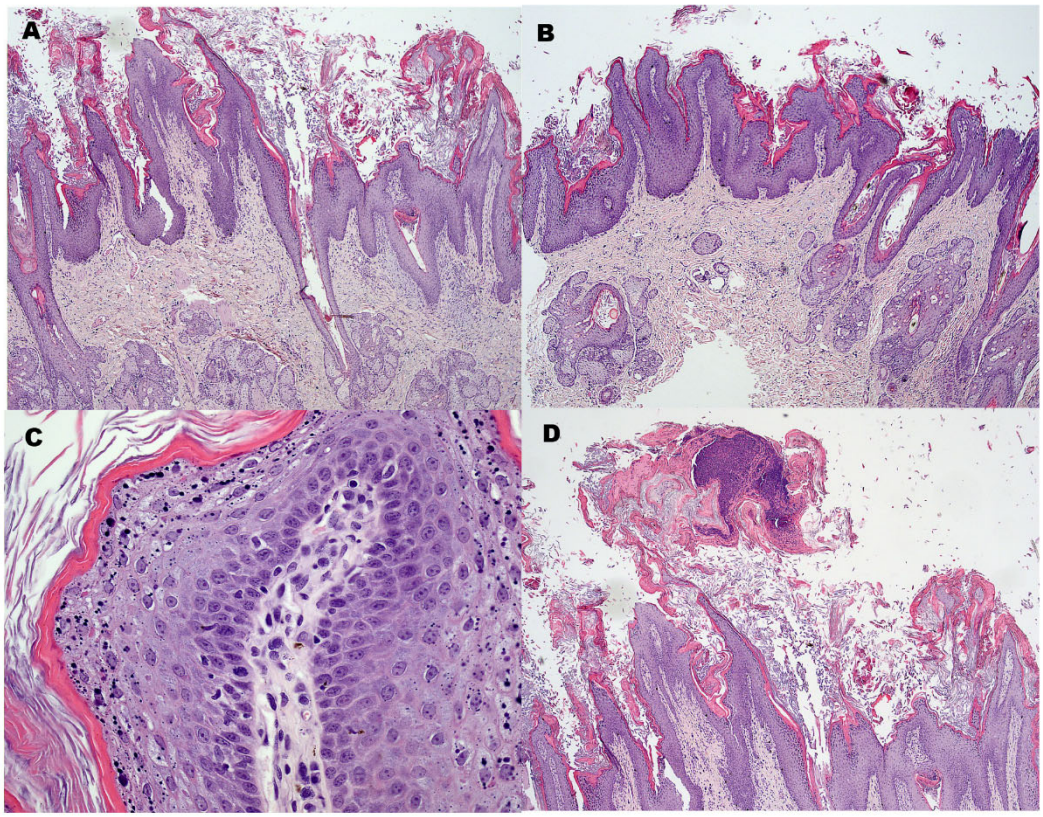

Fig 2. Histopathologic changes in the affected Chinese shar-pei. (A and B) The epidermis is severely hyperplastic with extensive compact and lamellar hyperkeratosis of the surface. The hyperkeratosis extends into the follicular lumina dilating the follicular openings (H&E; 40x). (C) There is a prominent granular layer with irregularly sized keratohyalin granules and common perinuclear vacuoles (H&E; 400x). (D) Multifocally, there were neutrophilic crusts indicating secondary pyoderma.

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ichthyosis (S2 Table), which were exclusively present in the affected dog and absent from 793 control genomes (Tables 1 and S1 and S3).

Subsequently, we performed a trio analysis and compared the genotypes in the affected dog with the genotypes of both parents (S3 Table). We considered two alternative scenarios for the putative causal variant: For an autosomal recessive trait, we expected the affected dog to be homozygous for the alternate allele and both parents heterozygous. Alternatively, for a

Table 1. Variants detected by whole genome sequencing of the affected Chinese shar-pei.

Filtering step	heterozygous variants	homozygous variants
Variants in the whole genome	4,261,447	2,920,513
Private variants[a]	82,046	9,522
Private protein-changing variants[a]	503	39
Private protein-changing variants in 36 candidate genes[a]	2	0

[a]The parents of the affected dog were excluded for these filtering steps.

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dominant trait that could only have been caused by a *de novo* mutation event, the affected dog should be heterozygous and both parents should be homozygous for the reference allele. The results of the trio analysis are summarized in Table 2.

Taken together these analyses identified a single protein-changing variant in a known ichthyosis candidate gene, for which the genotypes of the parents were compatible with a pathogenic effect. The variant was a heterozygous in frame deletion in the first exon of *KRT1* (NM_001003392.1:c.567_569del) (Fig 3A), removing three nucleotides coding for an asparagine of the 1A coil domain (NP_001003392.1:p.(Asn190del), Fig 3B). The formal genomic designation of the variant is Chr27:2,422,716_2,422,718del (CanFam3.1).

The trio analysis comparing the variants in the affected dog with the genomes of both parents revealed that *KRT1*:c.567_569del represented a *de novo* variant as the mutant allele was absent from leukocyte DNA of both parents. The correct parentage of sire and dam in this family was confirmed based on the genome sequence data.

We used Sanger sequencing to confirm the identified candidate *KRT1*: c.567_569del variant and to genotype the rest of the Chinese shar-peis from our study. The deletion was only present in heterozygous state in the ichthyosis affected puppy whereas both parents and all remaining Chinese shar-peis were homozygous for the wild type allele.

Expression of KRT1

Given the *de novo* variant in the *KRT1* gene in this Chinese shar-pei, KRT1 expression of the tissue was evaluated by light microscopy using immunohistochemistry. The intensity of KRT1 expression in the epidermis and the follicular epithelia of the affected dog was visually comparable to normal skin samples from Chinese shar-pei dogs (Fig 4) and other breeds. Keratinocytes revealed strong membranous and cytoplasmic KRT1 expression.

Discussion

Reported canine hereditary cornification disorders are due to genetic variants affecting either keratins or components involved in crosslinking of peptides or disruption of lipids within the cornified envelope [1]. All of them lead to interruption of successful cornification or desquamation. Most well documented forms of canine ichthyosis are non-epidermolytic, involving genetic variants in *ABHD5* or *PNPLA1* in golden retrievers, *TGM1* in Jack Russell terriers, *NIPAL4* in American bulldogs, and *ASPRV1* in a German shepherd dog [3–11, 21].

Marked follicular fronds in addition to prominent surface scales observed in this Chinese shar-pei indicated a cornification disturbance affecting both epidermis as well as follicles. Follicular fronds appear to be less prominent in other breeds with non-epidermolytic ichthyosis [3, 5, 6, 21].

Associated with the marked epidermal and follicular hyperkeratosis was a rather prominent acanthosis. The latter was also present in areas with no evidence of secondary pyoderma. This is somewhat in contrast with non-epidermolytic ichthyosis in other dog breeds, where marked

Table 2. Trio analysis of the affected Chinese shar-pei and its parents.

Filtering step	heterozygous variants	homozygous variants
Private variants that were absent from 793 control genomes	82,046	9,522
Protein-changing & genotypes of parents compatible with a pathogenic effect	27	19
Protein-changing & genotypes of parents compatible & in 36 candidate genes	1	0

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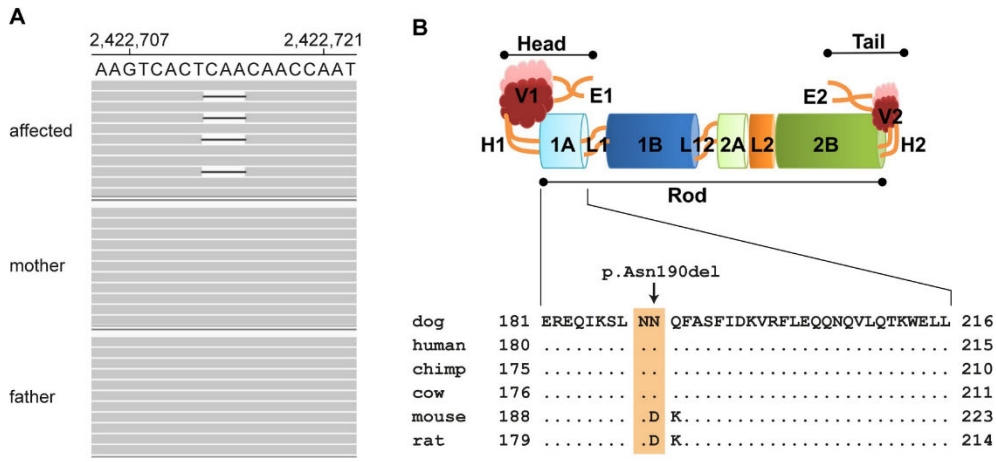

Fig 3. Details of the *KRT1*:c.567_569del, p.(Asn190del) variant. (A) Integrative Genomics Viewer (IGV) screenshot showing the short-read alignments of the ichthyosis affected puppy and its non-affected parents at the position of the deletion. A deletion of one copy of the allele is visible in the case but not in the parents. Note that in the IGV screenshot bases 2,422,713–2,422,715 are deleted, whereas the 3'-rule of HGVS nomenclature requires to designate this variant as Chr27:2,422,716_2,422,718del (CanFam3.1). (B) Schematic representation of the protein domain structure of a keratin dimer [20] with the highly conserved amino acid sequence of the coil 1A subdomain shown below. The variant is predicted to delete an asparagine residue from coil 1A, which is located within the rod domain of KRT1.

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hyperkeratosis is often disproportionate to the degree of epidermal acanthosis [5, 6, 21]. However, the marked hypergranulosis with irregularly sized keratohyalin granules as well as the presence of mild perinuclear vacuolization of keratinocytes was a finding consistent with features seen in other breeds. Electron microscopy revealed curvilinear membranous material within the granular layer cytosol, also in particular in the perinuclear swellings seen on light microscopy [6]. Electron microscopic evaluation was not performed in the Chinese shar-pei investigated by us.

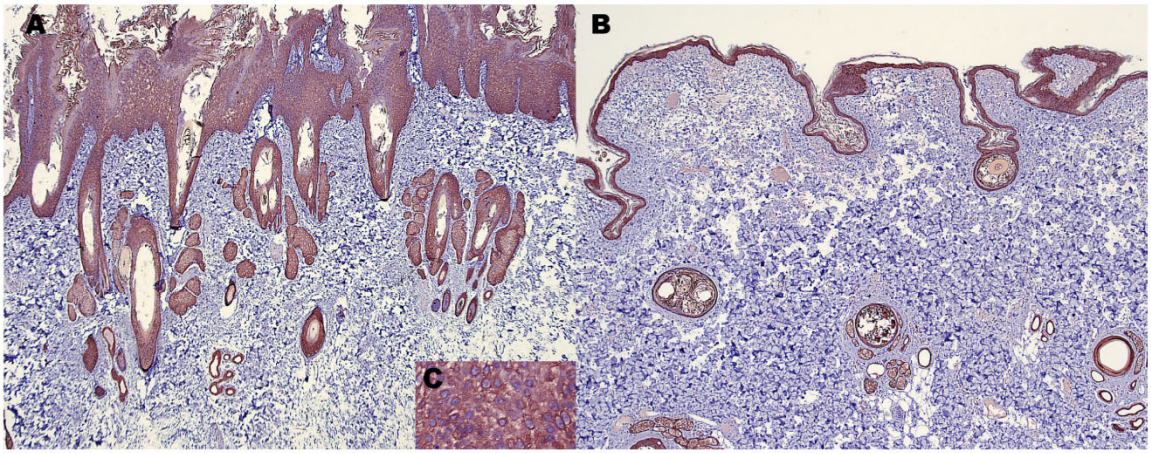

Fig 4. KRT1 expression in Chinese shar-pei skin (immunohistochemistry with anti-KRT1 antibody). (A) Intensity of KRT1 expression in the epidermis and follicular epithelia of the affected Chinese shar-pei is comparable to (B) normal Chinese shar-pei skin (40x). (C) Strong KRT1 expression in the epidermis of the affected Chinese shar-pei (400x).

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Genetic variants in *KRT1* or *KRT10* in humans typically present with an epidermolytic ichthyosis [12, 22–24]. The *KRT1*-related epidermolytic hyperkeratosis presents with or without palmo-plantar keratoderma, while *KRT10*-related epidermolytic ichthyosis typically does not involve palmo-plantar keratoderma. A *KRT10* variant in Norfolk terriers results in epidermolytic ichthyosis [2], but the *KRT1* variant in the Chinese shar-pei of this investigation was not associated with epidermolytic changes. Altered KRT1/KRT10 dimer formation due to variants affecting the structure of the paired 2B and V2 domains leads to severe acanthosis and hyperkeratosis in humans [25], reflecting the histologic features observed in the affected Chinese shar-pei. In human literature this is also referred to as "epidermolytic ichthyosis sine epidermolysis" [26].

The identified variant in the affected Chinese shar-pei leads to a deletion of 3 bases coding for an asparagine in the coil 1A motif of KRT1, which is part of the central rod domain of the KRT1/KRT10 heterodimer. The sequence of the central rod domain is highly conserved amongst epidermal keratins [20]. Three different missense variants affecting the homologous asparagine-188 of KRT1 in human patients have previously been described to cause epidermolytic hyperkeratosis [22, 27, 28]. This strongly suggests that changes in this region are intolerable for functional keratin filament formation.

In the affected Chinese shar-pei, the mutant KRT1 protein lacking asparagine-190 is apparently expressed within the acantholytic epidermis and the follicular epithelia as demonstrated by the immunohistochemistry experiment. The expression level was comparable to control skin tissue from an unaffected Chinese shar-pei and dogs from other breeds. This might indicate that the single amino acid deletion possibly interferes with dimerization or another function of the KRT1 molecule.

Our genetic analysis revealed *KRT*:p.(Asn190del) as a highly plausible candidate causal variant for the observed phenotype. According to the ACMG/AMP consensus standards for the interpretation of sequence variants in human patients [29], our data provide one strong, three moderate and one supporting criteria for pathogenicity, which is sufficient to classify the variant as pathogenic. The strong criterion is the demonstration of a *de novo* variant in an affected dog born out of two healthy parents. The three moderate criteria are the absence of the mutant allele from a relatively large control cohort, the protein length change due to an in-frame deletion and the fact that missense variants affecting the same amino acid have been established as pathogenic in humans. We consider the highly specific disease phenotype with known monogenetic etiology as supporting criterion for pathogenicity. Although our analysis yielded a variant fulfilling diagnostic criteria for pathogenicity, we have to caution that we did not investigate structural variants. Furthermore, the analysis relied on the accuracy and completeness of the CanFam3.1 genome assembly and NCBI annotation release 105. Therefore, we cannot formally exclude the possibility that other potentially plausible variants were missed. NCBI annotation release 105 agrees with a manually curated annotation of the canine *KRT1* gene [30].

In summary, to the best of our knowledge, this is the first case of a *KRT1*-related ichthyosis reported in domestic animals and the first case of ichthyosis in a Chinese shar-pei dog.

Supporting information

S1 Table. Accession numbers of 796 dog/wolf genome sequences. The affected dog is highlighted in red and the non-affected parents are highlighted in blue. Both parents and the affected dog were used for a trio analysis. The other 793 genome sequences were used as controls in filtering for private variants.
(XLSX)

S2 Table. 36 candidate genes for ichthyosis. List of non-syndromic and syndromic forms of ichthyosis.
(XLSX)

S3 Table. Private variants in the affected Chinese shar-pei. Variants are listed multiple times, if they have predicted effects on more than one transcript. Variants with a SnpEff predicted impact of "high" or "moderate" were considered protein-changing variants. For private variant filtering, the genomes of the case and 793 controls (excluding the parents) were considered. The KRT1:c.567_569del variant is highlighted in yellow (line 176,297).
(XLSX)

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Heterozygous *ATP2A2* missense variant identified in a Shih Tzu with Darier disease

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Heterozygous *ATP2A2* missense variant identified in a Shih Tzu with Darier disease

Abstract

Darier disease is caused by heterozygous loss of function variants in the *ATP2A2* gene encoding the endoplasmic/sarcoplasmic reticulum Ca^{2+} pump ATP2A2. Defective intracellular calcium signaling in the epidermis results in a loss of desmosomal adhesion and the development of characteristic skin lesions. In this study, we investigated a Shih Tzu that developed erythematous papules on the ventrum and, over time, the dorsal neck and a nodule in the right ear canal with secondary ear infection. Histopathologic examination demonstrated discrete foci of acantholysis affecting suprabasal layers of the epidermis. Whole genome sequencing of the affected dog identified a heterozygous missense variant, p.N809H, affecting an evolutionarily conserved amino acid residue of the ATP2A2 protein. The highly characteristic clinical and histopathologic findings together with a plausible variant in the only known functional candidate gene establish the diagnosis of canine Darier disease in the studied dog and highlight the potential of genetic analyses as complementary diagnostic approach in veterinary medicine.

Darier disease is a rare autosomal dominantly inherited skin disease in humans with keratotic papules in seborrheic areas, palmo-plantar pits and nail dystrophy (MIM #124200; Beck et al., 1977; Burge & Wilkinson, 1992). Clinical signs result from a separation between keratinocytes as well as abnormal cornification due to loss of desmosomal connections (Foggia & Hovnanian, 2004). The disease is caused by heterozygous variants in the *ATP2A2* gene, encoding the endoplasmic/sarcoplasmic reticulum Ca^{2+}-transporting ATPase 2, also termed SERCA2 (Nellen et al., 2017; Sakuntabhai et al., 1999). This calcium pump plays a key role in intracellular calcium signaling which in turn is central to the regulation of cell-to-cell adhesion, differentiation, and cornification in the epidermis (Foggia & Hovnanian, 2004). A canine form of Darier disease has been reported in an Irish Terrier with a heterozygous intronic SINE insertion in *ATP2A2* that resulted in a near-complete loss of functional transcripts from the mutant allele (OMIA 002265-9615; Linek et al., 2020).

This study investigated a 9-year-old, spayed female Shih Tzu from the USA that was referred to a dermatology specialty clinic. One year prior to referral, the dog was treated for bilateral otitis externa that improved but failed to completely respond to topical medication. The patient subsequently developed intense non-seasonal pruritus that was attributed to atopic skin disease and secondary superficial bacterial infection. Culture-based antibiotics, topical antimicrobials, antifungal medication, oclacitinib (Apoquel, Zoetis), and lokivetmab (anti-canine IL-31 monoclonal antibody, Cytopoint, Zoetis) were unsuccessful.

Dermatologic examination revealed erythematous papules on the ventrum, which frequently had a depressed center that was filled with keratin plugs (Figure 1a). The right external ear canal contained a pink-tan nodule with purulent exudate. Cytologic examination of the papules on the ventrum revealed numerous acantholytic cells with few scattered non-degenerative neutrophils (Figure 1b). Six-millimeter skin punch biopsies obtained under general anesthesia from the ventrum and the otic mass revealed discrete foci of marked epidermal hyperplasia with extensive acantholysis affecting the suprabasal layers of the epidermis. A broad cleft containing acantholytic keratinocytes separated the adherent basal layer from the overlying layers of the epidermis (Figure 1c). The acantholytic keratinocytes were either large, rounded cells with perinuclear halos and eosinophilic cytoplasm ('corps ronds') or small, ovoid cells with pyknotic flattened nuclei and intensely eosinophilic cytoplasm ('corps grains'; Figure 1d).

Although the late age of onset was unusual, the histopathologic features were typical for Darier disease. Therefore, EDTA whole blood was collected for genetic investigations. Genomic DNA was isolated with the Maxwell RSC Whole Blood Kit on a Maxwell RSC instrument and whole genome sequencing was performed on an Illumina Novaseq 6000 instrument with 2×150-bp paired-end reads at 18× coverage. The data was processed as described in Jagannathan et al. (2019); however, here we used the UU_Cfam_GSD_1.0 reference assembly

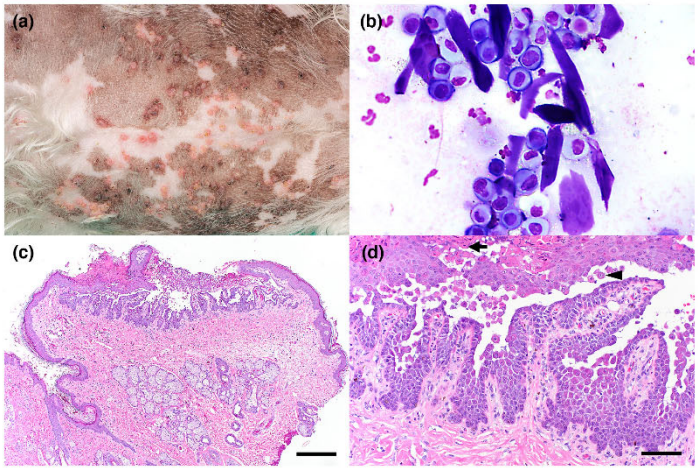

FIGURE 1 Clinical lesions, cytologic findings, and histopathologic features in a 9-year-old Shih Tzu with Darier disease. (a) Erythematous coalescing papules containing central keratin plugs. (b) Cytologic examination of the papules reveals numerous acantholytic cells with scattered non-degenerative neutrophils and keratin. Diff-Quik. (c) On histopathology the papule consists of discrete and marked epidermal hyperplasia with acantholytis of the spinous and granular cell layers. The basal layer remains adhered to the basement membrane zone. Hematoxylin and eosin, scale bar = 500 µm. (d) The corps ronds are acantholytic keratinocytes that are large and round with perinuclear halos and eosinophilic cytoplasm (arrowhead). The corps grains are acantholytic keratinocytes that are small, ovoid cells with pyknotic flattened nuclei and intensely eosinophilic cytoplasm (arrow). Hematoxylin and eosin, scale bar = 100 µm.

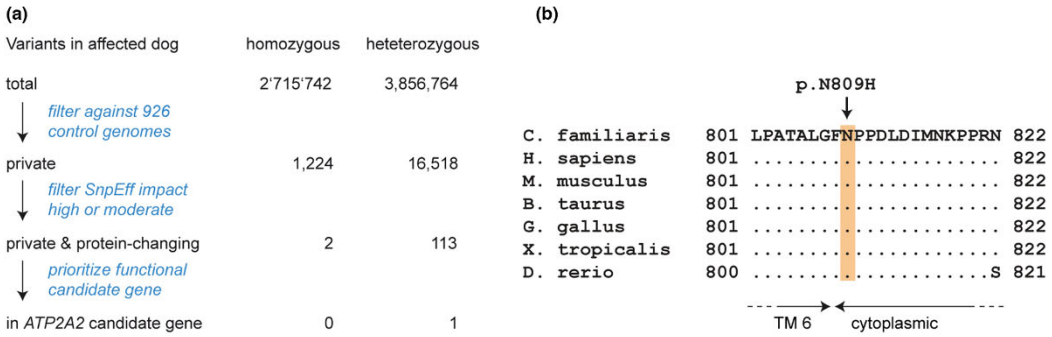

FIGURE 2 Details of the genetic analysis. (a) Workflow to identify the candidate causative *ATP2A2* missense variant from whole genome sequence data of the investigated case. Details of the identified variants are given in Table S2. (b) Multiple-species alignment of ATP2A2 amino acid sequences in the region harboring the p.N809H variant. The variant affects a perfectly conserved asparagine residue in the cytoplasmic domain following the sixth transmembrane helix. Accession numbers: dog (*Canis lupus familiaris*) NP_001003214.1; human (*Homo sapiens*) NP_733765.1; mouse (*Mus musculus*) NP_001103610.1; cattle (*Bos taurus*) XP_024833179.1; chicken (*Gallus gallus*) NP_001258903.1; frog (*Xenopus tropicalis*) XP_004910568.1; zebrafish (*Danio rerio*) NP_957259.1.

as described in Kiener et al. (2022). The sequencing data were submitted to the European nucleotide archive under the accession number SAMEA110175951. Private variants were filtered by comparing the sequencing data of the affected dog to 926 canine control genomes from diverse dog breeds including three Shih Tzus (Table S1). This resulted in 113 heterozygous and two homozygous private protein changing variants (Figure 2; Table S2). Only one of these variants was in the functional candidate gene for Darier disease, *ATP2A2*. This heterozygous missense variant, Chr26:8434781A>C or NM_001003214.1:c.2425A>C, is predicted to change a neutral asparagine to a positively charged histidine, NP_001003214.1:p.(Asn809His). The predicted amino acid exchange is located in a short cytoplasmic domain of the ATP2A2 protein, immediately after the sixth of 10 transmembrane domains. This region of the protein is evolutionarily conserved among vertebrates (Figure 2). In silico predictors, such as PredictSNP (Bendl et al., 2014), Provean (Choi & Chan, 2015), and MutPred2 (Pejaver et al., 2020), all classify this amino acid exchange as deleterious or pathogenic.

The clinical and histopathological findings in the studied Shih Tzu largely resembled previous descriptions of confirmed or suspected Darier disease in dogs (Linek et al., 2020; Müller et al., 2006; Olivry & Linder, 2009; Sueki et al., 1997). In humans, Darier disease is characterized by waxing-and-waning development of warty papules and plaques in seborrheic areas (central trunk, flexures, scalp, and forehead) associated with distinctive nail abnormalities. Skin lesions may be triggered by environmental factors such as heat, sweating, sunlight, and stress. Disease onset in human patients is highly variable with reports ranging from age 3 to 75 years, but most frequently starts around puberty and usually before the third decade (Foggia & Hovnanian, 2004; Li et al., 2017; Nellen et al., 2017). Clinical signs in the previously reported Irish Terrier with Darier disease started with a lesion in the ear canal and subsequent ear infection at age 4 months (Linek et al., 2020). The present case also started with ear infection but had a much later age of onset, with the 8-year-old dog resembling the huge variability in disease onset seen in human patients. While warty dyskeratoma described in dogs may have similar histopathologic changes (Gross et al., 2005), the multifocality of lesions and identification of an *ATP2A2* variant supported the diagnosis of Darier disease with a later onset. Therefore, genetic analysis in addition to histopathology was required for accurate diagnosis of Darier disease.

The genetic analysis of the affected Shih Tzu identified a heterozygous missense variant, p.N809H, of a conserved amino acid of the ATP2A2 protein. Darier disease is inherited as an autosomal dominant trait caused by haploinsufficiency of *ATP2A2* (Foggia & Hovnanian, 2004). We speculate that the variant has arisen by a de novo mutation event in the germline of the parents or during early embryonic development of the affected dog. Unfortunately, no phenotype information or samples of the parents were available to confirm this hypothesis. We did not experimentally assess the functional impact of the detected variant and, so far, no human patient with the homologous variant has been found. However, the ATP2A2 protein is highly conserved across vertebrates and missense variants in the same region of the protein have been reported in human patients with Darier disease, e.g. p.N796S or p.A838P (Li et al., 2017; Nellen et al., 2017). The highly characteristic phenotype and the finding of a missense variant in the only known functional candidate gene mutually support the diagnosis of Darier disease in the studied dog.

In conclusion, this study describes clinical, histopathologic and genetic findings in a Shih Tzu with a late-onset canine form of Darier disease. The identified heterozygous missense variant in *ATP2A2* represents a plausible candidate causative variant for the observed phenotype and corroborates the clinical diagnosis.

KEYWORDS

Canis lupus familiaris, dermatology, dog, precision medicine, skin

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are freely available. Accession numbers for the whole genome sequence data are given in Table S1.

ETHICS STATEMENT

All dogs in this study were privately owned and samples were collected with the consent of their owners. The collection of blood samples from control dogs was approved by the 'Cantonal Committee For Animal Experiments' (Canton of Bern; permit 71/19; Approval date: 09-09-2019). The collection of samples from the affected dog was performed for diagnostic or therapeutic reasons and did not constitute an animal experiment in the legal sense.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

SDR9C7 missense variant in a Chihuahua with non-epidermolytic ichthyosis

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SDR9C7 missense variant in a Chihuahua with non-epidermolytic ichthyosis

Abstract

Ichthyoses represent a heterogeneous group of cornification disorders that are associated with skin barrier defects. We investigated a 9-month-old Chihuahua showing excessive scale formation. Clinical and histopathological examinations revealed non-epidermolytic ichthyosis and a genetic defect was suspected. We therefore sequenced the genome of the affected dog and compared the data with 564 genetically diverse control genomes. Filtering for private variants identified a homozygous missense variant in *SDR9C7*, c.454C>T or p.(Arg152Trp). *SDR9C7* is a known candidate gene for ichthyosis in humans and encodes the short-chain dehydrogenase/reductase family 9C member 7. The enzyme is involved in the production of a functional corneocyte lipid envelope (CLE), a crucial component of the epidermal barrier. Pathogenic variants in *SDR9C7* have been described in human patients with autosomal recessive ichthyosis. We assume that the identified missense variant in the affected Chihuahua of this study impairs the normal enzymatic activity of SDR9C7 and thus prevents the formation of a functioning CLE, resulting in a defective skin barrier. To the best of our knowledge, this is the first report of a spontaneous *SDR9C7* variant in domestic animals.

Ichthyoses represent a group of genetic skin disorders that are characterized by dry, thickened and scaly skin. Various forms of ichthyosis have primary causes associated with skin barrier function (Akiyama, 2017; Akiyama & Shimizu, 2008; Oji et al., 2010). The skin barrier is fundamental for protection from environmental insults and maintaining body hydration (Mauldin & Elias, 2021). During epidermal terminal differentiation transglutaminases crosslink protein products (e.g. loricrin, involucrin, envoplacin, periplacin, small proline-rich protein family) at the plasma membrane, resulting in the formation of the cornified cell envelope. Subsequently, the intercellular lipid bilayer, composed of ceramides, free fatty acids, cholesterol, proteases and antimicrobial peptides, forms. This lipid bilayer is connected to the cornified envelope by the corneocyte lipid envelope (CLE), which serves as a bond between these two structures. The CLE is a monolayer mainly composed of acylceramides of the EOS class (cerEOS), a combination of esterified ω-hydroxy ultra-long-chain fatty acids and sphingosines (Akiyama, 2021).

A defective or absent CLE constitutes a prime structural defect in many diseases with impaired skin barrier function (Akiyama, 2017; Elias et al., 2014). In human patients, several genes associated with either biosynthesis or the processing of ceramides that form the CLE have been described to cause different forms of ichthyosis (Akiyama, 2021; Crumrine et al., 2019). Variants in two of these genes, *PNPLA1* and *ABHD5*, have also been reported in dogs with ichthyosis (Grall et al., 2012; Kiener et al., 2022).

Next-generation sequencing technologies have seen huge advances in recent years with concomitant decreases in sequencing costs. Whole genome sequencing, with the ability to identify the underlying genetic defect of inherited diseases, has become more accessible in veterinary medicine (Leeb, Bannasch, et al., 2022a). Together with clinical and histopathological examinations, genetic investigations offer a unique opportunity for a relatively fast and low-invasivity precise diagnosis, which in turn enables a more accurate prognosis and potentially even targeted therapy (Leeb, Roosje, et al., 2022b; Park et al., 2022). The objective of this study was to clinically and histopathologically characterize a cornification disorder in a Chihuahua and to investigate a possible underlying genetic defect.

A 9-month-old Chihuahua was presented owing to progressive abnormal scale formation since adoption at 3 months of age (Figure 1). On the first visit, the dog had scales all over the haircoat, mild to moderate pruritus associated with mild erythema and malodorous skin. A trichogram did not show any *Demodex* spp. and a tape test showed various *Malassezia* yeasts. Antiparasitic treatment (Nexgard®, afoxolaner) and twice weekly shampoos (Sebolytic® Zinc gluconate and Malaseb®, miconazol) were prescribed. Fungal culture results were

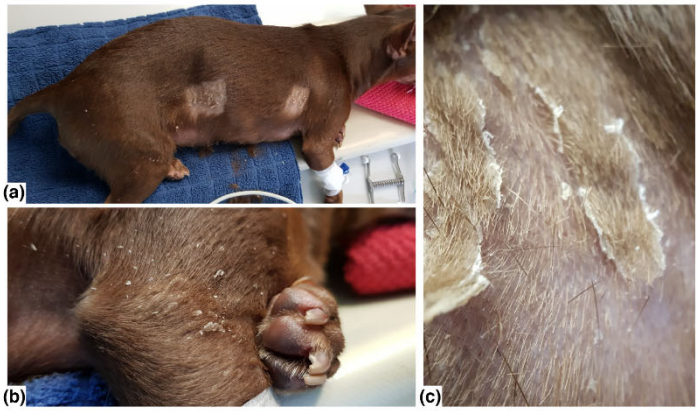

FIGURE 1 Clinical phenotype of the Chihuahua affected with non-epidermolytic ichthyosis. (a) The dog presented with generalized scale formation, clearly visible on the clipped areas. (b) Scales were thick and large and often adherent to the epidermis or the hair shafts. Paw pads and claws were normal. (c) Higher magnification of the clipped area. The scales were white-gray, thick and adhered to the hair coat. The edges were often elevated.

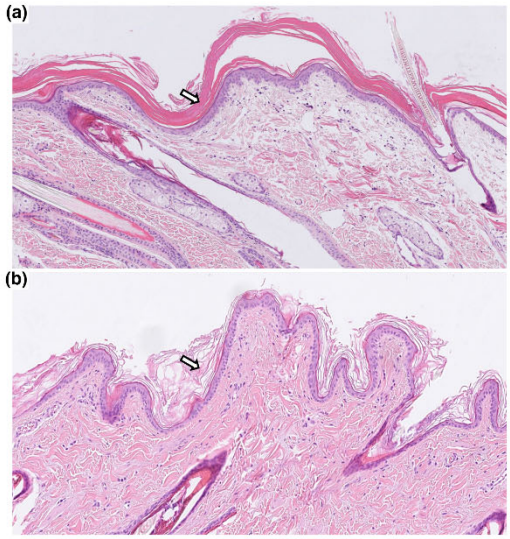

FIGURE 2 Histopathological findings. (a) Skin biopsy of the affected dog. The epidermis is covered by a thick layer of compact orthokeratotic keratin (arrow) which is extending into the follicular ostia. The dermis is mildly edematous, the lymph vessels are dilated and the number of mast cells is mildly increased in the superficial dermis. (b) Skin biopsy of a control Chihuahua. The epidermis is covered by basket-weave orthokeratotic keratin (arrow) which represents the normal stratum corneum.

negative for dermatophytes and positive for *Malassezia pachydermatis* (numerous colonies). A recheck was made 1 month later, when pruritus and erythema were absent, but little improvement was noticed concerning the scale formation.

Two 6 mm skin punch biopsies were taken under general anaesthesia and prepared for histopathological examination. In both biopsies, the epidermis was multifocally mildly hyperplastic and covered by a thick layer of mostly compact orthokeratotic keratin, which was multifocally detaching from the epidermis. The compact keratin was extending into the follicular ostia. In addition, the superficial dermis was oedematous with ectactic lymph vessels and a mildly increased number of mast cells (Figure 2). These findings together with the clinical history led to the diagnosis of a non-epidermolytic ichthyosis.

Given the clinical and histopathological findings together with the early age of the onset, an underlying genetic defect was suspected. We therefore took EDTA blood samples from the affected dog and its unaffected full sibling and extracted genomic DNA with the Maxwell RSC Whole Blood DNA Kit using a Maxwell RSC instrument (Promega). The affected dog's genome was sequenced at 25× coverage on an Illumina Novaseq 6000 instrument. Mapping and variant calling with respect to the UU_Cfam_GSD_1.0 reference genome assembly were performed as described (Jagannathan et al., 2019). Comparing the sequencing data with 564 canine control genomes resulted in 143 heterozygous and eight homozygous private protein changing variants (Tables S1, S2). Among these was a homozygous missense variant in *SDR9C7*, which is a known functional candidate gene for ichthyosis (Shigehara et al., 2016). The single nucleotide variant, Chr10:1471341G>A (UU_Cfam_GSD_1.0) or XM_038549505.1:c.454C>T, is predicted to change a conserved arginine to a tryptophan, XP_038405433.1:p.(Arg152Trp), removing a positive charge from the surface of the protein (Figure S1). The amino acid exchange was categorized as deleterious by the variant impact predictors PROVEAN (Choi & Chan, 2015) and PREDICTSNP (Bendl et al., 2014).

The genomic variant was located in a ~14 Mb homozygous segment (Chr10:30014-13939246). Sanger sequencing confirmed the homozygous genotype in the affected dog. The unaffected brother carried the mutant allele in a heterozygous state and 38 control Chihuahuas from the Vetsuisse Biobank were all homozygous wildtype.

Only recently, *SDR9C7* has been identified as functional candidate gene for ichthyosis owing to its essential role in the formation of the CLE. Several causative variants in *SDR9C7* have been reported in

human patients with autosomal recessive congenital ichthyosis (ARCI13; OMIM #617574; Hotz et al., 2018; Karim et al., 2017; Mohamad et al., 2017; Mazereeuw-Hautier et al., 2019; Seidl-Philipp et al., 2019; Shigehara et al., 2016; Takeichi et al., 2017; Youssefian et al., 2019). The clinical features described in these human patients were characterized by silvery white to brownish scales covering the entire body and in some cases palmoplantar hyperkeratosis. In some cases, the severity of the phenotype decreased with age. Human patients with *SDR9C7* variants were described to frequently suffer from recurrent fungal infections and it was suggested that the dysfunctional skin barrier facilitates this type of infection (Takeichi et al., 2017).

The affected Chihuahua initially presented with a *Malassezia* dermatitis, which could be successfully controlled by antifungal topical therapy. The dermal edema and the increased number of mast cells in the superficial dermis were also compatible with an impaired skin barrier function.

SDR9C7 is encoding the short-chain dehydrogenase/reductase family 9C member 7, which is involved in the production of a functional CLE. The SDR9C7 enzyme generates a highly reactive epoxy-enone that facilitates covalent binding of oxidized acylceramide to cornified cell envelope proteins (Takeichi et al., 2020). Studies in human patients demonstrated that a missense variant, Arg276Cys, resulted either in lower transcription or in an unstable protein that was degraded rapidly in the differentiated keratinocytes (Takeichi et al., 2020). Expression of SDR9C7 in the skin of patients with another missense variant, Ile200Thr, was significantly decreased compared with normal skin (Shigehara et al., 2016). We therefore hypothesize that the identified missense variant in the affected Chihuahua, Arg152Trp, also impairs the proper enzymatic activity of SDR9C7 and thus prevents the formation of a functioning CLE, resulting in a defective skin barrier. Such defects are known to play an important role in the pathogenesis of various types of ichthyosis (Akiyama, 2017).

The unaffected brother of the affected Chihuahua carried the mutant allele in a heterozygous state and was clinically completely normal. These results are consistent with an autosomal recessive mode of inheritance and suggestive of a recent inbreeding event.

In conclusion, this study describes the clinical, histopathological and genetic details of a Chihuahua with ichthyosis. The identified homozygous missense variant in *SDR9C7* represents a plausible candidate causative variant. To the best of our knowledge, this is the first report of a spontaneous *SDR9C7* variant in a domestic animal.

KEYWORDS

animal model, *Canis lupus familiaris*, dermatology, dog, genodermatosis, precision medicine, skin, veterinary medicine

ACKNOWLEDGEMENTS

The authors would like to thank the dog owner for donating samples and information. The authors also wish to thank the Next Generation Sequencing Platform of the University of Bern for performing whole-genome sequencing experiments and the Interfaculty Bioinformatics Unit for providing high-performance computing infrastructure. We acknowledge the DBVDC consortium, the Dog10K genomes project and all researchers who deposited dog or wolf whole genome sequencing data into public databases. This study was funded by the Swiss National Science Foundation, grant number 310030_200354. Open access funding provided by Universitat Bern.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interests.

FUNDING INFORMATION

This study was funded by grant 310030_200354 from the Swiss National Science Foundation. Open access funding provided by Universitat Bern.

ETHICS STATEMENT

The dogs in this study were privately owned and samples were collected with the consent of their owners. The collection of blood samples from control dogs was approved by the 'Cantonal Committee For Animal Experiments' (Canton of Bern; permit 71/19; approval date 9 September 2019). The collection of samples from the affected dog was performed for diagnostic or therapeutic reasons and did not constitute an animal experiment in the legal sense. The biopsy for the control dog also represented a diagnostic sample from the tissue archive of the Institute of Animal Pathology, Vetsuisse Faculty, University of Bern.

DATA AVAILABILITY STATEMENT

All data are freely available. Accessions for the whole genome sequence data are given in Table S1.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Heterozygous *KRT10* missense variant in a Chihuahua with severe epidermolytic ichthyosis

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ANIMAL GENETICS WILEY

BRIEF REPORT

Heterozygous *KRT10* missense variant in a Chihuahua with severe epidermolytic ichthyosis

BACKGROUND

Ichthyoses are a group of heritable cornification disorders. Various different subtypes are known and they can be classified based on the genetic background and underlying molecular mechanisms (Gutiérrez-Cerrajero et al., 2023). A rare form of ichthyosis is termed epidermolytic hyperkeratosis, which is mainly caused by variants in *KRT1* and *KRT10* (Fuchs & Green, 1980; Peter Rout et al., 2019). The encoded proteins, keratin 1 and keratin 10, assemble into intermediate filaments that form the cytoskeleton in differentiated keratinocytes, providing mechanical resilience (Fuchs & Green, 1980; Gutiérrez-Cerrajero et al., 2023; Syder et al., 1994).

Epidermolytic hyperkeratosis in humans predominantly follows an autosomal dominant mode of inheritance, with half of the cases resulting from *de novo* mutation events (Chipev et al., 1994; Peter Rout et al., 2019). In dogs, one occurrence of *KRT10*-associated recessive epidermolytic hyperkeratosis has been described in Norfolk Terriers owing to a homozygous splice-site variant (Credille et al., 2005).

ANALYSES

An 11-month-old male Chihuahua was presented with severe skin lesions gradually progressing from 5 months of age. Clinical examination revealed severe, multifocal hyperkeratosis, mainly affecting paw pads, axillas and the skin around the anus, lips and eyes (Figure 1). Three skin punch biopsies from axilla, lip and paw pad were taken under general anesthesia for diagnostic purposes. All samples displayed similar changes, characterized by marked epidermal hyperplasia and orthokeratotic hyperkeratosis with hypergranulosis, forming papillary

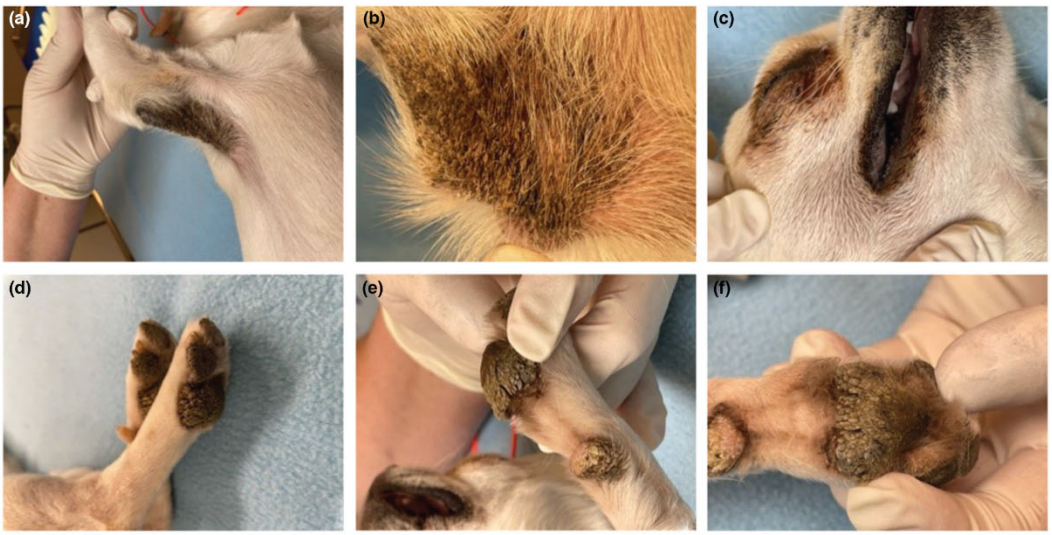

FIGURE 1 Clinical phenotype of the affected Chihuahua. (a) Severe, focal and macroscopically laminar hyperkeratosis in the axillae with concurrent severe hair loss and moderate seborrhoeic changes. (b) Close up of right axilla. (c) Rim of hyperkeratosis around lip commisure and rima. (d–f) Severe paw pad hyperkeratosis and mild palmar and plantar erythema affecting all pads on all four paws.

projections on the skin surface These histopathological findings, together with the anamnesis and clinical findings, were characteristic for an epidermolytic hyperkeratosis.

Whole genome sequencing at 28× coverage was performed on genomic DNA extracted from leukocytes. Data processing was done as previously described (Jagannathan et al., 2019), using the genome reference assembly UU_Cfam_GSD_1.0. We identified 72 heterozygous and five homozygous private protein changing variants in the case by comparing its sequence data with 926 genetically diverse control genomes (Tables S1 and S2). One of these variants was located in *KRT10*, a known ichthyosis candidate gene. The heterozygous missense variant, Chr9:21814695G>A, XM_038547368.1:c.437G>A, was predicted to change an evolutionarily conserved arginine in the coil 1A domain, XP_038403296.1:p.(Arg146His). The presence of the heterozygous genotype at the position of the variant was confirmed by Sanger sequencing. The genotypes of 40 additional Chihuahuas from the Vetsuisse Biobank were homozygous wild type. We speculate that this variant has most likely arisen *de novo* in the affected dog; however, samples from the parents to confirm this hypothesis were not available.

CONCLUSIONS

The identified *KRT10* missense variant is most likely causative for the observed epidermolytic hyperkeratosis in the Chihuahua. The variant affects a conserved CpG dinucleotide within an arginine codon. Heterozygous variants affecting human Arg156, homologous to canine Arg146, have been described in several human patients with epidermolytic hyperkeratosis, suggesting this residue to be a mutational hot spot (Cheng et al., 1992; Chipev et al., 1994; Rothnagel et al., 1992, 1993; Syder et al., 1994).

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FUNDING INFORMATION

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All underlying raw data are freely available. Accession numbers of the whole genome sequences used in this study are listed in Table S1.

ETHICS STATEMENT

The examinations of the affected dog were done with the consent of the owner in the framework of veterinary care and diagnostics and did not constitute an animal experiment. The collection of blood samples from control dogs was approved by the *Cantonal Committee for Animal Experiments* (Canton of Bern; permit BE94/2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Overall discussion and outlook

In this thesis, I investigated the genetic background of six different inherited skin diseases in cats and ten in dogs using next-generation sequencing technologies. This approach allowed the identification of genes harboring compelling candidate variants for all 16 inherited skin diseases (Table 5). The investigated disorders affect various structures in the skin and belong to distinct categories of genodermatoses (Figure 3).

Table 5. Summary of the 16 variant harboring genes identified in the investigated skin diseases from this thesis. The corresponding OMIA number is provided, as well as the OMIM number of the orthologous human phenotype, if known.

Gene	Phenotype	MOI[1]	Species	Breed	OMIA# [20]	Human disorder (OMIM#) [19]
ABHD5	Ichthyosis	AR	Dog	Golden Retriever	002368	275630
ADAMTS2	Dermatosparaxis Ehlers-Danlos syndrome	AR	Cat	Domestic shorthair cat	000328	225410
ATP2A2	Darier disease	AD (*de novo*)	Dog	Shih Tzu	002265	101900 124200
COL17A1	Junctional epidermolysis bullosa	AR	Cat	Domestic shorthair cat	000342	619787 122400
COL5A1	Classical Ehlers-Danlos syndrome	AD (*de novo*)	Cat	Bengal Bombay Domestic shorthair cat	002165	130000 619329
COL5A2	Classical Ehlers-Danlos syndrome	AD (*de novo*)	Dog	Chihuahua	002295	130010
COL7A1	Dystrophic epidermolysis bullosa	AR	Dog	Basset Hound	000341	226600 131750 132000 604129 131850 607523 131705
DSG1	Footpad hyperkeratosis	AR	Dog	Rottweiler	002266	615508 148700
DSG4	Hair shaft dysplasia	AR	Cat	Domestic shorthair cat	002452	607903

KRT1	Ichthyosis	AD (*de novo*)	Dog	Shar Pei	002425	113800 146590 620148 607654 620411 600962
KRT10	Ichthyosis	AD (*de novo*)	Dog	Chihuahua	001415	146600 620150 609165 607602
KRT5	Epidermolysis bullosa simplex	AD (*de novo*)	Dog	Welsh Corgi (Cardigan)	002081	179850 619555 619588 619594 619599 609352 131960
LAMB3	Junctional epidermolysis bullosa	AR	Dog	Australian Shepherd	002269	104530 226650 226700
SDR9C7	Ichthyosis	AR	Dog	Chihuahua	002659	617574
SLC39A4	Acrodermatitis enteropathica	AR	Cat	Turkish Van	000593	201100
SOAT1	Sebaceous gland dysplasia	AR	Cat	Domestic shorthair cat	002669	?

[1]MOI = mode of inheritance: AR = autosomal recessive, AD = autosomal dominant

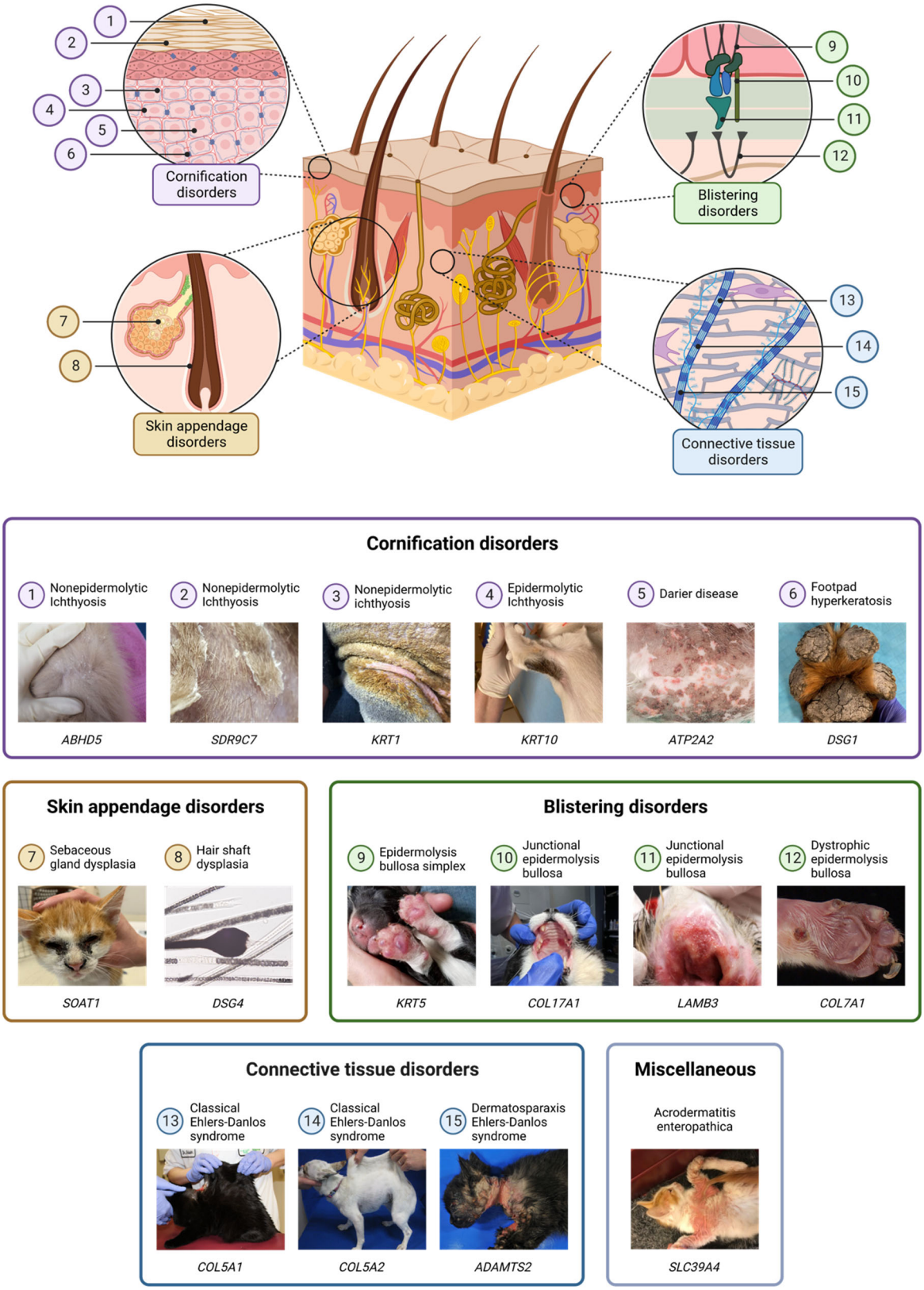

Figure 3. Overview of the 16 investigated skin diseases, displaying the different affected structures, clinical presentation and genes harboring the identified variants in each of the disorders. Illustrations are adapted from “Anatomy of the Skin” and “Desmosomal Protein Distribution in the Epidermis” by BioRender (2023) [10].

Six of the investigated phenotypes belonged to the group of **cornification disorders**. The first study focused on ichthyosis in Golden Retrievers and comprised the largest cohort of all investigated disorders. The phenotype was very similar to the probably most common canine ichthyosis, the *PNPLA1*-associated autosomal recessive ichthyosis also in Golden Retrievers. However, none of the 14 affected dogs from our study were homozygous for the mutant *PNPLA1* allele. This suggested that another pathogenic *PNPLA1* allele or a new pathogenic variant in another gene had arisen in the investigated cohort by spontaneous mutation, representing either allelic or locus heterogeneity. Through positional cloning and whole genome sequencing, a candidate causative variant in *ABHD5* could be identified, present in homozygous state in all 14 cases. In humans, variants in *ABHD5* cause Chanarin-Dorfman syndrome (CDS), a neutral lipid storage disorder with ichthyosis [59–61]. The new Golden Retriever ichthyosis was designated as Golden Retriever ichthyosis type 2 (ICH2).

The second study involved a Shih Tzu with Darier disease in which I identified a heterozygous missense variant in *ATP2A2*, a well-known candidate gene for Darier disease. *ATP2A2* encodes an endoplasmic/sarcoplasmic reticulum Ca^{2+} pump and defective intracellular calcium signaling in the epidermis results in a loss of desmosomal adhesion and the development of characteristic skin lesions [62–64].

The third study analyzed a single Rottweiler with footpad hyperkeratosis and a homozygous candidate causative variant in *DSG1* was identified. The encoded desmoglein-1 is important for cell-cell adhesion between keratinocytes. In humans, *DSG1* is a known gene for autosomal dominant striate palmoplantar keratoderma, while biallelic *DSG1* variants lead to severe dermatitis, multiple allergies, and metabolic wasting (SAM) syndrome [65–68]. This compares to the observed phenotype of the Rottweiler from our study that at later age additionally developed skin infections and allergy predisposition.

The fourth and fifth studies examined single cases of epidermolytic and non-epidermolytic ichthyosis in a Chihuahua and a Shar Pei, respectively. The presented clinical signs in both dogs resulted from disruption of keratin filaments that form the cytoskeleton in differentiated keratinocytes, caused by the identified underlying variants in the candidate genes *KRT10* in the Chihuahua and *KRT1* in the Shar Pei. Both variants were present in heterozygous state. In the Shar Pei, a *de novo* mutation event could be confirmed through sequencing analyses of both parents. In the Chihuahua, the same scenario was suspected, however, samples of the parents were not available to confirm this hypothesis.

The sixth study focused on a Chihuahua that presented with excessive scale formation. After clinical and histopathological examination, the dog was diagnosed with non-epidermolytic ichthyosis. The genetic analyses revealed a homozygous missense variant in *SDR9C7*. This known candidate gene for ichthyosis in humans encodes the short-chain dehydrogenase/reductase family 9C member 7, an enzyme involved in the production of crucial components of the epidermal barrier [69]. This study represented the first report of a spontaneous *SDR9C7* variant in domestic animals.

The group of **blistering disorders** consisted of four different studies of EB. Firstly, two unrelated domestic shorthair cats with EB. Both affected cats carried individual homozygous variants in *COL17A1* encoding the collagen type XVII alpha 1 chain. The transmembrane protein links the epidermis to the basal membrane and is crucial for the integrity of healthy skin [70]. The first cat, presented with a very severe phenotype, had a splice site variant that most likely resulted in complete loss of function of collagen XVII. The splice region variant in the second cat was experimentally shown to result in both wildtype and mutant transcript, suggesting some preserved residual collagen XVII function. This might explain its milder phenotype.

Secondly, a close inbreeding loop directly contributed to the emergence of the severe DEB phenotype in three Basset Hound puppies. The genetic analysis revealed a homozygous variant in *COL7A1*, consistent with an autosomal recessive mode of inheritance. The variant likely leads to an absence of functional type VII collagen, which is the main component of the anchoring fibrils in the basement membrane zone [71]. This deficiency resulted in the observed severe blistering due to dermo-epidermal cleavage.

Thirdly, we investigated a Welsh Corgi (Cardigan) with congenital blisters and erosions. The puppy carried a heterozygous variant in a highly conserved motif in *KRT5* that had arisen due to a *de novo* mutation event. The homologous variant has been described in human patients [72,73]. The molecular analysis refined the initial diagnosis in the affected dog from EB to EBS.

Fourthly, a litter of Australian Shepherds, which resulted from a father-daughter mating and included three puppies with JEB, was studied. The identified homozygous *LAMB3* variant was hypothesized to change the tertiary structure of the encoded laminin β3. This subunit of laminin 322 is a critical component of hemidesmosomes, adhesion complexes that bind the epidermis to the underlying dermis [37,74,75]. Defects in laminin β3 are a known cause of JEB in humans [37].

I further investigated different types of EDS in three studies, representing **disorders of connective tissue**. Study number one investigated four domestic shorthair kittens with dermatosparaxis type of EDS, in which we identified a defect in the candidate gene *ADAMTS2*. The encoded N-proteinase cleaves the propeptides of procollagen, which afterward assemble into mature collagen molecules [46].

Study number two included one Bengal, one domestic shorthair cat, and two sibling Bombays with classical EDS. We identified three individual heterozygous variants in *COL5A1* that all presumably led to nonsense-mediated mRNA decay. The resulting haploinsufficiency of *COL5A1* is in line with other results in the literature stating that in classical EDS, about 75% of the identified pathogenic variants are loss-of-function variants in *COL5A1.* These lead to haploinsufficiency, resulting in decreased type V collagen in the extracellular matrix [46].

Study number three involved a Chihuahua with clinical signs suggestive of classical EDS. The identified heterozygous *de novo* deletion variant in *COL5A2* most likely resulted in a dominant-negative gain-of-function in the mutant allele and thus defective type V collagen molecules in the skin.

Next, I studied two **disorders affecting skin appendages.** In the first study, we examined two unrelated domestic shorthair cats exhibiting abnormal hair shaft swellings, similar to the lanceolate hair phenotype in mice with loss-of-function variants in *Dsg4*. In both of our studied cats, an individual homozygous variant could be identified in the candidate gene *DSG4*, which is crucial for normal hair shaft formation [76,77].

In the second study, two sibling domestic shorthair kittens with sebaceous gland dysplasia were investigated. The identified candidate causative variant was a homozygous missense variant in *SOAT1* encoding an intracellular enzyme that catalyzes the formation of cholesteryl esters, an essential component of sebum and meibum [78]. So far, there is no human phenotype associated with this gene. However, two mouse models support our hypothesis that the identified feline *SOAT1* variant could influence the development and function of sebaceous glands, thereby causing the observed phenotype [78–81]. Our results propose a new candidate gene for sebaceous gland dysplasia phenotypes in veterinary and human medicine.

Finally, one investigated disorder had to be classified as **miscellaneous**. Three Turkish Van kittens from one litter presented with severe signs of skin disease, coupled with diarrhea and systemic signs of stunted growth. The identified underlying candidate causative variant was a homozygous missense variant in *SLC39A4* that perfectly co-segregated in the extended

family. The encoded zinc transporter is most likely nonfunctional due to this variant, resulting in zinc malabsorption and the observed devastating clinical signs of acrodermatitis enteropathica (AE).

The candidate genetic variants identified in my PhD studies were predominantly located in known candidate genes with correlating phenotypes in humans. Knowledge of the functional effects of genes in other species often provided substantial support that the identified variants may be causative for the disorders we studied. However, establishing causality in genetics is a complex task and requires the fulfillment of several criteria [82]. The presence of the causal variant in affected animals and a functional and pathogenic effect of it are two of these conditions. To better characterize the functional consequence, several bioinformatic tools for computational prediction are available, but *in silico* methods have their limitations [83–86]. For the evaluation of variants affecting exon splicing, the most straightforward and reliable method is the analysis of RNA samples [85]. This was successfully applied in one of the cats with JEB and the splice region variant in *COL17A1*. We demonstrated that the variant indeed impaired splicing and resulted in both mutant and wildtype *COL17A1* transcripts. Functional assays at the protein level and biochemical analyses are additional methods to complement *in silico* predictions. Such analyses were planned for the kittens with sebaceous gland dysplasia, as the identified *SOAT1* variant was presumed to alter the composition of sebum. Similarly, for the Chihuahua with *SDR9C7*-related ichthyosis, as prior studies of the homologous human phenotype suggested an altered skin lipid composition [69]. Unfortunately, the examinations of sebum and skin lipids could not be conducted due to practical limitations. The kittens were euthanized before the genetic analysis could be completed, leaving no samples for these follow-up experiments, and the Chihuahua was not accessible for sample collection following the completion of the genetic analysis. This underlines a major constraint in studying privately owned animals: the obtainment of all required samples is not always feasible, and the cooperation of the owners is an essential factor. For variant interpretation and evaluation of its effect, reliance on consensus criteria can be a guiding tool. Although no such criteria are defined for animals, they have been established for humans and have proven highly useful to provide a standardized classification reflecting the strength of evidence for pathogenicity [87]. Applying these criteria to our results allowed us, for instance, to classify the *ADAMTS2* frameshift variant in the cats with Ehlers-Danlos syndrome, and the *de novo* variant in *KRT1* in the Shar Pei with ichthyosis, as pathogenic. Definite proof of causality would finally involve more sophisticated methods using experimental models and targeted gene editing, where introduction of the variant should cause the phenotype and removal (deletion or silencing) of the candidate causal variants should reverse the phenotype [82,88].

Understanding the genetic cause of a disease provides insight into the mode of inheritance and enables genetic testing, both being important factors for making strategic breeding decisions [18]. In the case of ICH2 in Golden Retrievers, genetic testing was established to prevent the unintentional breeding of affected puppies and eradicate ICH2 from the population as a long-term goal. Generally, every genetic variant identified in this thesis can now be genotyped in a fast, simple way. These tests can be used if a cat or dog presents with clinical signs suggestive of the corresponding disease. Since genodermatoses in purebred animals are often less heterogeneous than in humans, affected individuals from the same breed typically carry the same deleterious variant. Therefore, a positive test result can offer a clear diagnosis, potentially avoiding more invasive diagnostic procedures like biopsies. However, a negative result does not exclude the possibility of another, yet unidentified, disease-causing variant [28]. Furthermore, our published results and the descriptions of our clinical findings linked to the identified underlying variants provide valuable information to veterinary clinicians. For many disorders, the current classification criteria are based on human patients. Thus, this work expands the understanding of these conditions in cats and dogs and can help to evaluate and manage such disorders more effectively.

Another benefit of understanding the underlying molecular pathomechanism is the potential for targeted or individualized therapy. If future clinical studies show that ICH2 also affects other organs in affected Golden Retrievers, as CDS does in humans, then dietary management could be a potential approach. This is suggested by studies in human CDS patients which indicate that a low-fat diet can help manage lipid droplet accumulation in tissues [89]. Another example is AE in Turkish Vans caused by a defective intestinal zinc transporter. Unfortunately, these kittens died before the genetic investigations were completed. Otherwise, treatment would most likely have been possible as previous studies have shown that zinc deficiency in AE patients can be successfully addressed with sufficient supplementation of oral zinc [90–97].

Additionally, the knowledge gained is also beneficial from a One Health perspective, as findings can be extrapolated to human medicine. Domestic animals can serve as excellent models for studying inherited diseases [98]. Notably, the significance of many genes in disease manifestation was initially discovered in dogs [17,99]. Two examples of such genes with dermatological relevance are *PNPLA1* and *ASPRV1*. Identified defects in these genes were found to be responsible for subtypes of ichthyosis in Golden Retrievers and a German Shepherd Dog, respectively. It was only later that genetic variants in *PNPLA1* and *ASPRV1* were identified in human patients with previously unexplained ichthyoses [30,100,101]. One of the most important results of my study was the discovery of the *SOAT1* gene in cats with

sebaceous gland dysplasia. Currently, there is no known human phenotype associated with defective *SOAT1*. Thus, our results introduce a new candidate gene for similar sebaceous gland dysplasia phenotypes in humans.

In conclusion, the results from this thesis highlight the great potential of whole genome sequencing analysis for investigating monogenic skin diseases in cats and dogs. This assessment is supported by other similar studies in companion animals that have successfully applied these techniques to a broader array of inherited diseases [102–106]. Knowledge about the disease-causing variant often results in a definitive diagnosis for the patient. This insight can improve disease management and owner counseling, provide thoughtful breeding recommendations, and may guide potential new targeted therapies [18,107]. Through continuous further development of genetic and sequencing technologies, a precision medicine approach can be applied more broadly in veterinary medicine. Notably, such advances not only benefit the animals but also broaden our understanding of rare diseases, underlying pathomechanisms, and gene functions. The gained insights can be transferred to human medicine, demonstrating the value of a One Health approach.

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Curriculum vitae

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List of publications

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Barsh GS, Kaelin CB, McGowan KA, Hutcherson AD, Delay JM, Li JH, **Kiener S**, Jagannathan V, Leeb T, Murphy WJ. Ancestry dynamics and trait selection in a designer cat breed. *Current Biology*. Submitted

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Declaration of Originality

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